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Editorial Board

All correspondence to Editor-in-Chief
Department of Medical Microbiology,
LAUTECH Teaching Hospital,
PMB 4007, Ogbomoso, Nigeria
Email: afrjcem@gmail.com and ajcem2019@gmail.com
Mobile: +234 (0) 8033436344
OR
Department of Medical Microbiology,
College of Health Sciences,
LAUTECH, PMB 4000, Ogbomoso, Nigeria
E-mail: sstaiwo@lautech.edu.ng

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Review Article **Open Access**

Emerging infectious disease preparedness and response in healthcare: perspectives from COVID-19 and the role of College-Learnt Microbiology

*[1]Tonui, J., [2]Chepkutto, W., and [3]Rotich, J.

[1]Department of Biomedical Sciences-Medical Microbiology, Faculty of Medicine, Kabarak University, Nakuru, Kenya
[2]Department of Public Health, Faculty of Medicine, Kabarak University, Nakuru, Kenya
[3]Department of Preventive and Promotive Health, Ministry of Health, Nairobi, Kenya
*Correspondence to: josephat@kabarak.ac.ke

Abstract:

Coronavirus disease 2019 (COVID-19) pandemic began in December 2019 in Wuhan City China where it is believed to have been transmitted to humans from an unknown animal species. The public health, social and economic impact of the pandemic world over is detrimental. Health care providers at the frontline in the fight against COVID-19 are at the greatest risk of infection and so far, many have been infected and some have already died from the disease. Thus, it is imperative that healthcare providers have adequate knowledge of infectious diseases and microbial pathogens to comprehend the scale of risk for better recognition and response. Microbiological concepts of infection prevention and control, hand hygiene and aseptic techniques are essential in slowing down the spread of the virus. COVID-19 has proven that infectious agents can emerge from any region in the world and can spread rapidly with ominous consequences to all humanity. This narrative review discusses the role of college-learnt microbiology in health care provider preparedness for emerging infectious diseases in light of the current pandemic.

Keywords: Emerging; Infections; Preparedness; Response; Microbiology; COVID-19; Training

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Préparation et réponse aux maladies infectieuses émergentes dans les soins de santé: perspectives du COVID-19 et le rôle de la microbiologie universitaire

*[1]Tonui, J., [2]Chepkutto, W., et [3]Rotich, J.

[1]Département des sciences biomédicales-microbiologie médicale, Faculté de médecine, Université Kabarak, Nakuru, Kenya
[2]Département de santé publique, Faculté de médecine, Université de Kabarak, Nakuru, Kenya
[3]Département de la santé préventive et promotionnelle, ministère de la Santé, Nairobi, Kenya
*Correspondance à: josephat@kabarak.ac.ke

Abstrait:

La pandémie de maladie à coronavirus 2019 (COVID-19) a commencé en décembre 2019 dans la ville de Wuhan en Chine, où elle aurait été transmise à l'homme par une espèce animale inconnue. L'impact sanitaire, social et économique de la pandémie dans le monde est préjudiciable. Les prestataires de soins de santé en première ligne dans la lutte contre le COVID-19 sont les plus exposés au risque d'infection et jusqu'à présent, beaucoup ont été infectés et certains sont déjà morts de la maladie. Ainsi, il est impératif que les prestataires de soins de santé aient une connaissance adéquate des maladies infectieuses et des agents pathogènes microbiens pour comprendre l'ampleur du risque pour une meilleure reconnaissance et une meilleure réponse. Les concepts microbiologiques de prévention et de contrôle des infections, d'hygiène des mains et de techniques aseptiques sont essentiels pour ralentir

la propagation du virus. Le COVID-19 a prouvé que les agents infectieux peuvent émerger de n'importe quelle région du monde et se propager rapidement avec des conséquences inquiétantes pour toute l'humanité. Cet examen narratif traite du rôle de la microbiologie acquise au collège dans la préparation des fournisseurs de soins de santé aux maladies infectieuses émergentes à la lumière de la pandémie actuelle.

Mots clés: émergent; Les infections; Préparation; Réponse; Microbiologie; COVID-19; Formation

Introduction:

Emerging infectious diseases (EIDs) represent significant threats to global public health. Frequent outbreaks and epidemics continue to occur and exert immense pressure on healthcare systems all over the world (1). Over 75% of EIDs are zoonotic in nature, transmitted by direct contact with animals or their products (1). Human-to-animal interaction remains the major risk factor, and perceptions on the impact of the increasing incidence of these infections to the general public and health care workers (HCWs) influence the health system preparedness and policy interventions (2). Emerging infectious pathogens range from viruses, bacteria, fungi and protozoa (3). The emergence and global spread of antimicrobial resistant (AMR) organisms also threatens the clinical use of available antimicrobial agents for prophylaxis and management of infections (4).

