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Commentary **Open Access**

Applying lessons learnt from Ebola for effective COVID-19 response in Africa

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Abstract:

The Ebola virus is transmitted to people from wild animals and spreads in the human population through human-to-human transmission via direct contact with blood, secretions, organs or other bodily fluids of infected people, and with surfaces and materials contaminated with these fluids. In December 2019, a novel coronavirus disease (COVID-19) caused by severe acute respiratory syndrome-coronavirus-2 (SARS-COV-2) emerged in Wuhan, China, attracting the notice of regional authorities and rapidly drawing global attention. In less than 4 months, COVID-19 spread through almost all countries and regions. The COVID-19 pandemic is wreaking havoc on the world economy, in addition to creating the current global public health crisis. According to the World Health Organization (WHO), 28,616 cases of Ebola were detected, and 11,310 people died during the outbreak in Guinea, Liberia and Sierra Leone. As of 17th December 2020, COVID-19 has killed 1,658,062 people, and positive cases have topped 74 million globally. Africa has suffered several outbreaks of Ebola Virus Disease (EVD); learning from the past is a good way to prepare for the future. We hope to highlight some of the lessons learnt from Africa's response to previous epidemics that can help in the fight against the ravaging coronavirus pandemic.

Keywords: Ebola, COVID-19, WHO, transmission, global

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Appliquer les leçons tirées d'Ebola pour une réponse efficace au COVID-19 en Afrique

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Abstrait:

Le virus Ebola est transmis aux humains par des animaux sauvages et se propage dans la population humaine par transmission interhumaine par contact direct avec du sang, des sécrétions, des organes ou d'autres fluides corporels de personnes infectées, et avec des surfaces et des matériaux contaminés par ces fluides. En décembre 2019, une nouvelle maladie à coronavirus (COVID-19) causée par le syndrome respiratoire aigu sévère-coronavirus-2 (SRAS-COV-2) est apparue à Wuhan, en Chine, attirant l'attention des autorités régionales et attirant rapidement l'attention mondiale. En moins de 4 mois, le COVID-19 s'est propagé dans presque tous les pays et régions. La pandémie de COVID-19 fait des ravages sur l'économie mondiale, en plus de créer la crise mondiale actuelle de santé publique. Selon l'Organisation mondiale de la santé (OMS), 28616 cas d'Ebola ont été détectés et 11310 personnes sont décédées au cours de l'épidémie en Guinée, au Libéria et en Sierra Leone. Au 17 décembre 2020, le COVID-19 avait tué 1658062 personnes et les cas positifs dépassaient 74 million dans le monde. L'Afrique a souffert de plusieurs flambées de maladie à virus Ebola

(MVE); apprendre du passé est un bon moyen de préparer l'avenir. Nous espérons mettre en évidence certaines des leçons tirées de la réponse de l'Afrique aux épidémies précédentes qui peuvent aider à lutter contre la pandémie ravageuse de coronavirus.

Mots clés: Ebola, COVID-19, OMS, transmission, mondial

Applying lessons of Ebola outbreak response to the COVID-19 pandemic response

In the wake of COVID-19 pandemic, Hossain et al., (1) believes that fake news, rumours and doubts have been disseminated from all angles especially the social media, giving rise to mass panic and lack of confidence in the COVID-19 response by many communities. According to the Medicins Sans Frontieres (2), community engagement must form the cornerstone of any response as it was key in ending previous Ebola outbreaks in different parts of Africa. The World Health Organization (WHO) states that community engagement is very necessary in discovering new cases of Ebola virus disease (EVD) and in contact tracing of people sick with Ebola and community members need training in many aspects of the response so that they can contribute in ending the transmission of EVD. This was demonstrated when Ebola outbreak began in Sierra Leone in 2014, the ministry of health began a massive community-based intervention where community and religious leaders, volunteers and activists were trained on Ebola response from public sensitization to contact tracing, which was very helpful as 70.8% of 72 cases reported in three communities was done by trained local community members (3).

In responding to the 2014-2016 West Africa Ebola outbreak, the International Federation of Red Cross (IFRC) and Red Crescent Societies (RCS) mobilized 600,000 community members as volunteers across 10 countries in Africa. This was counted as a big success in the fight against Ebola epidemic as was corroborated my Mrs Khadidiatou Baro, an Ivorian, who said, "*we did not know what quarantine was and why it is important for stopping Ebola. Many people would not allow any of their family members to be quarantined. But the Red Cross community engagement volunteers convinced us about the importance of quarantine and we now accept it in our communities"* (4).

To achieve the goal of flattening the global SARS-COV-2 infection curve, community leaders, health workers, volunteers and various social groups within the communities must be made to champion the fight against COVID-19. Various communities especially in low -and - middle - income-countries (LMICs) without access to adequate information must realize the importance of social distancing and hygienic practices in breaking the chain of community transmission; they must learn to report cases immediately and also participate in contact tracing. This training will come by community engagement.

Vaccines have been crucial in the eradication of some deadly diseases such as smallpox and in reducing harms caused by these pathogens (5). The WHO states that 2013-2016 West Africa Ebola outbreak which killed more than 11,300 people has exposed the importance of a vaccine. The necessity of a vaccine warranted the approval of an experimental vaccine for use by the regulatory and ethics review boards in the Democratic Republic of Congo on May 29, 2017 (6). Therefore, following the resurgence of Ebola in DRC in April 2018, the ministry of health sprang into action, and on May 21, 2018 began a radical but important ring vaccination using rVSV-ZEBOV to vaccinate primary and secondary contacts of diagnosed cases. This proved very helpful as 3,796 individuals who were immediately vaccinated were not infected. Lesson learnt is that 'what has worked before can work again' as ring vaccination has been used before in the eradication of smallpox (7). The rVSV-ZEBOV vaccine produced by Merck went through clinical trials during the 2013-2015 West Africa Ebola epidemic and was found to be 100% efficacious, and was 97.5% effective in the 2018 Kivu outbreak (8).

According to the Global Alliance for Vaccines and Immunization (GAVI) (9), *"developing a vaccine against COVID-19 is the most pressing challenge of our time".* COVID-19 vaccine could be the game changer in the global struggle against COVID-19 pandemic, a view that was reaffirmed by Dr Seth Berkley, CEO of GAVI who believes that *"COVAX is the only truly global solution to COVID-19 pandemic*" (10). Calls have been made by funders, manufacturers and scientists to speed up the vaccine development, so far, 70 vaccines and numerous therapeutics are in clinical trials in different parts of the world (11). To show the importance of effective vaccine, more than 150 countries have agreed to be part of the COVID-19 vaccine global access facility which will ensure the equitable distribution of the vaccines to participating countries through COVAX using the criteria of need, vulnerability and level of threat to determine further distribution of the vaccine when ready (10). Knowledge derived from Ebola and SARS-COV responses has contributed in some measure in designing innovative approaches for COVID-19 vaccine

development which is expected to be the quickest vaccine development in history (12). Just like in DRC Ebola outbreak of 2018, a COVID-19 vaccine will assist in breaking the chain of transmission in the primary and secondary contacts of infected persons. It is expected that by the end of 2021, COVAX would have delivered more than 2 billion doses of safe and effective vaccines (10).

According to the World Health Organization 2016 [13], *"a well-functioning health system working in harmony is built on having trained and motivated health workers, a well maintained infrastructure and a reliable supply of medicines and technologies backed by adequate funding, strong health plans and evidence -based policies."* The weak health system in Africa was heavily exposed during the Ebola outbreak that began in Guinea in 2013, and spreading to Liberia and Sierra Leone in 2014 (14,15). The Republic of Guinea suffers from a critical shortage of healthcare workers with a health workforce density less than 1.5 per 10,000 (14). Apanga and Akparigbo (15) reported that 80% of Ebola cases in the early days of the outbreak who were diagnosed of Ebola were not admitted by the hospital authorities due to lack of infrastructure (accommodation). Poor infrastructure and weak health systems continue to plague many LMICs in the face of the recent COVID-19. To improve the COVID-19 response, national governments must pay more attention to their health systems by increasing funding, quality and quantity of their health workforce (16). This view is reemphasized by Bill Gates (17) who believes that *"it's essential to help LMICs strengthen their primary health care systems. When you build a health clinic, you are also creating part of the infrastructure for fighting epidemics."*

The 2014-2016 outbreak in West Africa has been described severally as the largest and most complex of all Ebola outbreaks (18), the first case was reported on March 23, 2014 in South Eastern Guinea (19). An important lesson learnt is that outbreak response requires concerted effort and should not be left for any one government or organization (20). The importance of international collaboration was demonstrated when the international community committed USD 100 million to fight the Ebola epidemic of 2014-2016 which enabled the deployment of more medical personnel to assist in combating Ebola (21). The cross-border collaboration on preparedness and response to Ebola virus and other disease outbreaks which DRC entered in 2019 with 9 neighboring countries is a sign of strong international commitment and willingness to pull resources which will strengthen disease surveillance among the participating countries and improve health security [22].

A joint international collaboration is necessary in the wake of COVID-19 pandemic (23,24). Thanh et al., (25) emphasized the importance of strong international coordination between vaccine developers, regulators and other relevant stakeholders. Momtazmanesh et al., (24) made 2 key observations; first, the quick exchange of genomic sequence of COVID-19 by many researchers globally, and second, the launch of the solidarity trial which has at least 10 countries in participation with the WHO at the helm of the study. Those two observations are marks of international coordination and cooperation. During this COVID-19, we have seen countries help each other; from donating ventilators to taking in very critical patients (26). Furthermore, the United Nations (UN) believes that "*the only way to fight COVID-19 is through a global approach*" and in demonstration of its commitment to humanitarian response has donated USD 2 billion to assist 51 countries across the globe to cushion the effect of COVID-19 (27). More international collaborations will be needed to facilitate vaccine delivery and break the barriers to vaccine licensing. LMICs will require great help to bounce back from the heavy blow of COVID-19, and this can only come through international solidarity.

Conclusion:

By joining together and engaging communities, COVID-19 can be contained just like the EVD. It is important to note that the world will only be safe when we are all safe. The COVID-19 pandemic has exploited any and all cracks in humanity. In the war against COVID-19, health system resilience, accountability and integrity are more important than ever. The health systems of some high-income-countries have become overwhelmed by the rising number of infected cases and deaths from the disease. The weaker, corruption-prone and less resilient health systems of many LMICs are even more vulnerable, and some may even collapse.

Research has underscored the vulnerability of Africa's health system. A consistently solid and accountable health system has eluded the continent. The requisite health resources are also in short supply. Also, Ebola and COVID-19 have demonstrated the need for universal health coverage. According to Tedros Adhanom (WHO Director General), "*global health is only as strong as its weakest link"*. Whether it is Ebola, COVID-19, measles, chickenpox or another pathogen, universal health coverage is our best defense. This means investing in surveillance, health workforce and primary health care as key compo-

nents to ensuring quality health services for all.

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Viewpoint **Open Access**

Neglect of common infectious disease outbreaks during the COVID-19 pandemic: an impending crisis in Nigeria?

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Abstract:

Infectious diseases are major challenges of healthcare system in Nigeria. The coronavirus disease-19 (COVID-19) pandemic has disrupted many systems including healthcare at all levels by creating disparities in the treatment, prevention, resource allocation and control of diseases in Nigeria. Premised on the foundation of circulating news and fact-checking platforms, this paper provides empirical evidence on varying perceptions on COVID-19 pandemic and apparent neglect of other infectious diseases while giving a critical analysis and comparison between them.

Keywords: COVID-19; infectious diseases; neglect; Nigeria

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Négliger les flambées de maladies infectieuses courantes pendant la pandémie COVID-19: une crise imminente au Nigeria?

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Abstrait:

Les maladies infectieuses sont des défis majeurs du système de santé au Nigeria. La pandémie de coronavirus-19 (COVID-19) a perturbé de nombreux systèmes, y compris les soins de santé à tous les niveaux, en créant des disparités dans le traitement, la prévention, l'allocation des ressources et le contrôle des maladies au Nigéria. Fondé sur la diffusion d'informations et de plates-formes de vérification des faits, cet article fournit des preuves empiriques sur les différentes perceptions de la pandémie de COVID-19 et la négligence apparente d'autres maladies infectieuses tout en fournissant une analyse critique et une comparaison entre elles.

Mots clés: COVID-19; maladies infectieuses; négligence; Nigeria

Introduction:

Coronavirus disease-19 (COVID-19) caused by severe acute respiratory syndrome-coronavirus-2 (SARS-COV-2) is a pandemic disease burden that has affected millions of people all over the world. In Nigeria, infectious diseases constitute significant disease burden in the country. Prior to the onset of the global pandemic of SARS-COV2 infection, national reports and statistics regarding specific infectious diseases in Nigeria were already alarming, and while plans were being made to mitigate these, the results have been at best moderately effective (1).

Tuberculosis (TB), caused by acid fast bacillus, *Mycobacterium tuberculosis*, affects more than 63,000 people in Nigeria yearly, coupled with the existing 407,000 people already living with the tuberculous disease (2). Although largely preventable, malaria cases in Nigeria contributes up to 25% of all global malaria deaths (3) while the country has the second largest HIV epidemic in the world, affecting more than 3.2 million people, mostly young people but including women, children, people who inject drugs (PWIDs) and sexual minorities such as men who have sex with men (MSM) (4).

Other common diseases such as meningitis and Lassa fever are already endemic in Nigeria. Interestingly, some of these diseases

are associated with one another, some with direct proportional relationships. For example, malaria increases risk for HIV infection and causes a temporary increase in viral load (5) while there is 30 times increased risk of developing TB in people living with HIV than non-HIV infected persons (6). This is baffling for a developing country like Nigeria that has public health issues occasioned by political instability and social insecurity, inadequate or delayed funding and poor standard of living of residents, which eventually affect campaigns aimed at eliminating these infectious diseases (7).

This viewpoint critically analyses the perspectives of whether an impending crisis is possible using current facts and historical data while seeking to inspire researchers to develop quantitative surveys on the direct correlation of the COVID-19 pandemic and common infectious diseases in Nigeria. To fully understand the probability of an impending crisis in the country, a brief history in time of the most prevalent and current infectious diseases in Nigeria, Lassa Fever and Yellow Fever, was undertaken.

Current infectious diseases with epidemic potentials in Nigeria:

Lassa fever is an acute viral haemorrhagic fever caused by the Lassa virus, a member of the *Arenaviridae* family of viruses. The disease is transmitted through urine and droppings of infected multimammate rats of the *Mastomyss* genus and is characterized by fever, bleeding and headaches. Shortly before the report of first case of COVID-19 in Nigeria, Lassa fever had already prompted calls for declaration as a national health emergency. This was because as at February 23, 2020, the disease had spread through 27 States of the country, with 689 confirmed cases and 118 deaths, as against the cumulative 381 confirmed cases and 83 deaths for the same time period of February 2019 (8). This became alarming and the immediate response by the Nigeria Center for Disease Control (NCDC) was to activate the national emergency operation centre (EOC) that would coordinate activities at the State level, activate State public health emergency operation centre, and scale up community engagement and risk communications. For typical infectious diseases such as this, extreme precaution and coordinated responses are necessary to prevent a national epidemic disaster. Public stakeholders have therefore been involved in controlling this potentially dangerous disease by localizing its spread.

After 21 years without occurrence, Yellow fever re-emerged in Ifelodun Local Government Area (LGA) of Kwara State, Nigeria on September 12th 2017 with an index case, a 7–year-old who presented with fever, jaundice and vomiting of blood, with no record of travel or vaccination (1). By the end of the year, the number of suspected cases had risen to 337 with 13.6% case fatality rate and 23.3% for suspected cases (1). At this time, it was already confirmed in 4 States (Kwara, Kogi, Kano and Zamfara). Strategies were deployed to tackle this outbreak, which included surveillance by active case search and entomological surveys, sample collection for laboratory testing, immunization and vaccination (1). Even with this scheme, there were series of re-emergence between 2018 and 2020.

A deeper comparative analysis of these two common infectious diseases and the COVID-19 in terms of control strategy has shown that the recent pandemic has taken more attention than these other infectious diseases.

Was there increased awareness and alertness of COVID-19?

The index case of COVID-19 in Nigeria was reported on February 28, 2020 (9) and since then, many strategies have been developed to contain the disease. SARS-COV-2 is transmitted through droplet secretions and direct contact but could also be airborne when aerosols are generated. Elderly and immuno compromised people are more prone to the disease. Therefore, containment of the disease solely depends on containing the virus spread as a preventive measure and not just its treatment. There is no general agreement on the direct impact of the pandemic and existing infectious diseases in Nigeria as there are insufficient data regarding clinical and community management of infectious diseases apart from the SARS-COV-2 infection. With varying perceptions of the pandemic, the impacts for now can only be speculative.

COVID-19 affects individuals of any age and while asymptomatic patients have been reported, the most common clinical symptoms are fever, dry cough and upper respiratory tract symptoms such as sore throat, headache and myalgia (10). The differential diagnoses include a wide range of diseases such as any type of respiratory viral infections (influenza, adenovirus, coxsackie), bacteria pneumonia and respiratory infections by atypical organisms. Because SARS-COV-2 infection presents like many other respiratory diseases and cannot be identified by routine laboratory tests, it is almost impossible to distinguish COVID-19 from other respiratory infections without specific molecular tests (10).

Following the index case and rise of the infections rate, healthcare workers in the country became more careful in relating to

patients especially suspected cases of any infection. All suspected infections especially respiratory infections were ultimately treated as high alert. Since infections are on high alert, detection and diagnosis of any suspected infection were fast-tracked and improved in order to eliminate false positives and false negatives. Infections then were promptly treated and managed. If high priority was put on all types of infections in order to diagnose COVID-19, the main question therefore is "are infectious diseases really neglected?". It could be argued that infections are in fact, not neglected. The nexus between common infections and COVID-19 is however inextricable.

A more popular school of thought proposes that the issues regarding healthcare in Nigeria is chronic in nature and as such, more evident during the pandemic, especially that Nigeria is one of the five WHO African Region countries that has five public health events per annum (7). From an historical perspective, the key principles Nigeria has utilized in controlling major outbreaks such as Lassa fever, Yellow fever and Ebola have involved contact tracing, local intervention by community engagement and sensitization, constant monitoring of diseases, resilience and urgency in action (7). These have however shown to be ineffective because of the many limitations hindering the development of these strategies. These limitations include shortages of human personnel, poor healthcare funding, inadequate diagnostic capacity, political instability and technological limitations, which are still much in existence and consequential today (7). Prior to the COVID-19 pandemic, there was already resource scarcity and deficit, and controlling one outbreak seems entirely difficult. The onset of COVID-19 simply created a reallocation of already limited financial and human resources and an eventual neglect in others.

Recent data show that the Ebola outbreak in three West African countries; Guinea, Sierra Leone and Liberia, led to the disruption of healthcare and an eventual 10,900 deaths due to malaria (12). Even the latest prediction of WHO explained how global number of TB deaths could increase by 0.2-0.4 million people in 2020 alone if health services continue to be disrupted, and affects diagnosis and treatment rate by about 50% (6). Two questions are therefore important in perspective; (i) does Nigeria have adequate capacity to mitigate only one health crisis? and (ii) is Nigeria capable of diversifying resources while also tackling a pandemic?

As at May 2020, while the pandemic progressed, there was a growing concern for capacity to handle other diseases as resources necessary for mitigating the crisis became depleted. In the bid to accommodate preemptive resource fallouts as well as limit contacts as a preventive strategy, clinic consultations and elective procedures were halted and patients with other medical illnesses were discharged from the wards and intensive care units. This subsequently served as barrier for accessing healthcare outside of COVID-19 and as such, a neglect for other diseases.

The framework in preventing a full-blown crisis led to increasing demand of personal protective equipment (PPE) for healthcare workers (HCWs) and the populations, which ultimately led to shortages of these materials. This is also partly because of the dearth in local production of these materials in Nigeria and the hike in price of PPEs fuelled by heightened public anxiety. The resultant effect is the over 1,000 HCWs testing positive for COVID-19 as at June 2020 (13). Many centres were subjected to rationing and sharing PPEs as a method of avoiding infection. Following this again, was a nationwide strike of resident doctors as a protest for the non-payment of the special COVID-19 hazard and inducement allowance of 50% consolidated basic salary, which the Federal Government of Nigeria had promised the frontline HCWs in fighting the pandemic (14).

While it is possible that monitoring of other diseases has been active by the major stakeholders like the Nigeria Centre for Disease Control (NCDC), the resilience, urgency and resource mobilization can be argued to have shifted to the pandemic, creating a doorway for possible re-emergence of other diseases. From an historical view, we can see how a simple re-allocation of resources led to the neglect of another and eventually caused a significant damage in it. It is therefore recommended that while plans are being made to mitigate the impending crisis, resources are also allocated in an effort to buffer the neglect.

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Review Article **Open Access**

A review of COVID-19 vaccines strategies and anti-vaxxers theories

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Abstract:

In what is a global record time of getting the COVID-19 vaccines available within 11 months, the world has equally been faced with several myths and conspiracy theories dissuading the public from accepting vaccination as an important measure in the response to the pandemic. We reviewed the leading conspiracy theories and balanced these with the scientific basis of viral transmission and replication and the broad role of vaccination in tackling this challenge. We briefly examined the design of the leading vaccines, and provided recommendations for worldwide COVID-19 distribution, acceptance and use.

Keywords: COVID-19, vaccine, anti-vaxxer, review

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Un examen des stratégies de vaccins COVID-19 et des théories anti-vaxxers

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Abstrait:

Dans ce qui est un temps record mondial pour obtenir les vaccins COVID-19 disponibles en 11 mois, le monde a également été confronté à plusieurs mythes et théories du complot dissuadant le public d'accepter la vaccination comme une mesure importante dans la réponse à la pandémie. Nous avons passé en revue les principales théories du complot et les avons équilibrées avec la base scientifique de la transmission et de la réplication virales et le rôle général de la vaccination dans la lutte contre ce défi. Nous avons brièvement examiné la conception des principaux vaccins et formulé des recommandations pour la distribution, l'acceptation et l'utilisation du COVID-19 dans le monde.

Mots clés: COVID-19, vaccin, anti-vaxxer, revue

Introduction:

'The world hates change, yet it is the only thing that has brought progress' (1). 'Progress is impossible without change, and those who cannot change their minds, cannot change anything' (2). In 1882, the introduction of electricity was rejected. Several decades after Edison had formed the Edison Electric Illuminating Company of New York, most Americans still used gas lights and candles. In year 2020, anti-vaxxers are also doubting the science of the COVID vaccine and sponsoring theories to discredit its use. The social media is brimming with the unscientific personal opinions of a few political, community, and religious leaders asking their followers to reject the COVID-19 vaccine. One of the consequences of rejection of the efforts of researchers and drug-makers in terms of public health is the slowdown of coronavirus vaccination campaigns in countries where these false claims are deeply rooted.

This indirect effect of anti-vaxxers'

influence may therefore weigh on mortality from coronavirus globally. This has apparently also downplayed the highly commendable feat accomplished in getting COVID-19 vaccines available within a record time of 11 months from when the first genome sequence of SARS-CoV-2 was made public and the emergence of the vaccines in December 2020. In this review, we briefly examined common conspiracy theories, and the design of the leading vaccines, and provided recommendations for acceptable worldwide COVID-19 vaccines distribution and use.

Common COVID-19 conspiracy/ anti-vaxxer theories

Pfizer/BioNTech and Moderna mRNA vaccines will alter human DNA

This theory is untrue. The messenger RNA (mRNA) vaccines cannot change human DNA as this is located and tucked away inside the nucleus. The nucleus is a small rounded area found close to the centre of human cells, with the cytoplasm in the periphery (Fig 1).

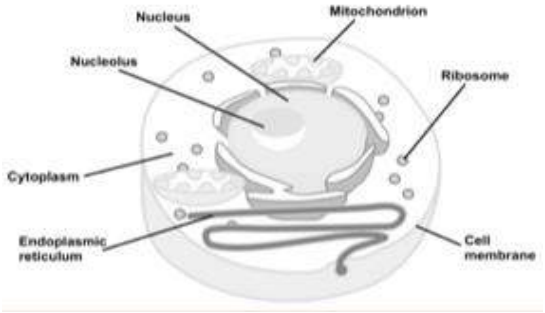

Fig 1: Human cell

To transport the vaccines into the cytoplasm, mRNA is enveloped in smart lipid-made layers called nanoparticles, where the mRNA is translated to spike proteins by a process known as ribosome processing (Fig 2).

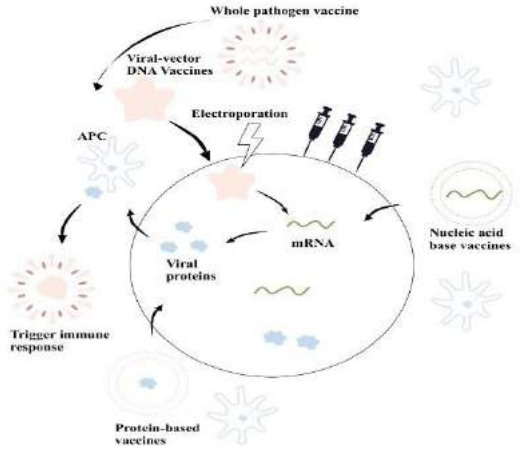

Fig 2: Virus vector DNA and mRNA vaccines' ribosome processing in cells after vaccination

This subsequently results in the SARS-COV-2 antigen presentation for the body's immune system to respond by making antibodies against the spike proteins needed to ward off future infections with COVID-19 virus after creating a T cell memory immune response. Therefore, the mRNA molecules cannot penetrate the nucleus to reach the DNA and alter it as widely acclaimed by the social media conspiracy theorists. It is also not possible for these mRNA vaccines to integrate into the host genome (3).

New vaccine's DNA would form chimers

Conspiracy theorists believe that the new vaccine causes a mixture between animal and viral DNA and the human DNA to form a chimera i. e. a single organism having cells with more than one genotype. In the real sense, the human DNA is already a natural form of chimera which is made of DNA of endogenous retroviruses and other inherent animal species. Thus, no new chimera is formed after the vaccine is given to a person.

Vaccines created to reduce Africa's population

This is particularly a major issue among people of African descent in general. In 2021, there is a higher level of ethical requirements and transparency about human subjects in clinical trials, drug safety policies, and vaccine formulation processes before they are used for disease prevention among the general public. The conspiracy theory which suggests that the newly approved COVID-19 vaccines have been created to decimate the Africa's population is not true. What is true is the fact that neither Pfizer plant which are located in Puurs, Belgium, or Michigan in the US nor the Lonza Moderna's factory plant in the Swiss Alps have been primed to make a segregated vaccine for Africa alone.

What is needed to complement the use of the current vaccines would be for researchers in Africa to gather scientific data through polymerase chain reaction (PCR) assays, genomics, and antibody tests, to tell the African's story about COVID-19. Such objectivity would lay the premise for evidence-based vaccine protocols that would attests to the required dose and timing of vaccination to achieve herd immunity peculiar to Africa.

Metals and toxins in vaccines are capable of human 5G radiation susceptibility

The ingredients in the vaccines as listed by the manufacturers show no metallic contents that could be toxins or cause human to be susceptible to 5G radiations. The only metals in vaccines are found in the buffer salts with neutral charges in salt, their quantities are too inconsequential to mount such false claim effects.

Religious theories

A number of Christians view the Bill and Melinda Gates patent 060606 as the technology carrying the microchips that will also emblem the lipid nanoparticles in the COVID-19 vaccines. It is believed that the recipients of the COVID-19 vaccine potentially have the mark of the anti-Christ that will be used as cryptocurrency for the future. From scientific point of view, the nanoparticles used in the preparation of vaccines definitely do not carry microchips.

Nanoparticles are mere carriers or vehicles to convey and protect the genetic information as they travel to the target cells (6). Several other medications use nanoparticles technology right now and their use will increase more because of their effectiveness in delivery medications to targeted sites without collateral damages to unrelated sites or organs (7-9).

Ionizable cationic lipids, phospholipids, cholesterol, and polyethylene glycol (PEG)-lipids are the main constituents of lipid nanoparticles (LNP) (Fig 3). Each component is responsible for payload protection, and enables effective intracellular delivery. Ionizable cationic lipids are fundamental drivers for nucleic acid entrapment, and determine the potency for intracellular delivery (10).

Nanoparticles may be referred to as smart-nanoparticles but they cannot be considered as microchips. In the Pfizer and Moderna vaccines, these nanoparticles are composed of lipids, most of them originally present in human cytoplasmic membranes and are serving as carriers to merely transport mRNA in the case of COVID-19 vaccines to target cells cytoplasm (11).

Box 1: Vaccine contents

Pfizer covid-19 vaccine: "Each dose has mRNA, lipids (4-hydroxybutyl) azanediyl) bis (hexane-6, 1-diyl) bis (2-hexyldecanoate), 2 [(polyethylene glycol)-2000]-N, N-ditetradecylacetamide, 1, 2-Distearoyl-sn-glycero-3-phosphocholine, and cholesterol), potassium chloride, monobasic potassium phosphate, sodium chloride, dibasic sodium phosphate dihydrate, and sucrose" (4).

Moderna COVID-19 vaccine: "Each dose has a total lipid content of 1.93 mg (SM-102, polyethylene glycol [PEG] 2000 dimyristoyl glycerol [DMG], cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphocholine [DSP C]), 0.31 mg tromethamine, 1.18 mg tromethamine hydrochloride, 0.043 mg acetic acid, 0.12 mg sodium acetate, and 43.5 mg sucrose" (5).

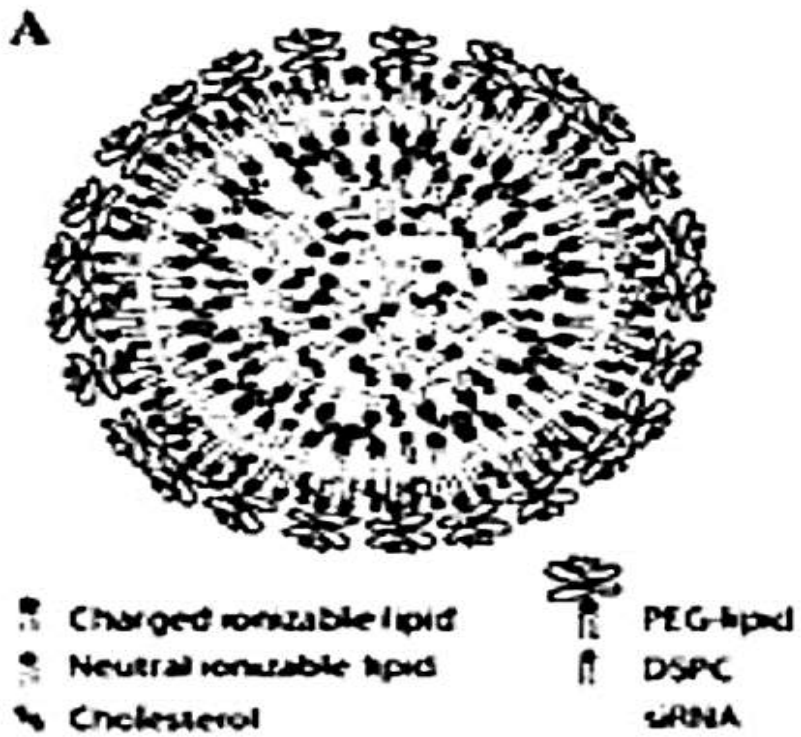

Fig 3: Structure and Internal morphology of lipid nanoparticles containing siRNA

Meanwhile, some Muslims believe vaccines could be a form of 'halal' because of their usefulness in protecting humans from serious infections and diseases (12). Furthermore, because some of these vaccines are made from components that were originally 'halal', Pfizer and Moderna vaccines could be named 'halal vaccines' if declared to be so, based on fatwa i. e. a ruling on a point of Islamic law given by a recognized authority. Malaysia is currently conducting such a ruling on the COVID-19 vaccines.

Many scriptural references are in support of medicinal approaches to preventing and curing diseases or sicknesses (13). For example, Biblical and Hebrew healings have some of their bases and roots in natural elements such as balm, earth (dirt), oil, and salt.

Luciferase technology use on COVID-19 vaccinated persons

Infrared scanners and Luciferase technology are typically used to verify if an individual has had previous measles vaccination. It is based on ability to exhibit bioluminescence. There is no evidence to support that the current COVID-19 vaccines exhibit such bioluminescence technology. No approval has been granted to use luciferase technology on COVID-19 vaccinated patients. What is being advocated is a COVID-19 vaccine passport for travel and other verification purposes.

COVID-19 vaccines

Traditionally, whole pathogen vaccine contains the virus or bacteria that cause the disease in a subdued form. Measles vaccine for example contains measles virus, and Hib vaccine contains *Haemophilus influenza* type b bacteria. There are two ways to make whole pathogen vaccines; the microbes are either deactivated (weakened) or exterminated (killed) to the point that they cannot make a person receiving the vaccine sick.

Vaccines work by stimulating the immune system to produce antibodies, exactly like it would if exposed to the disease-causing pathogen. After being vaccinated, the individual is expected to develop immunity to that disease such that should the vaccinated individual be exposed to the disease again, s/he would not suffer severe disease. The current COVID-19 vaccines in addition to this traditional approach utilised nucleic acid, viral vector, protein-based approaches to make current candidate vaccines.

Pfizer-BioNTech vaccine

The vaccines from Pfizer and Moderna both work in the same way. The two vaccines use the tiny snippets of human genetic code, called mRNA, to prompt the immune system into producing antibodies to the coronavirus. These are the first such vaccines to be authorized for use.

Moderna vaccine

Moderna vaccines like Pfizer-BioNTech are made from nucleic acids (mRNA) that are encapsulated into lipid nanoparticles prepared from mainly phospholipids and cholesterol. The synthetic phospholipids used in the process resemble the natural phospholipids found in the human cells.

Oxford/AstraZeneca vaccine

The Oxford/AstraZeneca vaccine is another coronavirus vaccine, but differs in its preparation when compared with the Pfizer and Moderna vaccines. The Oxford/AstraZeneca vaccine has its roots in the adenovirus-5, a virus taken from Chimpanzee. During the vaccine formulation, the adenovirus-5 in a virus vector capsid is genetically modified to transport genetic information in form of virus DNA to the human cells for the subsequent translation to produce spike proteins (Fig 2). Spike proteins are the distinguishing feature of the corona virus. Upon vaccination, it is expected that the human cells will start to produce the spike proteins and the spike proteins will lead to the generation of antibodies against these spike proteins to confer immunity against the coronavirus.

Efficacy of the approved vaccines

As emergency use authorization (EUA) approvals continue to roll out throughout the world, the United Kingdom, Bahrain, Mexico, Saudi Arabia were trail blazers granting early authorizations for the use of the Pfizer BioNTech COVID-19 vaccine in their countries. Moderna vaccine was first granted emergency use authorization (EUA) in the United States. The first shot of the Moderna vaccine confers 50% protection against the risk of developing severe COVID-19. The second dose, scheduled to be taken 28 days later, increases vaccine effectiveness to 94.1%. The Pfizer BioNTech COVID-19 vaccine first shot confers 52% protection against mild to severe disease, with the second dose taken after 21 days, offering up to 95% effectiveness (17).

Box 2: Development of mRNA vaccines

Messenger RNA (mRNA) was discovered and announced with fun fare as the foundation towards new vaccines and drugs development in 1961. For decades, the efforts and contributions mRNA therapeutics by Katalin Karikó, a biochemist at the University of Pennsylvania was not given its due regards (14). Adjunct Associate Professor Karikó researched extensively the technology to use mRNA as life's building block in creating a new set of therapeutic agents in form of vaccines and drugs for unmet clinical needs. Karikó was one of the first to understand the concept of using mRNA molecules copy instructions from DNA in the cell nucleus, and transport them to the cytoplasm for ribosome processing to make vital therapeutic proteins.

Nanoparticles-driven vaccine development process
In a Petri dish, artificial mRNAs are made and transported in nanoparticles to cytoplasm' ribosomes of human cells to make therapeutic proteins, corona spike proteins in the case of COVID-19 vaccines. These proteins presented as antigens to our immune system to generate specific antibodies to fight off a future exposure to the same virus should the vaccinated person become exposed again to the virus.

Emergence of PCR
In 1984, the polymerase chain reaction (PCR), was invented by the American biochemist, Kary Mullis (15). PCR was designed to amplify very small amounts of DNA, thus in vast use in genetics, genomics, crimes investigation etc. Few years down the lane, other life science researchers joined the train of creating catalytic actions of RNA polymerase enzyme to create mRNA molecules, thus generating mRNA from PCR by amplifying and multiplying DNA strands.

Fatal immune response obstacle
For years, studies on use of the artificial mRNA stopped because of the severe inflammatory reactions on the test animals. To overcome this obstacle, Karikó collaborated with Drew Weissman, a respected immunologist (now a Professor of Infectious Diseases in Penn's Perelman School of Medicine). They forged ahead when they created a form of mRNA incapable of provoking the immune system after Karikó discovered that uridine of the RNA's genetic code could trigger certain immune receptors likely causing the fatal immune response.

The success of 2005 led to today's mRNA vaccines
In 2005, Karikó and Weissman published a study replacing uridine to generate a specifically modified form of mRNA, which led to a non-fatal immune response (16). More extensive studies with the mice injected with this modified mRNA revealed a non-fatal immune response as the mice in the study survived. In 2010, Derrick Rossi co-founded a biotech company called Moderna, with the goal of this using modified mRNA to create vaccines and therapeutics on the premise of the study published by Karikó and Weissman. Using this technology, Moderna initially developed a potential Zika and Influenza vaccines. Therefore, when COVID-19 emerged, the company rode on this success story to develop their versions of COVID-19 mRNA vaccines. In 2020 these mRNA-based vaccines; Moderna's showed 94% efficacy, while Pfizer-BioNTech revealed 95% efficacy in a Phase III clinical trial.

Side effects or adverse events post-vaccination

In clinical use round the world, the common side effects reported post vaccination typically are fever, injection site redness and pain. Others are itching, diarrhoea, vomiting, lethargy, muscle aches, headache, vertigo, and dizziness. These symptoms are usually worse after second dose and during the last few days. Pfizer BioNTech, Moderna, Astra-Zeneca have put in place post-vaccination pharmacovigilance and other safety surveillance systems in their bid to monitor the potential reactions that people might develop after taking these vaccines.

In the post-approval vaccination with Pfizer BioNTech vaccine, a few health workers in the UK were reported to have developed anaphylaxis. Anaphylactic reactions to vaccinations usually occur in people with pre-existing allergies or multiple allergies to any or all of the ingredients used in preparing the vaccines. In the light of this, the recommendation is for individuals prior to receiving vaccinations to answer all allergy questions truthfully. Users are expected to check the lists of ingredients in the vaccine and do not proceed if any known allergy is stated. It is recommended that individuals with multiple allergies are cautious while considering taking the Pfizer/ BioNTech vaccination.

The COVID-19 vaccines would be reviewed in due course to correctly ascertain if it poses some risks of birth defects to expectant mothers because the trials did not include pregnant or lactating women. It is worthy to note that according to UK gov.uk website (18), the safety and efficacy of the COVID-19 mRNA vaccine BNT162b2 in children under 16 years of age have not yet been established.

Importance of T-cell tests post-vaccination

T-cells originate from stem cells in the bone marrow. As they develop, they migrate to the thymus gland, hence their name "T" cells. In the thymus, they display antigens that are eliminated down to those that recognize self. Antigens distinguish subtypes;

CD4+ T cells (helper T cell) recognize foreign antigens on macrophages, stimulate B cells to produce antibodies, secrete cytokines, and activate $CD8^+$ T cells (cytotoxic T lymphocytes or CTLs). $CD8^+$ T functions to burst virally-infected cells.

In COVID-19, T cells appear a day or two after symptoms start; they bind the virus at several sites, and persist. Tracking $CD8^+$ T cells in recovered COVID-19 patients or post vaccination of individuals using investigative longitudinal studies, can help to ascertain if memory $CD8^+$ T cell response is sustained for an extended period. This would help to ascertain whether the current 6 months dosing vaccination regimen should be sustained long term.

A cohort study (19) revealed that out of 94 patients with blood COVID-19 antibodies tested during a follow-up study reported an initial seropositivity rate of 96·2% which later declined to 58·5% after 6 months. Likewise, the median titres of the neutralising antibodies dropped from 19·0 in the acute phase to 10·0 after 180 days. This is likely to be the reason for reported incidence of reinfection in patients after 5-6 months seen in some previously infected COVID-19 patients worldwide.

Discussion

The long-term safety data on the COVID-19 vaccine is not available at the moment. Readers and users of the vaccines may rely on the great knowledge and advances that have materialized in the field of science and on the vaccine platforms. Credits go to remarkable and resilient Moderna researchers such as Kanika Karikó in collaboration with Weissman and in the UK, Professor Sarah Gilbert, the brainchild of AstraZeneca Oxford Vaccine.

From 8 days after receiving the 1st shot to ten weeks post 2nd dose vaccination, scientists would be eager to know whether initial immune response has been conferred, and studies are on ongoing on the Pfizer vaccine in Israel to answer these questions. In the coming months further down the lane, Immunologists are expected to perform complex T-cell tests to study T-cell memory vaccine-generated response to the spike proteins and how long lived the B-cell antibody production to these vaccine-induced spike proteins would be. It is expected that if individuals previously vaccinated become infected with SARS-COV-2, the effect would only be a mild or asymptomatic course of COVID-19 disease.

Scientists would also want to know whether sufficient anti–nucleocapsid IgG antibody and anti-coronavirus spike antibody titres produced in response to the vaccination are sustainable long enough to get a patient through the long pandemic haul. It is at the moment uncertain if several multiple doses would be needed at intervals to build a sustained immunity.

The news of the expedited clinical trials of Oxford/AstraZeneca vaccine which began in April 2020 and approved for clinical patient use by 30th of December 2020 is refreshing in the space of life sciences in the UK. The approval granted though quick did not sacrifice the integrity and safety of the vaccine science. Furthermore, everything about the Oxford vaccine is a plus; the cost, storage, UK-made, and 100 million doses available for the UK population. The question now is how long before the herd immunity becomes a reality all over the world.

Vaccine distribution, availability and storage issues

Beyond hoarding toilet paper in 2020 is the challenge of effective and safe storage for vaccine in 2021 worldwide. Recommended temperature for Pfizer BioNTech vaccines is -80°C, while the Moderna vaccines is -20°C. Distribution and safe storage conditions are expected to be the major obstacles in Africa, South America, and South East Asia.

Conclusions:

The world has suffered a significant amount of physical, emotional, and financial losses from dealing with the unforeseen challenges of COVID-19. At the time of writing this article, more than 84 million confirmed cases with close to 1.84 million deaths have been recorded worldwide as at 3rd of January 2020 (20). A review of echocardiograms of 1,261 patients from 69 countries by researchers at Edinburgh University revealed abnormal scans for more than half of hospitalized COVID-19 patients; 55% of the patients in the study had abnormality in the pumping function of the heart, with one in seven showing evidence of severe dysfunction (21). Another study showed that 29.4% of 47,780 COVID-19 patients who needed inpatient hospital treatment were readmitted within 140 days after the initial discharge home, with one in ten (12.3%) during the readmission being elderly (over 65 years of age), and Black, Asian and Minority Ethnic (BAME) backgrounds had the highest re-admission rates (22). These data attest to the devastating cardiovascular effects of COVID-19 among the BAME elderly and the need for an immediate use of vaccination for rescue.

With the arrival of Pfizer, AstraZeneca, Moderna and other candidate vaccines, the uncertainties and questions around the world are; how long will the pandemic last? should I get the vaccines? what will our lives look like

when the pandemic is over? could I still be infected post vaccination in the light of the fact that frontline health workers in the US and UK have been reported to develop severe COVID-19, a month after receiving vaccination? Should this happen there is no need to panic, as it is estimated to take 8 days after 1st dose to 10 weeks of 2nd dose post vaccination for the much-desired immunity the vaccine confers to reach climax. Therefore, responsible citizens should continue with stringent measures to slow down the spread of SARS-CoV-2, the virus that causes COVID-19. This means the wearing of facemasks, frequent handwashing, avoiding large gatherings or crowds and practicing social distancing. The take-home message is not rest on your oars despite the emergence of the new COVID-19 vaccines.

This review is an attempt to address the doomsday conspiracy theories offered by poorly-informed social media anti-vaxxers who may have a large listening audience in less developed countries of the world. Anti-vaccination has been around a long time, such as anti-fertility and anti-tetanus toxoid vaccines which have had severe impact on the willingness of people to take life-saving vaccines. There is therefore the need to debunk such outrageous claims and get the world in one voice to support scientists and researchers so that collectively we may retrace our footsteps and get our normal lives back.

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PreciseMed is a bespoke drug development research company based in Scotland with special interests in developing topical, oral, nebulised and parenteral formulations for unmet clinical needs mainly as delivered in organ-targeted nanoparticles to reduce systemic toxicity and optimise efficacy, as well as conduct specialised preclinical studies and clinical trials on novel therapies.

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Review Article **Open Access**

Prognostic implication of hypocalcaemia in COVID-19: a systematic review

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Abstract:

Coronavirus disease-2019 (COVID-19) has been declared as a pandemic affecting several millions of people worldwide. It has varied clinical manifestations ranging from asymptomatic to critical illness. It has led to the mortality of several affected individuals. However, the prognosis seems to vary from one person to the other and efforts are being made to identify the prognostic factors. Hypocalcaemia has been identified as a poor prognostic factor with a high frequency among individuals affected with COVID-19. This review aims to estimate the prevalence of hypocalcaemia among COVID-19 patients and identify the poor prognostic factors associated with the presence of hypocalcaemia in COVID-19 patients. Electronic medical databases were searched for publications on the prognostic implications of hypocalcaemia in COVID-19 infection, and relevant articles were selected for systematic review following PRISMA algorithm. The prevalence of hypocalcaemia among patients with COVID-19 was 40.0-74.4%. There was a significant association between the rate of hospital admission, intensive care unit (ICU) admission as well as septic shock and hypocalcaemia in patients with COVID-19. Hypocalcaemia is also associated with a higher mortality rate in these patients. COVID-19 patients with hypocalcaemia tend to have elevated C-reactive protein, interleukin-6, alanine transaminase, procalcitonin, serum creatinine and low albumin. Hypocalcaemia is common in COVID-19 patients and is a poor prognostic factor in these patients. Presence of hypocalcaemia is associated with a severe illness and even death.

Keywords: COVID-19; hypocalcaemia; prognosis; systematic review

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Implication pronostique de l'hypocalcémie dans COVID-19: une revue systématique

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Abstrait:

La maladie à coronavirus-2019 (COVID-19) a été déclarée pandémie affectant plusieurs millions de personnes dans le monde. Il a des manifestations cliniques variées allant de la maladie asymptomatique à la maladie grave. Cela a

conduit à la mortalité de plusieurs personnes touchées. Cependant, le pronostic semble varier d'une personne à l'autre et des efforts sont faits pour identifier les facteurs pronostiques. L'hypocalcémie a été identifiée comme un facteur de mauvais pronostic avec une fréquence élevée chez les personnes atteintes de COVID-19. Cette revue vise à estimer la prévalence de l'hypocalcémie chez les patients COVID-19 et à identifier les facteurs de mauvais pronostic associés à la présence d'une hypocalcémie chez les patients COVID-19. Les bases de données médicales électroniques ont été recherchées pour des publications sur les implications pronostiques de l'hypocalcémie dans l'infection à COVID-19, et les articles pertinents ont été sélectionnés pour une revue systématique suivant l'algorithme PRISMA. La prévalence de l'hypocalcémie chez les patients atteints de COVID-19 était de 40,0 à 74,4%. Il y avait une association significative entre le taux d'hospitalisation, l'admission en unité de soins intensifs (USI) ainsi que le choc septique et l'hypocalcémie chez les patients atteints de COVID-19. L'hypocalcémie est également associée à un taux de mortalité plus élevé chez ces patients. Les patients atteints de COVID-19 souffrant d'hypocalcémie ont tendance à avoir une protéine C-réactive élevée, l'interleukine-6, l'alanine transaminase, la procalcitonine, la créatinine sérique et un faible taux d'albumine. L'hypocalcémie est fréquente chez les patients atteints de COVID-19 et constitue un facteur de mauvais pronostic chez ces patients. La présence d'une hypocalcémie est associée à une maladie grave et même à la mort.

Mots clés: COVID-19; hypocalcémie; pronostic; Revue systématique

Introduction:

Coronavirus disease-2019 (COVID-19) is an acute viral infection of public health importance caused by the severe acute respiratory syndrome coronavirus-2 (SARS CoV-2) (1). The SARS CoV-2 emerged from Wuhan, the largest and capital city of Hubei Province in Central China (1). The first outbreak was reported as an unexplained pneumonia among persons connected to a seafood market in Wuhan (2). It later spread all over China and across the globe. Bats are believed to be the natural reservoir of the virus but some researchers tend to dispute this (3). Transmission is commonly from person to person via respiratory droplets which are released during coughing, sneezing, talking, laughing and singing. However, transmission through contaminated fomites and aerosol, in specific situations, have been documented (4). As at the 20th of November 2020, over 56 million individuals have been affected worldwide (5). The distribution of number of infected cases across continents, as at 20th November 2020, is shown in Fig 1.

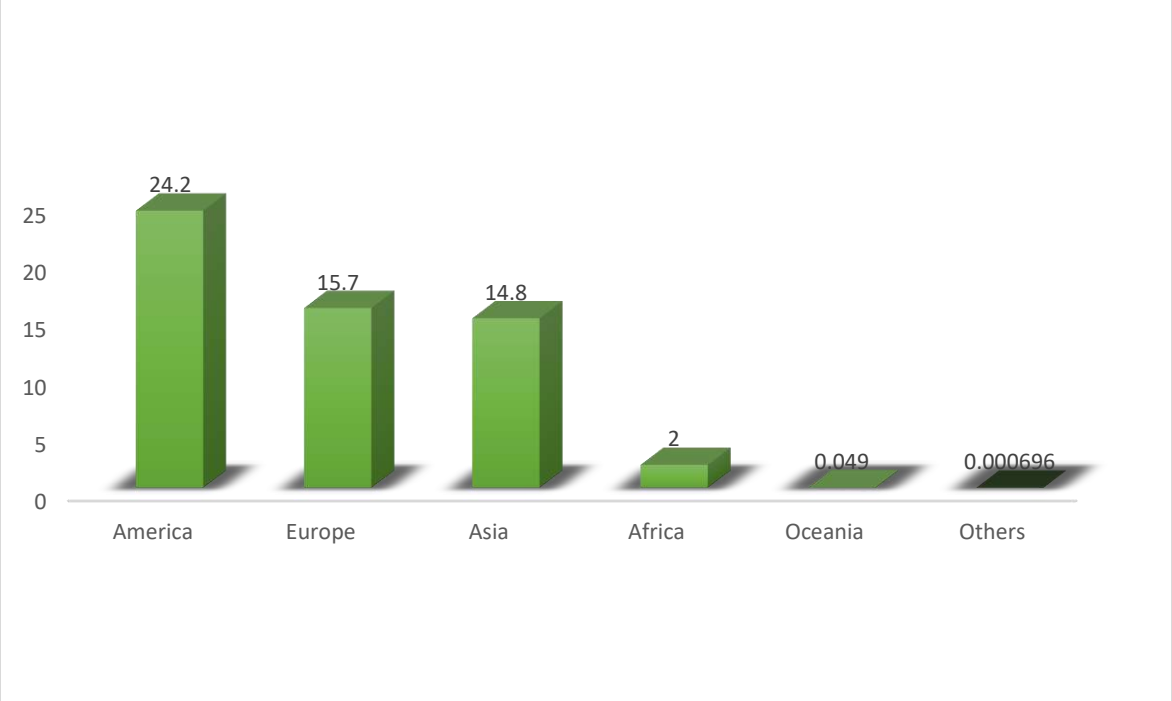

Fig 1: Distribution of COVID-19 cases across the continents of the world

The median incubation period of COVID-19 infection is about 5 days and by the 12th day, almost 100% of symptomatic infected individual would have started manifesting symptoms hence the adoption of 14 days to quarantine individuals who have been apparently exposed (6). A significant portion of the infected individuals are asymptomatic. Table 1 shows the percentages of asymptomatic cases reported from various studies, which range from 30.8-97.5%.

Table 1: Proportion of asymptomatic COVID-19 cases

Studies	Asymptomatic cases (%)
Nishiura et al., (7)	30.8
Lavezzo et al., (8)	41.0
Moriarty et al., (9)	46.5
Arons et al., (10)	52.2
Jung (11)	62.0
Ing et al., (12)	81.3
Baggett et al., (13)	87.8
Sutton et al., (14)	87.9
Lytras et al., (15)	97.5

In symptomatic cases, the most prominent symptoms are fever, cough and breathlessness (2). Others include fatigue, myalgia, headache, sore throat, abdominal pain and diarrhoea (16). Loss of taste, loss of smell, joint pain and chest pain have also been described (16,17). Essentially, differentiating COVID-19 from other causes of acute respiratory infection may be very difficult (18). Respiratory failure, sepsis, septic shock and multi-organ dysfunctions are some of the reported acute complications in those with progressive illness (19).

In diagnosing COVID-19, samples such as nasopharyngeal swab, oropharyngeal swab, tracheal aspirate and bronchoalveolar lavage are collected for reverse transcriptase-polymerase chain reaction (RT-PCR) assay, which is the diagnostic procedure of choice for detection of SARS-COV-2 (19). However, naso and oropharyngeal swabs are the commonest specimens (19). Documented abnormalities in other laboratory test parameters are leukopenia or leukocytosis, thrombocytopenia, deranged electrolytes, urea creatinine as well as elevated D-dimer, C-reactive protein (CRP), lactate dehydrogenase (LDH) and ferritin (20,21).

The commonest cause of death in COVID-19 patients is respiratory failure secondary to acute respiratory distress syndrome (22). The mortality pattern across the conti nents is shown in Fig 2 (5). The global case fatality rate, at the time of writing this manuscript, was 2.4% and the case fatality rates of different countries, ranging from 1.5-9.7%, are shown in Table 2 (23).

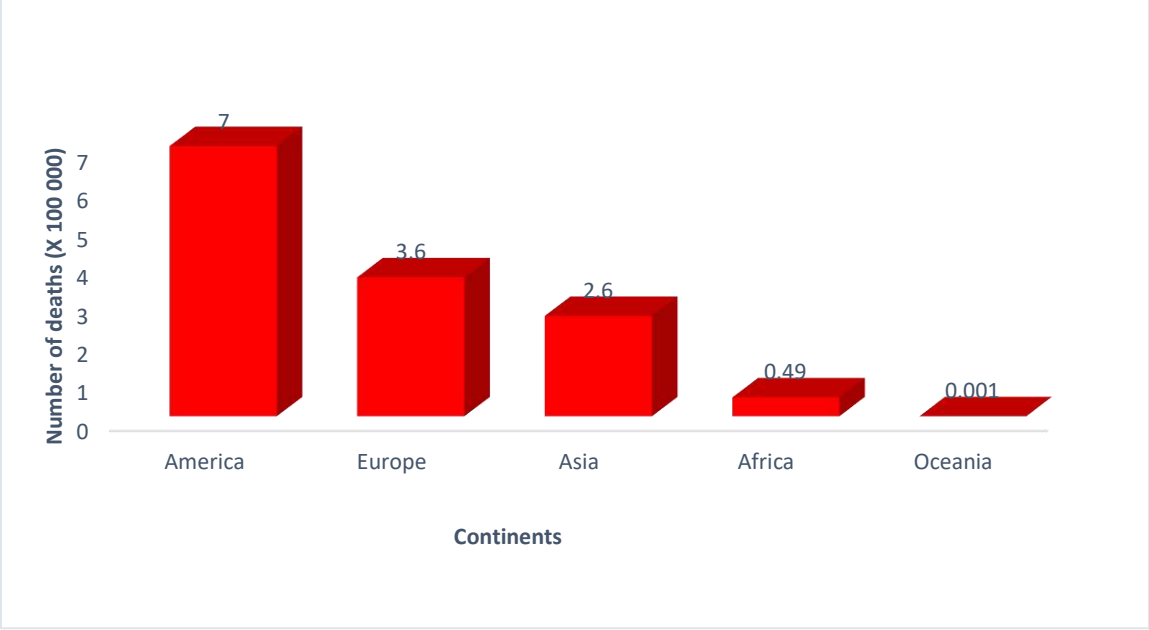

Fig 2: Distribution of COVID-19 deaths across continents

Table 2: Case fatality rates of COVID-19 in the 20 most affected countries in the world

No	Countries	Number of cases (Millions)	Case fatality rate (%)
1	USA	12.8	2.1
2	India	9.2	1.5
3	Brazil	6.1	2.8
4	France	2.1	2.3
5	Russia	2.1	1.7
6	Spain	1.6	2.7
7	UK	1.5	3.6
8	Italy	1.4	3.5
9	Argentina	1.4	2.7
10	Colombia	1.3	2.8
11	Mexico	1.0	9.7
12	Germany	0.95	1.5
13	Peru	0.95	3.7
14	Poland	0.91	1.6
15	Iran	0.88	5.2
16	South Africa	0.77	2.7
17	Ukraine	0.65	1.7
18	Belgium	0.60	2.8
19	Chile	0.54	2.8
20	Iraq	0.54	2.2

Several markers of poor prognosis in COVID-19 have been published. Old age and the male gender have been found to be associated with poor prognosis in COVID-19 patients (24). Smoking and co-morbidities such as hypertension, diabetes, chronic obstructive pulmonary disease and malignancy have been reported as prognostic factors (24). Examination findings of poor prognostic implication include tachypnoea, tachycardia, hypotension and reduced arterial saturation of oxygen using the pulse oximeter (24). Haematological parameters associated with poor prognosis are lymphopaenia, leukocytosis, neutrophilia and thrombocytopenia (24). Biochemical parameters that have been reported in COVID-19 patients with more severe illness include raised C-reactive protein, elevated D-dimer, lactate dehydrogenase, procalcitonin and raised cytokines such as interleukin-6 (24). Radiologically, consolidative or infiltrative changes as well as pleural effusion on chest imaging have also been reported to correlate with poor prognosis (24).

Calcium is required for the fusion of coronavirus to the human cells before they can gain entry into the cells (25). Generally, hypocalcaemia is not an uncommon finding in critically ill patients (26). Some of the possible explanations for this include vitamin D deficiency, reduced dietary intake and hypomagnesemia (27). Vitamin D deficiency has been documented to be highly prevalent in patients with COVID-19 (28). Studies have also shown that COVID-19 patients with hypocalcaemia also tend to have other poor prognostic factors such as lymphopenia, elevated D-dimer, raised C-reactive protein and increased alanine transaminase (ALT) (26). Patients with hypocalcaemia were found to have higher incidence of acute respiratory distress syndrome (ARDS) (26). Liu et al., (29) also found that hypocalcaemia is also associated with poor outcome in patients with COVID-19.

Some authors have reported that unsaturated fatty acids released during COVID-19 infection is responsible for the hypocalcaemia seen in patients with the illness, and the process is independent of the vitamin D status of patients (30). In support of this is the finding of Thomas et al., (42) who reported a high level of unsaturated fatty acids in patients with severe COVID-19 infection. This is a systematic review of studies reporting the prevalence and prognostic implications of hypocalcaemia in COVID-19 patients.

Methodology:

Electronic online accessible medical data bases were searched for studies on the prognostic implications of hypocalcaemia in COVID-19 infections. The online databases searched were Google Scholar, Public Library of Medicine (PubMed), African Journals Online (AJOL), Scopus and Web of Science. The terms searched were 'hypocalcaemia', 'prognostic factors', and 'COVID-19 infection'. Boolean operators such as 'AND' as well as 'OR' were used during the data search so as to improve the quantity and specificity of the articles retrieved. Grey literature was also searched. The Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) flow diagram of the literature search and selection is shown in Fig 3.

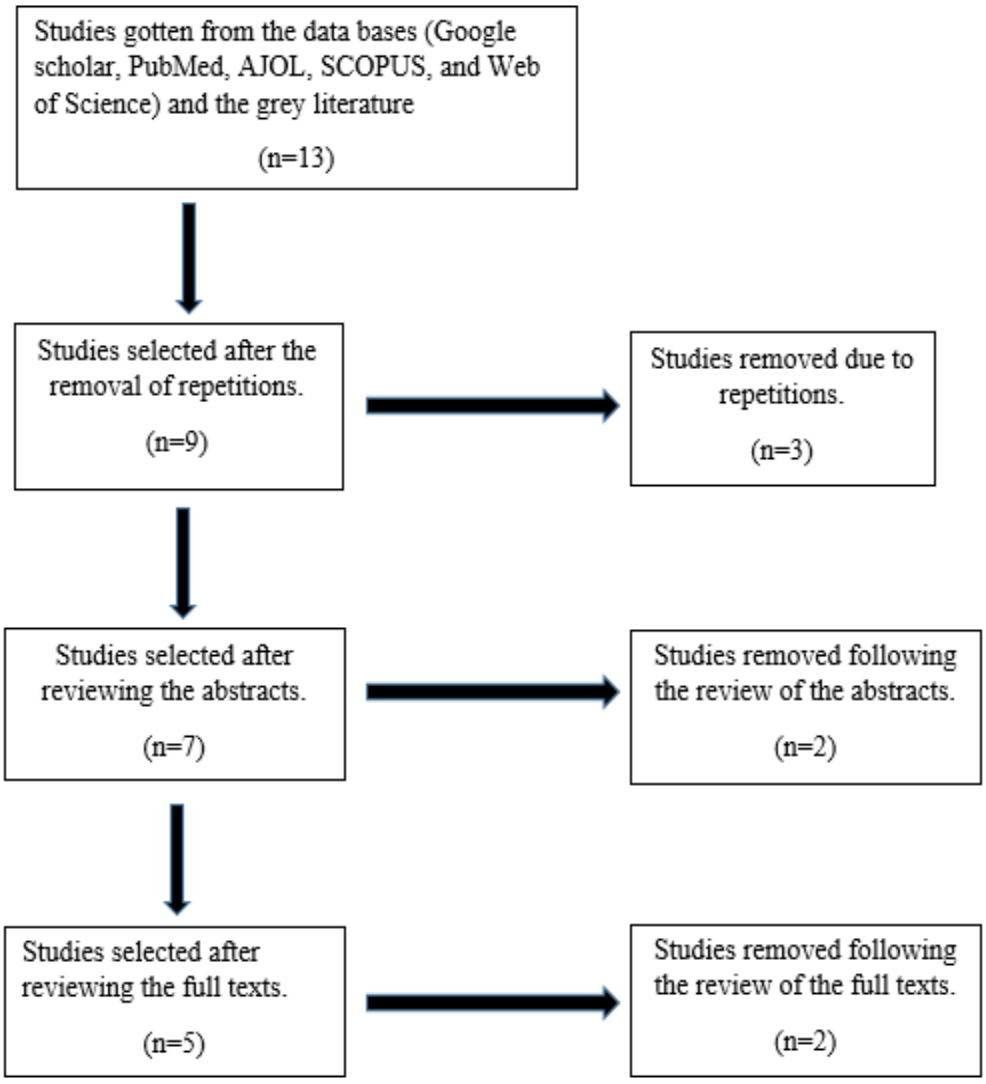

Fig 3: PRISMA flow diagram of the literature search and selection

The inclusion criteria were; studies done between 1st January, 2020 and 10th December, 2020 to determine the association between hypocalcaemia and poor prognostic factors in patients infected with COVID-19 done, and studies which abstracts and or full text were available at the searched databases or from the grey literature. The exclusion criteria included studies on COVID-19 not focused on the prognostic implications of hypocalcaemia, and studies which abstracts or main texts were not available for review. The databases were searched independently by the authors and the included studies were deemed appropriate by at least three of the five authors. Relevant data were extracted and presented in texts, tables and charts.

Results:

Table 3 shows the sample sizes in the selected studies, with a total sample size of 775 in the systematic review. The prevalence rates of hypocalcaemia among patients with COVID-19 patients in the various studies selected for

Table 3: Sample sizes of the selected studies

Study	Sample size
Sun et al., (26)	241
Filipo et al., (31)	20
Liu et al., (29)	107
Torres et al., (32)	316
Raesi et al., (33)	91
Total	775

this systematic review are shown in Table 4, which shows that the prevalence of hypocalcaemia among patients with COVID-19 was 40.0 -74.4%.

Table 4: Prevalence of hypocalcaemia among patients with COVID-19

Study	Prevalence rate (%)
Sun et al., (26)	74.4
Filipo et al., (31)	40.0
Liu et al., (29)	62.6
Torres et al., (32)	63.0
Raesi et al., (33)	59.3

Table 5 below shows the various poor prognostic factors associated with the presence of hypocalcaemia in COVID-19 patients across the selected studies. It shows the association between rate of hospital admission, intensive care unit (ICU) admission as well as septic shock with hypocalcaemia in patients with COVID-19. Hypocalcaemia is also associated with a higher mortality rate in patients with COVID-19 (26, 31,33).

Table 6 below shows laboratory parameters that are often deranged in hypocalcaemic COVID-19 patients (26,29,31-33). Commonly measured laboratory parameters such as the C-reactive protein (CRP), D-dimer, lactate dehydrogenase (LDH), albumin and others are statistically significantly associated with the presence of hypocalcaemia in patients with COVID-19 (26,29,31-33).

Among the selected studies, only the study by Sun et al., (26) measured the parathyroid hormone and vitamin D levels in a cohort of patients with COVID-19 even though all the selected patients did not have hypocalcaemia. Serum calcium was found to positively correlate with parathyroid hormone and negatively with vitamin D.

Table 5: Poor prognostic factors associated with hypocalcaemia among patients with COVID19

Prognostic factor	Sun et al., (26)	Filipo et al., (31)	Liu et al., (29)	Torres et al., (32)	Raesi et al., (33)
Septic shock	X				X
MODS	X				
ICU admission	X		X		X
ARDS	X				
Liver injury	X				
AKI	X				
Need for hospitalization	X	X	X		
Need for oxygen support	X	X	X	X	X
ICU admission		X	X	X	X

X=Presence; MODS=Multiple Organ Dysfunction Syndrome; ARDS=Acute Respiratory Distress Syndrome; AKI=Acute Kidney Injury; ICU=Intensive Care Unit

Table 6: Laboratory parameters often affected in hypocalcaemic COVID-19 patients

Laboratory parameters often elevated	Laboratory parameters often elevated
Erythrocyte sedimentation rate (ESR)	Lymphocyte
C-reactive protein (CRP)	Platelet
Lactate dehydrogenase (LDH)	Haemoglobin concentration
Alanine transaminase (ALT)	Albumin
Aspartate transaminase (AST)	Arterial partial pressure of oxygen (PaO_2)
D-dimer	
Procalcitonin	
Interleukin-6	
Total bilirubin	
Blood urea nitrogen (BUN)	
Creatinine	

Discussion:

In this systematic review, the prevalence rate of hypocalcaemia among patients with COVID-19 was high (40-74.4%). High prevalence rate of hypocalcaemia was also documented for severe acute respiratory syndrome (SARS) caused by the coronavirus, SARS-COV (34). Hypocalcaemia has also been found to be highly prevalent and is associated with poor prognosis in other viral infections such as measles and the viral haemorrhagic fever (35,36). Generally, hypocalcaemia has been extensively reported to be common among very ill patients and the causes are said to be multifactorial (37,38). The reported explanations for this observation include dysregulated secretion of parathyroid hormone, transient vitamin D deficiency, effects of catecholamines, multiple transfusion of citrated blood as well as the effects of certain drugs (38,39).

In a review by Mikhail et al., (40), the frequency of hypocalcaemia among patients with COVID-19 was reported to be 9.5-78%. In the general population, common causes of hypocalcaemia include chronic kidney disease, vitamin D deficiency, hypomagnesaemia, drugs and hypoparathyroidism (41). So far, the exact cause (s) of hypocalcaemia in COVID-19 patients is/ are not known (40). However, some plausible hypotheses have been proposed. One of the hypotheses was put forward by Singh et al., (30), who stated that COVID-19 is associated with the release of a large amount of free fatty acids into the circulation. It is believed that these free fatty acids bind to the circulating plasma calcium thereby rendering the patient hypocalcaemic. Thomas et al., (30) also corroborated this assertion by observing high levels of free fatty acids among patients with severe COVID-19 (42), a process said to be unrelated to the vitamin D status of the patient (30). Although vitamin D levels of hypocalcaemic patients in the selected studies for this systematic review were not determined, some other studies have documented the presence of vitamin D deficiency in patients with COVID-19 and this may account for the hypocalcaemia seen in these patients (28,43).

Hypocalcaemia has been extensively linked with the severity of COVID-19 infection (26,31-33). This systematic review demonstrates that hypocalcaemia is associated with septic shock in patients with COVID-19. The association between septic shock and hypocalcaemia is explained by certain observations such as, alteration in plasma pH which affects calcium ion binding to albumin, alteration of parathyroid hormone production by inflammatory mediators and deranged concentrations of plasma calcium binders namely, citrate, fatty acids and phosphate (44).

Acute respiratory distress syndrome (ARDS) is a common pathway leading to death in patients with severe COVID-19 and this systematic review clearly shows a significant association between hypocalcaemia and development of ARDS among COVID-19 patients (26). Even among patients without COVID-19, Thongprayoon et al., (45) observed that several case reports have documented a relationship between hypocalcaemia and ARDS. Some of the documented explanations for this observation include respiratory muscle weakness, laryngeal and bronchospasm and possibly tetany (45). Hypocalcaemia enhances contraction and tetany of airway smooth muscles by lowering the threshold for action potential (46). Also, hypocalcaemia has been reportedly linked with a high respiratory infection rate, higher inflammatory markers in the lungs and resultant progression of respiratory diseases (47).

This systematic review found an association between hypocalcaemia and acute kidney injury (AKI) as a marker of poor prognosis in patients with COVID-19. Hypocalcaemia was documented to be a poor prognostic index in patients with AKI secondary to COVID 19 infection (26). Hypocalcaemia has also been reported to be associated with the development of AKI in other critically ill patients without COVID-19 (48). Vitamin D deficiency has been documented in AKI and this has been put forward as one of the mechanisms by which AKI is associated with hypocalcaemia (49). Hyperphosphatemia, which is a common finding in AKI, is also a reported mechanism of hypocalcaemia in AKI (48). Furthermore, parathyroid hormone resistance in the bones has been documented in patients with AKI and this has been suggested as a possible mechanism of hypocalcaemia in AKI.

In this review, an association was found between hypocalcaemia and the risk of ICU admission. Hypocalcaemia has been extensively documented to affect different regulatory systems in the body (50). Hypocalcaemia is not an uncommon finding in critically ill patients (51). Hypocalcaemia has been reported to have a prognostic implication in critically ill individuals and is associated with increased mortality rate (52). This study also found a relationship with markers of inflammation such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), interleukin 6(IL-6), albumin and markers of specific organ dysfunction such as creatinine and alanine transaminase (ALT). The link between hypocalcaemia and inflammation has been

attributed to vitamin D deficiency which is a common finding in inflammatory and infectious diseases as vitamin D has some anti-inflammatory properties (28,53,54).

This review has two limitations; (i) the number of studies that met the eligibility criteria was rather scanty, and (ii) most of the studies did not assay for vitamin D and parathyroid hormone levels which are important cofounders.

Conclusion:

In conclusion however, hypocalcaemia is common among patients infected with COVID-19 and is associated with the progression of the illness. The risk of mortality is higher in COVID-19 patients with hypocalcaemia. The presence of deranged levels of some biochemical parameters such as CRP, IL-6, serum creatinine and ALT is associated with low serum calcium levels. Therefore, hypocalcaemia is not only prevalent in COVID-19 patients but it is a marker of poor prognosis. In view of this, it is recommended to routinely measure serum calcium in patients with COVID-19.

Conflict of interest:

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Review Article **Open Access**

Recent advances in the pathophysiology and management of sepsis: a review

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Abstract:

Sepsis is a syndrome consisting of physiological, pathological and biochemical anomalies caused by infectious agents. It causes clinical organ dysfunction, which is identified by an acute increase in the Sequential (sepsis-related) Organ Failure Assessment (SOFA) score of two or more points. SOFA score is a score of three components that can be easily used at the bedside to track the clinical status of a patient while on admission, and these are altered respiratory rate of ≥ 22 breaths/minute, altered mental status, and systolic blood pressure of ≤ 100 mmHg. A patient with SOFA score of ≥ 2 has an attributable 2 - 25-fold increased risk of mortality compared to a patient with SOFA score of < 2. This present review provides information on the new definition of sepsis and septic shock, aetiology, pathophysiology, biochemical, pathological and haematological changes, morbidity and mortality parameters, management, and prognostic factors in patients with sepsis.

Key words: Sepsis, septic shock, SOFA score, pathophysiology, management bundles

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Progrès récents dans la physiopathologie et la gestion de la septicémie: une revue

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Abstrait:

La septicémie est un syndrome constitué d'anomalies physiologiques, pathologiques et biochimiques causées par des agents infectieux. Il provoque un dysfonctionnement des organes cliniques, qui est identifié par une augmentation aiguë du score d'évaluation séquentielle (liée à la septicémie) de la défaillance d'organe (SOFA) d'au moins deux points. Le score SOFA est un score de trois composants qui peuvent être facilement utilisés au chevet du patient pour suivre l'état clinique d'un patient lors de son admission, et il s'agit d'une fréquence respiratoire altérée ≥ 22 respirations / minute, d'un état mental altéré et d'une pression artérielle systolique de ≤ 100 mmHg. Un patient avec un score SOFA ≥ 2 a un risque de mortalité attribuable 2 à 25 fois plus élevé qu'un patient avec un score SOFA de < 2. Cette revue fournit des informations sur la nouvelle définition de la septicémie et du choc septique, l'étiologie, la physiopathologie, changements biochimiques, pathologiques et hématologiques, paramètres de morbidité et de mortalité, prise en charge et facteurs pronostiques chez les patients atteints de septicémie.

Mots clés: septicémie, choc septique, score SOFA, physiopathologie, faisceaux de prise en charge

Introduction:

Sepsis is a major public health concern at individual, health system and societal levels both in the developed and developing nations of the world (1). It is characterized by syndrome of physiological, pathological and biochemical anomalies caused by infectious agents (2). The incidence of sepsis over the years is on the increase. About 49 million cases of sepsis and 11 million sepsis-related deaths were reported globally in the year 2017 (3), which constitute about 20% of all deaths globally.

On annual basis, estimated 3 million and 1.2 million cases of sepsis are reported globally among newborns and children respectively. About 30% of neonatal sepsis-related deaths are due to multidrug resistant organisms (4). The total cost of managing sepsis in the US hospitals in 2011 amounted to 20 billion US dollars, constituting 5% of US total hospital bill (3,5).

This present review provides information on the new definition of sepsis and septic shock; aetiology, biochemical, pathological and haematological changes in patients with sepsis, morbidity and mortality parameters, and management and prognostic factors of sepsis.

Methodology:

Two electronic databases (PubMed and Google Scholar) were examined for list of publications written in English Language relating to pathophysiology and management of sepsis. Additional publications were obtained from textbooks and other hardcopy journals. After removing duplicate and non-relevant publications, a total of 87 publications were included for the review. Fig 1 depicts the process by which publications used for the review were selected.

Pathophysiology of sepsis:

Sepsis is a condition of dysregulated host response that was *ab-initio* initiated to exterminate the invading pathogens into the body (6). The host response is mediated by the innate and adaptive immune systems. The various cell types including monocytes, macro phages, neutrophils, dendritic and epithelial cells involved in innate immune system possess pattern-recognition receptors (PRRs). The PRRs are essential for recognizing invading bacteria and subsequent initiation of immune response. They recognize both pathogen-associated molecular patterns (PAMPs) which are conserved motif expressed by pathogens and damage-associated molecular patterns (DAMPs) also called alarmins, which are host cell molecules released during inflammatory stress (7).

Following the invasion by the pathogen and its interaction with the host innate immune cells, there is a coordinated host response characterized by both proinflammatory and anti-inflammatory/immunosuppressive reactions that are directed to eliminate the pathogens and restore normal homeostasis. When the pathogen

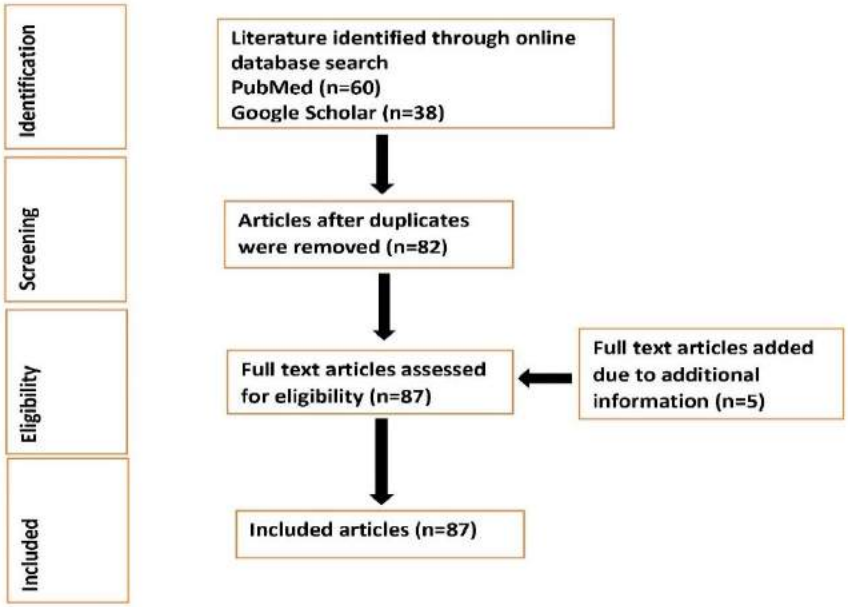

Fig 1: Process for selection of articles for the review

succeeds in circumventing the protective host immunity, there is continuous stimulation of host cells resulting in excessive and injurious immune response, and inability to restore homeostasis. This is exactly what transpired in a host with sepsis (4).

Hyperinflammation

Initiation of inflammation

The first line of defense against invading pathogens include the anatomical barriers such as the skin and mucosal lining of the respiratory, gastrointestinal and urogenital tracts. This is followed by interaction with the innate immune system that comprise of complement system, sentinel phagocytic cells, and natural killer cells, which serve to activate the adaptive immune system. The innate immune system broadly recognizes various antigens by sensing the carbohydrate and fatty acid molecules present on the surface of most pathogens, collectively known as pathogen associated molecular patterns (PAMPs) or host cell molecules expressed during inflammatory stress called damage associated molecular patterns (DAMPs). PAMPs or DAMPs bind to pattern-recognition receptors (PRRs) on the cells of these innate immune system resulting in signaling cascade that leads to sustained immune activation and dysfunction from hyperproduction of cytokines. The PRRs are grouped into various families such as Toll-like receptor (TLRs), C-lectin receptors (CLRs), retinoic acid-inducible gene-like (RAG) receptors, and nucleotide-binding oligomerization domain-like (NOD) receptors (4). The most widely studied of the PRRs is the TLR.

In pathogenesis of sepsis, implicated proinflammatory cytokines include IL-1β, IL-12, IL-18, and TNF. Also involved are high mobility group box 1 (HMGB1), a nuclear protein actively secreted following inflammatory stimuli, and S100A8/9 (calprotectin), a heterodimer protein expressed in neutrophil. Both HMGB1 and S100A8/9 levels are elevated in sepsis. HMGB1 can act as cytokine or chemotactic factor via TLR4 signaling while S100A8/9 stimulates systemic inflammation also via TLR signaling. If these processes are not curtailed promptly, and local response spread systemically, the TLR-signaling activation of the immune system will result in harmful hyperinflammatory response initiated by proinflammatory cytokines which is called the "cytokine-chemokine storm". This is believed to be responsible for all the various consequences seen in early phase of sepsis, which has therefore been the target of many clinical trials with the use of antibodies or inhibitors of cytokines such as TNF-α and β, IL-1β and other anti-inflammatory strategy (4,8). The primary aim of the innate immune system is complete extermination of the pathogen through this primary inflammatory reaction which is then followed by resolution of the immunological process by the adaptive immune system.

Activation of complement system

Complement system is the second most important contributor to the inflammatory reaction that occurs in the pathogenesis of sepsis. Complement system is made up of small proteins that are primarily synthesized by the liver and are triggered by the presence of PAMPs and DAMPs in the host body via interaction with complement components C1q, mannose-binding lectin (MBL), and ficolins. Complement system can be activated through classical, alternate or lectin pathways. However, various intracellular (elastase, neutral proteases) and extracellular (thrombin, activated clotting factors) proteases are known to also generate complement proteins such as C3a and C5a, which are small protein fragments called anaphylatoxins that exert strong proinflammatory effect on leukocytes, endothelial cells and platelets. C5b protein mediates formation of membrane attack complex (MAC) that results in lysis of bacteria. In addition, complement proteins once activated multiply the effects of local immune reactions by putting yet more cytokines into play (9).

Activation of coagulation system and vascular endothelium

Activation of the coagulation system during host-pathogen interaction elicits immune defense mechanisms such as induction of immune response via protease-activated receptors (PARs) activation, release of antimicrobial peptides, and recruitment and activation of phagocytic cells (10). In recent time, the term "immunothrombosis" has been hypothesized to demonstrate the independence of the innate immune and coagulation systems (11). The high-level activation of coagulation seen in sepsis occurs through the tissue factor-extrinsic pathway, resulting in net procoagulant state with high risk of microvascular thrombosis. Disseminated intravascular coagulopathy (DIC) characterized by microvascular thrombosis and haemorrhage is one of the most important complications of sepsis. Vascular injury in sepsis exposes tissue factors to coagulation factors in blood, leading to clotting. Endothelial cells and macrophages are triggered to express large amount of tissue factors by both PAMPs and proinflammatory cytokines. Coagulation can also be activated in sepsis via the intrinsic

pathway and stimulate inflammation through the kallikrein-kinin system. This is enhanced by the reduced activity of the main anticoagulant pathways such as the tissue factor pathway inhibitor (TFPI), antithrombin, and the protein C system (4).

Interactions of the complement and coagulation systems

There is an increasing evidence from clinical and experimental studies which showed that coagulation proteases can activate the complement system and vice vasa. In sepsis, the functions of coagulation and complement systems are closely intertwined. Some components of coagulation and fibrinolytic system such as factors IXa, Xa, XIa and thrombin, and the central fibrinolytic protease plasmin can convert C3 and C5 proteins into C3a and C5a respectively. Conversely, C5a and membrane attack complex can stimulate exposure of tissue factors in endothelial cells due to the structural alteration caused by the complement products (4,12).