COVID-19 pandemic is believed to have originated from a sea food market in Wuhan, China from an unknown animal source in December 2019 when the first case was reported. Further investigations revealed that the infection was caused by a novel strain of beta-coronavirus (CoV) that was subsequently named Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-COV-2) (5). The disease quickly spread from the epicenter and on January 15, 2020, the first death was reported from Wuhan. Infection among HCWs was discovered on January 20, 2020 suggesting possible human to human transmission. The World Health Organization (WHO) declared the outbreak a Public Health Emergency of International Concern (PHEIC) on January 30, 2020 based on the imminent potential of the disease to spread internationally (6). Since the beginning of the COVID-19 pandemic, the impacts on all facets of human life including economic, political and social disruptions have been catastrophic. The pandemic has evolved tremendously and new variants of concern (VOC) have emerged in various countries, although the impact of the new strains in the human population is not yet clear. However, the virus has become more adaptive and highly virulent with marked transmissibility (7). The UK variant of SARS-CoV-2 (VOC 202012/01) for instance has been reported in over 80 countries prompting the institution of new restrictions and lockdowns to curb its spread.

At the time of concluding the manuscript for this review, the number of confirmed cases of COVID-19 globally stood at 130,459,184 with 2,842,325 cumulative deaths (8). Healthcare systems in various countries were ill prepared for huge influx of patients with pneumonia requiring intensive care services and ventilation (9). COVID-19 exhibits severe disease in vulnerable individuals who already have various underlying conditions that have weakened the immune system. This in part coupled with accompanying health system constraints (e. g. slow diagnostic rollout processes) have resulted in high death toll in some countries (10).

At the forefront in the fight against the pandemic are HCWs, who are mostly doctors, nurses, pharmacists, public health experts, medical laboratory personnel, medical microbiologists and other responders providing the much-needed services. It is indisputable that these professionals need a solid knowledge of the nature of infectious diseases (e. g. transmission and pathogenesis) and fundamental principles of microbiology, which are essential for diagnosis, treatment, management, epidemiology, biocontainment and infection prevention and control (IPC) (11). Substantial contents of microbial pathogens and infectious diseases are entrenched in the respective HCW training curriculum, and for non-microbiology majors, these courses are usually taught at the preclinical level (12). HCWs need to constantly adapt and respond safely to changing infectious disease patterns and the unprecedented public health emergencies occasioned by COVID-19 pandemic and other EIDs that may arise in the future.

Overview of emerging infectious diseases:

An EID is primarily defined as one that has existed before but is rapidly increasing in incidence or geographic scope or one that has newly appeared in the population. Other definitions include drug resistant pathogens or new emerging infections which incidence has increased in the last two decades or which incidence is likely to increase in the future (3). The definition may however vary depending on classifications adopted by various experts as a result of the complex nature of these infections. Neverthe-

less, these emerging infectious diseases remain a serious threat to global health and security.

Over the years, epidemiological and geospatial information have demonstrated a steady increase in the incidence and geographic distribution of emerging pathogens. This phenomenon is thought to be driven mainly by socioeconomic, ecological and environmental factors. The peak incidence of EID was recorded in 1980s associated with the emergence of the human immunodeficiency virus (HIV) pandemic (13). Previous attempts to understand the trends and biology of EIDs have classified viral pathogens (particularly RNA viruses) as the most significant threat due to lack of a proof-reading error-correction mechanism in their genome replication cycle. As a result, they possess higher capacity to acclimatize to new hosts including humans (1). One study analyzed a database of 355 EIDs between 1940 and 2004 consisting events caused by newly emergent strains of pathogens such as multi drug resistant tuberculosis (MDR-TB) and the severe acute respiratory syndrome (SARS) coronavirus. The report showed that the majority of pathogens involved in EID events are bacterial or rickettsial (54.3%) due to the emergence of antibacterial resistant strains. Viral pathogens or prions constituted only 25.4% of the EID events. The report further concurred with previous information regarding the incidence of EIDs due to other pathogen types which were found to be 10.7% for protozoa, 6.3% for fungi and 3.3% for helminths (13).