Vascular endothelial and multiorgan dysfunction

Following entry of pathogens into the host body, platelets and leukocytes migrate to the sites of proliferating microbes through the vascular endothelium. There is a significant nexus between sepsis and disruption of the integrity of endothelial barrier. This results in exposure of the underlying collagen fibres and tissue factors to blood cells in circulation, triggering activation of platelets and the complement system. Thrombin, cathepsin G, matrix metalloproteinase (MMP), trypsin and plasmin are known activators of protease-activated receptors (PARs). Activated PARs cause dysfunction of endothelial lining via derangement of cytoskeletal system, contraction and rounding of endothelial cells resulting in disruption of cell-to-cell contact and vascular hyperpermeability. There is usually high level of circulating MMP in sepsis (13).

The primary cause of death in septic shock are either refractory hypotension or multi-organ failure. The vascular hyperpermeability results in severe hypotension refractory to intravenous fluid resuscitation and vasopressor therapy. In addition, there is inability to sustain high cardiac output. These are the major causes of refractory hypotension (14). Local and systemic inflammatory responses cause microvascular injury that result in organ failure. The number of failed organ systems determine the outcome of sepsis. About 70% mortality seen in sepsis is due to multiorgan (≥3 organ systems) failure, and about 18% of patients develop respiratory failure. Renal failure resulting from renal hypoperfusion, intra-renal shunting, and use of nephrotoxic agents (such as antibiotics, and radiologic imaging dye) is seen in about 15% of septic patients (14).

Platelets activation

Recent evidence showed that platelets, which are small circulating anucleate cells, play a vital role in inflammation and immunity. Activated platelets secrete various molecules including thromboxane A2 and adenosine diphosphate which have positive feedback effects on platelets activation (15). In sepsis, activated platelets trigger formation of platelet-leukocyte complex, platelet-platelet aggregation as well as release of granular cellular components such as chemokines, adhesive proteins, coagulation factors, mitogenic factors and regulators of angiogenesis. Sepsis therefore results in platelets consumption (thrombocytopenia) with high risk of mortality. Also, there is decline in the levels of ADAMTS 13 (disintegrin and metalloproteinase with thrombospondin type1 motif 13) which is a cleaver of large von Willebrand factor. This results in enhanced platelet-endothelium (injured) complex, promoting the development of vaso-occlusive thrombi in the microvasculature that contribute to most organ failure seen in sepsis (15). Platelet activation in sepsis occurs through many mechanisms; thrombin, a coagulation activation product, is a great activator of platelets through the PARs. In the same vein, platelets can also be activated by von Willebrand factor via binding platelet glycoprotein Ibα (4).

Anti-inflammatory induction and host immunosuppression

Induction of anti-inflammatory reaction in sepsis is meant to counteract/limit excessive and harmful inflammatory process as well as stimulate tissue repair. However, immunosuppression results from persistence of anti-inflammatory reactions seen among septic patients who required intensive care, a condition referred to as "persistent inflammatory, immunosuppression and catabolism syndrome" (6,16). Secondary infections with less virulence organisms such as *Acinetobacter* spp., *Enterococcus* spp., *Stenotrophomonas*, *Candida* spp., cytomegalovirus and herpes simplex virus reactivation can occur as a sequel of persistent immunosuppression (17).

Suppression of innate immune cell functions

Neutrophil extracellular traps (NETs), a product of neutrophil made up of DNA, histones, and neutrophil-derived protease, play an important role in protective immunity through captu-

ring and killing of microbes. In sepsis however, there is defect in neutrophil chemotaxis and recruitment to the sites of infection, and depressed capacity of neutrophils to produce essential effector molecules such as reactive oxygen species (ROS) and cytokines. Moreover, there is impaired expression of HLA-DR and reduced ability to release pro-inflammatory cytokines by antigen presenting cells (APCs) such as monocytes, macrophages, and dendritic cells. This condition is referred to as "immuno-paralysis" but their ability to express anti-inflammatory mediators such as IL-10, is however retained (4).

Suppression of adaptive immune cell functions

Significant decline in $CD4^+$ and $CD8^+$ T-lymphocytes, B lymphocytes and dendritic cells due to programmed cell death (apoptosis) is a common finding in sepsis (17,18). In many studies with experimental models, administration of inhibitors of lymphocyte apoptosis results in profound improvement in treatment outcome. The interaction of programme cell death 1 (PD-1), which are immune checkpoint (IC) molecules on the surface of T-cells, with its corresponding ligands (PD-L1) on the surface of antigen presenting cells (APCs) results in high degree of immunosuppression of the adaptive immune system. The functions of monocytes and neutrophils are also inhibited by regulatory T cells (Tregs) (17).

Biochemical, hormonal and organelle dysfunctions

Some Gram-positive bacteria are capable of producing and releasing exotoxins, which are often products of plasmids and episomes, that act as superantigens (19). The cell wall of Gram-negative bacteria are endotoxins, the glycoprotein components of which act as toxins, that cause pyrexia, vasodilatation, sepsis, and endotoxic shock. Bacterial toxins are known to play a pivotal role in pathophysiology of sepsis (20).

Oxidation of plasma components

Oxidation of the components of the blood is one of causes that provide distant tissues injury and dysregulation in sepsis. Oxidation of plasma components is caused by oxygen release to plasma, and it destroys humoral regulation of different cells, tissues and organs (21). The stimulation of surface receptors of erythrocytes by bacteria causes the oxygen release. The higher the concentration of oxygen released from erythrocytes to the arterial blood, the more severe the sepsis. There are many complications which arise as a result of the release of a large amount of oxygen. Erythrocytes are unable to transport oxygen. This results into general multi-organ hypoxia. Secondly, the released oxygen is highly reactive, and tends to destroy and transform plasma proteins, peptides, immune complexes, hormones, amino acids, fatty acids, vitamins and many other substances necessary for cell nutrition, proliferation, protection, and energy production and functioning (21).

Reduction of enzymatic activities and intravascular coagulation

Oxidative changes to proteins can lead to diverse functional consequences such as inhibition of enzymatic and binding activities, increased susceptibility to aggregation (intravascular coagulation) and proteolysis, increased or decreased uptake by cells, and altered immunogenicity (22). The most important aspect of this oxidation is inactivation of regulatory substances, in particular, hormones including the pituitary gland hormones (23).

Impairment of growth hormonal function

The growth hormone and insulin-like growth factor-1 (IGF-1) axis plays a pivotal role in critical illnesses. Derangement in the functions of these hormones leads to profound changes in metabolism such as protein wasting, with loss of skeletal muscle, delay in wound healing, and impairment in recovery of organ systems (23,24).

Insulin resistance

Inactivation of other proteins such as insulin impairs the ability of cells to uptake glucose, amino acids, and other essential substances. Formation of dityrosine decreases and abolishes insulin biological activity (25). Insulin is necessary for glucose to enter many cell types therefore inactivation of insulin causes hyperglycemia, which is one of the metabolic derangements that influence sepsis outcome (26,27).

Adrenal and thyroid hormonal insufficiency

The oxidation of blood components by reactive oxygen species (ROS) released from erythrocytes may affect the hypothalamic-pituitary-adrenal axis (28) resulting in primary and secondary adrenal insufficiency in patients with sepsis, and is associated with a poor outcome (29,30,31). The oxidation of the blood components can also affect the hypothalamo-pituitary-thyroidal axis, leading to inactivation of thyrotropin, the thyroid gland hormones (triiodothyronine TT3 and thyroxine TT4), and their binding proteins. The thyroid hormones

regulate metabolism and they have impacts on sepsis prognosis. The level of TT4 is lower in patients with septic shock than in patients without septic shock (32,33).

Generalized vasodilatation and shock

Vasopressin (an antidiuretic hormone) is also oxidized by the reactive oxygen species released from erythrocytes. Vasopressin plays a role in circulatory homeostasis and serum osmolality. Oxytocin and vasopressin are oxidized, with the formation of dityrosine (34). Vasopressin oxidation and depletion cause vasodilatory shock, a situation associated with high mortality (35). Low expression of angiotensin II and angiotensin converting enzyme (ACE) are valuable in predicting the mortality of patients with severe sepsis (36). Systemic vasodilatation and arterial hypotension are landmarks of septic shock.

Development of oedema

Oxidation of albumin causes hypoalbuminemia in sepsis (37,38). Albumin is the main determinant of plasma oncotic pressure. It plays an important role in modulating the distribution of fluids between compartments, transport of endogenous and exogenous compounds, modulation of capillary permeability, neutrophil adhesion and activation, haemostasis and free radical scavenging (39). Hypoalbuminaemia is a poor prognostic factor in sepsis (40).

Immunoglobinopathies

Reactive oxygen species released from erythrocytes also destroys other proteins, including immune complexes and immunoglobulins, particularly IgG and IgM. The initial steps of oxidation may change the specificity and avidity of immunoglobulins due to chemical alteration of the hypervariable region. Oxidation of IgG significantly changes the immunoreactivity and specificity of IgG fractions (41). Oxidized immunoglobulins have autoimmune and proinflammatory activity (42,43). Low levels of immunoglobulins are frequent in severe sepsis and septic shock (44). However, intravenous immunoglobulins (IVIG) as adjunctive therapy have not shown benefits in treatment of sepsis (45).

Anaemia

When oxygen is released from erythrocytes into the plasma in arterial blood, they cause failure of oxygen delivery to cells resulting in tissue hypoxia, which can lead to anaemia (46). Many factors contribute to the development of anaemia in sepsis and these include blood sampling, decreased erythrocyte synthesis, bone-marrow suppression, reduced production of erythropoietin, and increased erythrocyte uptake (47). The main factor of anaemia in sepsis is increased destruction of erythrocytes caused by; (i) erythrocyte membrane injury caused by bacteria on the surface of erythrocytes; (ii) erythrocyte membrane pores with haemoglobin pouring out as a result of bacterial penetration into the inner space of erythrocyte; and (iii) increased destruction of injured erythrocytes and bacteria containing erythrocytes in plasma and reticulo-endothelial system (RES) particularly spleen (46,48,49).

Mitochondrial dysfunction

Tissue-related hypoxic injury results from hypoxaemia, hypoperfusion and cytokine-mediated mitochondrial dysfunctions, termed cytopathic hypoxia (50,51,52). The lack of oxygen transforms cell metabolism from aerobic to anaerobic. As a result, the Krebs cycle is suppressed and with anaerobic metabolism, lactic acid accumulation occurs. Lack of oxygen delivery to the tissues results in decreased cellular metabolism and increase in cellular lactate production (42). Elevated lactic acid is a marker of suboptimal supply of oxygen to the tissues. High levels of lactic acid are associated with increased mortality in sepsis (53), and its non-clearance in sepsis is a significant independent predictor of death (53,54).

Clinical manifestations of sepsis

The clinical features of sepsis and septic shock are non-specific, and include fever or hypothermia, tachypnoea, tachycardia, and organ dysfunctions which manifest as altered mental status, decrease capillary refill, and cyanosis (2,55). These presentations are largely dependent on the presence or absence of co-morbidities.

Haematological manifestations in sepsis

Frequent haematological findings seen in patient with sepsis include anaemia, leukocytosis, low platelets, as well as haemostatic system activation (56). Patients with sepsis often develop anaemia. Factors responsible for anaemia in sepsis includes inhibition of erythropoiesis secondary to repressive consequence of inflammatory cytokines, impaired iron homeostasis, reduced life span of the red blood cells and haemolysis. Other causes of anaemia in patients with sepsis include nutritional deficiencies due to reduced intake, bleeding secondary to disseminated intravascular coagulopathy and frequent phlebotomy (57). The haematologic manifestations are varied and include anaemia, leukopaenia, leukocytosis and DIC.

Anaemia

The effect of pro-inflammatory cytokines on erythropoietin production has been reported. Such cytokines include TNF-α, IL-1 and IL-6. Apoptosis of the colony forming unit erythroid and the burst forming erythroid by interferon gamma (IF-γ) and IL-1 contributes significantly to anaemia seen in patients with sepsis. Bleeding in this category of patients secondary to DIC and gastrointestinal bleeding from stress ulcers (57) could cause anaemia.

Iron metabolism is impaired in patient with sepsis. The altered iron metabolism is secondary to increased levels of hepcidin (58), which is produced mainly in the liver, and its level is elevated secondary to increased expression of IL-6 in patients with sepsis (20). There is also inhibition of iron release from the RES, inhibition of iron absorption from the intestinal mucosa as well as inhibition of iron incorporation into maturing erythrocyte, with increased levels of hepcidin, leading to iron deficit erythropoiesis (59). Sepsis reduces erythrocyte lifespan by causing alteration in erythrocyte metabolism, and by reducing 2,3-bisphosphoglycerate, it can cause significant increase in production of deformed red cells. The cumulative effects of these alterations affect the membrane of the red blood cells, and reduce its survival (60).

Neutrophilia

The major haematologic findings in patients with sepsis are leukocytosis and neutrophilia. These are usually accompanied with peripheral blood film features of toxic granulation of neutrophils and vacuolations. Left shift are often seen, in which maturing myeloid cells, including myelocytes, metamyelocytes and band forms, appear in the peripheral blood. Sometimes few promyelocytes and blasts are observed (61). High leucocyte count (up to $50x10^9$/L) referred to as leukaemoid reaction, may be seen in some bacteria sepsis and may need to be differentiated from chronic myeloid leukaemia (CML). One of the criteria for diagnosis of sepsis includes left shift, with immature myeloid cells greater than 10. Neutropaenia has also been noted in some patients with sepsis, usually in the elderly and young children (62).

Lymphopaenia

Lymphopaenia has been documented in patients with sepsis, usually due to apoptosis of the lymphocytes and lymphocyte redistribution from the compartment of blood to tissues of lymphoid origin (62).

Thrombocytopaenia

Thrombocytopaenia (low platelet count) occurs commonly in patients with sepsis. The cause and contributing factors to thrombocytopaenia in patients with sepsis include reduced platelets production, consumption of platelets in DIC, platelet destruction via immune mediated mechanism, and haemodilution (63). Aggregation of platelets as well as its attachment to the endothelium and leukocytes may contribute to thrombocytopaenia that is observed in patients with sepsis. Immunoglobulin of the IgG class has been associated with immune mediated thrombocytopaenia and reported in up to 30%-40% of the patients with sepsis (64).

Reported findings of coagulopathy in patients with sepsis are common. The spectrum may range from subtle initiation of coagulation cascade to DIC. Patients with sepsis may present with venous forms of thromboembolism or DIC. Contributory factors to coagulopathy in patients with sepsis include increased tissue factor expression, endothelial cell dysfunction with inhibition of the natural anticoagulant process, and inhibition of the fibrinolytic pathways (65,66).

Disseminated Intravascular Coagulation (DIC)

The maintenance of steady blood flow is enhanced by balance between the procoagulant and anticoagulant systems. Coagulopathy in patients with sepsis is associated with increased levels of procoagulant proteins and reduced levels of naturally occurring anticoagulants. There is increased tissue factor expression in patients with sepsis, which is responsible for initiation of the coagulation cascade (67). The natural anticoagulant system is impaired in sepsis, with reduced levels of anti-thrombin III and protein C. Thrombin production is increased with subsequent generation of fibrin, and with consumption of the coagulation factors, DIC may occur (68).

Biochemical changes in sepsis

Inflammatory cytokines (IL-6, IL-8 and TNF-α) are elevated in patients with sepsis and septic shock, so are the levels of nitrous oxide and lactic acid (69). Only elevated levels of IL-6 and IL-18 are associated with increased mortality in patients with sepsis and septic shock, while elevated levels of TNF-α, lactate and nitrous oxide (though observed in patients with sepsis) are not associated with increased mortality (58). Creatinine levels are only elevated when there is an associated liver damage.

Biomarkers, which are genes, molecules or other host factors help in the identification of health and disease processes. Biomarkers of importance in patients with sepsis can be grouped into diagnostic and prognostic markers. Diagnostic biomarkers provide sensitive and specific information that can be used to differentiate sepsis from all other forms of non-infectious critical illnesses. This enhances antibiotic stewardship program as it helps reduce unnecessary use of antibiotics where they are not required (55). However, prognostic biomarkers are used to monitor patients with sepsis on treatment and also in predicting prognosis including complications.

Monocyte anergy which presents with decreased expression of monocyte HLA-DR, is has been reported to be an independent predictor of poor prognosis in patient with sepsis. Recently, the term theranostics was introduced and entails biomarker tests that help in the selection and monitoring of treatment response, a step towards personalized medicine. According to the Surviving Sepsis Campaign (SSC) guidelines, procalcitonin is the only biomarker with potential usefulness in the choice of antibiotics in critically ill patients. Other biomarkers with lower sensitivity and specificity include C-reactive protein (CRP), IL–6, LPS binding proteins (LBPs) and lactate (5,55).

Complications of sepsis

The main complication of sepsis is septic shock. However, there are other sets of possible complications, which include kidney failure, multiple organ failure, and heart dysfunction. Dialysis may be needed temporarily or permanently if the kidneys fail, and death can occur from multiple organ systems dysfunctions and failure. Stroke due to low blood pressure on the brain and cognitive decline have been reported in some patients with septic shock (70).

Management of sepsis

The major principles of management of sepsis include; timely identification of sepsis features, titrated fluid resuscitation, adequate source control, obtaining blood culture samples prior to commencement of antimicrobials (this is the gold standard for diagnosis of sepsis), and organ support (77).

Clinical assessment and management

In the management of sepsis, clinical assessment is carried out initially to detect the risk factors for infection. These include age, chronic disease, immunosuppressive therapy, AIDS, pre-existing co-morbidities such as diabetes mellitus (DM), renal failure, and bleeding disorders. The aim of physical examination is to identify the possible foci of infection. The vital signs are used to evaluate clinical improvement during hospital admission (77,78).

Serious cellular, circulatory, and metabolic abnormalities occur in patients with septic shock. Clinically, patients with septic shock require a vasopressor to maintain mean arterial blood pressure of ≥ 65 mmHg and serum lactate level >2 mmol/L (>18 mg/dl) in the absence of hypovolemia. A patient with suspected infection can be rapidly identified as being more likely to have poorer outcomes typical of sepsis if they have at least 2 of the following clinical criteria which together constitute a new bedside clinical score termed quick SOFA (qSOFA); respiratory rate ≥22/min; altered consciousness (Glasgow coma score < 13) and systolic blood pressure (SBP) ≤ 100 mm/Hg (78).

The particular site of infection in the body should be identified as soon as possible, at least within 6 hours of presentation. This is referred to as source control (79), and helps define early goals of management e. g. presence of invasive devices suspected to be source of infection can be promptly removed. Distinction should be made between sepsis and septic shock in order to guide management of patient. During the first 6 hours of presentation, initial resuscitation involves achieving the following goals; central venous pressure of 8-12 mmHg, mean arterial pressure of 65 mmHg, urine output of 0.5 ml/kg/Hr, and central venous/ mixed venous oxygen saturation of 70%/65% respectively.

The fluid of choice for initial resuscitation of patients with sepsis/septic shock is crystalloids with the aim of achieving a minimum of 30 ml/kg of crystalloids (4). Blood culture samples and urine culture or sputum samples should, as much as possible, be obtained before administration of antibiotics. However, treatment should not be delayed if this is not possible. Intravenous antibiotics should be administered within the first hour of recognition of sepsis/septic shock (3). A broad-spectrum antibiotic that can penetrate the tissues in adequate concentration should be used. If the cause of sepsis/septic shock is suspected to be of viral origin, appropriate antiviral therapy (if available for the virus) should be initiated (70).

Laboratory assessment

Blood culture

This is the "gold standard" for diagnosis

of sepsis. A minimum of two sets of blood cultures (aerobic and anerobic) should be collected as much as possible before initiating antimicrobial therapy. Urine, pus, ascitic fluid, pleural fluid, cerebrospinal fluid, and other body fluids may be collected as well.

Ancillary laboratory studies

Other laboratory studies essential for diagnosis and monitoring of progress of patients with sepsis (58,80) include; complete blood count (total and differentials), electrolytes, urea and creatinine (E, U, Cr), prothrombin time, liver function tests, arterial blood gas analysis, urinalysis, chest X-ray (to identify source of pulmonary infection and respiratory distress), computerized tomography (CT) scan, positron emission tomography (PET-CT) and magnetic resonance imaging (MRI) techniques to identify source of primary infections

Estimation of biomarkers of sepsis

In recent years, biomarkers that allow early diagnosis of sepsis have been sought for in order to allow early diagnosis of sepsis, since culture-based diagnosis is slow. These biomarkers include C-reactive protein, lactate and procalcitonin. C-reactive protein (CRP) is an acute phase protein produced by the liver and alveolar macrophages. Its level increases after trauma and inflammation. Bacterial infections are powerful stimuli which produce rapid rise in CRP levels in few hours. Its production is stimulated by IL-1, IL-6 and TNF-α. Changes in plasma levels of CRP are useful in diagnosis/ prognosis of infection, a fall in plasma levels indicates infection resolution and its assay is less expensive (71).

Procalcitonin (PCT) has been popularly referred to as the most useful marker of severe systemic inflammation (72). It is normally found in blood at very low level but its production can be stimulated by inflammatory cytokines and bacterial endotoxins, causing its release in higher concentration in response to infection and to systemic infections in patients with viral disease (73). It provides an indicator of risk of sepsis, the higher the level of PCT, the greater the likelihood of systemic infection and sepsis. It is regarded as the most sensitive biomarker to help diagnose bacterial sepsis. This can help allow earlier diagnosis of sepsis and better monitoring of its progression.

Lactate is another biomarker of sepsis. An increase in its level implies poor tissue perfusion and progression to organ dysfunction and are related to or associated with increases mortality rate from 35% to 70%. The use of lactate as a marker for diagnosis, prognosis and treatment of tissue hypoxia in septic shock has been established to be important. Therefore, a patient with sepsis and significant lactic acidosis should receive early antibiotics, haemodynamic monitoring, and adequate resuscitation (73). Absence of lactate clearance in the blood predicts death of sepsis patient. A high level of lactate may be a manifestation of organ dysfunction because its clearance is dependent on the liver and kidney functions. Lactate has been reported to be very useful as a prognostic indicator of state of septic shock (74,75,76).

Venous-arterial pressure difference (dPCO2) is another important biomarker of septic shock. The measurement of dPCO2 is a good prognostic indicator in septic shock, as it provides an index of tissue oxygenation (76).

Sepsis treatment care bundles

The mortality rate in septic shock is high. Over the years, due to the high incidence and mortality rate of sepsis and septic shock, the Surviving Sepsis Campaign (SSC) was set up by a group of international critical care and infectious disease experts. The SSC third revised guidelines, published in 2017, aims to improve the outcome of sepsis and septic shock (55).

In the revised guideline, the previous 6-hour and 3-hour bundles have been compressed into a one-hour bundle with 5 steps initiated at time zero of patient presentation. The one-hour Surviving Sepsis Campaign Bundle of Care (81,82) includes; (i) measure lactate level with repeat measurement if baseline lactate is > 2 mmol/L; (ii) obtain blood culture samples prior to initiation of antibiotics; (iii) use of broad-spectrum antibiotics; (iv) commence rapid administration of crystalloid at 30 ml/kg for hypotension or lactate level of ≥ 4 mmol/L; and (v) administer vasopressors if patient remains hypotensive during or after resuscitation to ensure mean arterial pressure (MAP) at ≥65mmHg. The "time-zero" or "time of presentation" is defined as the time of triage at the emergency department or if presenting from another care facility, from the earliest chart annotation consistent with all elements of sepsis or septic shock ascertained through chart review (82).

Discussion:

About 49 million cases of sepsis, and 11 million sepsis-related deaths were reported globally in the year 2017 (3). These sepsis-related deaths constitute about 20% of all deaths globally. Sepsis is often seen in patients with background of previous medical conditions,

and are caused by opportunistic organisms from patient's own microbiota (55,83). The SOFA score, consisting of altered respiratory rate of 22 breaths/minute or more, altered mental status, and systolic blood pressure of 100mmHg or less, is designed to measure the severity and prognosis from sepsis and septic shock (55).

Sepsis is a life-threatening organ dysfunction caused by dysregulated host response to infection. Clinically, organ dysfunction is identified by an acute increase in the SOFA score of two or more points. A patient with SOFA score of ≥2 has an attributable 2 - 25-fold increased risk of mortality compared to a patient with < 2 SOFA score (61). The most commonly isolated Gram-positive bacteria are *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae,* and *Enterococcus* spp., while the most commonly implicated Gram-negative bacteria causes are *Escherichia coli, Klebsiella* spp*., Pseudomonas* spp*.,* and *Acinetobacter* spp. Among patients with background co-morbidity or hospital-acquired sepsis, the isolated pathogens are mostly multidrug resistant staphylococci*, Pseudomonas, Acinetobacter* and *Candida* spp (84). Other organisms such as *Burkholderia pseudomallei* and *Salmonella enterica* have been reported (85,86).

The pathophysiology of sepsis is a complex event involving hyperinflammatory syndrome that results in 'cytokine-chemokine storm', anti-inflammatory response and host immunosuppression that can lead to secondary infection, and a variety of host biochemical, hormonal and organelle dysfunctions (4,8,16, 17,18,19,20). The clinical symptoms and signs of sepsis and septic shock are tachypnoea, fever or hypothermia, tachycardia, and organ dysfunctions manifested as altered mental status, decrease capillary refill, and cyanosis (2,4). Frequent haematological findings seen in patient with sepsis include anaemia, leukocytosis, thrombocytopaenia and activation of haemostatic system (56). Anaemia in sepsis is mainly due to increased levels of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, apoptosis of the colony forming unit erythroid and burst forming erythroid by IF-γ IL-1, and impairment of iron metabolism secondary to increased levels of hepcidin (57,58).

Leukocytosis and neutrophilia are also major findings in patients with sepsis, along with peripheral blood film features of toxic granulation of neutrophils and vacuolations. Left shift with maturing myeloid cells, myelocytes, metamyelocytes and band forms, appear in the peripheral blood. Sometimes few promyelocytes and blasts are visible (61). High leucocyte count (up to $50x10^9$/L) referred to as 'leukaemoid reaction', may be seen in some bacteria sepsis and should to be differentiated from chronic myeloid leukaemia (63).

Thrombocytopaenia that is observed in patients with sepsis is due to the aggregation of platelets, as well as its attachment to the endothelium and leukocytes. Immunoglobulin of the IgG class has also been associated with immune mediated thrombocytopaenia, and was recorded in up to 30%-40% of the patients with sepsis (64). Inflammatory cytokines (IL-6, IL-8, IL-10 and TNF-α) are elevated in patients with sepsis and septic shock, so are levels of nitrous oxide and lactic acid (69). Elevated IL-6, IL-8 and IL-18 levels are associated with increased mortality in patients with sepsis and septic shock but elevated levels of TNF-α, lactate and nitrous oxide (though observed in patients with sepsis) are not associated with increased mortality, and creatinine levels are only elevated when there is an associated liver damage (58). The main complications of sepsis are septic shock, multiple organ failures, DIC and cardiac dysfunction. Death occur when multiple organ systems become dysfunctional and shutdown. Stroke due to low blood pressure on the brain and cognitive decline can also complicate septic shock (72).

Clinical assessment is based on the risk factors for infection which include age, underlying chronic disease, immunosuppressive therapy, HIV/AIDS, pre-existing co-morbidities such as diabetes mellitus (DM), renal failure, and bleeding disorders. There is an urgent need to identify the possible foci of infection, and vital signs are measured and used to evaluate clinical improvement during admission (77,78). A patient with suspected infection can be rapidly identified as being more likely to have poorer outcomes from sepsis if they have at least 2 SOFA score (qSOFA); respiratory rate ≥22/min, altered consciousness with GCS score of < 13, and systolic blood pressure (SBP) of 100mm/Hg (78,79,81).

Treatment of sepsis is based on intravenous fluid administration and correction of acidosis, identification of primary site and agent of infection, administration of antimicrobial agents based on the local susceptibility pattern of the causative agents, and implementing the 1-hour 'Sepsis-3 Bundles' or 'The SSC 2018 Update' as described by the Surviving Sepsis Campaign, depending on the clinical assessment of the patient (80,81,82,88).

Conclusion:

Sepsis is a medical emergency resulting from infection by bacteria, fungi and viruses

including severe acute respiratory syndrome coronavirus-2 (SARS-COV-2), the aetiology of the ongoing COVID-19 pandemic. The SOFA score is used for the initial assessment and monitoring of such patients, and management is based on identification of primary site and agent of infection, intravenous fluid administration and correction of acidosis, administration of anti-microbial agents determined by the local susceptibility pattern of the causative agents, and implementation of the 1-hour 'Sepsis-3 Bundles' or 'The SSC 2018 Update' described by the Surviving Sepsis Campaign, depending on the clinical assessment of the patient.

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Original Article **Open Access**

Quality of metagenomic DNA extracted for molecular identification of microorganisms from CSF samples of patients with suspected cerebrospinal meningitis in northern Nigeria

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Abstract:

Background: Following an increase in the practice of starting antimicrobial therapy prior to clinical sample collection, the ability to confirm pathogenic microorganisms of bacterial meningitis has decreased by approximately 30%. Culture results may be false negative when fastidious or culture-resistant bacteria are involved or when patient samples are obtained after antimicrobial therapy has started. Molecular diagnosis using PCR can be performed directly on clinical samples after metagenomic DNA (mDNA) extraction not requiring live organisms for a positive result. The specific objectives of this study are to perform mDNA extraction directly from cerebrospinal fluids (CSF) using appropriate spin column method, and to determine the quality of the mDNA elute.
Methodology: Cerebrospinal fluid specimens were collected from 210 patients with suspected acute cerebrospinal meningitis (CSM) in the Federal Capital Territory and some States in Northern Nigeria during the 2017 and 2018 outbreak seasons. Metagenomic DNA was extracted from approximately 200µL of CSF specimens using the Qiagen QIAamp$^{(R)}$ DNA Mini kit specific for bacterial agents only. DNA quality check was performed on all DNA elutes using fluorometric, spectrophotometric and agarose gel electrophoresis methods.
Results: Of the 210 CSF samples analyzed microbiologically, Gram reaction was positive in 94 cases (44.8 %) but only 17 (8.1 %) were culture positive for two of the three major bacterial causes of meningitis. One hundred and eighty (85.7%) samples had DNA concentrations ≥ 0.005 ng/µL, 55 (30.6 %) of these had DNA purity (A_{260}/A_{280}) of ≥ 1.7, 103 (57.2%) had purity value between 1.0 - 1.69, 14 (7.8%) had value of 0.57 - 0.99, and 8 (4.4%) failed purity evaluation with value of 0.00 at A_{260}/A_{280}.
Conclusion: The essence of mDNA extraction is multipurpose. A multiplex PCR can be performed on the extracted mDNA to interrogate the presence of microbial pathogens of interest using specific primers and probes (when applicable). Quality mDNA from CSF samples will ensure successful qPCR results for rapid and accurate detection of bacterial pathogens in meningitis. This will eliminate the challenges associated with traditional culture methods.

Keywords: Meningitis, CSF, DNA Quality Check, Fluorometry.

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Qualité de l'ADN métagénomique extrait pour l'identification moléculaire des microorganismes à partir d'échantillons de LCR de patients suspectés de méningite cérébrospinale dans le nord du Nigéria

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Abstrait:

Contexte: Suite à une augmentation de la pratique de commencer un traitement antimicrobien avant le prélèvement d'échantillons cliniques, la capacité à confirmer les microorganismes pathogènes de la méningite bactérienne a diminué d'environ 30%. Les résultats de la culture peuvent être faux négatifs lorsque des bactéries exigeantes ou résistantes à la culture sont impliquées ou lorsque des échantillons de patients sont prélevés après le début du traitement antimicrobien. Le diagnostic moléculaire par PCR peut être réalisé directement sur des échantillons cliniques après extraction d'ADN métagénomique (ADNm) ne nécessitant pas d'organismes vivants pour un résultat positif. Les objectifs spécifiques de cette étude sont d'effectuer l'extraction de l'ADNm directement à partir des fluides céphalo-rachidiens (LCR) en utilisant la méthode de colonne de rotation appropriée, et de déterminer la qualité de l'élution d'ADNm.
Méthodologie: Des échantillons de liquide céphalo-rachidien ont été collectés auprès de 210 patients suspectés de méningite cérébrospinale aiguë (MSC) dans le Territoire de la capitale fédérale et dans certains États du nord du Nigéria au cours des saisons d'épidémie 2017 et 2018. L'ADN métagénomique a été extrait d'environ 200µL d'échantillons de LCR en utilisant le kit Qiagen QIAamp$^{(R)}$ DNA Mini spécifique pour les agents bactériens uniquement. Un contrôle de la qualité de l'ADN a été effectué sur tous les élues d'ADN en utilisant des méthodes d'électrophorèse sur gel fluorométrique, spectrophotométrique et d'agarose.
Résultats: Sur les 210 échantillons de LCR analysés microbiologiquement, la réaction de Gram était positive dans 94 cas (44,8%), mais seulement 17 (8,1%) étaient positives en culture pour deux des trois principales causes bactériennes de la méningite. Cent quatre-vingt (85,7%) échantillons avaient des concentrations d'ADN ≥ 0,005 ng/µL, 55 (30,6%) d'entre eux avaient une pureté d'ADN (A_{260}/A_{280}) ≥ 1,7, 103 (57,2%) avaient une valeur de pureté comprise entre 1,0 et 1,69, 14 (7,8%) avaient une valeur de 0,57 à 0,99, et 8 (4,4%) ont échoué l'évaluation de la pureté avec une valeur de 0,00 à A_{260}/A_{280}.
Conclusion: L'essence de l'extraction d'ADNm est polyvalente. Une PCR multiplex peut être effectuée sur l'ADNm extrait pour interroger la présence d'agents pathogènes microbiens d'intérêt en utilisant des amorces et des sondes spécifiques (le cas échéant). Un ADNm de qualité provenant d'échantillons de LCR assurera des résultats de qPCR réussis pour une détection rapide et précise des bactéries pathogènes dans la méningite. Cela éliminera les défis associés aux méthodes de culture traditionnelles.

Mots clés: méningite, LCR, contrôle de la qualité de l'ADN, fluorométrie

Introduction:

The continuous yearly outbreak of acute meningitis over the years no doubt had left behind very sad memories, moments in the minds and life of individuals (sufferers), family members, friends, communities and nations alike especially in the Meningitis Belt of Africa. Meningitis has been reported as one of the deadliest diseases that has been plaguing West Africa for decades. The sub-Saharan Africa was been plagued by large epidemics of meningococcal meningitis for a century, leading to it being labelled the 'meningitis belt' (1), spanning 26 countries (Fig 1).

Epidemics usually occur in the dry season which commences from December to June, with an epidemic wave that can last two to three years but dies out during the intervening raining seasons (2). In 1998, the World Health Organization attributed several factors to be associated with the development of epidemics in the Africa's meningitis belt. These factors include medical conditions (immunological susceptibility of the population), demographic conditions (travel and large population displacements), socioeconomic conditions (overcrowding, poor hygiene and living conditions), climatic conditions (drought and dust storms), and concurrent acute respiratory infections (3).

In Nigeria, about 5000 cases of meningitis occur every year with loss of many lives (4). The Nigeria Centre for Disease Control (NCDC) and Federal Ministry of Health (FMoH) Weekly Epidemiological Report from 2012 to 2018 revealed that this problem persists. Suspected cerebrospinal meningitis (CSM) cases within these seven years were 21,353; of which only

Fig 1: African Meningitis Belt

Source: African meningitis Belt/CDC. http://wwwn.cdc.gov/travel/yellowbook/2010/chapter-2/meningococcal-disease.aspx

643 cases (3.01 %) were laboratory confirmed. The number of deaths (case fatality ratio, CFR) was 1,347 (6.31 %) (5).

Bacterial meningitis remains a serious global health problem as well as a life-threatening condition that requires prompt recognition and treatment. It is also documented that, over 1.2 million cases of bacterial meningitis are estimated to occur worldwide each year (3). Without treatment, the case-fatality rates vary from 10% to greater than 50% (6), and can be as high as 70%, with one in five (20%) survivors left with permanent sequelae including hearing loss, neurologic disability, or loss of a limb (7).

The diagnosis of bacterial meningitis rests heavily on examination of CSF collected through lumber puncture (8). The presumptive identification of *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* as well as other bacteria can be made on the basis of cytological examination of the CSF, specific colony morphology on blood and/or chocolate agar, staining properties on Gram stain or by detection of specific antigens in the CSF by latex agglutination test or a rapid diagnostic test (9). Although culture technique has been recommended as the 'gold standard' test because cultured bacteria are sources of data for antibiotic susceptibility, complete sub-typing, expression of antigens that are to be included in future vaccines, and understanding the pathophysiology of isolates, specimens that do not yield any culture growth can still be analyzed by molecular methods using metagenomic DNA (mDNA) extracted from clinical samples (9). A further probing into this statement by the CDC (9), revealed that they were actually referring to "metagenomic protocol" (either consciously or otherwise) aimed at tackling the 'yielded no growth syndrome' or abysmal low yield of CSF culture results. Culture was referred to as the 'gold standard' before now because it was the only method that provided evidence for the presence of any aetiological agent which can be used for further down-stream activities. In low income resource countries that do not have molecular diagnostic methods such as PCR, CSF culture method remains the best option.

Metagenomics is being described as the direct study of genetic materials recovered or extracted from microbial communities present in environmental samples, which take advantage of the rich diversity of genes and biochemical reactions of millions of non-cultivated and uncharacterized microorganisms (10). Metagenomics has been in the practice of microbiology for a while. Far away in 1935, Henrici and Johnson reported the known age long standard methods in bacteriology of "*pure culture isolation and observation upon artificial media which often yield only an incomplete knowledge of a particular microbial flora*" (11). Truly, microbial cultures have always been used to determine the microbial composition, but at the present, reports state that a large proportion of microorganisms in each ecosystem cannot be cultured with traditional tools, and their detection is only possible with DNA sequencing of their genetic fingerprints, the so-called metagenome (12). However, it should be clear at this point that the detection of bacteria in clinical or environmental samples by way of metagenomic approach is not only possible with DNA sequencing, but with molecular detection using multiplex PCR protocol with specific primers and probes (when applicable). Reports on estimates have it that cultured microorganisms account for less than 20% of the real phylogenetic diversity of pro-

karyotes (13). Bacteria may be recalcitrant to culturing for diverse reasons such as lack of necessary symbionts, nutrients, or surfaces; excess inhibitory compounds; incorrect combinations of temperature, pressure, or atmospheric gas composition; accumulation of toxic waste products from their own metabolism; and intrinsically slow growth rate or rapid dispersion from colonies (14).

Due to the increase in the practice of starting antimicrobial therapy prior to clinical sample collection (15-18), the confirmation of aetiological agents of bacterial meningitis has been reported to decrease by about 30% (16, 17). The use of molecular diagnostic protocol (especially PCR analysis) offers the advantages of detecting the DNA of serogroup-specific *N. meningitidis* as well as other implicating microorganisms, and not requiring live organisms for a positive result (19). In 2012, Foxman reported that advanced molecular techniques have provided the opportunity to detect trace amounts of genetic materials of a pathogen in various specimens with sensitivity that is far beyond culture-based methods (20).

Molecular techniques especially PCR-based assay, have become available to provide an early and accurate diagnosis of bacterial meningitis (21). This assay can detect as few as 10-100 CFU/mL of bacteria in CSF (22). PCR can be performed directly on clinical samples; the viability or otherwise of any organism present does not affect the result, this being that DNA can be extracted from clinical samples (typically blood and CSF) (9). The use of molecular assays for detection of aetiological agents of meningitis directly from CSF is now an established protocol.

The efficient extraction/preparation of DNA template is a necessary step for any real-time PCR (9) to meet the required DNA quality in terms of concentration, purity and quantity. The goal of DNA extraction is to lyze the bacterial cells in the specimens to maximize bacterial DNA yield and quality while removing any PCR inhibitors (i.e. salts, proteins), and dissolve the DNA in a buffer compatible with the enzymes used in the next analytical step while concentrating the extracted DNA at the same time (9). Commercial DNA extraction kits are available for culture, blood and body fluids (CSF inclusive). One of such DNA extraction kit is the Qiagen QIAamp$^{(R)}$ DNA Mini Kit which provides fast and easy method for purification of total genomic DNA for reliable PCR, and can purify total DNA (e.g. metagenomic) from whole blood, plasma, serum, buffy coat, bone marrow, CSF, lymphocytes, cultured cells, tissue, and forensic specimens (23). The Qiagen QIAamp DNA Mini kit is a spin column technology in which DNA is selectively absorbed onto silica membrane (24). DNA purified using QIAamp kits is up to 50 kb in size, with fragments of approximately 20-30kb predominating. DNA of this length denatures completely during thermal cycling and can be amplified with high efficiency.

It is mandatory to determine the quality (concentration and purity) of extracted metagenomic DNA before use for PCR assays. DNA yield can be assessed using various methods including absorbance (optical density), agarose gel electrophoresis, or fluorescent DNA-binding dyes. The Eppendorf BioPhotometer Plus instrument used for quality check measures optical density (absorbance values) at 230, 260 and 280 nanometres and converts optical densities to concentrations. Dependent on the method, the results can be calculated through fixed factors, standards, or curve calibration. In addition to the results, the device also displays the absorbance values and some other important details, such as the common absorbance quotients e.g. A_{260}/A_{280} and A_{230}/A_{280} ratio for nucleic acid calculations. Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA (good quality DNA) has A_{260}/A_{280} ratio of 1.7–1.9, although reading lower than 1.6 does not render the DNA unsuitable for any application, but lower ratios may indicate the presence of more contaminants (25). Elution buffer for genomic DNA is usually 1 x Tris EDTA buffer at pH 8.0.

For checking DNA quality and yield, RNase/DNase free water (nuclease free water) is used to dilute samples and to zero the spectrophotometer while the absorbance is measured at A_{260} and A_{280} nm. Both DNA and RNA are measured with spectrophotometer, however, to measure only DNA, a fluorometer must be used (23). Quality check of DNA extract by agarose gel electrophoresis is one of the most frequently used techniques in life sciences (26). DNA fragments loaded on agarose gels would have been stained with ethidium bromide and detected by an ultraviolet (UV) transilluminator system (27). Agarose gel electrophoresis is also another way to quickly estimate DNA concentration.

Fluorescence measurement of nucleic acids is based on the use of fluorogenic dyes that bind selectively to DNA or RNA. Dyes only emit signal when bound to the target, and signal is measured by fluorometers. Sample is excited with filtered light (at the excitation wavelength), and the emitted light (at the emission wavelength) is recorded by a detector (28). The objective of this study is to evaluate the quality of mDNA (concentration, purity, and amount) extracted by spin column technique (Qiagen

QIAamp) from CSF samples and control strains using fluorometric, spectrophotometric and gel electrophoresis methods.

Materials and method:

Study settings

The study sites were in Federal Capital Territory (FCT): National Hospital, Abuja; all District/General Hospitals in the FCT (Asokoro, Wuse, Maitama, Garki, Gwarinpa, Bwari, Kubwa, Kuje, and Nyanya), and some States in Northern Nigeria. However, CSF samples were not received from any District/General Hospital in the FCT; but from National Hospital, Abuja and also from Kebbi, Plateau, Sokoto and Zamfara States in Northern Nigeria during outbreak seasons of February – May 2017 and January – April, 2018.

Ethical consideration

Ethical approvals were obtained from the Ethics Committees of National Hospital, Abuja (NHA/EC/034/2015), Federal Capital Development Authority Health Services (FHREC/ 2017/01/27/03-04-17), Kebbi State Ministry of Health (MOH/KSREC/VOL.1/56/No 101.3/2015), Plateau State Ministry of Health (MOH/MIS/ 202/VOL.T/X,2017), Sokoto State Ministry of Health (SMH/1580/V.IV, 2017), and Zamfara State Ministry of Health (ZSHREC/02/03/2017).

A letter of introduction from the Nigeria Centre for Disease Control (NCDC) of the Federal Ministry of Health (Ref. MH/2768/S.162/III) was obtained to cover for all outbreak sites in the country. Written informed consent for storage and future use of unused samples, sample materials and data transfer agreement, were also obtained.

Subjects

All hospitalized patients (all ages and gender) with clinical symptoms of meningitis as reviewed by the attending physicians were included in the study. Patients who did not give informed consent and sites that did not grant approval were excluded from the study.

Sample size and sampling method

The sample size was determined using the Cochran formula (29) for calculating simple proportion; $n_o=z^2pq/e^2$, where 'n_o' is the minimum required sample size, 'z' is the selected critical value of desired confidence level at 95% (standard value of 1.96), 'p' is the estimated proportion of an attribute that is present in the population [estimated prevalence of meningitis in Zamfara State of 13.7% (30)], 'q' is 1-p and 'e' is the desired level of precision (margin of error at 5%; standard value of 0.05). Therefore, the estimated sample size was 181.7 which was adjusted to 210 samples after calculating for 10% attrition. The subjects were recruited consecutively until the sample size was attained.

Collection and transportation of CSF specimens

CSF was collected into sterile containers by experienced physicians after performing lumbar puncture under aseptic conditions. The samples were transported to the laboratory at the various sites and kept at -20^{O}C before being transported in ice-packs to Abuja for onward transfer to the Safety Molecular Pathology Laboratory, Enugu, where the samples were kept at -80^{O}C until mDNA extraction was carried out.

Metagenomic DNA extraction

The Qiagen QIAamp$^{(R)}$ DNA Mini kit was used for mDNA extraction of the CSF samples, bacterial isolates and three ATCC control strains (*N. meningitidis* serogroup B ATCC 13090, *H. influenzae* Type B, Biotype 1 ATCC 10211, and *S. pneumoniae* serotype 19F ATCC 49619). Approximately 200µL each of the CSF, bacterial isolates from the CSF (kept in 10% Skim milk with 15% glycerol) and ATCC bacterial control strains in TE buffer, were transferred into 2.0mL microcentrifuge (Eppendorf) tubes. 20µL of proteinase K was added to all samples, vortexed at 2000 rpm for 5 seconds, and incubated at 56^{O}C for 15 minutes. 60µL buffer AL was added, mixed thoroughly by vortexing for 15 seconds, incubated at 70^{O}C for 10 minutes and briefly centrifuged to remove drops from the lid of the tube. 200µL ethanol (96–100%) was added, vortexed for 15 seconds and briefly centrifuged to remove drops from the lid of the tube.

The mixture was pipetted onto the QIAamp Mini spin column (in a 2ml collection tube), centrifuged at 6000xg (8000rpm) for 1 min, and the flow-through and collection tube discarded. The QIAamp Mini spin columns were placed in a new 2ml collection tube, 500µL buffer AW1 was added, centrifuged at 6000xg (8000rpm) for 1 min and the flow-through and collection tube discarded. The QIAamp Mini spin columns were placed in a new 2ml collection tube, 500µL buffer AW2 was added, centrifuged at full speed of 20,000 x g (14,000 rpm) for 3 min and the flow-through and collection tube discarded. The QIAamp Mini spin columns were then placed in a new 1.5 ml collection tube and 60µL buffer AE added, incubated at room temperature for 1min and centrifuged at 6000xg (8000rpm) for 1min to elute the mDNA. Eluted DNA samples were labelled accordingly for quality check and appropriately stored for later use.

Metagenomic DNA extract quality check

The quality of the extracted mDNA was performed by the fluorometric method (for concentration) and spectrophotometric method (for purity). The agarose gel electrophoresis method was used to access few DNA samples for the presence or absence of bands only.

Fluorescence method for DNA concentration

The Qubit 3.0 fluorometer instrument (Invitrogen Life Technologies, now Thermo-Fisher) was used to determine the concentration of the extracted mDNA from the samples. Assay components were equilibrated at room temperature; the Qubit(R) working solution was prepared by diluting Quant - iT™ dsDNA HS reagent 1:200 in Quant - iT™ dsDNA HS buffer. 200µL of working solution was prepared for each standard and sample. The assay tubes were prepared according to Table 1.

All tubes were vortexed for 2–3 sec. The tubes were incubated for 2 minutes at room temperature. The tubes were inserted in the Qubit 3.0 fluorometer and readings taken. The Qubit 3.0 fluorometer was calibrated using the readings of the Standard Assay Tubes (8 in number) with concentrations range of 0.0 ng/µl to 10.0ng/µl, and prepared a standard curve to determine DNA amounts in user samples (unknown DNA sample concentrations). For the calibration curve, data from Qubit 3.0 were entered into GraphPad Prism and linear regression of DNA standards was determined (Table 2) and used in reading the relative fluorescence unit of samples (Table 3).

Table 1: Protocol for preparing assay tubes for fluorometric method

Volume of solution/analyte	Standard Assay Tubes	Unknown DNA (user) samples Assay Tubes
Volume of working solution (from step 2) to add	190 µl	195 µl
Volume of standard (from kit) to add	10 µl	-
Volume of user sample to add	-	5 µl
Total volume in each Assay Tube	200 µl	200 µl

Thin–walled, clear 0.5ml PCR tubes were used. Acceptable tubes include Qubit(R) assay tubes (set of 500 – Cat No. Q32856) or Axygen PCR – 05 – C tubes (VWR, Part No. 10011 - 830). The minimum assay volume must be 200 µl.

Table 2: Characteristics of the Linear Regression for DNA check by Fluorometric Method (Qubit 3.0)

Best-fit values	Relative Fluorescent Unit (RFU)
Slope	1651 ± 53.21
Y-intercept when X= 0.0	487.1 ± 268.9
X-intercept when Y= 0.0	- 0.2950
1/slope	0.0006056
95% Confidence Intervals	
Slope	1503 to 1799
Y-intercept when X= 0.0	-259.4 to 1234
X-intercept when Y= 0.0	-0.8004 to 0.1478
Goodness of Fit	
R square	0.9959
Sy.x	464.5
Is slope significantly non-zero?	
F	962.9
DFn, DFd	1.000, 4.000
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	6
Maximum number of Y replicates	1
Total number of values	6
Number of missing values	0

Table 3: Fluorometric readings of standards

	Concentration (ng/µl)	RFU value
Std1	10.00	17203.03
Std2	8.00	9922.97
Std3	6.00	9735.85
Std4	4.00	7538.76
Std5	1.00	2423.75
Std6	0.50	989.15
Std7	0.00	532.96

Table 4: Spectrophotometer Method – Operation

Method group	Method	Description	Wavelength
DNA	dsDNA	Calculating the concentration of DNA with evaluation via factor. Already pre-programmed factor ex-factory	Measuring wavelength: 260nm Secondary wavelength to check for purity: 280nm

Spectrophotometric method for DNA purity

In the spectrophotometric method, the Eppendorf BioPhotometer Plus instrument was used to determine the purity of the extracted metagenomic DNA from the samples. Measuring procedure was according to literature insert in the manufacturer's manual, with the instrument set for dsDNA and sample dilutions of 5µL sample + 95µL diluent for reading at A_{260}/A_{280}. The Eppendorf BioPhotometer Plus instrument switched-on to initialize. Sample preparation (95µL of diluent distilled water) was pipetted into appropriately labelled tubes. 5µL of mDNA extract was added to the corresponding labelled tubes. The sample was transferred into a clean cuvette shaft of outside diameter 12.5mm x 12.5mm. The instrument was set at blank (zero) before reading at A_{260}/A_{280} wavelength (Table 4). Result was recorded for purity value at A_{260}/A_{280} and dsDNA concentration in µg/mL but readings of the dsDNA concentration were disregarded because of inconsistent values (non-reproducibility of readings).

Gel electrophoresis method

In the gel electrophoresis method, the Biorad Horizontal Gel Electrophoresis tank was used in running of some samples. The set gel (1.5% agarose) was well placed into the tank and filled to the brim with 0.5xTris Borex EDTA (TBE). 5µL of mDNA extract was pipetted into microtitre plate wells appropriately. 3µL of loading dye (10 x Dream Taq Green Buffer which includes 20mM $MgCl_2$) was added into all samples in the microliter plate wells and well mixed. The loading dye helps the DNA extract (sample) to sink into the well of the gel. Each gel well was loaded with the sample and covered with adequate 0.5xTBE. The electrophoresis tank is connected to the power pack with positive and negative terminals, and switched on. The gel was run at 100 volts for 45–60 min. The gel was then transferred to the UV trans-illuminator that is fitted with a camera system which is viewed on a computer connected to the camera system. Using the GenoSpot programme and EOS Utility (for the Camera) that are installed on the computer, snap shot gel pictures of the mDNA bands were taken, saved and labelled appropriately.

Calculating amount of mDNA extracts

The mDNA concentration (ng/µL) was measured by the Qubit Fluorometer 3.0g. The DNA yield = DNA concentration x eluted volume (60µL) per 200µL of CSF. The amount of DNA = DNA concentration x 5µL per qPCR reaction.

Results:

Of the 210 subjects recruited into the study, 129 (61.4 %) were males, comprising 104 (49.5%) children (<15 years of age) and 25 (12%) adults while females were 81 (38.6%), comprising 66 (31.4%) children (<15 years) and 15 (7.1%) adults (Table 5). Following microbiological analysis, Gram reaction was positive in 94 (44.8%) samples while only 17 (8.1%) were culture positive for two of the three bacteria under study (Table 6).

Table 7 shows the summary of results obtained from linear regression for mDNA concentration by fluorometric method (Qubit 3.0) on the 210 CSF samples. Metagenomic DNA was extracted from 180 (85.7%) samples with concentrations of ≥ 0.005 ng/µl [relative fluorescent unit (RFU) value of 537.32] being the lowest limit of detection (LOD), while 30 (14.3%) had DNA concentration less than 0.005

ng/µl. The amounts of mDNA present in the 180 (85.7%) samples were DNA concentration of 0.03–50.5ng/µl, DNA yield of 1.8–3030 µg and DNA amount of 0.15–252.5 ng/µl. Table 8 is the summary results of mDNA purity values at A_{260}/A_{280} and concentration of ≥ 0.005 ng/µl.

Table 5: Age group and gender distribution of subjects recruited for the study

Age group (years)	Male (%)	Female (%)	Total (%)
< 15	104 (49.5)	66 (31.4)	170 (81.0)
> 15	25 (11.9)	15 (7.1)	40 (19.0)
Total	129 (61.4)	81 (38.6)	210 (100)

Table 6: Results of Gram reaction, culture and metagenomic DNA on the CSF samples

Test	CSF samples	
	Number positive	Percentage
Gram reaction	94	44.8
Culture	17	8.1
Metagenomic DNA (≥0.005ng/ul)	180	85.7

CSF = cerebrospinal fluid

Table 7: Linear regression for mDNA concentration by fluorometric method (Qubit 3.0) on CSF samples

Concentration (ng/ul)	RFU value	No of samples	Percentage
0.005 - ≥ 10.00	537.32 - ≥ 17203.03	180	85.7
0.00 - < 0.005	532.96 - < 537.32	30	14.3
Total		210	100

RFU = Relative Fluorescent Unit; mDNA = metagenomic DNA

Table 8: Spectrophotometric results of mDNA purity at A_{260}/A_{280} and concentration of ≥ 0.005 ng/µL

Purity @ A_{260}/A_{280}	Number of samples	Percentage
≥ 1.7	55	30.6
1.0 – 1.69	103	57.2
0.57 – 0.99	14	7.8
0.00	8	4.4
Total	180	100

Fig 2 shows the linear regression plot for mDNA concentration. Fig 3 shows gel picture of ATCC bacterial control strains that had DNA purity at A_{260}/A_{280} of 1.53 (*N. meningitidis*), 1.48 (*H. influenzae*), and 1.57 (*S. pneumoniae*) and Fig 4 shows the gel electrophoresis picture of mDNA of samples that had purity range of 1.0–4.37 at A_{260}/A_{280}.

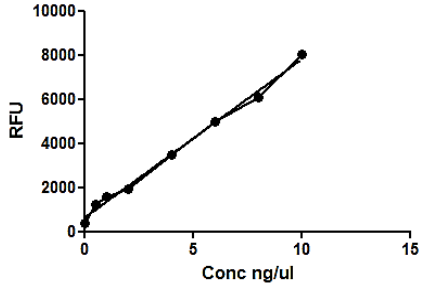
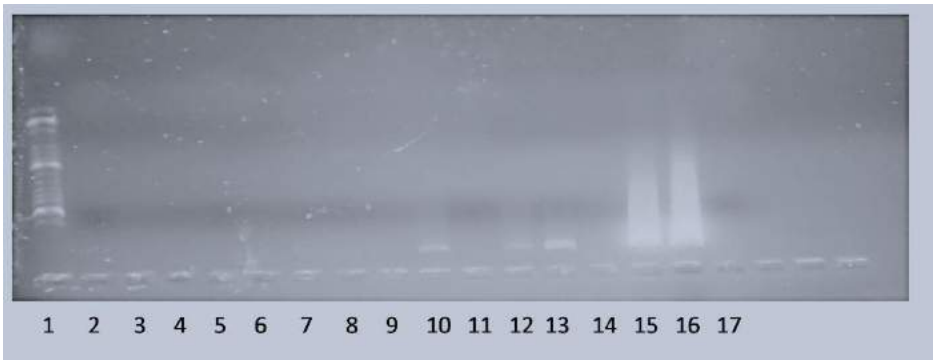


Fig 2: Linear Regression Plot for DNA Concentration

Lane 1 – DNA ladder; Lane 2 – *N. meningitidis* (DNA purity @ A_{260}/A_{280} - 1.53); Lane 3 – *H. influenzae* (DNA purity @ A_{260}/A_{280} – 1.48); Lane 4 – *S. pneumoniae* (DNA purity @ A_{260}/A_{280} – 1.57); Lane 5 - *N. meningitidis* (DNA purity @ A_{260}/A_{280} - 1.53); Lane 6 - *H. influenzae* (DNA purity @ A_{260}/A_{280} – 1.48)

Fig 3: Gel electrophoresis picture of ATCC bacterial control strains



Lane 1: DNA Ladder (50 bp); Lane 2: IP01N (DNA purity @ A_{260}/A_{280} – 1.23; DNA Conc. 1.3 ng/µL); Lane 3: IP07N (DNA purity @ A_{260}/A_{280} – 0.95; DNA Conc. 10.8 ng/µL); Lane 4: IP026N (DNA purity @ A_{260}/A_{280} – 1.3; DNA Conc. 0.14 ng/µL); Lane 5: IP35N (DNA purity @ A_{260}/A_{280} – 3.1; DNA Conc. 0.04 ng/µL); Lane 6: IP62N (DNA purity @ A_{260}/A_{280} – 1.9; DNA Conc. 2.3 ng/µL); Lane 7: IP95S (DNA purity @ A_{260}/A_{280} – 1.0; DNA Conc. 5.9 ng/µL); Lane 8: IP101N (DNA purity @ A_{260}/A_{280} – 1.2; DNA Conc. 1.2 ng/µL); Lane 9: IP128H (DNA purity @ A_{260}/A_{280} – 1.1; DNA Conc. 0.97 ng/µL); Lane 10: IP147N (DNA purity @ A_{260}/A_{280} – 1.2; DNA Conc. 5.8 ng/µL); Lane 11: IP157H (DNA purity @ A_{260}/A_{280} – 8.1; DNA Conc. 6.1 ng/µL); Lane 12: IP178S (DNA purity @ A_{260}/A_{280} – 1.4; DNA Conc. 1.8 ng/µL); Lane 13: IP189N (DNA purity @ A_{260}/A_{280} – 1.8; DNA Conc. 4.7 ng/µL); Lane 14: IP209N (DNA purity @ A_{260}/A_{280} – 2.0; DNA Conc. 1.1 ng/µL); Lane 15: Nm ATCC (DNA purity @ A_{260}/A_{280} – 1.5; DNA Conc. 15.1 ng/µL); Lane 16: Hi ATCC (DNA purity @ A_{260}/A_{280} – 1.48; DNA Conc. 20.0 ng/µL); Lane 17: Sp ATCC (DNA purity @ A_{260}/A_{280} – 1.6; DNA Conc. 16.6 ng/µL)

Fig 4: Gel electrophoresis picture of mDNA samples (DNA purity @ $A_{260/280}$ of 1.10 – 4.37)

Discussion:

The findings of our study showed that Gram reaction was positive in 44.8% (94/210) of CSF samples of the patients and culture in 8.1% (17/210) while 85.7% (180/210) yielded mDNA concentrations of ≥ 0.005ng/ml. Of the 180 samples, spectrophotometric reading for DNA purity value of ≥ 1.7-12.20 (good quality DNA) was recorded in 55 (30.6%), 1.0–1.69 (quality DNA) in 103 (57.2%), 0.57–0.99 (low quality DNA) in 14 (7.8%) with only 8 samples (4.4%) failing purity evaluation (with value of 0.00 at $A_{260/280}$). In a recent metagenomic study by Zhang et al., (32) on 135 patients, 26 (19.3%) were culture positive while 32 (23.7%) were identified by metagenomic next generation sequencing (mNGS). This is the closest method to the one we used. While their method was mNGS, ours is still at the level of mDNA extraction from CSF samples and subsequent molecular identification and characterization by qPCR of the three bacteria of interest (*N. meningitidis, H. influenzae,* and *S. pneumoniae*). Our study therefore provides a strong baseline data for processing CSF samples for qPCR without the need for culture, and shows that majority of samples would yield quality DNA material (> 1.0 ng/µL).

The gel electrophoresis results showed spatial bands, that could be linked to the percentage of agarose in the gel used (1.5%), which is good in resolving linear DNA molecules size range of 300–3000 bp (31) as against the amplicon size of the bacteria of interest; *N. meningitidis* (127 bp), *H. influenzae* (113 bp), and *S. pneumoniae* (51 bp). The strength of our research lies on the fact that metagenomic protocol does not rely on bacterial culture and isolation for extraction of mDNA for use in the detection of aetiological agents of meningitis, but rather on the constituent DNA concentration present in the sample. However, one limitation to our study is that we did not include viral pathogens, being that RNA was not extracted. Another limitation is that we did not perform restriction digest of the extracted mDNA or PCR amplification of 16S rDNA on the extract, which would have confirmed the suitability of the extracted mDNA for downstream processing.

Conclusion:

Quality mDNA from CSF samples will ensure successful qPCR results for rapid and accurate detection of bacterial pathogens in meningitis, beginning first at molecular detection using multiplex real-time PCR (rt-PCR) down to species-specific singleplex rt-PCR. This will eliminate the time and labour consuming traditional culture methods often associated with "yielded no growth syndrome" or abysmal low yield of CSF culture output resulting in the very poor outcome of laboratory confirmed cases of cerebrospinal meningitis (CSM).

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Authors contributions:

PIC and IEI conceived, and led the design and writing of the manuscript. PIC, NUP, UYB, DLD, OCN, and MR processed the CSF samples at the various sites for phenotypic methods, storage, and transportation of the samples to Abuja and finally to Safety Molecular Pathology Laboratory, Enugu for storage at -80^{O} C. NE and NCR were responsible for molecular biology techniques (PCR) orientation, training and processing of CSF samples extraction and quality check of DNA. PIC fully participated in performance of the PCR activities. PIC, IEI, and NE were responsible for the final editing of the manuscript.

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Original Article **Open Access**

Faecal carriage of extended-spectrum beta-lactamase-producing Enterobacteriaceae in healthy volunteers and hospitalized patients in Ouagadougou, Burkina Faso: prevalence, resistance profile, and associated risk factors

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Abstract:

Background: Extended spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-PE) are a serious challenge to patients' treatment. The aim of this study is to determine the prevalence of ESBL-PE, investigate the associated resistance, and analyze the associated risk factors for acquisition of ESBL-PE.
Methodology: A cross-sectional study was conducted on healthy volunteers and inpatients. After obtaining informed consent, rectal swabs were collected from each participant for isolation of Enterobacteriaceae on Hektoen enteric agar containing 4µg/L cefotaxime. The Enterobacteriaceae isolates were identified using biochemical tests and ESBL production was confirmed by the double-disc synergy test of amoxicillin and clavulanic acid. Antibiotic susceptibility test of each isolate was done by the disc diffusion method and interpreted using the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints version 5.0.
Results: During the study period, prevalence of faecal ESBL-PE among the study participants was 54.5% (103/189); 53.5% among healthy volunteers and 55.7% among inpatients (p=0.87). The major ESBL-PE isolates was *Escherichia coli* (71%) followed by *Klebsiella pneumoniae* (16%). The isolates in hospitalized patients were resistant to norfloxacin (84.2%), cotrimoxazole (89.5%), and gentamicin (7.0%). The isolates from healthy volunteers were resistant to norfloxacin (86.2%), cotrimoxazole (82.8%), and gentamicin (1.7%). Gender, age, and previous antibiotic use were not significantly associated with carriage of ESBL-PE (p=0.51).
Conclusion: The high prevalence of ESBL-PE in this study is worrying. There is an urgent need to develop measures to monitor and limit the spread of these multidrug-resistant organisms in healthcare facilities and the community in Burkina Faso.

Keywords: faecal carriage, ESBL-PE, healthy volunteers, inpatients, Burkina Faso

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Portage fécal d'entérobactéries productrices de bêta-lactamases à spectre étendu chez des volontaires sains et des patients hospitalisés à Ouagadougou, Burkina Faso: prévalence, profil de résistance et facteurs de risque associés

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Résumé:

Contexte: Les entérobactéries productrices de bêta-lactamases à spectre étendu (EP-BLSE) représentent un défi majeur pour le traitement des patients. Le but de cette étude est de déterminer la prévalence de EP-BLSE, d'étudier la résistance associée et d'analyser les facteurs de risque associés pour l'acquisition de EP-BLSE.
Méthodologie: Une étude transversale a été menée sur des volontaires sains et des patients hospitalisés. Après avoir obtenu le consentement éclairé, des écouvillons rectaux ont été prélevés sur chaque participant pour isoler les entérobactéries sur gélose entérique Hektoen contenant 4µg/L de céfotaxime. Les isolats d'Enterobacteriaceae ont été identifiés à l'aide de tests biochimiques et la production de BLSE a été confirmée par le test de synergie double disque de l'amoxicilline et de l'acide clavulanique. Le test de sensibilité aux antibiotiques de chaque isolat a été réalisé par la méthode de diffusion sur disque et interprété en utilisant les recommandations de la version 5.0 des seuils cliniques du Comité européen sur les tests de sensibilité aux antimicrobiens (EUCAST).
Résultats: Au cours de la période d'étude, la prévalence de EP-BLSE fécale parmi les participants à l'étude était de 54,5% (103/189); 53,5% parmi les volontaires sains et 55,7% parmi les patients hospitalisés (p= 0,87). Les principaux isolats de EP-BLSE étaient *Escherichia coli* (71%) suivis de *Klebsiella pneumoniae* (16%). Les isolats des patients hospitalisés étaient résistants à la norfloxacine (84,2%), au cotrimoxazole (89,5%) et à la gentamicine (7,0%). Les isolats de volontaires sains étaient résistants à la norfloxacine (86,2%), au cotrimoxazole (82,8%) et à la gentamicine (1,7%). Le sexe, l'âge et l'utilisation antérieure d'antibiotiques n'étaient pas significativement associés au portage de EP-BLSE (p=0,51).
Conclusion: La forte prévalence de EP-BLSE dans cette étude est préoccupante. Il est urgent de développer des mesures pour surveiller et limiter la propagation de ces organismes multirésistants dans les établissements de santé et la communauté au Burkina Faso.

Mots clés: portage fécal, EP-BLSE, volontaires sains, patients hospitalisés, Burkina Faso

Introduction:

Antibiotic resistance (AMR) is a threat to global health and has tremendous impact on human development. It is associated with prolonged patients' hospitalization, increase health expenditure, morbidity and mortality (1). According to a study in Senegal, the cost of treatment of infection due to extended-spectrum beta-lactamase-producing Entero-bacteriaceae (ESBL-PE) may be up to 100 € (2). The multi-drug resistance (MDR) issue according to the World Health Organization (WHO) involves several families of bacteria including the family Enterobacteriaceae (3). Acquisition of MDR is favored by several factors such unfavorable socio-demographic conditions, inappropriate use of antibiotics, absence of regulations for acquisition of antibiotics, and varying quality of antibiotics among others (4).

Carriage of ESBL-PE is associated with high risk of developing ESBL-PE infection (5), and also constitutes an important reservoir for MDR diffusion. This is a serious public health problem because treatment of infections caused by ESBL-PE entails the use of high ceiling antibiotics which are often unavailable in low-income-countries. Thus, the emergence of ESBL-PE has become a daily concern for treatment of infections in these countries. In Burkina Faso, the first study on carriage of ESBL-PE reported an overall ESBL-PE prevalence rate of 32% in western Bobo Dioulasso (4). This present study focuses on ESBL-PE carriage among healthy volunteers and hospitalized patients in Ouagadougou, the capital city of Burkina Faso.

Materials and method:

Study setting

The study was conducted in the Tengandogo University Hospital, which has 600 beds, distributed across different specia-lized acute care units; medicine, surgery, gynaecology, obstetrics, and paediatrics.

Study design and period of study

This was a cross-sectional study conducted during the period July 1 to November 30, 2017.

Subject participants

The study subjects were 88 patients who were hospitalized at the Tengandogo University Hospital for more than 48 hours (people with digestive pathologies were excluded from the study), and 101 healthy volunteers (healthy personnel and accom-panying persons).

Ethical considerations

The study was approved by the Tengandogo University Hospital Board (Authorization No. MS/SG/CHUBC/DG/DSM 2017-569, October 27, 2017). Written informed consent was obtained from all subjects and at least one parent for each child before enrollment. Participants were enrolled voluntarily. The confidentiality of the obtained information from the subjects was respected, as the participants and their samples were codified.

Samples and data collection

Each participant was interviewed by a healthcare professional using a questionnaire to obtain information on age, gender and antibiotic use/treatment during the previous 3 months before the study. Rectal samples were taken by swabbing the rectum of each subject, using sterile swab soaked in sterile physiological saline, which was inserted into the rectum to about 2 cm and rotated 2 to 3 times. The swab was then put back into the swab container, and transported immediately to laboratory for microbiological analysis (6).

Microbiological culture and isolate identification

All rectal samples were immediately seeded on Brain Heart Infusion (BHI) broth and incubated for 5 hours at 37°C to improve bacteriological yield (7). After this enrichment phase, 100 µL of the broth was transferred to Hektoen enteric agar (Oxoid, UK) supplemented with 4 µg/L cefotaxime and incubated at 37°C for 24 hours. Predominant colonies of different morphotypes were identified to species level by using in-house biochemical test panels including Triple Sugar Iron (TSI) agar, Sulfur-Indole-Motility test, Simmons´s citrate agar, and urease test.

Detection of ESBL-PE

The detection of ESBL production was routinely performed by the disc diffusion synergy method using third generation (3GC) discs of cefotaxime (30µg), ceftriaxone (30µg), and ceftazidime (30µg) (HiMedia, Ltd, India) placed at a distance of 20-30 mm apart around a disc of amoxicillin+clavulanic acid (20+10µg) as recommended by the Antibiogram Committee of the French Microbiology Society (8,9). ESBL production was detected by the presence of synergy between the third generation cephalosporins and the inhibitor (clavulanic acid) (8–10). In case of high-level cephalosporinase production, the combined double-disc synergy test was performed using cloxacillin–supplemented agar medium. *Klebsiella pneumoniae* ATCC 700 603 (ESBL-positive) and *Escherichia coli* ATCC 25922 (ESBL-negative) were used as control strains.

Antibiotic susceptibility test

Antimicrobial susceptibility test (AST) was performed on each isolate by the disc diffusion (Kirby–Bauer) method. A suspension in saline solution (0.9% NaCl) equivalent to 0.5 McFarland standards (or to an optical density OD of 0.08-0.10 read at 625 nm) was used. The incubation was carried out at 37°C aerobically for 18 to 24 hours. Reading and interpretation were carried out according to the recommendations of the Antibiogram Committee of the French Microbiology Society (8).

The following single discs were used for the AST assay; amoxicillin+clavulanic acid (30µg), ceftriaxone (30µg), ceftazidime (30 µg), cefotaxime (30µg), meropenem (10µg), gentamicin (30µg), norfloxacine (10µg), and sulfamethoxazole-trimethoprim (25µg). The inhibition zone diameters were used to categorize isolates as sensitive (S), intermediate (I) or resistant (R). *Escherichia coli* ATCC 25922 was used as the control strain in the AST assay.

Statistical analysis

Data entry was performed on Excel 2013 and statistical analysis of the data was done using XLSTAT 2017 version 19.5. The distributions of the variables were compared by the χ^2 independence test. The significance level was set at 5%.

Results:

Prevalence of faecal carriage of ESBL-PE

A total of 103 subjects were faecal carriers of ESBL-PE, giving an overall prevalence rate of 54.5% (103/189). Forty-nine of the 88 (55.7%) inpatients and 54 of 101 (52.4%) healthy volunteers were carriers of ESBL-PE. The prevalence of ESBL-PE carriage was slightly higher among hospitalized patients than among healthy volunteers but the difference was not statistically significant (p=0.87).

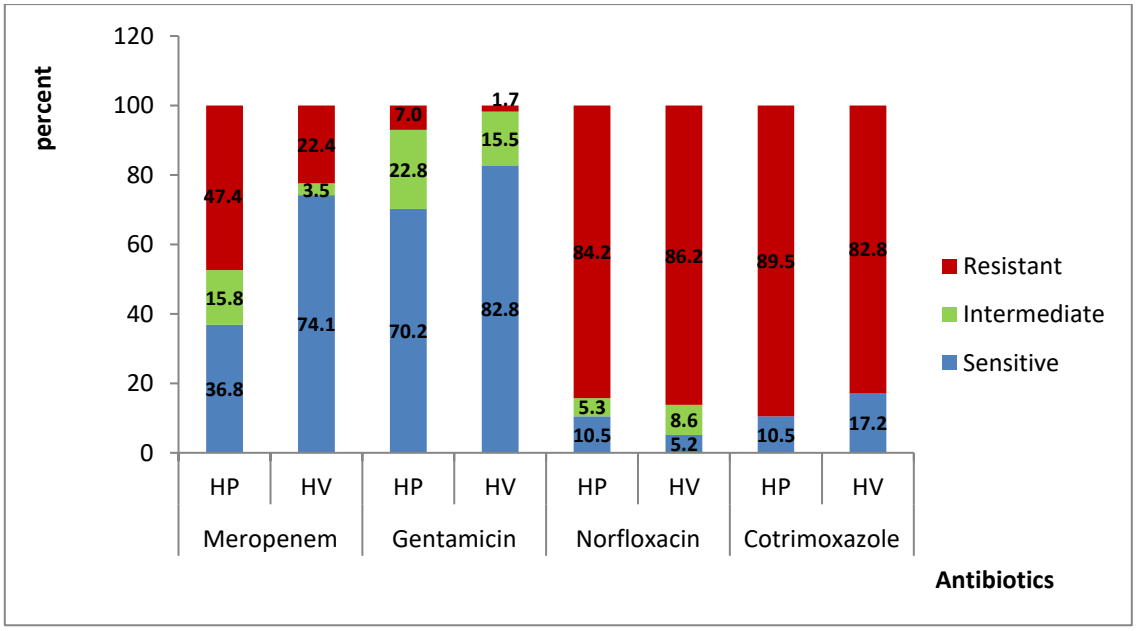
Distribution of ESBL-PE in the study population

ESBL-PE isolates were identified as *Escherichia coli* (71.3%), *Klebsiella pneumoniae* (15.7%), *Klebsiella oxytoca, Enterobacter cloacae, Enterobacter agglomerans, Citrobacter freundii* and *Proteus mirabilis* (Table 1).

Table 1: Distribution of ESBL-PE in the study population

ESBL-PE	In-patients		Healthy volunteers		Total	
	n	%	n	%	n	%
Escherichia coli	38	66.7	44	75.9	82	71.3
Klebsiella pneumoniae	7	12.3	11	18.9	18	15.7
Klebsiella oxytoca	5	8.8	1	1.7	6	5.2
Enterobacter cloacae	2	3.5	1	1.7	3	2.6
Enterobacter agglomerans	3	5.3	1	1.7	4	3.5
Citrobacter freundii	1	1.8	0	0	1	0.9
Proteus mirabilis	1	1.8	0	0	1	0.9
Total	57	100	58	100	115	100

ESBL-PE = Extended Spectrum Beta-Lactamase-Producing Enterobacteriaceae; n = number



HP: Hospitalized Patients, HV: Healthy Volunteers

Fig 1: Susceptibility and resistance profile of ESBL-PE to selected antibiotics

Antibiotic resistance profile of ESBL-PE

Resistance rates of ESBL-PE to amino-glycosides, fluoroquinolones and sulfon-amides were similar among inpatients and healthy volunteers, with 89.5% and 82.8% resistant to cotrimoxazole and 84.2% and 86.2% to norfloxacine respectively (Fig 1). However, resistance rate to meropenem of 57.4% among inpatients was significantly higher than 22.4% among healthy volunteers (p=0.007).

Association of participant's characteristics and risk factors to ESBL-PE carriage

Analysis of participant's characteristics and risk factors by $\chi 2$ test showed that none was associated with ESBL-PE carriage (Table 2). Antibiotics usage in the last 3 months (p=0.21) and types of antibiotics used (p=0.51) were not significantly associated with ESBL-PE carriage.

Table 2: characteristics and risk factors for ESBL-PE in the study population

	Inpatients (n=88)			**Healthy volunteers (n=101)**		
Variables	ESBL positive (n=49)	ESBL negative (n=39)	*p* value	ESBL positive (n=54)	ESBL negative (n=47)	*p* value
Mean age (±SD) (years)	45.91 (±23.02)	46.78 (±23.65)		39.75 (±8.94)	41.29 (±10.24)	
Gender n (%)						
Male	29 (59.2)	26 (66.7)	0.47	12 (22.2)	18 (38.3)	0.077
Female	20 (40.8)	13 (33.3)		42 (77.8)	29 (61.7)	
Age group (years)	n (%)					
≤1	6 (12.2)	7 (17.9)		0	0	NA
>1 – 20	4 (8.2)	3 (7.7)		1 (1.9)	0	
>20 – 40	16 (32.7)	12 (30.8)	0.078	33 (61.1)	23 (48.9)	
>40 – 60	9 (18.4)	5 (12.8)		18 (33.3)	21 (44.7)	
>60	14 (28.6)	12 (30.8)		2 (3.7)	3 (6.4)	
Antibiotic use/treatment in last 3 months, n (%)						
Yes	24 (48.9)	14 (35.9)	0.21	13 (24.1)	14 (29.8)	0.51
No	25 (51. 0)	25 (64.1)		41 (75.9)	33 (70.2)	
Type of antibiotics used n (%)						
β-lactams	21 (87.5)	12 (85.7)	0.87	11 (84.6)	9 (64.3)	0.22
Others	3 (12.5)	2 (14.3)		2 (15.4)	5 (35.7)	

Discussion:

In this study, β-lactams were the most frequently used antibiotics among the subjects, with a frequency of approximately 80%. The same observation was made by Ouédraogo et al., (4) in 2016, who reported 56.25% of β-lactams among antibiotics used by patients in the 3 months before detection of ESBL-PE carriage. Indeed β-lactams are available for patients use because they are less expensive, and easy to use by oral administration. This high rate could increase the pressure of the selection of resistant mutants in the country. Indeed, excessive consumption of penicillins and cephalosporins can cause mutations in bacterial populations, and genes encoding the synthesis of penicillinases, cephalosporinases or extended spectrum β-lactamases are transferable to other bacteria, thus increasing resistance to these antibiotics.

The prevalence of 54.5% of ESBL-PE carriage (55.7% among in-patients and 53.5% among volunteers) is high, and this could predispose individuals to increase risk of infections because these enterobacteria may be found in the urinary tract or on an operating wound, resulting in infections that are difficult to treat. This prevalence is higher than those observed in the northern countries where the faecal carriage of ESBL-PE was in the range of 0.6% to 11.6% (11,12). However, our results are similar to those found in several countries in sub-Saharan Africa like Cameroon, where prevalence rate ranges between 16% and 55%. The prevalence of colonization by ESBL-PE in healthy subjects in West African countries varies between 10 and 100% (13). This high prevalence may be explained as consequences of excessive use of antibiotics, poor quality of antibiotics, poor hygiene, and absence of surveillance system networks for these multi-resistant bacteria. We did not find any significant difference between prevalence in hospitalized patients (55.7%) and healthy volunteers (53.5%), which is contrary to the study by Ouédraogo et al., (4) in Bobo Dioulasso, where there significant difference was reported. However, the prevalence rate both studies is high and indicates a need for urgent action by way of antimicrobial stewardship and infection prevention and control.

Among the ESBL-PE, the most frequent isolates were *E. coli* and *K. pneumoniae* both among hospitalized patients and healthy volunteers with frequencies of 71.3% and 15.7% respectively. Our results are similar to those reported by Ouédraogo et al.,

(4) in Burkina Faso and Tellevik et al., (14) in Tanzania, who both reported predominance of *E. coli* and *K. pneumoniae*. *Escherichia coli* is responsible for about 80% of urinary tract infections but is also implicated in suppurative infections while *K. pneumoniae* is frequently implicated in respiratory and post-operative infections. The high proportion of these two species among ESBL-PE is the cause of treatment failure for the infections that they may cause. Most studies on ESBL-PE carriage have shown a predominance of *E. coli* which may carry genes located on plasmids encoding the production of ESBLs, that could facilitate transfer to other enterobacteria (4,15).

The ESBL-PE showed resistance to meropenem of 47.4% and 22.4% respectively among hospitalized patients and healthy volunteers. This resistance to carbapenems is worrying because these are exclusive antibiotics used as last resort in the treatment of infections caused by ESBL-PE. This high resistance must have resulted from excessive prescription of carbapenems with the arrival of these antibiotics in generic forms. The ESBL-PE also showed high resistance to norfloxacine (86.2% among volunteers and 84.2% among inpatients) and sulfamethoxazole–trimethoprim (89.5% for inpatients and 82.8% for volunteers). The high resistance rate to fluoroquinolones and sulfamethoxazole–trimethoprim may have resulted from overuse due to easy access, and lack of control of these antibiotics in the markets. Plasmids carrying genes encoding ESBLs are known to also carry other genes conferring resistance to fluoroquinolones, aminoglycosides and cotrimoxazole (16). Our results are similar to those reported by Ouédraogo et al., (4) in Burkina Faso who showed that there were associated resistance of ESBL-PE to other families of antibiotics.

Analysis of the characteristics of the subjects and risk factors for faecal ESBL-PE carriage did not show any significant association with respect to age group, gender, previous use of antibiotics, and the types of antibiotics consumed by both the healthy volunteers and hospitalized patients. These findings are similar to those of Isendahl et al., (13) in 2012 in Guinea Bissau who did not observe any association between ESBL-PE carriage and previous consumption of antibiotics. However, our results contradicted those reported by Rodriguez-Bano et al., in Spain, Wu et al., in Hong Kong, and Ouédraogo et al., in Burkina Faso, who found association between ESBL-PE carriage and antibiotic use (4,17).