Over 75% of EIDs are due to zoonotic pathogens transmitted to humans from a non-human animal species. Wildlife sources have previously been implicated in numerous emergent EIDs, for instance the occurrence of Nipah virus in Perak, Malaysia and SARS in China, Guangdong Province (14). A number of vectors are also responsible for EID events, previous estimates reporting EIDs attributed to vector-borne diseases was put at 22.8% and 28.8% in the last decades (13). The beginning and scale of an EID is often unpredictable. Many EIDs have been identified while many remained to be discovered. The HIV/AIDS pandemic has caused great human suffering and till the present time, there has been no cure for the disease. The impacts on the healthcare system globally have been detrimental particularly in developing countries with limited resources, high population growth rate, and poverty. In 2010, the global deaths reported were 1.5 million (15). Other EIDs identified in the past years include the 2009 H1N1 influenza, enterovirus 68, hepatitis C and E, *Bordetella pertussis*, poliovirus, and rubeola virus (16).

Several factors are responsible for the emerging and reemerging infectious diseases. Primarily, the interface between humans, animal and the environment. Human encroachment into new environments is one key factor fueling exposure to new infectious agents such the Ebola Virus Disease in 2014/2015 in West Africa (17). Chikungunya and Zika are other examples of emerging viruses that have made headlines in past few years. Chikungunya, a mosquito-borne virus which was first reported in 1952, recently mutated causing infections in several regions mainly Africa, Asia and the Americas with more than 1.25 million cases (17). In 2009, an emerging fungal pathogen *Candida auris* was identified in a Japanese patient. The organism exhibited extreme antifungal resistance and caused severe invasive disease among individuals with underlying co-morbidities (16).

Other factors responsible for the emergence and re-emergence include changes in human social behavior (cultural, sexual and war). Increased international travel in particular has fueled the spread of such pathogens from their source to new environments and vast regions which has been a key factor in the current COVID-19 pandemic (18). Evolution of microbial pathogens, the emergence of antibiotic resistant or antigenic microbial variants, intentional release of infectious agents (bioterrorism), lack of political will coupled with breakdown of public health systems are other key drivers (3). The classic epidemiological triad depicted in Fig 1 below explains the interaction between microorganism, host and environment, which determine disease outcome (19).

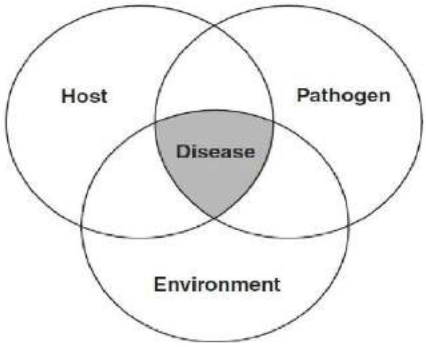

Fig 1: The classic epidemiologic triad explaining the interaction of pathogen, host and environment. Source: (19)

General features of coronaviruses and SARS-COV-2

Coronaviruses are enveloped consisting of a single stranded positive sense RNA genome with up to 32 kilobases. These viruses belong to

the family *Coronaviridae*, subfamily *Coronavirinae* in the order *Nidovirales* according to the International Committee on Taxonomy of Viruses (ICTV). The viruses are named after the arrangement of spike proteins on the envelope having a crown like appearance (20). Coronaviruses were not considered highly virulent until the outbreak of severe acute respiratory syndrome (SARS) in 2002 and 2003 in Guangdong Province of China. A highly pathogenic strain Middle East respiratory syndrome coronavirus (MERS-CoV) appeared later in 2012. It was established that direct transmission of SARS-CoV to humans occurred from market civets. Animal contact was consistent in all index cases and later discoveries identified SARS-CoV-related viruses in horseshoe bats of the genus Rhinolophus, suggesting that bats are the natural reservoirs of SARS-CoV. Related strains have so far been identified in various regions globally including Africa, South East Asia and Europe (21). Similarly, the index cases of MERS-CoV had a history of contact with dromedary camels from which direct transmission to humans originated. However further investigations identified MERS-CoVs in at least 14 species of bats from two bat families, *Nycteridae* and *Vespertilionidae* confirming that camels were intermediates (21). Patients infected by MERS-CoV present with fever and cough, which progress to severe pneumonia and in some cases diarrhea is the first symptom to appear following an incubation of up to 14 days (17). Similar to the COVID-19 pandemic caused SARS-CoV-2, MERS-CoV causes severe disease in individuals with weak immunity and those with underlying conditions such as diabetes and chronic lung disease.

Severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) is a beta-coronavirus that was initially named novel coronavirus-2019 (nCoV-2019). The virus is responsible for the worst global pandemic in the current civilization. The disease began in Wuhan, Hubei Province, China, around December 2019 where the first cases of flu-like disease with pneumonia of unknown aetiology were reported (22). The likely source of the infections was traced to Huanan seafood market where it was believed to have been transmitted to human from an animal species. The virus primarily targets the respiratory system leading to complication of acute respiratory distress syndrome (ARDS). Patients with COVID-19 have shown abnormal respiratory symptoms such as pneumonia, ground glass opacities and cardiac injury as well as increased leukocyte counts and pro-inflammatory cytokines which promote disease severity (10).

COVID-19 has exhibited unique features particularly with the possibility of asymptomatic individuals to transmit the virus before any symptom becomes apparent. The virus is also highly transmissible with some studies reporting an average basic reproductive number (R_o) of 3.28 and a median of 2.79 (23). The new virus variants of concern (VOC) present a more complex scenario with the UK variant for instance exhibiting a 43–90% (95% CI 38–130) higher reproduction number (7). Globally, the number of cases and deaths are increasing and as of 4th April 2021, over 4 million new cases were reported in a week, with 11% increase in mortality rate (8).

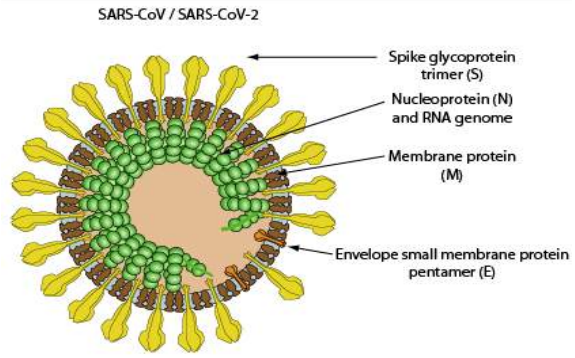

Fig 2: Schematic representation of coronavirus
Source: https://viralzone.expasy.org/764?outline=all_by_species

Consequences of emerging and re-emerging infectious diseases on healthcare workers (HCWs)

Emerging infectious agents such as the SARS-CoV-2, MERS-CoV, Ebola virus and multi-drug resistant (MDR) organisms exhibit high virulence in humans. This is mainly due to well adapted pathogen virulence factors and lack of immunity in the population. The COVID-19 pandemic has had its greatest impact on frontline healthcare workers ever witnessed in the recent years. As of March 2020, there were 3300 infections of HCWs in China and by February at least 22 HCWs had died. In Italy, over 20% of HCWs responding to the pandemic have acquired the infection and some had died (5). The trend is the same in other regions across the world and keeping HCWs safe is now a top priority for most governments. In part this has been attributed to shortages of personal protective equipment (PPE) such as aprons, gloves, medical masks, goggles, respirators, gowns and face shields. The shortage has been occasioned by the high-rate consumption due to huge influx of COVID-19 patients to healthcare settings increasing exposure rates for HCWs.

The WHO estimated through modeling that 89 million medical masks and 76 million examination gloves are needed for COVID-19 response each month with international demand for goggles standing at 1.6 million per month (24, 25).

During the outbreak of Ebola Virus Disease (EVD) in 2014-2015, many HCWs were infected and the WHO reported 874 cases of EVD with 509 deaths among HCWs in West Africa (26). A systematic review including 94 articles linked to 22 outbreaks exploring the infection rates and associated risk factors for infection among HCWs during Ebola and Marburg virus outbreaks highlighted inadequate and inappropriate use of PPEs and exposure to individuals with asymptomatic Ebola and Marburg infection (27). The highest of HCWs exposed were nurses (61%) and medical staff, hence over half of the cases were in this occupation. A similar trend has been observed in China where approximately 60% and 30% of HCWs infected were nurses and clinical personnel (28). However, with continued integration of healthcare services and patient management in healthcare delivery, there is an increased risk to other occupations which should be considered in infection prevention and control (IPC) efforts (27). With the emergence of new pathogens such as the current COVID-19 pandemic, lessons learnt from previous outbreaks should be implemented appropriately and best practices adopted to reduce the impact on health care works and the entire population.

Some of the important measures implemented in the current COVID-19 pandemic include social distancing, hand hygiene, HCW training on IPC and supply of adequate PPEs. Many countries have also implemented lockdowns to restrict movement and human interactions as well as cancellation of international travels and extensive screening at entry points. As new infections emerge, there is a greater need for sufficiently trained IPC specialists for proper response to these extraordinary events (29).