Conclusion:

Our study revealed the prevalence of rectal carriage of ESBL-PE to be 54.5% which showed that rectal carriage of ESBL-PE among hospitalized (55.7%) and healthy volunteers (53.5%) is high in Burkina Faso. The ESBL-PE were mainly represented by *E. coli* and *K. pneumoniae* with high resistance to norfloxacine and cotrimoxazole in both hospitalized and healthy volunteers. However, none of the factors analysed was significantly associated with the carriage of ESBL-PE. In light of these findings, it is desirable to establish surveillance program for multi-resistant bacteria, and institute antimicrobial stewardship in the country.

Authors' contributions:

SS, SI, OAS and IKS conceived and design of the study, SS and BS collected and analyse the samples, SS, SI, OAS, SaS, SY, PA., ZJ, DSNP, DMD and OB analyse and correct the results. All authors read and approved the final manuscript.

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Original Article **Open Access**

Characterization of biofilm formation in clinical urinary isolates of *Staphylococcus aureus* from five hospitals in Lagos State, Nigeria

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Abstract:

Background: Biofilm formation by pathogens is of great clinical importance as it mediates persistence and resistance to antibiotics, hence posing difficulty in treatment and management of diseases. The aim of this study was to evaluate the biofilm forming potential of *Staphylococcus aureus* isolated from urine samples of females with urinary tract infection and to detect the presence of clumping factor (*clfA*) and intracellular adhesion (*icaA*) encoding genes.
Methodology: A total of 50 *S. aureus* were obtained from urine samples of women in five hospitals in Lagos State, Nigeria. Isolates were confirmed by standard biochemical and novobiocin susceptibility tests. The isolates were screened for biofilm formation using three methods; Congo-red agar (CRA), tube, and tissue culture plate (TCP) methods. Detection of *clfA* and *icaA* genes was done by PCR.
Results: The Congo red agar method showed that 39 (78%) of the isolates were biofilm producers while 11 (22%) were non-biofilm producers. However, the tube method indicated that 12 (24%) were strong biofilm producers, 26 (52%) were moderate biofilm producers, and 12 (24%) were non-biofilm producers. The standard TCP assay showed that strong biofilm producers (OD > 0.240) were 13 (26%), moderate biofilm producers were 22 (44%), and weak or non-biofilm producers (OD < 0.120) were 15 (30%). The tube method showed a good correlation with the TCP method for strong biofilm production. Ten (20%) isolates possessed *clfA* gene and 31 (62%) possessed *icaA* gene.
Conclusion: The ability of *S. aureus* to form biofilm is a key risk factor that can increase morbidity and mortality from infections they cause. Hence, rapid and sensitive phenotypic methods can be used in screening for biofilm formation thereby providing data that can guide therapy and control of the pathogen

Keywords: *Staphylococcus aureus*, Biofilm, Clumping factor, Intracellular adhesion

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Caractérisation de la formation de biofilm dans des isolats urinaires cliniques de *Staphylococcus aureus* provenant de cinq hôpitaux de l'État de Lagos, Nigéria

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Abstrait:

Contexte: La formation de biofilm par des agents pathogènes est d'une grande importance clinique car elle intervient dans la persistance et la résistance aux antibiotiques, ce qui pose des difficultés dans le traitement et la gestion des maladies. Le but de cette étude était d'évaluer le potentiel de formation de biofilm de *Staphylococcus aureus* isolé à partir d'échantillons d'urine de femmes atteintes d'une infection des voies urinaires et de détecter la présence de gènes codant pour le facteur d'agglutination (*clfA*) et l'adhésion intracellulaire (*icaA*).
Méthodologie: Un total de 50 *S. aureus* a été obtenu à partir d'échantillons d'urine de femmes dans cinq hôpitaux de l'État de Lagos, au Nigéria. Les isolats ont été confirmés par des tests standard de sensibilité biochimique et à la novobiocine. Les isolats ont été criblés pour la formation de biofilm en utilisant trois méthodes; Méthodes de gélose au rouge Congo (CRA), de tubes et de plaques de culture tissulaire (TCP). La détection des gènes clfA et icaA a été réalisée par PCR.
Résultats: La méthode de l'agar rouge du Congo a montré que 39 (78%) des isolats étaient des producteurs de biofilm tandis que 11 (22%) n'étaient pas des producteurs de biofilm. Cependant, la méthode du tube a indiqué que 12 (24%) étaient de puissants producteurs de biofilm, 26 (52%) étaient des producteurs de biofilm modérés et 12 (24%) n'étaient pas des producteurs de biofilm. Le test TCP standard a montré que les producteurs de biofilm forts (DO > 0,240) étaient 13 (26%), les producteurs de biofilm modérés étaient de 22 (44%) et les producteurs de biofilm faibles ou non (DO < 0,120) étaient de 15 (30%). La méthode du tube a montré une bonne corrélation avec la méthode TCP pour une forte production de biofilm. Dix (20%) isolats possédaient le gène clfA et 31 (62%) possédaient le gène icaA.
Conclusion: La capacité de *S. aureus* à former un biofilm est un facteur de risque clé qui peut augmenter la morbidité et la mortalité dues aux infections qu'elles provoquent. Par conséquent, des méthodes phénotypiques rapides et sensibles peuvent être utilisées dans le dépistage de la formation de biofilm, fournissant ainsi des données qui peuvent guider la thérapie et le contrôle du pathogène.

Mots clés: *Staphylococcus aureus*, Biofilm, Facteur d'agrégation, Adhésion intracellulaire

Introduction:

Biofilms are communities of bacteria embedded in a self-produced extracellular matrix made of exopolysaccharides (EPSs) containing proteins and some macromolecules such as DNA. They can form on both biotic and abiotic surfaces (1). Studies have shown using scanning electron microscopy (SEM) and other molecular techniques that human tissues and medical implants are colonized by biofilms. Biofilm protects the microorganism from host defences and impedes delivery of antibiotics thereby resulting in the persistence of infections (2,3).

Staphylococcus aureus is an opportunistic pathogen implicated in skin and soft tissue infections. It exists in the nasopharynx, skin, eye, intestine and urogenital tract as normal flora. However, it can breach the skin barriers through the wound or surgical incision and cause infection. Furthermore, it has the ability to adhere to and form a biofilm on tissues or medical indwelling devices (4). *S. aureus* initially adheres to a solid substrate, after which cell–cell adhesion occurs; and the bacteria then multiply to form a multilayered biofilm encased in EPS.

Biofilm formation involves the production of polysaccharide intercellular adhesin, which depends on the expression of the intercellular adhesion molecule operon (*ica*ADBC) which encodes three membrane proteins (IcaA, IcaD, and IcaC) and one extracellular protein (IcaB). In addition, several surface proteins have been involved in the biofilm formation process in staphylococci including biofilm-associated protein; surface protein G, fibronectin-binding proteins and staphylococcal protein A (5). Biofilm formation by *S. aureus* can lead to delay in re-epithelialization of infected tissues, ultimately increasing healing time. *S. aureus* biofilms have been associated with chronic wounds such as diabetic foot ulcer, pressure sores and venous ulcers (6). Detachment of matured biofilm of *S. aureus* is a prerequisite for the dissemination of wound infection (7).

In Nigeria, there is paucity of data on studies that characterize biofilm formation in pathogens of clinical origin. Hence this study is aimed at characterizing biofilm formation in clinical *S. aureus* isolates.

Materials and method:

Study setting and subjects

The subjects were non-pregnant women presenting with urinary tract infection in five public hospitals; Lagos University Teaching hospital, General Hospital Marina, General Hospital Gbagada, General Hospital Shomolu, and General Hospital Orile-Agege in Lagos State, Nigeria. Samples were collected between February 2015 and April 2017.

Ethical approval

Ethical approval for this study was obtained from the Human Research and Ethical Committee (HREC) of the Lagos University Teaching Hospital with code number ADM/

DCST/HREC/879 and Health Research Ethics Committee of the Gbagada General Hospital with code number GBGH/705/100

Bacterial isolates

One millilitre of the urine sample was inoculated into 9mL of brain heart infusion broth (Oxoid, Basingstoke, UK) and incubated for 24 hours at 37°C. A loopful of broth culture was then streaked onto mannitol salt agar (Oxoid, Basingstoke, UK) and incubated for 24 hours at 37°C. Yellow colonies were picked and sub-cultured on nutrient agar to obtain pure colonies. Standard biochemical tests and novobiocin susceptibility test to phenotypically confirm *S. aureus* were carried out according to Cheesbrough (8), with *S. aureus* ATCC 29213 as quality control strain.

Biofilm formation assay

Biofilm formation assays were performed according to Mathur et al., (9) and Cavant et al., (10) with slight modifications. All 50 *S. aureus* isolates were tested by three *invitro s*creening procedures for their ability to form biofilm

Tube method

Staphylococcus aureus isolates were inoculated into brain heart infusion broth (Oxoid, Basingstoke, UK) and incubated for 24 hours at 37°C. Thereafter, spent broth were decanted and tubes were washed with phosphate buffered saline (pH 7.3) and allowed to air dry, and then stained with 0.1% crystal violet solution. Excess stain was discarded and tubes were washed three times with sterile distilled water and dried. A positive result was indicated by the presence of visible film lining the wall and bottom of the tube. The absence of a film or presence of only a stained ring at the liquid air interface was considered as a negative result. Biofilm forming potential were categorized base on the intensity of film as strong (+++), moderate (++), weak (+) (9). *S. aureus* ATCC 29213 was used as positive control

Congo-Red Agar (CRA) method

Suspension of *S. aureus* isolates were inoculated onto brain heart infusion agar (Oxoid, Basingstoke, UK) supplemented with 5% sucrose and 0.8 g/L Congo red. This was done in triplicate and incubated at 37°C for 24 to 48 hours. Culture plates that displayed black colonies with a dry crystalline consistency were noted as positive for biofilm formation while plates that had pink colonies with occasional darkening at the center were considered as weak or non-biofilm formers.

Tissue Culture Plate (TCP) method

Staphylococcus aureus isolates were inoculated into brain heart infusion broth (Oxoid, Basingstoke, UK) supplemented with 2% sucrose and incubated for 18 hours at 37°C. A dilution of 1in 100 with fresh medium was done and 200µL was dispensed into individual wells of sterile 96 well tissue culture plates. Positive control *S. aureus* ATCC 29213 strain was included in separate wells, while negative control was sterile broth medium. The tissue culture plates were incubated for 18 hours at 37°C, after which the content of each well was gently removed by tapping the plates. The wells were washed four times with 200µL of phosphate buffer saline (pH 7.2) to remove free-floating bacteria. Biofilm formed by adherent organisms in plate were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed off gently with deionized water and dye incorporated by the adherent cells was solubilized by adding 200µL of 33% glacial acetic acid (Merck, Darmstadt, Germany).

The optical density (OD) of each well was determined with an Emax® Plus Microplate Reader (Molecular Devices San Jose, CA) at wave length 570nm. The experiment was performed in triplicate and repeated three times. Absorbance was calculated by subtracting the OD_{570} of control from that of the test assays OD_{570} with mean value determined for each isolate. Data obtained were used to classify OD_{570} values < 0.120 as non-biofilm producers, OD_{570} values between 0.120-0.240 as moderate biofilm producers, and OD_{570} values > 0.240 as strong biofilm producers.

Molecular detection of *icaA* and *clfA* genes

DNA extraction was done using the method of Suvajdžić et al., (11). PCR was performed by targeting specific primers listed in Table 1. A 20µL PCR reaction containing 4µl (5x) FIREPol master mix [7.5mM $MgCl_2$, 1Mm

Table 1: List of primers targeted to detect *icaA* and *clfA* genes

Primer	Sequence (5'-3')	Product Size	Reference
icaA-FW	GAGGTAAAGCCAACGCACTC	151	(12)
icaA-RV	CCTGTAACCGCACCAAGTTT		
clfA-FW	ACCCAGGTTCAGATTCTGGCAGCG	165	(12)
clfA-RV	TCGCTGAGTCGGAATCGCTTGCT		

dNTPs, 0.4M Tris-HCl, 0.1M $(NH_4)_2SO_4$, 0.1% Tween-20, FIREPol DNA Polymerase) (Solis BioDyne, Estonia)], 0.6µL forward primer, 0.6µL reverse primer, 4µL DNA template and 10.8µL nuclease free water, was carried out in a Master cycler (Eppendorf AG, Hamburg, Germany). Amplification conditions consist of initial DNA denaturation at 95°C for 5min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 45°C for *clfA* and 54°C for *IcaA* for 30s and extension at 72°C for 2min, followed by a final extension at 72°C for 10min. Amplification products were analysed by electrophoresis at 100V for 60min in 2% agarose gel stained with ethidium bromide and visualized under ultraviolet trans-illuminator (Cleaver Scientific Ltd). A 100bp DNA ladder (Solis BioDyne, Estonia) was used as a molecular weight marker.

Statistical analysis

Microsoft Excel (Microsoft Cooperation, 2013 USA) was used to generate graphics and determine percentages

Results:

The three phenotypic biofilm characterization methods used yielded varying results. The CRA method indicated that 39 (78%) of the isolates were biofilm producers while 11 (22%) of the isolates were non-biofilm producers as shown in Fig 1. However, the tube method indicated that 12 (24%) were strong biofilm producers, 26 (52%) were moderate biofilm producers and 12 (24%) were non-biofilm producers. The standard TCP assay showed that strong biofilm producers (OD > 0.240) were 13 (26%), moderate biofilm producers were 22 (44%) and weak or non-biofilm producers (OD < 0.120) were 15 (30%). The tube method showed a good correlation with the TCP for strong biofilm producers as shown in Fig 2.

Despite the phenotypic display of biofilm formation by the *S. aureus* isolates, only 10 (20%) harboured clumping factor encoding gene (*clfA*) while the intracellular adhesion encoding gene (*icaA*) was detected in 31 (62%) isolates as shown in Fig 3.

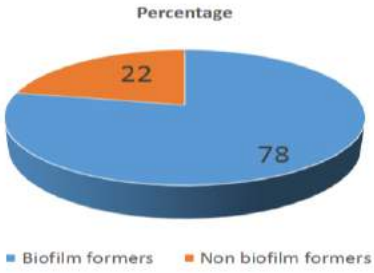


Fig 1: Frequency (%) of biofilm formation among *Staphylococcus aureus* isolates by Congo red agar method

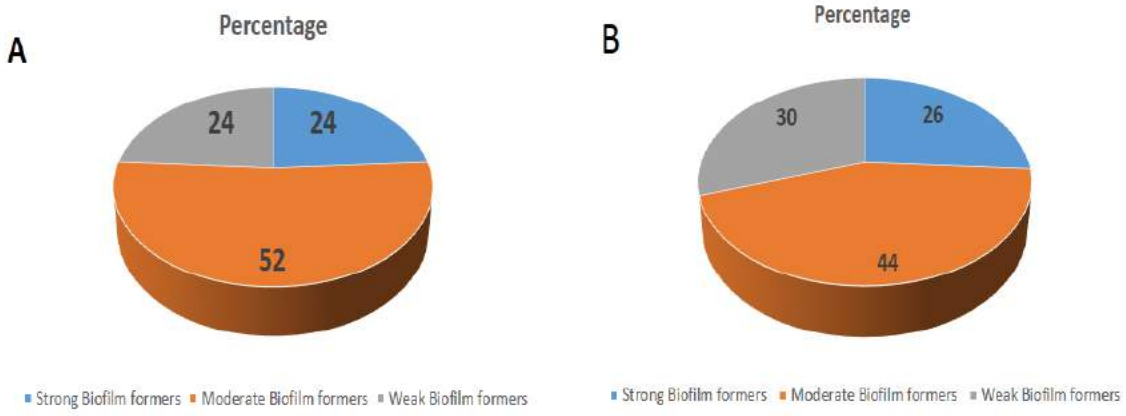
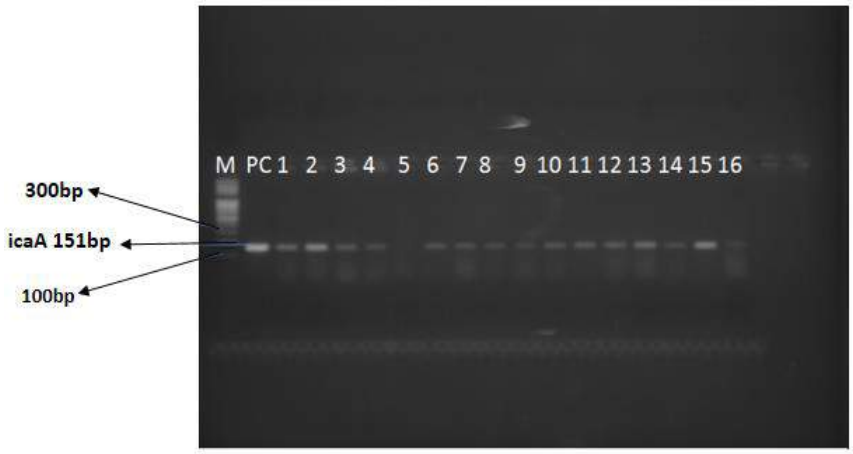


Fig 2: Frequency (%) of strong, moderate and weak biofilm formation among *Staphylococcus aureus* isolates by the tube (A) and TCP (B) methods



Lane M: 100bp molecular marker, Lane PC: Positive control, Lane 1-16 with the exception of Lane 5 are positive for *icaA* gene

Fig 3: Agarose gel image showing the 151 bp *icaA* gene detected in *Staphylococcus aureus* isolates

Discussion:

Biofilm forming *S. aureus* has been linked to life threatening infections that defies antimicrobial therapy due to resistance, which encourage persistence and easy dissemination (12). In this study, the TCP assay method revealed that 13 (26%) of the *S. aureus* isolates screened were strong biofilm formers, 44% were moderate biofilm formers, and 30% were weak or non-biofilm formers. This is similar to the findings of Mathur *et al*. (9) who reported 14.47%, 39.4%, and 46.0% as strong, moderate and weak *S. aureus* biofilm formers respectively. This method gives the best discrimination between strong, moderate and non-biofilm formers as it used cut-off values.

Although the tube method correlated well with the TCP strong biofilm formers in this study, weak formers were difficult to discriminate from non-biofilm formers. The tube method detected slightly lower number of strong biofilm formers with 24%, which was lower in comparison to the TCP method with 26%. However, the observation of Flemming et al., (13) showed that TCP method detected 69% of biofilm formers, whereas, TM detected only 36%. The authors further opined that this method could discriminate between strong and moderate biofilm formers since it is a quantitative method.

In this study, CRA detected 78% *S. aureus* isolates that were biofilm formers and 22% non-biofilm formers. However, this is at variance with previous observations made by Mathur et al., (9) and Taj et al., (14) who reported very low percentage 5.3%, and 3.4% of biofilm formers respectively by the CRA method. In a similar study by Sharvari and Chitra (15), a higher percentage (25.3%) was reported. The higher percentage reported in our study using the CRA method could be attributed to a modification in the preparation of the CRA in our laboratory by filter sterilization of the Congo red before addition to the molten autoclaved brain heart infusion agar.

Clumping factor A (clfA) has been reported to mediate adhesion to fibrinogen thereby enhancing the formation of biofilm in *S. aureus*. In the report of Wolz et al., (16), staphylococcal *clfA* cloned into *Streptococcus gordonii* and *Lactococcus lactis* which lack surface adhesion potentially gained the ability and were able to cause endocarditis in an animal model. PCR assay of *clfA* in this study revealed that 24% of the *S. aureus* isolates possessed the gene. In a similar study, Nourbakhsh and Namvar (17) reported a slightly higher carriage rate (41.4%) of *clfA* gene in *S. aureus* isolated from nosocomial infection in Isfahan Iran. As opposed to the low occurrence of *clfA* genes in our isolates, majority (62%, n=31) of them carried *icaA* gene. Kifaya et al., (18) reported that all *S. aureus* isolates assayed for *icaA* gene were positive for the gene and they all phenotypically formed biofilm, which agrees with our study where all 31 isolates carrying *icaA* gene phenotypically formed biofilm. The correlation of biofilm formation and the possession of *icaA* gene was also reported by Namvar et al., (12).

Conclusion:

Biofilm formation by microbial pathogens remains a great threat in the healthcare sector because infections caused by them are

associated with increased morbidity and mortality, as well as contributing to significant financial burden on the economy. Hence, the use of nanoparticles that have been shown to compromise integrity or destroy biofilms, may be explored in tackling the challenges of biofilm forming pathogens.

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Original Article **Open Access**

Comparative analysis of poliovirus-specific IgA and cytokine levels in the sera of *Ascaris lumbricoides*-infected and helminth-negative Nigerian children after oral poliovirus vaccination

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Abstract:

Background: Intestinal helminth infection is associated with altered immune responses and compromised vaccine efficacy in infected children. Altered immune response due to *Ascaris lumbricoides* infection may compromise efficacy of oral poliovirus vaccination in children. There is no information on humoral immune response during oral poliovirus (OP) vaccination of *A. lumbricoides*–infected Nigerian children. The objective of this study is to determine the serum levels of cytokines (tumour necrosis factor–alpha TNF-α, interferon-gamma IFN–γ, interleukins -4, -6, -8, -10) and poliovirus-specific IgA (PV-IgA) antibody in children infected with *A. lumbricoides* compared with helminth-negative children (control) before and after oral poliovirus vaccination.
Methodology: Twenty-three *A. lumbricoides*-infected children between ages 5-15 years (13 males and 10 females) and 23 age (4-15 years) and sex-matched helminth-negative children who met selection criteria were enrolled into the study after ethical approval and informed consent. Their stool samples were examined for helminth ova using concentration technique. Sera were collected before and 3 weeks after OP vaccinations, and serum concentrations of IFN–γ, TNF–α, IL-4, -6, -8, -10, and poliovirus-specific IgA concentrations were determined by enzyme-linked immunosorbent assay. The level of statistical significance was set at $\alpha_{0.05}$.
Results: Pre-vaccination serum levels of IFN–γ, IL–4, IL-6 and IL-8 were significantly higher in *A. lumbricoides*–infected children compared with pre-vaccination levels in helminth-negative children. Post-vaccination serum levels of IFN–γ, IL–4 and IL-8 were significantly higher in *A. lumbricoides*–infected children compared with post-vaccination serum levels in helminth-negative children. In the *A. lumbricoides*-infected children, pre-vaccination serum levels of IL-6 and IL-8 were significantly higher compared with post vaccination levels while pre-vaccination serum levels of IFN–γ, IL–4 and IL-8 were significantly higher in helminth-negative children compared with the post-vaccination levels. There was no significant reduction in post-vaccination median serum level of PV-IgA compared with level before vaccination in *A. lumbricoides*-infected children. Also, there was no significant increase in post-vaccination median serum level of PV-IgA compared with level before vaccination in helminth-negative children.
Conclusion: Oral polio vaccine administration caused decrease expression of inflammatory cytokines (IL-6 and IL-8) in *A. lumbricoides*-infected school children, and *A. lumbricoides* infection may reduce PV-IgA production following OP vaccination.

Keywords: *Ascaris lumbricoides* infection, cytokines, children, poliovirus vaccination

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Analyse comparative des taux d'IgA et de cytokines spécifiques du poliovirus dans les sérums d'enfants Nigérians infectés par *Ascaris lumbricoides* et négatifs pour les helminthes après vaccination orale contre le poliovirus

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Abstrait:

Contexte: L'infection intestinale par les helminthes est associée à des réponses immunitaires modifiées et à une efficacité du vaccin compromise chez les enfants infectés. Une réponse immunitaire modifiée due à une infection à *Ascaris lumbricoides* peut compromettre l'efficacité de la vaccination antipoliomyélitique orale chez les enfants. Il n'y a pas d'informations sur la réponse immunitaire humorale lors de la vaccination antipoliomyélitique orale (OP) des enfants nigérians infectés par *A. lumbricoides.* L'objectif de cette étude est de déterminer les taux sériques de cytokines (facteur de nécrose tumorale–alpha TNF-α, interféron-gamma IFN–γ, interleukines -4, -6, -8, -10) et IgA spécifiques du poliovirus (PV-IgA) chez les enfants infectés par *A. lumbricoides* par rapport aux enfants anti-helminthes (témoin) avant et après la vaccination antipoliomyélitique orale.
Méthodologie: Vingt-trois enfants infectés par *A. lumbricoides* âgés de 5 à 15 ans (13 hommes et 10 femmes) et 23 enfants (4 à 15 ans) et enfants de sexe masculin négatifs pour les helminthes qui répondaient aux critères de sélection ont été inclus dans l'étude après approbation éthique et consentement éclairé. Leurs échantillons de selles ont été examinés pour les ovules d'helminthes en utilisant une technique de concentration. Les sérums ont été collectés avant et 3 semaines après les vaccinations OP, et les concentrations sériques d'IFN–γ, de TNF–α, d'IL-4, -6, -8, -10 et d'IgA spécifiques du poliovirus ont été déterminées par un test d'immunosorbant lié à une enzyme. Le niveau de signification statistique a été fixé à α0,05.
Résultats: Les taux sériques d'IFN–γ, d'IL–4, d'IL-6 et d'IL-8 avant la vaccination étaient significativement plus élevés chez les enfants infectés par *A. lumbricoides* par rapport aux niveaux avant la vaccination chez les enfants négatifs pour les helminthes. Les taux sériques d'IFN–γ, d'IL–4 et d'IL-8 après la vaccination étaient significativement plus élevés chez les enfants infectés par *A. lumbricoides* par rapport aux taux sériques après vaccination chez les enfants négatifs pour les helminthes. Chez les enfants infectés par *A. lumbricoides*, les taux sériques d'IL-6 et d'IL-8 avant la vaccination étaient significativement plus élevés par rapport aux niveaux après vaccination, tandis que les taux sériques d'IFN–γ, d'IL–4 et d'IL-8 avant la vaccination étaient significativement plus élevés plus élevé chez les enfants négatifs aux helminthes par rapport aux niveaux post-vaccination. Il n'y a pas eu de réduction significative du taux sérique médian de PV-IgA après la vaccination par rapport au niveau avant la vaccination chez les enfants infectés par *A. lumbricoides*. En outre, il n'y avait pas d'augmentation significative du taux sérique médian de PV-IgA après la vaccination par rapport au niveau avant la vaccination chez les enfants négatifs pour les helminthes.
Conclusion: L'administration du vaccin antipoliomyélitique oral a entraîné une diminution de l'expression des cytokines inflammatoires (IL-6 et IL-8) chez les écoliers infectés par *A. lumbricoides*, et l'infection par *A. lumbricoides* peut réduire la production de PV-IgA après la vaccination OP.

Mots clés: infection à *Ascaris lumbricoides*, cytokines, enfants, vaccination contre le poliovirus

Introduction:

Ascaris lumbricoides (also known as roundworms) belongs to a group of intestinal parasitic worms known as soil-transmitted helminths (STH) (1). STH infects over one and half billion people worldwide which correspond to about 24% of population of the world with very high prevalence in China, the Americas, sub-Saharan Africa and East Asia (1). It was approximated that *A. lumbricoides* alone infects about 819 million people worldwide (2). In Nigeria, it is the commonest among the STH infection that affect children who usually require extensive vaccination (3). Morbidity is related to the worm intensity. Minor infection with the parasite often gives no symptoms but heavy infection may result in symptoms such as passing of worms in faeces, abdominal discomfort, intestinal ulceration, cough, bloody sputum and fever (4).

Ascaris lumbricoides infection is associated with mast cells hyperplasia, eosinophilia and high levels of circulating immunoglobulin E which confers protective immune response against the invading larvae, but may also be a marker of enhanced type–2 immune response (5,6). Some studies have also demonstrated marked Th-2 cytokine responses in *A. lumbricoides* infection and the roles of IL-4, IL-5, IL-9 and IL-13–associated pathways in the mediation of resistance to the infection and the expulsion of the helminthic parasite (7).

Polioviruses are RNA viruses that colonize the gastrointestinal tract particularly the intestine and oropharynx. They infect humans alone and are of three serotypes; poliovirus type 1 (PV1), type 2 (PV2), and type 3 (PV3), with slight differences between each based on the make-up of their capsid protein (8). Vaccination against poliovirus infection came about in 1955 with the introduction of live-attenuated oral polio vaccine (OPV) (Sabin types 1, 2 and 3) and 1961, with inactivated (killed) polio vaccine (IPV). OPV has however been the choice vaccine for the global eradication programme based on its action on mucosal immunity, very low cost, and the ease of oral administration of the vaccine.

OPV produces antibodies to all the three poliovirus strains in the blood and protect individuals against nerve paralysis if infected with poliovirus by preventing the spread of the virus to the nervous system but also potect individuals from being infected with poliovirus by producing local immune response in the mucous lining of the intestine, which is the primary poliovirus multiplication site (9). Vaccination against poliovirus induces strong Th-1 response (IL-2

and IFN-γ) which confers protective immune response in vaccinated hosts (10). However, helminth infection may affect responses to vaccine through the expression of regulatory cytokines (IL-10) or cause Th-1 to Th-2 shift, which down-regulates the expression of Th-1 cytokines following vaccination (11). Studies that investigate factors influencing the efficacy of OP vaccines among Nigerian children are sparse and are therefore necessary.

Low immunogenic responses to routine vaccinations have been reported among populations in low-income-countries when compared with those in the developed countries (12,13). Several factors such as maternal trans-plancental antibody titres, micronutrient malnutrition, breast-feeding practices, stomach acidity and interfering gut flora have been reported to be responsible for these observations (14). STH infections have generally been proposed to contribute to malnutrition and low intelligent quotient in children, as a result of reduction in digestion and absorption, helminth induced chronic inflammation, and loss of nutrients (15). Unfortunately, little attention has been paid to the possible effect of STH, especially *A. lumbricoides* infection on vaccine efficacy, specific micronutrients levels or specific vaccine immune factors. There is therefore the need to assess vaccine-specific immune status of Nigerian children in whom vaccine administration is compulsory and helminth infection is common. This comparative study determined the serum poliovirus-specific IgA antibody and cytokine levels (TNF–α, IFN–γ, IL-4, IL-6, IL-8, and IL-10) in *Ascaris lumbricoides*-infected and helminth-negative school-aged children before and three weeks after oral poliovirus vaccinations.

Materials and method:

Study setting and participants

The study center, participant selection, collection and examination of stool have earlier been reported (6). Briefly, of 349 preschool and school aged children who were screened for intestinal helminth infection, 23 children age 5–15 years, whose stool microscopic examination revealed only *A. lumbricoides*, and met other inclusion criteria were selected as the study subjects. Twenty-three gender matched children age 4–15 years, whose stool sample revealed no eggs or larvae of any stool helminth were selected as controls and regarded as helminth–negative.

All study participants were apparently healthy and with no sign of any infection. The parents claimed that the children received oral polio vaccines at infancy. Any child on medication, with malaria parasites and whose parent refused participation were excluded from the study.

Ethical consideration

Ethical clearance was obtained from the University of Ibadan/University College Hospital Joint Ethics Committee (UI/EC/ 13/0331) and Oyo State Ministry of Health (AD/13/479/517). Participants were enrolled following town-hall and the parents-teachers association's health awareness meetings carried out in the communities and schools respectively. Children whose parents consented to participate in the study were recruited. A general de-worming exercise was carried out at the end of the exercise.

Stool specimen collection and processing

Faecal specimen was scooped using spatula into a labeled screw cap polystyrene bottle and tightly screwed. The stool specimens were examined microscopically within 12 hours of collection using the formol-ether concentration technique for helminth identification (16). The magnifications of x10 and x40 objectives of the light microscope were used to identify characteristic ova of the intestinal helminth.

Blood sample collection and processing

Blood sample collection was carried out before vaccination and three weeks after vaccination from each child. At each instance, five millilitres of venous blood was obtained from the antecubital vein and dispensed into plain polysterene bottle. The blood samples were allowed to clot, and the clotted samples retracted and spun at 3000rpm for 10 min. The sera were removed into plain sterile cryo-precipitate tube and frozen at -20^{o}C until analysis.

Procedure for oral poliovirus vaccination

The oral poliovirus vaccine (Sabin, GlaxoSmithkline) was supplied in glass vials with droppler and stored at 4^{o}C inside a thermos flask. It was allowed to attain normal temperature before administration. The child's mouth was opened gently between fingers to make the child's lips point outward. The dropper was held over the child's mouth at an angle of about 45 degrees and two drops of the vaccine was dropped onto the rear part of the child's tongue. Each child was allowed to resume normal sitting position observed for about 10 minutes after the administration of the vaccine, to ensure it was not vomited.

Laboratory estimation of cytokines and IgA by ELISA

The serum levels of IFN–γ, TNF–α, IL-4, IL-8, IL-6, IL-10 and PV-specific IgA

were determined using enzyme linked immunosorbent assay (ELISA) as described by the manufacturers (Abcam, MA, USA; AssayPro, MO, USA; Calbiotech, USA, and Sunlong Biotech, Hangzou, China) and as previously carried out (6). The ELISA was based on direct antigen-antibody interaction. Protein antigens present in patient's sample were allowed to bind in wells of plate pre-coated with antibodies. The plate was washed after a period of incubation to remove the remaining sample components and reduce interference. To this plate, a corresponding second enzyme-linked antibody was added, which catalyzed the conversion of a suitable substrate to produce a colour reaction. The colour produced was measured as a function of antigens present in the sample.

Statistical analysis

The data generated were expressed as median (interquartile range) and represented as figures in percentages and tables. Statistical data evaluation was carried out using the Statistical Package for the Social Sciences (SPSS) version 21.0. The Mann-Whitney U test was used to compare differences in levels of serum cytokines between *Ascaris lumbriocides* infected and helminth-negative subjects. Wilcoxon Signed Ranks test was used to compare pre- and post-vaccinated cytokines levels in both groups. The level of statistical significance was set at $\alpha_{0.05}$.

Results:

Table 1 shows the serum cytokine levels of *A. lumbricoides*-infected children compared with helminth-negative children before OP vaccination. Serum levels of IFN–γ (113.41 [IQR 68.41-146.52] vs 67.21 [IQR 23.46–93.29]pg/ml, p=0.014), interleukin-4 (191.2 [IQR 127.9-320.6] vs 92.7 [IQR 66.8-151.1]pg/ml,p=0.001), interleukin-8 (1211.1 [IQR 696.4-1226.7] vs 778.4 [IQR 232.9–899.9]pg/ml, p=0.014) and interleukin-6 (13.12 [IQR 9.37-22.15] vs 5.54 [IQR 3.02-7.29] pg/ml, p=0.000) were significantly higher in *A. lumbricoides*-infected children compared with helminth-negative children. The serum levels of TNF–α (50.90 [IQR 40.41-69.34] vs 40.09 [IQR 32.97 - 58.30] pg/ml, p=0.145) and IL-10 (0.11 [IQR 0.05-0.61] vs 0.12 [IQR 0.06 - 0.43]ng/ml, p= 0.720) in *A. lumbricoides*-infected children compared with helminth-negative children were not statistically significant.

Table 2 shows the serum cytokine levels in *A. lumbricoides*-infected children compared with helminth-negative children after oral polio vaccination. Post vaccination serum levels of IFN–γ (96.23 [IQR 74.83-123.29] vs 25.86 [IQR 17.01-30.57]pg/ml, p=0.000), IL-4 (170.8 [IQR 133.0-199.3] vs 41.1 [IQR 31.2-64.4]pg/ml, p=0.000) and IL-8 (805.6 [IQR 603.2-821.4] vs 233.4 [IQR 205.0-251.7]pg/ml, p=0.000) were significantly higher in *A. lumbricoides*-infected children compared with post-vaccination levels in helminth-negative children. The post -vaccination serum levels of TNF–α (44.19 [IQR 35.41 - 54.59] vs 33.53 [IQR 29.12-51.56]pg/ml, p=0.063), IL-6 (7.58 [6.01-10.55] vs 6.33 [IQR 3.87 - 8.17]pg/ml, p=0.174) and IL-10 (0.08 [IQR 0.06-0.21] vs 0.09 [IQR 0.08-0.19]ng/ml, p=0.800) in *A. lumbricoides*–infected children compared with helminth-negative children were not statistically significant.

Table 1: Serum cytokine levels *Ascaris lumbricoides* -infected children compared with helminth-negative childrenbefore oral polio vaccination

Serum values	*A. lumbricoides*-infected (n=23)	Helminth - negative (n=23)	U	p-value
IFN–γ (pg/ml)	113.81 (68.41-146.52)	67.21 (23.46-93.29)	36.000	0.014*
TNF–α (pg/ml)	50.90 (40.41-69.34)	40.09 (32.97-58.30)	56.000	0.145
IL–4 (pg/ml)	191.2 (127.9-320.6)	92.7 (66.8-151.1)	21.000	0.001*
IL–10 (ng/ml)	0.11 (0.05-0.61)	0.12 (0.06-0.43)	78.000	0.720
IL-8 (pg/ml)	1211.1 (696.4-1226.7)	778.4 (232.9-899.9)	36.000	0.014*
IL-6 (pg/ml)	13.12 (9.37-22.15)	5.54 (3.02-7.29)	12.000	0.000*

*Significantat $\alpha_{0.05}$; U - Mann Whitney U Test; *A. lumbricoides – Ascaris lumbricoides*

Table 2: Serum cytokine levels in *Ascaris lumbricoides* -infected children compared with helminth-negative children after oral polio vaccination

Serum values	*A. lumbricoides*-infected (n=23)	Helminth -negative (n=23)	U	p-value
IFN–γ (pg/ml)	96.23 (74.83-123.29)	25.86 (17.01-30.57)	0.000	0.000*
TNF–α (pg/ml)	44.19 (35.41-54.59)	33.53 (29.12-51.56)	48.000	0.063
IL–4 (pg/ml)	170.8 (133.0-199.3)	41.1 (31.2 - 64.4)	4.000	0.000*
IL–10 (ng/ml)	0.08 (0.06-0.21)	0.09 (0.08-0.19)	80.000	0.800
IL-8 (pg/ml)	805.6 (603.2-821.4)	233.4 (205.0-251.7)	6.000	0.000*
IL-6 (pg/ml)	7.58 (6.01-10.55)	6.33 (3.87-8.17)	58.000	0.174

*Significant at $\alpha_{0.05}$; U - Mann Whitney U Test; *A. lumbricoides – Ascaris lumbricoides*

Table 3: Serum cytokine levels in *Ascaris lumbricoides*-infected school children before and after oral polio vaccination

Serum values	Pre-vaccination (n=23)	Post-vaccination (n=23)	Z	p-value
IFN–γ (pg/ml)	113.81 (68.41-146.52)	96.23 (74.83-123.29)	0.260	0.795
TNF–α (pg/ml)	50.90 (40.41-69.34)	44.19 (35.41-54.59)	0.876	0.381
IL–4 (pg/ml)	191.2 (127.9-320.6)	170.8 (133.0-199.3)	1.444	0.149
IL–10 (ng/ml)	0.11 (0.05-0.61)	0.08 (0.06-0.21)	1.642	0.101
IL-8 (pg/ml)	1211.1 (696.4-1226.7)	805.6 (603.2-821.4)	2.208	0.027*
IL-6 (pg/ml)	13.12 (9.37-22.15)	7.58 (6.01-10.55)	3.011	0.003*

*Significant at $\alpha_{0.05}$; Z - Wilcoxon Signed Ranks Test

Table 3 shows the serum cytokine levels in *A. lumbricoides*-infected children before and after oral polio vaccination. Pre-vaccination serum levels of IL-8 (1211.1 [IQR 696.4 - 1226.7] vs 805.6 [IQR 603.2-821.4]pg/ml, p=0.027) and IL-6 (13.12 [IQR 9.37-22.15] vs 7.58 [IQR 6.01-10.55]pg/ml, p=0.000) were significantly higher compared with post-vaccination levels. The pre-vaccination serum levels of IFN–γ (113.81 [IQR 68.41-146.52] vs 96.23 [IQR 74.83-123.29] pg/ml, p=0.795), TNF–α (50.90 [IQR 40.41-69.34] vs 44.19 [IQR 35.41-54.59]pg/ml, p=0.381), IL-4 (191.2 [IQR 127.9-320.6] vs 170.8 [IQR 133.0-199.3]pg/ml, p=0.149) and IL-10 (0.11 [IQR 0.05-0.61] vs 0.08 [IQR 0.06-0.21]ng/ml, p=0.101) compared with post vaccination levels were not statistically significant.

Table 4 shows the serum cytokine levels in helminth-negative children before and after oral poliovirus vaccination. Pre-vaccination serum level of IFN–γ (67.21[IQR 23.46-93.29] vs 25.86 [IQR 17.01-30.57] pg/ml, p=0.037), IL-4 (92.7 [IQR 66.8-151.1] vs 41.1 [IQR 31.2-64.4]pg/ml, p= 0.013) and IL-8 (778.4 [IQR 232.9-899.9] vs 233.4 [IQR 205.0-251.7]pg/ml, p=0.012) were significantly higher compared with post-vaccination levels. The pre-vaccination serum levels of TNF–α (40.09 [IQR 32.97-58.30] vs 33.53 [IQR 29.12-51.56]pg/ml, p=0.114), IL-10 (0.12 [IQR 0.06-0.43] vs 0.09 [IQR 0.08-0.19]ng/ml, p=0.201), and IL-6 (5.54 [IQR 3.02-7.29] vs 6.33 [IQR 3.87-8.17] pg/ml, p=0.241) compared with post vaccination levels were not statistically significant.

Table 4: Serum cytokine levels in helminth uninfected school children before and after oral polio vaccination

	Pre-vaccinated (n=23)	Post-vaccinated (n=23)	Z	p-value
IFN–γ (pg/ml)	67.21 (23.46-93.29)	25.86 (17.01-30.57)	2.090	0.037*
TNF–α (pg/ml)	40.09 (32.97-58.30)	33.53 (29.12-51.56)	1.580	0.114
IL–4 (pg/ml)	92.7 (66.8-151.1)	41.1 (31.2 - 64.4)	2.497	0.013*
IL–10 (ng/ml)	0.12 (0.06-0.43)	0.09 (0.08-0.19)	1.279	0.201
IL-8 (pg/ml)	778.4 (232.9-899.9)	233.4 (205.0-251.7)	2.505	0.012*
IL-6 (pg/ml)	5.54 (3.02-7.29)	6.33 (3.87-8.17)	1.174	0.241

*Significant at $\alpha_{0.05}$; Z - Wilcoxon Signed Ranks Test

Table 5: Serum levels of Poliovirus-Specific IgA in school aged children with and without helminth *Ascaris lumbricoides* before and after oral polio vaccination

		Poliovirus specific –IgA (U/ml)
Pre-vaccination	*A. lumbricoides*-positive(n=23)	1.831 (1.609 – 2.575)
	Helminth-negative (n=23)	1.983 (1.368 – 4.234)
Post-vaccination	*A. lumbricoides*-positive(n=23)	1.782 (1.381 – 2.979)
	Helminth-negative (n=23)	2.488 (1.597 – 3.641)
Z, p-value[a]		0.213, 0.831
Z, p-value[b]		0.153, 0.878
U, p-value[c]		0.025, 0.980
U, p-value[d]		0.209, 0.223

*Significant at $\alpha_{0.05}$; [a]*A. lumbricoides*-positive pre-vaccination vs *A. lumbricoides*-positive post-vaccination; [b]Helminth-negative pre-vaccination vs Helminth-negative post–vaccination; [c]*A. lumbricoides*-positive pre-vaccination vs Helminth-negative pre-vaccination; [d]*A. lumbricoides*-positive post-vaccination vs Helminth-negative post–vaccination; U=Mann Whitney U Test; Z=Wilcoxon Signed Ranks Test

Table 5 shows the serum levels of poliovirus-specific IgA (PV-IgA) in *A. lumbricoides*-infected and helminth-negative children before and after oral poliovirus vaccination. The post-vaccination median serum level of PV-IgA compared with pre-vaccination level in *A. lumbricoides*-infected children (1.782 [1.381–2.979] vs 1.831 [1.609–2.575] U/ml) was not statistically significant (p=0.831). Similarly, the post-vaccination median serum level of PV-IgA compared with pre-vaccination serum level in helminth-negative children (2.488 [1.597–3.641] vs 1.983 [1.368–4.234] U/ml) was not statistically significant (p=0.878). Also, post-vaccination median serum level of PV-IgA in *A. lumbricoides*-infected children compared with post vaccination serum level in helminth negative children (1.782 [1.381–2.979] vs 2.488 [1.597–3.641] U/ml) was not statistically significant (p=0.233). There was also no significant difference (p=0.980) in serum PV-IgA levels of helminth-negative children compared with *Ascaris lumbricoides*-infected school aged children (1.983 [1.368–4.234] vs 1.831 [1.609–2.575] U/ml).

Discussion:

Global efforts at eradicating poliomyelitis through vaccination has recorded successes but not without challenges. The dramatic progress in reducing the virus incidence had been made as at year 2000 (17) but polio has remained endemic in few countries (8,17). Failure of the oral poliovirus vaccine and the emergence of circulating vaccine-derived polioviruses (cvPV) are part of the main challenges facing global eradication of the disease (18). While several reasons and solutions have been postulated as means of improving poliovirus immuni-

zation outcome, studies that investigate the immuno-modulatory effects of intestinal helminth infection as possible reason for failure of OPV in the affected countries were not encountered. Our study therefore focused on the dynamics of the interplay between pro-inflammatory and anti-inflammatory cytokine responses following vaccination, which conversely may give insight into the effectiveness of OPV in *Ascaris lumbricoides*-infected Nigerian children.

Significantly higher serum levels of IFN–γ and IL-4 were observed in *Ascaris lumbricoides*-infected children compared to helminth-negative children before vaccination (Table 1) and after vaccination (Table 2). IFN-γ is a cytokine that is critical for the innate and adaptive immunity against viral, some bacterial and protozoal infections. It is produced predominantly by natural killer and natural killer T-cells as part of the innate immune response, and by CD4 Th-1 and CD8 cytotoxic effector T-lymphocytes in cases of specific immunity (19). IL-4 production by leukocytes is a key regulatory event that occurs early in the type-2 immune response, which induces allergic reactions and mediates expulsion of parasites. $CD4^+$ T-cells and basophils are thought to be the key cell types that produce IL-4 during a type-2 response (20). Studies have demonstrated the reciprocal roles of IFN-γ and IL-4 in worm expulsion. Depletion of IFN-γ and increased expression of IL-4 with significant IgE secretion is required for worm expulsion (21). The increased IFN-γ in *A. lumbricoides* infected children in this study is not in consonance with earlier findings (21) but the increased expression of IL-4 agrees with what was observed in a previous study (22). The raised IFN-γ seen in our study may be attributed to a systemic inflammation in the *Ascaris lumbricoides*-infected children since the children considered for this study were apparently healthy, with no known laboratory confimed viral, bacterial and protozoal infections. However, raised IL-4 level might be attempt by the host to expel the worm.

IL-8 is a pro-inflammatory cytokine, produced by a wide variety of cells including neutrophils, T-lymphocytes, monocytes, vascular endothelial cells, dermal fibroblasts, hepatocytes and keratinocytes. It functions majorly in neutrophil activation and recruitment (23). Th-2 lymphocyes also contribute to eosinophil differentiation and recruitment which in turn secrete IL-8 (24). Increased eosinophils are observed in acute helminth infection through helminth-induced IL-5 secretion which induces eosinophil proliferation and differentiation (25). Significantly higher serum IL-8 levels in *A. lumbricoides*-infected children compared with helminth-negative children in this study agrees with with earlier findings (26), and may be associated with helminth–associated eosinophilia, because IL-8-mediated neutrophil proliferation and activation may occur due to *A. lumbricoides*–induced hypersensitivity reaction (27). This may also be associated with post-vaccination occurence.

IL-6 is a highly pleiotropic molecule, with diverse pro- and anti-inflammatory properties depending on prevailing circumstance (28). It is involved in the induction of switch from neutrophil to monocytes recruitment by suppressing neutrophil-attracting chemokines and enhancing neutrophil apoptosis thereby contributing to the resolution of acute neutrophil infiltration (29). IL-6 has been reported to limit Th-2 responses, modifies T*reg*-cell phenotype, and promotes host susceptibility following helminth infection (30). The higher serum IL-6 levels in *A. lumbricoides*-infected children compared with helminth-negative children in our study is similar to that of Nagy et al., (31) who reported elevated IL-6 level in children with *Toxocara canis* infection. This may be attributed to the role of IL-6 as an enhancer of Th-2 cell differentiation involved in the control of helminth infection. The observed lower post vaccination serum level of IL-6 in OP-vaccinated *A. lumbricoides*–infected children compared with the pre-vaccination level might suggest inhibitory effect of *A. lumbricoides*–induced Th-2 immunity on IL-6. This may also support the function of IL-6 as promoting host susceptibility to helminth infection (30). The reduced serum levels of IL-6 and IL-8 in *Ascaris lumbricoides*-infected children after oral polio vaccination compared with serum levels before polio vaccination is an indication that decreased expression of inflammatory cytokines may be one of the mechanisms by which *A. lumbricoides* reduces efficacy of oral polio vaccine. This finding will require further investigation.

Vaccines induce immune effector cells which are majorly antibodies produced by B-lymphocytes and cytotoxic $CD8^+$ T lymphocytes that may recognise and kill evaded cells or secrete specific antiviral cytokines (32). These antibodies productions are supported by factors and signals made available by the $CD4^+$ T helper cells, which are of T helper 1 (Th-1) and T helper 2 (Th-2) subtypes (33). However, the major limitation of our study is the small sample size which compelled us to cautiously conjectured that *A. lumbricoides* infection may reduce efficacy of poliovirus vaccine because of reduction, albeit stastically insignificant, in post-vaccination serum level of PV-IgA antibody in *A. lumbricoides*-infected children compared with serum PV-IgA antibody level before vaccination.

Conclusion:

In this study, oral poliovirus (OP) vaccination caused decrease expression of inflammatory cytokines (IL-6 and IL-8) in *A. lumbricoides*-infected school children, and *A. lumbricoides* infection may reduce PV-IgA production following OP vaccination.

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Authors declare no conflict of interest

Authors contributions:

KSA collected the data, carried out the study and ran the data analyses. GOA conceived the study, designed the study and edited the final manuscript.

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Original Article **Open Access**

Haemoglobin phenotypes and the risk of asymptomatic malaria parasitemia among blood donors in northwest Nigeria: clinical implications in the practice of tropical transfusion medicine

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Abstract:

Background: In malaria-endemic populations, sickle cell trait (SCT) protects against both severe and non-severe malaria, but inconsistencies exist about protective effect of SCT on asymptomatic malarial parasitemia (AMP). Surprisingly, the effect of Hb-phenotypes on AMP has not been explored among blood donors in Nigeria or other malaria-endemic countries, where risks of AMP and transfusion transmitted malaria (TTM) are high. The objective of this study is to determine risk of AMP with respect to donor Hb-phenotypes (SCT versus HbAA), and elucidate clinical implications of AMP with respect to risk of TTM vis-à-vis the practice of transfusion medicine in Nigeria, and by implication other malaria-endemic tropical countries.
Methodology: Analysis of 100 blood donors with AMP (cases) and 100 donors without AMP (controls) was performed. Frequencies of SCT and HbAA (determined by Hb electrophoresis) among cases and controls were compared by X^2-test. Risks of AMP (detected by microscopy) with respect to Hb-phenotypes were expressed as Odds ratios (OR) by case-control logistic regression.
Results: In comparison with blood donor without AMP (controls), donors with AMP had lower frequencies of SCT (12% vs 28%, $p<0.05$) with corresponding higher frequencies of HbAA (88% vs 72%, $p<0.05$). HbAA is associated with high risk of AMP (OR=2.91, 95%CI: 2.10-3.48, $p=0.021$), while SCT is associated low risk of AMP (OR=0.49, 95%CI: 0.27-0.73, $p=0.032$).
Conclusion: This finding shows that donor SCT is a surreptitious mitigator of the risk of AMP and TTM in the tropics. Therefore, patients who are selectively transfused with HbAA blood (e. g. neonates and sickle cell disease patients) could be at greater risks of TTM, and such patients need closer post transfusion monitoring. The risk of TTM calls for diligent post transfusion haemovigilance in Nigeria and other malaria endemic tropical countries in Africa

Keywords: blood donors, sickle cell trait, asymptomatic malaria parasitemia, transfusion transmitted malaria

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Phénotypes d'hémoglobine et risque de parasitémie asymptomatique du paludisme chez les donneurs de sang dans le nord-ouest du Nigéria: implications cliniques dans la pratique de la médecine transfusionnelle tropicale

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Abstrait:

Contexte: Dans les populations d'endémie palustre, le trait drépanocytaire (SCT) protège à la fois contre le paludisme grave et non sévère, mais des incohérences existent quant à l'effet protecteur de la SCT sur la parasitémie asymptomatique du paludisme (AMP). De manière surprenante, l'effet des phénotypes Hb sur l'AMP n'a pas été exploré chez les donneurs de sang au Nigéria ou dans d'autres pays d'endémie palustre, où les risques de PMA et de paludisme transmis par transfusion (TTM) sont élevés. L'objectif de cette étude est de déterminer le risque d'AMP par rapport aux phénotypes Hb des donneurs (SCT versus HbAA), et d'élucider les implications cliniques de l'AMP en ce qui concerne le risque de TTM vis-à-vis de la pratique de la médecine transfusionnelle au Nigéria, et par implication d'autres pays tropicaux d'endémie palustre.
Méthodologie: Une analyse de 100 donneurs de sang avec AMP (cas) et 100 donneurs sans AMP (témoins) a été réalisée. Les fréquences de SCT et d'HbAA (déterminées par électrophorèse Hb) parmi les cas et les témoins ont été comparées par test X2. Les risques d'AMP (détectés par microscopie) par rapport aux phénotypes Hb ont été exprimés en odds ratios (OR) par régression logistique cas-témoins.
Résultats: En comparaison avec les donneurs de sang sans AMP (témoins), les donneurs avec AMP avaient des fréquences plus faibles de SCT (12% vs 28%, p<0,05) avec des fréquences plus élevées correspondantes d'HbAA (88% vs 72%, p<0,05). L'HbAA est associée à un risque élevé d'AMP (OR=2,91, IC à 95%: 2,10-3,48, p=0,021), tandis que la SCT est associée à un faible risque d'AMP (OR=0,49, ICà95%: 0,27-0,73, p=0,032).
Conclusion: Cette découverte montre que le donneur SCT est un atténuateur subreptice du risque d'AMP et de TTM dans les tropiques. Par conséquent, les patients qui sont sélectivement transfusés avec du sang HbAA (par exemple, les nouveau-nés et les patients atteints de drépanocytose) pourraient être plus à risque de TTM, et ces patients ont besoin d'une surveillance post-transfusionnelle plus étroite. Le risque de TTM appelle une hémovigilance post-transfusionnelle diligente au Nigéria et dans d'autres pays tropicaux endémiques du paludisme en Afrique

Mots clés: donneurs de sang, trait drépanocytaire, parasitémie asymptomatique du paludisme, paludisme transmis par transfusion

Introduction:

Haemoglobin S (HbS) is the best characterized human genetic polymorphism that is strongly associated with resistance to malaria (1). HbS is a structural variant of HbA that arose as a result of GAG > GTG base transition at codon-6 of the β-globin gene on chromosome-11, which corresponds to the substitution of glutamic acid (polar, hydrophilic amino acid) by valine (neutral, hydrophobic amino acid) at position-6 of the β-globin chain of the haemoglobin molecule (1). Consequently, HbS has less anionic potential, slower electrophoretic mobility and reduced deoxygenated solubility that leads to polymerization, and red cell sickling (2). The sickle cell trait (SCT) refers to the heterozygous inheritance of the sickle β-gene (3). The SCT protects against severe falciparum malaria and confers survival advantage in populations living in malaria endemic countries (4). This is attained through the process of natural selection (5), mediated by the phenomenon of balanced polymorphism (6), and executed by immunological and biochemical mechanisms that protect individuals with SCT from malaria (7). Consequently, the prevalence of SCT in Nigeria and other tropical African countries is up to 25-30% in the general population (4).

In Nigeria, the eligibility of prospective donors is determined by pre-donation assessment of health status. A significant part of the assessment takes the form of verbal and/or questionnaire screening with reliance on answers to simple standard questions relating to general health, medical and social history, and simple general physical examination including the measurements of weight and blood pressure (8,9). Persons who are between the ages of 18 and 65 years, and have passed the pre-donation medical assessment with negative test results for HIV, hepatitis B and C viruses, and syphilis, with haemoglobin (Hb) levels of more than 13.5g/dl for males or 12.5 g/dl for females are acceptable as donors (9).

Individuals with SCT are genetically heterozygous for the sickle β-globin gene and their red cells have the HbAS phenotype expressing both HbS (20-40%) and HbA (60-80%) (1,10). The relative abundance of HbA prevents undue sickling and haemolysis under physiological conditions, hence the red cell life span is normal in SCT and affected individuals are symptomless, non-anaemic, and have normal life expectancy (10,11). Therefore, SCT does not in any way reduce the chances of passing routine pre-donations tests in affected individuals. Consequently, persons with SCT constitute a significant proportion of eligible blood donors in tropical African countries such as Nigeria, where up to one quarter (21-27%)

of blood donors carry the SCT (12,13). Unfortunately, the SCT blood has certain disadvantages; it is unsuitable for some blood banking procedures (e. g. leuco-filtration) and also inappropriate for transfusing certain vulnerable patients (e. g. foetuses, neonates and sickle cell disease patients) (9). Therefore, the World Health Organization (WHO) does not consider SCT as a contraindication for blood donation as long as the blood is not subjected to leuco-depletion or used for transfusing foetuses (intra-uterine), neonates and patients with sickle cell disease (SCD) (9).

Extensive systematic and meta-analytical review revealed that various studies had consistently shown that SCT definitively protects against both severe and uncomplicated malaria in the tropics, wherein SCT confers greater than 90% protection from severe malaria and up to 50% protection against symptommatic uncomplicated malaria (7). However, inconsistencies exist regarding whether or not the SCT protects against asymptomatic malarial parasitemia (AMP) in tropical populations. While some studies (7,14) suggested that SCT was protective and associated with low prevalence of AMP, other studies (7,15) found no such protective association between SCT and AMP in tropical populations. These inconsistencies call for further studies. Surprisingly, to the best of our knowledge, the effect of Hb-phenotypes on the prevalence of AMP has not been explored among blood donors in Nigeria or other malaria-endemic countries, where transfusion safety is low and the risk of transfusion transmitted malaria (TTM) is high (16,17). We therefore intended to study the effect of Hb phenotypes (SCT versus HbAA) on the prevalence and risk of AMP among apparently healthy blood donors in Nigeria.

As in many other tropical countries, donor screening for AMP and post transfusion haemovigilance of patients are not routinely conducted in Nigeria, hence the exact incidence of TTM among transfused patients in Nigeria is unknown (18,19). However, the incidence of TTM is presumably high because the prevalence of AMP among Nigerian donors was reported to range from 6% to as high as 45.8% (16,17). Nonetheless, we predicted that SCT would protect donors against AMP, while HbAA would increase donors' susceptibility to AMP. We thus hypothesized that SCT and HbAA would be associated with low and high risks of AMP among blood donors respectively. If our prediction and hypothesis are correct, AMP positive blood donors will have significantly lower relative frequencies of SCT and higher relative frequencies of HbAA phenotypes in comparison with randomly selected normal (AMP negative) control donors. In order to test our prediction and hypothesis, we conducted an analysis of the pattern and frequencies of haemoglobin phenotypes (SCT versus HbAA) among AMP positive blood donors in comparison with randomly selected normal (AMP negative) control donors.

The aim of this study was two-folds; first, to determine the risk of AMP with respect to Hb phenotypes (SCT versus HbAA) of blood donors, and secondly, to elucidate and review the clinical implications of AMP positivity among blood donors with respect to the risk of TTM vis-à-vis the practice of transfusion medicine in Nigeria and, by implication, in other malaria endemic tropical African countries.

Materials and method:

Study setting and design

This is a 'case-control' study with a total of 200 apparently healthy blood donors; 100 with AMP (as case) and 100 without AMP (as control). The study was conducted to investigate the risk of AMP with respect to Hb phenotypes (SCT versus HbAA) and carried out during the year 2017 at Rasheed Shekoni Teaching Hospital, Dutse, North-West Nigeria, and Federal Medical Centre, Birnin Kudu, North-West Nigeria. The study was conducted after obtaining informed consent of the donors and approval of the ethical committees of the hospital.

Subject participants, inclusion and exclusion criteria

Apparently healthy donors who passed the pre-donation clinical evaluation and Hb estimation, and tested negative for infectivity markers of hepatitis B and C, HIV and syphilis were consecutively recruited at the time of blood donation in the blood bank of the study hospital. Prospective donors who failed any of the aforementioned pre-donation assessments were excluded from this study.

Microscopic evaluation and speciation of AMP

All recruited donors were investigated for AMP by manual technique based on microscopic examination of Giemsa-stained thick blood smears and Leishman-stained thin blood smears using standard techniques (22). If no parasite was found in 100 oil-immersion microscopic fields of a thick smear, the sample was considered negative for malaria parasite (22). Samples positive for malaria parasite by thick smear were further tested by thin smear for morphological identification of parasite species,

while parasite density was microscopically estimated on the thick smear by counting the number of asexual forms of the parasites per high power field (HPF) (22). Parasites density levels were documented by using the plus (+) grading system (22); count of 1-10 parasites per 100 HPF = (1+), 11-100 parasites per 100 HPF = (2+), 1-10 in single HPF = (3+), and >10 parasites in single HPF = (4+) (22).

Donor categorization on the basis of microscopy results for AMP

Donors with AMP (n=100) were categorized as 'case'. Equal number of subjects were randomly selected from donors without AMP (n=100) to serve as 'control'.

Determination of ABO blood groups

The ABO blood groups were determined by standard manual techniques using monoclonal anti-A and anti-B against donors' red cells in saline tubes at room temperature, and read for agglutination after 15 min incubation. On the basis of the pattern of agglutination, donor red cells were categorized as group O, A, B or AB (23).

Determination of Hb phenotypes

The Hb phenotypes were determined by Hb electrophoresis at a pH of 8.6 on cellulose acetate paper. On the basis of electrophoretic patterns, the Hb phenotypes were categorized as normal (HbAA) or SCT (HbAS) (24).

Statistical analysis

Statistical analyses of data were performed using the Statistical Package for the Social Sciences (SPSS) software version 15.0 (SPSS Inc., Chicago, IL, USA). Frequency distribution of ABO blood groups and Hb phenotypes among study donors study cohort were presented as percentages. Age of study donors study cohort was calculated in years and presented as mean and standard deviation, while gender profile of the study donors study cohort was presented as proportions (percentages) of male and female donors. Mean values of data were compared between case donors (with AMP) and control donors (without AMP) by Student's *t*-test and proportions (percentages) by X^2-test. P value less than 0.05 was considered statistically significant.

The risks of AMP associated with SCT and HbAA were calculated as Odds ratios (OR) and separately determined by age and gender adjusted case-control logistic regression analysis using the following inputs; OR for the risk of AMP associated with SCT = number of cases with SCT/number of cases with HbAA) / (number of controls with SCT/number of controls with HbAA), and OR for the risk of AMP associated with HbAA = number of cases with HbAA/number of cases with SCT) / (number of controls with HbAA/number of controls with SCT). A 'low risk OR' (i. e. OR<1) was considered to be statistically significant if the upper limit of 95% confidence interval (95% CI) was less than 1.0 with $p<0.05$; and a 'high risk OR' (i. e. OR>1) was considered to be statistically significant if the lower limit of the 95% CI was greater than 1.0, with $p<0.05$. An OR was considered statistically insignificant if the range of its 95% CI included 1.0, with $p>0.05$.

Results:

All of the 100 donors with AMP had low parasite density level of (1+), and the parasite morphology was consistent with *P. falciparum* in all cases. The distribution of donor age, gender and relative frequencies of Hb phenotypes and ABO blood groups among donors with AMP and control donors are shown in Table 1. There were no significant differences between them with respect to mean age (28.5 years vs. 27.7 years, $p>0.05$), gender (male; 98% vs 97%, $p>0.05$ and female; 2% vs 3%, $p>0.05$), relative frequencies of blood group-O (51% vs 53%, $p>0.05$), and non-O blood groups (49% vs 47%, $p>0.05$).

However, the relative frequencies of Hb phenotypes revealed that donors with AMP had significantly lower frequencies of SCT (12% vs 28%, $p<0.05$) and higher frequencies of HbAA (88% vs 72%, $p<0.05$) compared to control donors. The Odd Ratios for the risk of AMP with respect to donor Hb phenotypes are shown in Table 2, wherein HbAA is associated with a high OR of 2.91 (95% CI: 2.10-3.48, $p=0.021$), while SCT is associated low OR of 0.49 (95% CI: 0.27-0.73, $p=0.032$).

Table1: Age, gender, ABO groups, and Hb phenotypes among blood donors with AMP and control blood donors

Parameters	No of donors with AMP (%) (n=100)	No of donors without AMP (control donors) (%) (n=100)	*p* value
Mean age (years) (± SD)	28.5±3.2	27.7±2.3	>0.05
Male	98 (98)	97 (97)	>0.05
Female	2 (2)	3 (3)	>0.05
Blood group-O	51 (51)	53 (53)	>0.05
Blood non-O groups [A+B+AB]	49 (49)	47 (47)	>0.05
HbAA	88 (88)	72 (72)	<0.05
SCT	12 (12)	28 (28)	<0.05

AMP: Asymptomatic Malarial Parasitemia. SCT: Sickle Cell Trait (HbAS)

Table 2: Odds ratios (OR) for the risk of AMP with respect to donor Hb phenotypes

Hb phenotypes	No of donors with AMP (%) (n=100)	No of donors without AMP (control donors) (%) (n=100)	OR values (95% CI)	*P* value	Inference
HbAA	88 (88)	72 (72)	2.91 (2.10-3.48)	0.021	HbAA is associated with high risk of AMP in blood donors
SCT	12 (12)	28 (28)	0.49 (0.27-0.73)	0.032	SCT is associated with low risk of AMP in blood donors

AMP: Asymptomatic Malarial Parasitemia. SCT: Sickle Cell Trait (HbAS)

Discussion:

With the largest back population of over 200 million, SCT frequency of 25-30% and SCD prevalence of 1-3%, Nigeria carries the heaviest burden of the sickle cell gene in the world (20). Moreover, malaria is endemic in Nigeria, which has a year-round transmission with up to 97% of the population being at risk of malaria infection (21). Thus, virtually the entire Nigerian population is at risk of malaria infection, which is predominantly caused by *P falciparum* specie (21). Inspite of the fact that the prevalence rates of SCT and AMP are high in Nigeria (12,13,16,17), and despite the WHO recommendation that countries with high prevalence of SCT and malaria infection should screen their donors (9), Nigerian blood banks do not routinely screen prospective donors for SCT or AMP. Therefore, both SCT and AMP are prevalent among apparently healthy blood donors in Nigeria.

Nigerian blood donor panels are predominated by young people as revealed by the donor mean ages of less than 30years reported in this study. This is consistent with the demographic profiles of Nigerian blood donors as reported in previous studies (25). This pattern is a manifestation of the demographic structure of Nigeria, which is a developing country with a relatively young population in comparison to the developed countries (26). In addition, younger people are relatively more educated (26), and are therefore more amenable to donor recruitment campaigns. The overwhelmming preponderance of male donors as seen in this study is a reflection of the general low level of blood donation among the female population in Nigeria (27). Despite the fact that blood donation is acceptable from healthy females that are not pregnant or breast feeding (9), there is a misconception in the general Nigerian population that women are not eligible to donate blood (27). There is therefore the need to rectify this misconception by re-configuring our donor mobilization strategy in order to target and sensitize the female sector (28), which constitutes about half of the Nigerian population (26). Lack of significant differences in donor age, gender and ABO blood group distributions between donors with AMP and control donors suggested that these parameters did not affect the risk of AMP among the blood donors studied in this study, which is consistent with the findings of previous studies (17). However, we found striking and significant differences between donors with AMP and control donors with respect to the distribution of Hb phenotypes among the study population.

The distribution of Hb phenotypes among our control donors (without AMP) revealed that HbAA and SCT occurred with

relative frequencies of 72% and 28% respectively, which is consistent with the relative frequencies of Hb phenotypes in the general population in Nigeria (20). In contrast, donors with AMP showed a distorted distribution of Hb phenotypes with significantly higher relative frequency of HbAA (88%) and lower relative frequency of SCT (12%). Logistic regression analysis of the differences in the relative frequencies of Hb phenotypes vis-à-vis the risk of AMP among blood donors revealed OR of 2.91 and 0.45 for HbAA and SCT respectively. These ORs suggested that donors with HbAA were about 3 times more likely to have AMP than control donors, while donors with SCT were about 50% less likely to have AMP than control donors. Therefore, the two ORs implied that HbAA was associated with high risk of AMP, while SCT was associated (and protective) with low risk of AMP among blood donors.

Our findings are at variance with previous studies, which reported that SCT did not protect against AMP (7,15). However, our findings are consistent with our working hypothesis, and are in conformity with previous studies, which reported that SCT protected against AMP (7,14). Moreover, these findings are in keeping with the fact that persons with SCT resist malaria infection through innate, immunological and biochemical mechanisms, which include reduced red cell invasion by the parasite, low intra-red cell parasite proliferation, parasite-induced red cell sickling and phagocytosis, reduced rosetting and cytoadherence of parasitized red cells, and enhanced cellular and humoral immune response against the malaria parasite (7).

The result of our study has triple clinical implications with respect to the risk of TTM in the practice of transfusion medicine in the tropics. First, the results suggest that blood donated by persons with HbAA is associated high risk of TTM. Second, blood donated by persons with SCT is associated with low risk of TTM, hence we believe that donor SCT is an important but surreptitious mitigator of the risk of TTM in the tropics. Third, patients who are selectively transfused with HbAA blood would be at increased risk of acquiring TTM. Suffices to say that TTM is a serious but inadequately quantified (i. e. exact incidence is unknown due to lack of post transfusion haemovigilance) complication of blood transfusion in Nigeria and other malaria endemic countries (18,19). Nonetheless, the risk of acquiring TTM would certainly be higher among patients with various forms of vulnerabilities and immune incompetence as may be encountered in the elderly, pregnant women, neonates and children, and patients with cancers, HIV/AIDS and SCD (29). However, two categories of patients (neonates and SCD patients) deserve careful consideration because in addition to their individual vulnerabilities, they are selectively transfused with HbAA blood, which we found (in this study) to be associated with high risk of AMP and by implication, high risk of TTM.

Nigeria has one of the highest birth rates in the world (26), with a commensurate high frequency of neonatal anaemia, jaundice and transfusion due to high prevalence of sepsis, prematurity, and G6PD-deficiency (30). The standard of care for best practice for neonatal transfusion requires selective use of HbAA blood (9,31), which unfortunately is associated with high risks of AMP and TTM as seen in this study. It is well known that neonates are naturally protected from malaria by two barriers viz maternal antibodies and HbF (32,33). Nevertheless, these neonatal protective barriers can be easily overcome by direct transfusion of malaria infected blood for four reasons. First, neonates are selectively transfused with HbAA, which carries higher risk of AMP as found in this study. Second, neonates are often transfused with fresh blood (31), and malaria parasites remain viable in stored refrigerated blood for only about two weeks (34), hence fresh blood is more likely to transmit malaria than old stored blood (19). Third, relative to the size of the neonate, the parasite dose in infected donor blood is often massive, and it has been suggested that a massive infective dose would easily overwhelm the protective barriers and lead to the establishment of malaria (35). Fourth, neonatal transfusions are often given by exchange blood transfusion (EBT) procedures (30), and EBT invariably removes the protective maternal antibodies (32) and HbF (33) in the discarded neonatal blood. For the reasons above, TTM is an important complication of neonatal transfusion in Nigeria and other malaria endemic countries (36). Therefore, neonates that are transfused must be closely monitored for any clinical manifestations of TTM.

Nigeria carries the heaviest burden of SCD in the world (20). The management of SCD is transfusion intensive (37), and in similarity with neonatal transfusion, the standard of care for best practice for SCD transfusion requires selective use of HbAA blood (9,38). Although SCD patients are fundamentally vulnerable to malaria by virtue of their immune compromised status (39), we believe that the selective transfusion of HbAA blood would increase their risks of acquiring TTM.

Consequently, TTM is an important complication seen among SCD patients who had received blood transfusion from donors within or originating from malaria endemic countries (40,41). Therefore, SCD patients and their parents should be adequately counseled to strictly comply with their routine anti-malarial chemo-prophylaxis as is usually incorporated in the standard of care for SCD patients who are resident in their native malaria-endemic tropical countries (42). Nonetheless, SCD patients who are transfused must be closely monitored for any clinical manifestations of TTM.

One limitation in our study was the use of the plus (+) grading system to estimate malaria parasitaemia as opposed to the more accurate but time-intensive parasitemia estimation per microlitre of blood, which is usually done in research laboratories. However, the plus (+) grading system is the commonest method for parasitemia estimation and recommended by the WHO (22) for high turnover routine clinical laboratories such the one from which our study was conducted.

Conclusion:

This study showed that HbAA is associated with high risk of AMP among blood donors, while SCT was protective and associated with low risk of AMP. These findings imply that blood donated by persons with HbAA is associated high risk of TTM, while blood donated by persons with SCT was protective and associated with low risk of TTM. There is the need to validate the findings of this study by conducting larger studies. Meanwhile, the result of this study suggests that donor SCT is an important but surreptitious mitigator of the risk of AMP and TTM in the tropics. Therefore, patients who are selectively transfused with HbAA blood (e. g. neonates and SCD patients) would be at greater risks of acquiring TTM, and such patients need closer post transfusion monitoring. The risk of acquiring TTM by neonates and patients with SCD, and indeed all other transfusion dependent patients calls for greater post transfusion haemovigilance, which unfortunately is lacking in Nigeria and most other tropical countries.

Conflict of interest:

Authors declare no conflict of interest.

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Intestinal schistosomiasis in an apparently healthy rural population in Bayelsa State, Nigeria

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Abstract:

Background: Schistosomiasis is endemic in Nigeria and three species; *Schistosoma haematobium, Schistosoma mansoni,* and *Schistosoma intercalatum* have been reported in Niger Delta, Nigeria. This study aimed to determine the prevalence of schistosomiasis in rural communities of Bayelsa State, Nigeria.
Methodology: Four rural homogeneous communities; Otuegala, Immiringi, Otuesega, and Ibelebiri in Ogbia Local Government Area of Bayelsa State, Nigeria, were randomly selected for the study. A structured questionnaire was administered to each participant in their native language and used to collect participant's biodata and swimming history. Stool samples collected from all participants were examined qualitatively by wet preparation and after formol-ethol concentration. Data were analyzed using SPSS version 20.0 software and results presented in proportion and tables.
Results: A total of 829 participants (age range 1 - 80 years) were recruited for the study. Helminth ova were identified in the stool samples of 218 (26.3%) participants. Among 380 males examined, 82 (21.6%) were infected, while out of 449 females examined, 138 (30.3%) were infected. The ova of seven helminths identified and their frequency of occurrence were; *S. intercalatum* 86 (10.4%), *Ascaris lumbricoides* 53 (6.4%), *S. mansoni* 35 (4.2%), *Trichuris trichiura* 22 (2.6%), hookworm 20 (2.4%) and *Taenia* spp 2 (0.2%). *Schistosoma haematobium* was identified in non-urine contaminated stool sample of an eight-year old boy. A total of 11 (1.3%) participants had double infections, affecting 7 (63.6%) females and 4 (36.4%) males, with the commonest combination being *S. intercalatum* and *A. lumbricoides* 6 (0.7%), followed by *S. intercalatum* and hookworm 4 (0.5%), and *S. mansoni* and hookworm 1 (0.1%).
Conclusion: *S. intercalatum* was the most prevalent intestinal helminthic infection in this study, which is a rare finding in most epidemiological investigations. The affinity of *Schistosoma* species to establish double infections with hookworm and other intestinal helminths should be taken into account during chemoprophylaxis.

Keywords: Schistosomiasis, Chemoprophylaxis, Prevalence, Rural Population

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Schistosomiase intestinale dans une population rurale apparemment en bonne santé dans l'État de Bayelsa, Nigéria

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Abstrait:

Contexte: La schistosomiase est endémique au Nigeria et dans trois espèces; *Schistosoma haematobium*, *Schistosoma mansoni* et *Schistosoma intercalatum* ont été signalés dans le delta du Niger, au Nigeria. Cette étude visait à déterminer la prévalence de la schistosomiase dans les communautés rurales de l'État de Bayelsa, au Nigéria.
Méthodologie: Quatre communautés rurales homogènes; Otuegala, Immiringi, Otuesega et Ibelebiri dans la zone de gouvernement local d'Ogbia de l'État de Bayelsa, au Nigéria, ont été sélectionnés au hasard pour l'étude. Un questionnaire structuré a été administré à chaque participant dans sa langue maternelle et utilisé pour recueillir les données biographiques et l'histoire de la natation des participants. Les échantillons de selles prélevés sur tous les participants ont été examinés qualitativement par préparation humide et après concentration de formol-éthol. Les données ont été analysées à l'aide du logiciel SPSS version 20.0 et les résultats ont été présentés sous forme de proportions et de tableaux.
Résultats: Un total de 829 participants (tranche d'âge 1 - 80 ans) ont été recrutés pour l'étude. Des ovules d'helminthes ont été identifiés dans les échantillons de selles de 218 participants (26,3%). Sur 380 hommes examinés, 82 (21,6%) étaient infectés, tandis que sur 449 femmes examinées, 138 (30,3%) étaient infectées. Les ovules de sept helminthes identifiés et leur fréquence d'apparition étaient; *S. intercalatum* 86 (10,4%), *Ascaris lumbricoides* 53 (6,4%), *S. mansoni* 35 (4,2%), *Trichuris trichiura* 22 (2,6%), ankylostome 20 (2,4%) et *Taenia* spp 2 (0,2%). *Schistosoma haematobium* a été identifié dans un échantillon de selles non contaminé par l'urine d'un garçon de huit ans. Un total de 11 participants (1,3%) ont eu une double infection, touchant 7 femmes (63,6%) et 4 hommes (36,4%), la combinaison la plus courante étant *S. intercalatum* et *A. lumbricoides* 6 (0,7%), suivis de *S. intercalatum* et ankylostome 4 (0,5%), et *S. mansoni* et ankylostome 1 (0,1%).
Conclusion: *S. intercalatum* était l'infection helminthique intestinale la plus répandue dans cette étude, ce qui est une découverte rare dans la plupart des enquêtes épidémiologiques. L'affinité des espèces de Schistosoma pour établir des doubles infections par l'ankylostome et d'autres helminthes intestinaux doit être prise en compte au cours de la chimioprophylaxie.

Mots clés: schistosomiase, chimioprophylaxie, prévalence, population rurale

Introduction:

Schistosomiasis is a parasitic disease caused by blood fluke of the genus *Schistosoma* (1). In humans, the infection of schistosomiasis outranks all parasitic diseases except malaria, causing great morbidity with profound economic and public health importance (2). The disease exists in two forms; urogenital schistosomiasis caused by *S. haematobium* and intestinal schistosomiasis caused by four species: *Schistosoma mansoni*, *S. mekongi*, *S. japonicum*, and *S. intercalatum* (3). However, *S. mansoni* is the most widely distributed in endemicity and is found in sub-Saharan Africa, the Middle East, and Latin America. *Schistosoma intercalatum* is endemic in the rainforest area of Sao Tome and Equatorial Guinea and in the Central Africa Republic (4). *Schistosoma mekongi* and *S. japonicum* are found in China, the Philippines, and Cambodia districts, while in Africa, *S. mansoni* and *S. intercalatum* are the most prevalent species of intestinal schistosomiasis (5). *Schistosoma intercalatum* causes human rectal schistosomiasis in Africa (4) and has been associated with clinical non-typhoidal salmonella (NTS) septicaemia in children (6). Additionally, *S. mansoni* infection causes bloody diarrhea (7).

In Nigeria, both urinary and intestinal schistosomiasis are endemic but the degree of endemicity is low (8). *Schistosoma haematobium* is the most widely distributed species in Nigeria with 79.8% area coverage. All three species; *S. haematobium*, *S. mansoni*, and *S. intercalatum* known in Nigeria have been reported in the southern part of the country (9). According to epidemiological reports, most transmission sites of intestinal schistosomiasis were an agrarian settlement and among people living along water bodies (2).

The vegetations in Bayelsa State, Nigeria is that of freshwater swamps and lowland rain forest in most areas, which creates an ideal transmission site for schistosomiasis. In light of this, over the past 10 years, there have been continuous community-based health intervention programs targeted at prevention and control of infectious diseases and soil-transmitted helminths in the State. We, therefore, conducted this study with the intention to have better understanding of the prevalence of schistosomiasis and soil-transmitted helminthic infections in Bayelsa State.

Materials and methods:

Study setting:

The study was conducted in four rural communities located along the bank of Kolo Creek, within latitude/longitude N04.74960 E00 639553/N04.93480 E00 641840, in Ogbia Local Government Area of Bayelsa State. In the study areas are eutrophic water bodies and several inbuilt and natural factors in the environment that can sustain the transmission of intestinal helminths. These include absence of pit latrines and water closet system, culture of defaecating in the bush and into the creek water as well as

the presence of freshwater swamps, lowland rain forest vegetation with much clay soil such that a greater part of the land is flooded during the rainy season. The inhabitants are peasant farmers, who are into fish and crop farming.

Both children and adults recreate in the creek and the stagnant pool of water, which also serve the purpose for washing of clothes, dishes and in some cases, for drinking. There are many freshwater snails in the water bodies in the neighborhood (Plate 1 & 2). Although rain falls every month of the year, the heavy downpour is typical of the tropical rain forest belt during the raining season (April-October).

Bayelsa State generally has the heaviest rainfall density in Nigeria with a short dry season (from November to March). It has a uniform mean annual temperature ranging from 25°C to 31°C throughout the year. The relative humidity is usually high and slightly higher during the rainy season (11).