The impact of EIDS to countries and the global community are enormous as witnessed in the current COVID-19 pandemic. Therefore, every country should prepare and build the necessary capacity and infrastructure in disease surveillance, diagnostics, education, training, research, communication and strategic planning to be able to quickly recognize and respond adequately to any such events (30). Prompt and well-structured response limits the overall mortality and morbidity as well as economic implications. Newly EIDS often compete with other healthcare priorities and existing diseases hence vital resources in the healthcare system are overstretched (2). The extensive systematic review highlighted the risk factors associated with HCW infections during Ebola and Marburg outbreaks. Key among them was insufficient and inappropriate use of PPEs, lack of training in PPE and hand hygiene, multiple use of disposable PPEs and HCW refusing PPEs while caring for patients. Other important factors included inappropriate risk assessment and lack of environmental controls (27).

Significance of basic microbiology training on infection control practices by healthcare providers

Medical microbiology is a subject of medicine concerned with the study of human infectious diseases and responses to such infections. The main focus includes aetiology, pathogenesis, laboratory diagnosis, treatment, epidemiology and infection control. The subject is generally taught as a pre-requisite basic science in medical, nursing, public health, clinical medicine and other relevant paramedical courses. The subject is meant to equip learners with the knowledge of medically important microorganisms such as bacteria, viruses, protozoa, fungi and other infectious agents. The pre-clinical curricular for non-microbiology courses (nurses, doctors and public health) covers most of the infectious diseases. However, there is a continuously expanding content in this subject as new infectious agents emerge and more discoveries are made on the existing organisms (31). Some of the most important areas of concern to medical practice currently include IPC, antimicrobial resistance (AMR), EIDs, and global health, which are supposed to be taught along with the foundational microbiology course (32). In this regard, there is a greater need for adaptability in the teaching and training as well as medical microbiology content evaluation from time to time.

In recent years, there has been a surge in the frequency of healthcare associated infections which has greatly impacted patient care as well as HCWs. Healthcare associated infections (HAIs) are mainly caused by highly resistant microorganisms such as methicillin-resistant S*taphylococcus aureus* (MRSA) and multi-drug resistant tuberculosis (MDR-TB). The microbes colonize the hospital environment and the cycle of transmission and outbreaks can occur for an extensive period of time. The sources are either endogenous (from the patient's own flora) or exogenous (from healthcare worker's hands) (33). Prevention of HAIs is now considered part of safe and quality healthcare delivery which has

created new demands in the health care system with significant cost implications when implementing enhanced IPC measures and the resulting longer hospital stays for infected individuals (34). Occurrence of HAIs may also have judicial consequences for responsible health facilities (35). It is estimated that 10% of hospitalized patients in the developed countries will develop a HAI while the risk in developing countries is 2-20 times more (33). EIDs such as COVID-19 (SARS-CoV-2 infection) are often noticed when HCWs have already been infected and other patients within the healthcare setting are always at risk.

In view of the emerging complexities regarding infectious diseases, it is crucial that HCWs involved in patient care possess sufficient IPC knowledge and skills in order to address these challenges in a safe manner. Basic microbiology training should empower medical students to understand the nature of infectious diseases (e. g. transmission, pathogenesis and diagnosis) and the risk level for adoption of proper IPC measures in their future clinical practice. The relevant topics include appropriate use of antibiotics, safe collection of specimen for microbial analysis, preparation of specimens for shipment and delivery, communicating microbiological results to patients and healthcare teams, and appropriate care for patients based on microbiological results and their immune status (36).

Regarding IPC, the most vital concepts include use of PPEs, hand hygiene, handling and safe disposal of sharps, aseptic techniques, clean clinical environment and decontamination (37). A number of studies have highlighted challenges in IPC compliance among nurses and clinicians. One online survey sought to evaluate the experience of student nurses in clinical placement where they were presented with a range of questions as to whether they had witnessed some lapses in IPC and the frequency of such lapses. All participants reported to have witnessed lack of compliance particularly for hand hygiene and over 75% reporting failure to clean hands between patients contact. Other lapses were witnessed in isolation precautions, poor cleaning of patient environment, changing of PPE between patients, and improper handling of sharp instruments (38). Another study that sought to evaluate the effectiveness of a special IPC training offered to nursing students before graduation reported a positive influence of the program on the students IPC knowledge, attitudes and compliance. The study used an experimental design where participants were randomly assigned to two groups with pre and post-tests which demonstrated the importance of such programs in supplementing basic curricula training (37). It has been demonstrated that nursing students have a positive attitude towards hand hygiene and IPC signifying their concern for safety and patient care.