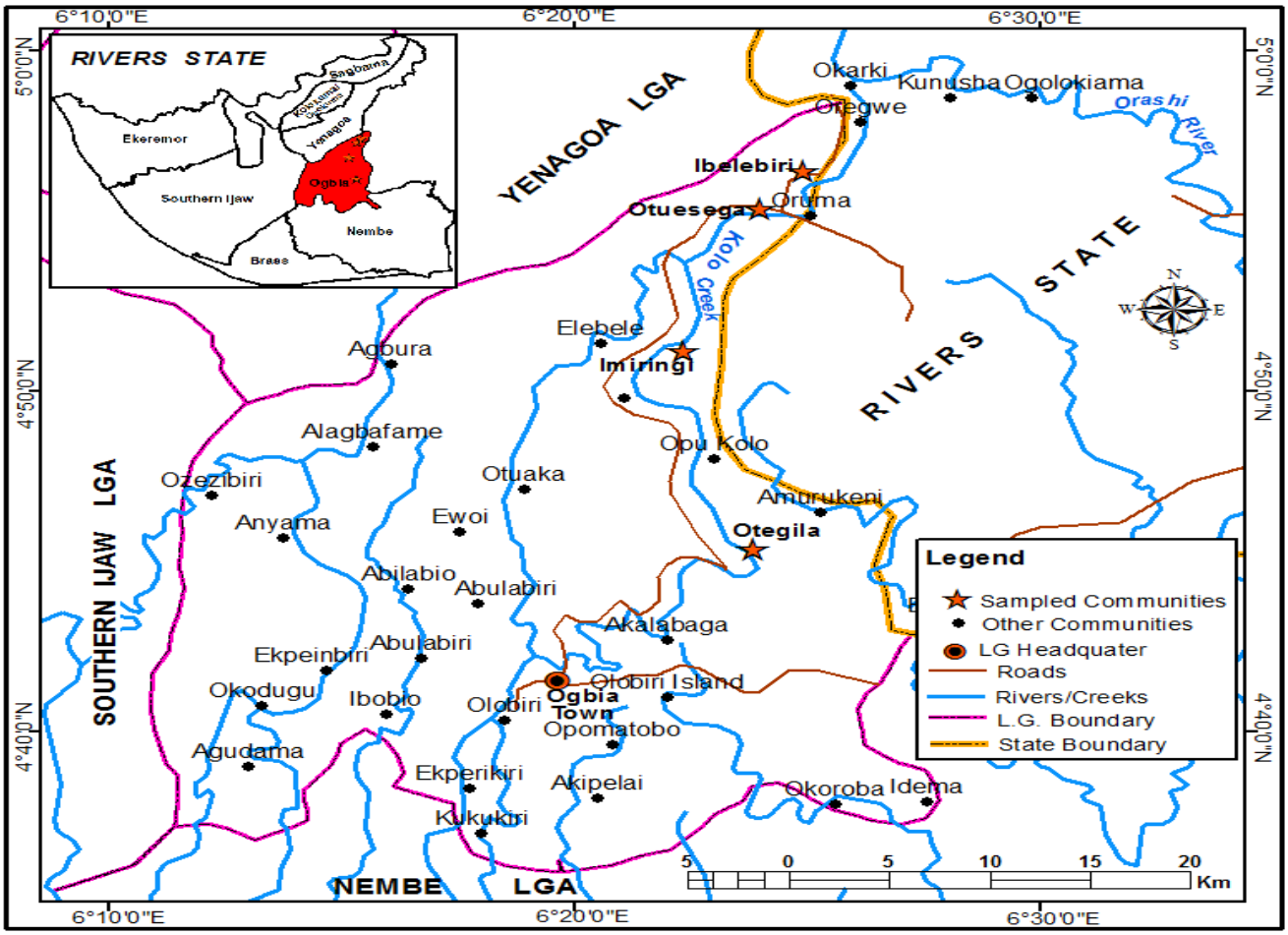

Fig 1: Ogbia Local Government Area Showing Sampled Communities
(Adapted from Mapsandmap.com, 2017)

PLATE 1: Swimming activities in a stagnant pool in Ibelebiri Community.

PHOTO CREDIT: Odoya

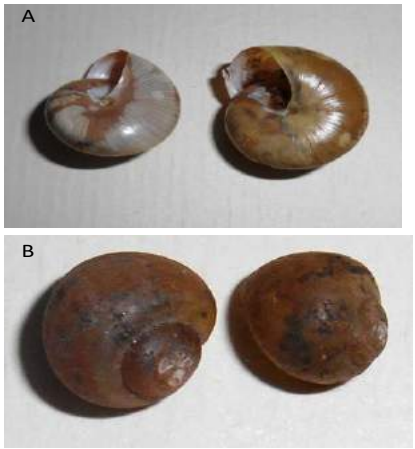

Plate 2. Snail vectors of schistosomiasis in the study locations.
A. *Biomphalaria* sp.; B. *Bulinus* sp.

Study population and subject participants

The study was cross-sectional in design and was conducted between May and June 2018. A simple random sampling technique (10) was used to select four out of the eleven communities in the Kolo district, and these were Ibelebiri, Otuesega, Otuegala, and Immiringi. All apparently healthy volunteers 4-80 years of age who had lived in the selected communities for more than three months were voluntarily enrolled into the study. Pregnant women and children less than four years of age were excluded.

Sample size determination

The sample size (n) was determined using the statistical formula, $n = z^2 p (1-p)/d^2$ (10), where n = minimal sample size, z = confidence level of 95% (standard value is 1.96), p = expected prevalence 50%, and d = margin of error of 5% (11). The final sample size was multiplied by a design effect of 2 due to the randomization technique used in community selection (12). Additionally, due to an expected attrition rate of 5%, the final sample size was adjusted to 808, but 829 participants were recruited.

Ethical approval

Ethical approval was obtained from the Ethics Committee, College of Medicine, University of Benin, Benin City, Edo State, Nigeria, and the Ministry of Health, Bayelsa State. Approval of leaders of the four communities was obtained as well as informed consent of each participant or parents/guardians of children.

Data collection and questionnaire administration

A structured questionnaire was administered to each participant in their native language and responses were appropriately marked in the given boxes. The information in the questionnaire included age, gender, history of swimming, fishing, and recreation in water bodies in the neighborhood.

Sample collection and laboratory examination of stool

A clearly labeled container and clear instruction on the process for collecting stool samples were provided for each subject participant. Participants were instructed to pass faeces on spread-out paper and with a spatula collect about a quarter filled faeces into the container. Stool samples were immediately fixed with 10% formalin, and sent to the postgraduate laboratory of the Department of Animal and Environmental Biology, University of Benin for analysis.

The examination of helminthes ova was qualitative and was done by wet preparation and formol ethol method (13). About 1g of well-mixed faeces was collected using emulsified stick into a tube containing about 4 ml of 10% formol-water mixture. A little more formol-water was added to the tube and covered with a cap. Tube content was agitated gently for more thorough mixing. The emulsified faecal samples were sieved, and the suspensions collected in a beaker, which was transferred into a centrifuge tube and about 3-6ml of ethyl acetate added. The tube was stoppered and mixed for 1 minute. The stopper was loosened and centrifuged immediately at 3000 rpm for 1 minute. The tube was slightly inverted to discard the ether, faecal debris, and formol-water while leaving the sediment. After mixing the sediment in the tube, it was transferred to a slide and covered with a slip. Specimens were examined under the light microscope using 10x and 40x objective lens, which give a final magnification of 100 and 400 respectively. Three experienced laboratory technologists were employed for the examination and agreement of two of three technologists was accepted in the reading of the slides.

Statistical analysis

Data were analyzed using SPSS version 20.0 software. Descriptive statistics were used and results presented in proportion and tables.

Results:

A total of 829 participants (380 males 46%, 449 females 54%) were recruited for the study. The population-based on age and gender in each community is shown in Table 1. The prevalence of intestinal helminths infection in the four communities is indicated in Table 2 with a total of 218 (26.3%) participants infected and slightly different prevalence rates between the communities. The frequency of identification of helminthes ova in the faecal specimens were; *S. intercalatum* (10.4%), *A. lumbricoides* (6.4%), *S. mansoni* (4.2%), *T. trichiura* (2.6%), hookworm (2.4%) and *Taenia* spp (0.2%) (Tables 2). The prevalence of *S. intercalatum* of 12% and hookworm of 11% were highest in Ibelebiri community. Ova of *Schistosoma mansoni* were found in all the communities with the highest prevalence in Immiringi (11%) and Otuesega (10.5%). The prevalence of tapeworm infection was low (0.5%, n=1) in each of Otuesega and Immiringi (Table 2).

The prevalence of helminthiasis in males is 21.6% (82/380) while the prevalence in the female is 30.3% (136/449) (Table 3). The prevalence of *S. intercalatum* in the females is 13% compared to 7.6% in the males. Similarly, the prevalence of hookworm in females is 5.2%, which is much higher than in males 0.8% (Table 3). All age groups were affected by *Schistosoma* species, although the frequency was highest in the 71-80 years age group (25.0%) infected by *S. intercalatum* and 33.3% in 61-70 years age group infected by *S. mansoni* (Table 4).

Eleven subjects (7 females and 4 males) had double helminth infections representing a prevalence of 1.3% (Table 5), with a combination of *S. intercalatum/A. lumbricoides* (n=6), *S. intercalatum*/hookworm (n=4) and *S. mansoni* /hookworm (n=1) identified in their stool samples. The age group 41-50 years had the highest frequency (n=4). *Schistosoma haematobium* was identified in the stool sample of an eight-year old male (Plate 2).

Table 1: Distribution of the study population for helminthiasis in four rural communities of Bayelsa State, Nigeria, with respect to age group and gender

Parameter	Gender		Total (%)
	Male (%)	Female (%)	
Age group (years)			
<10	168 (50.1)	167 (49.8)	335 (40.4)
10-19	106 (52.7)	95 (47.2)	201 (24.2)
20-29	40 (39.2)	62 (60.7)	102 (12.3)
30-39	25 (30.8)	56 (69.1)	81 (9.7)
40-49	22 (33.8)	43 (66.1)	65 (7.8)
50-59	10 (38.4)	16 (61.5)	26 (3.1)
60-69	6 (40.0)	9 (60.0)	15 (2.0)
≥70	3 (75.0)	1 (25.0)	4 (0.4)
Community			
Otuegela	86 (40.9)	124 (59.1)	210 (25.3)
Ibelebiri	82 (39.0)	128 (61.0)	210 (25.3)
Otuesega	114 (54.5)	95 (45.5)	209 (25.2)
Immiringi	98 (49.0)	102 (51.0)	200 (24.2)
Total	380 (45.8)	449 (54.2)	829 (100)

Table 2: Prevalence of helminthiasis in the study populations in four rural communities of Bayelsa State, Nigeria

Community	No examined	No positive (%)	*S. intercalatum* n (%)	*A. lumbricoides* n (%)	*S. mansoni* n (%)	*T. trichiura* n (%)	*Taenia* sp n (%)	Hookworm n (%)
Otuegala	210	45 (21.4)	17 (8.1)	5 (2.4)	16 (7.6)	6 (2.8)	-	1 (0.5)
Ibelebiri	210	59 (28.1)	25 (12.0)	12 (5.7)	8 (4.0)	2 (1.0)	1 (0.5)	11 (5.2)
Otuesega	209	60 (28.7)	22 (10.5)	16 (7.6)	8 (3.8)	8 (3.8)	1 (0.5)	5 (2.4)
Immiringi	200	54 (27.0)	22 (11.0)	20 (10.0)	3 (1.5)	6 (3.0)	-	3 (1.5)
Total	829	218 (26.3)	86 (10.4)	53 (6.4)	35 (4.2)	22 (2.6)	2 (0.24)	20 (2.4)

S = Schistosoma, A = Ascaris, T = Trichuris

Table 3: Prevalence of helminthiasis in the study population with respect to gender in rural communities of Bayelsa State, Nigeria

Gender	No Examined	No positive (%)	*S. intercalatum* n (%)	*A. lumbricoides* n (%)	*S. mansoni* n (%)	*T. trichiura* n (%)	*Taenia* sp n (%)	Hookworm n (%)
Male	380	82 (21.6)	29 (7.6)	25 (2.4)	12 (3.2)	9 (2.3)	2 (0.5)	3 (0.8)
Female	449	136 (30.3)	57 (13.0)	28 (5.7)	23 (5.1)	13 (2.9)	-	17 (5.2)
Total	829	218 (26.3)	86 (10.4)	53 (6.4)	35 (4.2)	22 (2.6)	2 (0.24)	20 (2.4)

S = Schistosoma, A = Ascaris, T = Trichuris

Table 4: Prevalence of helminthiasis with respect to age groups among the study population in four rural communities of Bayelsa State, Nigeria

Age group (years)	No examined	No positive (%)	*S. intercalatum* n (%)	*A. lumbricoides* n (%)	*S. mansoni* n (%)	*T. trichiura* n (%)	*Taenia* sp n (%)	Hookworm n (%)
1-10	335	100 (29.8)	36 (10.7)	29 (8.6)	20 (6.0)	12 (3.6)	-	3 (0.9)
11-20	201	34 (16.9)	17 (8.4)	5 (2.5)	4 (2.0)	5 (2.5)	-	3 (1.5)
21-30	102	24 (23.5)	8 (7.8)	8 (7.8)	1 (0.9)	1 (0.9)	-	6 (5.9)
31-40	81	23 (28.4)	7 (8.6)	7 (8.6)	3 (3.7)	2 (2.5)	1 (1.2)	3 (3.7)
41-50	65	20(30.7)	10 (15.3)	4 (6.1)	1 (1.5)	1 (1.5)	1 (1.5)	3 (4.6)
51-60	26	4 (15.4)	4 (15.4)	-	-	-	-	-
61-70	15	11 (73.0)	3 (20.0)	-	5 (33.3)	1 (6.7)	-	2 (13.3)
71-80	4	2 (50.0)	1 (25.0)	-	1 (25.0)	-	-	-
Total	829	218 (26.3)	86 (10.4)	53 (6.4)	35 (4.2)	22 (2.6)	2 (0.4)	20 (2.4)

S = Schistosoma, A = Ascaris, T = Trichuris

Table 5: Prevalence of mixed helminthic infection among the study population with respect to age group and gender in four rural communities of Bayelsa State, Nigeria

Parameters/infection	No infected with *S. intercalatum* and Hookworm (%)	No infected with *S. intercalatum* and *A. lumbricoides* (%)	No infected with *S. mansoni* and Hookworm (%)	Total
Age group (years)				
1-10	-	-	-	-
11-20	1	-	1	2
21-30	-	2	-	2
31-40	-	1	-	1
41-50	2	2	-	4
51-60	-	-	-	-
61-70	-	1	-	1
71-80	1	-	-	1
Gender				
Male	1	2	1	4
Female	3	4	0	7
Total	4	6	1	11

S = Schistosoma, A = Ascaris, T = Trichuris

Schistosoma haematobium *Schistosoma intercalatum* *Schistosoma mansoni*; Scale bars = 0.05mm

Fig 2: Ova of helminthes identified from stool samples of subjects from Ibelebiri community of Ogbia LGA, Bayelsa State, Nigeria

Discussion:

Identified in this study were ova of six species of intestinal helminths; *S. intercalatum*, *S. mansoni*, *A. lumbricoides*, *T. trichiura*, *Taenia* spp, hookworm, and *S. haematobium* in stool samples. This distribution differs from the commonly reported triad of intestinal helminths, involving *A. lumbricoides*, hookworm, and *T. trichiura* (14). The species of helminths identified is known to vary depending on environmental factors such as temperature, rainfall, humidity, soil moisture, and others such as personal hygiene and level of contamination of the environment (15). In the south-west, the commonest species of helminths reported were *A. lumbricoides* and *T. trichiura* while hookworm and *Strongyloides stercoralis* were the highest prevalence in north-east and north-central regions. A study among primary school children in Aniocha, south-south Nigeria reported three helminths species; *A. lumbricoides*, *T. trichiura* and hookworm (16). In a study of school-age children in Kwara State, four species of intestinal helminths; *Hymenolepis* species, *S. mansoni*, and *Enterobius vermicularis* were identified

in stool samples (17). However, across Nigeria, the highly prevalent species of helminths reported are; *A. lumbricoides*, *S. stercoralis*, *T. trichiura* and hookworms (15).

The occurrence of all species of helminths followed a similar trend of declining prevalence with increasing age but the highest prevalence of 73% (11/15) reported in the 61-70 years age group in our study, is contrary to other studies where higher prevalence are reported in younger children as a result of poor personal hygiene practices such as eating with unwashed hands, and spending more time playing out-door without adult supervision (11). As the school age children (< 18 years) were dewormed about 5 weeks before our study, this could account for the much lower prevalence of 29.8% (100/335) in children under 10 years of age. However, the generally low prevalence of helminths infection in adults could be attributed to the relative consciousness of good hygiene practice and less contact with the dirty environment (10).

The prevalence of 6.4% for *A. lumbricoides* reported in our study is lower than 15.0% reported by a study in Edo (18) and 13.1% by another study in Osun State (19). Hookworm infection rate was also low (prevalence of 2.4%) and affected more adults, which could have been acquired by children that walk barefooted, with hookworm larvae actively penetrating the exposed skin. Although, the presence of adequate moisture and optimal temperature allows for larval activity and migration (20), heavy rainfall characteristic of the study settings may have carried infective larvae away into a runoff, which could explain the low infectivity of hookworm in the study areas. The prevalence of taeniasis was 0.2% affecting only adult population in the study. Tapeworm infection is usually associated with consumption of poorly cooked beef or pork meat. However, with the availability of fresh-fish and snails and the peasantry life of the people, consumption of roasted "*suya*" meat or pork is reduced, and this may account for the low prevalence of taeniasis in the study areas.

Most studies have reported *A. lumbricoides* as the most prevalent intestinal helminths in the world (15). This contrasts our finding in this study where the helminthes with the highest prevalence was *S. intercalatum* (10.4%), followed by *A. lumbricoides* (6.4%) and *S. mansoni* (4.2%). Three species of *Schistosoma* were identified; *S. intercalatum*, *S. mansoni* and *S. haematobium* in that order. Ekpo et al., (21) identified all three species of *Schistosoma* in Rivers State, a neighboring State to the study area. Intestinal schistosomiasis was identified in all age groups, reflecting the exposure of the study participants in snail-infested water bodies in the environment. *Schistosoma haematobium* was identified in a stool sample of an eight-year old male. The aberrant presence of species of *Schistosoma* is not uncommon. In a study of urinary and intestinal schistosomiasis among 1,709 children (5-15 years of age) in Port Harcourt, Nigeria (22), ova of *S. intercalatum* were identified in urine samples only, although with a low prevalence of 5.7%, and neither ova of *S. mansoni* nor *S. haematobium* were identified both in stool and urine samples. Eggs of *Schistosoma* or parasite migration can be lodged in strange sites such the central nervous system with may cause compressing of the site with clinical manifestations of pyrexia, headache, vomiting, blurred vision, and Jacksonian epilepsy (23). In Nigeria, the geographical distribution of *Schistosoma* infections depends on availability of the right intermediate hosts (fresh water snails), with *S. mansoni* requiring freshwater *Biomphalaria* snail while *S. haematobium* requires *Bulinus* snail as vectors for transmission (24). Multiple infections in this study involved most commonly *A. lumbricoides* and *S. intercalatum*, followed by *S. intercalatum* and hookworm.

Contact with infective ova of helminths depends on hygiene-behavioral disposition of the population such as hand washing, nail hygiene, and foot-wear practices (25). In the study area, parents, who were mostly peasant farmers, are believed to spend less time caring for their children at home. The children are hence not supervised on hygiene practices such as hand washing after defecation, and this could promote acquisition of helminthic infection. Also, children who were not properly supervised by parents/guardians spent more time recreating in snail-infested water bodies (as shown in Plate 1). Our observation of Kolo Creek showed that it was shallow and slow-flowing, invariably stagnant with dense vegetation, which favors the breeding of snail intermediate hosts (as shown in Plate 3) and the proliferation of miracidium. Although the sanitary condition of the study area was typical of a rural community in a developing country, it is most probable that human activities in snail-infested water bodies are responsible for sustained infection with intestinal platyhelminthes, despite ongoing chemoprophylaxis. This is in agreement with a previous study (26) which showed that persistent transmission of schistosomiasis had cultural affiliation with the use of river water for "drinking" and swimming, implying that behavioral changes are needed to reduce the transmission of schistosomiasis.

The primary objective of any control program is to reduce morbidity (5). Our study observed a lopsided control strategy, where chemoprophylaxis with praziquantel was the only measure adopted by the health intervention program. Praziquantel has been reported to be effective for adult *Schistosoma* parasites only (26), hence combination of some anti-malarial agents such as the artemisinin derivatives (such as artemether) with demonstrated clinical potency in eliminating young stage *Schistosoma* infection (26), may be a better chemoprophylactic strategy.

Conclusion:

The epidemiological implication of combined infection of *Schistosoma* and hookworm in human health is significant in this study. The combination of praziquantel and artemether as chemoprophylaxis for prevention and control of schistosomiasis in the study area should be considered. Additionally, a school-based intervention program should be established to create a platform for screening and treatment of infected pupils. Provision of portable water along with integration of snail control in the Kolo Creek and the surrounding water bodies are highly recommended.

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Original Article **Open Access**

Antimicrobial resistance patterns and transferable traits in Enterobacteriaceae isolates from poultry in Tlemcen, Algeria

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Abstract:

Background: Antibiotics are overused in poultry industry, and this has resulted in the emergence of multidrug resistant (MDR) bacteria. The current study is aimed at determining antimicrobial resistance (AMR) patterns of Enterobacteriaceae isolates from poultry in the west of Algeria.
Methodology: Different chicken samples (kidney, bone and intestine) were collected and processed for culture using standard microbiological methods to isolate Enterobacteriaceae. Isolates were identified biochemically using API 20E, while isolated *Escherichia coli* was typed for O1, O2 and O78 antigens using slide agglutination with specific antisera. All identified isolates were tested against 26 antibiotic disks using the Kirby Bauer disk diffusion method according to the CLSI standards. The minimum inhibitory concentrations (MICs) of chloramphenicol, tetracycline, nalidixic acid, ofloxacin and ciprofloxacin were determined for selected isolates. Conjugative plasmid transfer, plasmid incompatibility and colicin tests were used to detect transferable resistance traits in 48 selected *E. coli* isolates.
Results: One hundred and thirty-eight bacteria species were isolated, which included *Escherichia* coli (n=107), *Salmonella* spp (n=11), *Klebsiella* spp (n=8), *Enterobacter* spp (n=7), *Pseudomonas* spp (n=3) and *Citrobacter* spp (n=2). Serotyping identified 24 agglutinable *E. coli* isolates with O78:K80 (n=11), O1:K1 (n=9) and O2:K1 (n=4). Antibiotic susceptibility showed high frequency of *E. coli* resistance to nalidixic acid (89.7%), tetracycline (82.2%), streptomycin (82.2%), nitrofurantoin (68.2%), ampicillin (45.8%), ticarcillin (44.9%), piperacillin (42.1%), and chloramphenicol (15.9%). The percentage of multi-drug resistance isolates (resistance to more than 3 antibiotic classes) was 87.9%. The results of conjugative transfer in 48 *E. coli* isolates shows that the most important resistance traits transferred by plasmids are *ASTeSuTmp* (18.5%) and *SuTmp* (12.3%).
Conclusion: This study confirmed the presence of multiple antibiotic resistant *E. coli* and other members of family *Enterobacteriaceae* in poultry in Algeria, and showed that these antibiotic resistance traits are easily disseminated by plasmids, with dire consequences on human health.

Keywords : Poultry, Enterobacteriaceae, antimicrobial resistance, conjugation, plasmid.

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Profils de résistance aux antimicrobiens et caractères transférables des isolats d'entérobactéries provenant de volailles à Tlemcen, Algérie

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Abstrait:

Contexte: Les antibiotiques sont surutilisés dans l'industrie de la volaille, ce qui a entraîné l'émergence de bactéries multirésistantes (MDR). L'étude actuelle vise à déterminer les profils de résistance aux antimicrobiens (RAM) des isolats d'Enterobacteriaceae provenant de volailles dans l'ouest de l'Algérie.

Méthodologie: Différents échantillons de poulet (rein, os et intestin) ont été prélevés et traités pour la culture en utilisant des méthodes microbiologiques standard pour isoler les Enterobacteriaceae. Les isolats ont été identifiés biochimiquement en utilisant l'API 20E, tandis que *Escherichia coli* isolé a été typé pour les antigènes O1, O2 et O78 en utilisant l'agglutination sur lame avec des antisérums spécifiques. Tous les isolats identifiés ont été testés contre 26 disques antibiotiques en utilisant la méthode de diffusion sur disque de Kirby Bauer selon les normes CLSI. Les concentrations minimales inhibitrices (CMI) du chloramphénicol, de la tétracycline, de l'acide nalidixique, de l'ofloxacine et de la ciprofloxacine ont été déterminées pour certains isolats. Des tests de transfert plasmidique conjugatif, d'incompatibilité plasmidique et de colicine ont été utilisés pour détecter des traits de résistance transférables dans 48 isolats sélectionnés d'*E. coli*.
Résultats: Cent trente-huit espèces de bactéries ont été isolées, parmi lesquelles *Escherichia coli* (n=107), *Salmonella* spp (n=11), *Klebsiella* spp (n=8), *Enterobacter* spp (n=7), *Pseudomonas* spp (n=3) et *Citrobacter* spp (n=2). Le sérotypage a identifié 24 isolats d'*E. coli* agglutinables avec O78: K80 (n=11), O1: K1 (n=9) et O2: K1 (n=4). La sensibilité aux antibiotiques a montré une fréquence élevée de résistance d'*E. coli* à l'acide nalidixique (89,7%), à la tétracycline (82,2%), à la streptomycine (82,2%), à la nitrofurantoïne (68,2%), à l'ampicilline (45,8%), à la ticarcilline (44,9%), à la pipéracilline (42,1%) et le chloramphénicol (15,9%). Le pourcentage d'isolats de résistance multi-médicaments (résistance à plus de 3 classes d'antibiotiques) était de 87,9%. Les résultats du transfert conjugatif dans 48 isolats d'*E. coli* montrent que les traits de résistance les plus importants transférés par les plasmides sont *ASTeSuTmp* (18,5%) et *SuTmp* (12,3%).
Conclusion: Cette étude a confirmé la présence de multiples *E. coli* résistants aux antibiotiques et d'autres membres de la famille des Enterobacteriaceae chez les volailles en Algérie et a montré que ces traits de résistance aux antibiotiques sont facilement disséminés par les plasmides, avec des conséquences désastreuses sur la santé humaine.

Mots clés: volaille, entérobactéries, résistance aux antimicrobiens, conjugaison, plasmide.

Introduction:

With a lower price than red meat, poultry is the most widespread meat consumed in Algeria. Poultry meat, like other meat can provide a good environment for microbial growth. Most members of the family Enterobacteriaceae have been known to be major cause of food-borne diseases and spoilage of a variety of foods, including poultry products (1,2). A large diversity of antibiotics is used in veterinary medicine to raise poultry in many countries (3,4), mostly through the oral route of antibiotic administration as prophylaxis or for the treatment of infectious diseases or in animal nutrition to promote growth and productivity (5,6). The excessive and misuse of such antimicrobials had led to increase in antibiotic resistance (7), which is considered critical and of high importance for human medicine (8,9,10).

Antimicrobial resistant pathogens in poultry infections may result in treatment failure, leading to economic losses, but also can be a source of resistant bacteria/genes that present a significant risk to human health (11). In the last decades, epidemics have been associated with resistant strains of food-borne Enterobacteriaceae (12). Avian Enterobacteriaceae are considered as secondary pathogens and mostly involved *Escherichia coli*. However, recently in Algeria, they are considered as one of the most important causes of economic losses in the poultry sector (13).

According to some reports, *E. coli* commonly found in raw meats, has the potential to transfer antibiotic resistance to other intestinal organisms and may act as transport medium for antimicrobial resistant genes to other pathogens (14,15,16,17). Antimicrobial resistant *E. coli* strains pose a serious problem for public health, since these strains could be passed to humans via the food chain or by direct contact with infected chicken (18). Therefore, the objectives of this study are to determine antimicrobial resistance patterns of Enterobacteriaceae isolates from poultry in Tlemcen, Algeria, and to detect the plasmids responsible for potential dissemination of resistant traits present in these isolates.

Materials and method:

Samples and isolation of bacteria species

Different chicken samples (kidneys, bones and intestines) were collected between 2018 and 2019 from various locations in western Algeria including Tlemcen, Oran, Sidi bellabes, Saida, Ain Temouchent and Naâma. From each sample, 1g was mixed with 9ml of Rappaport Vassiliadis broth (BioMérieux, Marcy l'Étoile, France), vortexed, and incubated overnight at 37°C. To isolate *E. coli* and *Salmonella* spp, a drop of the broth was streaked on Hektoen agar medium (Biokar, Diagnostics, Beauvais, France). Bromocresol purple lactose agar (Bio-Rad Laboratories Inc., California, USA) was used to isolate the other Enterobacteriaceae

Biochemical identification and serotyping

The isolates were identified biochemically using the API 20E system (BioMérieux, Marcy l'Étoile, France). All confirmed *E. coli* isolates were serotyped by the slide agglutination with specific antisera (Biovac, Angers,

France) for O1, O2, and O78 antigens in accordance with Qrskov and Orskov (19). The isolates confirmed as *Salmonella* were also serotyped (20) using an array of pooled and factor *Salmonella* antisera (Bio-Rad Laboratories Inc., California, USA).

Antibiotic susceptibility test (AST) assay

All identified isolates were tested for susceptibility to 26 antibiotics using the disk diffusion Kirby–Bauer standard method, with the following antibiotics; ampicillin (10µg), amoxicillin-clavulanic acid (10µg), ticarcillin (75µg), piperacillin (100µg), cefazoline (30 µg), cefoxitin (30µg), cefotaxime (30µg), cefepime (30µg), cefpirome (30µg), moxalactam (30µg), imipinem (10µg), gentamicin (10µg), amikacin (30µg), netilmycin (30µg), streptomycin (10µg), kanamycin (10µg), nitrofurantoin (300µg), nalidixic acid (30µg), ofloxacin (5µg), ciprofloxacin (5µg), colistin (10µg), tetracycline (30µg). chloramphenicol (30 µg), sulfonamide (300µg), trimethoprim (5µg), and sulfamethoxazole-trimethoprim (1.25/23.75 µg).

Isolates were categorized as sensitive or resistant to each antibiotic according to the Clinical and Laboratories Standards Institute guidelines (21). *E. coli* strain ATCC 25922 and *Pseudomonas aeruginosa* strain ATCC 27853 were used for quality control.

Minimum inhibitory concentrations

The minimum inhibitory concentrations (MICs) of chloramphenicol (Roussel, UCLAf), tetracycline (Sigma), nalidixic acid (Serva), ofloxacin (Roussel, UCLAf) and ciprofloxacin (Bayer) were determined for *E. coli* isolates (n=83) by the broth dilution technique according to Andrews (22).

Conjugative transfer experiment

A colony of selected donor isolates (isolates resistant to chloramphenicol and some multi-drug resistant *E. coli*) and a colony of the reference *E. coli* C600 Rif (host recipient strain) were put in each Brain Heart Infusion Broth (BHIB) tube and incubated for 4 hours at 35°C with stirring. 1 ml of donor and 1 ml of recipient cultures were mixed with a spreader in a Petri dish of Mueller Hinton broth and incubated overnight. 1 ml of sterile BHIB was added to the incubated mixtures and mixed with a spreader, and the supernatant containing the transconjugants was collected. A loopful of the supernatant was inoculated as a line on a quarter of the selective agar (which contain 2 antibiotics, one corresponding to the suspected resistant plasmid trait of the donor and the other to the chromosomal trait of the recipient) in Petri dish, and the mixture streaked along the remaining three quarters of the agar. After incubation for 18 hours at 35°C, an antibiogram was carried out on the transconjugants on non-selective agar in order to determine the characters transferred.

Test for plasmid incompatibility

In the test for incompatibility, the transconjugant was crossed with an *E. coli* which carries a reference plasmid, in order to determine the group to which the studied plasmid belongs. All the reference plasmids were from the "Institut Pasteur d'Algérie" (IPA) and includes; Com1 lfi 14R 525 resistant to kanamycin, Com1 PPED I resistant to chloramphenicol and trimethoprim, Com1 PPED 2 resistant to kanamycin, gentamicin, tobramycin and netilmycin, and FI Fi' R 386 resistant to tetracycline.

Test for colicin

The test isolate and control strain (*Escherichia coli* F3, which produces colicin) were stirred in BHIB for 4 hours at 35°C. A drop of the cultures was placed on Trypticase Soy agar (TSA, Bio-Rad Laboratories, Inc., California, USA), and incubated for 48 hours at 35°C. *Escherichia coli* J 5 Azide was grown in BHIB to obtain a slight cloudiness. A loopful of the growth was then diluted in 10 ml of physiological water. A drop of this dilution was added next to the test isolate and control strain on TSA, and agar incubated for 48 hours at 35°C. The production of colicin results by the isolate (and control strain) results in the formation of a zone of inhibition around *Escherichia coli* J 5 Azide, next to the isolate being studied and the positive control strain.

Statistical analysis

Statistical analysis of data and graphical representations were performed using XLSTAT Statistical Software version 2020.5.1 (www.xlstat.com).

Results:

Bacterial isolates

A total of 138 bacteria species were isolated; *E. coli* (n=107), *Salmonella* spp [n=11 with *S.* Gallinarum (n=7), *S.* Enteritidis (n=2), *S.* Infantis (n=1) and *S.* Brunei (n=1)], *Klebsiella* spp [n=8 with *K. oxytoca* (n=7) and *K. pneumoniae* (n=1)], *Enterobacter* spp [(n=7) with *E. cloacae* (n=4), *E. asburiae* (n=2) and *E. auraginus* n=1)], *Pseudomonas aeruginosa* (n=3) and *Citrobacter amalonaticus* (n=2). Serotyping of the *E. coli* identified 24 agglutinable isolates;

O78:K80 (n=11), O1:K1 (n=9) and O2:K1 (n=4).

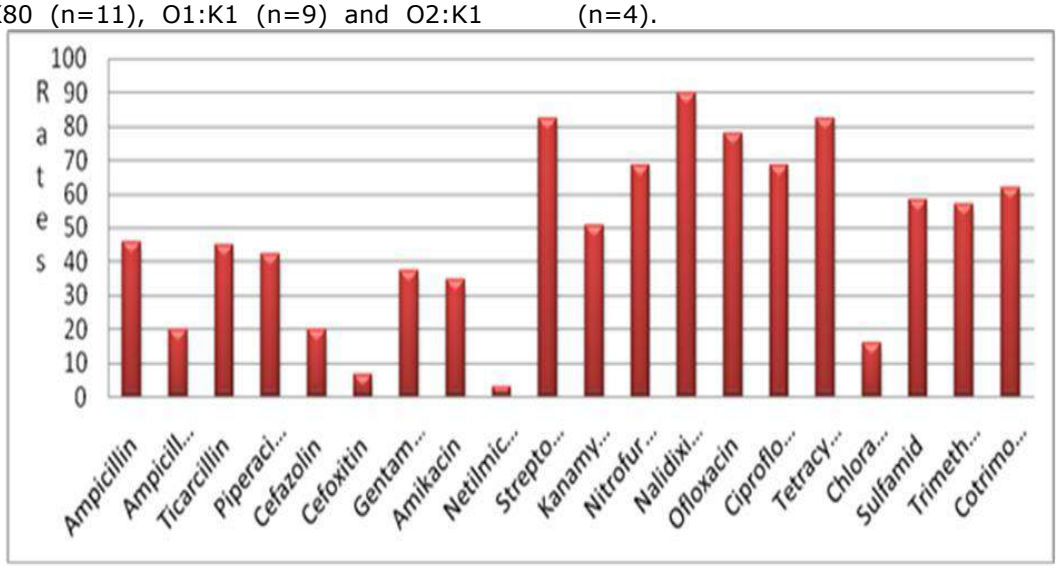

Figure-1 : Resistant patterns of *E.coli* isolated from poultry, n=107.

Results of antibiotic susceptibility test

The results of the disk diffusion AST on 107 *E. coli* isolates are shown in Fig 1. Resistance to aminoglycosides varied from 2.8% for netilmicin to 82.2% for streptomycin. Most of the *E. coli* isolates were resistant to tetracycline (82.2%) and nitrofurantoin (68.2%). There was also high resistance rate to ampicillin (45.8%), ticarcillin (44.9%), and piperacillin (42.1%). A worrying 15.9% resistance rate to chloramphenicol was obtained inspite of the fact this antibiotic is no longer used in veterinary medicine. Resistance rate to sulfonamides was 57.9%, fluoroquinolones 78.5%, and nalidixic acid 89.7%. All *E. coli* isolates were sensitive to cefepime, cefpirome, moxalactam, imipinem, ceftazidime and colistin. Multi-drug resistant isolates (resistance to more than 3 antibiotic classes) represented 87.9%

Salmonella isolates were resistant to nalidixic acid (63.6%), ciprofloxacin (63.6 %), ofloxacin (63.6%), nitrofurantoin (63.6 %), and streptomycin (27.3%). All *Enterobacter* isolates were resistant to ampicillin, amoxicillin-clavulanic acid, cefoxitin, cefazolin and nitrofurantoin, however no resistance to gentamicin, amikacin and kanamycin was observed, while 42.9% were resistant to nalidixic acid and ciprofloxacin. Regarding *Klebsiella* isolates, there was no resistance to gentamicin, amikacin and kanamycin, however all the isolates were resistant to ampicillin and tetracycline, 62.5% to streptomycin, 87.5% to nalidixic acid and nitrofurantoin, 75% to ciprofloxacin and 50% to ofloxacin. The minimum inhibitory concentrations of ciprofloxacin and tetracycline were respectively, 0.063 and 8µg/ml. At these MICs, 95.2% of tested isolates were resistant to ciprofloxacin and 95.3% to tetracycline. For nalidixic acid MIC of 8µg/ml, 40.6% of isolates were resistant and for ofloxacin MIC of 0.258 µg/ml, 15.6% isolates were resistant. For chloramphenicol MIC of 8µg/ml, 86.4% of isolates were resistant.

Transfer of resistant trait by conjugation

From the conjugation experiment on isolates resistant to chloramphenicol and multi-drug resistant *E. coli*, a total of 48 isolates transferred one or more markers. The results of the transfer showed that the most frequently transferred markers were *ASTeSuTmp* (18.5%) and *SuTmp* (12.3%) (Fig 2). However, *Tmp* was detected in 86.2%, *Te* in 50.8%, *Su* in 78.5% and *A* in 43.1% of the transconjugants.

The grouping of the plasmids allowed us to determine the *Inc* group to which all the plasmids belonged. With the exception of four plasmids (Figs 3, 4, 5, 6), all the plasmids were grouped into Com1 and F1 family. The colicin test revealed that of the 48 wild type isolates, 17 (35.4%) produced colicins while only 3 (6.3%) transconjugants were colicin positive.

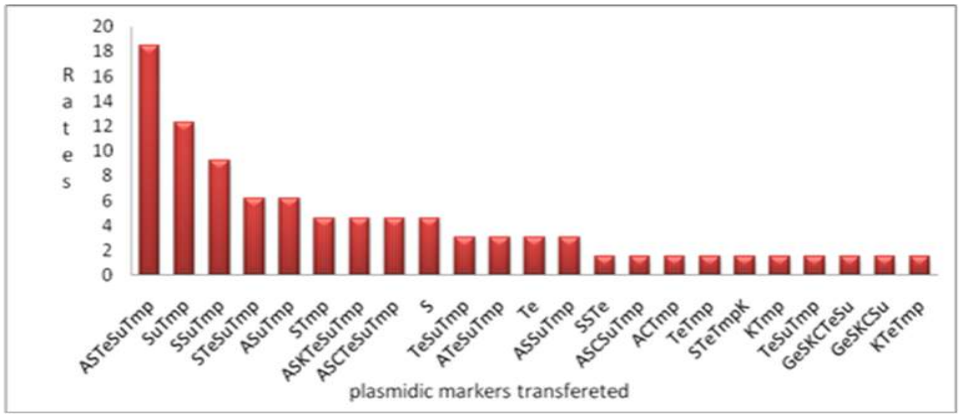

Figure-2 : Rates of plasmidic markers transfereted

A =Ampicillin, S=Streptomycin, Te=Tetracyclin, Su=Sulfamid, Tmp=Trimethoprim, K=Kanamycin, C=Chloramphenicol, Ge=Gentamycin

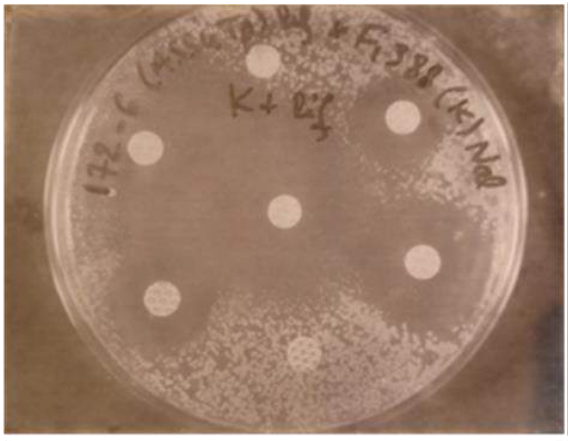

Figure-3: Grouping transconjugant *E.coli* 172-6 (ASSuTmp) in *E.coli* C600Rif with F1 386 (K) in *E.coli* K12 Nal, Selection dish K+Rif

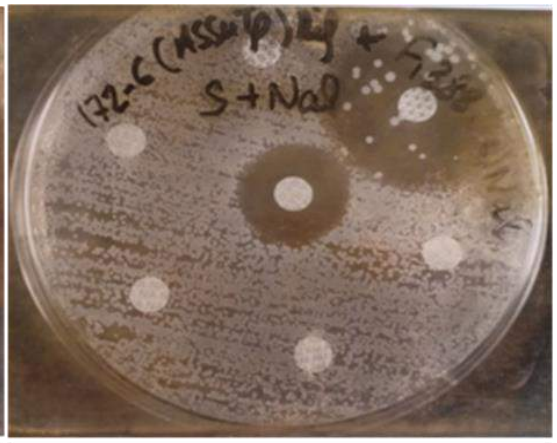

Figure-4: Grouping transconjugant *E.coli* 172-6 (ASSuTmp) in *E.coli* C600Rif with F1 386 (K) in *E.coli* K12 Nal, Selection dish S+Rif

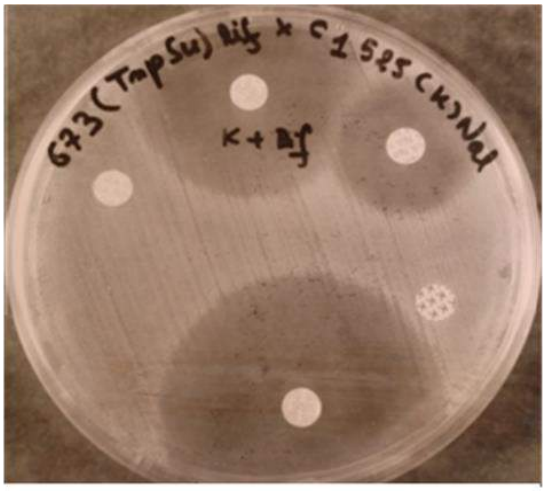

Figure-5: Grouping transconjugant *E.coli* 673 (SuTmp) in *E.coli* C600Rif with Com1 525 (K) in *E.coli* K12 Nal.

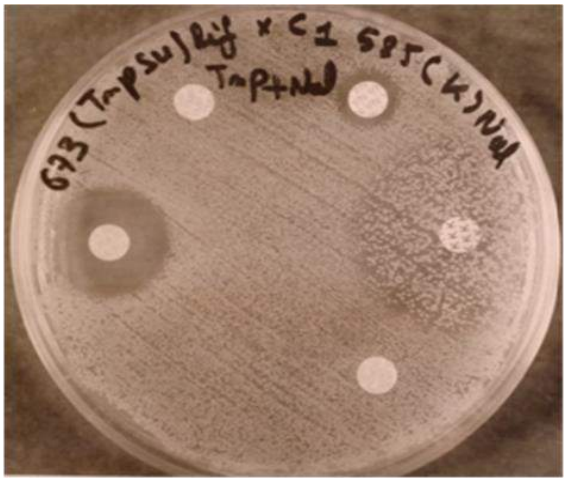

Figure-6: Grouping transconjugant *E.coli* 673 (SuTmp) in *E.coli* C600Rif with Com1 525 (K) in *E.coli* K12 Nal, in the opposite direction.

Discussion:

In this study, a total of 138 Enterobacteriaceae were isolated from different organs of poultry, with predominance of *E. coli*, and others such as *Salmonella*, *Klebsiella*, *Enterobacter*, *Pseudomonas* and *Citrobacter* in that order, similar to the results of the study by Boutaiba et al., (23). Of the 24 *E. coli* agglutinable isolates in our study, serotypes O78, O1 and O2 were identified at frequencies of 45.8%, 37.5% and 16.7% respectively, similar to the results observed in Algeria and Egypt (13,24). However, Ibrahim et al., (18) reported a lower prevalence of O78 (23.8%), O1 (14.9%) and O2 (12.6%) in their study. In Northern Ireland, *E. coli* serotype O78 was the predominant serotypes reported in chicken colibacillosis (25).

Most of the *E. coli* isolates exhibited multi-drug resistance phenotypes. The highest resistance rate was to nalidixic acid (89.7%) which is similar to the rate reported by Benameur et al., (26). Resistance to tetracycline, which is used as growth promoter or treatment of infections in domestic animals (27), is high at 82.2%. There was also high resistance of *E. coli* isolates to streptomycin (82.2%), ofloxacin (77.8%), ciprofloxacin (68.2%), nitrofurantoin (68.2%), sulfamethoxazole-trimethoprim (61.7%) ampicillin (45. 8%), ticarcillin (44.9%) and piperacillin (42.1%), which is similar to the studies by Bakhshi et al., (28) and Kim et al., (29) who reported that more that 60% of their isolates were resistant to tetracycline, streptomycin and ampicillin.

However, no resistance was detected for cefotaxime, cefepime, cefpirome, moxalactam, imipenem, ceftazidime and colistin, which is not unexpected given the fact that these classes of cephalosporins (and colistin) are not used in poultry industry. A 2012 study by Obeng et al., (33) in Australia reported a relatively lower resistance rates to tetracycline (40.6%), ampicillin (26.7%), and sulfamethoxazole-trimethoprim (12.4%), with no resistance (0%) to ceftiofur, ciprofloxacin and gentamicin, which is an indication of appropriate usage of these antibiotics in Australia. These findings however contradicted the report of a study in Zambia which showed 100% resistance to cefotaxime and ceftazidime (30).

The resistance rate of 37.4% to gentamicin in our *E. coli* isolates is also lower than the rates of 57.2% reported by Sciberras et al., (31) and 75.6% reported by Ahmed et al., (32). However, resistance rate of 15.9% to chloramphenicol in our isolates is rather too high for an antibiotic which use has been forbidden in animals, although higher resistance rates to chloramphenicol were recently reported in studies from Algeria (13, 23). The rate of multi-drug resistance in our *E. coli* isolates (resistance to more than 3 antibiotic classes) was extremely high at 87.9% but similar results were reported by Boutaiba et al., (23), where resistance rate of *E. coli* in the region of Tlemcen was higher compared to the other regions except for β-lactams where the region of Saida had the highest rate.

The resistance rate of *Salmonella* spp was 63.6% each to nalidixic acid, ciprofloxacin, ofloxacin and nitrofurantoin (furanes), and 27.3% to streptomycin. This may indicate an overuse of fluoroquinolones and furans antibiotics in the empirical treatment of suspected cases of salmonellosis on the part of breeders (34,35). All the *Enterobacter* isolates were resistant to ampicillin, amoxicillin-clavulanic acid, cefoxitin, cefazolin and furanes. On the other hand, no resistance was observed for gentamicin, amikacin and kanamycin, while 42.9% of the isolates were resistant to nalidixic acid and ciprofloxacin, which is similar to the findings of Halfaoui et al., (13). For *Klebsiella* isolates, no resistance was reported for gentamicin, amikacin and kanamycin but all the strains were resistant to ampicillin and tetracycline, 87.5% to furanes and nalidixic acid, 62.5% to streptomycin, 75% to ciprofloxacin, and 50% to ofloxacin, which are similar findings to the study by Burtram et al., (36).

With regards to the minimum inhibitory concentration of chloramphenicol, ciprofloxacin, tetracycline, nalidixic acid and ofloxacin, the resistance breakpoints were 8µg/ml, 0.0063µg/ml, 8µg/ml, 8µg/ml, and 0.25 µg/ml respectively. By this, the isolates were sensitive to the antibiotics tested except for tetracycline where intermediate resistance was found. The most frequently transferred resistance markers were *ASTeSuTmp* (18.5 %) and *SuTmp* (12.3%). However, the *Tmp* trait was present in 86.2%, *Te* (tetracycline) in 50.8%, *Su* (sulfamid) in 78.5% and *A* (ampicillin) in 43.1% of the transconjugants, similar to what Poirel et al., (38) reported. The high presence of these traits in the transconjugants can be explained by the fact that they are carried by easily disseminated characters, and the misuse of antibiotics as growth promoters and prophylaxis in animal husbandry, implies that there is always a reservoir of resistance and dissemination of the plasmids. However, traits such as nalidixic acid, ciprofloxacin and furans appeared not easily transferable as previously reported (38,39).

The grouping of the plasmids allowed us to determine the *Inc* group to which the plasmids belong, which are groups of the Com1 and F1 family, already described by Chaslus-Dancla et al., (40), with exception of 4 plasmids that we could not group. The colicin test was carried out on all the isolates which transferred antibiotic resistance trait. With the knowledge that the gene encoding colicin can be attached to antibiotic resistance gene (R plasmid), we investigated the production of colicin in the transconjugants obtained from the transfer of antibiotic resistance, and their wild type isolates. Of the 48 wild type isolates, 35.4% (n=17) produced colicins but only 3 (6.3%) transconjugants were colicins positive. These results led us to suppose that the antimicrobial resistance and production of colicin, are two different character traits carried by the same plasmid, even if it occurs at low frequency (41).

Conclusion:

This study confirmed the presence of multiple antibiotic resistant *E. coli* and other members of the family Enterobacteriaceae in poultry in Algeria, and showed that these transferable antibiotic resistance traits are easily disseminated by plasmids.

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Original Article **Open Access**

Impact of decalcification on antibacterial properties of eggshell against selected poultry pathogens

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Abstract:

Background: Eggshell which is primarily composed of more than 98% calcium carbonate crystal, serves as the physical protective and active barrier structure of egg content. Recently, antimicrobial properties of eggshell are fast becoming center of interest among stakeholders of poultry industry. However, few studies have focused on the rigidity factor of calcium components of eggshell as antimicrobial agent. Thus, this study was designed to determine the effect of decalcification on the ability of eggshell to inhibit common poultry and egg bacterial pathogens.
Methods: Raw eggshell denoted as calcified eggshell (CES) and decalcified eggshell (DES) were extracted and made into fine powder. Standard protocol was used for preparations of CES and DES at concentrations of 10, 5, 2.5 and 1.25 mg/ml, and their antibacterial assays on selected bacterial pathogens (*Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella* Typhi) were performed by agar diffusion method. Gentamicin 80mg solution (CC1) and distilled water (CC2) served as controls. Data were analysed with SPSS version 20.0 and presented as mean±SD for descriptive statistics. Friedman's two-way test ANOVA was used to compare the differences in mean values between CES, DES, CC1 and CC2 at significance level of $p<0.05$.
Results: The mean zone diameter of inhibition produced by DES (range 13–28mm) for the isolates was significantly higher ($p<0.05$) than that produced by CES (range 10-21mm). However, the mean zone diameter of inhibition produced by CC1 (gentamicin) (range 16-40mm) was higher than that produced by DES or CES ($p<0.05$). The concentrations of DES and CES have no significant antibacterial effect on *B. subtilis* and *K. pneumoniae* ($p>0.05$), but had inverse effect on *P. aeruginosa*. Overall, DES had a better inhibitory effect than CES against *B. subtilis*, *K. pneumoniae* and *P. aeruginosa*, but notably, neither DES nor CES had inhibitory effect on *E. coli* and *S.* Typhi.
Conclusion: Poor antibacterial effect of CES may be attributed to the calcium-protein interactions within bacterial cell membrane, which hinders absorption or mobility mechanism of the antibacterial factor of the eggshell, but decalcification had significant impact on the antibacterial profile of the eggshell for some bacterial isolates. However, *S.* Typhi and *E. coli* were totally resistant to both DES and CES. Breed of eggs with minimal calcified eggshell to withstand transportation fragility, may enhance antibacterial index and shelf-life of table eggs.

Keywords: Decalcification; Antibacterial; Eggshell; Poultry; Pathogens.

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Impact de la décalcification sur les propriétés antibactériennes de la coquille d'œuf contre certains pathogènes de la volaille

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Abstrait:

Contexte: La coquille d'œuf, qui est principalement composée de plus de 98% de cristaux de carbonate de calcium, sert de structure de protection physique et de barrière active du contenu en œufs. Récemment, les propriétés

antimicrobiennes de la coquille d'œuf sont devenues rapidement un centre d'intérêt parmi les intervenants de l'industrie avicole. Cependant, peu d'études se sont concentrées sur le facteur de rigidité des composants calciques de la coquille d'œuf en tant qu'agent antimicrobien. Ainsi, cette étude a été conçue pour déterminer l'effet de la décalcification sur la capacité de la coquille d'œuf à inhiber les pathogènes bactériens courants de la volaille et des œufs.

Méthodologie: La coquille d'œuf crue dénommée coquille d'œuf calcifiée (CES) et la coquille d'œuf décalcifiée (DES) ont été extraites et transformées en poudre fine. Le protocole standard a été utilisé pour les préparations de CES et DES à des concentrations de 10, 5, 2,5 et 1,25 mg/ml, et leurs dosages antibactériens sur des pathogènes bactériens sélectionnés (*Bacillus subtilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli* et *Salmonella* Typhi) ont été réalisés par méthode de diffusion d'agar. Une solution de gentamicine 80mg (CC1) et de l'eau distillée (CC2) ont servi de témoins. Les données ont été analysées avec SPSS version 20.0 et présentées sous forme de moyenne ± écart-type pour les statistiques descriptives. Le test bidirectionnel ANOVA de Friedman a été utilisé pour comparer les différences de valeurs moyennes entre CES, DES, CC1 et CC2 au niveau de signification de p <0,05.

Résultats: Le diamètre moyen de la zone d'inhibition produite par le DES (gamme 13-28 mm) pour les isolats était significativement plus élevé (p<0,05) que celui produit par le CES (gamme 10-21 mm). Cependant, le diamètre moyen de la zone d'inhibition produite par CC1 (gentamicine) (gamme 16-40 mm) était plus élevé que celui produit par DES ou CES (p<0,05). Les concentrations de DES et CES n'ont pas d'effet antibactérien significatif sur *B. subtilis* et *K. pneumoniae* (p>0,05), mais ont eu un effet inverse sur *P. aeruginosa.* Dans l'ensemble, le DES avait un meilleur effet inhibiteur que le CES contre *B. subtilis, K. pneumoniae* et *P. aeruginosa*, mais notamment, ni le DES ni le CES n'avaient d'effet inhibiteur sur *E. coli* et *S.* Typhi.

Conclusion: Le faible effet antibactérien du CES peut être attribué aux interactions calcium-protéines au sein de la membrane cellulaire bactérienne, ce qui entrave l'absorption ou le mécanisme de mobilité du facteur antibactérien de la coquille d'œuf, mais la décalcification a eu un impact significatif sur le profil antibactérien de la coquille d'œuf pour certaines bactéries isoler. Cependant, *S.* Typhi et *E. coli* étaient totalement résistants au DES et au CES. Race d'œufs avec une coquille d'œuf calcifiée minimale pour résister à la fragilité du transport, peut améliorer l'indice antibactérien et la durée de conservation des œufs de table.

Mots-clés: décalcification; Antibactérien; Coquille d'oeuf; La volaille; Les agents pathogènes.

Introduction:

Eggshell matrix is primarily composed of calcium carbonate crystals (98%) held together by protein and biominerals accounting for the remaining 2% (1). Rigidity of the eggshell depends on the calcium component that confers active physical barrier against penetration by pathogenic microbes. However, within the average of 30 days shelf-life of table eggs, proteolytic enzymes synthesized by these microbes gradually collapses the eggshell matrix, thereby compromising the active barrier (2,3,4). Bacteria penetration and egg deterioration rates directly correlate with eggshell thickness (5). Perhaps, this indicates, directly or indirectly, that antimicrobial property of eggshell is a function of calcium. Exclusive from the seeming antimicrobial influence of calcium components of eggshell, recent studies have shown that calcium component of eggshell can be an economic alternative to lime as soil stabilizer (6), and can be a combined nutritional therapy for patients with osteoporosis without significantly increasing the level calcium in blood (4).

Antimicrobial properties of eggshell are not prominent but there are prospects on the rigidity factor of calcium components of eggshell as antimicrobial agent. Atee et al., (7) successfully reduced the growth rate of *Agrobacterium tumefaciens* by 7-10% and completely inhibited growth using calcium carbonate and nano-calcium carbonate respectively. The increasing prevalence of some of these pathogens and their biotoxins are posing serious threat to the egg industry (2,8). Enterotoxins from *E. coli*, *Salmonella* spp, *Shigella* spp, *Campylobacter* spp, *Listeria monocytogenes* and carcinogenic biotoxins from *Aspergillus flavus* and *Aspergillus parasitiscus* are among the threats to the global market of poultry and egg industry (2,5,9). Unfortunately, effective management of these pathogens are hampered by antibiotics resistance hype and consequences of antibiotics use/misuse in poultry industry (10). These have necessitated the search for alternative antimicrobial approaches. Thus, this study aims to assess the impact of calcium component on the antimicrobial properties of eggshell.

Materials and method:

Collection of eggshells

Eggshells used for this study were from local chicken breed of Fulani ecotype. The local chickens were fed with household wastes, supplemented with worms and insects in a semi-intensive management system.

Preparation of calcified eggshell (CES)

Five fresh table eggs were cleansed with sterile cotton wool soaked in 70% alcohol. The posterior ends of the eggs were cracked to allow complete drainage of the egg contents, leaving behind the eggshell and shell membrane which were air-dried at room temperature. The egg-

shells with membrane were crushed with mortal, pestle into fine powder, and labelled as calcified eggshell (CES). About 1g of CES was added into test tube containing 9 ml of distilled water and labelled 100mg/ml. This was vigorously agitated for about 2-3mins and used as stock solution for preparation of 10mg/ml, 5mg/ml, 2.5mg/ml and 1.25mg/ml concentrations using distilled water as diluent.

Preparation of decalcified eggshell (DES)

The theory of decalcifying an egg is based on acid-base reaction. Vinegar (acetic acid) react with calcium carbonate crystal (base) to release carbon dioxide (gas) and calcium residues. Five fresh table eggs were immersed separately into 250ml beakers containing acetic acid and kept standing for 24 hours for the gas bubbles to escape. Clean spoons were to use to scoop the eggs into fresh 250ml beakers containing acetic acid and kept for another 24 hrs. After this, the eggs were rinsed in distilled water. The eggs appeared cooked and the inner eggshell of the decalcified eggs were carefully extracted and allowed to dry at room temperature. The extracts were crushed with mortar and pestle into fine powder and labelled as decalcified eggshell (DES). About 1g of DES was added into test tube containing 9ml of distilled water and labelled 100mg/ml. This was vigorously agitated for about 2-3 mins and used as stock solution for preparation of 10mg/ml, 5mg/ml, 2.5mg/ml and 1.25mg/ml concentrations using distilled water as diluent.

Preparation of gentamicin control (CC1)

One milliliter of commercial gentamicin (80mg/mL) solution was added to 7ml of distilled water to obtain stock solution of 10mg/ml, which was serially diluted with distilled water to prepare 5mg/ml, 2.5mg/ml and 1.25mg/ml solutions.

Antibacterial activity of test and control samples

The antibacterial activities of DES, CES, CC1 (gentamicin positive control) and CC2 (distilled water negative control) were evaluated against *Pseudomonas aeruginosa, Salmonella* Typhi*, Escherichia coli, Klebsiella pneumoniae,* and *Bacillus subtilis* using agar diffusion technique. The microbial strains were obtained from the Bacterial Bank of the Department of Microbiology, Ibrahim Badamasi Babangida University, Lapai.

Each test bacterial isolate was pre-enriched in Mueller-Hinton broth (MHB) for 24 hours at 37^{o}C and suspension equivalent to 0.5 McFarland standard solution (10^{8} CFU/ml) was prepared. One ml of the suspension was inoculated onto wells of Mueller-Hinton agar (each for DES, CES, CC1 and CC2) that bad been bored on each plate using a sterile 4 mm cock-borer. The inoculated plates were incubated aerobically at 37^{o}C for 18-24 hours after which the diameter of zone of inhibition (in mm) around each well was measured with a calibrated meter rule. The assay was performed in triplicates, and the mean zone diameters of inhibition produced by the test and control samples was calculated for each bacterial isolate.

Statistical analysis

Statistical analysis was performed using SPSS software version 20.0. Data were presented as mean±standard deviation and Friedman's two-way test ANOVA was used to compare the difference between the mean zone diameter of inhibition produced by DES, CES and CC1. *P* value less than 0.05 was considered statistically significant.

Results:

Bacillus subtilis was relatively inhibited at all the concentrations of DES and CES with mean zone diameters of inhibition ranging from 15±2.38mm to 24±4.20mm. Comparably, the mean zone diameters of inhibition of this bacterium to DES and CES were not significantly different ($p>0.05$) but were significantly ($p<0.05$) lower than that for gentamicin control (Fig 1).

The zone diameters of inhibition by DES and CES for *P. aeruginosa* were trendy but did not correlate with the different concentrations of DES and CES. The mean zone diameters of inhibition were significantly different ($p<0.05$) between DES (25-28±1.29mm) and CES (17-21 ±1.70mm) and from the gentamicin control (28-34±2.65mm) (Fig 2).

Similarly, *K. pneumoniae* showed trendy inhibition zone diameters to DES and CES. The mean zone diameters of inhibition of DES (13-15±0.96mm) and CES (10-15±2.65mm) were only significantly different ($p<0.05$) at 2.5mg/l while all other test concentrations (10 mg/l, 5 mg/l and 1.25 mg/l) did not show significant difference ($p>0.05$). However, the mean zone diameter of inhibition to the gentamicin control sample (16-27±4.65mm) was significantly different ($p<0.05$) compared to the DES and CES test samples (Fig 3).

The DES and CES had no effect on *S.* Typhi and *E. coli*, as no zones of inhibition were produced. Expectedly, the concentrations of the gentamicin control sample were inversely proportional to the mean zone diameters of inhibition (Figs 4 and 5).

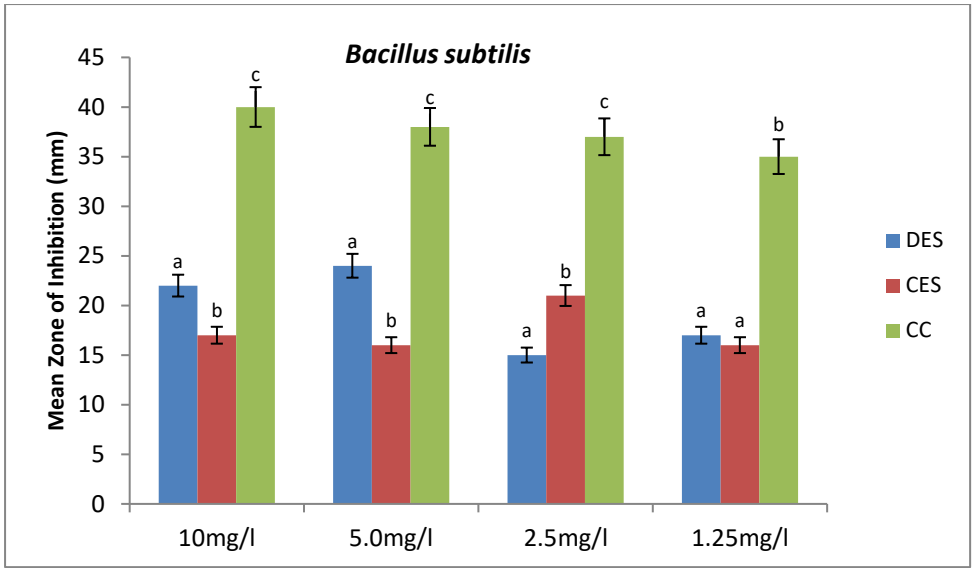

Fig. 1: Susceptibility profile of *Bacillus subtilis* to DES and CES

DES = Decalcified Eggshell; CES = Calcium containing Eggshell; CC = Control Gentamicin (80mg/ml);
*Bars bearing different alphabets are significantly different (p<0.05) across each concentration subsets;
**‡ = Standard deviation (±) of the zones of inhibitions values

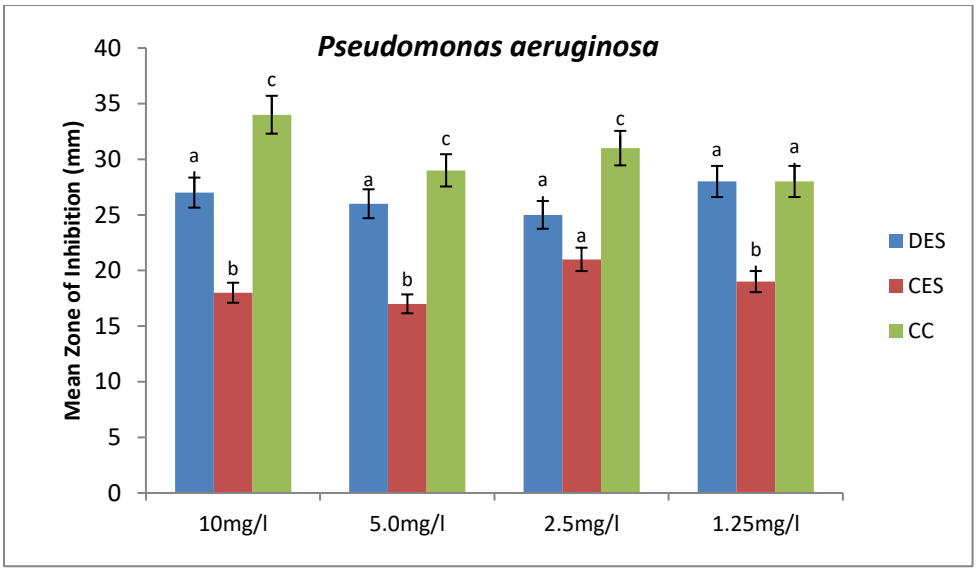

Fig. 2: Susceptibility profile of Pseudomonas *aeruginosa* to DES and CES

DES = Decalcified Eggshell; CES = Calcium containing Eggshell; CC = Control Gentamicin (80mg/ml);
*Bars bearing different alphabets are significantly different (p<0.05) across each concentration subsets;
**‡ = Standard deviation (±) of the zones of inhibitions values

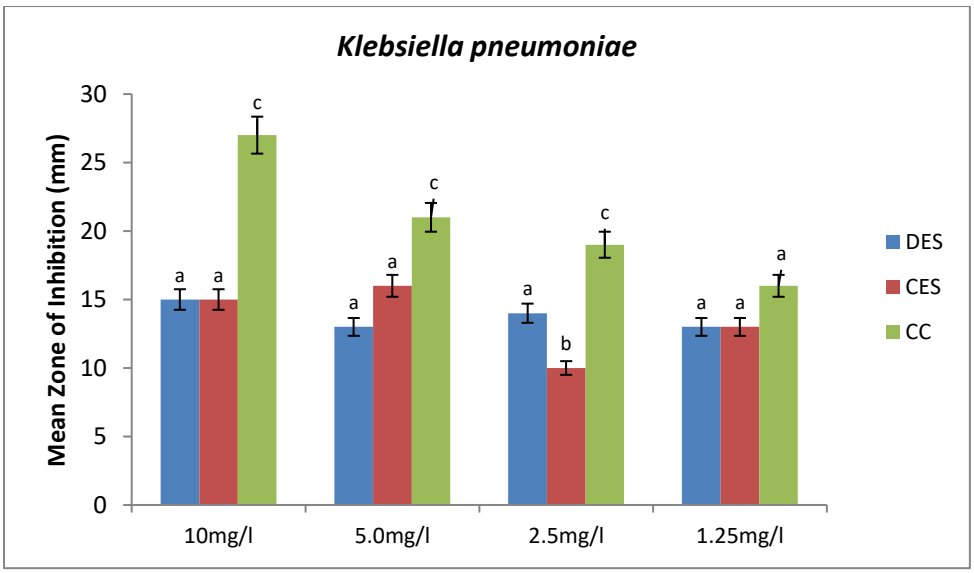

Fig. 3: Susceptibility profile of *Klebsiella pneumoniae* to DES and CES

DES = Decalcified Eggshell; CES = Calcium containing Eggshell; CC = Control Gentamicin (80mg/ml)
*Bars bearing different alphabets are significantly different ($p<0.05$) across each concentration subsets
**‡ = Standard deviation (±) of the zones of inhibitions values

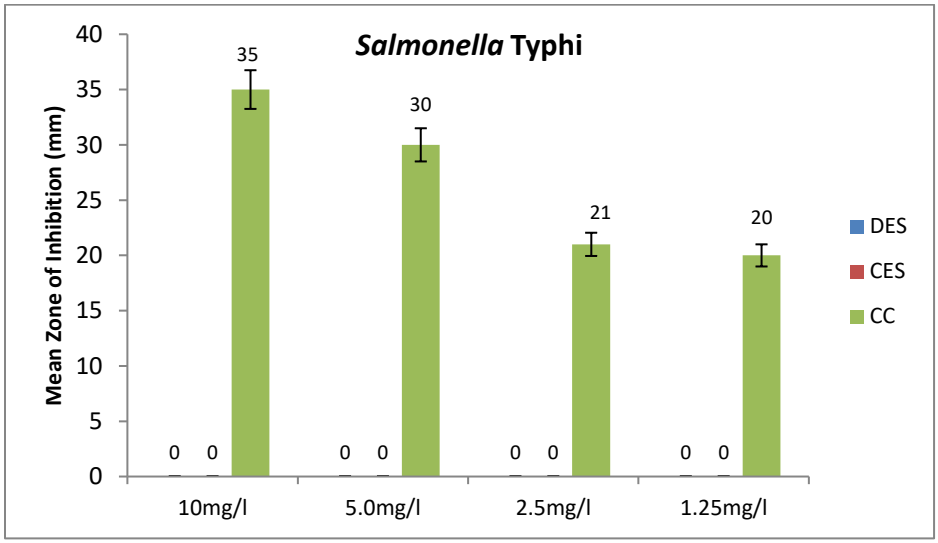

Fig. 4: Susceptibility profile of *Salmonella* Typhi to DES and CES

DES = Decalcified Eggshell; CES = Calcium containing Eggshell; CC = Control Gentamicin (80mg/ml)
*Bars bearing different alphabets are significantly different ($p<0.05$) across each concentration subsets
**‡ = Standard deviation (±) of the zones of inhibitions values

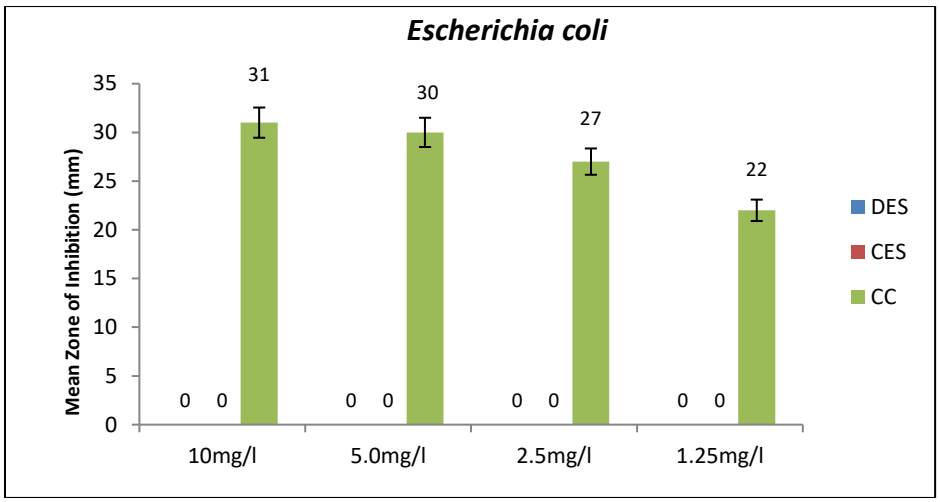

Fig. 5: Susceptibility profile of *Escherichia coli* to DES and CES

DES = Decalcified Eggshell; CES = Calcium containing Eggshell; CC = Control Gentamicin (80mg/ml)
*Bars bearing different alphabets are significantly different ($p<0.05$) across each concentration subsets
**‡ = Standard deviation (±) of the zones of inhibitions values

Discussion

Susceptibility of *B. subtilis* and *P. aeruginosa* to purified protein extracts (ansocalcin and ovocleidin-17) of eggshell (11) have been documented, where the authors reported that calcium enhances antibacterial activity, but this is contrary to the findings of our study. Perhaps, extraction and purified nature of these eggshell proteins require calcium binding to be effective unlike the whole eggshell used in our study. *Klebsiella* spp as opportunistic pathogens are common isolates of poultry products with relative average resistance rate of 50-60% to the common antimicrobial drugs (12,13). Based on the efficiency of eggshells to inhibit this opportunistic pathogen, proper hygiene is advocated to avoid compromising the integrity of eggshells during handling and processing.

Total resistance of *S.* Typhi to DES and CES was not a surprising observation in our study as several reports spanning decades have implicated *Salmonella* as an infamous poultry product contaminant (14), agent of disease outbreaks (15), and significant economic losses (9). Perhaps, the ability of *Salmonella* to infect eggs prior to shell formation enables it to avert most antimicrobial agents popularly used within the poultry industry, as shown by *S.* Typhi and *E. coli* total resistant to all concentrations of DES and CES in our study. Similarly, more than 95% trans shell penetrative ability of *E. coli* was observed by Cook et al., (8), which connotes a strong resistance of *E. coli* to antibacterial factor of eggshells. The authors also concluded that bacterial trans-shell invasions were not proportional to the thickness of eggshell, which supports the inference of our study that calcium (thickness factor of eggshells) is not a determinant factor of antimicrobial activity of eggshell.

Although high calcium content indicates superior protective structural and physical strength of eggshell, it significantly decreases the antibacterial profiles of eggshell. The absorption or mobility mechanism of antimicrobial factor of eggshell may be limited by calcium-protein interactions within bacterial cell membrane. This may explain why eggs with less calcium content in shell have extended shelf-life compared to those with higher calcium content (16). Comparative variations of calcium content of eggshell (17) is known to add more validity to notable shelf-life differences of eggs.

Conclusion:

The poor antibacterial effects of CES in our study may be attributed to the calcium-protein interactions within bacterial cell membrane, which hinders absorption or mobility mechanism of antibacterial factor of the eggshell. Decalcification have significant positive impact on the antibacterial profile of eggshell. However, *S.* Typhi and *E. coli* were totally resistant to both DES and CES. Thus, this study opined that combination of proper hygiene and special breed of eggs with moderate calcified eggshell (to withstand transportation fragility) will enhance microbial shelf-life of table eggs.

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Original Article **Open Access**

Salmonella Dublin associated with abortion in dairy cattle in Algiers and comparison of different diagnostic methods

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Abstract:

Background: In cattle, many serotypes of *Salmonella enterica* are responsible for a wide variety of clinical manifestations, which can cause considerable economic loss. Some serotypes can cause cows to abort sporadically, such as the Dublin serotype. This study was carried out on different cattle farms in the Algiers region to determine the prevalence of *Salmonella* Dublin using bacteriological and immunological methods.
Methodology: The prevalence of *Salmonella* was determined by bacteriological analysis in accordance with the reference method AFNOR NF U 47-100 on faecal samples collected from 184 cattle belonging to 19 different farms, and serotyping for *S.* Dublin. Immunological analysis by enzyme-linked immunosorbent assay (ELISA) for *S.* Dublin was carried out on milk samples collected from 91 cattle. A survey of case (n=5) and control (n=14) farms for comparative analysis was performed to demonstrate a link between abortion in cows and prevalence of *S.* Dublin with both bacteriological and immunological methods. Sensitivity, specificity, Cohen Kappa coefficient, McNemar test odds ratios, and confidence intervals were calculated using Winepiscope 2.0 and StatA 9.1 software, and $p<0.05$ was considered as statistically significant.
Results: The bacteriological results showed a prevalence of 7.6% (95%CI: 3-10), for *Salmonella* and serotyping revealed a prevalence for *S.* Dublin of 2.7%. The immunological analysis of milk by the ELISA technique revealed a prevalence of 13.2% (95%CI: 5-20) for *S.* Dublin. The comparative study between immunological results from milk and bacteriological results from faeces for detecting *S.* Dublin showed poor agreement between the two tests (k=0.25), with enzyme immunoassay being significantly more sensitive than the bacteriological test ($p<0.05$). The results of the survey did not demonstrate a clear association between bacteriological detection of *S.* Dublin in faeces and abortion in cows (OR=8.66, 95%CI: 0.58-130.12). However, with the immunological analysis of milk for *S.* Dublin, there was a significant positive association (OR=62.33, 95%CI: 2.13-18.22) between a positive antibody response to *S.* Dublin in milk and the presence of abortions on the farm.
Conclusion: In view of these results, we can conclude that *Salmonella* infections should systematically feature in the differential diagnosis of abortions in dairy cattle in Algeria.

Keywords: *S.* Dublin, cattle, faeces, milk, abortion, immunology, bacteriology, Algiers

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Salmonella Dublin associée à l'avortement chez les bovins laitiers à Alger et comparaison de différentes méthodes de diagnostic

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Résumé:

Contexte: Chez les bovins, de nombreux sérotypes de *Salmonella enterica* sont responsables d'une grande variété de manifestations cliniques, ce qui peut entraîner des pertes économiques considérables. Certains sérotypes peuvent provoquer des avortements sporadiques chez les vaches, comme le sérotype Dublin. Cette étude a été réalisée dans différents élevages bovins de la région d'Alger pour déterminer la prévalence de *Salmonella* Dublin à l'aide de méthodes bactériologiques et immunologiques.

Méthodologie: La prévalence de *Salmonella* a été déterminée par analyse bactériologique selon la méthode de référence AFNOR NF U 47-100 sur des échantillons fécaux prélevés sur 184 bovins appartenant à 19 exploitations différentes, et sérotypage pour *S.* Dublin. Une analyse immunologique par dosage immunoenzymatique (ELISA) pour *S.* Dublin a été réalisée sur des échantillons de lait prélevés sur 91 bovins. Une enquête sur des cas (n=5) et des fermes témoins (n=14) pour une analyse comparative a été réalisée pour démontrer un lien entre l'avortement chez les vaches et la prévalence de *S.* Dublin avec des méthodes bactériologiques et immunologiques. La sensibilité, la spécificité, le coefficient Cohen Kappa, les Odds ratios du test de McNemar et les intervalles de confiance ont été calculés à l'aide des logiciels Winepiscope 2.0 et StatA 9.1, et $p<0,05$ a été considéré comme statistiquement significatif.
Résultats: Les résultats bactériologiques ont montré une prévalence de 7,6% (IC 95%: 3-10), pour *Salmonella* et le sérotypage a révélé une prévalence pour *S.* Dublin de 2,7%. L'analyse immunologique du lait par la technique ELISA a révélé une prévalence de 13,2% (IC à 95%: 5-20) pour *S.* Dublin. L'étude comparative entre les résultats immunologiques du lait et les résultats bactériologiques des fèces pour la détection de *S.* Dublin a montré une mauvaise concordance entre les deux tests (k=0,25), le dosage immunoenzymatique étant significativement plus sensible que le test bactériologique ($p<0,05$). Les résultats de l'enquête n'ont pas démontré une association claire entre la détection bactériologique de S. Dublin dans les fèces et l'avortement chez les vaches (OR = 8,66, IC à 95%: 0,58-130,12). Cependant, avec l'analyse immunologique du lait pour *S.* Dublin, il y avait une association positive significative (OR=62,33, IC 95%: 2,13-18,22) entre une réponse anticorps positive à S. ferme.
Conclusion: Au vu de ces résultats, nous pouvons conclure que les infections à *Salmonella* devraient systématiquement figurer dans le diagnostic différentiel des avortements chez les bovins laitiers en Algérie.

Mots clés: *S.* Dublin, bovins, fèces, lait, avortement, immunologie, bactériologie, Alger

Introduction:

Salmonella infections are major concern in animal husbandry and public health. Ruminants, in particular, cattle are victims of salmonellosis, which has serious economic consequences on animal production (1). Cattle are the main reservoir for *Salmonella enterica* subsp *enterica* serovar Dublin (*Salmonella* Dublin) which is considered to be the most common cause of *Salmonella* infections in cattle (2). *S.* Dublin is the serotype of most economic concern due to its particularly invasive nature, causing diarrhoea, sepsis, and mortality, mainly in calves aged 2 weeks to 3 months, as well as affecting reproduction, and causing abortions in cattle (3). As a host-adapted strain in cattle, animals infected with *S.* Dublin can become a chronic subclinical reservoir that has the potential to excrete large numbers of bacteria in the environment. These reservoirs also play an important role in maintaining infection within a herd by excreting the germ not only in faeces, but also in milk and colostrum (4). This serotype can be difficult to detect due to asymptomatic carrier status with intermittent bacteraemia and shedding (4,5).

Several studies have shown that bacteriological method for detection of *S.* Dublin in infected cattle suffers limitations in terms of sensitivity compared to serological methods (6). Therefore, the most widely used tests for the detection of *S.* Dublin include the enzyme-linked immunosorbent assays (ELISA) in the serum and in milk (7,8,9). Despite its importance, this disease has so far been very little studied in the Algerian context, and the epidemiology of *S.* Dublin infections in cattle remains largely unknown, either in terms of the prevalence of the infection or its impact on abortions on the farms. The objective of this study is to provide information on the epidemiological situation of this disease in Algeria and particularly in the Wilaya of Algiers.

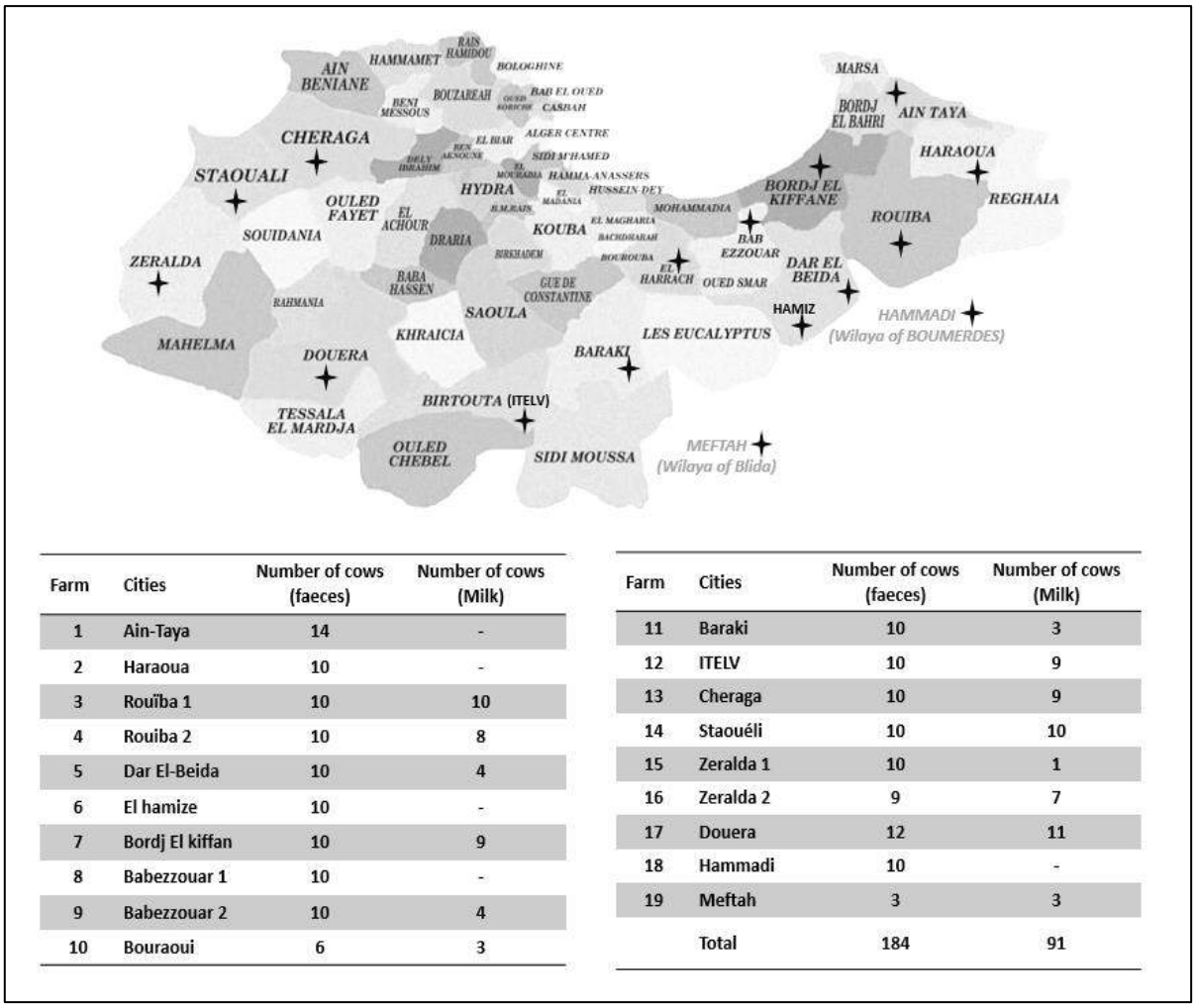
Materials and method:

Study area and sampling technique

We carried out our samples in different regions of the Wilaya of Algiers (Fig. 1). The region studied has 1,281 breeders with 13,115 herd of cattle, including 7,514 herds of dairy cows (10). The selection of farms was done by random sampling method, using a list of cattle breeders in the Wilaya of Algiers, to ensure homogeneous distribution of the farms in the study area. Subsequently, the number of cattle to be included in the study from each farm was defined according to the total number of cattle present. When the farm had less than 10 cattle, all cattle were included. When the farm contained more than 10 cattle, the number of cattle included was at least 10, the objective of which was to have a sample representing at least 10% of all cattle present in the farms selected (11,12).

Survey of 'case' and 'control' farms

A pre-validated epidemiological questionnaire was administered to herd owners from the selected 19 farms; 5 as 'case' and 14 as 'control' farms. The questionnaire was interviewer-administered on the day of sample collection to determine whether or not the cattle in the farm had experienced abortion episodes. The questionnaire contained information related to the herds visited (management system, type and size of herd) and the cows enrolled (breed, age, pregnancy, month of gestation, history of abortion, stage of pregnancy at which abortion occurred, pathological history and clinical signs observed at the time of sampling). 'Case' farms were those where episodes of abortions had occurred in the last 5 years, a phenomenon not observed in the 'control' farms. A farm was considered positive if at least one animal was positive.



Farm	Cities	Number of cows (faeces)	Number of cows (Milk)
1	Ain-Taya	14	-
2	Haraoua	10	-
3	Rouïba 1	10	10
4	Rouiba 2	10	8
5	Dar El-Beida	10	4
6	El hamize	10	-
7	Bordj El kiffan	10	9
8	Babezzouar 1	10	-
9	Babezzouar 2	10	4
10	Bouraoui	6	3

Farm	Cities	Number of cows (faeces)	Number of cows (Milk)
11	Baraki	10	3
12	ITELV	10	9
13	Cheraga	10	9
14	Staouéli	10	10
15	Zeralda 1	10	1
16	Zeralda 2	9	7
17	Douera	12	11
18	Hammadi	10	-
19	Meftah	3	3
	Total	184	91

Fig. 1: Location of farms studied and number of cows sampled from each farm

Sample collection

Faeces were collected from the rectum of 184 cows; 43 from the 5 'case' farms and 141 from the 14 'control' farms. The faeces were stored in sterile jar with a capacity of 100 ml, and then sent for laboratory analysis on the same day. In addition, milk samples were collected from 91 of the 184 cows; 34 from the 5 'case' farms and 57 from 9 of the 14 'control' farms and then transferred to sterile tubes with a capacity of 10 ml. The samples were stored in a cool place at -20°C and analyzed in the microbiology laboratory of the National Veterinary School of El Alia (ENSV), Oued Smar, Algeria.

Bacteriological analysis

This method was based on the application of the Association française de normalisation (AFNOR), NF U 47-100 standard (18). This technique is a standard method of research by isolation and identification of any specified serovar(s) of *Salmonella* in the environment of animal production (Fig 2) as itemized in the following steps;

Pre-enrichment with buffered peptone water:

25g of faeces were added to 250ml of buffered peptone water (Pasteur Institute of Algeria, EPT) at room temperature and incubated for 18 (±2) hours in an incubator set at 37°C.

Enrichment in liquid and semi-solid media:

This step allowed the growth and selection of bacteria of the genus *Salmonella*, with the use of two media selective enrichments in parallels; MSRV (Modified Semi-solid Rappaport-Vassiliadis) medium (BioRad, France) and MKTTn (Müller-Kauffmann Tetrathionate) medium (Bio-Rad, Marnes-La-Coquette, France). Three drops (total of about 0.1mL) of the pre-enrichment broth were transferred and inoculated on the semi-solid agar dishes of the MSRV medium. The medium was supplemented with novobiocin solution before pouring into the Petri dishes to inhibit the growth of Gram-positive bacteria. The plates were incubated at 41.5°C (±1) for 24 hours cover up, and then examined. If the migration is greater than 20mm

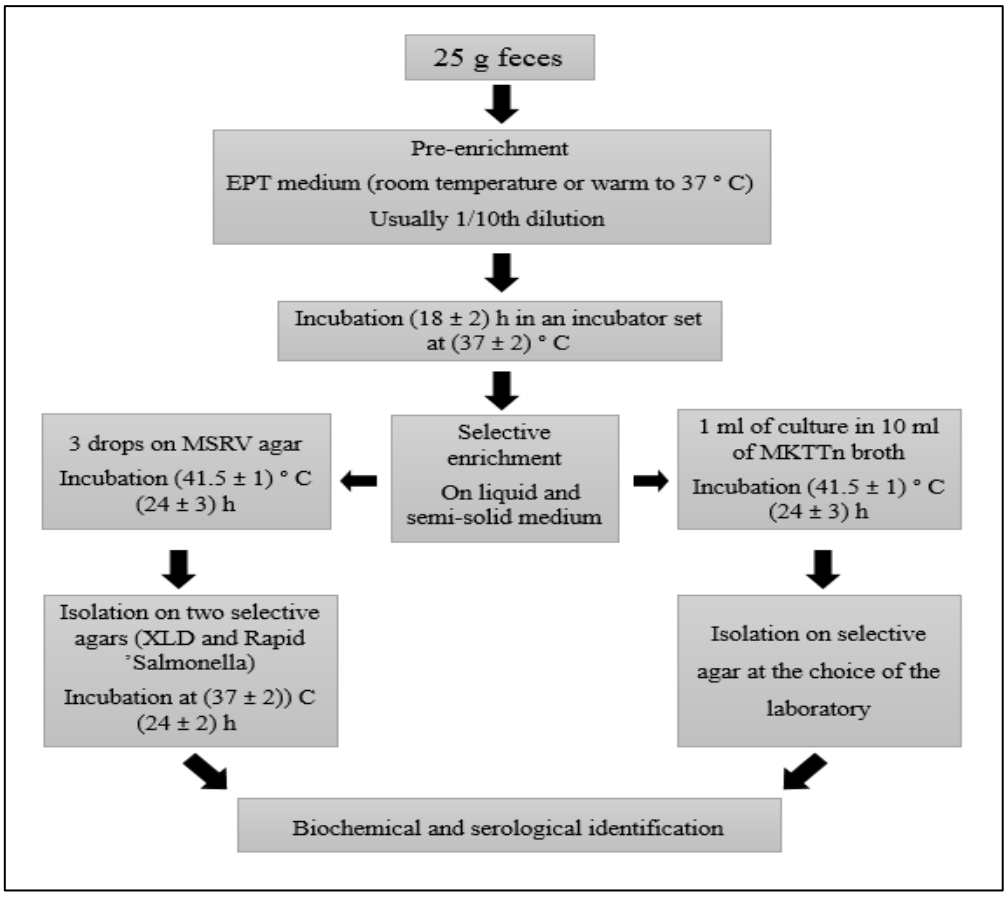


Fig 2: Analysis diagram according to the reference method AFNOR, NF U 47-100 (18)

from the point of inoculation, an inoculum was taken from the periphery of the migration zone and then inoculated on the RAPID *Salmonella* chromogenic medium (Bio-Rad, Marnes-La-Coquette, France) and the XLD medium (XLD: Condalab, Madrid, Spain) by an appropriate isolation technique. For the MKTTn medium, 1ml of the pre-enrichment broth was transferred to a 10 ml tube of MKTTn broth, and then incubated at 41.5°C (±1) for 24 (±3) hours.

Isolation of *Salmonella*

Each typical *Salmonella* colony was taken from each of the selective media (XLD and chromogenic agar). The typical *Salmonella* colony appeared red with black centers on XLD and on RAPID *Salmonella* chromogenic medium, *Salmonella* formed characteristic magenta colonies. The colonies recovered were then purified on nutrient agar (GN: Pasteur Institute of Algeria) after incubation at 37°C for 18-24 hours.

Identification and serotyping of *Salmonella*

Confirmation of suspected *Salmonella* colonies was carried out using Triple Sugar Iron agar (TSI: Pasteur Institute of Algeria) and API 20E gallery (BioMérieux, France). *Salmonella* serovars were identified by serotyping with slide agglutination reaction using diagnostic polyvalent and monovalent O and H *Salmonella* antisera according to Kauffman-White scheme (13).

Serological analysis on milk samples

For the detection of specific antibodies against *S.* Dublin, we used an indirect ELISA test for the detection of antibodies directed against the O antigen (part of the lipo-polysaccharide LPS); 1, 9 and 12 of S. Dublin, and performed according to the manufacturer's instructions (Prio CHECK *Salmonella* Antibody ELISA Dublin; Thermo Fisher Scientific, Waltham, MA). Briefly, milk samples were first heated for one hour at 37°C. The upper layer of fat was pulled out, and the undiluted skim milk samples were inoculated in microtiter plate and the optical density (OD) was measured at 450 nm using ELISA reader (Bio-Rad, USA).

Statistical analysis

Sensitivity, specificity, accuracy, Cohen Kappa coefficient, McNemar test Odds ratios and confidence intervals were calculated for comparison of bacteriological and immunological methods using Winepiscope 2.0 and Stat A 9.1 softwares. $P<0.05$ was considered as statistically significant.

Results:

Prevalence of *Salmonella* spp and *S.* Dublin by bacteriological analysis of the cattle faeces

The results obtained show that of the 184 faecal samples, 14 (7.6%) were positive for *Salmonella* spp., and 5 (2.7%) were positive for *S.* Dublin. Of the 19 farms studied, *Salmonella* spp was isolated in 6 (31.6%) and *S.* Dublin in 3 (15.8%) (Table 1).

Seroprevalence of *S.* Dublin by ELISA assay on the cow milk

Of the 19 selected farms, 14 were analyzed for antibodies to *S.* Dublin in the milk samples of 91 cows. Twelve milk samples of cow were positive for *S.* Dublin, which represents a prevalence of 13.2% (Table 2).

Table 1: Prevalence of *Salmonella* spp and *S.* Dublin in the farms by bacteriological results of faeces

Farm/Municipality	Number of cattle	*Salmonella* spp (%)	*S.* Dublin (%)
Babezzouar 1	10	6 (60.0)	3 (30.0)
Babezzouar 2	10	1 (10.0)	1 (10.0)
Bordj El kifane	10	1 (10.0)	-
Bouraoui	6	1 (16.7)	-
ITELV	10	4 (40.0)	-
Meftah	3	1 (33.3)	1 (33.3)
Other 13 farms	135	-	-
Total	184	14 (7.6%)	5 (2.7%)

Table 2: Prevalence of *S.* Dublin by bacteriological method on faeces and immunological method on milk of the cattle from the various farms/municipality

Farm/Municipality	Bacteriological test on faeces		Immunological test on milk	
	Number of cattle	Positive for *S.* Dublin	Number of cows	Positive for *S.* Dublin
Rouiba 2	10	0	8	0
Babezzouar 1	10	3	0	0
Babezzouar 2	10	1	4	3
Bordj El kifane	10	0	9	1
Cheraga	10	0	9	3
Staouéli	10	0	10	1
ITELV	10	0	9	2
Meftah	3	1	3	2
Other farms	111	0	39	0
Total	184	5 (2.7%)	91	12 (13.2%)

Comparison of the bacteriological and serological methods of S. **Dublin detection**

The immunological results of 91 milk samples from 184 cows were compared with bacteriological results of 184 faeces from the same cows (Table 3). The results obtained showed that bacteriological analysis had a sensitivity of 16%, specificity of 100% and accuracy of 89%, compared to the immunological assay. The Cohen's Kappa coefficient of 0.25 and McNemar test of 0.004 showed that the two methods gave significantly different values (p <0.05).

Results of survey with respect to bacteriological results of S. **Dublin** ***at the farm level***

The association between exposure to *S.* Dublin and the presence of abortions on the farm as calculated is shown in Table 4. The survey shows farm exposure rate of 40% for the case farms compared to 7.14% for the control farms. However, given the low number of farms tested, the Odds ratio was not significantly different from 1 (p=0.12). As a result, there was no association between *S.* Dublin exposure and the presence of abortions in the case and control farms.

Results of survey with respect to bacteriological results of S. **Dublin** ***for individual cattle***

From the survey at individual cattle level, the calculation of the Odds Ratio revealed a value of 2.2 (Table 5), which is not significantly different from 1 (p=0.38). Analysis of the table shows cattle exposure rate of 4.65% for the case farms, and 2.12% for the control farms. As a result, there was no association between *S.* Dublin in cattle and presence of abortion in case and control farms.

Table 3: Comparison of bacteriology and immunology methods (as gold standard) for identification of *S.* Dublin

		Immunology		**Total**
		Positive	Negative	
Bacteriology	Positive	2	0	2
	Negative	10	79	89
Total		12	79	91

95% CI (4.56 – 17.41); p = 0.0020

Table 4: Result of the survey for bacteriological identification of *S.* Dublin in case and control farms

	Farms	**Case**	**Control**	**Total**
Bacteriology	Positive	2	1	3
	Negative	3	13	16
	Total	5	14	19
	Exposure rate	40%	7.14%	
	Odd	0.66	0.07	
	Odds Ratio (95% CI)	8.66 (0.58- 130.12)		

Table 5: Results of the survey for bacteriological identification of *S.* Dublin in the cattle from case and control farms

Bacteriology	**Animals**	**Cattle from case farms**	**Cattle from control farms**	**Total**
	Positive	2	3	5
	Negative	41	138	179
	Total	43	141	184
	Exposure rate	4.65%	2.12%	
	Odd	0.04	0.02	
	Odds Ratio (95% CI)	2.2 (0.36-13.88)		

Results of survey with respect to immunological results of S.* Dublin *in milk at farm level

The survey at the farm level revealed exposure rate in the case farms to be 100%, in contrast to the control farms which was 11.11%. The OR ratio normally has an infinite value due to the presence of zero. In this case, 0.5 was added to all the values according to Deeks and Higgins, and Addis et al., (14,15). With this modification, we obtain an OR value of 62.33 (2.13-1822) (Table 6), which was significantly different from 1 (p<0.05). As a result, there was a positive association between farm exposure with *S.* Dublin antibody presence in milk and the presence of abortions on the farms.

Results of survey with respect to immunological results of S.* Dublin *in milk at individual cattle level

From the survey of the individual cattle, it revealed the OR of 26.78 (Table 7), which was significantly different from 1 (p< 0.01). As a result, there was positive association between positive *S.* Dublin antibody presence in milk of individual cattle and presence of abortions on the farm. This was further underscored by the *S.* Dublin exposure rate of 32.35% for the cattle in the case farms compared to 1.75% for the cattle in the control farms.

Table 6: Results of the survey for immunological identification of *S.* Dublin in case and control farms

ELISA PrioCHECK	Farms	Case farms	Control farms	Total
	Positive	5 (5.5)*	1 (1.5)*	6
	Negative	0 (0.5)*	8 (8.5)*	8
	Total	5	9	14
	Exposure rate	100%	11.11%	
	Odd	∞ (11)*	0.12 (0.176)*	
	Odds Ratio (95% CI)	62.33 (2.13-1822)		

*The numbers in brackets are the modified values for the calculation of the Odds ratio as described above

Table 7: Results of the survey for immunological identification of *S.* Dublin in the milk of cattle from case and control farms

ELISA PrioCHECK	Animals	Cattle from case farms	Cattle from control farms	Total
	Positive	11	1	12
	Negative	23	56	79
	Total	34	57	91
	Exposure rate	32.35%	1.75%	
	Odd	0.47	0.01	
	Odds Ratio (95% CI)	26.78 (3.27-219.57)		

Table 8: Different studies around the world illustrating the prevalence of *S.* Dublin in milk

Country	Number of samples (Milk) at		*S.* Dublin Prevalence	References
	Herd	Cattle		
California (USA)	/		14.1%	(49)
California (USA)	/		3.5%	(49)
Denmark	1464		9.9%	(51)
Pays-Bas	79		54.5%	(8)
Denmark	4326	/	11%	(52)
Ireland	158	/	49% (78)	(53)
Suede	/	1069	17%	(54)
Suede	/	4683	3% (142)	(55)
New York (USA)	4896	5219	1% (50/5219) 0.9% (46/4896)	(56)

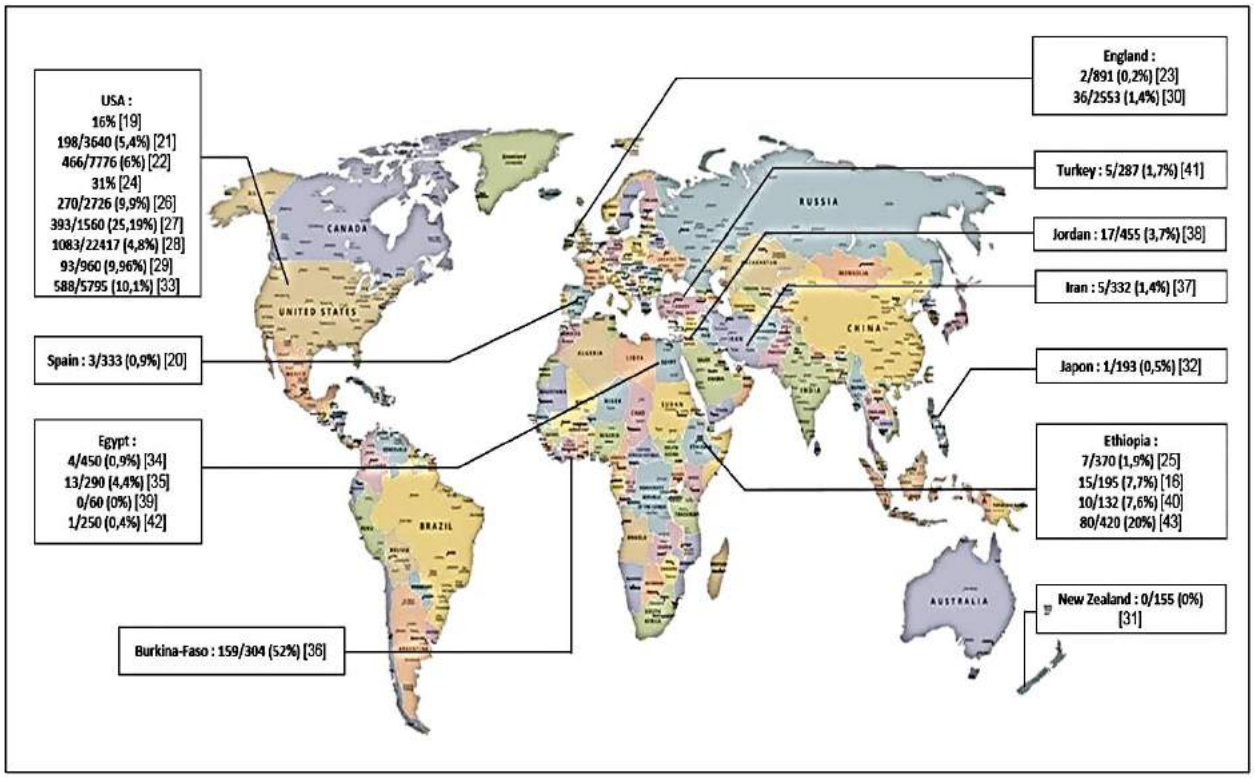


Fig 3: Studies around the world illustrating the prevalence of faecal excretion of *Salmonella* spp in cattle

Discussion:

Salmonellosis remains a significant public health problem around the world, particularly in developing countries (16). In addition, *Salmonella* are emerging pathogens responsible for many diseases in cattle. *S*. Dublin infection is of concern in several countries because of its ability to cause abortions and reduced milk production, as well as the significant economic losses it causes (17). Of the 184 faecal samples analyzed by the AFNOR NF U 100-47 reference method (18) in our study, 14 were positive for *Salmonella* spp or an overall prevalence of 7.60%, while the positivity rate for *S.* Dublin was 2.71%. Numerous epidemiological studies carried out worldwide on faecal excretion of *Salmonella* spp in cattle (16,19-43) showed prevalence of between 0% and 52% (Fig 3). These differences in the prevalence of *Salmonella* could be explained by the seasonal variation in faecal excretion of *Salmonella* in animals. Some studies showed that *Salmonella* excretion was highest in cows sampled from spring through summer (February through September) (22,29) while *Salmonella* excretion in cows sampled during the winter was found to be low (44). Likewise, the serotype and prevalence of the *Salmonella* serotype may vary from farm to farm and within the same farm from one sampling period to another (44). Other factors that could be responsible for the wide differences include size and age of the herd, region which can influence the frequency of isolation from one study to another (29), clinical condition of the animals, amount of sample used, individual laboratory skills, differences in culture methods, presence of inhibitory factors in faeces contaminated with other microorganisms, and differences in the data collected from the population studied (45).

The absence of *Salmonella* in healthy adult cattle may be explained by the fact that the bacteria is not detectable in some samples which contain small number of organisms (39). In addition, it is important to note that the detection limit for the enrichment methodology is approximately 1 CFU/g of faeces. Therefore, a negative result does not necessarily indicate that the animal is negative, but simply that the *Salmonella* population is present at less than 1 CFU/g of faeces (29). In addition, none of the farms included in our study reported clinical salmonellosis cases before taking the sample. The prevalence of salmonellosis in animals is difficult to assess due to the lack of an epidemiological surveillance system in place, which is the case in most developing countries. In Algeria, only few studies have been carried out on the presence of *Salmonella* spp in

lactating cows on dairy farms.

The prevalence of *S.* Dublin serotype from faeces was 2.71% (5 of 184), which is similar to the study carried out in Denmark with a rate ranging from 0.3 to 2.8% (46), and 1% of 393 samples in the USA study (27). The Dublin serotype was weakly detected in these studies, despite being the most frequently excreted serotype in the faeces of cows. Nevertheless, some authors have reported higher prevalence, in a study carried out in Denmark with a prevalence of 6%-14% in 4531 faecal samples (47), and by Pacer et al., in California who reported prevalence of Dublin serotype of 10.7% among 16% of *Salmonella* detected (19). It should be noted that *S.* Dublin is the most frequently isolated serotype in Danish cattle and is responsible for the economic losses reported in infected herds. As a result, a national surveillance program was launched in Denmark in October 2002 which lowered the prevalence by 12% in 2009 (48).

In this study, the positivity rate of *S*. Dublin from milk collected from 91 cows was 13.18%. Numerous studies on prevalence of *S.* Dublin in milk in cattle conducted worldwide (8, 49-56), show prevalence rate between 0.9% and 54.5% (Table 8). The differences in the prevalence rates from these studies may be explained by differences in geographical locations and herd size. These two parameters can significantly influence the seroprevalence of salmonellosis in dairy cattle (57). The comparison between direct detection of *S.* Dublin by faecal culture and indirect detection by ELISA test on milk samples in our study gave different results. The sensitivity of faecal detection of *Salmonella* was low compared to detection of antibodies directed against the bacterium, which are present in the milk. This may be explained by the fact that the duration of the Dublin antibodies presence in the milk is longer compared to the duration of excretion of Dublin serovar in the faeces. The existence of latent carriers with persistent antibody titers and intermittent or even absent excretion of Dublin serovar in faeces may also influence the results (49,50). Therefore, we can say that the bacteriological method is less sensitive than the immunological method. Some other studies have shown that bacteriological culture methods for the detection of *S.* Dublin in infected animals suffer from severe limitations in terms of sensitivity (6, 58).

The sensitivity was very low at around 16% in our study, which is similar to that reported by Nielsen and al., (47) with a sensitivity of 6-14% and that reported by Nielsen (46) with a sensitivity of 20%. Dublin serovar is difficult to detect because of its poor growth in commonly used culture media (46,59). The most common technique used to detect *Salmonella* is the traditional microbiological technique but this detection method is insensitive due to the large number of Gram-negative organisms present in the faeces, which often hamper the isolation of *Salmonella* colonies. In addition, these methods are generally labor intensive and time consuming, requiring a minimum of 4 to 6 days, thus increasing the risk of transmission of this pathogen (60). It has also been reported that culture methods show low sensitivity following low level contamination (61). Diagnostic laboratories use enrichment media to promote *Salmonella* growth and inhibit other faecal flora. Enriched samples are then spread over *Salmonella* selective media, and suspect colonies are tested using a series of biochemical tests and *Salmonella* antisera. In the case of active carriers of *S*. Dublin or other serotypes, faecal crops grown three times at intervals of 7 to 14 days are recommended to confirm the diagnosis (62). Bacteriological culture of large numbers of individual faecal samples is however expensive and time consuming (26).

Morphological descriptions and biochemical tests can also produce ambiguous results (63). Bacteriological culture tests can lead to suboptimal detection of excretors with many false negative results (64). They require collecting samples repeatedly over a long period of time to differentiate acutely infected animals from persistently infected animals. The substantial economic cost of this procedure necessitates the use of a less expensive and easier method to detect persistently infected animals (50). *Salmonella* serotyping is generally performed by reference laboratories, and is based on the identification of somatic (O) and flagellar (H) antigens using specific sera according to the Kauffmann-While Le Minor scheme (65). It could therefore be a difficult task as it requires many antisera and expertise to interpret the results of agglutination, not to mention that serotyping is also laborious, complicated and very time consuming. It should be noted that, carriers are easier to detect by use of the serological ELISA technique than acutely infected animals. The sensitivity of ELISA is therefore much higher for carriers than acutely infected animals (66). Carriers frequently have consistently elevated levels of immunoglobulins in serum and milk (67,68).

In order to verify whether *S.* Dublin is a cause of abortion in cows, we conducted a survey of 'case' and 'control' farms with *S.* Dublin positivity as an exposure factor. The results of the survey at the farm level based on bacteriological analysis for *S*. Dublin, show an exposure rate of 40% for the 'case' farms compared to 7.14% for the 'control' farms, however the Odds ratio was not significantly different from 1. Therefore, there was no association between *S.* Dublin positivity and

the presence of abortions on the farms, hence we suggest that more farms would have to be tested. For a disease prevalence of 10%, at least 86 farms should be tested for a relative risk of 4 with a proportion of one to three controls per case (69). The results of the survey of individual cattle based on bacteriological analysis gave an Odds Ratio value of 2.2, which was not significantly different from 1 (p=0.24), and analysis showed an exposure rate of 4.65% for cattle in the 'case' farms, and 2.12% in the 'control' farms, which implied no association between *S.* Dublin positivity in the cattle on the farms and abortions.

The results of the survey of 'case' and 'control' farms based on immunological analysis of milk at the farm level, gave an OR of 62.33 (95%CI: 2.13-1822), which value was significantly different from 1 (p<0.05), indicating an association between *S.* Dublin seropositivity in milk and the presence of abortions on the farms. This was further underscored by the exposure rate of 100% of the 'case' farms compared to only 11.11% in the 'control' farms. The results of the survey for individual cattle in both 'case' and 'control' farms gave an Odds Ratio of 26.78, which was significantly different from 1 (p<0.01) indicating an association between *S.* Dublin seropositivity in milk and the presence of abortions on the farms. This was further underscored by the exposure rate of 32.35% for the cattle in the 'case' farms compared to only 1.75% in the 'control' farms. From these results, we concluded that there was a clear association between *S*. Dublin seropositivity and the presence of abortions. A similar study carried out on cattle from the Algiers region, also demonstrated the existence of a close relationship between *S*. Dublin seropositivity and presence of abortions with an Odds ratio of 14.12, an exposure rate of 4.9% for the case farms, and 0.4% for the control farms (70). Indeed, several other studies have demonstrated the abortive effect of *S*. Dublin in cows (4,58,71-74).

Conclusion:

This study provided new information on bovine salmonellosis and particularly on salmonellosis caused by *S*. Dublin serovar among cattle in Algeria. *S*. Dublin identification pose challenges during laboratory diagnostic process. The use of more sensitive and less expensive method is important in order to monitor this pathogen. As *S.* Dublin is associated with abortions in cattle, we recommend that it should be systematically included in the differential diagnosis of abortions in cows in Algeria.

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Original Article **Open Access**

Prevalence, characteristics and antibiogram profile of *Escherichia coli* O157:H7 isolated from raw and fermented (nono) milk in Benin City, Nigeria

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Abstract:

Background: Most *Escherichia coli* strains are harmless commensals, but some serotypes can cause serious food poisoning in their hosts, and are infrequently responsible for product recalls due to food contamination. The present study was carried out to determine the occurrence of *E. coli* O157:H7 and other *E. coli* strains from raw and fermented (nono) milk in Benin City, Nigeria.
Methodology: A total of 66 (33 raw and 33 nono) milk samples were obtained from retailers from 3 different stations in Aduwawa market, Benin City, Nigeria between January and June, 2017. Samples were analysed by cultural methods for faecal coliforms using M-Fc agar, *E. coli* using Chromocult coliform agar, and *E. coli* O157:H7 using sorbitol MacConkey agar supplemented with cefixime and potassium tellurite. Presumptive *E. coli* and *E. coli* O157:H7 isolates were confirmed by polymerase chain reaction (PCR) assay using specific primers. Antimicrobial susceptibility profile of confirmed isolates was performed using the Kirby-Bauer disk diffusion method, with zones of inhibition interpreted according to the guidelines of Clinical and Laboratory Standards Institute (CLSI). Data were analysed using the SPSS version 21.0.
Results: From the 66 nono and raw milk samples assessed in this study, all (100%) were phenotypically positive for *E. coli* O157:H7. A total of 19 *E. coli* O157:H7 and 41 other strains of *E. coli* were confirmed by PCR. The resistance profile of the 19 *E. coli* O157:H7 isolates showed 100% (19/19) resistance to penicillin G and ampicillin; 94.7% (18/19) to chloramphenicol; 89.5% (17/19) to erythromycin; and 78.9% (15/19) to sulfamethoxazole and oxytetracycline, while the sensitivity profile showed that 100% (19/19) *E. coli* O157:H7 isolates were sensitive to gentamicin and ofloxacin. The resistance profile of other 41 *E. coli* isolates showed 100% (41/41) resistance to penicillin G and ampicillin; 97.6% (40/41) to chloramphenicol; and 92.7% (38/41) to erythromycin, while 97.6% (40/41) were sensitive to gentamicin and kanamycin. Ten *E. coli* O157:H7 isolates (52.6%) showed extensive drug resistance pattern to 11 antibiotics in 7 antimicrobial classes with multiple antibiotic resistance (MAR) index of 0.46.
Conclusion: Findings from the present study clearly indicated that the safety and quality of fresh and fermented milk were not satisfactory and could be of public health concern.

Key words: Nono, *Escherichia coli*; Pathotypes, Resistance index, Public health, Milk

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Prévalence, caractéristiques et profil antibiogramme d'*Escherichia coli* O157:H7 isolé à partir de lait cru et fermenté (nono) à Benin City, Nigéria

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Abstrait:

Contexte: La plupart des souches d'*Escherichia coli* sont des commensaux inoffensifs, mais certains sérotypes peuvent causer de graves intoxications alimentaires chez leurs hôtes et sont rarement responsables de rappels de produits en raison de la contamination des aliments. La présente étude a été réalisée pour déterminer la présence d'*E. coli* O157:H7 et d'autres souches d'*E. coli* provenant de lait cru et fermenté (nono) à Benin City, au Nigeria.
Méthodologie: Un total de 66 échantillons de lait (33 cru et 33 nono) ont été obtenus auprès de détaillants de 3 stations différentes sur le marché d'Aduwawa, Benin City, Nigeria entre janvier et juin 2017. Les échantillons ont été analysés par des méthodes de culture pour les coliformes fécaux en utilisant de la gélose M-Fc, E. coli en utilisant de la gélose Chromocult coliforme et *E. coli* O157:H7 en utilisant de la gélose MacConkey au sorbitol supplémentée en céfixime et tellurite de potassium. Des isolats présomptifs d'*E. coli* et d'*E. coli* O157:H7 ont été confirmés par un test de réaction en chaîne par polymérase (PCR) en utilisant des amorces spécifiques. Le profil de sensibilité aux antimicrobiens des isolats confirmés a été réalisé en utilisant la méthode de diffusion sur disque de Kirby-Bauer, avec des zones d'inhibition interprétées selon les directives du Institut des normes cliniques et de laboratoire (CLSI). Les données ont été analysées à l'aide de la version 21.0 de SPSS.
Résultats: Parmi les 66 échantillons de lait nono et de lait cru évalués dans cette étude, tous (100%) étaient phénotypiquement positifs pour *E. coli* O157: H7. Un total de 19 *E. coli* O157: H7 et 41 autres souches d'*E. coli* ont été confirmés par PCR. Le profil de résistance des 19 isolats d'*E. coli* O157: H7 a montré une résistance de 100% (19/19) à la pénicilline G et à l'ampicilline; 94,7% (18/19) au chloramphénicol; 89,5% (17/19) à l'érythromycine; et 78,9% (15/19) au sulfaméthoxazole et à l'oxytétracycline, tandis que le profil de sensibilité a montré que 100% (19/19) des isolats d'*E. coli* O157: H7 étaient sensibles à la gentamicine et à l'ofloxacine. Le profil de résistance des 41 autres isolats d'*E. coli* a montré une résistance de 100% (41/41) à la pénicilline G et à l'ampicilline; 97,6% (40/41) au chloramphénicol; et 92,7% (38/41) à l'érythromycine, tandis que 97,6% (40/41) étaient sensibles à la gentamicine et à la kanamycine. Dix isolats d'*E. coli* O157: H7 (52,6%) ont montré un profil de résistance aux médicaments étendu à 11 antibiotiques dans 7 classes d'antimicrobiens avec un indice de résistance aux antibiotiques multiples (MAR) de 0,46.
Conclusion: Les résultats de la présente étude indiquent clairement que la sécurité et la qualité du lait frais et fermenté ne sont pas satisfaisantes et pourraient être préoccupantes pour la santé publique.

Mots clés: Nono, *Escherichia coli*; Pathotypes, Indice de résistance, Santé publique, Lait

Introduction:

Escherichia coli is a Gram-negative, rod-shaped, facultative anaerobic coliform bacterium of the genus *Escherichia*, usually found in the lower intestine of warm-blooded organisms (1). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts (2), and are infrequently responsible for product recalls due to food contamination. Some types of *E. coli* bacteria cause disease when they make a toxin called Shiga-toxin. The most common type of Shiga toxin-producing *E. coli* (STEC) is *E. coli* O157 while other STECs are called non-O157. STEC has been known to produce two major classes of toxins, shiga-toxin 1 and shiga-toxin 2. These strains produce verotoxin 1 and 2 and their variants. They can catalytically inactivate the 60s ribosomal subunits of most eukaryotic cells, blocking mRNA translation, which leads to cell death (3).

They also possess intimin which is a protein that enables the intimate attachment of enterohaemorrhagic *E. coli* (EHEC) to the epithelial cells of the intestine, causing attachment and effacement (AE) lesions of the intestinal cells (4). The death of these cells leads to intestinal function disruption and intestinal bleeding. The toxic damage to the intestines can lead to kidney damage, anaemia, platelet segregation and death (3). The prominence of *E. coli* O157:H7 serotype dates back to 1982 when it was first discovered in an outbreak traced to contaminate hamburgers (5). Bovine food products and produce contaminated with bovine waste are a reservoir of *E. coli* O157:H7. Consumption of contaminated raw milk and unpasteurized dairy products made from raw milk is a known risk factor for *E. coli* infections (6).

Escherichia coli and other pathogens which are shed in the faeces of livestock such as cows and goats can contaminate milk during the milking process. Milk is an important source of nutrients for humans and animals (7), but milk for human consumption should be free from any pathogenic organisms (8). Contamination of milk can occur through various means such as during the milking process of an infected lactating animal with the use of unsanitary equipment, improper milk handling, transportation, and storage (6). Contamination can also occur through improper handling and storage of milk (7). About 90% of all dairy-related human diseases are as a result of ingestion of contaminated milk, even though milk and milk products are minor constituents in most diets (9).

Escherichia coli O157:H7 has been known to survive well in the environment and can adapt to a variety of conditions. This is possible because it possesses certain virulence factors such as shiga toxins, which are products of the pathogenicity island, *locus of enterocyte effacement* (4), and products of the F-like plasmid pO157 (10). The extensive use of antibiotics in both human medicine and agricultural settings, especially in disease

prevention and promotion of growth in animal production, is the major cause of selection of antibiotic resistant *E. coli* (11).

Multi-drug resistant *E. coli* isolates characteristically exhibit non-susceptibility to at least one agent in three or more antibiotic categories (12). Surveillance data on antibiotic-resistant *E. coli* shows clearly a higher resistance for older generation human and veterinary antibiotics including ampicillin, streptomycin, and tetracycline and an increasing resistance to new generation antibiotics such as fluoroquinolones and cephalosporins (13). Monitoring the emergence and spread of resistant pathogens in animal reservoirs is very important, especially for those with zoonotic importance (14). The aim of this study is to characterize *E. coli* O157:H7 isolates of raw and "nono" milk samples retailed by street vendors in Benin City, Edo State, Nigeria, using conventional microbiological and molecular methods.

Materials and method:

Study setting and sample collection

Raw (n=33) and fermented (nono) (n=33) milk samples were purchased from retail sellers in an open market (Aduwawa market) in Benin City, Edo State, Nigeria between January and June, 2017. Aduwawa market is the major market where raw and fermented milk are sold to retailers who then sell to other markets in Benin City. Three randomly selected locations within the market were sampled and described as stations (station 1, station 2, and station 3). A total of 22 samples (11 raw and 11 fermented) were obtained per station.

Milk samples were collected in 50 mL sterile bottles, and transported to the Applied and Environmental Microbiology Research Group Laboratory of the University of Benin, in cooler boxes within two hours of collection, for microbial analyses.

Enumeration and isolation of heterotrophic bacteria, faecal coliform, *Escherichia coli* and *Escherichia coli* O157:H7

One millilitre of the raw and 'nono' milk samples was serially diluted, and 0.1 mL immediately spread-plated on nutrient agar plates (Lab M, UK), Chromocult coliform agar (Lab M, UK), and Sorbitol MacConkey agar (Lab M, UK) supplemented with cefixime (50 µg/L) and potassium tellurite (25mg/L). All plates were incubated aerobically at 37°C for 18-24 hours. Likewise; an aliquot of 0.1mL was also spread-plated on M-Fc agar plates (Merck, Darmstadt, Germany) and incubated at 45°C for 24 hours.

Discrete dark blue to violet colonies from Chromocult coliform agar (probable *E. coli*), colourless or beige colonies from Sorbitol MacConkey agar plates supplemented with cefixime and potassium tellurite (probable *E. coli* O157:H7), and blue colonies from M-Fc agar (faecal coliforms), were enumerated and expressed in colony forming units per millilitre (CFU/mL). Discrete colonies of probable *E. coli* and *E. coli* O157:H7 were purified on nutrient agar and stored on nutrient agar slant at 4°C for further laboratory analysis.

Identification of bacterial isolates

Identification of isolates was carried out using methods outlined in Cheesbrough (15), on the basis of cultural, morphological, Gram reaction, and biochemical tests such as indole, oxidase, catalase, and 3% potassium hydroxide.

Antibiotic susceptibility test (AST) of isolates

Antimicrobial susceptibility testing of isolates was carried out by the Kirby-Bauer disk diffusion method in accordance with the Clinical and Laboratory Standards Institute guidelines (16). A 0.5 MacFarland suspension of each isolates was aseptically streaked on Mueller-Hinton (MH) agar plates, and single antibiotic disks were aseptically placed on the inoculated agar. The disks utilized included; penicillin G (10units), ampicillin (10µg), amoxicillin (25µg), ampicillin/sulbactam (30µg), amoxicillin/clavulanate (30µg), gentamycin (10µg), kanamycin (30µg), streptomycin (25 µg), tobramycin (10µg), doxycycline (30µg), tetracycline (30µg), oxytetracycline (30µg), imipenem (10µg), meropenem (10µg), cephalothin (30µg), cefotaxime (30µg), erythromycin (15µg), trimethoprim (25µg), sulfamethoxazole (30µg), polymyxin (300 units), colistin (20µg), chloramphenicol (30µg), ofloxacin (5µg), and ciprofloxacin (10µg). Five antibiotics were used equidistant apart on each plate.

The agar plates were allowed to dry for about 10 minutes and then incubated aerobically at 37°C for 24 hours. The diameter of the inhibitory zone was measured using a transparent meter rule and interpreted as resistant (R), intermediate resistant (I) or sensitive (S), in accordance with the recommended standards established by CLSI (16).

Genetic confirmation of *E. coli* O157:H7 and non-*E. coli* O157: H7

Extraction of genomic DNA

DNA extraction from each bacterial isolate was done using the boiling method previously described by Igbinosa et al., (17) with modifications. Briefly, purified isolates were inoculated into sterile Tryptone Soy Broth and incubated at 37°C overnight. At the end of the incubation, about 2ml from the previously grown culture was transferred into

sterile 2ml Eppendorf tubes and centrifuged at 11000 rpm for 10min. The obtained pellet was washed twice using sterile distilled water before re-suspending into 200µl of sterile distilled water. The mixture was then boiled for 10 minutes at 100°C. The cell lysate was immediately cooled at -20°C for 10 minutes, followed by centrifugation at 12000g for 5 minutes. The supernatant was then carefully transferred into new sterile microfuge tubes and used as template genomic DNA for PCR assay. *E. coli* O157:H7 ATCC 35150 was used as positive control strain.

Species–specific identification by PCR assay

PCR assay was performed in a 25µl reaction cocktail in a 200µl tube with 12.5µl of Master Mix, 0.25 µl each of forward and reverse primers, 2 µl of nuclease free water and 10µl of template DNA. The amplification was performed in a Peltier-Based Thermal Cycler. Primer pairs used in the amplification are shown in Table 1.

The thermal cycling conditions for *uidA* gene of *E. coli* were initial denaturation at 95°C for 2 min followed by 25 cycles of denaturation at 94°C for 1 min, 58°C annealing for 1 min and 72°C extension for 1 min, final extension at 72°C for 2 min and amplicons held at 4°C after the cycles. The thermal cycling conditions for *rfbE* of *E. coli* O157 as well as *fliC* of H7 were initial denaturation at 95°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C for 90 sec, extension at 72°C for 90 seconds, and initial extension at 72°C for 5 minutes and the amplicons were held at 4°C until ready for electrophoresis.

Electrophoresis of amplicons was performed with 1% agarose gel (CLS-AG100, Warwickshire, United Kingdom) containing ethidium bromide (Merck, SA) with 0.5 mg/L for 1 hour at 100 V in 0.5×TAE buffer (40mM Tris-HCl, 20mM Na acetate, 1mM EDTA, pH 8.5) and visualized under UV transilluminator (EBOX VX5, Vilber Lourmat, France), and photographed.

Statistical analysis

All data were analysed using IBM SPSS version 21.0. Population densities of the isolates were analysed using descriptive statistics and expressed as mean ± standard deviation of the mean. One-way analysis of variance (ANOVA) was used to analyse the data across sampling months and sampling points while Duncan Multiple Range test was used to show significant difference between mean. The probability-values less than 0.05 were considered statistically significant.

Results:

Population counts from milk samples

The mean heterotrophic bacterial counts of nono and raw milk samples is presented in Table 2. The mean bacterial counts for nono milk in respective stations range as follows; station 1 ($6.40\times10^4\pm0.14$ - $2.88\times10^{10}\pm0.02$ CFU/ml), station 2 ($3.10\times10^4\pm0.28$ - $2.40\times10^{10}\pm0.14$ CFU/ml), station 3 ($5.00\times10^4\pm0.28$ - $2.58\times10^{10}\pm1.40$ CFU/ml), while that of the raw milk ranges as follows; station 1 ($2.64\times10^5\pm0.14$ - $2.84\times10^{10}\pm0.14$ CFU/ml), station 2 ($2.16\times10^5\pm0.01$ - $2.29\times10^{10}\pm0.04$ CFU/ml), station 3 ($2.94\times10^5\pm0.00$ - $2.56\times10^{10}\pm0.01$ CFU/ml). There was no significant difference ($p>0.05$) across the months (January - June) for stations 2 and 3 (nono milk). There was also no significant difference ($p>0.05$) across stations for raw and nono milk (station 1 - 3) for the months of February, April, May and June (Table 2).

Table 1: Primer sequences and expected size of PCR amplified genes targeted in the isolates

Primer	Primer sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
Escherichia coli	F- AAAACGGCAAGAAAAAGCAG R- ACGCGTGGTTAACAGTCTTGCG	*uid*A	147	Bej et al., (37)
*Fli*CH7	F-TACCATCGCAAAAGCAACTCC R-GTCGGCAACGTTAGTGATACC	$fliC_{H7}$	247	Wang et al., (38)
*Rfb*E	F-CTACAGGTGAAGGTGGAATGG R-ATTCCTCTCTTTCCTCTGCGG	$rfbE_{O157}$	327	Nazemi et al., (39)

Table 2: Distribution of total heterotrophic bacterial counts on the milk samples over 6 months period of study

Months	Nono Milk			Raw Milk			*p*-value
	Station 1	Station 2	Station 3	Station 1	Station 2	Station 3	
January	$6.40\times10^{4}\pm0.14^{A}_{b}$	$3.10\times10^{4}\pm0.28^{A}_{a}$	$5.00\times10^{4}\pm0.28^{A}_{ab}$	$2.64\times10^{5}\pm0.14^{A}_{d}$	$2.16\times10^{5}\pm0.01^{A}_{c}$	$2.94\times10^{5}\pm0.00^{A}_{e}$	0.000
February	$1.50\times10^{5}\pm0.04^{A}_{a}$	$2.00\times10^{5}\pm0.14^{A}_{a}$	$2.50\times10^{5}\pm0.14^{A}_{a}$	$2.70\times10^{5}\pm0.40^{A}_{a}$	$2.30\times10^{5}\pm0.14^{A}_{a}$	$3.00\times10^{5}\pm0.14^{A}_{a}$	0.571
March	$2.88\times10^{10}\pm0.02^{C}_{c}$	$2.40\times10^{10}\pm0.14^{B}_{a}$	$2.58\times10^{10}\pm1.40^{B}_{b}$	$2.84\times10^{10}\pm0.14^{D}_{c}$	$2.88\times10^{10}\pm0.01^{C}_{c}$	$2.56\times10^{10}\pm0.01^{C}_{b}$	0.001
April	$4.07\times10^{9}\pm0.10^{A}_{a}$	$2.21\times10^{10}\pm0.16^{B}_{b}$	$1.33\times10^{10}\pm0.12^{AB}_{ab}$	$1.43\times10^{10}\pm0.93^{BC}_{ab}$	$1.07\times10^{10}\pm0.11^{AB}_{ab}$	$1.04\times10^{10}\pm0.27^{B}_{ab}$	0.223
May	$9.57\times10^{9}\pm0.01^{AB}_{a}$	$1.81\times10^{10}\pm1.55^{AB}_{a}$	$1.83\times10^{10}\pm0.11^{AB}_{a}$	$2.50\times10^{10}\pm0.49^{CD}_{a}$	$2.29\times10^{10}\pm0.04^{BC}_{a}$	$1.43\times10^{10}\pm0.48^{B}_{a}$	0.511
June	$2.16\times10^{10}\pm0.04^{BC}_{a}$	$1.18\times10^{10}\pm0.88^{AB}_{a}$	$1.13\times10^{10}\pm0.12^{AB}_{a}$	$8.80\times10^{9}\pm0.11^{AB}_{a}$	$1.16\times10^{10}\pm0.17^{AB}_{a}$	$1.17\times10^{10}\pm0.47^{B}_{a}$	0.559
p-value	0.015	0.056	0.139	0.002	0.005	0.000	

Values are in mean ± standard deviation of the mean. Values across column with different superscript uppercase alphabets show significant difference across column (months); while values across rows with different subscript lowercase alphabets show significant difference across rows (stations). *P*-values less than 0.05 were considered statistically significant

Table 3: Distribution of faecal coliform counts on the milk samples over the 6 months period of study

Months	Nono Milk			Raw Milk			*p*-value
	Station 1	Station 2	Station 3	Station 1	Station 2	Station 3	
January	$2.84\times10^{5}\pm0.14^{A}_{d}$	$2.80\times10^{5}\pm0.14^{B}_{d}$	$9.60\times10^{4}\pm0.14^{A}_{a}$	$1.68\times10^{5}\pm0.01^{A}_{b}$	$2.10\times10^{5}\pm0.14^{A}_{c}$	$2.16\times10^{5}\pm0.01^{A}_{c}$	0.000
February	0^{A}_{a}	0^{A}_{a}	0^{A}_{a}	0^{A}_{a}	$1.20\times10^{5}\pm0.14^{A}_{c}$	$8.00\times10^{4}\pm1.41^{A}_{b}$	0.000
March	$2.00\times10^{5}\pm0.14^{A}_{ab}$	0^{A}_{a}	0^{A}_{a}	$5.00\times10^{5}\pm1.41^{B}_{c}$	$3.00\times10^{5}\pm1.41^{A}_{bc}$	0^{A}_{a}	0.011
April	$2.00\times10^{5}\pm0.10^{A}_{a}$	$2.00\times10^{5}\pm1.73^{AB}_{a}$	$1.33\times10^{5}\pm1.52^{A}_{a}$	$1.33\ 10^{5}\pm1.15^{A}_{a}$	$6.00\times10^{5}\pm8.71^{A}_{a}$	$2.33\times10^{5}\pm2.08^{A}_{a}$	0.678
May	$1.87\times10^{7}\pm1.62^{A}_{b}$	$4.00\times\ 10^{3}\pm4.0^{A}_{a}$	$9.33\times10^{3}\pm9.71^{A}_{a}$	$2.06\times10^{4}\pm3.40^{A}_{a}$	$3.16\times10^{4}\pm4.89^{A}_{a}$	$2.83\times10^{5}\pm4.14^{A}_{a}$	0.023
June	$5.50\times10^{3}\pm6.30^{A}_{a}$	$1.50\times10^{4}\pm0.14^{A}_{a}$	$5.95\times10^{3}\pm7.41^{A}_{a}$	$5.00\times10^{3}\pm1.41^{A}_{a}$	0^{A}_{a}	$1.05\times10^{4}\pm1.20^{A}_{a}$	0.366
p-value	0.123	0.028	0.294	0.001	0.643	0.136	

Values are in mean ± standard deviation of the mean. Values across column with different superscript uppercase alphabets show significant difference across column (months); while values across rows with different subscript lowercase alphabets show significant difference across rows (stations). *P*-values less than 0.05 were considered statistically significant

The mean faecal coliform count of the nono and raw milk is presented in Table 3. The mean coliform count for nono milk in respective stations ranges as follows; station 1 (0 - $1.87\times10^{7}\pm1.62$ CFU/ml), station 2 (0 - $2.80\times10^{5}\pm0.14$ CFU/ml), station 3 (0 - $1.33\times10^{5}\pm1.52$ CFU/ml), while that of the raw milk ranges as follows; station 1 (0 - $5.00\times10^{5}\pm1.41$ CFU/ml), station 2 (0 - $6.00\times10^{5}\pm8.71$ CFU/ml), and station 3 (0 - $2.83\times10^{5}\pm4.14$ CFU/ml). There was no significant difference ($p>0.05$) across months (January - June) for stations 1 and 3 (nono milk) as well as stations 2 and 3 (raw milk). In addition, there was no significant difference ($p>0.05$) across stations for raw and nono milk (station 1 - 3) for April, May and June.

The mean *E. coli* O157:H7 count of the nono and raw milk is presented in Table 4. The mean *E. coli* O157:H7 count for nono milk in respective stations ranges as follows: station 1 ($1.00\times10^{5}\pm0.14$ - $2.82\times10^{7}\pm0.14$ CFU/ml), station 2 ($4.00\times10^{5}\pm0.14$ - $3.00\times10^{7}\pm0.14$ CFU/ml), station 3 ($2.00\times10^{5}\pm0.14$ - $2.30\times10^{7}\pm0.14$ CFU/ml), while that of the raw milk ranges as follows; station 1 ($3.55\times10^{5}\pm4.03$ - $2.35\times10^{7}\pm0.14$ CFU/ml), station 2 ($5.10\times10^{5}\pm2.68$ - $2.52\times10^{7}\pm0.14$ CFU/ml), and station 3 ($2.66\times10^{5}\pm4.44$ - $2.40\times10^{7}\pm0.14$ CFU/ml). There was no significant difference ($p>0.05$) across the stations for both raw and nono milk (station 1 - 3) for the months of March, April, May and June.

Table 4: Distribution of *Escherichia coli* counts on the milk samples over the 6 months period of study

Months	Nono Milk			Raw Milk			*p*-value
	Station 1	Station 2	Station 3	Station 1	Station 2	Station 3	
January	$1.00\times10^{5}\pm0.14^{A}_{a}$	$4.00\times10^{5}\pm0.14^{A}_{a}$	$2.00\times10^{5}\pm0.14^{A}_{a}$	$1.24\times10^{7}\pm0.14^{B}_{c}$	$9.60\times10^{6}\pm0.14^{C}_{b}$	$1.44\times10^{7}\pm0.14^{B}_{c}$	0.000
February	$7.00\times10^{5}\pm0.14^{A}_{a}$	$3.00\times10^{7}\pm0.14^{C}_{c}$	$1.50\times10^{6}\pm0.14^{A}_{ab}$	$2.40\times10^{6}\pm0.14^{A}_{b}$	$2.40\times10^{6}\pm0.14^{B}_{b}$	$1.20\times10^{6}\pm0.14^{A}_{ab}$	0.000
March	$2.82\times10^{7}\pm0.14^{B}_{b}$	$2.60\times10^{7}\pm0.14^{B}_{ab}$	$2.30\times10^{7}\pm0.14^{B}_{a}$	$2.35\times10^{7}\pm0.14^{C}_{a}$	$2.52\times10^{7}\pm0.14^{D}_{ab}$	$2.40\times10^{7}\pm0.14^{C}_{ab}$	0.071
April	$8.30\times10^{5}\pm5.96^{A}_{a}$	$1.13\times10^{6}\pm0.71^{A}_{a}$	$6.36\times10^{6}\pm5.43^{A}_{a}$	$1.15\times10^{6}\pm0.84^{A}_{a}$	$1.32\times10^{6}\pm7.52^{AB}_{a}$	$1.08\times10^{6}\pm0.85^{A}_{a}$	0.873
May	$1.44\times10^{6}\pm1.29^{A}_{a}$	$1.26\times10^{6}\pm0.98^{A}_{a}$	$1.70\times10^{6}\pm1.47^{A}_{a}$	$1.68\times10^{6}\pm0.73^{A}_{a}$	$8.56\times10^{5}\pm6.64^{AB}_{a}$	$2.66\times10^{5}\pm4.44^{A}_{a}$	0.497
June	$2.30\times10^{5}\pm0.28^{A}_{a}$	$4.30\times10^{5}\pm3.81^{A}_{a}$	$5.30\times10^{5}\pm0.98^{A}_{a}$	$3.55\times10^{5}\pm4.03^{A}_{a}$	$5.10\times10^{5}\pm2.68^{A}_{a}$	$5.85\times10^{5}\pm4.31^{A}_{a}$	0.862
p-value	0.000	0.000	0.000	0.000	0.000	0.000	

Values are in mean ± standard deviation of the mean. Values across column with different superscript uppercase alphabets show significant difference across column (months); while values across rows with different subscript lowercase alphabets show significant difference across rows (stations). *P*-values less than 0.05 were considered statistically significant

Table 5: Distribution of the counts of other *Escherichia coli* strains on the milk samples over the 6 months period of study

Months	Nono Milk			Raw Milk			*p*-value
	Station 1	Station 2	Station 3	Station 1	Station 2	Station 3	
January	$1.00\times10^{5}\pm0.14^{A}_{a}$	$2.00\times10^{5}\pm0.28^{B}_{a}$	0^{A}_{a}	$1.40\times10^{7}\pm0.14^{B}_{b}$	$1.68\times10^{7}\pm0.14^{B}_{c}$	$2.08\times10^{7}\pm0.01^{D}_{d}$	0.000
February	0^{A}_{a}	$1.60\times10^{6}\pm0.14^{C}_{a}$	$4.00\times10^{5}\pm0.14^{A}_{a}$	$2.80\times10^{7}\pm0.14^{C}_{c}$	$1.80\times10^{7}\pm0.14^{B}_{b}$	$1.82\times10^{7}\pm0.01^{C}_{b}$	0.000
March	$6.00\times10^{5}\pm0.14^{B}_{d}$	0^{A}_{a}	0^{A}_{a}	$1.00\times10^{5}\pm0.14^{A}_{b}$	$2.00\times10^{5}\pm0.14^{A}_{c}$	0^{A}_{a}	0.000
April	$7.00\times10^{4}\pm2.00^{A}_{a}$	$4.66\times10^{4}\pm8.08^{A}_{a}$	$3.40\times10^{5\pm}5.37^{A}_{a}$	$5.33\times10^{5}\pm4.88^{A}_{a}$	$4.76\times10^{5}\pm7.65^{A}_{a}$	$4.33\times10^{4}\pm6.65^{AB}_{a}$	0.559
May	$1.50\times10^{5}\pm2.17^{A}_{a}$	$1.00\times10^{4}\pm1.73^{A}_{a}$	$6.33\times10^{4}\pm10.9^{A}_{a}$	$6.00\times10^{4}\pm10.3^{A}_{a}$	$5.33\times10^{4}\pm9.23^{A}_{a}$	$4.66\times10^{4}\pm7.23^{AB}_{a}$	0.800
June	$1.70\times10^{5}\pm2.12^{A}_{a}$	$6.00\times10^{4}\pm1.41^{AB}_{a}$	$2.15\times10^{5}\pm1.20^{A}_{a}$	$6.00\times10^{4}\pm2.82^{A}_{a}$	$2.40\times10^{5}\pm0.42^{A}_{a}$	$5.00\times10^{5}\pm5.37^{B}_{a}$	0.536
p-value	0.019	0.000	0.525	0.000	0.000	0.000	

Values are in mean ± standard deviation of the mean. Values across column with different superscript uppercase alphabets show significant difference across column (months); while values across rows with different subscript lowercase alphabets show significant difference across rows (stations). *P*-values less than 0.05 were considered statistically significant

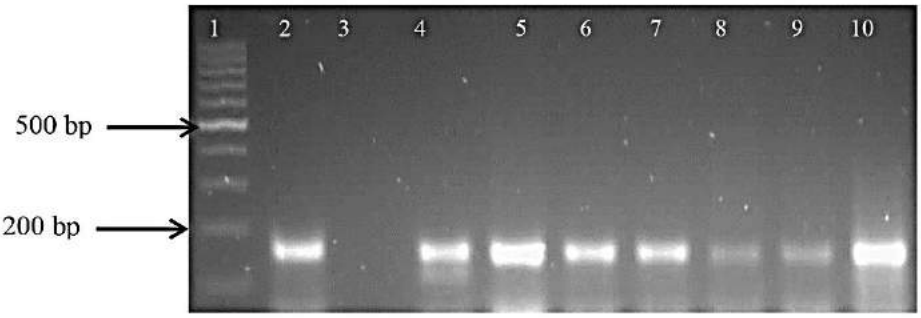
The mean counts of the other *E. coli* pathotypes for the nono and raw milk is presented in Table 5. The mean density of other *E. coli* pathotypes for nono milk in respective stations ranges as follows; station 1 (0 - $6.00\times10^{5}\pm0.14$ CFU/ml), station 2 (0 - $1.60\times10^{6}\pm0.14$ CFU/ml), station 3 (0 - $4.00\times10^{5}\pm0.14$ CFU/ml), while that of the raw milk ranges as follows; station 1 ($6.00\times10^{4}\pm2.82$ -$2.80\times10^{7}\pm0.14$ CFU/ml), station 2 ($5.33\times10^{4}\pm9.23$ - $1.80\times10^{7}\pm0.14$ CFU/ml), and station 3 (0 - $2.08\times10^{7}\pm0.01$ CFU/ml). There was no significant difference ($p>0.05$) across months (January - June) for stations 3 (nono milk). In addition, there was no significant difference ($p>0.05$) across stations for raw and nono milk (stations 1-3) for April, May and June.

Prevalence of faecal coliforms, *E. coli* O157:H7 and other *E. coli* pathotypes in raw and fermented milk samples

Of the nono milk samples assessed, all 33 (100%) were positive for *E. coli* O157, 22 (66.7%) were positive for other *E. coli* pathotypes, and 20 (60.6%) were positive for faecal coliforms. For the raw milk samples, all 33 (100%) were positive for *E. coli* 0157, and 24 (72.7%) for other *E. coli* pathotypes and faecal coliforms (Table 6). Nineteen *E. coli* 0157:H7 and 41 other *E. coli* pathotypes were confirmed by PCR assay (Figs 1, 2 & 3).

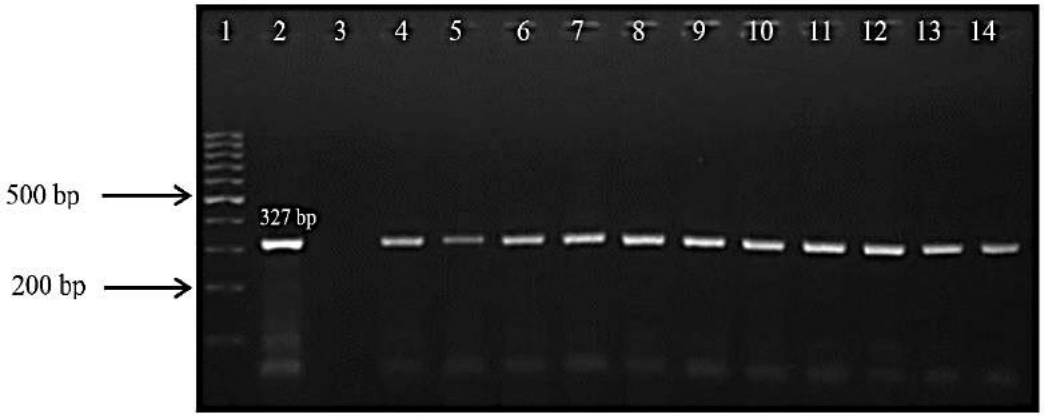
Table 6: Prevalence of *Escherichia coli* O157:H7, other *Escherichia coli* pathotypes and faecal coliforms from the milk samples

Milk samples	Stations	Bacteria	Number of samples examined	Number of positive samples	Percentage of positive samples
Nono Milk	Station 1	*E. coli* O157:H7	11	11	100
		Other *E. coli* pathotypes	11	9	81.8
		Faecal coliforms	11	8	72.7
	Station 2	*E. coli* O157:H7	11	11	100
		Other *E. coli* pathotypes	11	6	54.6
		Faecal coliforms	11	5	45.5
	Station 3	*E. coli* O157:H7	11	11	100
		Other *E. coli* pathotypes	11	7	63.6
		Faecal coliforms	11	7	63.6
Raw Milk	Station 1	*E. coli* O157:H7	11	11	100
		Other *E. coli* pathotypes	11	8	72.7
		Faecal coliforms	11	8	73.7
	Station 2	*E. coli* O157:H7	11	11	100
		Other *E. coli* pathotypes	11	8	72.7
		Faecal coliforms	11	7	63.6
	Station 3	*E. coli* O157:H7	11	11	100
		Other *E. coli* pathotypes	11	8	72.7
		Faecal coliforms	11	9	81.8



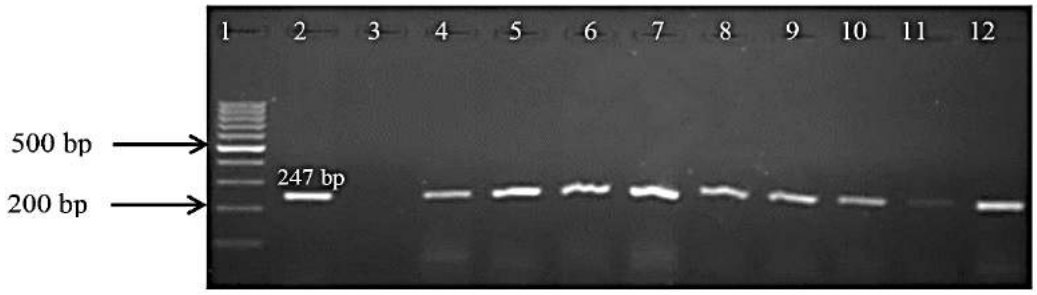
Lane 1: 100 bp Molecular marker; Lane 2: Positive control; Lane 3: Negative control; Lanes 4 to 10: Positive *E. coli* representative isolates obtained from raw and fermented milk samples.

Fig 1: Amplified *uid*A genes (147 bp) of *E. coli* isolates



Lane 1: 100 bp Molecular Marker; Lane 2: Positive control; Lane 3: Negative control; Lanes 4 to 14: Positive *E. coli* O157 representative isolates obtained from raw and fermented milk samples

Fig 2: Amplified $rfbE_{O157}$ genes (327 bp) of *E. coli* O157 isolates.



Lane 1: 100 bp Molecular marker; Lane 2: Positive control; Lane 3: Negative control; Lanes 4 to 12: Positive *E. coli* H7 representative isolates obtained from raw and fermented milk samples

Fig 3: Amplified *fliC*$_{H7}$ genes (247) of *E. coli* H7 isolates

Table 7: Antimicrobial susceptibility profile of *Escherichia coli* O157:H7 and other *Escherichia coli* isolates

Antimicrobial class	Antibiotics	*E. coli* O157:H7 strain (*n*=19)			Other *E. coli* strain (*n*=41)		
		R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Penicillin	Penicillin G	19 (100)	-	0	41 (100)	-	0
	Ampicillin	19 (100)	0	0	41 (100)	0	0
	Amoxicillin	12 (63.1)	3 (15.8)	4 (21.1)	34 (82.9)	5 (12.2)	2 (4.9)
β-lactam\Beta lactamase inhibitors	Ampicillin\Sulbactam	10 (52.6)	3 (15.8)	6 (31.6)	25 (61)	5 (12.2)	11 (26.8)
	Amoxicillin\Clavulanate	11 (57.9)	5 (26.3)	3 (15.8)	32 (78)	7 (17.1)	2 (4.9)
Aminoglycosides	Gentamycin	0	0	19 (100)	1 (2.4)	0	40 (97.6)
	Kanamycin	0	1 (5.3)	18 (94.7)	0	1(2.4)	40 (97.6)
	Streptomycin	2 (10.5)	2 (10.5)	15 (80)	3 (7.3)	4(9.8)	34 (82.9)
	Tobramycin	6 (31.6)	1 (5.3)	12 (63.1)	5 (12.2)	7 (17.1)	29 (70.7)
Tetracyclines	Doxycycline	6 (31.6)	1 (5.3)	12 (63.1)	11 (26.8)	3 (7.3)	27 (65.9)
	Tetracycline	7 (36.8)	8 (42.1)	4 (21.1)	21 (51.2)	12 (29.3)	8 (19.5)
	Oxytetracycline	15 (78.9)	4 (21.1)	0	26 (63.4)	10 (24.4)	5 (12.2)
Carbapenems	Imipenem	12 (63.1)	3 (15.8)	4 (21.1)	27 (65.9)	5 (12.2)	9 (22)
	Meropenem	0	3 (15.8)	16 (84.2)	1 (2.4)	1 (2.4)	39 (95.1)
Cephalosporins	Cephalothin	2 (10.5)	2 (10.5)	15 (78.9)	12 (29.3)	2 (4.9)	27 (65.8)
	Cefotaxime	12 (63.2)	7 (36.8)	0	30 (73.2)	8 (19.5)	3 (7.3)
Macrolides	Erythromycin	17 (89.5)	1 (5.3)	1 (5.3)	38 (92.7)	1 (2.4)	2 (4.9)
Folate pathway inhibitors	Trimethoprim	12 (63.2)	2 (10.5)	5 (26.3)	27 (65.9)	5 (12.2)	9 (21.9)
	Sulfamethoxazole	15 (78.9)	4 (21.1)	0	34 (82.9)	4 (9.8)	3 (7.3)
Polymyxins	Polymyxin B	7 (36.8)	10 (52.60	2 (10.5)	10 (24.4)	26 (63.4)	5 (12.2)
	Colistin	3 (15.8)	1 (5.3)	15 (78.9)	6 (14.6)	1 (2.4)	34 (83)
Phenicols	Chloramphenicol	18 (94.7)	0	1 (5.3)	40 (97.6)	0	1 (2.4)
Quinolone	Ofloxacin	0	0	19 (100)	2 (4.9)	1 (2.4)	38 (92.7)
	Ciprofloxacin	0	2 (10.5)	17 (89.5)	0	6 (14.6)	35 (85.4)

GEN: Gentamycin (10µg); KAN: Kanamycin (30µg); STR: Streptomycin (25µg); TOB: Tobramycin (10µg); AMP: Ampicillin (10µg); AMX: Amoxicillin (25µg); SAM: Ampicillin/Sulbactam (30µg); IPM: Imipenem (10µg); MEM: Meropenem (10µg); CTX: Cefotaxime (30µg); CEF: Cephalothin (30µg); SUL: Sulfamethoxazole (30µg); TMP: Trimethoprim (25µg); ERY: Erythromycin (15µg); PEN: Penicillin G (10 units); AMC: Amoxicillin/clavulanate (30µg); CHL: Chloramphenicol (30µg); CST: Colistin (20µg); PMB: Polymyxin B (300 units); DOX: Doxycycline (30µg); OXY: Oxytetracycline (30µg); TET: Tetracycline (30µg); CIP: Ciprofloxacin (10µg); OFX: Ofloxacin (5µg); R: Resistant; I: Intermediate; S: Sensitive; Values in parenthesis represent percentage; MAR: Multiple antibiotic resistance

Antibiotic resistance profiles of *E. coli* isolates

The antibiotic resistance profile of the 19 genetically confirmed *E. coli* O157:H7 isolates is shown in Table 7; all 19 (100%) isolates were resistant to penicillin G and ampicillin, 18 (94.7%) to chloramphenicol, 17 (89.5%) to erythromycin, 15 (78.9%) to sulfamethoxazole and oxytetracycline, 12 (63.1%) to amoxicillin, imipenem, cefotaxime and trimethoprim, 11 (57.9%) to amoxicillin/clavulanate, and 10 (52.6%) to ampicillin/sulbactam. All the 19 (100%) isolates were however sensitive to gentamicin and ofloxacin.

The resistance profile of the 41 other genotypically confirmed *E. coli* isolates is presented in Table 7; all 41 (100%) isolates are resistant to penicillin G and ampicillin, 40 (97.6%) to chloramphenicol, 38 (92.7%) to erythromycin, 34 (82.9%) to amoxicillin and sulfamethoxazole, 32 (78%) to amoxicillin/clavulanate, 30 (73.2%) to cefotaxime, and 27 (65.9%) to trimethoprim and imipenem. A total of 40 (97.6%) isolates were however sensitive to gentamicin and kanamycin.

Multidrug resistance (MDR) profiles of the 19 *E. coli* O157:H7 isolates shows that all 19 (100%) were resistant to 3 antibiotics (AMP^R, PEN^R, CHL^R) in 2 antimicrobial classes with multiple antibiotic resistance (MAR) index of 0.13. The extensive drug resistance profiles include resistance of 10 (52.6%) to 11 antibiotics (AMP^R, PEN^R, CHL^R, ERY^R, SUL^R, OXY^R, AMX^R, TMP^R, IMI^R, AMC^R, SAM^R) in 7 antimicrobial classes with MAR index of 0.46 (Table 8).

Multidrug resistance (MDR) profiles of 41 other *E. coli* isolates shows that 40 (97.6%) were resistant to 3 antibiotics (AMP^R, PEN^R, CHL^R) in 2 antimicrobial classes with MAR index of 0.13. The extensive drug resistance profiles include resistance of 25 (60.9%) to 11 antibiotics (AMP^R, PEN^R, CHL^R, ERY^R, SUL^R, AMX^R, AMC^R, CTX^R, SAM^R, IMI^R, TMP^R) in 7 antimicrobial classes with MAR index of 0.46 (Table 8).

Discussion:

Milk is highly nutritious; however, it is a well-thought-out high-risk food as it can serve as a good medium for bacterial growth (18). Several factors can contribute to milk contamination such as unhygienic milking conditions, tainted equipment, and poor hygiene of individual milk handlers (19). The high bacterial counts reported in this study certainly reveal the overall poor conditions of hygiene/cleanliness and temperature control under which the milk was produced and handled. Probable reasons for the high counts could be due to infection of cow udder, use of germ-contaminated equipment, lack of cooling after milking and absence of heat treatment. These can contribute to the low hygiene quality of the raw milk. Consequent on this, training in general milking, hygiene/germ-free practices and keeping of milk at low temperature should be given to the farmers and retailers to circumvent microbial growth and extend the shelf life of milk (20).

The high prevalence of faecal contamination reported in our study is higher than the rate reported in Plateau State, Nigeria, where 0.7% (5/350) of the nono samples and 3.0% (21/350) of the raw milk samples had *E. coli* O157 isolated (21). According to a study by Ahmed and Samer (22), where 50 raw and 50 pasteurized milk samples were investigated, *E. coli* O157 was isolated from 33 (66%) raw and 15 (30%) pasteurized milk samples. Another study by Garbaj et al., (23) on milk and dairy products in Libya revealed that *E. coli* O157 (6/28, 21.4%) was identified from fermented cow milk, which is far lower than the rates obtained in our study where 100% of the nono milk samples were positive for *E. coli* O157. Arafa and Soliman (24) in a study conducted in Egypt on raw milk and fresh cream reported that 2.6% and 1% were contaminated with *E. coli* O157:H7 respectively. Allerberger et al., (25) also reported 3% of milk samples tested in Austria to be positive for *E. coli* O157:H7, which is in tandem with the report of Arafa and Soliman (24). On the other hand, Chye et al., (26) detected *E. coli* O157:H7 in 33.5% of raw milk samples in Malaysia. The disparities observed in these studies might be due to dissimilarity in animal management, milking system, and handling practices in different regions. The ability of *E. coli* O157:H7 to survive in fermented milk samples could be the result of the organism's tolerance to high acidity (23).

The prevalence of *E. coli* from milk samples (66.7%) in our study is higher than the rate of 33.9% reported by Disassa et al., (20) in a study of 380 raw cow milk samples collected from marketed raw cow milk in and around Asosa Town, Western Ethiopia. However, the prevalence rate in our study is similar to those reported by Lingathurai et al., (27) in South India, Ali (28) in Britain, and Shunda et al., (29) in Mekelle town, with reported incidence of *E. coli* from raw milk of 70%, 63% and 44.4% respectively. The presence of *E. coli* is a strong indicator of poor sanitary practices during the milking process, transportation, production methods, and sale of milk and milk products. This portends a potential danger for individuals consuming such products (24). In Nigeria, it is a common practice to manufacture dairy products from raw milk. The isolation of *E. coli* O157:H7, other *E. coli* pathotypes, and faecal coliforms from large proportions of raw and fermented (nono) milk in our study could be a potential source for human diseases following their consumption. However, it is difficult to link these findings with any case of food poisoning that might have occurred in Benin City due to poor documentations of such cases of bacterial food poisoning.

Antimicrobial resistance in enteric bacteria has become a global burden these past years, particularly in developing countries like Nigeria, which has played a crucial role in restricting treatment options with evidential spread of resistant pathogenic strains to humans through food (30). The antibiotic resistance rates in this study is slightly different from an earlier report by Msolo et al., (31) which indicated a resistant rate of 85% for penicillin G, 45% for chloramphenicol, 70% for erythromycin, and 74% for sulfamethoxazole. The 100% resistance to penicillin observed in our study agrees with the study of Alam et al., (6) who reported high rate (100%) of resistance to penicillin among *E. coli* O157 isolates cultured from raw milk marketed in Chittagong, Bangladesh.

The high susceptibility rate (100%) to gentamicin and ofloxacin for genotypically confirmed *E. coli* 0157:H7 isolates obtained in this study is different from a report by Alam et al., (6) where 50% rate to gentamicin and ofloxacin was reported. High resistance rates to penicillin and tetracyclines in our study agrees with the antibiotic susceptibility test study by Reuben and Owuna (32) on *E. coli* O157 isolates recovered from milk samples.

In a similar study by Tadesse et al., (33), *E. coli* showed high resistance rates to ampicillin (70%), sulfamethoxazole-trimethoprim (60%), clindamycin (80%), erythromycin (60%), chloramphenicol (50%), and kanamycin (50%), which is slightly different from the findings of our study. Antibiotic resistance could be due to abuse of antibiotics in both human medicine and for agricultural purposes, predominantly in disease suppression and advancement of growth in animal production. The high susceptibility of *E. coli* to kanamycin in our study is different from the study of Tadesse et al., (33) which reported 50% resistance rate, although the study reported high susceptibility rates to some antibiotics such as gentamicin (100%), ofloxacin (100%), and ciprofloxacin (90%), which is similar to the findings in our study.

Multiple antibiotic resistance (MAR) index is a good tool for health risk assessment which identifies if isolates are from a region of high or low antibiotic use. MAR index values greater than 0.2 indicate high risk source of infection where antibiotics are often used. MAR indexing has been revealed to be a cost effective and usable method of bacteria source tracking. The high MAR index of the isolates from this study could be a potential detriment to human health in our environment. In accordance with the multidrug resistance (MDR) pattern reported by Igwe et al., (5), resistance pattern of their isolates showed that 54.5% were MDR and 18.2% were extensive drug resistance (EDR). This is however different from the high EDR (52.6%) obtained in our study, which might be attributed to misuse of antibiotics within Benin City. Sudda et al., (34) reported 87.5% MDR among *E. coli* isolates, with about 50% resistant to sulfamethoxazole/trimethoprim, penicillin, tetracycline, and amoxicillin/clavulanic acid. The MDR pattern of *E. coli* reported by Haque (35) in Bangladesh and by Memon et al., (36) in Eastern China also showed similar results. This MDR pattern could be the result of amassing of resistance genes on plasmids, each coding resistance to specific antibiotics and/or multi-drug efflux pumps (34). The accumulation of MDR bacteria is a significant threat to public health as this can lead to ineffective treatment of infections and poor recovery of patients (12).

Conclusion:

The findings of our present study clearly indicated that safety and quality of fresh and fermented milk in Benin City were unsatisfactory. The presence of fecal coliform bacteria denotes poor hygiene practices. The pathogenic bacteria such as *E. coli* O157:H7 and other *E. coli* strains may be transferred through the milk to consumers. Fresh and fermented milk is of a special concern since these organisms can proliferate at variable conditions using the milk as reservoir. In addition, the high MAR index observed in *E. coli* O157:H7 and other *E. coli* strains in our study is a threat to consumers' health.

Conflict of interest:

Authors declare no conflict of interest

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Original Article **Open Access**

Correlation between faecal indicator bacteria in diarrheagenic stools and hospital wastewaters: implication on public health

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Abstract:

Background: Hospital wastewaters contain blends of inorganic, natural constituents and contaminants that carry significant health risk when released directly into the environment. The aim of this study is to investigate the correlation between faecal indicator bacteria in diarrheagenic stools and wastewaters generated in University of Medical Sciences Teaching Hospital complex, Akure, Nigeria.
Methodology: Quantification of faecal indicator bacteria was carried out on diarrheagenic faecal samples collected from 55 hospitalized patients and 68 wastewater samples from the medical laboratory science and laundry units of the hospital over of period of 12 weeks. Standard membrane filtration technique was performed using membrane intestinal enterococcus (m-ENT), membrane faecal coliform (m-FC), membrane lauryl sulphate (MLSA), eosin methylene blue (EMB) and Salmonella-Shigella (SS) agar plates, which were incubated at 37ºC for 24 hours (MLSA, EMB and SSA), 44ºC for 24 hours (m-FC); and 37ºC for 48 hours (m-ENT). Bacterial colonies on agar plates were counted and expressed as colony forming units (CFU) per 100ml of diarrheagenic stool and wastewater. Pearson's correlation analysis was used to determine the relationship between the level of faecal indicator bacteria in diarrheagenic stools and wastewaters at $p<0.05$ level of significance (and 95% confidence interval).
Results: The faecal coliform counts (log 10 CFU/100ml) ranged from 1.18 to 1.54 in diarrheagenic stools, 1.32 to1.64 in laboratory wastewater and 1.08 to 2.19 in laundry wastewater. *Escherichia coli* count (log 10 CFU/100ml) ranged from 1.08 to 1.40 in diarrheagenic stools, 1.20 to 1.86 in laboratory wastewater and 0.30 to 1.81 in laundry wastewater. Intestinal enterococci count (log 10 CFU/100ml) ranged from 0 to 0.30 in diarrheagenic stools, 0.78 to 0.90 in laboratory wastewaters and 0.48 to 1.11 in laundry wastewaters. Pearson's correlation co-efficient showed that all the faecal indicator bacteria count in diarrheagenic faecal samples exhibited positive correlation with those in laboratory wastewaters, but not with those from laundry wastewaters.
Conclusion: The findings suggest that diarrheagenic stools should be properly disinfected after the performance of laboratory tests to prevent transmission of potential pathogens, and wastewater generated from hospitals should be treated prior to discharge into the environment, to prevent possible infections in the community.

Keywords: Correlation, faecal indicator bacteria, public health, transmission, wastewater

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Corrélation entre les bactéries indicatrices fécales dans les selles diarrhéiques et les eaux usées des hôpitaux: implication sur la santé publique

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Abstrait:

Contexte: Les eaux usées des hôpitaux contiennent des mélanges de constituants inorganiques et naturels et de contaminants qui présentent des risques importants pour la santé lorsqu'ils sont rejetés directement dans l'environnement. Le but de cette étude est d'étudier la corrélation entre les bactéries indicatrices fécales dans les selles diarrhéiques et les eaux usées générées dans le complexe de l'hôpital universitaire des sciences médicales, Akure, Nigeria.
Méthodologie: La quantification des bactéries fécales indicatrices a été réalisée sur des échantillons fécaux diarrhéiques prélevés sur 55 patients hospitalisés et 68 échantillons d'eaux usées du laboratoire médical scientifique et des unités de blanchisserie de l'hôpital sur une période de 12 semaines. La technique de filtration membranaire standard a été réalisée à l'aide de plaques de gélose à entérocoque intestinal membranaire (m-ENT), coliforme fécal membranaire (m-FC), laurylsulfate membranaire (MLSA), éosine bleu de méthylène (EMB) et gélose Salmonella-Shigella (SS), qui étaient incubé à 37°C pendant 24 heures (MLSA, EMB et SSA), 44°C pendant 24 heures (m-FC); et 37°C pendant 48 heures (m-ENT). Les colonies bactériennes sur des plaques de gélose ont été comptées et exprimées en unités formant colonies (CFU) pour 100 ml de selles diarrhéiques et d'eaux usées. L'analyse de corrélation de Pearson a été utilisée pour déterminer la relation entre le niveau de bactéries fécales indicatrices dans les selles diarrhéiques et les eaux usées à p <0,05 niveau de signification (et intervalle de confiance à 95%).
Résultats: Le nombre de coliformes fécaux (log 10 CFU/100ml) variait de 1,18 à 1,54 dans les selles diarrhéiques, de 1,32 à 1,64 dans les eaux usées de laboratoire et de 1,08 à 2,19 dans les eaux usées de lessive. Le nombre d'*Escherichia coli* (log 10 UFC/100ml) variait de 1,08 à 1,40 dans les selles diarrhéiques, de 1,20 à 1,86 dans les eaux usées de laboratoire et de 0,30 à 1,81 dans les eaux usées de lessive. Le nombre d'entérocoques intestinaux (log 10 UFC/100ml) variait de 0 à 0,30 dans les selles diarrhéiques, de 0,78 à 0,90 dans les eaux usées de laboratoire et de 0,48 à 1,11 dans les eaux usées de lessive. Le coefficient de corrélation de Pearson a montré que tous les comptages de bactéries fécales indicatrices dans les échantillons fécaux diarrhéiques présentaient une corrélation positive avec ceux des eaux usées de laboratoire, mais pas avec ceux des eaux usées de lessive.
Conclusion: Les résultats suggèrent que les selles diarrhéiques doivent être correctement désinfectées après la réalisation des tests de laboratoire pour éviter la transmission d'agents pathogènes potentiels, et que les eaux usées générées par les hôpitaux doivent être traitées avant d'être rejetées dans l'environnement, afin de prévenir d'éventuelles infections dans la communauté.

Mots clés: corrélation, bactéries indicatrices fécales, santé publique, transmission, eaux usées

Introduction:

Hospice discharges remain a specific instance of anthropogenic contaminants. The aqueous hospital wastes are intricate blends of inorganic and natural constituents which are frequently dislodged into the immediate vicinity (1). This assortment is the consequence of analytical research laboratory outcomes. Waste and drugs including active by-products from pharmaceutical exhibits and their matters, synthetics, sterilizing agents, specific cleaners, irradiated indicators, bacteria and their anti-microbial resistant genes are sometimes present in hospital wastewaters (1). These wastewaters spawn from all therapeutic and non-medical activities from the laboratory to laundry activities (2). They are also a catalogue of microorganisms, disinfectants and drug wastes (3).

Faecal indicator bacterial (FIB) species are commensal-like bacteria found in the alimentary canal of warm-blooded animals, including humans (4,5). FIB species are used to examine faecal contamination intensities and therefore the prospect of pathogens of faecal source in soils and water in equally humid and temperate systems (6). FIB species comprise *Escherichia coli*, *Salmonella* spp, *Enterococcus* spp, and the coliforms. Globally, these groups of microorganisms are acceptable as useful indicators of faecal contamination since they exhibit close relationship with health hazards associated with gastrointestinal symptoms (7). *Escherichia coli* is reflected to be a more explicit pointer of faecal pollution than faecal coliforms since the broader test for faecal coliforms also detects thermotolerant non-faecal coliform bacteria (8). Enterococci are currently the lone FIB endorsed by the United States Environmental Protection Agency (9) for salty and marine waters, since they compare superiorly with human wellbeing effects than further FIB such as faecal coliforms or *E. coli* (10,11).

Most bacteria consortia of gastro-intestinal infections such as *Shigella*, *Campy-lobacter*, *Salmonella* and *Escherichia coli* are pathogenic in nature (12), one of which *Shigella* plays a central role in the occurrence of inflammatory diarrhea (13). Hospital-acquired gastroenteritis is an ordinary snag in hospitalized patients but its basis and implications are often taken for granted. Diarrhea illnesses among these patients may be associated with considerable morbidity or mortality (14,15,16). Hospital - acquired gastroenteritis constitutes a heightened incidence of diarrhea

in hospitalized patients that was not experienced prior to admission but commences after more than three days of hospital duration stay (17,18,19). Medically, this description is constructive since there is a possibility of community-acquired viral, bacterial or parasitic gastroenteritis budding later than the 3-day duration of hospital stay (17), although, in hospitalized patients, administration of antimicrobials and other medical approaches may cause diarrhea by unsettling the host-intestinal microbiome association (18,20,21,22).

Hospital wastewater may contain undesirable latent pathogens including antibiotic-resistant bacteria, and viruses (23,24). These innocuous agents, which may linger in wastewater treatment plants can aggravate contamination of the normal milieu by instigating natural bio-disproportion (25). The effluents from hospice wastewater treatment plants (WWTPs) are often discharged into surface waters (e.g., rivers, streams, lakes). Potential hazards as a result of these effluents in aquatic environments depend largely on the composition of the effluents, degree of treatment of the effluents, absorption of composites in wastewater, and water current rate of the receiving-river or stream (26). This contamination may lead to multiple environmentally-related risks such as sewage pollution of rivers, streams and other surrounding waters (27,28).

The World Health Organization (29) stated that about 85% of hospice wastes in the United States are harmless, 10% infective and 5% non-infective (but harmful), and infectivity outcomes may differ due to factors related to environmental setup (28). In the absence of hospital WWTPs, pathogenic bacteria may be discharged directly into aquatic reservoirs. The release of untreated hospice effluents directly into the environment may increase the volume of biological materials and critical nutrients that can impact variations in the quality of the receiving environment (30). In addition, hospital wastewater effluents are not odorless and microbial decomposition processes in the effluents may lead to air pollution and cause respiratory problems in humans residing in the surrounding environment and ecosystem (31).

The rationale for this study was to investigate the correlation between faecal indicator bacteria in diarrheagenic stools and wastewaters generated from the laboratory and laundry of the University of Medical Sciences Teaching Hospital Complex, Akure, Nigeria. This is to gain an improved comprehension of the risk of onward transmission of potential pathogens in hospital settings as well as the impact of untreated or partially treated hospital wastewaters discharged into the surrounding milieu.

Materials and method:

Study area

The study setting, the University of Medical Sciences Teaching Hospital, Akure (UNIMEDTH) (coordinates 7.2421⁰ N, 5.1957⁰ E), is a state-owned teaching hospital located in Akure South Local Government Area of Ondo State, Nigeria (Fig 1).

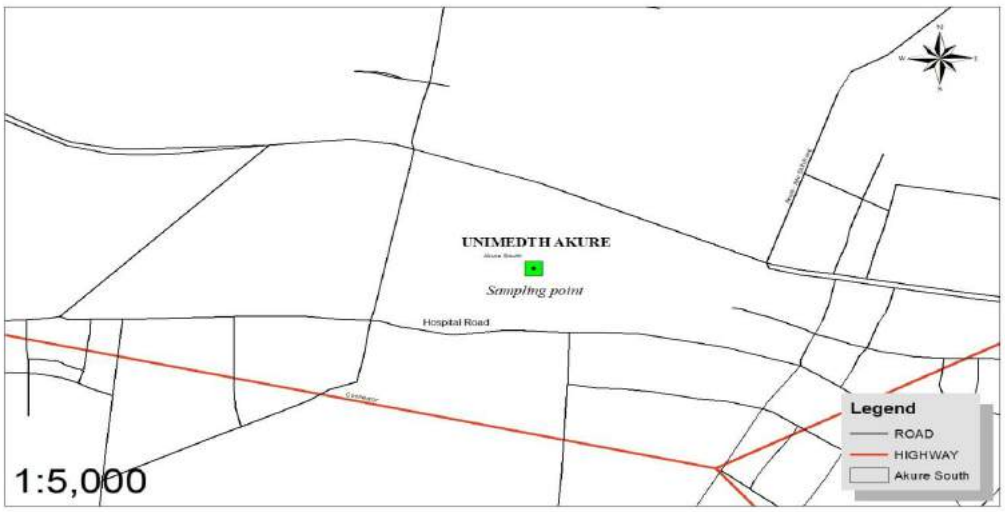

Fig. 1: Locality map showing University of Medical Sciences Teaching Hospital, Akure

Sample collection

Five millimeters of diarrheagenic faecal samples were collected from 55 hospitalized patients into sterile red-capped bottles and 500 ml of 68 wastewater samples were collected into sterile one litre plastic bottles from medical laboratory science and laundry units of the hospital over a period of 12 weeks. Samples were stored in ice packs at a temperature of 4ºC and analyzed within 1 hour of collection.

Enumeration of bacteria in diarrheagenic faecal stool and wastewater samples

Membrane filtration was performed according to the method of Maheux et al., (32), with slight modification. The selective media used; membrane intestinal enterococci (m-ENT), membrane faecal coliform (m-FC), membrane lauryl sulphate agar (MLSA), eosine methylene blue (EMB) and *Salmonella-Shigella* agar (SSA), were prepared according to the manufacturers' specification. The membrane filters (0.45µm, Delson Pascal Nig. Ltd) were positioned on the freshly prepared selective media. Agar plates were incubated at 37ºC for 24 hours (MLSA, EMB, SSA), 44ºC for 24 hours (m-FC) and 37ºC for 48 hours (m-ENT). Colonies (purple for faecal coliforms, metallic green for *E. coli*, dark brown for intestinal enterococci, black for *Salmonella* and colourless for *Shigella*) were counted using J 2 colony meter (Pec Medical, USA), calculated and expressed as colony-forming units (CFU) per 100ml of diarrheagenic stools and wastewaters (Fig 2).

Statistical analysis

Data obtained were converted to log10 and subjected to general descriptive statistics. Analysis of variance (ANOVA) and test of significance using Duncan's new multiple range test were undertaken using Statistical Package for the Social Sciences (SPSS) version 20.0. All data were analysed by Pearson's correlation at $p<0.05$ level of significance representing 95% confidence interval, to determine the relationship between the level of faecal indicator bacteria in diarrheagenic stools of hospitalized patients and wastewaters generated from the laboratory and laundry units.

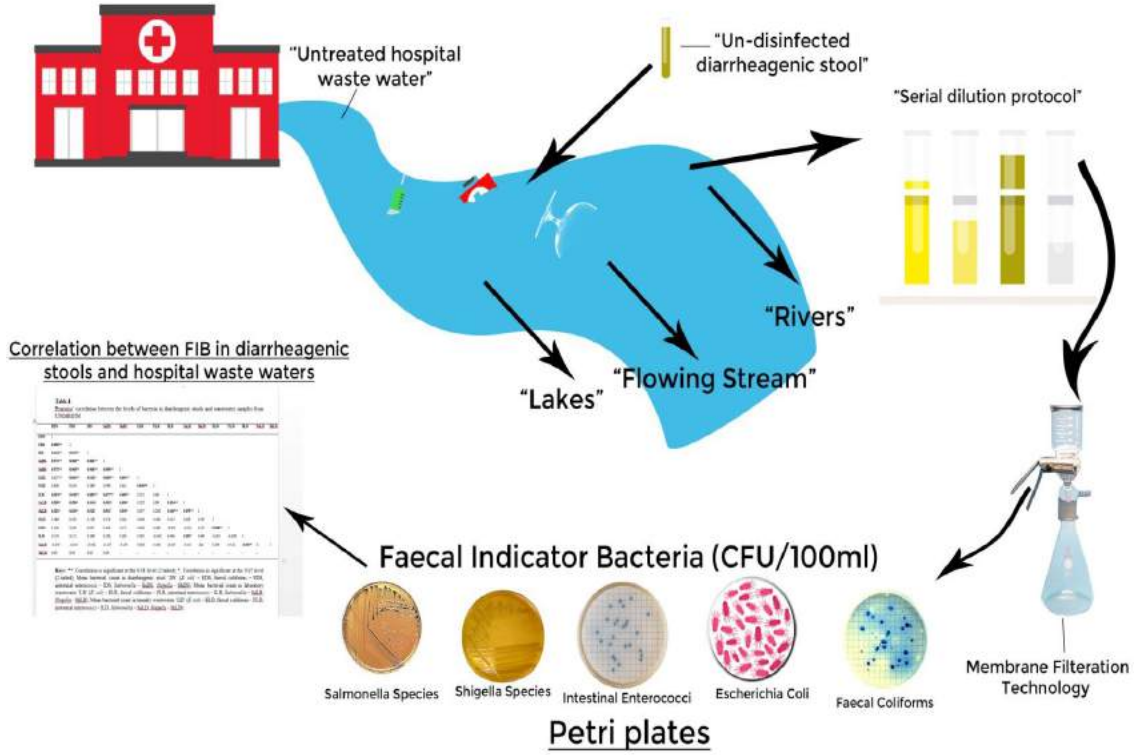

Fig. 2: A graphical illustration of the explanatory synopsis of the correlation of faecal marker bacteria in diarrheagenic stools and hospice effluents

Results:

Detection of bacteria in diarrheagenic faecal samples

Faecal coliforms had the highest mean count of 1.34 log10CFU/100ml while intestinal enterococci had the lowest mean count of 0.30 log10CFU/100ml in diarrheagenic stool. *Escherichia coli*, *Salmonella* spp, and *Shigella* spp had mean counts of 1.28 log10CFU/100ml, 1.04 log10CFU/100ml and 1.15 log10CFU/100 ml respectively (Fig 3).

Detection of bacteria in wastewater samples

Faecal coliforms had the highest mean count of 1.73 log10CFU/100ml and 1.72 log10 CFU/100ml in laboratory and laundry wastewater samples respectively while intestinal enterococci had the lowest mean count of 0.85 log10CFU/100ml and 0.95 log10CFU/100ml in laboratory and laundry wastewater samples respectively. The respective mean counts in laboratory and laundry wastewater samples for *E. coli* were 1.61 log10CFU/100ml and 1.69 log10CFU/100ml, *Salmonella* spp, 1.08 log10 CFU/100ml and 1.62 log10CFU/100ml, and *Shigella* spp 1.40 log10CFU/100ml and 1.53 log10CFU/100ml. The bacterial counts in the laundry wastewater samples were multiple than those in the laboratory wastewater samples (Fig 4).

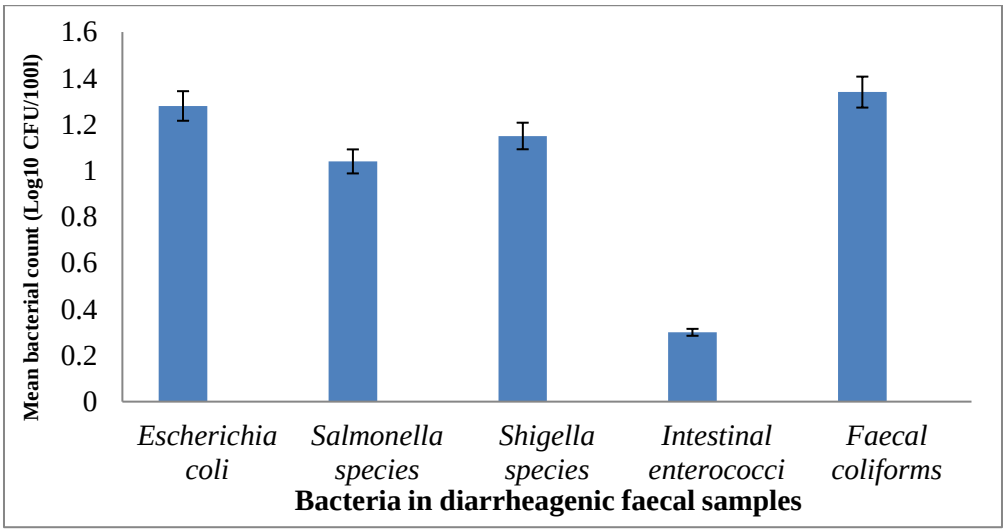

Fig. 3. Mean count of targeted bacterial indicators in diarrheagenic faecal samples

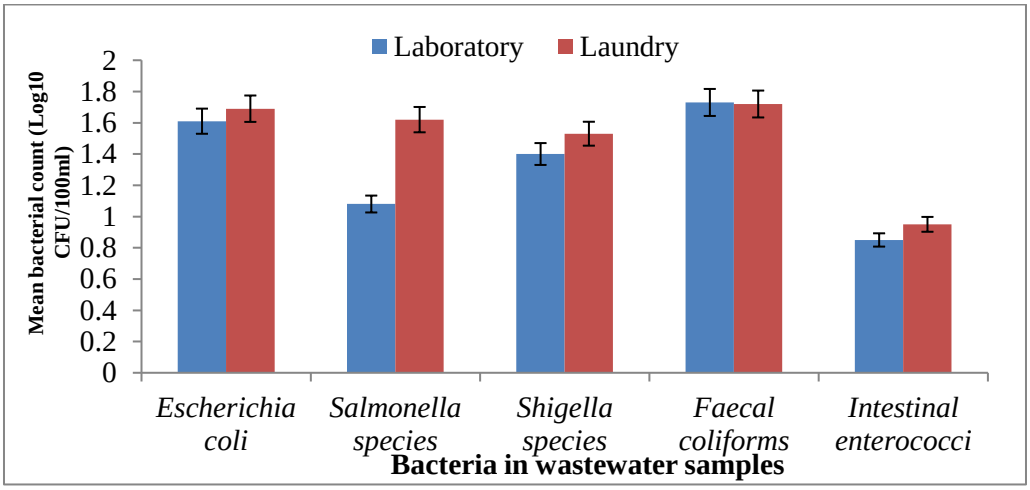

Fig. 4. Mean count of bacterial indicators in laboratory and laundry wastewater samples

Table 1: Pearson's correlation between bacteria counts in diarrheagenic stools and wastewater samples

	EDS	FDS	IDS	SaDS	ShDS	ELB	FLB	ILB	SaLB	ShLB	ELD	FLD	ILD	SaLD	ShLD
EDS	1														
FDS	0.999**	1													
IDS	0.944**	0.934**	1												
SaDS	0.972**	0.966**	0.981**	1											
ShDS	0.972**	0.968**	0.968**	0.998**	1										
ELB	0.657**	0.664**	0.542*	0.650**	0.694**	1									
FLB	0.408	0.419	0.269	0.390	0.442	0.940**	1								
ILB	0.654**	0.648**	0.680**	0.677**	0.668**	0.331	0.68	1							
SaLB	0.595*	0.590*	0.614*	0.615*	0.609*	0.322	0.89	0.894**	1						
ShLB	0.531*	0.530*	0.512	0.541	0.549*	0.457	0.300	0.669**	0.875**	1					
ELD	0.188	0.193	0.118	0.178	0.204	0.458	0.493	0.012	0.016	0.367	1				
FLD	0.156	0.162	0.087	0.146	0.172	0.438	0.485	-0.033	-0.015	0.347	0.998**	1			
ILD	0.276	0.272	0.299	0.288	0.280	0.383	-0.045	0.464	0.593*	0.493	-0.261	-0.298	1		
SaLD	-0.119	-0.116	-0.142	-0.127	-0.119	0.026	0.100	-0.247	-0.311	-202	0.293	0.312	-0.567*	1	1
ShLD	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-	-	-	

**. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed); Mean bacterial count in diarrheagenic stool 'DS' (*E. coli* - EDS, faecal coliforms - FDS, intestinal enterococci - IDS, *Salmonella* - SaDS, *Shigella* - ShDS); Mean bacterial count in laboratory wastewater 'LB' (*E. coli* - ELB, faecal coliforms - FLB, intestinal enterococci - ILB, *Salmonella* - SaLB, *Shigella* - ShLB); Mean bacterial count in laundry wastewater 'LD' (*E. coli* - ELD, faecal coliforms - FLD, intestinal enterococci - ILD, *Salmonella* - SaLD, *Shigella* - ShLD).

Relationship between levels of bacteria in diarrheagenic stools and wastewaters

The mean counts of *E. coli* in diarrheagenic stools showed positive correlation with the count of *E. coli* (r=0.657), intestinal enterococci (r=0.654), *Salmonella* (r=0.595), *Shigella* (r=0.531) in the laboratory wastewaters. In addition, the mean faecal coliform counts in diarrheagenic stools showed positive correlation with the count of *E. coli* (r=0.664), intestinal enterococci (r=0.648), *Salmonella* (r =0.590), *Shigella* (r=0.530) in the laboratory wastewaters.

Similarly, the mean bacterial counts of intestinal enterococci in diarrheagenic stools showed positive correlation with the count of *E. coli* (r=0.542), intestinal enterococci (r= 0.680), *Salmonella* (r=0.614), and *Shigella* (r= 0.512) in the laboratory wastewater. Also, the mean bacterial counts of *Salmonella* in diarrheagenic stools showed positive correlation with *E. coli* count (r=0.650), intestinal enterococci (r=0.677), *Salmonella* (r=0.615), and *Shigella* (r= 0.541) in the laboratory wastewaters.

The mean bacterial count of *Shigella* in diarrheagenic stools showed positive correlation with the count of *E. coli* (r=0.694), intestinal enterococci (r=0.668), *Salmonella* (r =0.609), and *Shigella* (r=0.549) in the laboratory wastewaters. There was no significant association between the levels of bacteria in diarrheagenic stools and the laundry wastewaters, with faecal coliforms (r=0.312), *Salmonella* (r=1) and *Shigella* (r=1) as shown in Table 1.

Discussion:

In this study, the correlation between faecal indicator bacteria (FIB) in diarrheagenic stools and hospital wastewaters with the implications and impacts of FIB on human health was evaluated. The high faecal coliform counts in diarrheagenic stools is in agreement with the observations of Sinton et al., (33) where the authors inferred that faecal coliforms are abundant in human faeces. The intestinal enterococci count in diarrheagenic stools also agrees with the reports of Boehm and Soller (11), and Boehm and Sassoubre (34), which noted that enterococci are found in high concentrations in human faeces but at a lower concentration when compared to the level of *E. coli*. Studies have reported *E. coli* as a predominant inhabitant of the gastrointestinal tract of humans (4,5), and in human faeces, 90-100% of coliform bacteria isolated are *E. coli* (35). The large numbers of *E. coli* inhabiting the human gut and their absence in other environments backed their continual usage as the utmost subtle marker of faecal contamination (36).

Similarly, García-Aljaro et al., (37) reported that intestinal microbiome in healthy individuals serves as pointer to varying pathological conditions which cause diarrhea. This may account for the high *E. coli* count in diarrheagenic faecal samples in this study. The relatively high concentration of *Salmonella* species agrees with the findings of Jafari et al., (38) who observed high count of *Salmonella* spp from acute diarrhea patients at some hospitals in Tehran, and reported that *Salmonella* spp constitutes significant human health hazard in that it presents diarrhea as one of its major clinical symptoms in humans. In the same vein, the high count of *Shigella* spp in diarrheagenic stools in this study agrees with the observations of Majalan et al., (39) who detected high *Shigella* count among diarrhea patients admitted to certain hospitals in Tehran. Moghanloo et al., (40) also detected *Salmonella* and *Shigella* species in stools of patients with diarrhea in Kashan City, Iran.

The high faecal coliform count observed in laboratory and laundry wastewater samples agrees with the findings of Chukwu et al., (41) who detected high total faecal coliform count (TFCC) of 2.9×10^3 and 2.4×10^2 CFU/ml respectively from laboratory and laundry wastewater samples collected from Abia State University Teaching hospital, Aba, Nigeria. Our observation is also in agreement with Sadek et al., (42) who detected significant levels of faecal coliforms in hospital discharges, thus indicating the level of faecal pollution of the receiving environment. Studies have demonstrated that the discharge of raw wastewater from hospices may have undesirable consequences and a considerable impact on public health as a result of constituents such as pathogenic organisms also including antibiotic resistant organisms, innocuous chemicals, chemotherapeutic remains, pharmaceutical materials, and irradiated isotopes (43,44).

In our study, the low count of intestinal enterococci in laundry and laboratory wastewater samples agree with Moges et al., (45) who observed low levels of enterococci in wastewater samples from hospital environment in Northern Ethiopia. However, *E. coli* counts in laundry and laboratory wastewater samples were higher than those observed by Moges et al., (45). Other studies have established the presence of *E. coli* in habitats outside the gastrointestinal tract such as hospital wastewaters (45,46).

The correlation between counts of enteric bacteria in diarrheagenic stools with those in wastewaters generated in the laboratory in this study may not be unconnected with the fact that faecal marker bacteria are usually commensals of the alimentary tract of many warm-blooded animals especially humans, and are passed out in faeces in large quantities. These enteric bacteria are also present in considerable quantity in faecally-contaminated waters in the hospital environment (47). The positive correlation between *E. coli* in diarrheagenic stools and *E. coli*, intestinal enterococci, *Salmonella* and *Shigella* spp in laboratory wastewater could be a consequence of high rate of uninterrupted discharge of faecal samples from the laboratory after necessary diagnostic procedures into wastewater. This bears some semblance with the findings of Patra et al., (48) who showed positive significant correlation of *E. coli* in stool samples with presumptive *E. coli*, *Shigella*, and *Salmonella* spp in laboratory wastewaters.

The positive correlation of faecal coliforms in diarrheagenic stools with *E. coli*, intestinal enterococci, *Salmonella* and *Shigella* spp in laboratory wastewater samples may indicate the discharge of un-disinfected faeces into hospital effluents (49). This also buttress the observations of Patra et al., (48) who showed positive significant correlation of faecal coliforms in diarrheagenic stools with presumptive *E. coli*, *Shigella*, *Salmonella* spp in laboratory wastewaters. The significant correlation observed between intestinal enterococci in diarrheagenic stool samples with intestinal enterococci, *Salmonella* and *Shigella* spp in

laboratory wastewaters is comparable to the findings of Schriewer et al., (4) who observed significant correlation between intestinal enterococci from diarrheagenic faecal samples and *Salmonella* spp in laboratory wastewater samples. In contrast, a study conducted by Hatha et al., (50), observed no significant correlation between high levels of faecal coliforms in diarrheagenic stools and incidence of specific pathogens such as *Salmonella* and *Shigella* spp in laboratory wastewaters. The positive correlation observed between *Salmonella* spp in diarrheagenic stools and *E. coli*, intestinal enterococci, *Salmonella* and *Shigella* spp in laboratory wastewaters agrees with the findings of Efstratiou et al., (51), where the authors observed a strong direct correlation between indicator bacteria (enterococci and *E. coli*) in wastewater samples and *Salmonella* spp in stool samples but contrastingly, the study by Polo et al., (52) reported little or no correlation of indicator bacteria in hospital wastewater samples with *Salmonella* spp from stool samples.

In the study by McEgan et al., (53), weak direct correlation between natural markers (*E. coli* and coliforms) in hospital wastewaters and *Salmonella* levels in stool samples was reported, which may suggests that *E. coli* and coliforms are not absolute markers of the presence of pathogenic enteric bacteria such as *Salmonella* and *Shigella* spp in diarrheagenic stool samples, because there could also be contaminations by pathogens from non-faecal sources of the wastewaters in the hospital environment (54). The positive correlation reported between *Shigella* spp in diarrheagenic stools and levels of *E. coli*, intestinal enterococci, *Salmonella* and *Shigella* spp in hospital wastewater samples is comparable to the results of Ferguson et al., (55), who reported statistically significant correlations of bacterial pathogens such as *Salmonella* and *Shigella* spp in hospital wastewater with faecal indicators in stool samples.

Conclusion:

Our study demonstrates significant correlation between faecal indicator bacteria in diarrheagenic stools and laboratory wastewaters. The findings suggest that diarrheagenic stools should be disinfected after the performance of laboratory diagnostic tests in order to prevent onward transmission of potential pathogens in hospital settings. In addition, wastewater generated from hospitals must be fully treated prior to discharge into the environmental milieu in order to protect human health.

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Ethical approval:

Ethical approval (NHREC/18/08/2016 and a protocol number of OSHREC/16/09/2019/245) for the study were obtained from the Ondo State Health Research Ethics Committee (OSHREC) of the Ministry of Health (MOH), Ondo State, Nigeria.

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Conflict of interest:

Authors declare no conflict of interest.

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Original Article **Open Access**

Microbial contamination of Naira notes circulating in Bauchi metropolis: prevalence, microbial load and detection of extended spectrum beta-lactamase producing Gram-negative bacteria

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Abstract:

Background: Globally, contamination of banknotes with various microbial species is increasingly being reported. This usually results from improper handling during exchange of goods and services. In the present study, we aimed to determine the microbial load, prevalence and the presence of Extended Spectrum Beta Lactamase (ESBL) among bacteria isolated from the Nigerian Naira notes circulating in Bauchi metropolis.
Methodology: A total of 400 Naira notes of various denominations were randomly collected aseptically, cultured and total viable counts determined. The isolated microbial species were identified using standard microbiological techniques. Antibiotic susceptibility of the isolates and detection of ESBL were determined by Kirby-Bauer's disc diffusion method and Double Disc Synergy Test (DDST), respectively.
Results: All the 400 samples collected were contaminated with various microbial species. The highest mean colony count was detected in 20 Naira notes (28.5%), while the least was observed in 1000 Naira note (3.3%). Fourteen different microbial species were isolated from the contaminated currency notes, predominantly *Escherichia coli* (25.0%*),* and *Staphylococcus aureus* (12.0%). Some fungal species mainly *Aspergillus flavus* and *Aspergillus niger* were also isolated. Majority of the bacteria isolates resistant to the third generation cephalosporins (72.1%) were ESBL positive.
Conclusion: The study shows that Naira notes circulating in Bauchi metropolis were heavily contaminated with various microbial species, and a high proportion of the isolated Gram-negative bacteria were ESBL producers. Efforts should thus be made to improve hygiene practices in the study area. Importantly, businesses should be encouraged to adopt the use of electronic transactions.

Keywords: Currency notes, Naira, Microbial contamination, ESBL

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Contamination microbienne des notes de Naira circulant dans la métropole de Bauchi: prévalence, charge microbienne et détection de la production de bêta-lactamase à spectre étendu Bactéries Gram-négatives

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Abstrait:

Contexte: À l'échelle mondiale, la contamination des billets de banque par diverses espèces microbiennes est de plus en plus signalée. Cela résulte généralement d'une mauvaise manipulation lors de l'échange de biens et de services. Dans la présente étude, nous avons cherché à déterminer la charge microbienne, la prévalence et la présence de bêta lactamase à spectre étendu (BLSE) parmi les bactéries isolées des notes nigérianes naira circulant dans la métropole de Bauchi.
Méthodologie: Un total de 400 billets Naira de différentes dénominations ont été collectés au hasard de manière aseptique, cultivés et le nombre total viable déterminé. Les espèces microbiennes isolées ont été identifiées à l'aide de techniques microbiologiques standard. La sensibilité aux antibiotiques des isolats et la détection des BLSE ont été déterminées respectivement par la méthode de diffusion sur disque de Kirby-Bauer et le test de synergie à double disque (DDST).
Résultats: Tous les 400 échantillons prélevés étaient contaminés par diverses espèces microbiennes. Le nombre moyen de colonies le plus élevé a été détecté dans 20 billets nairas (28,5%), tandis que le moins a été observé dans les billets 1000 nairas (3,3%). Quatorze espèces microbiennes différentes ont été isolées des billets de banque contaminés, principalement *Escherichia coli* (25,0%) et *Staphylococcus aureus* (12,0%). Certaines espèces fongiques, principalement *Aspergillus flavus* et *Aspergillus niger*, ont également été isolées. La majorité des isolats bactériens résistants aux céphalosporines de troisième génération (72,1%) étaient BLSE positifs.
Conclusion: L'étude montre que les notes de Naira circulant dans la métropole de Bauchi étaient fortement contaminées par diverses espèces microbiennes et qu'une forte proportion des bactéries Gram-négatives isolées étaient des producteurs de BLSE. Des efforts devraient donc être faits pour améliorer les pratiques d'hygiène dans la zone d'étude. Surtout, les entreprises devraient être encouragées à adopter l'utilisation des transactions électroniques.

Mots clés: billets de banque, naira, contamination microbienne, BLSE

Introduction:

Bank currency notes are the commonest means of exchanging goods and services particularly in developing countries (1). They are one of the most frequently passed items from one hand to the other during transactions (2). Improper handling practices such as concurrent handling of banknotes and food items, use of saliva to wet fingers during counting, placing or storing paper notes in or on dirty surfaces among others has led to widespread contamination of banknotes with various microbial species (3).

Globally, contamination of banknotes with microbial species is being reported. A large multi-national study involving 1280 banknotes obtained from 10 different countries showed that bacterial contamination of banknotes is greatly influenced by age of the notes and the nature of material used to produce the notes (polymer-based versus cotton-based) (4). In the United States, 94% contamination of circulating one dollar bill was reported in 2002 (5). A similar high contamination rate has been reported in Estonia (6), Pakistan (7), Croatia (2) and Ghana (8).

The contaminated banknotes have been demonstrated to be a viable source of cross-contamination and a vehicle for transmission of infectious agents in the community (9). Bacteria causing foodborne diseases such as typhoid fever, gastroenteritis, shigellosis, etc have been isolated from banknotes (10,11). Some other researchers have isolated parasites and viral particles from bank currency notes (12–14).

Studies across Nigeria have documented varying rate of microbial contamination of banknotes (15–17). In Bauchi, north-eastern Nigeria, the level of microbial bioburden and the prevalence of extended spectrum beta-lactamase (ESBL) among the circulating Naira notes is currently unknown. In the present study, we aimed to determine the microbial load, prevalence and the presence of ESBL among bacteria isolated from the Nigerian Naira notes circulating in Bauchi metropolis.

Materials and method:

Study setting

The study was carried out in the main market (Wunti market) in Bauchi metropolis, the capital of Bauchi State (geographic coordinates 10.7761° N, 9.9992° E). The city has an estimated population of 6,537,314, approximately 3.38% of the Nigerian population (18). The market is located in the heart of Bauchi metropolis, serving as point of exchange of goods and services for the inhabitants of the State. Field survey showed that the market has 2980 shops operating various businesses ranging from grocery stores, fashion houses, butcher houses, snack bars, canteens, and hardware shops.

Study design and sampling method

This was a descriptive cross-sectional study of Naira currency denominations among market shop owners, conducted between January and July 2019. Systematic random sampling method was used to select shops for participation in this study. Samples were collected from every 5th shop in the study area.

Ethical considerations

The approval to conduct this study was granted by the Bauchi State Ministry of Health (Ref No: NREC/12/05/2013/2017/38). Consent was also sought from the market men and women after carefully briefing them on the objective of the study.

Selection criteria

All the Naira currency denominations currently in use in Nigeria from the selected shop owners were included with the exception of the mutilated or grossly dirty notes. The samples were collected from only numbered shops. Constructed road side/temporary shops without numbering were excluded. Also, old notes that have been withdrawn from circulation were also excluded from the study.

Sample collection

Fifty (50) samples of each of the eight (₦5, ₦10, ₦20, ₦50, ₦100, ₦200, ₦500 and ₦1,000) Naira denominations in circulation total ling 400 samples were collected from different shop owners practicing different businesses of their choice in Wunti market in the metropolis of Bauchi State, Nigeria. The Naira note samples were collected aseptically by letting the owners drop them into sterile polythene bags, and the polythene sealed immediately. The sealed polythene bags were immediately taken to the Medical Microbiological Laboratory of the Abubakar Tafawa Balewa University Teaching Hospital in Bauchi.

Enumeration of microbial contaminants

The enumeration of microbial contaminants on the notes was carried out as previously described (17). Briefly, the collected Naira notes were placed aseptically into different sterile test tubes containing 10ml of sterile water and shaken for few minutes. After 3 minutes, the notes were removed from the test tubes and a 5 serial 10-fold dilution (10^{-1} to 10^{-5}) were made. An aliquot of 0.1 ml from the 10^{-4} dilution was aseptically inoculated on Blood, MacConkey and Mannitol salt agar plates which had been prepared according to manufacturer's instruction. All plates were incubated at 37°C for 24 hrs, and examined for bacterial growth, and the number of colonies on each plate was counted using a digital colony counter.

Identification of bacterial isolates was done using a combination of morphological characteristics such as shape, colour, elevation, consistency etc. and biochemical characteristics including coagulase, novobiocin, triple sugar iron (TSI) agar, oxidase, catalase, oxidation-fermentation (OF), urease, motility, indole, citrate, methyl red (MR) and Voges-Proskauer (VP) tests as previously described (19).

For isolation of fungi, a loopful of the culture was inoculated in duplicate onto Sabouraud's Dextrose agar (SDA) supplemented with 0.01% chloramphenicol. The first plate was incubated aerobically at 37°C for 48 hours and the second plate was incubated for five days at room temperature (25^{o}C). Identification was done on the basis of cultural (mould or yeast form, pigment production) and microscopic morphological characteristics following staining with lactophenol cotton blue as described (20).

Antibiotic susceptibility testing

Antimicrobial susceptibility pattern of bacterial isolates was determined using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (21). The tested antibiotics were as follow; ampicillin, ciprofloxacin, gentamicin, nalidixic acid, amoxicillin, aztreonam, cefoxitin, vancomycin, tetracycline and erythromycin (Mast company, UK). The standard strain of *E. coli* ATCC 25922 was used for quality control in susceptibility testing. Interpretation of results as susceptible, intermediate or resistant was done according to the criteria recommended by the CLSI guideline.

Screening test for ESBLs

Gram negative bacterial isolates resistant to third generation cephalosporins (3GC) were regarded as presumptive ESBL positive. This was confirmed by the double discs synergy test (DDST). Briefly, the confirmatory test was performed by placing a β-lactamase inhibitor (amoxicillin–clavulanic) disc between two third generation cephalosporins (3GCs) discs at a distance of 20 mm centre-to-centre on a plate

inoculated with a standardized inoculum of the test organism as previously described [22]. Formation of a characteristic keyhole effect or champagne-cork shaped zone of inhibition between the discs was considered as a phenotypic indication of ESBL production.

Statistical analysis

Data was analysed using IBM SPSS statistics software, version 24.0 (IBM Corporation, Armonk, NY) and presented as simple descriptive statistics or pictograms. Categorical variables were compared using Pearson's χ^2 test or Fisher's exact test. At 95% confidence interval, $p<0.05$ was considered as statistically significant.

Results:

Mean microbial counts on each of the currency denominations

A total of 400 Naira notes samples of eight denominations (₦5, ₦10, ₦20, ₦50, ₦100, ₦200, ₦500 and ₦1,000) were collected from traders within the market. All the samples analysed were contaminated with various microbial species. The result of mean colony counts of the different currency denominations sampled from the markets is presented in Table 1.

Table 1: Mean colony counts of microbial pathogens across the currency notes

Currency denominations	Mean colony counts (CFU/ml x 10^3)	
	Bacteria	Fungi
1000	7.0 ± 0.4	3.0 ± 0.9
500	9.0 ± 0.7	11.0 ± 0.3
200	15.0 ± 0.5	12.0 ± 0.4
100	24.0 ± 0.1	13.0 ± 0.5
50	21.0 ± 0.6	6.0 ± 0.1
20	48.0 ± 0.1	5.0 ± 0.7
10	39.0 ± 0.5	5.0 ± 0.2
5	34.0 ± 0.4	4.0 ± 0.1

CFU = Colony Forming Unit; ml = Millilitre

The result shows that 20 Naira notes had the highest mean count of 48.0±0.1x103 CFU/ml for bacteria, followed by 10 Naira note which had 39.0±0.5x103 CFU/ml while 1000 Naira notes had the least count of 7.0 ±0.4x103 CFU/ml. The fungal count of the Naira notes revealed that 100 Naira note has the highest count of 13.0±0.5 x103 CFU/ml, followed by 200 Naira with 12.0±0.4x103 CFU/ml while the least was 5 Naira note which had 4.0±0.2 x103 CFU/ml (Table 1).

Distribution of the microbial isolates according to different denominations

As shown in Table 2, the highest contamination rate was in 20 Naira notes 114 (28.5 %), followed by 50 Naira note 64 (17.5%), and the least rate was in 1000 Naira note 13 (3.3%).

Table 2: Distribution of the microbial isolates according to different currency denominations

Banknotes	Contamination
1000	13 (3.3)
500	23 (5.8)
200	40 (10.0)
100	53 (13.3)
50	64 (16.0)
20	114 (28.5)
10	56 (14.0)
5	37 (9.3)

Distribution of isolated microbial contaminants

The distribution of isolated organisms is presented in Table 3. Fourteen different microbial species were isolated from the contaminated currency notes. *Escherichia coli* (25.0%) and *Klebsiella pneumoniae* (24.5%), were the commonest Gram-negative bacteria isolated. This was followed by *Klebsiella oxytoca*, *Proteus* spp and *Pseudomonas aeruginosa*. Among the Gram-positive bacteria isolated, *Staphylococcus aureus* (12.0%) and *Bacillus* spp (3.5%) were the most frequently encountered. Some fungal species were also isolated, mainly *Aspergillus flavus*, *Rhizopus* spp and *Aspergillus niger*.

Table 3: Distribution of isolated microbial species

Isolates	Number (n)	Percentage (%)
Gram-negative bacteria (n=280)		
Escherichia coli	100	25
Klebsiella pneumoniae	98	24.5
Klebsiella oxytoca	40	10
Proteus spp	28	7
Pseudomonas aeruginosa	10	2.5
Enterobacter spp	2	0.5
Salmonella typhimurium	2	0.5
Gram-positive bacteria (n=140)		
Staphylococcus aureus	48	12
Staphylococcus saprophyticus	10	2.5
Bacillus spp	14	3.5
Micrococcus spp	2	0.5
Fungal isolates		
Aspergillus flavus	18	4.5
Rhizopus spp	18	4.5
Aspergillus niger	10	2.5
Total	**400**	**100**

Table 4: Distribution of ESBL producing among multidrug resistant *Enterobacteriaceae* isolates from Naira notes

Isolates	Number of 3GC resistant isolates	No of ESBL positive (%)
Escherichia coli	19	26 (30.2)
Klebsiella oxytoca	2	5 (5.8)
Klebsiella pneumoniae	44	22 (25.6)
Proteus spp	14	0 (0)
Salmonella typhimurium	1	0 (0)
Pseudomonas aeruginosa	5	8 (9.3)
Enterobacter spp.	1	1 (1.2)
Total	**86**	**62 (72.1)**

Antibiotic resistance pattern of the isolated bacteria

Out of the total of 280 Gram-negative bacterial isolated from various Naira notes, 86 (30.7%) were resistant to third generation cephalosporins (3GC). The 3GC resistant isolates comprise *E. coli* (n=19), *K. oxytoca* (n=2), *K. pneumoniae* (n=44), *Proteus* spp (n=14), *Salmonella typhimurium* (n=1), *P. aeruginosa*

(n=5), and *Enterobacter* spp (n=1). The result of phenotypic ESBL detection showed that 62 (72.1%) of the 3GC resistant bacteria were ESBL positive (Table 4). This was predominantly detected among *E. coil* 26 (30.2%) and *K. pneumoniae* 5 (5.8%) isolates.

Discussion:

The contamination of paper currency notes is almost inevitable due to poor handling practices. In this study, 100 % contamination rate was observed. This is consistent with high contamination rate reported by other researchers across the country (23). Similarly, in a neighbouring West Africa country, 100% contamination rate was reported (8). One hundred percent contamination rate of Pakistani and Euro currency notes have also been reported (24,25). The contamination may have aroused from simultaneous handling of the currency notes and various articles during exchange at selling points (26). This practice is particularly common among small businesses in the study area due to low level of penetration and acceptance of electronic transaction as a result of high internet cost and low-literacy level in the area (27,28). Additionally, activities of cyber-criminals popularly known as 'Yahoo-Yahoo' using deceptive banking tools to defraud unsuspecting clients makes the use of physical currency very popular, with attendant health risks (29). Though variation in mean colony counts among currency of the same denomination exist, the heavy contamination of lower denomination currencies (₦100 to ₦5) observed in this study concurs with the findings of other researchers (30,31). This may be attributed to high use and frequent exchange of lower denomination currencies than the higher denominations in daily cash transactions (31).

The contamination of the notes with several known human pathogens concurs with several other reports (30,32). The predominance of *E. coli* and other enteric Gram-negative bacteria among the isolated bacteria may be due to poor hygiene (both personal and environmental) in the study area. This is in contrast with a result of a study in Uganda where *E. coli* isolates were not detected among the bacteria isolated from their paper currency notes (30). Similar to the finding of this study, currency notes in both Nigeria and elsewhere have been shown to be contaminated with various fungal isolates (11,33,34). Among the Gram-positive bacteria, *Staphylococcus* spp predominates. This could have been shed from the skin of the hands during handling and exchange (34).

The high ESBL detection rate (72.1%) in the isolated bacteria is not surprising. This is in line with 7.5 93.3% rate reported in a systematic review on the prevalence of ESBL producing Gram-negative bacteria in Nigeria (35). Another study has reported contamination of automated teller machine (ATM) touch surfaces by ESBL producing bacteria (36). Bacteria harbouring other antibiotic resistance mechanisms have also been isolated from Nigerian currency notes (23). The high ESBL rate among the isolated bacteria may be due to overuse of beta-lactam antibiotics in the study area (37). In Nigeria generally, beta-lactam antibiotics are the most widely used in both hospital and community settings (38). Sometimes, these antibiotics are sourced over-the-counter for self-medication (39). This practice has over the years led to the emergence of beta-lactamase producing bacteria threatening the therapeutic efficacy of most the beta-lactam antibiotics.

The finding in this study has significant health implications. Majorly, the contaminated currency notes may serve as vehicle for transmission of infectious agents, playing significant role in the community transmission of infectious agents (3). This is worrisome particularly in this era of global pandemic due to SARS-CoV-2. Moreover, handlers of these notes especially women may put the currency notes in their brassieres or other areas where there is intimate contact with the skin and thus predisposing them to infections. Also, some individuals usually wet their fingers with saliva to ease the counting of the currencies, consequently resulting in cross-contamination. Food vendors too simultaneously handling monies and ready to eat food could also facilitate the transmission of potential pathogenic organisms from currencies to their clients.

Preventive measures aimed at breaking the chain of transmission of infections and contamination of the currency notes should therefore be instituted. This should include public enlightenment on personal and environmental hygiene and proper handling of Naira notes. Old and mutilated notes should be frequently withdrawn and replaced. Lastly and most importantly, electronic transactions such as the use of digital money, point of service (POS), electronic transfer of fund and others should urgently be promoted as a safer alternative.

This study was characterised by some limitations. First, the study was restricted to bacterial and fungal contaminants. Potential contamination by viruses and some pathogenic parasites could not be inferred. Also, the ESBL detection test was restricted to phenotypic method. As such, the predominant genotype of

the detected ESBL producing strains remains unknown. Recently, genotypic methods including meta-genomics is being explored for investigation of microbial pathogens and antibiotic resistance genes on currency notes (14). However, until now, no previous report on the microbial contamination of Naira notes in the State. This study thus provides an important baseline data on contamination of Naira notes in Bauchi State, North-eastern Nigeria.

Conclusion:

The study shows that Naira notes circulating in Bauchi metropolis were heavily contaminated with microbial species, predominantly *E. coli, K. pneumoniae* and *S. aureus*. A high proportion (72.1%) of the isolated Gram-negative bacteria were ESBL producers. Because of the potential role of banknotes in transmission of pathogenic organisms, advocacy to improve hygiene practices in the study area should be urgently undertaken. Most importantly, businesses should be encouraged to adopt safer alternatives such as the use of electronic transactions.

Authors' contributions:

The study was designed and conducted by MU and AI. AO analysed and interpreted the data and produced the first manuscript draft, which was revised by all authors. All authors read and approved the final manuscript.

Conflict of interest:

Authors declare no conflict of interest

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Point prevalence survey of antimicrobial consumption and resistance: 2015-2018 longitudinal survey results from Nigeria

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Abstract:

Background: Nigeria joined the global community in monitoring antimicrobial prescribing practices since 2015. Results of individual hospital Global Point Prevalence Survey (Global-PPS) have stimulated efforts at instituting hospital-based antimicrobial stewardship (AMS) programmes. We report the trends of antimicrobial prescribing rates and quality indicators for 3 surveillance periods; 2015, 2017 and 2018.
Methodology: The web-based Global-PPS for surveillance of antimicrobial use in hospitals (www.global-pps.com) was completed by each participating hospital site for all inpatients receiving antimicrobials on a selected day in 2015, 2017 and 2018. Data included details on antimicrobial agents, reasons and indications for treatment and a set of quality prescribing indicators. Data were validated by the web-based data management system of University of Antwerp, exported into Microsoft Excel and analyzed with EPI INFO version 7.2.
Results: Thirteen hospitals participated in the survey involving a total of 5,174 inpatients. Mean weighted overall antimicrobial prescribing prevalence was 70.7% which declined over the years from 71.7% in 2015 to 59.1% in 2018 (p<0.001). The rate of documentation of date for post prescription review improved from 27.9% in 2015 to 48.5% in 2018 (p<0.001) while the rates of targeted treatment declined from 12.0% in 2015 to 5.2% in 2018 (p<0.001). There was no significant change in the choice of parenteral drug administration (64.5% in 2015, 65.1% in 2017 and 62.6% in 2018; p=0.6803), and but there was significant increase in documentation of reasons for prescription in case notes (62.2% in 2015, 74.5% in 2017, and 70.9% in 2018; p=0.008). Overall, the main indications for therapeutic prescribing were skin and soft tissue infections (20.8%), sepsis (15.9%) and pneumonia (11.6%). The top three antibiotics for therapeutic use were ceftriaxone (18.2%), metronidazole (15.3%) and ciprofloxacin (10.4%).
Conclusions: The survey showed reduction in the overall antimicrobial prescribing rate especially in hospitals that had introduced AMS programmes. Among the quality prescribing indicators, documentation of post prescription review date showed improvement. The Global-PPS serves as a cost effective, flexible and user-friendly tool in instituting AMS programmes in hospitals.

Keywords: antimicrobial prescribing, hospital, global-point prevalence survey, quality indicators

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Enquête ponctuelle de prévalence de la consommation et de la résistance aux antimicrobiens: résultats de l'enquête longitudinale 2015-2018 au Nigéria

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Abstrait:

Contexte: Le Nigéria a rejoint la communauté mondiale pour surveiller les pratiques de prescription d'antimicrobiens depuis 2015. Les résultats de l'enquête sur la prévalence des antimicrobiens dans les hôpitaux individuels (Global-PPS) ont stimulé les efforts visant à instaurer des programmes de gestion des antimicrobiens dans les hôpitaux. Nous rapportons les tendances des taux de prescription d'antimicrobiens et des indicateurs de qualité pour 3 périodes de surveillance; 2015, 2017 et 2018.
Méthodologie: Le Global-PPS en ligne pour la surveillance de l'utilisation des antimicrobiens dans les hôpitaux (www.global-pps.com) a été complété par chaque site hospitalier participant pour tous les patients hospitalisés recevant des antimicrobiens un jour sélectionné en 2015, 2017 et 2018. Données incluses des détails sur les agents antimicrobiens, les raisons et les indications du traitement et un ensemble d'indicateurs de prescription de qualité. Les données ont été validées par le système de gestion de données en ligne de l'Université d'Anvers, exportées vers Microsoft Excel et analysées avec EPI INFO version 7.2.
Résultats: Treize hôpitaux ont participé à l'enquête portant sur un total de 5174 patients hospitalisés. La prévalence moyenne pondérée globale des prescriptions d'antimicrobiens était de 70,7%, ce qui a diminué au fil des ans, passant de 71,7% en 2015 à 59,1% en 2018 (p<0,001). Le taux de documentation de la date pour le réexamen post-prescription est passé de 27,9% en 2015 à 48,5% en 2018 (p<0,001) tandis que les taux de traitement ciblé sont passés de 12,0% en 2015 à 5,2% en 2018 (p<0,001). Il n'y a pas eu de changement significatif dans le choix de l'administration parentérale du médicament (64,5% en 2015, 65,1% en 2017 et 62,6% en 2018; p=0,6803), mais il y a eu une augmentation significative de la documentation des motifs de prescription dans les notes de cas (62,2 % en 2015, 74,5% en 2017 et 70,9% en 2018; p=0,008). Dans l'ensemble, les principales indications de prescription thérapeutique étaient les infections de la peau et des tissus mous (20,8%), la septicémie (15,9%) et la pneumonie (11,6%). Les trois principaux antibiotiques à usage thérapeutique étaient la ceftriaxone (18,2%), le métronidazole (15,3%) et la ciprofloxacine (10,4%).
Conclusions: L'enquête a montré une réduction du taux global de prescription d'antimicrobiens, en particulier dans les hôpitaux qui avaient mis en place des programmes AMS. Parmi les indicateurs de qualité de prescription, la documentation de la date de revue post-prescription a montré une amélioration. Le Global-PPS est un outil rentable, flexible et convivial pour la mise en place de programmes AMS dans les hôpitaux.

Mots clés: prescription d'antimicrobiens, hôpital, enquête de prévalence globale, indicateurs de qualité

Introduction:

Uncontrolled use of antibiotics has been attributed to be one of the main factors contributing to the development of antimicrobial resistance (AMR). The challenge of AMR is rapidly reversing the gains and the advances made in medical practice in past decades. The cause of AMR is multifactorial, and the control is of great public health importance. Previously susceptible organisms have developed multidrug resistance (MDR) to broad spectrum antibiotics thus limiting their clinical efficacy. AMR is associated with higher cost of treatment, increased morbidity and mortality (1–3).

Some intervention methods such as prospective audit with intervention and feedbacks have been found to be effective in reducing irrational use of antimicrobials by improving antimicrobial prescribing practices. Similarly, antimicrobial stewardship (AMS) programme combined with educational interventions have resulted in improved rational use of antimicrobials and resulted in lower rates of resistance, improved clinical outcome and reduced costs of care (4-7). However, these cannot be sustained without continuous surveillance on the pattern of antimicrobial use (AMU) and the resistance profiles of microorganisms.

Nigeria joined the global community

in monitoring antimicrobial prescribing practice using the Global-PPS in 2015. Since then, the number of hospitals participating in the G-PPS has increased, thus creating awareness and providing data to guide decision making. Some hospitals have established AMS programmes after they started monitoring the pattern of antimicrobial prescription and use in their facilities. Most of these surveys have been on single hospital (8–11) or two hospitals (12), and have reported varying frequencies of AMU in the individual hospitals. These individual hospital reports have stimulated the interest of more hospitals in establishing the baseline pattern of AMU and resistance. These findings were to guide the planning of interventions in the respective hospitals but there are still gaps on the pattern of AMU in the country.

In 2017, Nigeria joined the World Health Organization (WHO) Global Antimicrobial Resistance Surveillance System (GLASS), and commenced reporting to the global platform. Nigeria has also developed the national action plan on AMR (13), however, there is paucity of data to present a representative picture of AMU, AMR and trends in the country. In this study, we present the reports of antimicrobial prescribing trends and quality indicators in 2015, 2017 and 2018 from 13 hospitals across Nigeria. It is our belief that this information will help to bridge the data gap on AMU and AMR situations in the country.

Materials and method:

Study area

The study involved 13 (12 tertiary and 1 secondary level) participating hospitals across the geographical regions of Nigeria. The Nigeria health system is three-tiered involving the primary, secondary and tertiary levels of care. The tertiary level provides training and research in addition to advanced curative and rehabilitative services to the populace. Nigeria first joined the Global-PPS survey in 2015 with 4 participating hospitals, and the number increased to 13 in 2018. The hospitals were distributed across 5 of the 6 geopolitical regions, thus providing a good representative coverage of Nigeria (Fig 1).

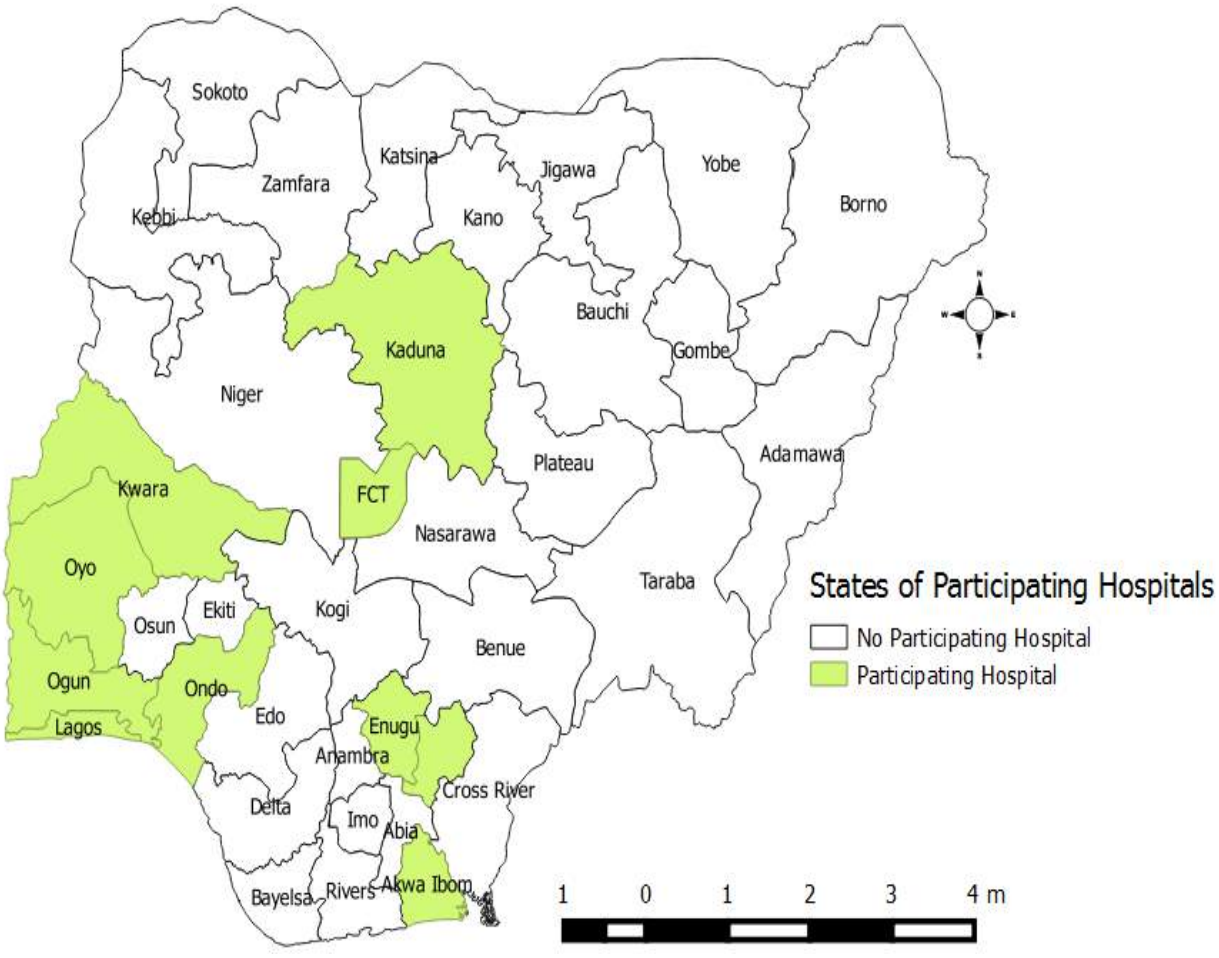

Fig 1: Map of Nigeria showing the states of participating hospitals

Study design, population and sampling

A longitudinal survey was conducted involving the total population of inpatients in each hospital at the time of the surveys. We included all inpatients admitted into any of the hospital wards before 08.00hrs on the day of the survey but surgical wards were not surveyed on Mondays and Fridays, and all patients admitted after 08.00hrs were excluded from the survey. All inpatients formed the denominator while all inpatients that had any antimicrobial treatment at the time of the survey formed the numerator.

Data extraction tool

We used pretested questionnaires (ward and patient forms) to extract information from the patients' folders. The ward form captured information on total number of inpatients, bed capacity of ward, category of ward (medical, surgical, or mixed), and date of the survey. The patient form captured information on the demographics of patients (gender, age, and weight), indication for treatment, antimicrobial agents (name, unit, dose, frequency and strength), prescribing quality indicators (stop/review date, adherence to guideline, route of administration, documented reason for antimicrobial use), type of antimicrobial biomarkers use and antimicrobial resistance type.

Data management and statistical analysis

The data were entered into the web-based data management system designed by University of Antwerp. The system has in-built data validation rules that ensure uniformity in data collection and other elements of data quality. After validation, the dataset was exported into Microsoft Excel and analyzed with EPI INFO version 7.2.

We estimated the mean weighted anti microbial prevalence rate for the participating hospitals over the period. The annual antimicrobial prevalence was calculated for each year to appreciate changes over the period. The proportion of the prescriptions that met the quality prescribing indicators (record of stop/review dates, adherence to guideline, targeted treatment and route of administration) was estimated and the trend of antimicrobial use in the various wards over the years was also described in a chart.

Ethical consideration

Ethical clearance for the conduct of the study was obtained from the various local research and ethics committees of the participating hospitals. There was no direct contact with patients, and no identifiers such as name, phone number and address were used. The hospital number was collected on the patient form but not on the online platform, the online platform automatically assigned a survey number to each participant.

Results:

Thirteen hospitals participated in the 2018 Global-PPS, of which 7 had participated at least twice since 2015 (4 in 2015, 10 in 2017 and 7 in 2018). There were 5,174 inpatients overall. The mean weighted antimicrobial prescribing prevalence over the period was 70.7%. This figure declined over the years from 71.7% in 2015 to 59.1% in 2018 ($p<0.001$). A high variation in prescribing practices among and within the hospitals was observed. While there were increased prescribing rates in some hospitals over the years, rates reduced in two hospitals that had initiated AMS programmes (Fig 2).

Generally, the antimicrobial prevalence rate was on the decline among the adult inpatients (medical and surgical inpatients) but not in paediatric and intensive care patients. However, the prevalence rate increased among haemato-oncology patients over the years (Fig 3). The main indications for therapeutic prescribing were skin and soft tissue infections (20.8%), sepsis (15.9%) and pneumonia (11.6%). Overall top three antibiotics for therapeutic use were ceftriaxone (18.2%), metronidazole (15.3%) and ciprofloxacin (10.4%)

With respect to quality of antimicrobial prescription, documentation of the stop/ review date showed some improvement over the three-year period, rising from 27.9% in 2015 to 48.5% in 2018, ($p<0.001$), however, it remained below 50% average. There was no change in documentation of switch from parenteral to oral route of administration through the three-year period (2015: 64.5%; 2017: 65.1%; 2018: 62.6%, $p=0.6803$) and slight change in documentation of reasons for antimicrobial prescription in notes at the start of the prescription (2015: 62.2%; 2017: 74.5%; 2018: 70.9%; $p=0.008$).

The use of laboratory services to guide antibiotic prescription was poor. The best observed targeted prescription was 12% in 2015 (Table 1) and declined over the three years period (2015: 12.0%; 2017: 2.9%; 2018: 5.2%; $p<0.001$). Metronidazole, ceftriaxone and ciprofloxacin were the most commonly used antibiotics among the inpatients in the participating hospitals (Table 2). Others were cefuroxime, amoxicillin with enzyme inhibitor, and levofloxacin (Table 2).

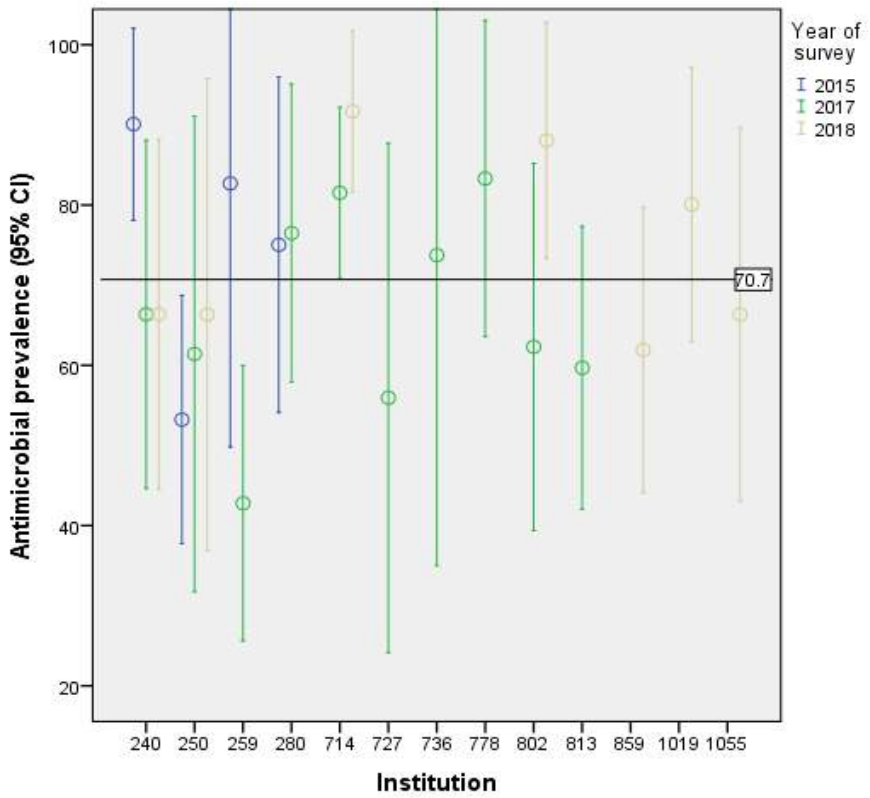

Fig 2: Variation of antimicrobial prevalence in participating Nigerian hospitals (2015, 2017 and/or 2018)

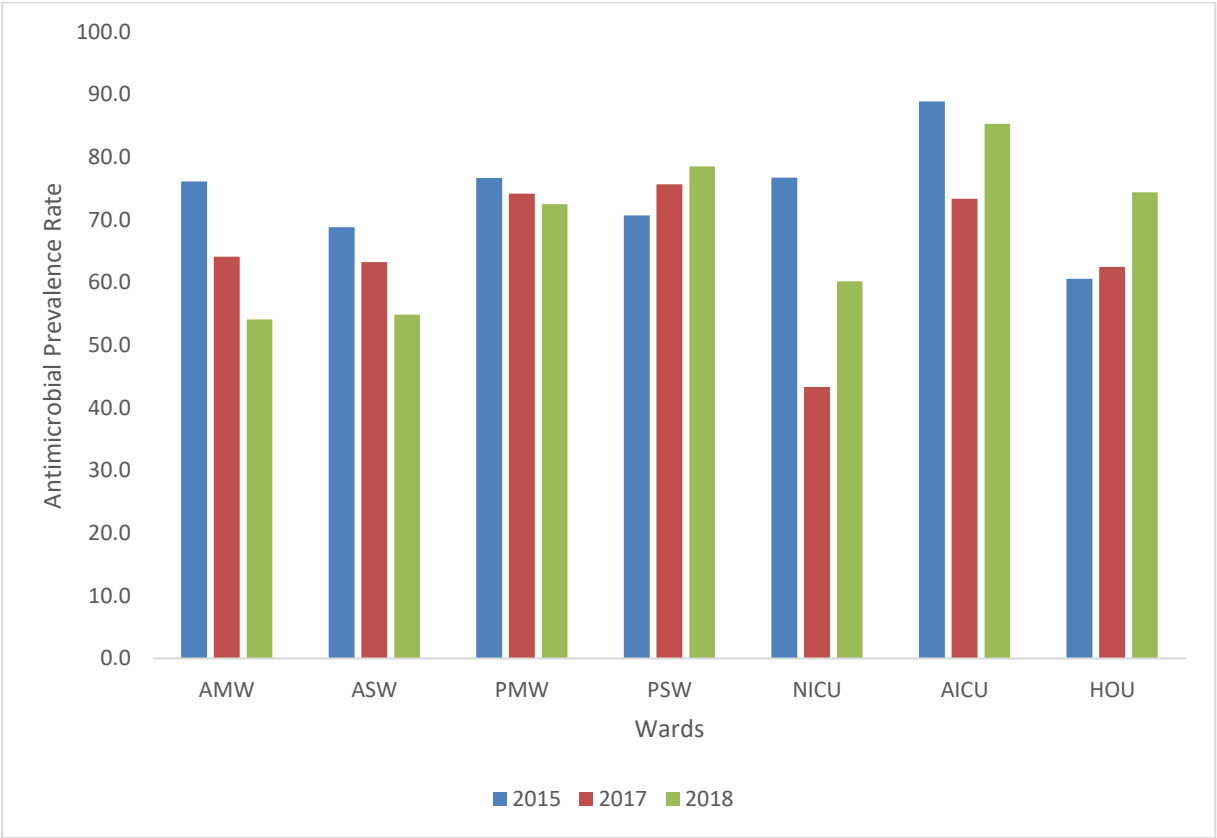

Fig 3: Antimicrobial prevalence rate in the different wards disaggregated by year of survey

AMW= adult medical ward, ASW=adult surgical ward, PMW= Paediatric Medical Ward, PSW = Paediatric Surgical Ward, NICU= Neonatal Intensive Care Unit, AICU = Adult Intensive Care unit, HOU = Haemato-Oncology Unit

Table 1: The antimicrobial prescription quality indicators in Nigerian hospitals 2015 – 2018

Variable	2015 (n=1085)		2017 (n=2500)		2018 (n=2612)	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Reason in notes						
Yes	675	62.2	1863	74.5	1851	70.9
No	410	37.8	637	25.5	761	29.1
Documented Stop/review date						
Yes	303	27.9	823	32.9	1266	48.5
No	783	72.1	1677	67.1	1344	51.5
Treatment						
Empirical	955	88.0	2427	97.1	2458	94.8
Targeted	130	12.0	73	2.9	136	5.2
Route of administration						
Oral	385	35.5	873	34.8	976	37.4
Parenteral	700	64.5	1627	65.1	1636	62.6
Guideline compliance						
Yes	128	11.8	152	6.1	341	13.0
No	179	16.5	170	6.8	104	4.0
NA/NI	778	71.7	2178	87.1	2167	83.0

NA/NI = Guidelines were either not available or could to accessible to make judgement to assess; n = no of inpatients

Table 2: Class of antimicrobials used in Nigerian hospitals (2015 – 2018)

Antimicrobial	AWaRe Class	2015 n (%)	2017 n (%)	2018 n (%)
Metronidazole	Access	241 (22.2)	583 (23.3)	579 (22.2)
Ceftriaxone	Watch	199 (18.3)	367 (14.7)	435 (16.7)
Ciprofloxacin	Watch	104 (9.6)	242 (9.7)	237 (9.1)
Cefuroxime	Watch	98 (9.0)	235 (9.4)	114 (4.4)
Amoxicillin and enzyme inhibitor	Access	31 (2.9)	155 (6.2)	118 (4.5)
Levofloxacin	Watch	41 (3.8)	95 (3.8)	124 (4.8)
Gentamicin	Access	35 (3.2)	131 (5.2)	104 (4.0)
Total		1085	2500	2612

n = no of inpatients; AWaRe = Access Watch and Reserve group of antibiotics according to the World Health Organization classification

Discussion:

Starting with four hospitals in 2015, we were able to enlarge and maintain a network, allowing sharing of experiences and knowledge to combat AMR issues in Nigeria. We found that the prevalence of antimicrobial use (AMU) declined especially among the hospitals that had commenced AMS programmes. These hospitals had formed AMS committees with the support of their hospital management. They had shared their findings and commenced sensitization of the physicians on the need to avoid irrational use of antimicrobial agents and the potential consequences of AMR in patients' management. In one of the hospitals, the development of departmental level antibiotic guideline had commenced with the technical support of the AMS committee.

The overall weighted antimicrobial prevalence rate remained high with wide variations existing within and among the participating hospitals. A closer look at the trend of the AMU by ward over the period showed that while the adult medical and surgical wards, and the paediatric medical ward had shown decline, this was the case for high dependency wards or intensive care units. The high prevalence of AMU reported among these Nigerian hospitals is similar to the high rates reported in other studies in Nigeria and other tropical countries (12,14, 15). This could be as a result of the generally low awareness on appropriate antibiotic use and AMR, high over-the-counter use of antibiotics, low level of implementation of AMS programmes such as awareness campaigns in the regions and lack of stringent guidelines and policy for AMU. The high level of infectious diseases as the major cause of hospitalization could also be contributory.

Community acquired infections (CAIs) were the most common indications for AMU over the period. This high prevalence of CAIs may not be unconnected with the high level of infectious diseases in the tropics due to poor personal hygiene, low environmental sanitation and poor housing. However, the low level of targeted treatment of these infections is a great concern. Most of the participating hospitals were tertiary level specialized hospitals with adequate laboratory support. One would have expected that CAIs could be promptly diagnosed on presentation and the treatment guided by the antimicrobial sensitivity patterns of prevalent microorganisms in the region. Alternatively, where the antibiogram of the community has been mapped out, a locally formulated anti-

microbial guideline could serve as guide for empirical treatment of some infections and syndromes common in those localities. Our findings however, revealed that AMU among Nigerian hospitals was neither based on antimicrobial guidelines nor laboratory diagnosis.

There was generally poor documentation in the patients' case note of the reasons for AMU and stop/review dates. Lack of documentation of reasons for AMU could encourage unnecessary use of these agents. Similarly, lack of documentation of the stop or review date of antimicrobial agents could lead to prolonged use of antimicrobials and increased risk of developing resistance. These practices varied widely across the participating hospitals, some had earlier reported improved documentation of reason for antimicrobial use (8,9). There is need for immediate intervention to enlighten the prescribers on the need to clearly indicate the reason for any antimicrobial use.

We observed a preference for broad spectrum antibiotics belonging to the Watch group of the WHO AWaRe classification such as the third generation cephalosporins (ceftriaxone) and fluoroquinolones. These antimicrobials have a higher risk of selecting for resistance and should therefore be monitored and prioritized in AMS programmes. There was also a preference for parenteral route of administration over oral route among the prescribers. Although, these prescribing practices may have some immediate advantage for the prescribers especially in absence of microbiological culture and sensitivity, they could expose these newer agents to high selection pressure for resistance. The prescribers preference for use of broad spectrum antimicrobials has been reported earlier (16).

This study to our knowledge, represents the largest collections of longitudinal data collection on AMU from multiple hospitals across regions in Nigeria over a three-year period. However, no hospital in the northeast region of the country participated in the survey, which implied that the unique antimicrobial prescribing practices in the region were not captured.

Conclusion:

We conclude that the level of AMU in Nigeria public hospitals remained high. There are poor documentations on reasons for the AMU and the stop/review date, as well as a suboptimal use of the available laboratory services. There is high prescribers' preference for broad-spectrum antibiotics and parenteral route of administration, and general lack of antibiotic guidelines among the hospitals. There is therefore urgent need for targeted interventions to reverse these trends. There is also the need to encourage the establishment of AMS programmes and the development of antibiotic guidelines in Nigerian hospitals.

Acknowledgements:

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Conflict of interest:

Authors declare no conflict of interest

Source of funding:

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Previous publication

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Original Article **Open Access**

Roll out of a successful antimicrobial stewardship programme in Lagos University Teaching Hospital Nigeria using the Global-Point Prevalence Survey

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Abstract:

Background: Antimicrobial resistance (AMR) has become a public health emergency with increasing rates and spread globally. Antimicrobial stewardship (AMS) has been advocated to reduce the burden of antimicrobial resistance, promote rational and appropriate use of antibiotics and improve clinical outcomes. Education and training are one of the AMS interventions to improve antimicrobial use. We present the roll out of a successful AMS programme with education and training using the Global-PPS as data collection tool to measure AMS interventions and impact.
Methodology: This was a cross sectional study on the implementation of an AMS programme at the Lagos University Teaching Hospital. Global PPS was conducted in 2015 to collect baseline data which was used to identify targets for quality improvement in AMS and was repeated in 2017 and 2018 to measure impact of AMS interventions. AMS interventions included education, feedback of Global-PPS result and writing of the hospital-wide antibiotic policy based on the baseline data.
Results: Out of the 746 inpatients surveyed, 476 (68.3%) had received at least one antimicrobial on the days of Global-PPS. The antimicrobial prescribing rates reduced significantly over the three time periods. In 2015, 82.5% were placed on antimicrobials, 65.5% in 2017 and 51.1% in 2018 (p<0.00001). The documentation of indication for treatment significantly improved from 53.4% in 2015 to 97.2% in 2018 (p<0.0001). Stop review date also significantly improved from 28.7% to 70.2% in 2018 (p<0.00001). Surgical prophylaxis for more than 24 hours reduced significantly from 93.3% in 2015 to 65.7% in 2018 (p=0.002) even though the prevalence was still high. The three most commonly administered antimicrobial groups were third generation cephalosporins, imidazole derivatives and quinolones. The most commonly prescribed antibiotics for surgical prophylaxis were ceftriaxone and metronidazole in 2015 and ceftriaxone in 2017.
Conclusion: The use of education and training as AMS intervention in a limited resource setting clearly made impact on antimicrobial prescribing patterns in the hospital. Global-PPS is useful to set quality improvement targets and for monitoring, evaluation and surveillance of an AMS programme.

Keywords: Antibiotic, Stewardship, Resistance, Education, Global-PPS

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Déploiement d'un programme réussi de gestion des antimicrobiens à l'hôpital universitaire de Lagos au Nigéria à l'aide de l'enquête sur la prévalence globale des points

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Abstrait:

Contexte: La résistance aux antimicrobiens (RAM) est devenue une urgence de santé publique avec des taux croissants et une propagation mondiale. La gestion des antimicrobiens (AMS) a été préconisée pour réduire le fardeau de la résistance aux antimicrobiens, promouvoir l'utilisation rationnelle et appropriée des antibiotiques et améliorer les résultats cliniques. L'éducation et la formation sont l'une des interventions AMS pour améliorer l'utilisation des antimicrobiens. Nous présentons le déploiement d'un programme AMS réussi avec éducation et formation en utilisant le Global-PPS comme outil de collecte de données pour mesurer les interventions et l'impact de l'AMS.
Méthodologie: Il s'agissait d'une étude transversale sur la mise en œuvre d'un programme AMS à l'hôpital universitaire de Lagos. Le PPS mondial a été mené en 2015 pour collecter des données de base qui ont été utilisées pour identifier les cibles d'amélioration de la qualité dans la MGS et ont été répétées en 2017 et 2018 pour mesurer l'impact des interventions de MGS. Les interventions d'AMS comprenaient l'éducation, le retour d'information sur les résultats de Global-PPS et la rédaction de la politique d'antibiotique à l'échelle de l'hôpital sur la base des données de base.
Résultats: Sur les 746 patients hospitalisés interrogés, 476 (68,3%) avaient reçu au moins un antimicrobien les jours de Global - PPS. Les taux de prescription d'antimicrobiens ont considérablement diminué au cours des trois périodes. En 2015, 82,5% étaient placés sous antimicrobiens, 65,5% en 2017 et 51,1% en 2018 (p<0,00001). La documentation de l'indication thérapeutique s'est significativement améliorée de 53,4% en 2015 à 97,2% en 2018 (p<0,0001). La date d'arrêt de l'examen s'est également considérablement améliorée, passant de 28,7% à 70,2% en 2018 (p<0,00001). La prophylaxie chirurgicale pendant plus de 24 heures a considérablement diminué, passant de 93,3% en 2015 à 65,7% en 2018 (p=0,002) même si la prévalence était encore élevée. Les trois groupes antimicrobiens les plus couramment administrés étaient les céphalosporines de troisième génération, les dérivés d'imidazole et les quinolones. Les antibiotiques les plus couramment prescrits pour la prophylaxie chirurgicale étaient la ceftriaxone et le métronidazole en 2015 et la ceftriaxone en 2017.
Conclusion: L'utilisation de l'éducation et de la formation comme intervention AMS dans un contexte de ressources limitées a clairement eu un impact sur les modèles de prescription d'antimicrobiens à l'hôpital. Global-Le PPS est utile pour fixer des objectifs d'amélioration de la qualité et pour le suivi, l'évaluation et la surveillance d'un programme AMS.

Mots clés: Antibiotique, intendance, résistance, éducation, mondial-PPS

Introduction:

Antimicrobial resistance (AMR) has become a public health emergency with increasing rates and spread globally. In Nigeria, the rate of carbapenem resistant *Klebsiella pneumoniae* increased from 5.2% in 2010 to 17.5% in 2015 (1,2). The increasing rate of AMR worldwide has led to increased length of hospital stay, antibiotic use, cost of hospitalization, morbidity and mortality. The drivers of AMR include irrational antimicrobial use, easy access to purchase of antibiotics over-the-counter, self-medication, poor compliance to prescribed antibiotics, lack of access to appropriate care and lack of adequate laboratory infrastructure for proper diagnosis (3). The global point prevalence survey (Global-PPS) of antimicrobial consumption and resistance showed high prevalence of antimicrobial use in teaching hospitals across Nigeria. In four teaching hospitals (in southwest, northcentral and northwest Nigeria), antimicrobial use was 76% (4), while in southeast Nigeria, it was 78.2% (5).

To combat this rise in AMR, the World Health Organization (WHO) advocates the adoption of antimicrobial stewardship (AMS) by health-care providers to check and reduce the burden of antibiotic resistance (6). This strategy involves the application of objective interventions to influence prescribing practices, thereby promoting rationale and appropriate antimicrobial use (6). Although AMS has been adopted and practiced in many high-income countries, there are challenges with the implementation of AMS in low-and-middle-income-countries (LMIC). In Africa, some hos-

pitals in South Africa, Kenya and Tanzania have implemented AMS programmes which has shown an overall reduction in antibiotic use (7), and reduction in surgical site infection for those who implemented interventions targeted at surgical antibiotic prophylaxis (8).

In Nigeria, there is a national action plan on AMR but no national AMS programme. A few healthcare facilities have instituted AMS interventions. The challenges identified in AMS in tertiary hospitals in Nigeria include lack of funding, poor awareness of the usefulness of AMS by staff, lack of information technology (IT) infrastructure and lack of leadership support (9). In two surveys of AMS among tertiary health care facilities in Nigeria, only 30-35% of hospitals have AMS committees in place (9,10). The success of AMS depends largely on support from the hospital leadership, however none of the hospitals in a survey of AMS in tertiary healthcare facilities had leadership support for AMS (10).

With respect to the AMS interventions commonly used in the country, a study found that 24% of hospitals had facility specific antibiotic treatment guide- lines (10) while another study found only 5% (9); 12% of hospitals use formulary restriction with pre-authorisation and 18% of hospitals use post prescription review and 5% used education and training (9,10). Monitoring and evaluation of AMS programmes can be done using the Global-PPS (11). In Nigeria, there are 19 hospitals conducting the Global-PPS and reporting data to the global network.

The aim of this study was to present the roll out of a successful AMS programme with education and training and use of antibiotic policy using the Global-PPS to design AMS interventions and measure impact at the Lagos University Teaching Hospital, Nigeria.

Materials and method:

Study location

The study was conducted at the Lagos University Teaching Hospital (LUTH), a tertiary care hospital with 761 beds and 10,600 admissions annually. It comprises of adult medical and surgical wards, adult intensive care unit, paediatric medical and surgical wards and neonatal wards.

Study design

This was a cross sectional study of audit of antimicrobial prescribing practices and resistance using the Global-PPS protocol (11). There was implementation of AMS programme and the Global-PPS conducted in 2015 was used to make a baseline assessment of antimicrobial prescribing practices to identify targets to improve quality of antimicrobial prescribing, design stewardship intervention to promote rational use of antimicrobials. The Global-PPS was repeated in 2017 and 2018 to monitor rates of antimicrobial prescribing in adults and children and measure the impact of the AMS interventions. Fig 1 outlines the steps for implementation of AMS programme at the Lagos University Teaching Hospital

Step 1: Formation of antimicrobial stewardship committee

In 2012, an AMS programme was set up in LUTH. A multidisciplinary AMS committee was formed and inaugurated by the Chief Medical Director with clear terms of reference. The committee consist of physicians, surgeons, paediatricians, gynaecologists, dentists, dental surgeons, epidemiologists, pharmacists, nurses, infection prevention and control specialists, clinical microbiologists, and staff from hospital administration. The committee has a physician leader who is a clinical microbiologist. A subcommittee was formed to develop a proposal to obtain baseline information on the burden of infection and antimicrobial use in the hospital but did not make any meaningful progress for three years due to lack of funds. The committee held meetings monthly. There was a 24-hour service clinical microbiology laboratory, infection prevention and control team and committee and department of pharmacy, with strong cooperation amongst members.

Step 2: Baseline data

In 2015, there was a call for Global-PPS which was open to all hospitals around the world. LUTH participated in the Global-PPS and obtained baseline data to start the AMS programme.

Step 3: Intervention (Education and feedback with use of antibiotic policy)

In 2016, a hospital grand round was held to present the result of the Global-PPS to the prescribers, pharmacists, nurses and other healthcare workers. This was an opportunity to educate the healthcare workers on rational antibiotic use and introduce them to the principles of antimicrobial stewardship. Education and awareness were done by the AMS team. More interventions were planned which included writing a hospital antibiotic policy and the requirement for each of the four major clinical departments (Medicine, Paediatrics, Surgery and Obstetrics and Gynaecology) to write their departmental antibiotic guidelines.

The AMS team visited each department to educate the prescribers and other healthcare workers on prudent antibiotic use, how to write guideline, and starting AMS. Each department was encouraged to write their guidelines which were to be ratified by the AMS committee and also to choose an AMS strategy. A hospital-wide antibiotic policy was formulated based on the hospital Global-PPS results which was distributed to all stakeholders (Appendix 1).

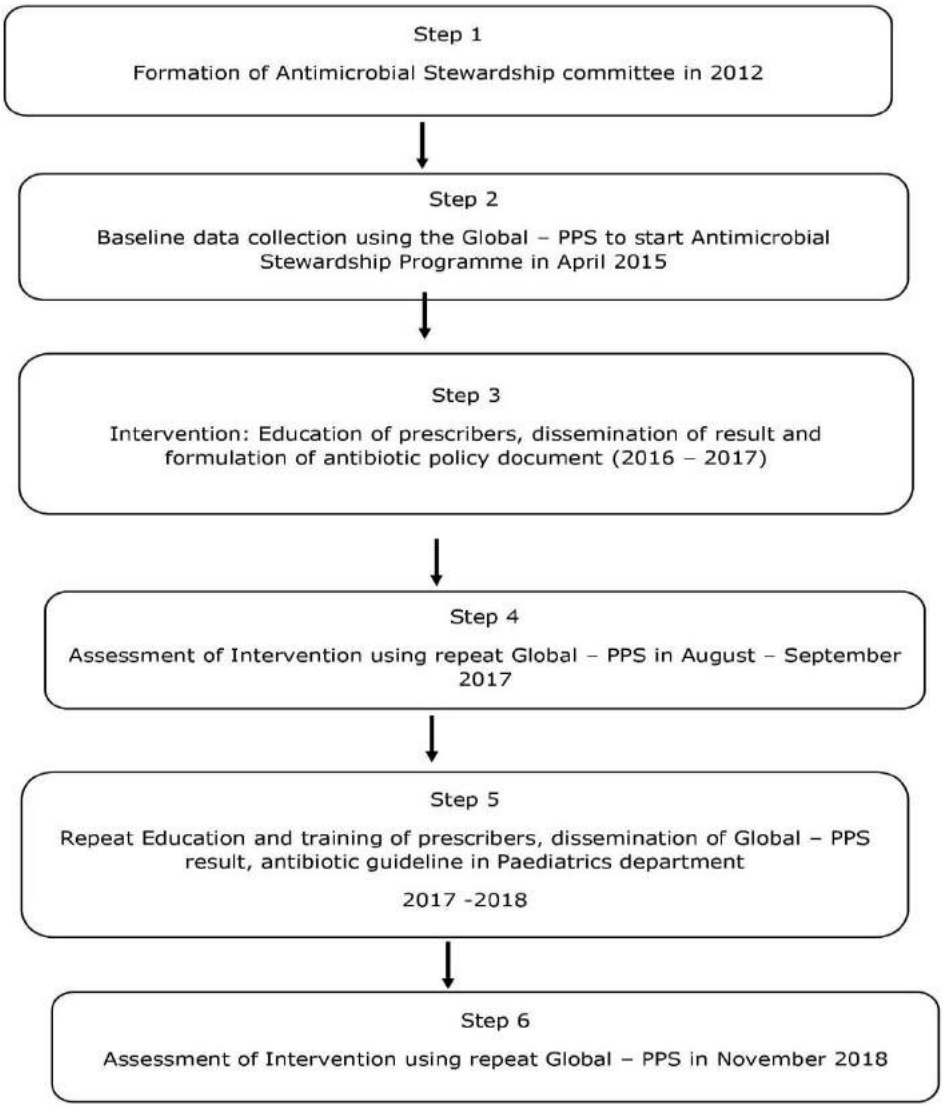


Fig 1: Steps for implementation of Antimicrobial Stewardship Programme at the Lagos University Teaching Hospital

Education involved presentation of the following topics at the hospital grand round; (i) Antimicrobial stewardship: choosing a strategy for LUTH; (ii) Feedback of the result of the Global-PPS in 2015; and (iii) Antibiotic policy/guideline and surveillance of multi-drug resistant organisms (MDRO). Education at the departments focused on; (i) the Global-PPS of antimicrobial consumption and resistance; (ii) results of antimicrobial prescribing in LUTH; (iii) antibiotic consumption in LUTH paediatric patients: trends in the last 3 years; (iv) antibiotic resistance pattern in LUTH in the last 2 years; and (v) management of infectious diseases: rational for antibiotic use.

Awareness was through celebration of the antibiotic awareness week in November each year. An antibiotic awareness week was celebrated in 2018, where some heads of departments were made antibiotic champions and took oath to protect the use of antibiotics in their sphere of influence.

Step 4: Assessment of effectiveness of intervention

In 2017, the second round of Global-PPS was called and LUTH participated in April 2017. The repeat PPS provided a means to assess the effectiveness of the intervention (education and awareness). The result of the 2017 Global-PPS was disseminated to each of the four clinical departments to give feedback on the monitoring of the antimicrobial prescribing pattern in the hospital. The department of paediatrics formed an antibiotic management team consisting of doctors, pharmacists and nurses. The antibiotic management team wrote an antibiotic guideline and chose prospective audit with feedback as their AMS strategy. The challenges noted were limited

staff to implement the strategy, healthcare workers resisting change, lack of information technology resources and lack of funds. The department of surgery has also formed an antibiotic management team but is still working on the development of their local antibiotic guidelines. In 2018, the third Global-PPS was done and the results were also disseminated to the prescribers. The department of Paediatrics has since started using prospective audit and feedback as an AMS strategy.

Data collection

Data was collected by resident doctors in clinical microbiology in 2015, 2017 and 2018 using the Global-PPS protocol. All wards in the hospital were included for all surveys which was conducted on a single day in order to calculate correctly the denominator (number of admitted patients). On the day of the survey, all inpatients admitted and stayed overnight on a ward at 8 o'clock in the morning on the day of survey were counted in the denominator. All in patients "on antimicrobial agents" at 8 o'clock in the morning on the day of survey were included in the numerator (i.e., a patient form is to be filled in for these patients only).

Exclusion criteria were; (i) day hospitalizations and outpatients. These were defined as ambulatory care patients, therefore, data from "day" surgery and "day" hospital units were excluded from the survey; and (ii) patients admitted after 8 o'clock on the day of the survey (even though these would be present by the time the survey is carried out). All patients/wards in the exclusion criteria were excluded from both the numerator and denominator data.

Data were collected using two forms, the ward form to collect denominator data and the patient form to collect numerator data. Data collected on the ward form included the date of survey, name of ward, ward type, ward activity, total number of admitted patients at 8am, total number of beds. Data collected on the patient form included hospital number, age, weight, ward activity, ward name, gender, treatment based on biomarker, whether culture was sent to the clinical microbiology laboratory.

The antimicrobial data included name of antimicrobial, dose, frequency, and route of administration. The others were diagnosis, reason in notes, stop/review date, type of indication, compliance to guidelines and information about empirical or targeted therapy. If therapy was based on microbiology data, then data was collected on targeted multidrug resistant organisms. The antimicrobials classification system used was the World Health Organization's (WHO) Anatomic Therapeutic Chemical (ATC) classification (www.whocc.no/atc_ddd_index). Antibiotics were further classified as 'Access', 'Watch', 'Reserve' or 'Not recommended' using the 2019 WHO AWaRe classification list (12). Antibiotics not listed in the AWaRe classification were listed as 'Unclassified'.

Data analysis

A web-based application was used for data-entry, validation and reporting as designed by the University of Antwerp (https://www.global-pps.com) (11) and exported as Microsoft Excel file. The prevalence of antibiotic prescription was calculated by dividing the number of patients treated with an antibiotic over the total number of inpatients surveyed. The prevalence of healthcare associated infection (HAI) was calculated by dividing the number of treated patients on at least one HAI over the number of inpatients surveyed. Data was analysed using the SPSS version 20 software. Data were presented as percentages or proportions. The Chi square test for trend was used to assess differences in the prevalence of antibiotic prescription and differences in quality indicators over the three PPS. The level of statistical significance was set at $p<0.05$.

Results:

The total number of eligible inpatients surveyed in the three time periods (2015, 2017 and 2018) was 746 of which 573 were admitted on adult wards and 173 on paediatric or neonatal wards. Table 1 provides the general patient characteristics, antimicrobial use and HAI prevalence for the three time periods.

The antimicrobial prescribing rates reduced significantly over the three time periods (82.5% in 2015, 65.5% in 2017 and 51.1% in 2018 ($p<0.00001$). Fig 2 provides the antimicrobial prescribing rates for adults and paediatric/neonatal wards which also reduced significantly over time ($p<0.00001$). Overall, the proportion of patients treated with combination of (multiple) antimicrobials was 65.6% in 2015, 55% in 2017 and 69.9% in 2018, with a combination of two antimicrobials accounting for 42.4%, 35.5% and 58.3% respectively in 2015, 2017 and 2018 and three or more antimicrobial combination accounting for 23.2%, 19.5% and 11.6% in 2015, 2017 and 2018 respectively. A total of 19 (10.4%), 36 (14%) and 20 (6.6%) patients in 2015, 2017 and 2018 respectively were treated with at least one antimicrobial for HAIs. The prevalence of HAIs was higher among children and neonates (Table 1).

Table 1: General characteristics of patients, antimicrobial use and prevalence of healthcare associated infection

Characteristic	**Year (%)**		
	2015	**2017**	**2018**
Number of inpatients	183	258	305
Adults	144 (78.7)	209 (81.0)	220 (72.1)
Children and neonates	39 (21.3)	49 (19)	85 (27.9)
Patients treated with at least one antimicrobial	151(82.5)	169(65.5)	156(51.1)
Gender*			
Male	85 (56.3)	87 (51.5)	87 (55.8)
Female	66 (43.7)	82 (48.5)	69 (44.2)
Adults treated with at least one HAI	14 (9.7)	27 (12.9)	5 (2.3)
Children and neonates treated with at least one HAI	5 (12.8)	9 (18.4)	15 (17.6)

*Defined for patients treated with at least one antimicrobial

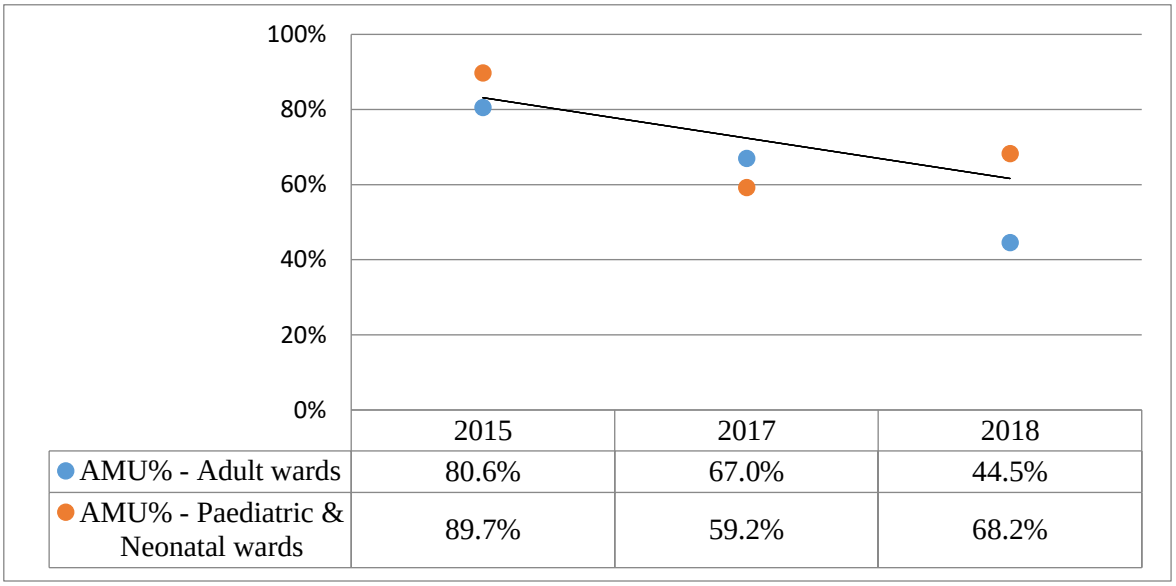


Fig 2: Antimicrobial use prevalence in adult and Paediatric/Neonatal wards (2015 – 2018)
AMU – Antimicrobial use

A total number of 895 antimicrobials were administered for the three time periods, 307 in 2015, 303 in 2017 and 285 in 2018. Parenteral use accounted for 70% in 2015, 76.9% in 2017 and 76.8% in 2018. Table 2 provides the indication for antimicrobial prescribing over time. Most of the antimicrobials were administered for community acquired infections. Antimicrobial prescribing for HAI was highest in 2017 (20.4%). Antimicrobial prescription for surgical prophylaxis significantly increased steadily. For medical prophylaxis, antimicrobial prescription was highest in 2015 (16%).

Table 3 shows the antimicrobial agents prescribed during the three time periods of Global-PPS. Antibacterials for systemic use accounted for most of the antimicrobial prescriptions. The three most commonly administered antimicrobials during the three time periods were third generation cephalosporins, imidazole derivatives and fluoroquinolones. The prescription of penicillins with extended spectrum, antimycobacterials and nitroimidazole derivatives (oral metronidazole) decreased over time, while the prescription of antimalarials and antivirals for systemic use increased over time.

The most commonly prescribed antibiotic for surgical prophylaxis were ceftriaxone and metronidazole in 2015 and 2018 (both rep resented 31.7% and 35.8% of prescriptions respectively) and ceftriaxone alone in 2017 (33.7%) (Fig 3). The ten most common diagnoses for which therapeutic antimicrobials were given are shown in Table 4. The most common diagnosis was skin and soft tissue infections. The proportion of antimicrobial prescription for sepsis and tuberculosis decreased over time while that of pneumonia and bone and joint infections increased over time from 2015 to 2018 (Table 4).

Table 2: Indications for antimicrobial use at three time periods (2015, 2017 and 2018)

Indication	**Year (%)**		
	2015 (n=307 AMP)	**2017 (n=303 AMP)**	**2018 (n=285 AMP)**
Community acquired infection	141 (45.9)	116 (38.3)	150 (52.6)
Health-care associated infection	30 (9.8)	62 (20.4)	33 (11.7)
Medical prophylaxis	49 (16)	24 (7.9)	28 (9.8)
Surgical prophylaxis	60 (19.5)	61 (20.1)	67 (23.5)
Other	0	7 (2.3)	7 (2.5)
Unknown	27 (8.8)	33 (10.9)	0

AMP = Antimicrobial prescriptions

Table 3: Antimicrobial agents prescribed during the three time periods (2015, 2017 2018)

ATC	**Antimicrobial**	**Year (%)**		
		2015(n=307)	**2017 (n=303)**	**2018 (n=285)**
J01	Antibacterials for systemic use	262 (85.3)	276 (91.1)	254 (89.1)
J01AA	Tetracycline	0	1 (0.3)	0
J01CA	Penicillins with extended spectrum	14 (4.6)	10 (3.3)	0
J01CE	Beta–lactamase sensitive penicillins	1 (0.3)	1 (0.3)	0
J01CF	Beta–lactamase resistant penicillins	2 (0.7)	2 (0.7)	2 (0.7)
J01CR	Combination of penicillins including beta–lactamase inhibitors	10 (3.3)	21 (6.9)	19 (6.7)
J01DC	Second generation cephalosporins	16 (5.2)	17 (5.6)	14 (4.9)
J01DD	Third generation cephalosporins	86 (28)	75 (24.8)	81 (28.4)
J01DE	Fourth generation cephalosporins	0	3 (1.0)	1 (0.4)
J01DH	Carbapenems	11 (3.6)	10 (3.3)	11 (3.9)
J01EE	Combination of sulphonamides and trimethoprim	3 (1.0)	1 (0.3)	1 (0.4)
J01FA	Macrolides	2 (0.7)	4 (1.3)	2 (0.7)
J01FF	Lincosamides	5 (1.6)	6 (2)	3 (1.1)
J01GB	Other aminoglycosides	25 (8.1)	13 (4.3)	32 (11.2)
J01MA	Fluoroquinolones	38 (12.4)	45 (14.9)	31 (10.9)
J01RA	Combination of antibacterials	2 (0.7)	0	1 (0.4)
J01XA	Glycopeptide antibacterials	6 (2.0)	2 (0.7)	3 (1.1)
J01XD	Imidazole derivatives	40 (13)	64 (21.1)	52 (18.2)
J01XE	Nitrofuran derivatives	1 (0.3)	1 (0.3)	1 (0.4)
J02	Antimycotics	7 (2.3)	8 (2.6)	3 (1.1)
J04	Antimycobacterials	16 (5.2)	8 (2.6)	4 (1.4)
J05	Antivirals	0	0	6 (2.1)
P01B	Antimalarial	7 (2.3)	0	9 (3.2)
P01AB	Nitroimidazole derivatives	15 (4.9)	11 (3.6)	9 (3.2)

ATC = Anatomical Therapeutic Chemical

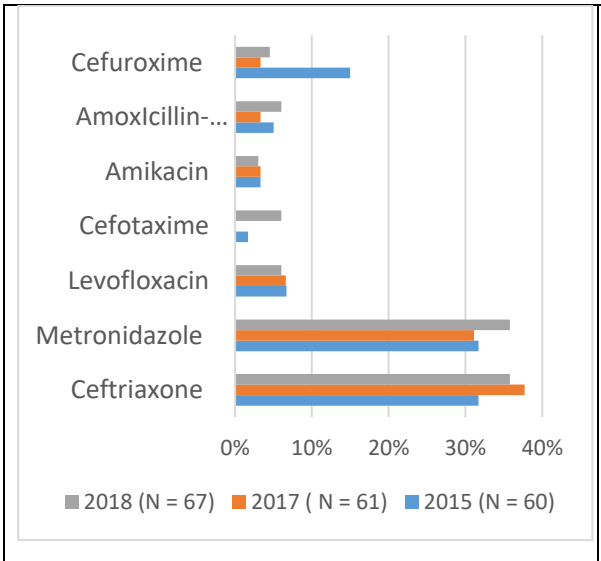


Fig 3a: Most frequently prescribed antibiotics for surgical prophylaxis

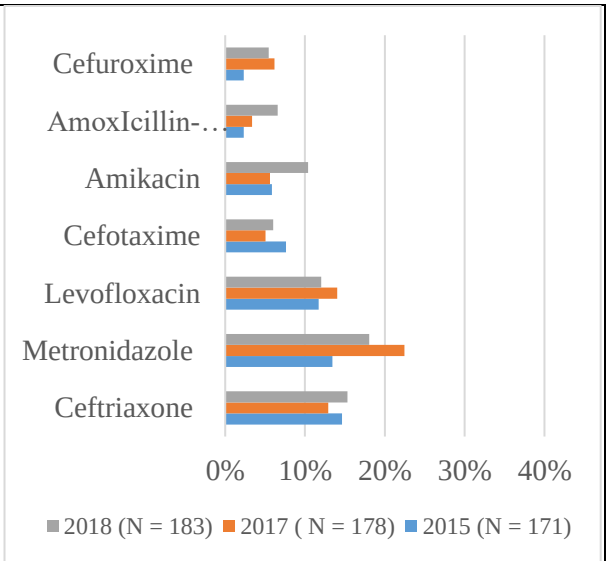


Figure 3b: Most frequently prescribed antibiotics for therapeutic use (community acquired infections and healthcare associated infections)

There was a general trend in favour of improvement in the quality indicators both for adult and paediatric/neonatal wards. The documentation of the reason for antimicrobial prescription (from 53.4% in 2015 to 97.2% in 2018) and stop-review date (from 28.7% to 70.2% in 2018) both significantly improved over time (p<0.0001) (Figs 4a & 4b). Prolonged surgical prophylaxis (for more than 24 hours) reduced significantly over time (from 93.3% in 2015 to 65.7% in 2018, p=0.002) mainly in the paediatric wards (Fig 4c). In the vast majority of cases, anti- microbials were administered empirically. Targeted therapy increased from 11% in 2015 to 13.1% in 2018 and the increase was mainly among paediatric and neonatal wards (p=0.0003) (Fig 4d). The use of procalcitonin as a biomarker was only observed in 2018 on the paediatric/neonatal wards and was used to guide treatment in only 2.6% of inpatients prescribed at least one antimicrobial. There was no antibiotic guideline available in 2015 and 2017. An antibiotic guideline was available in the department of Paediatrics in 2018. The overall Access/Watch ratio increased from 0.62 in 2015, 0.72 in 2017 to 0.77 in 2018. Fig 4e provides the proportion of total antibiotic use according to the AWaRe classification. There was no antibiotic prescribed belonging to the reserve list. Only a few 'not recommended' (n=6) and one 'unclassified' antibiotic were prescribed for the three survey periods.

Table 4: Ten most common diagnoses treated with at least one therapeutic antimicrobial

Diagnosis	**Year (%)**		
	2015 (n=84)	**2017 (n=96)**	**2018 (n= 100)**
SST	18 (21.4)	23 (24)	19 (19.0)
Intraabdominal	6 (7.1)	17 (17.7)	12 (12.0)
Sepsis	16 (9.8)	16 (16.7)	14 (14.0)
BJ	5 (19.0)	11 (11.5)	14 (14.0)
Pneu	6 (7.1)	10 (10.4)	9 (9.0)
CNS	7 (8.3)	5 (5.2)	13 (13.0)
Upper UTI	6 (7.1)	3 (3.1)	3 (3.0)
Cystitis	0	3 (3.1)	2 (2.0)
OBGY	0	3 (3.1)	0
TB	4 (4.8)	3 (3.1)	2 (2.0)

CNS=Infection of central nervous system; Pneu=Pneumonia or lower respiratory tract infection; TB=tuberculosis; SST= Skin & Soft Tissue: Cellulitis, wound including surgical site infection, deep soft tissue not involving bone e.g., infected pressure or diabetic ulcer, abscess; BJ= Bone joint Infection: Septic arthritis (including prosthetic joint), osteomyelitis; Cys=lower urinary tract infection; Upper UTI=upper urinary tract infection including catheter related urinary tract infection, pyelonephritis;; OBGY=obstetric/gynaecological infections; Intra-abdominal= Intraabdominal sepsis including hepatobiliary, intra-abdominal abscess etc; Sepsis= sepsis, sepsis syndrome or septic shock with no clear anatomic site;

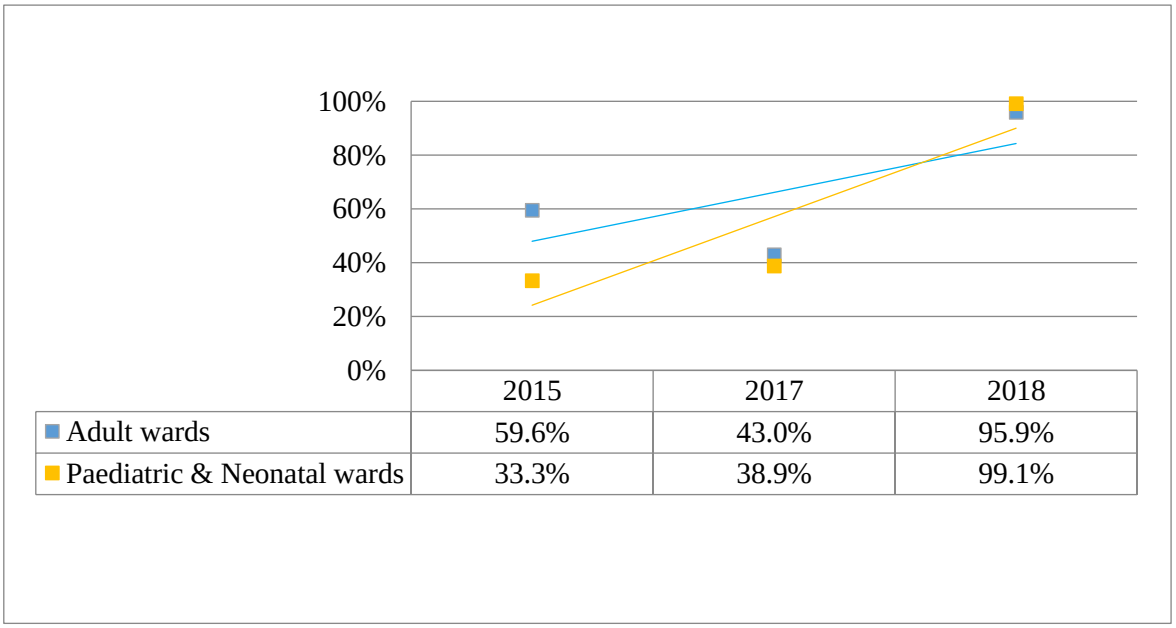
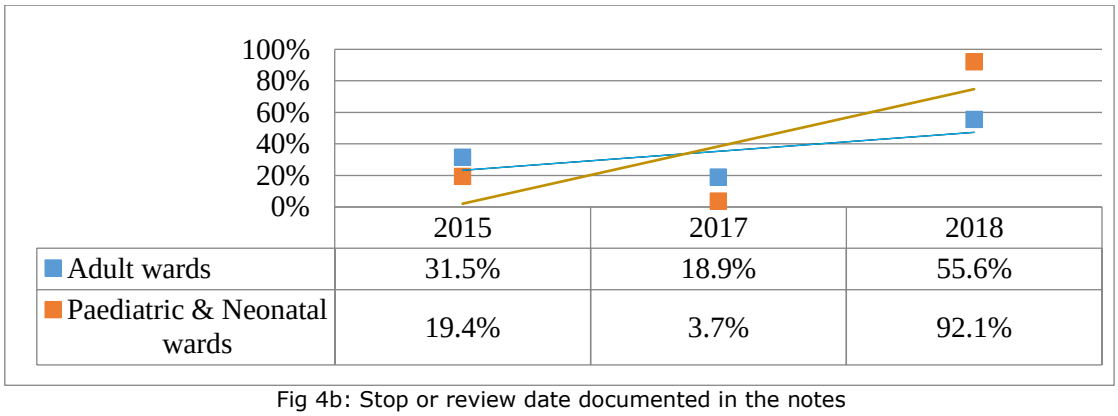
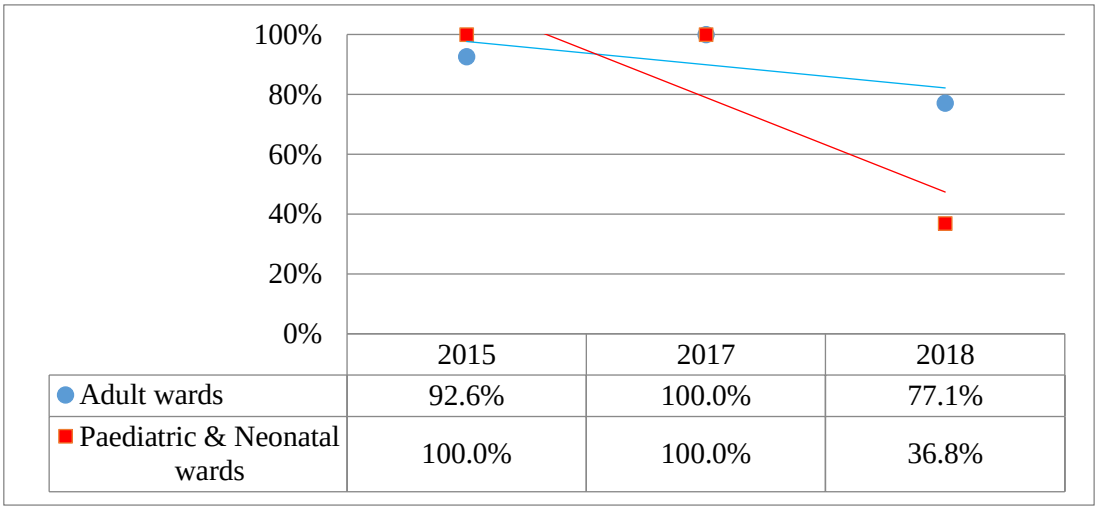


Fig 4a: Indication or reason documented in the notes



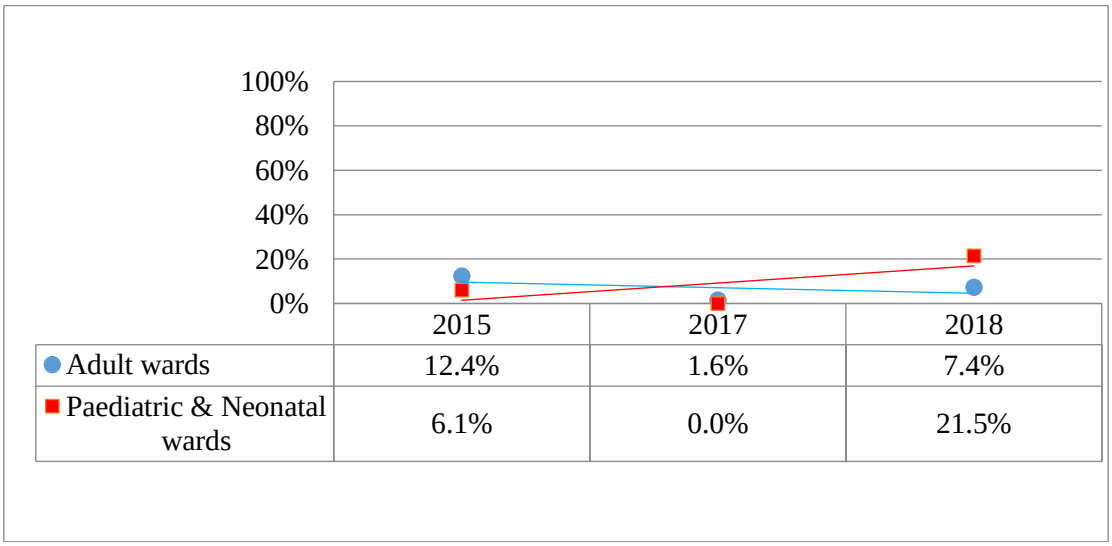
	2015	2017	2018
Adult wards	31.5%	18.9%	55.6%
Paediatric & Neonatal wards	19.4%	3.7%	92.1%

Fig 4b: Stop or review date documented in the notes



	2015	2017	2018
Adult wards	92.6%	100.0%	77.1%
Paediatric & Neonatal wards	100.0%	100.0%	36.8%

Fig 4c: Prolonged surgical prophylaxis (>24hrs)



	2015	2017	2018
Adult wards	12.4%	1.6%	7.4%
Paediatric & Neonatal wards	6.1%	0.0%	21.5%

Fig 4d: Targeted prescribing of antibiotics (J01 (prophylactic prescribing is excluded)) following a microbiological result for Adult and Paediatric/Neonatal wards.

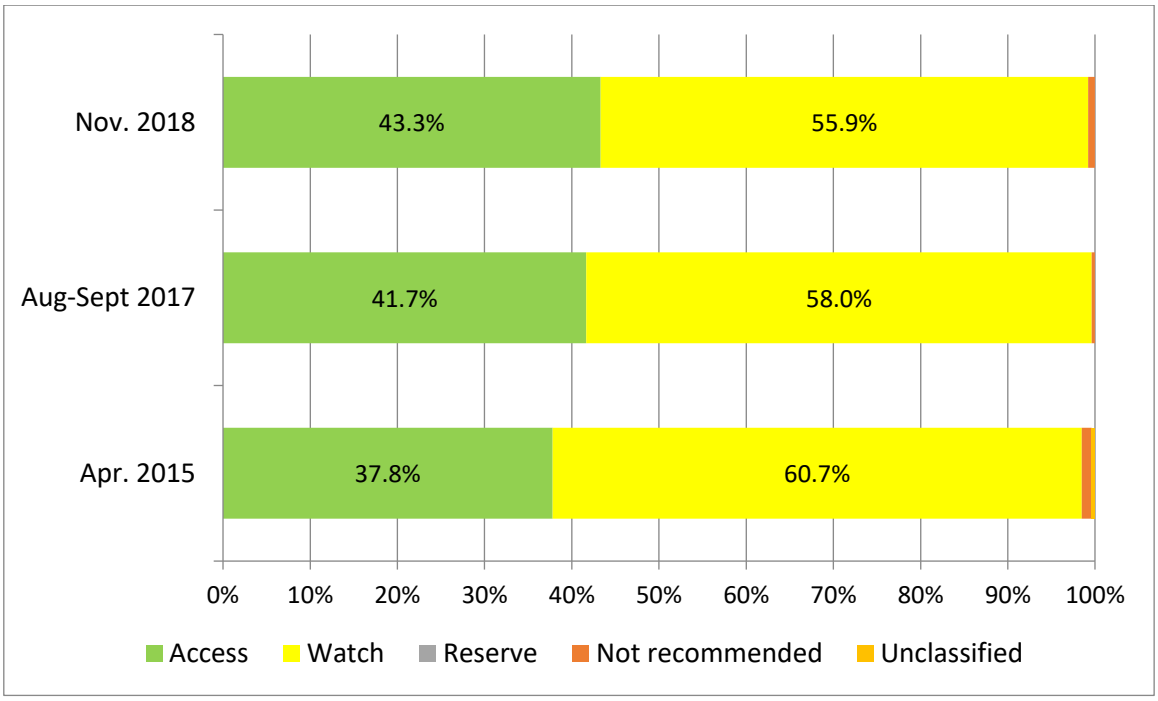


Fig 4e: Proportion of total antibiotic (ATC J01) use according to the AWaRe classification

Discussion:

We successfully rolled out AMS interventions and evaluated their effectiveness on antimicrobial prescribing patterns and quality indicators using the Global-PPS. The Global-PPS enabled us to collect data at three time periods (2015, 2017 and 2018) and served as an inexpensive and convenient monitoring and evaluation system rather than a robust, expensive system that requires substantial financial, human and IT resources. The AMS interventions included dedicated education and training, use of hospital antibiotic policy with feedback of result of Global-PPS to all clinical departments in the hospital.

There was a significant overall improvement in the prevalence of antimicrobial use with a reduction from baseline of 82.5% in 2015 to 51.1% in 2018 based on the implemented AMS interventions using education and training of prescribers and other healthcare workers. A study showed reduction in antibiotic use as an impact of education on AMS programme (13). The rate of antimicrobial use was very high at baseline comparable to most other teaching hospitals that have been surveyed in the Nigeria (4,5) and hospitals in Pakistan (14). The rate of 51.1% in 2018 is higher than 37.7% in South African hospitals (15), and 37.2% in Northern Ireland hospitals (16) and similar to the 50% among African hospitals taking part in the Global-PPS (17) and 52.2% in Brazil (18). The reason for the high rate of antimicrobial use may be due to non-availability of antibiotic treatment guidelines, reliance on empiric therapy, poor utilisation of the medical microbiology laboratory for the diagnosis of infections and in general, inappropriate antibiotic use.

We observed a reduction in the HAI rates from 10.4% in 2015 to 6.6% in 2018. The reduction was more significant in the adult wards. These HAI rates are in line with the main HAI prevalence found in Europe (17). Strikingly however, we observed much higher HAI prevalence in the children and neonatal wards with up to 18% of children/neonates been treated for at least one HAI in 2017 and 2018. These high rates in 2017 were mainly related to post-operative surgical site infections and in 2018, more children with an infection from another hospital were admitted (results not presented). More research needs to be done to further decrease these high HAI rates along with sustained infection prevention and control measures.

Most of the patients were placed on multiple antimicrobial therapy (55%-69.9%) while there was a significant decrease in the proportion of those placed on 3 or more antimicrobials from 23.2% in 2015 to 11.6% in 2018. Use of combination therapy was also high in studies from other parts of Nigeria (4,5), and lower in Pakistan (50%) (14) and Brazil (43.2%) (18). High rates of multiple antimicrobial therapy may lead to increased antimicrobial resistance and adverse drug events. Most of the antimicrobials were used for therapeutic purpose (56.7%-67.7%) similar to what has also been reported by other studies in Nigeria (4,5) and 66.9% among Africans taking part in Global-PPS (17). The proportion of antimicrobials used for

medical prophylaxis decreased over the time period from 16% in 2015 to 9.8% in 2018. This may have contributed to the reduction in overall antibiotic use in the hospital over the period. This is similar to reduction of proportion of medical prophylaxis over a 3-year period in Russian hospitals (19).

The top three prescribed antibiotics over the period were third generation cephalosporins mainly ceftriaxone (24.8% - 28.4%), followed by metronidazole and then the fluoroquinolones. This is similar to studies in Nigeria (4,5) Kenya, Brazil, Pakistan and Russia where ceftriaxone was the most frequently prescribed antibiotics (14,18-20). The increased use of third generation cephalosporins may be responsible for the high rate of endemic extended spectrum beta lactamase (ESBL) producing Enterobacteriaceae seen in different parts of Nigeria and Africa. Metronidazole is also found to be second most commonly used in other parts of Nigeria (4,5) and Ghana (21). It is important to note that the high use of metronidazole may be due to inappropriate use as most times it may not be for treatment of anaerobic bacterial infections and as part of redundant combination antibiotic coverage.

Ceftriaxone and metronidazole were the two most common antibiotic use for surgical prophylaxis and both are used in combination in about 90% of the time in the study. Ceftriaxone was the most commonly prescribed antibiotic for surgical prophylaxis in Eastern Europe, Southern Europe and Africa in the Global-PPS in 53 countries (17) and in Pakistan hospitals (14). It is very interesting to note that the high use of ceftriaxone and metronidazole is also driven by surgical prophylaxis; 32.7% – 44.4% of the total number ceftriaxone used and 25.3%-39.3% of the total metronidazole used were for surgical prophylaxis over the 3 time periods.

To our satisfaction however, we observed a decrease in prescription of antibiotics belonging to the Watch group (from 60.7% in 2015 to 55.9% in 2018) in favour of the Access group; a direct result of the educational training sessions to various prescribers. Strict follow up monitoring however will be needed if we want to continue to increase the proportion of the Access group. The Global-PPS allows for the analysis of additional quality indicators of antimicrobial use which can be set as targets for quality improvement in AMS programmes (11). The educational training sessions and use of antibiotic policy resulted in a significant improvement in most of the quality indicators of antimicrobial use. The documentation of the indication for antimicrobial prescription in notes improved significantly from 53.4% in 2015 to 97.2% in 2018 and this helps to ensure the communication of diagnosis and treatment among clinicians and other healthcare workers (17). The documentation of stop/review date also improved significantly with an increase from 28.7% in 2015 to 70.2% in 2018. Documenting the stop/review date helps the clinician to review continued use of antibiotics (with opportunities to deescalate, escalate, change or stop antibiotic use), reduce unnecessary prolonged duration of antibiotic use and adverse drug effects.

Furthermore, there was a significant reduction in surgical prophylaxis > 24 hours from 93.3% in 2015 to 65.7% in 2018. It is important to appreciate that though there was significant improvement, 65.6% is still high and targeting surgical prophylaxis as low hanging fruit in AMS will help to further reduce this figure. The proportion of 65.6% for prolonged prophylaxis is higher than rates in Europe (16,23). Evidence shows that surgical prophylaxis > 24 hours has no benefit in reducing surgical site infection (SSI) compared to single dose of antibiotic prophylaxis (24,25). Prolonged prophylaxis increases the likelihood of antimicrobial resistance and adverse drug effects. Prolonged surgical prophylaxis is often given out of fear of the implications of poor infection prevention and control practices in the operating room and surgical wards of hospitals. Therefore, AMS programmes should be strengthened with a strong infection prevention and control programme.

The use of parenteral antimicrobial therapy was very high (>70%) in all time periods and no significant change was noticed. This is similar to the result from studies in Nigeria (4,5), East and South Asia, North America and Africa from the Global-PPS study (17). An AMS intervention using intravenous (IV) to oral antibiotic switch may be a low hanging fruit to consider in our hospital to reduce the high rate of parenteral therapy. The IV to oral switch can help to reduce length of hospital stay, cost of healthcare, staff workload and risk of catheter associated infections (26-28).

Targeted antimicrobial therapy was very low in this study even though there was an improvement from 11% in 2015 to 13.1% in 2018. The low rate is similar to the 14.6% in Africa, Northern Europe and West and Central Asia from Global-PPS data (17). This means that clinical specimens for diagnosis of infections were not collected before antimicrobial therapy. A recent study showed that there is poor utilisation of medical microbiology laboratory by clinicians for the diagnosis of infections in Nigeria with only 15.9% of them always using the laboratory for diagnosis when treating suspected infections and infectious diseases (29). The reasons for the suboptimal use were perceptions that clinical diagnosis is sufficient, delay in getting the laboratory report and poor access to the

microbiology laboratory (29). The implication of a lack of targeted therapy is prolonged duration of empiric broad spectrum antibiotics with no opportunity to deescalate based on culture and antibiotic susceptibility test result leading to increased antimicrobial resistance and killing of the normal flora.

There was limited information on multidrug resistant organisms (MDRO) in the study because of the poor use of the microbiology laboratory. There was also very low use of biomarkers like procalcitonin and C-reactive protein though procalcitonin was observed in 2018 on the paediatric/neonatal wards and was used to guide treatment in only 2.6% of inpatients prescribed at least one antimicrobial. There was no antibiotic guideline for adult inpatients throughout the study period, however, an antibiotic guideline was available for children during the 2018 Global-PPS. Use of local antibiotic guidelines for empiric antibiotic therapy help to improve clinical outcomes like mortality, length of hospitalization, duration of treatment (30,31).

The limitations are inherent to a cross sectional survey showing a snapshot of antimicrobial prescription. There was no information on the duration of therapy, whether the antimicrobial prescription was justified, correction for comorbidity and other patient characteristics that may act as confounders.

Conclusion:

The study shows the usefulness and advantages of using the Global-PPS to set quality improvement targets and for monitoring, evaluation and surveillance of an AMS programme. The use of education and training and development of an antibiotic policy as AMS interventions in a limited resource setting clearly made an impact on antimicrobial prescribing patterns in terms of quantity and quality of antibiotic prescription in the hospital.

Further efforts are needed to increase the utilisation of the medical microbiology laboratory in order to increase targeted antimicrobial therapy. Identified low hanging fruit in AMS include IV to oral switch of antimicrobials, surgical prophylaxis along with improved infection prevention and control measures. With the successful roll out of the AMS programme, there will be need to consolidate the progress by identifying and implementing a core AMS strategy and other supplemental strategy by engaging the hospital leadership for support in terms of financial, human and information technology resources.

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Oral presentation:

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Original Article **Open Access**

Empirical antibiotherapy as a potential driver of antibiotic resistance: observations from a point prevalence survey of antibiotic consumption and resistance in Gombe, Nigeria

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Abstract:

Background: Empirical use of antibiotics is a standard practice in the treatment of infections worldwide. However, its over utilization without subsequent culture and antibiotic susceptibility testing could be a major driver of resistance. Over reliance on empirical antibiotherapy is common in most developing countries where antibiotic policies and availability or utilization of clinical microbiology laboratory are suboptimal. A standardized approach to point prevalence survey (PPS) on antimicrobial use (AMU) in hospitals was employed to assess the antimicrobial prescribing practices in Federal Teaching Hospital Gombe (FTHG), Nigeria.
Methodology: A PPS was conducted in April 2019 at FTHG by recruiting all in-patients present in the hospital on the day of survey. Data obtained from patients' records included details of the type and indication for antibiotherapy. A customized online application developed by the University of Antwerp (www.global-pps.be) was used for data-entry, validation, analysis and reporting.
Results: Of the total 326 patients who were on admission on the day of survey, 70.6% and 73.4% were on at least one antibiotic in adult and paediatric wards respectively. Most commonly used antibiotics include beta lactams such as cephalosporins (29.2%) and penicillins (22.8%), fluoroquinolones (12.4%), aminoglycosides (9.1%) and macrolides (3.4%). Among patients on antibiotics, route of administration was mainly parenteral (71.6%) while 44.8% were on more than one antibiotic. Overall, 91.3% of the antibiotic treatments were empirical with adults, children and neonates accounting for 96.4%, 77.6% and 100.0% respectively. Empirical antibiotic use is also high in medical wards (86.3%), surgical wards (89.9%) and intensive care unit (100.0%).
Conclusion: There is predominance and over-reliance on empirical antibiotherapy in our hospital. It further exposes the poor utilization of clinical microbiology laboratory and the potential for development of antibiotic resistance with resultant increase in morbidity/mortality and poor patient safety. There is need for further studies to highlight the dangers of over-reliance on empirical antibiotherapy and herald improvement in development and implementation of antibiotic stewardship programme.

Keywords: Empirical antibiotherapy, antimicrobial resistance, point prevalence survey, antimicrobial stewardship

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L'antibiothérapie empirique comme moteur potentiel de la résistance aux antibiotiques: observations d'une enquête ponctuelle de prévalence de la consommation et de la résistance aux antibiotiques à Gombe, au Nigéria

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Abstrait:

Contexte: L'utilisation empirique d'antibiotiques est une pratique courante dans le traitement des infections dans le monde entier. Cependant, sa surutilisation sans culture ultérieure ni test de sensibilité aux antibiotiques pourrait être un facteur majeur de résistance. Le recours excessif à l'antibiothérapie empirique est courant dans la plupart des pays en développement où les politiques d'antibiotiques et la disponibilité ou l'utilisation du laboratoire de microbiologie clinique sont sous-optimales. Une approche standardisée de l'enquête de prévalence ponctuelle (PPS) sur l'utilisation des antimicrobiens (AMU) dans les hôpitaux a été utilisée pour évaluer les pratiques de prescription d'antimicrobiens au Federal Teaching Hospital Gombe (FTHG), au Nigéria.
Méthodologie: Un PPS a été réalisé en avril 2019 au FTHG en recrutant tous les patients hospitalisés présents à l'hôpital le jour de l'enquête. Les données obtenues à partir des dossiers des patients comprenaient des détails sur le type et l'indication de l'antibiothérapie. Une application en ligne personnalisée développée par l'Université d'Anvers (www.global-pps.be) a été utilisée pour la saisie, la validation, l'analyse et le reporting des données.
Résultats: Sur les 326 patients au total qui étaient admis le jour de l'enquête, 70,6% et 73,4% prenaient au moins un antibiotique dans les services pour adultes et pédiatriques respectivement. Les antibiotiques les plus couramment utilisés comprennent les bêta-lactamines telles que les céphalosporines (29,2%) et les pénicillines (22,8%), les fluoroquinolones (12,4%), les aminosides (9,1%) et les macrolides (3,4%). Parmi les patients' sous antibiotiques, la voie d'administration était principalement parentérale (71,6%) tandis que 44,8% prenaient plus d'un antibiotique. Dans l'ensemble, 91,3% des traitements antibiotiques étaient empiriques, les adultes, les enfants et les nouveau-nés représentant respectivement 96,4%, 77,6% et 100,0%. L'utilisation empirique d'antibiotiques est également élevée dans les services médicaux (86,3%), les services chirurgicaux (89,9%) et les unités de soins intensifs (100,0%).
Conclusion: Il y a une prédominance et une dépendance excessive à l'antibiothérapie empirique dans notre hôpital. Il expose en outre la mauvaise utilisation du laboratoire de microbiologie clinique et le potentiel de développement d'une résistance aux antibiotiques avec une augmentation résultante de la morbidité/mortalité et une mauvaise sécurité des patients. Des études supplémentaires sont nécessaires pour mettre en évidence les dangers d'une dépendance excessive à l'antibiothérapie empirique et annoncer une amélioration dans le développement et la mise en œuvre d'un programme de gestion des antibiotiques.

Mots clés: antibiothérapie empirique, résistance aux antimicrobiens, enquête ponctuelle de prévalence, gestion des antimicrobiens

Introduction:

Antimicrobials which include antibiotics, antivirals, antifungals and antiparasitics are drugs used to prevent and treat infections in humans, animals and plants while antimicrobial resistance (AMR) occurs when bacteria, viruses, fungi and parasites change over time and no longer respond to previous medications thereby making them more difficult to treat, with resultant increase in risk of spread, morbidity and mortality (1). Multidrug resistant micro-organisms (MDROs) have remained one of the greatest challenges to patient safety affecting all facets of infection prevention and control (IPC), with poor patient outcome, increased cost of care and varying effects among different countries worldwide (1-4). The World Health Organization (WHO) declared AMR as one of the top ten (10) global public health threats facing humanity and mainly driven by misuse and overuse of antimicrobials with worsening effects in developing countries (1,5).

Inadequate and unnecessarily broad-spectrum empiric antibiotic therapy has been linked to poor patient outcome largely due to AMR (6,7). Although universally practiced, empirical antibiotherapy is among the most common drivers of AMR through the overuse of antibiotics with many MDROs being allowed to flourish through initial treatment with ineffective medications (6). The variations between empirical coverage and subsequent detection of MDROs is not an uncommon observation even in advanced countries (8). A study revealed a discordant empirical antibiotic therapy approximately 20% among patients with bloodstream infections in US hospitals which was closely associated with

infections with MDROs (7). This buttresses the need for timely isolation and identification of the correct pathogen, which is very critical in the management of infectious diseases and in avoiding misuse and abuse of antibiotics (9).

There is a link between MDROs/AMR and healthcare associated infections (HAIs) with empiric use of antibiotics, and even in settings where antibiotic guidelines are available (10). The ability of healthcare providers to accurately match their antibiotic use with prevalent nosocomial pathogens in most hospitals remains low; hence the discouragement of over-reliance on empirical antibiotherapy without appropriate/ timely culture results.[8]

Several studies carried out in Nigeria explored antimicrobial prescribing patterns in hospitals and have revealed the need for harmonized approach to combat AMR through institutionalization of antimicrobial stewardship programmes (ASPs) and other necessary IPC and patient safety measures (11-13). AMR is a complex challenge that requires collective multisectoral approach with the "One Health" approach which brings together multiple sectors and stakeholders required for all programmes, policies, legislation and research being considered as the most viable approach to tackling the menace (1,2). Rational use of antibiotics through reasonable use of empirical antibiotherapy in hospitals and ensuring optimal utilization of laboratories is one easily implementable measure that can be applied in most Nigerian hospitals at a relatively low cost or extra burden on the system.

This study used the Global Point Prevalence Survey (G-PPS) platform developed by the University of Antwerp, to explore empirical antibiotherapy in FTHG as a potential driver of AMR and the risk of increased HAIs and a threat to patient safety.

Materials and method:

This was a cross sectional hospital-based PPS study conducted in April 2019 at the FTHG; a 500-bedded tertiary hospital in north-eastern Nigeria with institutionalized IPC and patient safety programmes. All in-patients who have been on admission at least 24 hours before the day of the survey and were present by 8am, were included in the study. Data obtained from patients' records included details of the type and indication for antibiotherapy. Anonymized patients' data for the study included an automatically generated unique survey number, age, gender, antimicrobial agents, number of doses per day, route of administration, indications for treatment and documentation of indication, stop or review date of prescription, microbiological data and compliance with prescribing guidelines. The survey for all patients in each particular ward was completed on a single day.

Data-entry, validation, analysis and reporting were done using the customized online application developed by the University of Antwerp (www.global-pps.be). Frequencies and proportions were used to present qualitative variables while Chi-square test was used to determine associations. P-value of <0.05 was set to determine statistically significant associations among the variables. Ethical approval for the study was obtained from the Research and Ethics Committee of FTHG.

Results:

Of the total 326 patients who were on admission on the day of survey, 70.6% and 73.4% were on at least one antibiotic in adult and paediatric wards respectively. Most commonly used antibiotics include beta lactams such as cephalosporins (29.2%) and penicillins (22. 8%), fluoroquinolones (12.4%), aminoglycosides (9.1%) and macrolides (3.4%). Among patients on antibiotics, route of administration was mainly parenteral (71.6%). Almost half (44.8%) of the patients were on more than one antibiotic. The pattern of empirical antibiotherapy as observed among different patient groups/wards and among HAIs and community acquired infections (CAIs) were as presented in Fig 1.

Quality indicators for antibiotic use in our hospital revealed documentation of indication for prescription in case notes for all the patients but variable rate in documentation of stop/review dates, with total absence of antibiotic guidelines, as highlighted in Table 1.

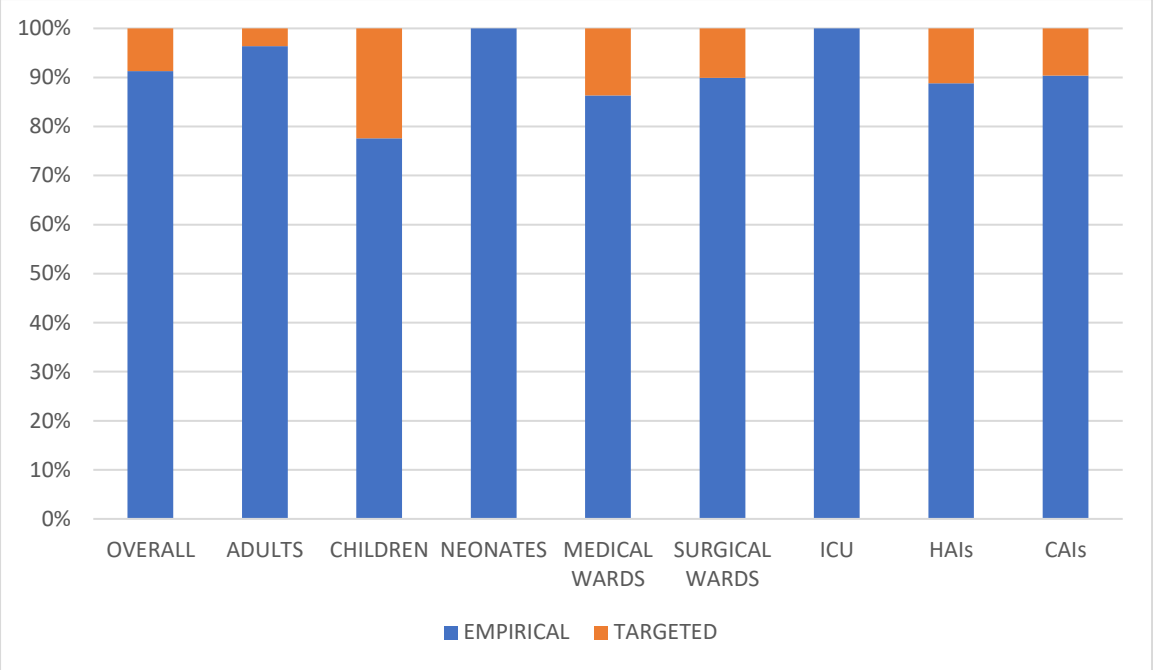

Fig 1: Rate of empirical and targeted antibiotherapy with respect to category of inpatients, ward and infection types

Table 1: Summary of some quality indicators for antibiotic use in Medical, Surgical and Intensive Care Unit

Quality indicator	Medical n (%)	Surgical n (%)	ICU n (%)
Reason in notes documented	81 (100)	184 (92.9)	19 (100)
Stop/review date documented	78 (96.3)	197 (99.5)	17 (89.5)
Guidelines absent	81 (100)	198 (100)	19 (100)

ICU = Intensive Care Unit; n = number of patients or cases

Discussion:

The overall rate of antibiotic prescription (>70%) in virtually all parts of the hospital observed in this study is not different from other reports on antimicrobial prescribing patterns and consumption in Nigeria (11-14). It is therefore obvious that majority of patients receives a minimum of one antimicrobial agent while on admission in Nigerian hospitals which is also a similar finding across other countries (15). Majority (>90%) of antibiotic therapy in our hospital were empirical and broad spectrum. This is higher than rates reported in studies from some parts of the world but reflects similar rates in other Nigerian studies (6,8,11-14).

Although the rate of inappropriate empirical antibiotic use could not be ascertained in this study, other reports have shown high rates that ranged between 14.1% to 78.9% (16,17). Approximately one in five patients with bloodstream infections in the US hospitals received discordant empirical antibiotic therapy with resultant rise in AMR (7). Inappropriate and discordant empirical antibiotic therapy has been shown to increase the 30-day and in-hospital mortality (7,16,17). Infection with antibiotic-resistant pathogens strongly predicted receiving discordant empirical therapy and therefore, misuse and overuse of broad-spectrum antibiotics prior to the availability of culture results may be counterproductive (7,18). It is therefore clear that empiric antibiotics may be irrelevant in many instances and should be cautiously handled (19). This re-emphasizes the need for institutionalization of ASPs for better patient safety and improved quality of care in all hospitals.

Appropriate selection of route of administration and switch from parenteral to oral antibiotics is one of the cardinal principles of antibiotherapy which may not be observed regularly as shown by high level (71.6%) of parenteral administration in our centre and

other local findings (9, 11-13). Most commonly used antibiotics in our hospital were beta lactams such as cephalosporins and penicillins, and the fluoroquinolones. This is similar to other findings from Nigeria, which could be due to similarities in training and approach to patient care without uniform/centre-based guidelines on antimicrobial prescribing (11-14). Findings from this study revealed that many (44.8%) patients were on more than one antibiotic which reflect the same pattern seen in many studies from Nigeria (11-14).

There was an observed excellent documentation of reasons for antibiotic prescription and variable documentation of the stop/review dates in this study, but there were absent antibiotic guidelines/compliance. This is similar to a recent report by Fowotade et al., (11) but different from the findings reported by Oduyebo et al., (13). Variations in the hospitals and differences in hospital policies and timing of the studies could explain the similarities and differences. This underscores the need for concerted efforts to have uniform antibiotic prescribing guidelines at both national and local levels in all hospitals in Nigeria, a task that the national antibiotic stewardship team, in collaboration with the G-PPS network, has started to establish to strengthen ASPs across the country.

Conclusion:

This PPS shows that majority of in-patients in FTHG are on empirical antibiotic therapy and on at least one antibiotic. Over-reliance on empirical antibiotherapy is potentially a major driver of AMR, which will worsen outcomes for both CAIs and HAIs. Strengthening of ASP is necessary for better patient safety in the hospital.

Conflict of interest:

Authors declare no conflict of interest.

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Previous publication:

Oral presentation of this paper was made at the Virtual Conference of the Clinical Microbiology and Infectious Disease Society of Nigeria (CLIMIDSON) via Zoom on 26th - 27th November 2020, and the abstract has been published in the conference programme.

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Original Article **Open Access**

Antibiogram of *Pseudomonas* species: an important tool to combat antibiotic resistance for patient safety in Gombe, Nigeria

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Abstract:

Background: *Pseudomonas* species are responsible for different healthcare-associated infections and are inherently resistant to many commonly used antibiotics. Hospital antibiograms are either absent or not regularly available in most healthcare facilities in Nigeria. The objective of this study is to present the antibiogram of *Pseudomonas* isolates in Federal Teaching Hospital Gombe (FTHG) in order to guide antibiotic prescription for better patient safety in the hospital.
Methodology: The is a hospital-based cross-sectional study. A total of 4309 bacterial isolates were recovered from aerobic cultures of routine clinical specimens including urine, sputum, blood, swabs, aspirates, biopsies, seminal fluids and cerebrospinal fluids at the Medical Microbiology laboratory of the hospital between January and December 2019. *Pseudomonas* species were identified by colony morphology, Gram-reaction and conventional biochemical tests. Antibiotic susceptibility testing was performed on each *Pseudomonas* isolate using the modified Kirby-Bauer disk diffusion method on Mueller-Hinton agar and results interpreted according to the guideline of the Clinical and Laboratory Standards Institute (CLSI). Data were analysed using the Statistical Package for Social Sciences (SPSS™) software version 23.0.
Results: Of the total 4309 bacterial isolates, 436 (10.1%) *Pseudomonas* species were identified, with majority (49.8%) from urine specimens. Antibiotic susceptibility test results revealed average susceptibility rates of 73.8%, 70.1%, 66.2%, 59.5%, and 34.3% to ciprofloxacin, gentamicin, levofloxacin ceftazidime, and carbenicillin respectively. These rates fluctuate only slightly for each of the antibiotic during the 12 months period of survey.
Conclusion: *Pseudomonas* species were most sensitive to ciprofloxacin and gentamicin among the first line antibiotics in FTHG in 2019. Regular updates and presentation of hospital antibiogram especially for intrinsically resistant bacteria such as *Pseudomonas* involved in healthcare associated infections, is an important tool in combating antimicrobial resistance and ensuring patient safety.

Keywords: antibiogram, *Pseudomonas*, antimicrobial resistance, antimicrobial stewardship, patient safety

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Antibiogramme des espèces de *Pseudomonas*: un outil important pour lutter contre la résistance aux antibiotiques pour la sécurité des patients à Gombe, Nigéria

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Abstrait:

Contexte: Les espèces de *Pseudomonas* sont responsables de différentes infections associées aux soins de santé et sont intrinsèquement résistantes à de nombreux antibiotiques couramment utilisés. Les antibiogrammes hospitaliers sont absents ou ne sont pas régulièrement disponibles dans la plupart des établissements de santé au Nigéria. L'objectif de cette étude est de présenter l'antibiogramme des isolats de Pseudomonas dans l'Hôpital Universitaire Fédéral de Gombe (FTHG) afin d'orienter la prescription d'antibiotiques pour une meilleure sécurité des patients à l'hôpital.
Méthodologie: Il s'agit d'une étude transversale en milieu hospitalier. Un total de 4309 isolats bactériens ont été récupérés à partir de cultures aérobies d'échantillons cliniques de routine, y compris l'urine, les crachats, le sang, les prélèvements, les aspirations, les biopsies, les fluides séminal et les fluides céphalo-rachidiens au laboratoire de microbiologie médicale de l'hôpital entre janvier et décembre 2019. Les espèces de *Pseudomonas* étaient identifié par la morphologie de la colonie, la réaction Gram et les tests biochimiques conventionnels. Des tests de sensibilité aux antibiotiques ont été réalisés sur chaque isolat de *Pseudomonas* en utilisant la méthode de diffusion sur disque Kirby-Bauer modifiée sur gélose Mueller-Hinton et les résultats ont été interprétés selon les directives du Clinical and Laboratory Standards Institute (CLSI). Les données ont été analysées à l'aide du logiciel Statistical Package for Social Sciences (SPSSTM) version 23.0.
Résultats: Sur les 4309 isolats bactériens au total, 436 (10,1%) espèces de *Pseudomonas* ont été identifiées, la majorité (49,8%) à partir d'échantillons d'urine. Les résultats des tests de sensibilité aux antibiotiques ont révélé des taux moyens de sensibilité respectivement de 73,8%, 70,1%, 66,2%, 59,5% et 34,3% à la ciprofloxacine, à la gentamicine, à la lévofloxacine ceftazidime et à la carbénicilline. Ces taux ne fluctuent que légèrement pour chacun des antibiotiques au cours de la période de 12 mois d'enquête.
Conclusion: les espèces de *Pseudomonas* étaient les plus sensibles à la ciprofloxacine et à la gentamicine parmi les antibiotiques de première intention dans le FTHG en 2019. Les mises à jour régulières et la présentation de l'antibiogramme hospitalier, en particulier pour les bactéries intrinsèquement résistantes telles que les *Pseudomonas* impliquées dans les infections associées aux soins, sont un outil important dans la lutte contre la résistance aux antimicrobiens et assurer la sécurité des patients.

Mots clés: antibiogramme, Pseudomonas, résistance aux antimicrobiens, gestion des antimicrobiens, sécurité des patients

Introduction:

Pseudomonas are non-fermentative oxidase-positive, motile, aerobic Gram-negative rods belonging to the family *Pseudomonadaceae,* with a large genome of approximately 6.3 million base pairs (Mbp), encoding 5567 genes(1). *Pseudomonas* commonly colonizes hospital environment, equipment, and have remained a prominent cause of healthcare-associated infections (HAIs) (2). They typically cause diseases in immunocompromised individuals but occasionally infects immunocompetent hosts, and are often associated with ventilator-associated pneumonia (VAP), central line-associated bloodstream infection (CLABSI), surgical site infection (SSI), respiratory infections in cystic fibrosis (CF) patients, and catheter-associated urinary tract infections (CAUTI) (3,4).

Pseudomonas generally possesses several virulence factors including lipopolysaccharide, type IV pili, type III and VI secretion systems, alginate, exotoxin A, proteases, quorum sensing, generation of oxidants in the airways, formation of biofilm and pigments (1). *Pseudomonas* generally are intrinsically resistant to several antibiotics, and recently, more resistance from acquired sources is progressively increasing (5).

Antibiogram is a summary of the periodic susceptibility pattern of organisms isolated within a specific area which is regularly communicated to the hospital community to guide appropriate use of antimicrobials and empirical treatment of infections (6), while patient safety is a health care discipline that focuses more on preventing, controlling, and minimizing risks, harm, and errors in healthcare (7). Patient safety together with antimicrobial stewardship and infection prevention and control (IPC) constitutes the major pillars of an integrated approach to health systems strengthening (7).

Inappropriate use of antimicrobials is the main driving force in the development of resistance by microorganisms, and these resistant organisms pose significant threat to patient safety globally (8). Antibiogram classically promote optimal use of antimicrobials by encouraging a targeted approach,thereby reducing treatment-related cost and also minimizing or slowing the development of antimicrobial resistance leading to improved quality of life and patient safety (7).

Studies specifically on *Pseudomonas* antibiogram are few in this part of the country. Communication and dissemination of

antiogram outcomes to health care workers, hospital community, and globally on regular basis, is also lacking. In this study, we present the FTHG 2019 *Pseudomonas* antibiogram, which is aimed at providing a guide for the empirical antibiotic prescribing practices among physicians to combat antimicrobial resistance for better patient safety.

Materials and method:

Study setting and ethical approval

This is a hospital-based cross-sectional study carried out in FTHG, a 457 beded tertiary hospital in north-eastern Nigeria. The hospital has a functional IPC programme and multidisciplinary patient safety team (MPST). Ethical approval for this study was obtained from the research and ethics committee of FTHG.

Identification and antibiotic susceptibility of *Pseudomonas* specie isolates

All *Pseudomonas* isolates recovered from clinical specimens (urine, sputum, blood, swabs, aspirates, biopsies, and seminal fluids) received and processed from at the Medical Microbiology laboratory of FTHG from January to December 2019, were included. Identification of *Pseudomonas* species was manually done using conventional methods including colonial morphology, Gram stain reaction, oxidase and motility tests.

Invitro susceptibility testing of the isolates to five commonest first-line anti-*Pseudomonas* antibiotics in our facility was performed by the modified Kirby-Bauer disk diffusion method on Mueller-Hinton agar using single antibiotic disks (ceftazidime 30µg, gentamicin 10µg, ciprofloxacin 5µg, levofloxacin 5µg and carbenicillin 100µg) (Oxoid, UK). The zone diameter of inhibition (measured in millimetres) for each isolate was interpreted as sensitive or resistant using the guidelines of the Clinical and Laboratory Standards Institute (CLSI) for *Pseudomonas* (9).

Data analysis

Data were analysed using the Statistical Package for Social Sciences (SPSS™) software version 23.0.

Results:

Of the total 4309 bacterial isolates recovered from clinical specimens in 2019 at the Medical Microbiology laboratory of FTHG, *Pseudomonas* species were 436 (10.1%), about half (49.8%) of which were isolated from urine specimens while others were as shown in Table 1. The monthly distribution and percentage antibiotic susceptibility of the *Pseudomonas* species are highlighted in Table 2.

The average sensitivity rates of *Pseudomonas* species against commonly used antibiotics in our facility were 73.8% (ciprofloxacin), 70.1% (gentamicin), 66.2% (levofloxacin), 59.5% (ceftazidime) and 34% (carbenicillin) (Fig 1).

Table 1: Distribution of *Pseudomonas* species isolated from different clinical specimens

Specimen type	No of *Pseudomonas* (%)
Urine	217 (49.8)
Swabs/ aspirates/biopsies	146 (33.5)
Sputum	53 (12.1)
Blood	20 (4.6)
Total	**436 (100)**

Discussion:

Pseudomonas species are organisms with a remarkable propensity to develop resistance to a wide range of antibiotics more commonly in hospital settings. It has inherent resistance to many commonly used antibiotics thereby making it a frequent agent responsible for inappropriate empirical antibiotic therapy which is associated with increased healthcare burden. Poor prescribing practices have been identified as a major driver in the development of resistance against antibiotics with *Pseudomonas* being very prominent.

This study revealed a relatively high prevalence of *Pseudomonas* (10.1%) among other bacterial isolates recovered from clinical specimens in Gombe, which is similar to 9.7% reported by Dash et al.,(10) in Peshawar, but higher than 5.1% reported in Nepal (11). This further highlights the significance of this pathogen and the importance of having an antibiogram to guide empiric antibiotic prescription especially given its inherent resistance to many commonly used drugs in the treatment of bacterial infections.

About half of the isolated *Pseudomonas* were from urine specimen which conforms to the findings by Shrestha et al., (11) and probably because urine is also the most common specimen received in our laboratory during the period Miscellaneous specimens such as swabs and aspirates usually from skin and soft tissue infections (SSTIs) and surgical site infection (SSIs) were very common sources of *Pseudomonas* isolates in our study, and similar to the findings of Dash et al., (12) in South Odisha, India.

The *invitro* susceptibility of *Pseudomonas* to ciprofloxacin was high at 73.8% average in this study, which was a similar

Table 2: Monthly distribution of *Pseudomonas* species recovered from clinical specimens and their antibiotic susceptibility at Federal Teaching Hospital, Gombe, Nigeria

Months in 2019	**No of *Pseudomonas* isolates (%)**	**No of *Pseudomas* isolates sensitive to the antibiotics (%)**				
		CIP	**LEV**	**CAZ**	**CN**	**CAR**
January	49 (11.2)	33 (67.3)	28 (57.1)	31 (63.3)	32 (65.3)	9 (18.4)
February	24 (5.5)	16 (66.7)	14 (58.3)	14 (58.3)	16 (66.7)	12 (50.0)
March	35 (8.0)	28 (80.0)	21 (60.0)	21 (60.0)	23 (65.7)	15 (42.9)
April	37 (8.5)	30 (81.1)	22 (59.5)	22 (59.5)	26 (70.3)	13 (35.1)
May	19 (4.4)	13 (68.4)	12 (63.2)	13 (68.4)	14 (73.7)	5 (26.3)
June	40 (9.2)	28 (70.0)	29 (72.5)	28 (70.0)	30 (75.0)	16 (40.0)
July	29 (6.7)	20 (69.1)	19 (65.5)	17 (58.6)	22 (75.9)	17 (58.6)
August	40 (9.2)	30 (75.0)	32 (80.0)	25 (62.5)	26 (65.0)	17 (42.5)
September	47 (10.8)	37 (78.7)	32 (68.1)	28 (59.6)	37 (78.7)	13 (27.7)
October	42 (9.6)	35 (83.3)	32 (72.2)	21 (50.0)	28 (66.7)	9 (21.4)
November	51 (11.7)	36 (70.6)	40 (78.2)	27 (52.9)	34 (66.7)	13 (25.5)
December	23 (5.2)	18 (78.3)	14 (61.0)	12 (52.2)	18 (78.3)	11 (49.0)
Total	**436 (100)**	**322 (73.8)**	**289 (66.2)**	**259 (59.5)**	**306 (70.1)**	**150 (34.3)**

CIP = Ciprofloxacin, LEV = Levofloxacin, CAZ = Ceftazidime, CN = Gentamicin, CAR = Carbenicilin

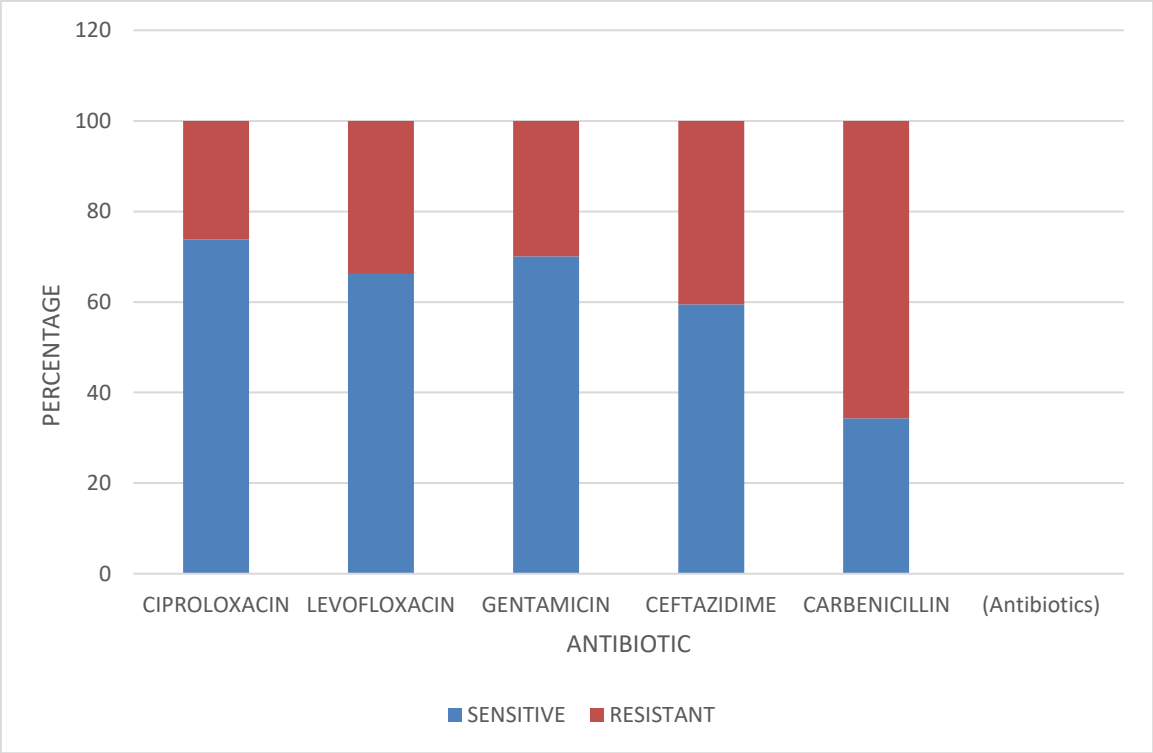

Fig 1: Average percentage sensitivity and resistance of *Pseudomonas* to first-line antimicrobials

pattern reported by Balogun et al., (13) in Maiduguri, which is in the same geographical region as Gombe in Nigeria. Geographical and social similarity, as well as prescribing habits and health indices between Gombe and Maiduguri, could play a role in this context. The susceptibility to gentamicin was also relatively high, similar to that reported by Shrestha et al.,(11). The 59.6% susceptibility rate to ceftazidime (a third generation cephalosporin) reported in this study is similar to the rate reported by Gill et al., (14) and Iregbu et al., (15). A study in Saudi-Arabia by Rashid et al., (16) reported 70.0% sensitivity of *Pseudomonas* to levofloxacin, which is similar to 66.2% reported by us, as against a higher resistance rate 36.1% reported by Dash et al.(12).

Carbenicillin, a carboxypenicillin used in the treatment of *Pseudomonas* infections, showed little *invitro* efficacy as only 34.3% of the *Pseudomonas* isolates were sensitive to it

in our study, similar to a study conducted by Jazani et al., in Iran with 35% sensitivity rate. This high resistance rate may be due to the increasing transfer of beta-lactamase genes between *Pseudomonas* species and members of the family *Enterobacteriaceae* (17).

The monthly susceptibility rates show very little variations of *Pseudomonas* isolates to each of the 5 commonly used anti-*Pseudomonas* antibiotics in our facility. These antibiogram results were discussed monthly and annually presented to the hospital community at the instance of the MPST, outlining the trend of infection, susceptibility, and resistant patterns of *Pseudomonas* species to guide and improve antibiotic prescribing practices.

Conclusion:

Antibiogram remains an important tool in combating AMR through improved prescribing practices at all levels of health-care delivery. *Pseudomonas* are among the common clinically important bacterial isolates in Gombe and most commonly recovered from urine and swabs/aspirates associated with healthcare-associated urinary tract and skin/soft tissue infections. Among the first line antibiotics at FTHG in 2019, *Pseudomonas* were most sensitive to ciprofloxacin and least sensitive to carbenicillin.

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Conflict of interest:

No conflict of interest is declared.

Previous publication

Oral presentation of this paper was made at the Virtual Conference of the Clinical Microbiology and Infectious Disease Society of Nigeria (CLIMIDSON) via Zoom on 26th - 27th November 2020, and the abstract has been published in the conference program.

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Original Article **Open Access**

Using longitudinal antibiotic point prevalence survey (PPS) to drive antimicrobial stewardship programmes in a Nigerian tertiary hospital

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Abstract:

Background: Antimicrobial stewardship (AMS) provides a means of tackling antimicrobial resistance (AMR). Unfortunately, in Nigeria, like in some other low-and-middle-income countries (LMICs), AMS practice has been lacklustre due to poor institutional support amongst other factors. Efforts were made to address this situation by engaging with the management of National Hospital Abuja, Nigeria, using antibiotic prescription information obtained through repeated point prevalence survey.
Methodology: Two rounds of antibiotic PPS were conducted in 2015 and 2017 using the Global Point Prevalence Survey (G-PPS) format. Data were collected from all inpatients receiving antibiotics on the selected day of study, including patient characteristics, antimicrobial prescription details, laboratory results and information on a set of quality indicators. The data were uploaded to an online G-PPS application hosted at the University of Antwerp in Belgium for validation, analysis and reporting.
Results: The PPS data showed that hospital-wide antibiotic use prevalence increased from 58% in 2015 to 61% in 2017. Surgical prophylaxis beyond 24 hours also increased from 88-90% in 2015 to 100% in 2017, and only minority of therapies were supported by laboratory input for diagnosis and monitoring; 22% in 2015 and 5% in 2017.
Conclusion: These results were used for evidence-based engagement with the management to formally support AMS activities in the hospital. Positive outcomes were the formal reconstitution and inauguration of AMS committee in 2018 as well as the issuance of a formal policy statement by the hospital in 2020. The ease and free availability of Global PPS methodology makes it ideal in driving antimicrobial stewardship programme (ASP) in LMICs like Nigeria.

Keywords: Antibiotic stewardship checklist**,** institutional support**,** point prevalence survey**,** policy statement

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Utilisation d'une enquête longitudinale sur la prévalence ponctuelle des antibiotiques (PPS) pour conduire des programmes de gestion des antimicrobiens dans un hôpital tertiaire nigérian

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Abstrait:

Contexte: La gestion des antimicrobiens (AMS) offre un moyen de lutter contre la résistance aux antimicrobiens (RAM). Malheureusement, au Nigéria, comme dans certains autres pays à revenu faible ou intermédiaire (PRFI), la pratique de la MGS a été médiocre en raison d'un soutien institutionnel insuffisant, entre autres facteurs. Des

efforts ont été faits pour remédier à cette situation en collaborant avec la direction de l'hôpital national d'Abuja, au Nigéria, en utilisant les informations sur les prescriptions d'antibiotiques obtenues grâce à une enquête ponctuelle répétée de prévalence.
Méthodologie: Deux séries d'antibiotiques PPS ont été menées en 2015 et 2017 en utilisant le format Global Point Prevalence Survey (G-PPS). Des données ont été recueillies auprès de tous les patients hospitalisés recevant des antibiotiques le jour sélectionné de l'étude, y compris les caractéristiques des patients, les détails de la prescription d'antimicrobiens, les résultats de laboratoire et les informations sur un ensemble d'indicateurs de qualité. Les données ont été téléchargées sur une application en ligne G-PPS hébergée à l'Université d'Anvers en Belgique à des fins de validation, d'analyse et rapports.
Résultats: Les données PPS ont montré que la prévalence de l'utilisation d'antibiotiques à l'échelle de l'hôpital est passée de 58% en 2015 à 61% en 2017. La prophylaxie chirurgicale au-delà de 24 heures est également passée de 88 à 90% en 2015 à 100% en 2017, et seule une minorité de thérapies a été soutenue par entrée de laboratoire pour le diagnostic et la surveillance; 22% en 2015 et 5% en 2017.
Conclusion: Ces résultats ont été utilisés pour un engagement fondé sur des données probantes avec la direction afin de soutenir officiellement les activités AMS à l'hôpital. Les résultats positifs ont été la reconstitution formelle et l'inauguration du comité AMS en 2018 ainsi que la publication d'une déclaration de politique formelle par l'hôpital en 2020. La facilité et la disponibilité gratuite de la méthodologie Global PPS la rend idéale pour conduire le programme de gestion des antimicrobiens (ASP) en PRFI comme le Nigéria.

Mots clés: liste de contrôle pour la gestion des antibiotiques, soutien institutionnel, enquête ponctuelle de prévalence, déclaration de politique

Introduction:

The role of organisational support is emphasised by the dedication of a section of both the WHO and CDC checklists of essential healthcare facility core elements for anti-microbial stewardship programme (AMS) to leadership, accountability and responsibilities (1,2). In certain situations, the support may be impaired by the management setting up AMS or selecting teams without recourse to the internationally recognised guidelines. AMS, a recognised systematic strategy to prevent and control the emergence of anti-microbial resistance (AMR) is most effective when applied under a structured organisa-tional arrangement (3,4). The creation and sustenance of this structure is strongly dependent on robust organisational support for AMS and plays a major role in determining the outcome of AMS interventions (4,5). Such organisational and management support includes formal recognition of the necessity to set up AMS committee, allocation of budget, and appointment of persons with requisite qualifications and experience to oversee AMS programme.

A common factor in low-and-middle-income-countries (LMICs) is the relatively low level of antimicrobial use (AMU) information and the related low level of AMS activity (6,7,8). Evidence, scientifically obtained and analysed, is the best way to provide proof of a problem. In the case of AMS, the prevalence and pattern of antimicrobial prescription are means to convince institution management of the problem of AMR (1,4,9,10,11) and engage in conversation on strategies for its contain-ment, especially in the form of a formal policy committing to AMS (4,5,6,8). This is further bolstered when such scientific evidence is from within the institution.

Periodic assessment of antimicrobial prescription using PPS presents a simple, objective and comprehensive methodology (1, 4,10). This information can be analysed and the results communicated and disseminated to key stakeholders to drive AMS (6,8). The aim of this study is to determine the antimicrobial prescription patterns among inpatients in the hospital through PPS and use this information to improve management support for AMS activity in National Hospital, Abuja, Nigeria.

Materials and method:

Study setting

This study was carried out in National Hospital, a 450-bed tertiary centre located in Abuja, Northcentral Nigeria. The hospital provides general services for adult (medical and surgical) and paediatric (medical and surgical) as well as a wide range of specialist diagnostic and intervention services including intensive care, trauma, oncology, and haema-tology among others.

Method of PPS

Two rounds of antibiotic PPS were conducted in 2015 and 2017 using Global-PPS format developed by the University of Antwerp (www.global-pps.com). Briefly, AMS data were collected from all inpatients on ward admission at 8:00 hours on the selected days of study in 2015 and 2017, but surgical wards were not surveyed on Mondays and Fridays, and all patients admitted after 08.00hrs were exclu-ded from the survey.

All inpatients on the day of the survey formed the denominator while all inpatients who had any antimicrobial treatment at the time of the survey formed the numerator. Information was gathered on antimicrobial use including prescription, indication and route of intake. Other information collected included but not limited to; age, gender, diagnosis, stop/review date, adherence to guidelines and laboratory results. The data were entered into

the G-PPS online application software of the University of Antwerp in Belgium for analysis.

Measurement of AMS activity in the hospital

The level of AMS activity in the hospital was objectively assessed annually from 2017 to 2020 using an AMS assessment toolkit adapted from the WHO and CDC checklists of essential healthcare facility core elements for AMS programmes (1,2). This kit assigned scores for various elements of AMS activity grouped into sections covering 'leadership commitment', 'accountability and responsibilities', 'AMS actions', 'education and training', 'monitoring and surveillance', and 'reporting and feedback'. The values obtained were used to measure changes in AMS activity within the hospital.

Results:

Antibiotic prevalence patterns:

The PPS results across adult (medical, surgical, obstetrics and gynaecology) and paediatric (medical and surgical) wards, and intensive care unit (ICU) showed that there was a hospital wide increase in prevalence of antibiotic use from 58% in 2015 to 61% in 2017.

The pattern of surgical prophylaxis showed that prescription beyond 24 hours increased from 88-90% in 2015 to 100% in 2017. Only a minority of cases had evidence of targeted therapy based on laboratory input for diagnosis and monitoring with 22% in 2015 and 5% in 2017 (Table 1).

Quality indicators:

Majority of prescriptions were via the parenteral route in both years surveyed (70% in 2015 and 82% in 2017) and 55% and 50% of patients were on two or more antibiotics in 2015 and 2017 respectively. Documentation of stop/review dates varied across the wards and ICU but in both years, it was lowest in the ICU (0% in 2015 and 10% in 2017) while documentation of reasons for therapy in notes was highest in the same ICU (97% in 2015 and 57% in 2017).

AMS activity:

The use of the toolkit adapted from the WHO AMS (1) showed that there was an improvement in AMS activity particularly in the sections covering 'leadership and commitment' and 'accountability and responsibilities' (Fig 1).

Table 1: Antibiotic prescription patterns in National Hospital, Abuja, Nigeria, 2015 and 2017

Antibiotic prevalence patterns			Year of survey	
			2015	2017
	Antibiotic prevalence in percentage		58	61
	Targeted therapy in percentage	Hospital average	22	5
		Paediatrics	10	5
		Adults	16	2
	Surgical prophylaxis >24 hours in percentage	Surgical	90	100
		O & G	88	100
Quality indicators	Parenteral route in percentage		70	82
	On > 2 antibiotics in percentage		55	50
	Documentation of reason in notes in percentage	Medical	88	57
		Surgical	95	50
		ICU	97	57
	Documentation of stop/review dates in percentage	Medical	45	22
		Surgical	82	42
		ICU	0	10

ICU = Intensive care unit; O & G = Obstetrics & Gynaecology

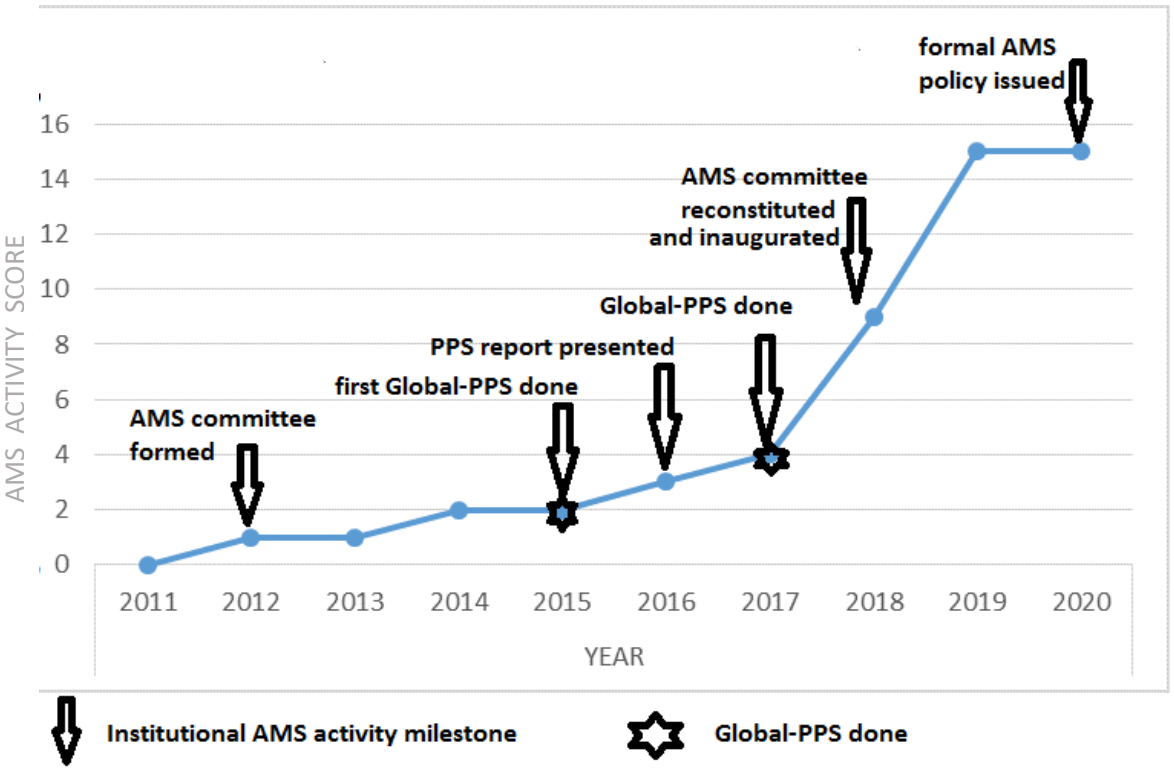

Fig 1: Time-line of AMS activities following presentation of Global-PPS reports to National Hospital management

Discussion:

The antimicrobial prescription rates in our institution for 2015 and 2017 (58% and 61%) were higher than the worldwide average of 34.4% (12). Rates are significantly lower in countries where AMS activities have been actively pursued such as the Netherlands and Belgium with rates at 22.9% and 27.1% respectively (13,14). This would suggest that antimicrobial misuse may be similarly high in this setting as it is linked to high prevalence rates (15). This was supported by the high levels of inappropriate surgical prophylaxis (88 -90%) which is far higher than 40.6-86.3% worldwide average (12). On the other hand, prevalence of targeted therapy (22% and 5%), stop/review dates (range 0-82%) and documented reasons for therapy in case notes (97% and 57%) were comparable to the global average of 19.8%, 38.3% and 76.9% for these parameters respectively (12).

These results suggest the extent of poor antimicrobial utilisation in our institution and the need for a robust antimicrobial stewardship programme. While the problem exists in more developed climes, efforts to establish AMS in many LMICs more often suffer from lack of institutional support (6,7). This has been demonstrated as major limitation to AMS activity in Nigerian healthcare institutions by Iregbu et al (16). In LMICs, AMS interventions are more effective when there is institutional support (17). An institution management does not necessarily set out to deny AMS the supports that it needs, however when there are competitions in the allocation of resources between overtly revenue generating hospital activities and non-revenue generating ones such as AMS, the latter usually gets short changed (8). Often AMS has failed to make a convincing case for itself and justify the resources it deserves from the management without which it cannot function (5,8). Behavioural change has been demonstrated to be one of the most difficult things to achieve but persistent reinforcement of the message increases the chances for success (11,18). This applies when trying to persuade managers and prescribers in healthcare providing institutions of the need to formally incorporate it into their healthcare policy and adopt new prescribing behaviours respectively (9).

The effectiveness of the AMS committee set up by our institution in 2012 was impaired by management's lukewarm support to AMS activities with no formal commitment or acknowledgement of AMR as an issue. Additionally, the committee was selected without adequate representation from key stakeholders. Information on anti-microbial prescription was needed to create local evidence for change. The results of our longitudinal Global-PPS were used for evid-ence-based engagement with the hospital management, which was done by presenting the PPS results to key policy makers and key stakeholders. Multiple opportunities were exploited to disseminate this information

including management meetings and grand rounds. Positive outcomes of these were the formal reconstitution and inauguration of AMS committee in 2018.

The management of the hospital used internationally accepted criteria in selecting the AMS committee makeup and clear terms of reference were issued. The current committee is multidisciplinary with key stakeholders under a qualified leadership of a consultant microbiologist with infectious disease training, and physicians, pharmacist and nurse as members. This is in line with recommended best practice (1). Additionally, office space was provided, and significantly, the AMS committee has formed a collaboration with the infection prevention and control (IPC) team. Furthermore, the hospital charged a team made up of upper management and experts experienced in AMS, with creating a policy statement on AMS in 2019. This document was reviewed and released by the top management as a formal policy statement in early 2020. This is one measure recommended by MacDougall and Polk to improve the chances of AMS achieving any measure of success (6).

Conclusion:

Institutional support is more forthcoming when evidence of antimicrobial misuse within the system is provided and used to engage management. The ease of Global PPS methodology makes it ideal in driving ASP in LMICs like Nigeria. AMS professionals should undertake PPS and use this to enhance formal institutional support for AMS. As more institutions adopt AMS, it behoves healthcare personnel to ensure its success by carrying out PPS and using it as evidence for AMS.

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Conflicts of interest:

Authors declare no conflicts of interest

Previous publication:

Oral presentation of this paper was made at the Virtual Conference of the Clinical Microbiology and Infectious Disease Society of Nigeria (CLIMIDSON) via Zoom on 26th - 27th November 2020, and the abstract has been published in the conference program.

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Communication **Open Access**

Implementation of biosafety in infection control: a 10-year review

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Abstract:

Biosafety is an important issue globally, as a line of defence that protects health personnel, public and the environment from exposure to hazardous agents. Most developing nations have weak health systems and consequently weak biosafety. Engaging in an external quality program such as an accreditation process will help build capacity and competence in all areas. The Centre for Human Virology and Genomics (CHVG) laboratory of the Nigerian Institute of Medical Research, Lagos, Nigeria started its biosafety program in compliance to international standards stipulated by ISO 15189:2012, in its journey towards laboratory accreditation. Accreditation is widely used to attest for laboratory competence. In the quest for laboratory accreditation, several processes were implemented to ensure compliance, one of which was biosafety. Simple remedial actions and policies that have worked in other climes were applied over a 10-year period in the CHVG laboratory. A significant drop in nonconforming incidences and laboratory accidents was seen while biosafety audits showed improvement in safety practices.

Keywords: Biosafety, Infection control, audit

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Mise en œuvre de la biosécurité dans la lutte contre les infections: un examen décennal

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Abstrait:

La biosécurité est une question importante à l'échelle mondiale, en tant que ligne de défense qui protège le personnel de santé, le public et l'environnement de l'exposition à des agents dangereux. La plupart des pays en développement ont des systèmes de santé faibles et, par conséquent, une faible sécurité biologique. S'engager dans un programme de qualité externe tel qu'un processus d'accréditation aidera à renforcer les capacités et les compétences dans tous les domaines. Le laboratoire du Centre de virologie humaine et de génomique (CHVG) de l'Institut nigérian de recherche médicale de Lagos, au Nigéria, a commencé son programme de biosécurité conformément aux normes internationales stipulées par ISO 15189: 2012, dans son parcours vers l'accréditation des laboratoires. L'accréditation est largement utilisée pour attester la compétence du laboratoire. Dans la recherche de l'accréditation des laboratoires, plusieurs processus ont été mis en œuvre pour assurer la conformité, dont l'un était la biosécurité. Des mesures correctives simples et des politiques qui ont fonctionné sous d'autres climats ont été appliquées sur une période de 10 ans dans le laboratoire du CHVG. Une baisse significative des incidents non conformes et des accidents de laboratoire a été observée tandis que les audits de biosécurité ont montré une amélioration des pratiques de sécurité.

Mots clés: Biosécurité, Contrôle des infections, audit

Introduction:

Accreditation is an authorized recognition of competency issued by a third party often following the results of a conformity assessment (1). It shows capabilities and proficiency in a specific field; for the laboratory, it is a certification of competency to perform specific tests, classes or types of tests mostly in conformity to international standards such as the International Organization for Standardization (ISO) and the International Electrotechnical Commission (IEC) (1,2).

Accreditation is an essential element by which implementing organizations can improve managerial and technical capability such as laboratory safety while achieving international best practices (1). The accreditation process must show full compliance with specific laws, regulations, standards, protocols, and agreements, this helps strengthens processes and technical expertise of participating bodies (2). In the journey towards achieving accreditation, the Centre for Human Virology and Genomics (CHVG), was introduced to Quality Management System (QMS) and Strengthening Laboratory Management Toward Accreditation (SLMTA) programmes. SLMTA is an initiative of the World Health Organization (WHO), a framework tool for public health-based laboratories to achieve ISO 15189 standards and prepare such laboratories for accreditation (3). Through the establishment of standardised processes, SLMTA measures and evaluates the progress of laboratory systems towards international accreditation via the Stepwise Laboratory Improvement Process Towards Accreditation (SLIPTA) checklist tool. The SLIPTA checklist is a framework of auditing tool that allows laboratories measure and evaluate the progress of its laboratory quality system. SLIPTA checklist is used to evaluate biosafety for conformity to ISO standards (3).

Biosafety is the safe handling and containment of infectious microorganisms and hazardous biological materials (4). It is an important issue in laboratory settings worldwide, especially in developing countries where standardized practices are lacking (5). It is a critical tool in the global fight against infectious diseases spread particularly in the face of highly contagious diseases of recent times such as Ebola and Lassa viruses (6-8). Most laboratory infections have been reported to occur as a result of inattention to safety practices, error, accident or carelessness in handling infectious agents (5,8). Hence laboratories are required to provide means to control infections and monitor these measures to ensure conformity to best practise and reduce laboratory accidents (8). This manuscript presents a 10 year (2006-2016) retrospective review of biosafety indicators, monitored in CHVG in the laboratory's pathway to accreditation to ISO 15189:2012 in 2017.

Methodology:

The CHVG created the biosafety process in 2006 as part of the quality essential processes required to achieve accreditation. Its biosafety program commenced with the training and appointment of a dedicated biosafety officer to implement and report on safety issues monthly. A biosafety manual to direct process activities was developed, which had references to the WHO safety regulations for a biosafety level 2 laboratories and also the local regulatory agency laws such as the Federal Environmental Protection Agency (FEPA) waste management laws.

Biosafety working documents which included standard operating procedures (SOPs) and job aids to address various safety areas such as waste management, fire emergency, microorganism transfer or transport, access control, sample spill checks, laboratory accidents (needle pricks and sample splash) and personal protective equipment (PPE) use were developed. Fire emergency trainings and drills were regularly conducted; spill and microorganism transfer and handling trainings were followed through. On-site training of all personnel on the use of these relevant documents and activities were done.

Key indicators for monitoring biosafety were identified and monitored monthly for compliance. Monitoring of noncompliance to policies and procedures were tracked and corrective actions were analysed for effectiveness, subsequent training from gaps identified were also initiated. Periodic safety audits were conducted to measure the effectiveness and implementation. Indicators of effective QMS such as rate of laboratory accidents and nonconformities were tracked to maintain the integrity of the quality of laboratory biosafety. All indicators were monitored in reviews and analysed annually to detect issues, trends and lessons for improvement.

Two key indicators were reviewed; laboratory accidents report and internal safety audit reports. Safety audits were conducted using section twelve of the standard SLIPTA

checklist to monitor biosafety implementation and effectiveness. Laboratory accidents were promptly managed using an established Post Exposure Prophylaxis (PEP) policy. This involved screening for a baseline HIV test and with a negative result obtained, prescription of antiretroviral drugs for a 28-day dosage period.

Results:

Key biosafety indicators

The results of the key biosafety indicators monitored in the 10-year period is displayed in Fig 1. During the 10 years under review, there were 15 laboratory accidents (12 needle pricks and 3 sample splashes on mucous membrane) in CHVG. The highest frequency of accidents occurred between 2008 and 2011. There was no sero-conversion after 3 months post exposure surveillance.

Scores of biosafety audit assessment results over four years

Fig 2 represents the aggregate scores of the biosafety process monitored in preparation for laboratory accreditation. From 2010 to 2013, the CHVG was audited using SLIPTA checklist on biosafety. The percentages for biosafety over this period were 92.5%, 95%, 93%, 97.6% in 2010, 2011, 2012 and 2013 respectively.

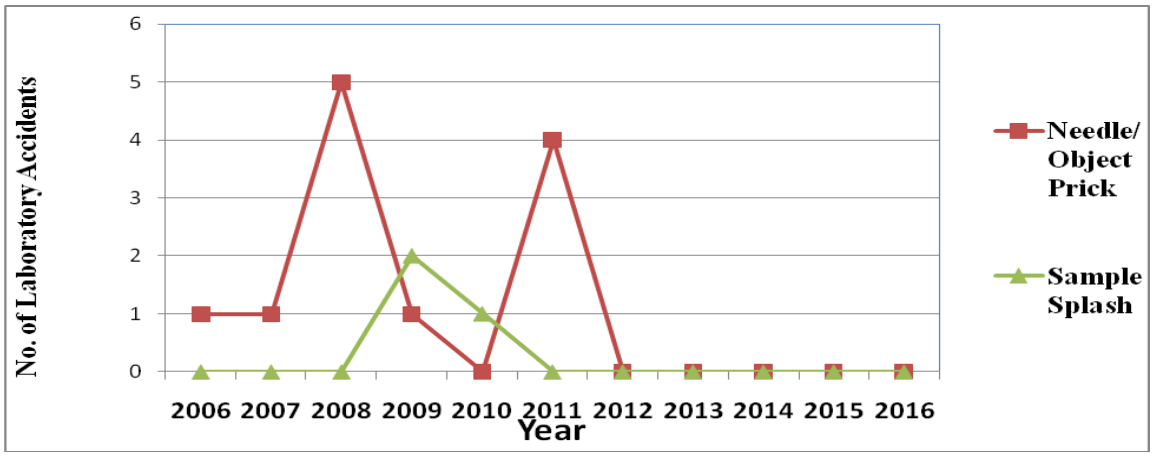

Fig 1: Needle prick and sample splash laboratory accidents in CHVG, NIMR, Lagos, Nigeria

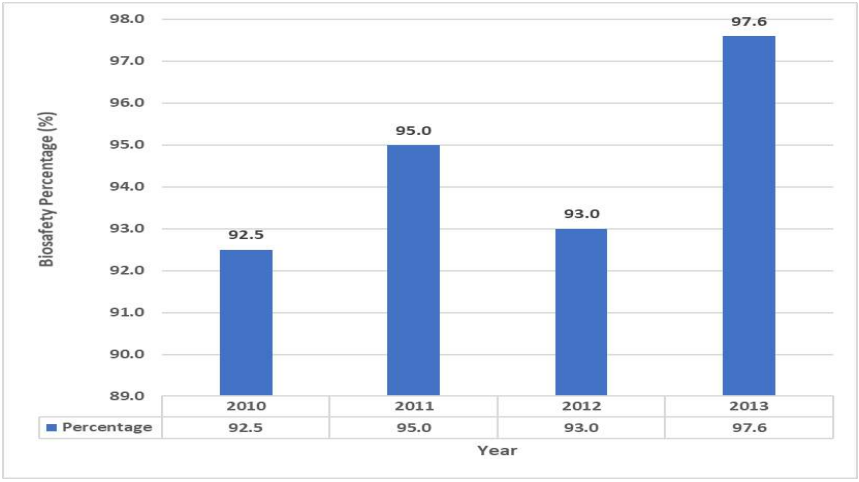

Fig 2: Audit scores from 2010 to 2013 using the SLIPTA checklist on biosafety in CHVG, NIMR, Lagos, Nigeria

Discussion:

Fifteen occurrences of laboratory accidents (12 needle pricks and 3 sample splashes) were reported during this period. The highest incidences (n=6) were observed in 2008 and 2009. Preventive policies to counter this were established and that helped achieve reduction in laboratory accidents. The laboratory was erstwhile utilizing syringes and needles for phlebotomy, which was abolished and a policy of "no recap of needle" was introduced in 2011. Training on the no-recap policy was done and use of needles was abolished and replaced by vacutainer use, which is a closed system of blood collection. The use of vacutainer helped stop needle pricks and was effective as a control measure as no further accidents were reported after 2011.

The World Health Organization (WHO) recommends the use of closed systems for blood collection in phlebotomy as they have proven to be safer than open systems such as syringe and needle (9). Various studies have shown that recapping of needles is a major cause of needle pricks while the use of vacutainer for venepuncture help reduce the risk of direct exposure to blood, prevent needle pricks and are safer. This was also found in our practice as the rate of laboratory accidents due to needle pricks reduced and became non-existent in subsequent years (10, 11). Vacutainer use also assisted in improving customer satisfaction as collection of multiple samples from a single venepuncture was an advantage in the system. The use of PEP is widely accepted and proven to be effective in occupational exposure to HIV prevention, as reported in many other studies (12).

The results of the biosafety audits were used as key indicators and were monitored for 4 years. A resultant average score of 95% was achieved during this period. This improvement was as seen in other studies that ascribed improvement to participating in improvement steps and processes toward accreditation (2). Initiation of these audits and monitoring of other key indicators such as fire emergencies and drills, sample spill checks and drills, waste disposal monitoring and PEP usage, helped the successful implementation of biosafety as a laboratory culture. The implementation of QMS and a dedicated safety officer with regular biosafety audits were instrumental in institutionalizing and sustaining the biosafety process in CHVG.

Conclusion:

The key factors that helped the implementation of biosafety standards were training, resources allocation and monitoring for continual improvement. Laboratory accreditation makes training, resource allocation, monitoring and institutionalization of quality processes available and these are essential elements for implementation and sustenance of an effective biosafety program. Enrolling laboratories for accreditation allow development of competence thereby strengthening the weak health systems in developing countries. Ultimately, this helps in the global fight against infectious diseases particularly the re-emerging highly contagious diseases of our times.

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Short Communication **Open Access**

Effects of rinsing on *Staphylococcus aureus* load in frozen meats and fish obtained from open markets in Benin City, Nigeria

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Abstract:

Background: *Staphylococcus aureus* is a ubiquitous bacterium present in the environment and one of the leading causes of superficial and deep infections. In the food industry, it is acclaimed to be globally responsible for several food-borne diseases. This study was designed to isolate methicillin-resistant *S. aureus* (MRSA) and determine the effect of rinsing on MRSA load in frozen meat and fish obtained from open market in Benin City.
Methodology: Forty frozen meat samples (15 beef, 10 fish and 15 chickens) were randomly obtained from five markets in Benin City. The samples were analysed before and after rinsing using standard culture-based techniques to determine heterotrophic bacterial count, isolation of *S. aureus,* MRSA, and antibiotic susceptibility testing. Data were analysed using SPSS version 21 and Microsoft excel 2016, and association between variables were measured using Student's *t*-test with a probability level of < 0.05.
Results: The natural logarithm (LN) of heterotrophic bacterial count (CFU/g) before rinsing were 11.53±1.25 (beef), 11.16±0.95 (fish) and 11.42±1.58 (chicken), while the counts after rinsing were 2.70±0.45 (beef), 2.68±0.25 (fish) and 2.79±0.49 (chicken) ($p<0.05$). Sixteen of the 40 (40%) were positive for *S. aureus*, of which 4 (10%) were MRSA. Amongst the frozen meat evaluated in the study, beef had the highest frequency of *S. aureus* contamination (46.7%) followed by chicken (40.0%) and fish (30.0%). The profile of antibiotic resistance of *S. aureus* showed that they were least resistant to ciprofloxacin (6%) but showed high resistance to erythromycin (94%), amoxicillin/clavulanic acid (87.5%) and trimethoprim-sulfamethoxazole (81%). Multiple antibiotic resistance index of *S. aureus* was calculated to be 0.63.
Conclusion: The findings in this study revealed that frozen foods could act as a vehicle for the dissemination of antibiotic-resistant bacteria (ARB) and potential health risks for consumers.

Keywords: *Staphylococcus aureus*; antibiotic-resistant bacteria; MRSA; frozen meat; rinsing

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Effets du rinçage sur les charge de *Staphylococcus aureus* dans les viandes congelées et les poissons obtenus sur les marchés ouverts de Benin City, Nigéria

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Abstrait:

Contexte: *Staphylococcus aureus* est une bactérie ubiquitaire présente dans l'environnement et l'une des principales causes d'infections superficielles et profondes. Dans l'industrie alimentaire, il est reconnu pour être globalement responsable de plusieurs maladies d'origine alimentaire. Cette étude a été conçue pour isoler *S. aureus* résistant à la méthicilline (SARM) et déterminer l'effet du rinçage sur la charge de SARM dans la viande et le poisson congelés obtenus sur le marché libre de Benin City.
Méthodologie: Quarante échantillons de viande congelée (15 bœuf, 10 poissons et 15 poulets) ont été obtenus au hasard sur cinq marchés de Benin City. Les échantillons ont été analysés avant et après le rinçage en utilisant des techniques de culture standard pour déterminer le nombre de bactéries hétérotrophes, l'isolement de *S. aureus*, le SARM et les tests de sensibilité aux antibiotiques. Les données ont été analysées à l'aide de SPSS version 21 et de Microsoft Excel 2016, et l'association entre les variables a été mesurée à l'aide du test t de Student avec un niveau de probabilité <0,05.
Résultats: Le logarithme naturel (LN) du nombre de bactéries hétérotrophes (UFC/g) avant rinçage était de 11,53±1,25 (bœuf), 11,16±0,95 (poisson) et 11,42±1,58 (poulet), tandis que les comptages après rinçage étaient de 2,70±0,45 (bœuf), 2,68±0,25 (poisson) et 2,79±0,49 (poulet) (p<0,05). Seize des 40 (40%) étaient positifs pour *S. aureus*, dont 4 (10%) étaient SARM. Parmi les viandes congelées évaluées dans l'étude, le bœuf présentait la fréquence la plus élevée de contamination par *S. aureus* (46,7%), suivi du poulet (40,0%) et du poisson (30,0%). Le profil de résistance aux antibiotiques de *S. aureus* a montré qu'ils étaient les moins résistants à la ciprofloxacine (6%) mais présentaient une résistance élevée à l'érythromycine (94%), à l'amoxicilline/acide clavulanique (87,5%) et au triméthoprime-sulfaméthoxazole (81%). L'indice de résistance aux antibiotiques multiples de *S. aureus* a été calculé à 0,63.
Conclusion: Les résultats de cette étude ont révélé que les aliments surgelés pourraient servir de vecteur de dissémination de bactéries résistantes aux antibiotiques (ARA) et de risques potentiels pour la santé des consommateurs.

Mots clés: *Staphylococcus aureus*; bactéries résistantes aux antibiotiques; SARM; viande congelée; rinçage

Introduction:

Staphylococcus aureus is a ubiquitous bacterium in the environment as well as one of the leading causes of superficial infection in the hospital environment. In the food industry, it is acclaimed to be globally responsible for several food-borne diseases precisely ranked as the third most important foodborne pathogen (1). Frozen meats, fish or chicken are an important source of protein in diets such that day-to-day consumption of either of beef, fish or chicken is a common phenomenon across people of all social strata. It is worthy of note that none of the aforementioned frozen food is consumed directly without prior rinsing before cooking. Nonetheless, it is also on record that following poor storage conditions, unhygienic practices and when not properly cooked, frozen food might become a time-bomb for community infection by pathogenic bacteria. The above has been proven to be true because certain bacteria, particularly MRSA are capable of producing toxins which are heat stable (2).

Moreover, some strains of bacteria isolated from food-producing animals have been reported to be pathogenic, with potential public health implications in humans (3,4). Individuals with compromised immune system such as the elderly and children (with under-developed immune system) are majorly at high risk of infection with pathogenic *S. aureus*. Infections caused by the bacterium are either through active invasion of cells and tissues leading to abscess formation in the affected areas or through production and release of toxins that can kill cells and destroy tissues (5). Treatments of infections due to *S. aureus* have been a challenge especially because of multi-resistant strain such as the methicillin-resistant *S. aureus* (MRSA).

MRSA is a pathogen of increasing significance in the hospital and community (3). The strain of *S. aureus*, which is resistant to methicillin and related antibiotics are usually very difficult to treat and manage. Till date, several strains of MRSA have successfully been isolated from different sources just as Petinaki and Spiliopoulou (6) opined that they can be categorized according to their sources of isolation, which could either be in the hospital settings (hospital-associated MRSA, HA-MRSA, food animals or livestock (livestock-associated MRSA, LA-MRSA) or from the community (community-associated MRSA, CA-MRSA). The presence of MRSA or any bacteria of public health importance in frozen food products can pose a potential health hazard for consumers and by extension the entire community thereby resulting in serious economic loss, as well as low human productivity (2).

Most times, detection of staphylococci in meat is often connected to poor hygienic practices during slaughtering, transportation, chopping, storage and point of sale, by persons involved in the chain of production. Pathogenic bacteria from contaminated frozen foods can be transferred to other high-risk surfaces which

could further serve as source of infection in the community. More so, stainless steel surfaces, polythene surfaces, and bags, can harbour large number of bacteria from contaminated frozen meats (7,8).

Increasing levels of antimicrobial resistance have been reported in staphylococci, which make this pathogen become an important challenge in disease control (3). Despite the enormous public health challenge posed by this pathogen, there are limited informations available on their occurrence and characteristics in frozen foods such as beef, fish and chicken in Benin City. In this study, we attempt to evaluate the presence of MRSA and the effect of rinsing on the bacterial burden of frozen animal food products sold in different markets within Benin metropolis to examine the public health significance.

Materials and method:

Study setting and sample collection

Random samples of poultry meat (n= 15), ice fish (n=10) and beef (n=15) were collected from five randomly selected open markets in Benin City: Oliha, Ogida, Useh, Oluku and Egor markets. The samples were transported in clean polythene bags in cooler boxes to the Research Laboratory of Applied Microbial Processes and Environmental Health, University of Benin. All samples were analyzed within 4 hours of collection.

Sample analysis

Samples were analyzed in triplicates both in unwashed (without rinsing) and washed (properly rinsed with distilled water) forms. The standard bacteriological technique was employed using a ten-fold serial dilution for the unwashed samples while a five-fold serial dilution was used for the washed samples. For the unwashed frozen meat samples, 30 g of the meat (generic) was weighed into a flask containing 270 ml of peptone water. For the washed or the rinsed sample, the same quantity of meat (30 g) was weighed into 120 ml of sterile peptone water. The formula employed for the demystification of dilution factor is given as; dilution factor = final volume/aliquot volume, where final volume = aliquot volume (sample volume) + diluent volume.

The stock solution was then serially diluted into a set of five tubes maintaining the dilution factors for unwashed meat samples. After serial dilution, 100 µl (for the unwashed samples) and 1000 µl (for the rinsed sample) of inoculum volumes from the third, fourth and fifth tubes of the sets were plated in triplicates on nutrient agar supplemented with fluconazole (to prevent fungal growth). The plates were incubated at room temperature 28±2°C for 24 –48 hours. Bacterial colonies were counted using a colony counter and results recorded. Enumeration of the isolates was conducted using the formula by Willey et al., (5); $\frac{cfu}{g} = \frac{number\ of\ colonies\ x\ dilution\ factor}{volume\ of\ inoculum}$

Isolation of *Staphylococcus aureus* from frozen food samples

Following enumeration of heterotrophic bacteria (total bacterial load) from the meat samples, colonies were first sub-cultured on nutrient agar. Colonies presumptively identified as *S. aureus* from positive Gram stain, and catalase and coagulase tests positive, were sub-cultured on selective media; mannitol salt agar, Vogel Johnson, and oxacillin-resistant screening agar plates (Oxoid, Basingstoke, Hampshire, England) to phenotypically confirm their identity as *S. aureus* and methicillin-resistant *S. aureus* (MRSA).

Antibiotic susceptibility testing

Antibiotic susceptibility test (AST) was performed on confirmed isolates by the Bauer-Kirby disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guideline (9), using standard antibiotic disks (Oxoid, Basingstoke, England).

For the AST, bacterial cultures (18-24 hours) were standardized to 0.5 McFarland standards (~1.5×10^8 CFU/ml) and streaked on Mueller Hinton agar (MHA) using a sterile loop. Antibiotic disks were placed on the plates using a sterile forcep and incubated aerobically for 24 hours at 37ºC. The diameter of inhibition zone around each disk was measured using a meter rule and interpreted as resistant (R), intermediate resistant (I) and sensitive (S) according to the CLSI guideline (9).

The antibiotic disks used were ciprofloxacin CIP (5µg), amoxicillin/clavulanic acid AMC (20/10µg), erythromycin E (15µg), ceftriaxone CRO (30µg), trimethoprim/sulfamethoxazole (1.25/23.75µg), meropenem MEM (10µg) and gentamicin CN (10 µg).

Multiple antibiotic resistance (MAR) index

The MAR index was determined using the method described by Chitanand et al., (10), which defines MAR index as percentage of antibiotics isolate is resistant to divided by the sum of the percentages of total antibiotics tested against the isolate. According to Davis and Brown (11), MAR index of isolates ≥ 0.2 is indicative of high antibiotic use.

Statistical analysis of data

Data were entered into Microsoft Excel 2016 and analysed using SPSS version 21.0. Descriptive statistics (means, standard deviation and percentages) and Students' '*t*' test were used for comparative analysis of HBC of unwashed and rinsed frozen food samples using a probability level of < 0.05.

Results:

The mean (±SD) of logarithmic heterotrophic bacterial counts for beef, fish and chicken samples before rinsing were respectively 11.53±1.25 CFU/g, 11.16±0.95 CFU/g and 11.42±1.58 CFU/g compared to respective counts of 2.7±0.45, 2.68±0.25 and 2.79±0.45 after rinsing (Fig 1), which showed significant differences ($p<0.05$) in the bacterial burden of frozen meat samples before and after rinsing (Table 1).

The overall prevalence of *S. aureus* and MRSA in the frozen meat samples were 40% (16/40) and 10% (4/40) respectively. Of the frozen meat evaluated, beef had the highest frequency of *S. aureus* contamination (46.7%), followed by chicken (40.0%) and fish (30.0%). The antibiotic resistance profile of *S. aureus* showed high resistance to erythromycin (94%), amoxicillin/clavulanic acid (87.5%) and trimethoprim-sulfamethoxazole (81%). The MARI of *S. aureus* was 0.63, which is above the permissible value of 0.2.

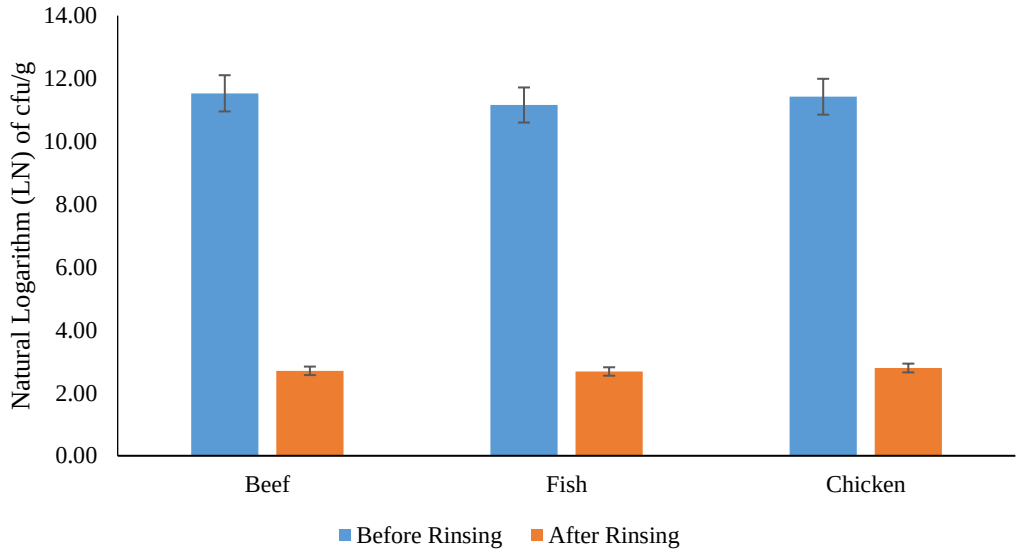

Fig 1: Mean logarithm heterotrophic bacterial count (CFU/g) of frozen food samples before and after rinsing

Table 1: Mean logarithm heterotrophic bacteria counts of frozen food samples before and after rinsing

Parameters	Before rinse	After rinse	*p* value	T. crit	T. cal
Beef	11.53±1.25	2.70±0.45	0.001	2.365	5.356
Fish	11.16±0.95	2.68±0.25	0.036	2.365	2.598
Chicken	11.42±1.58	2.79±0.49	0.013	3.182	5.303

Tcrit= T critical; Tcal= T calculated

Table 2: Prevalence of *S. aureus* and MRSA from frozen meat samples from markets in Benin City

Samples	Total number of samples	*Staphylococcus aureus* (%)	MRSA (%)
Beef	15	7 (46.7)	2 (13.3)
Fish	10	3 (30.0)	0 (0)
Chicken	15	6 (40.0)	2 (13.3)
Total	40	16 (40.0)	4 (10.0)

Table 3: Antibiotic susceptibility of staphylococcal isolates from meat samples

Antibiotics	Susceptibility profile (n=16)	
	Sensitive (%)	Resistant (%)
Meropenem (10µg)	11 (69.0)	5 (31.0)
Erythromycin (15µg)	1 (6.0)	15 (94.0)
Amoxicillin/clavulanic acid (20/10µg)	2 (12.5)	14 (87.5)
Ceftriaxone (30µg)	9 (56.0)	7 (44.0)
Trimethoprim/sulfamethoxazole (1.25/23.75µg)	3 (19.0)	13 (81.0)
Gentamicin (10µg)	6 (37.5)	10 (62.5)
Ciprofloxacin (5µg)	15 (94.0)	1 (6.0)

Discussion:

The detection of *S. aureus* in frozen meat (beef, fish and chicken) samples is to pave way for identifying possible source of contamination in case there is an outbreak of infection due to improperly cooked frozen meats. It is also very necessary because pathogenic *S. aureus* has been known to produce several heat-stable toxins capable of causing harm in humans. The latter is such that the bacterium produces the toxin in the meat samples and while the bacterium could be eliminated by heat (high temperature), the heat-stable toxin would still be potent to cause injury after consumption of such contaminated products.

The heterotrophic bacterial counts of the frozen food samples (beef, fish & chicken) before rinsing were found to be above LN 11.10 CFU/g. The results from this study agree with that of Islam et al., (12) who reported bacterial counts of LN 11.5 CFU/g from frozen chicken samples. The values obtained in our study is also similar to the study by Al-Tarazi et al., (13) who reported mean bacterial count of meat samples in the range of LN 6.27 to LN 10.67. Also closely related to the counts obtained in our study, Fahim et al., (14) reported bacterial count of LN 11.33 CFU/g. The bacterial counts in these studies could be related to microbiology of the meat samples, especially with reference to the microbiome of the animals. Environmental factors as well as hygiene status of food handlers also have a role to play in reference to the heterotrophic count of bacteria in meat.

Technically speaking, the bacterial counts after rinsing was significantly different from the counts before rinsing. This emphasize the importance of rinsing of food materials prior to cooking and consumption as this practice reduce the number of potential micro-organisms capable of causing infection in the host. Although there is no standard guide on how proper rinsing should be done, the mere act of rinsing can reduce microbial population and the risk of injury to humans.

In this study, a total of 40 % of frozen meats were contaminated with *S. aureus* while 10% were MRSA isolates. This finding is in agreement with several studies in literatures which had reported the presence of *S. aureus* in frozen meat samples. Wu et al., (15) reported that 35% of frozen meat samples and meat products in China were contaminated with *S. aureus* and a further 7.5% of the isolates being confirmed MRSA. Savariraj et al., (16) isolated the bacterium in 76.7% of frozen retail pork outlets in India. Higher prevalence of *S. aureus* has also been reported from frozen meat and fresh meat samples in Karbala province (Iraq) where 64% of the frozen meat samples were contaminated with this bacterium (17). In Nigeria, the situation is not different as Oranusi et al., (18) posited that while freezing is meant to preserve food by halting the growth of microbes, the reports of illness involving frozen food is definitely a function of microbial activity. Igbinosa et al., (3) also made similar

observations with regards to *S. aureus* and strains of MRSA where it was reported that 20% and 28% of chicken and beef samples respectively were contaminated with MRSA. Moreover, Igbinosa et al., (3) reported multi-drug resistance profile (which is described as resistance to antimicrobial agent in more than two antibiotic classes) for MRSA strains which were of public health importance. Our finding is similar to this, where *S. aureus* isolates were tested against antibiotics in eight different classes. One of the isolates was resistant to all eight antibiotic classes while majority of the isolates (>80%) were resistant to the following antibiotics; erythromycin (94%), amoxicillin/ clavulanic acid (87.5%), and trimethoprim-sulfamethoxazole (81%). Similar multi-drug resistance pattern was reported by Islam et al., (12) and Al-Tarazi et al., (12) in *S. aureus* isolates from frozen meats.

The multiple antibiotic resistance (MAR) index is used to evaluate usage of antibiotics in an environment. The MAR index of 0.63 for *S. aureus* isolates in our study is an indication of overuse of antibiotics in our environment, and health risks associated with transmission and spread of MDR bacteria pathogens. MDR raises a health concern because it limits treatment options available for microbial infections (3), which can lead to prolongation of illness, higher cost of therapy, and increased risks of death.

Conclusion:

The assessment of MRSA from frozen foods in Benin City shows that obvious frozen foods can be home or habitation for bacterial pathogens of public health importance. Proper rinsing, which can eliminate plethora of patho-genic bacteria isolates from frozen food before preparation should be emphasized. With the increase in prevalence of antibiotic-resistant bacteria in frozen foods, public health aware-ness is essential as a preventative measure.

Conflicts of interest:

Authors declare no conflict of interest

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Short Communication **Open Access**

Improved *Cryptosporidium* case findings using immunofluorescent microscopy on concentrated stool

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Abstract:

Background: Diarrhoea is a major cause of morbidity in Cape Town, South Africa, and mortality is attributed to a failure to recognize the severity of the condition. *Cryptosporidium* and *Giardia* are increasingly recognized as important causes of diarrhoea in Africa however, suboptimal diagnostic techniques may lead to underappreciation of their significance. Our objectives are to compare the diagnostic yield of direct immunofluorescent antigen (DFA) microscopy on concentrated stool samples for *Cryptosporidium* and *Giardia*, with the current approach of wet mount microscopy for *Giardia* and auramine fluorescent stain for *Cryptosporidium* on unconcentrated stool.
Methodology: Stool specimens (n=104) received at our hospital laboratory for routine microbiological investigations were used for the study. Direct wet-mount auramine-phenol fluorescent microscopy (auramine) detection of *Cryptosporidium* oocysts and wet mount iodine microscopy for *Giardia* detection, were performed on unconcentrated stool samples, while DFA stain for simultaneous detection of *Cryptosporidium* and *Giardia* was performed on sodium-acetate formalin concentrated stool samples. The diagnostic yields of the tests were compared using the MEDCALC® version 18.0
Results: Of the 104 stool specimens received for microbiological analysis, only 66 (63.5%) had specific *Cryptosporidium* requests while 38 (36.5%) had no *Cryptosporidium* specific requests. Of the 66 specimens, 9 (13.6%) were positive for *Cryptosporidium* oocysts with DFA while only 1 (1.5%) was positive with auramine staining (p=0.013). The one auramine-positive specimen was also positive by DFA. Auramine stain microscopy gave a sensitivity of 11.1% (95%CI: 0.28-48.25%) and specificity of 100% (95%CI: 93.7%-100%) when compared to DFA. Of the 38 stool specimens without specific *Cryptosporidium* request, DFA yielded 5 (13.2%) additional positive results. Taken together, *Cryptosporidium* was detected in 14/104 (13.5%; 95%CI: 8.36–21.7%) specimens and only 1 of 14 (7.1%) specimens with the current routine laboratory testing approach. *Giardia* was detected by DFA in 3/104 (0.9%) specimens, while direct iodine wet mount microscopy did not yield any positive results (0%). All 3 *Giardia*-positive specimens had *Cryptosporidium* oocysts detected by DFA.
Conclusion: These data suggest that a large proportion of *Cryptosporidium* cases remain undetected by the laboratory due to suboptimal testing methods, and failure by clinicians to specifically request for *Cryptosporidium* detection. There is need to periodically assess the effectiveness of diagnostic microbiology laboratory approaches to diarrhoea, and access to improved diagnostic laboratory techniques will contribute to more accurate differential diagnosis and a broadened understanding of local aetiology of diarrhoea diseases in Africa.

Keywords: *Cryptosporidium*, *Giardia*, diarrhoea, stool concentration, DFA, microscopy

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Amélioration des découvertes de cas de *Cryptosporidium* à l'aide de la microscopie immunofluorescente sur des selles concentrées

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Abstrait:

Contexte: La diarrhée est une cause majeure de morbidité au Cap, en Afrique du Sud, et la mortalité est attribuée à l'incapacité de reconnaître la gravité de la maladie. *Cryptosporidium* et *Giardia* sont de plus en plus reconnus

comme des causes importantes de diarrhée en Afrique, cependant, des techniques de diagnostic sous-optimales peuvent conduire à une sous-estimation de leur importance. Nos objectifs sont de comparer le rendement diagnostique de la microscopie à antigène immunofluorescent direct (DFA) sur des échantillons de selles concentrées pour *Cryptosporidium* et *Giardia*, avec l'approche actuelle de la microscopie à montage humide pour Giardia et la coloration fluorescente auramine pour *Cryptosporidium* sur des selles non concentrées.
Méthodologie: Des échantillons de selles (n=104) reçus au laboratoire de notre hôpital pour des examens microbiologiques de routine ont été utilisés pour l'étude. La détection directe par microscopie fluorescente auramine-phénol à montage humide (auramine) des oocystes de *Cryptosporidium* et la microscopie à l'iode à montage humide pour la détection de *Giardia*, ont été effectuées sur des échantillons de selles non concentrées, tandis que la coloration DFA pour la détection simultanée de *Cryptosporidium* et de Giardia a été réalisée sur de l'acétate de sodium formaline concentré échantillons de selles. Les rendements diagnostiques des tests ont été comparés à l'aide de MEDCALC® version 18.0
Résultats: Sur les 104 échantillons de selles reçus pour l'analyse microbiologique, seuls 66 (63,5%) avaient des demandes spécifiques de *Cryptosporidium* tandis que 38 (36,5%) n'avaient pas de demandes spécifiques de *Cryptosporidium*. Sur les 66 échantillons, 9 (13,6%) étaient positifs pour les oocystes de *Cryptosporidium* avec DFA tandis que seulement 1 (1,5%) était positif avec coloration à l'auramine (p=0,013). Le seul échantillon positif à l'auramine était également positif au DFA. La microscopie à l'auramine a donné une sensibilité de 11,1% (IC 95%: 0,28-48,25%) et une spécificité de 100% (IC 95%: 93,7% -100%) par rapport au DFA. Sur les 38 échantillons de selles sans demande spécifique de *Cryptosporidium*, le DFA a donné 5 (13,2%) résultats positifs supplémentaires. Pris ensemble, *Cryptosporidium* a été détecté dans 14/104 (13,5%; IC à 95%: 8,36–21,7%) et seulement 1 des 14 échantillons (7,1%) avec l'approche actuelle des tests de routine en laboratoire. *Giardia* a été détecté par DFA dans 3/104 (0,9%) échantillons, tandis que la microscopie directe à l'iode sur monture humide n'a donné aucun résultat positif (0%). Les 3 échantillons positifs à *Giardia* avaient des oocystes de *Cryptosporidium* détectés par DFA.
Conclusion: Ces données suggèrent qu'une grande proportion des cas de *Cryptosporidium* ne sont pas détectés par le laboratoire en raison de méthodes de test sous-optimales et de l'échec des cliniciens à demander spécifiquement la détection de *Cryptosporidium*. Il est nécessaire d'évaluer périodiquement l'efficacité des approches de laboratoire de microbiologie diagnostique pour la diarrhée, et l'accès à des techniques de laboratoire de diagnostic améliorées contribuera à un diagnostic différentiel plus précis et à une compréhension élargie de l'étiologie locale des maladies diarrhéiques en Afrique.

Mots clés: *Cryptosporidium, Giardia*, diarrhée, concentration des selles, DFA, microscopie

Introduction:

Diarrheal disease in developing countries account for up to 21% of deaths in children less than 5 years. Approximately 78% of these occur in Africa and South East Asia (1). In South Africa, diarrhea is the third leading cause of infant mortality (16%), trailing behind deaths in the neonatal period (27.5%) and HIV/AIDS (19.5%) (2). In the Western Cape Province, diarrhoea is the third leading cause of under-five mortality (11%); almost half (42.9%) of child diarrheal deaths in the Cape Town metro sub-district occur at home (3). Among those who seek primary healthcare, some are locally managed, while others are referred to tertiary care; a main contributing factor for diarrheal death among those referred to tertiary care in South Africa is a failure to correctly assess the severity of the child's condition (4).

The enteric coccidian parasites, *Cryptosporidium parvum* and *Cryptosporidium hominis*, and the protozoan parasite, *Giardia duodenalis* are important causes of diarrhea globally. *Cryptosporidium* is increasingly recognized as a leading cause of moderate-to-severe diarrhea in both immunocompetent and immunosuppressed subjects (5). The Global Enteric Multicenter Study (GEMS) showed that *Cryptosporidium* is second only to Rotavirus as a contributor to moderate-to-severe diarrhea in sub-Saharan Africa (6). *Cryptosporidium* is transmitted via contaminated food, water, and from person-to-person particularly where suboptimal sanitation and limited access to safe drinking water prevail. Similarly transmitted, *Giardia* is a major cause of intestinal disease globally, with a higher prevalence in Africa (7). Chronic infection can lead to weight loss and malabsorption and is associated with stunting, wasting and cognitive impairment in children (8).

Clinical management guidelines highlight the need, importance and calls for improved effectiveness of test and treat approaches to diarrhoea (1,9,10). Availability of accurate diagnostic laboratory tests are neglected, and evaluation and improvements are much needed (1,11,12). Our objectives were to employ stool concentration and immunofluorescent stain for the detection of *Cryptosporidium* and *Giardia* on all stool samples received for microbiological investigations at an academic pathology laboratory in Cape Town, South Africa, and compare findings to those obtained with the current methods employed by the laboratory.

Materials and method:

Study setting and specimens

Unpreserved diarrheal stool specimens (n=104) received for routine microbiological investigations at the National Health Laboratory Services (NHLS), University of Cape Town Department of Pathology at Groote Schuur Hospital, were collected from 12 June through

6 August 2014. The NHLS laboratory receives specimens for testing from hospitalized patients as well as specimens from secondary hospitals and clinics in the Southern Cape Town area, including the Red Cross Children's Hospital, a national paediatric referral hospital. Current routine laboratory investigations include direct wet mount with iodine microscopic analysis of stool specimens. Auramine-phenol fluorescent microscopy is performed when *Cryptosporidium* or *Isospora* testing is specifically requested. The remains of stool specimens after routine testing were collected by the researchers who were blinded to testing results.

Ethical approval

This study was approved by the Human Research Ethics Committee, Faculty of Health Sciences, University of Cape Town (Ref: 240/2014).

Microscopic examination of specimens

A grape sized amount, or a 5ml aliquot of liquid stool, was added to 40ml of sodium acetate-acetic acid-formalin (SAF) in a 50ml conical centrifuge tube and mixed by inversion until homogenous mixture was attained. The preserved stool samples were concentrated using a commercial kit (Para-Pak® Spin-Con® Stool Concentration System, Meridian Bioscience, Cincinnati, OH) according to the manufacturer's package insert (13). A direct immunofluorescent assay (DFA) (Merifluor® *Cryptosporidium/Giardia* Direct Immunofluorescent Assay, Meridian Bioscience) was performed according to the manufacturer's instructions (14).

Each slide was viewed under an LED fluorescent microscope with an excitation wavelength of 490-500nm under the 40x objective lens with a calibrated eyepiece 10x ocular lens with micrometer (total magnification 400x) for specific size measurements. Samples were screened for *Cryptosporidium* oocysts and *Giardia* cysts based on colour, shape and size. Both internal kit controls and external control slides were included in each staining process. External control slides comprising stool specimens previously shown to contain *Cryptosporidium* oocysts and *Giardia* cysts were kindly provided by the Centre for Opportunistic, Hospital and Tropical Diseases, National Institute for Communicable Diseases, South Africa.

Statistical analysis

Statistical calculations for diagnostic test evaluation were performed using MEDCALC® version 18 (www.medcalc.org) and McNemar's test for significance was applied. P value less than 0.05 was considered to be statistically significant.

Results:

Of the 104 stool specimens received for microbiological analysis, only 66 (63.5%) had specific *Cryptosporidium* request. Of the 66 stool specimens tested for *Cryptosporidium* oocysts using both the routine direct wet-mount auramine-phenol fluorescent microscopy and the DFA microscopy on concentrated specimen, only 1 (1.5%) specimen yielded a positive auramine finding compared to 9 (13.6%) with DFA (p=0.013). The one auramine-positive specimen was also positive by DFA. Direct wet-mount auramine microscopy yielded a sensitivity of 11.1% (95% CI: 0.28%-48.25%) and specificity of 100% (95% CI: 93.7%-100%) when compared to DFA.

Of additional 38 stool specimens received for microbiological analysis without a specific request for *Cryptosporidium* testing (no direct auramine), DFA yielded 5 (13.2%) additional positive results. Taken together, *Cryptosporidium* was detected in 14/104 (13.5%; 95%CI: 8.36–21.7%) specimens, and only 1 of 14 (7.1%) cases was successfully detected with the current routine laboratory testing approach. *Giardia* was detected by DFA in 3/104 (0.9%) specimens, while direct iodine wet mount microscopy did not yield any positive results (0%). All 3 *Giardia*-positive specimens had *Cryptosporidium* oocysts detected in them by DFA.

Discussion:

Case findings of *Cryptosporidium* in concentrated stool combined with DFA microscopy was significantly higher (13.6%) than those obtained using unconcentrated stool microscopy with auramine (1.5%). Similarly, case findings of *Giardia* were enhanced with DFA on concentrated stool (4.5%) compared to direct iodine microscopy (0%). An additional 5 of 38 cases of *Cryptosporidium* were detected in stool without a request for *Cryptosporidium* investigation. These data suggest that a large proportion of *Cryptosporidium* cases remain undetected by the laboratory due to suboptimal testing methods used, as well as a failure by clinicians to specifically request testing for *Cryptosporidium*.

The *Cryptosporidium* prevalence of 13.5 % (14/104) reported in this study is in line with findings elsewhere in South Africa. In the Northern metro district of Cape Town Nel et al., reported 10.4% of children under 5 years of age with diarrhoea yielded *Cryptosporidium* (15). In Limpopo province, *Cryptosporidium* was reported in 25.2% of hospital patients and 17.9% of diarrheal samples in school children (16). Our findings are consistent with the recognized laboratory standards

recommending concentration of stool for recovery of intestinal parasites, and the improved sensitivity and specificity of DFA for the detection of *Cryptosporidium* (17,18).

This study was limited to the months of June to August representing the winter/rain season in the Western Cape. The etiological causes of diarrhea may be affected by seasonality. However, *Cryptosporidium* is more commonly found in cases of diarrhoea than is suspected. Relying on unconcentrated stool for detection of *Cryptosporidium* is therefore suboptimal. DFA detection on concentrated stool improves case findings. These findings suggest the need to periodically assess the effectiveness of the microbiological diagnostic approach into the causes of diarrhoea.

Authors contributions:

FJLR conceptualized the study, obtained funding, and provided supervision. DC performed the investigation and curated the data. Both authors contributed to the analysis of data and writing of the manuscript.

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Conflicts of interest:

No conflicts of interest are declared.

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Short Communication **Open Access**

Non-tuberculous mycobacteria isolated from patients with suspected tuberculosis in Abidjan, Ivory Coast

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Abstract:

Background: Apart from tuberculosis caused by *Mycobacterium tuberculosis* complex (MTBc) species, there are many other mycobacterial infections due to nontuberculous mycobacteria (NTM). These are rarely identified in many low resource settings in Africa because of the lack of accurate identification methods. The aim of the study is to identify NTM species involved in respiratory infections in Abidjan, Ivory Coast.
Methodology: Isolates routinely identified as NTM by the detection of MPT64 antigen between 2015 and 2018 at the Centre for Diagnosis and Research on AIDS and other Infectious Diseases (CeDReS) of the University Hospital of Treichville, were included in the study. Bacterial strains were sub-cultured on three different Lowenstein-Jensen media in order to determine their cultural characteristics, and molecular identification of the strains was performed first by polymerase chain reaction (PCR) assay followed by reverse hybridization (GenoType Mycobacterium CM and AS kits, Hain Lifescience, Germany). The Cohen's kappa statistical coefficient was used to evaluate the degree of agreement of the phenotypic with the molecular method.
Results: Of 62 NTM isolates tested with the molecular method, 54 (87.1%) tested positive and the main species identified were *Mycobacterium fortuitum* (52%), followed by *Mycobacterium abscessus* (13%) alone or in combination with other species. Thirty-six (58.1%) of the 62 NTM isolates were identified phenotypically, out of which 31 (86.1%) were correctly identified by molecular method. The comparison of molecular and phenotypic methods revealed a good concordance, allowing the use of cultural patterns as identification tests in resource limited settings. However, MTBc isolates were identified among the NTM isolates, indicating that even if the rapid test for detection of MPT64 antigen is quite accurate, it could lack sensitivity and specificity in some cases.
Conclusion: *Mycobacterium fortuitum* and *M. abscessus* were identified as the main NTM species circulating in Abidjan but there is need for additional evaluation of MPT64 antigen detection assay for MTBc.

Keywords: non-tuberculous mycobacteria, identification, PCR, GenoType CM/AS, culture

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Mycobactéries non tuberculeuses isolées chez des patients suspects de tuberculose à Abidjan, Côte d'Ivoire

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Résumé:

Contexte: Outre la tuberculose causée par les espèces du complexe *Mycobacterium tuberculosis* (MTBc), il existe de nombreuses autres infections mycobactériennes dues à des mycobactéries non tuberculeuses (MNT). Ceux-ci sont rarement identifiés dans de nombreuses régions à ressources limitées, notamment en Afrique en raison du manque de méthodes d'identification précises. Le but de l'étude était d'identifier les espèces de MNT impliquées dans les infections respiratoires à Abidjan, en Côte d'Ivoire.
Méthodologie: Des isolats identifiés en routine comme étant des MNT par la détection de l'antigène MPT64 entre

2015 et 2018 au Centre de diagnostic et de recherche sur le sida et autres maladies infectieuses (CeDReS) sis au sein du CHU de Treichville, ont été inclus dans l'étude. Les souches bactériennes ont été réisolées sur trois milieux de Lowenstein-Jensen différents afin de déterminer leurs caractéristiques culturales, et l'identification moléculaire des souches a d'abord été réalisée par un test réaction de polymérisation en chaîne (PCR) suivi d'une hybridation inverse (kits GenoType Mycobacterium CM et AS, Hain Lifescience, Allemagne). Le test statistique kappa de Cohen a été utilisé pour évaluer le degré d'accord entre le phénotype et la méthode moléculaire. **Résultats:** Sur 62 isolats de NTM testés avec la méthode moléculaire, 54 (87,1%) ont été trouvés positifs les principales espèces identifiées étant *Mycobacterium fortuitum* (52%), suivi de *Mycobacterium abscessus* (13%) seul ou en association avec d'autres espèces. Trente-six (58,1%) des 62 isolats de MNT ont été identifiés phénotypiquement, parmi lesquels 31 (86,1%) ont été correctement identifiés par la méthode moléculaire. La comparaison des méthodes moléculaires et phénotypiques a révélé une bonne concordance, permettant l'utilisation de caractères culturaux comme tests d'orientation dans des zones à ressources limitées. Cependant, des isolats de MTBc ont été identifiés parmi les isolats de MNT, indiquant que même si le test rapide de détection de l'antigène MPT64 est assez précis, il pourrait manquer de sensibilité et de spécificité dans certains cas. **Conclusion:** *Mycobacterium fortuitum* et *M. abscessus* ont été identifiés comme les principales espèces de MNT circulant à Abidjan mais il est nécessaire de procéder à une évaluation supplémentaire du test de détection de l'antigène MPT64 pour l'identification des MTBc.

Mots clés: mycobactéries non tuberculeuses, identification, PCR, GenoType CM/AS, culture

Introduction:

The fight against mycobacterial infections is mainly focused on tuberculosis (TB) which is caused by species belonging to the *Mycobacterium tuberculosis* complex (MTBc). However, many other species called non-tuberculous mycobacteria (NTM) or mycobacteria other than tuberculosis (MOTT) are generally encountered in the environment and can also be involved in human infections (mycobacterioses) (1). NTM were previously considered as occurring mostly in patients with immune deficiency, and particularly in persons living with HIV/AIDS (PLHIV) (2). However, NTM are currently known to cause various diseases, also in immunocompetent people, including ulcerative forms such as Buruli ulcer (3). NTM can be responsible for respiratory tract infections as well (1). Therefore, there is a need for accurate identification of NTM and discrimination from pulmonary tuberculosis.

In Ivory Coast, as in many parts of Africa where microscopy is the main tool for the diagnosis of TB and other mycobacterial infections, only few studies have investigated the implication of NTM in pulmonary diseases and a few of them have identified species involved in those infections (4). Indeed, phenotypic identification methods using cultural and biochemical characteristics have low sensitivity, while molecular tests are more accurate but costly. Thus, the identification process is mainly limited to the differentiation between MTBc and NTM strains in these settings. As a consequence, there are usually no options provided to clinicians for the management of patients with suspected NTM infections (5). However, the determination of the major NTM species circulating in a given area can be useful as specific treatments have been identified for most of them (6). The aim of the study is to determine NTM species involved in respiratory infections in Abidjan, Ivory Coast.

Materials and method:

Study setting

The study was conducted at the mycobacteriology unit of the Centre for Diagnosis and Research on AIDS and other Infectious Diseases (CeDReS), located at the University Hospital of Treichville, Abidjan, Ivory Coast.

Routine isolation of *Mycobacteria* strains

From January 2015 to January 2019, mycobacterial strains isolated from patients with suspected TB and routinely collected in our laboratory were tested. These strains were isolated following processing of the patients' samples (respiratory and non-respiratory) first by decontamination/digestion process and then inoculating both Lowenstein-Jensen (LJ) (Biorad, Marne-La-Coquette, France) and BACTEC MGIT (Becton Dickinson, Sparks, MD, USA). The LJ tubes were incubated in aerobic conditions at 37°C while MGIT tubes were incubated in MGIT 960 analyzer (Becton Dickinson, Sparks, MD, USA). Bacterial growths on LJ medium were monitored daily during the first week and then weekly for 6 weeks, and hourly on BACTEC MGIT 960 automated incubator.

For each positive culture, the isolate was identified by Ziehl-Neelsen (ZN) staining and detection of MPT64 antigen (SD BIOLINE TB Ag MPT64 Rapid, Standard Diagnostics, Seoul, South Korea) according to the manufacturer's instructions. This lateral flow assay is based on the detection of MPT64 antigen specific for MTBc isolates. Briefly, 100 µl of the liquid culture was put unto the sample well. After 15 min, a reactive result (MTBc) was characterized by the presence of two pink bands, the test "T" band, and "C" zone (control band) that confirmed the test validity. The presence of only the "C" (control band) indicated a non-reactive result, which in the presence of acid-fast bacilli on ZN staining, indicated the presence of NTM. *M. tuberculosis*

H37Rv strain and a well-characterized external quality assessment (EQA) isolate of *M. fortuitum* obtained from the South African National Health Laboratory Service (NHLS) TB culture proficiency testing, were used as positive and negative controls respectively. All the isolates displaying non-reactive results for the determination of MPT64 antigen were identified as NTM and selected isolates out of these were included in the study.

Phenotypic identification and characterization of NTM isolates

The phenotypic characterization performed on 62 consecutively selected isolates, consisted the analysis of cultural patterns i. e. time of growth and aspects of the colonies (eugonic or dysgonic, presence or absence of pigment) following inoculation of three Lowenstein-Jensen media, with two of the culture tubes covered with aluminum foil in order to allow growth in the absence of light. The LJ tubes were incubated in aerobic conditions at 37°C for 6 weeks during which bacteria culture growth was monitored daily.

Molecular identification of NTM isolates

The molecular identification of the 62 NTM isolates was carried out by using GenoType Mycobacterium CM Ver 2.0 and GenoType Mycobacterium AS kits (Hain Lifescience, Manheim, Germany) according to the manufacturer's instructions. Briefly, molecular identification of the strains started by DNA extraction, followed by an amplification step with a polymerase chain reaction, and then reverse hybridization.

DNA extraction:

The GenoLyse^{R} kit was used for extraction by adding into 2 ml Eppendorf tube, 5 µl of internal control (IC) to 1 ml of liquid culture. After centrifugation at 10000 rpm for 15 mins, the pellet was resuspended in 100 µl of lysis buffer and incubated at 95°C for 5 minutes. One hundred microliters of the neutralizing buffer were then added and the mix spined at 13000 rpm for 5 minutes. The supernatant, containing DNA was used for the PCR.

PCR assay

For the amplification using GenoType MTBDR*plus* Ver 2.0 test in a thermal cycler, 10 µl of amplification mix A (AM-A, containing buffer, nucleotides and Taq polymerase), 35 µl of amplification mix B (AM-B, containing salts, specific primers and dye) and 5 µl of DNA in a final volume of 50 µl was used. The amplification protocol included 15 min of initial denaturation at 95°C, followed by 10 cycles, comprising 30 secs at 95°C and 2 mins at 58°C plus 20 additional cycles comprising 25 secs at 95°C, 40 secs at 53°C, and 40 secs at 70°C, and a final extension at 70°C for 8 mins.

Hybridization and detection:

Hybridization and detection were performed in TwinCubator system (Hain Life science, Manheim, Germany), with a total of 17 probes in the GenoType Mycobacterium CM and GenoType Mycobacterium AS kits, including amplification and hybridization controls for quality check of the procedures. The first step consisted mixing 20 µl of the amplicons with 20 µl of denaturing reagent for 5 min in separate wells of a plastic reaction plate. The strips were then put in each well and 1 ml of prewarmed hybridization buffer added, allowing the hybridization process to occur at 45°C for 30 mins, followed by a stringent washing step. Streptavidin conjugated with alkaline phosphatase was added for 30 mins at room temperature and washed before addition of the substrate for 5 mins. A final washing was performed and strips were air dried and sticked on a paper form.

Statistical analysis

The Cohen's kappa statistical coefficient was calculated to evaluate the degree of accuracy and reliability of the phenotypic method. The test was interpreted as follows; 0.01–0.20 slight agreement; 0.21–0.40 fair agreement; 0.41–0.60 moderate agreement; 0.61–0.80 substantial agreement; 0.81–1.00 almost perfect or perfect agreement

Ethical clearance

The study was conducted on bacterial strains routinely collected in the laboratory. Therefore, no ethical clearance was needed.

Results:

During the period of study, 4750 samples were routinely analyzed for the diagnosis of mycobacterial infections and 1121 were positive on culture, including 155 NTM isolates. Sixty-two of the 155 NTM isolates were sub-cultured for phenotypic identification and characterization on the three LJ media, and 36 (58.1%) of these were successfully sub-cultured. Of these 36 isolates, 31 (86.1%) were correctly identified by molecular method. In 23 of the 31 (74%) isolates, dysgonic colonies were observed, and 94% (29/31) of the colonies were non-chromogenic while 6% (2/31) produced pigment after exposure to light (photochromogenic strains). Most of the isolates had a growth period between 1 and 7 days (84%, 26/31).

Of the 62 NTM isolates tested by the molecular method, 54 (87.1%) isolates were confirmed to be valid NTM strains. Of these, the main species identified were *M. fortuitum* (52%, 28/54), followed by *M. abscessus* (13%, 7/54), *M. intracellulare* (11%, 6/54) and *M. avium* (6%, 3/54). Surprisingly, MTBc strains were identified in four cases (alone or

in association) and species combination were observed in five cases (Fig 1). The comparison of phenotypic and molecular methods gave a kappa value of 0.94 for both pigment production (Table 1) and growth time (Table 2).

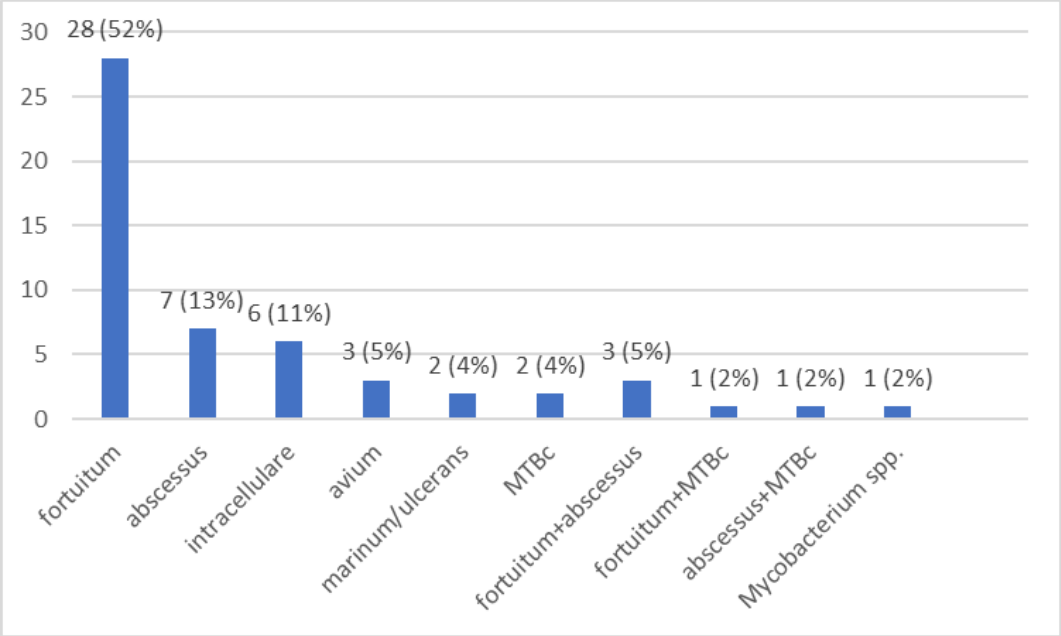

Fig 1: Non tuberculous mycobacteria species identified

Table 1: Molecular identification and pigments production

Mycobacteria	***fort***	***absc***	***intra***	***avium***	***marin/ ulcer***	***Tb cplx***	***absc/ Tb cplx***	***absc/ fort***	**Total**
Non chromogenic	19	2	3	0	1	0	1	3	29
Photochromogenic	1	0	1	0	0	0	0	0	2
Scotochromogenic	0	0	0	0	0	0	0	0	0
Total	**20**	**2**	**4**	**0**	**1**	**0**	**1**	**3**	**31**

fort=*fortuitum*; absc=*abscessus* ; intra=*intracellulare* ; marin/ulcer=*marinum/ulcerans* ; Tb cplx=*tuberculosis* complex; Grayed cell=discordance

Table 2: Molecular identification and growth delay

Days	*Fort*	*absc*	*Intra*	*Avium*	*marin/ ulcer*	*Tb cplx*	*absc/ Tb cplx*	*absc/ fort*	Total
1 – 7	19	2	1	0	0	0	1	3	26
8 – 14	0	0	0	0	0	0	0	0	0
15 – 21	1	0	2	0	0	0	0	0	3
22 – 28	0	0	1	0	1	0	0	0	2
> 29	0	0	0	0	0	0	0	0	0
Total	**20**	**2**	**4**	**0**	**1**	**0**	**1**	**3**	**31**

fort = *fortuitum*; absc = *abscessus*; intra = *intracellulare*; marin/ulcer = *marinum*/*ulcerans*; Tb cplx = *tuberculosis* complex; Grayed cell = discordance

Discussion:

This study provides a more recent insight about the distribution of NTM in Ivory Coast as the previous study was conducted more than 15 years ago (7). It also revealed that most of the isolates were fast growing mycobacteria (*M. fortuitum* and *M. abscessus*), slow growing MTN being less frequent (*M. intracellulare* and *M. avium)*. These results are quite similar to those observed in a metanalysis related to NTM isolates from respiratory samples in sub-Saharan African countries. Indeed, all these pathogens belonged to the top four species identified in various studies conducted in this area, with *M. avium* complex (MAC) being more frequently isolated (4).

Among the species belonging to this complex, *M. intracellulare* and *M. avium* are those frequently involved in opportunistic mycobacterial infections in PLHIV (2). It is noteworthy that although these mycobacteria were previously identified by the routine laboratory cultures as NTM, some isolates belonging to MTBc were also identified among them. This indicates that even if lateral flow antigen detection tests are quite accurate, they could lack sensitivity and specificity in some cases as reported by different authors (8). A relatively good concordance between the molecular test and phenotypic cultural patterns was also observed in our study, which implies that phenotypic culture method could be used as identification tests in low resource settings.

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Authors contributions:

TO designed the study, performed the tests and drafted the manuscript, MSNK and KAK performed the tests and revised the manuscript. All authors read and approved the final manuscript.

Previous presentation:

The findings of this study were presented in part at the first congress of the Societé Ivoirienne de Microbiologie (Ivorian Society for Microbiology), in Yamoussoukro, Ivory Coast on November, 29th 2019.

Conflicts of interest:

Authors declare no conflict of interest.

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Doctors do not use the medical microbiology laboratory when infectious diseases are suspected

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Les médecins n'utilisent pas le laboratoire de microbiologie médicale en cas de suspicion de maladies infectieuses

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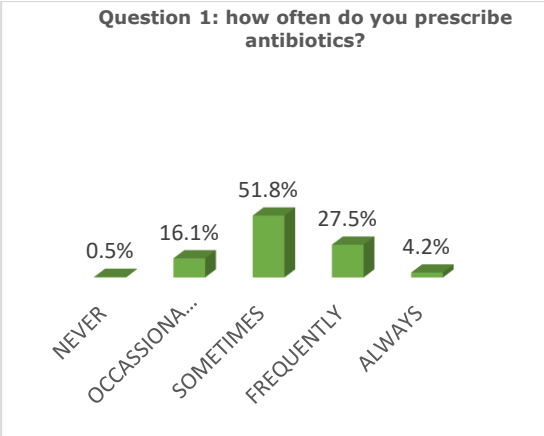
Dear Editor,

There has been a lot of talk in recent years about infection prevention and control, rational use of antibiotics (1), antimicrobial stewardship (2), antimicrobial resistance and reports of multi-resistant organisms (3) from isolates in hospitalized patients. Looking at the body of evidence available, we took a survey of doctors, their prescription patterns, request for microbiological investigations before prescription and some of the things that informed their decisions when treating patients suspected to have infections.

Of the 198 doctors in Port Harcourt, Nigeria who responded to the online survey between July and October 2019, 149 (75.3 %) had been in practice for more than 10 years, the majority working in government facilities, and 101 (52.3%) were physicians in various sub-specialities. Majority of the respondents 191 (98.9%) prescribed antibiotics; 83 (43%) prescribed daily while 51 (26.4%) prescribed 'occasionally'. Thirty-one respondents (16.1%) 'occasionally' request for microbiology laboratory input while 100 (51.8%) 'sometimes' request for microbiological tests before prescribing antibiotics, and only 8 (4.2%) 'always' request for microbiological tests before prescribing antibiotics.

The commonest reasons for not requesting for microbiological tests were the long turn-around-time in 93 (50.5%), and the fact that they 'knew what drug to use' in 43 (23.4%) using previously seen cases as guide. Some doctors had specific drugs they preferred to use in 41 (22.3%) of the respondents. Interestingly some responded that the cost of microbiological tests was too high in 30 (16.3%) respondents. A few 2 (1.1%) however responded they did not request for these tests because they did not know how to collect the samples required. Interestingly too, many of the doctors were satisfied with the test menu provided by their medical microbiology laboratory at the time of the survey, while some doctors would have loved to have more tests done such as automated microscopy, culture and sensitivity, molecular tests for viruses (including viral haemorrhagic fevers) and serological tests for fungal infections.

The findings from this one-minute survey are quite revealing and takes us back to the undergraduate training of the doctor. We understand that antibiotics are very important weapons in the doctor's arsenal, there had been a steady rise in the range of available antibiotics and a concomitant rise in mutations and acquisition of resistance genes in microorganisms (4) thus making antibiotics ineffective over time and increasing morbidity and mortality in our patients. As doctors, we will need to think 'patient safety', and from training to practice, establish the ground rules for the rational use of antimicrobial agents. This responsibility lies in the hands of medical microbiologists, pharmacologists and every



Answer choices	Responses (%)
I know what drugs to use	23.4
There are antibiotics we prefer to use in my team	22.3
The time to get results from the lab is too long	50.5
The test result is not usually in sync with clinical picture	16.9
I sometimes need the input of a Consultant Microbiologist and there is none	6.5
The microbiological tests are too expensive	16.3
The staff are sometimes harsh to my patients	4.9
I don't know how to collect some samples for microbiological tests	1.1
Other (please specify)	35.87

Fig 1: Responses from respondents on the frequency of prescription and use of the microbiology laboratory

doctor involved in training medical students. There is also a need to build capacity and interest in our young doctors, pharmacists and biomedical scientists so that we can do more in prompt diagnosis of infectious diseases and be better stewards of antibiotics.

One of the things that may drive inappropriate use of antibiotics in our setting is the 'corridor consultation', where doctors and 'non-medics' prescribe medications for symptoms described by patients on phone without any physical examination and/or laboratory evaluation (5). Indiscriminate empirical prescription of antibiotics without appropriate laboratory investigation should be discouraged in this era of antimicrobial resistance, and antibiotic stewardship should be promoted in every sphere of medical education and service. Some of the responses to Q1 as shown in Fig 1 are not acceptable in this era of antimicrobial stewardship. Healthcare managers, consultants in different specialities of medicine and laboratory managers must take steps to fill these gaps and make better use of the medical microbiology services.

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