Infection prevention and control is deeply anchored in a thorough understanding of microbiological principles, particularly pathogen characteristics (transmission and pathogenesis). Therefore, IPC decisions should be based on these factors. It has been shown that HCWs often make decisions regarding IPC based on their perceived (often inaccurate) judgment of risk rather than rational application of microbiological principles (39). Therefore, failure to correctly assess the risk may compromise safety of the HCWs. Some reasons for the significantly high HCWs infections in the COVID-19 pandemic apart from the shortage of PPEs include lack of understanding of the pathogen, poor knowledge of IPC, exposure to a large number of infected patients for a long period of time, work intensity, lack of rest and pressure, lack of training on IPC for respiratory borne pathogens and lack of professional supervision and guidance on the pandemic response (28). These factors contributed to substantial HCWs exposure and infections at the onset of the outbreak.

Infectious agents are generally categorized into four biosafety levels which determine the level of containment and safety precautions needed when handling them. One factor that influences the adoption and compliance to IPC precautions has been explained through the Health Believe Model (HBM), a theory developed to explain health related behaviors. The HBM suggests that the likelihood of an individual to engage in a health related behavior is determined by their perception of four important variables; susceptibility, severity, benefit and barriers (40). Threat perception is based on two beliefs; perceived susceptibility of the individual to a certain disease and the perceived severity of the disease to the individual. Based on this concept, there is need to influence behavior change and health beliefs of HCWs to enhance the adoption of safe practices (41).

Strengthening medical microbiology in academic medical training institutions

The discovery of microorganisms in the 17th century by Robert Hooke and Antoine van Leeuwenhoek due to their interest in microscopes laid the foundation for today's technological advancements in microbial studies (42). The world over, medical training institutions are the epitome of knowledge that can be leveraged to develop vital remedies in times of public

health emergencies. As the pandemic rages, a number of vaccines have been developed and universities such Oxford in the UK have played a key role in this endeavor. Despite the development and rollout of COVID-19 vaccines, constraints still exist particularly the availability of enough doses for the entire population as well variants of concern (VOC) that could impact on the efficacy of the existing vaccines. Continuous research on the virology of SARS-CoV-2 is paramount for better control strategies. One significant milestone was the successful genomic sequencing of the virus which enabled further informed experiments for vaccine development (5).

Academic medical institutions are mandated to train competent healthcare personnel who can diligently serve in the community. Medical students are future HCWs and as such, sufficient training to inculcate sound knowledge of medical microbiology and IPC competency is indispensable. To facilitate compliance with set safety precautions among nursing students, it has been proposed that IPC competence should entail theoretical, practical, and decision-making competence (39). Based on this proposed approach, students would need to understand, analyze, synthesize, and apply the knowledge of microbiology. The decision-making competence would be essential for effective response in the event of an emerging infectious agent such as the current SARS-CoV-2, MERS-CoV or Ebola virus which requires an in-depth understanding of microorganisms (e. g. mode transmission and portals of entry) rather than simple memorization of procedures. Another approach is the provision of additional microbiology classes and special programs focusing on important IPC concepts. Data have shown that adequate understanding of IPC and consequences of breach among HCWs improves the overall compliance (43).

To be able to recognize and respond adequately to future emerging and re-emerging infectious diseases, and to address the rising challenge of AMR, medical students should be exposed to these important concepts early in their career. The concept of "One Health" is critical considering that the majority (>70%) of EIDs are zoonotic and mainly related to wild animals (44). Medical students need to have understandings of the interface between human, animal, and environmental health. This will promote future interactive multidisciplinary efforts both locally and internationally to address AMR and zoonotic diseases (2,45). Medical students, nurses and clinicians should be given opportunities to participate in multidisciplinary teams involved in community and patient education on "One Health" to promote better understandings and real-life applications. This can be augmented by mentorship programs to stimulate interests in this important subject.

Antimicrobial stewardship (AMS) has been embraced as the means to address the rapidly emerging resistance to antimicrobials and to promote accountability and rational use of these medicines which are vital global resources that need to be safeguarded (46,47). One of the key challenges to this initiative that has been highlighted include communication barriers between microbiology laboratories and other care units within health settings. In essence, the clinicians and the nursing staff have had challenges interpreting some of the microbiology reports while the laboratory could miss vital information in request forms mainly due to the lack of in-depth knowledge into each other's areas of expertise (48). This impediment could be addressed by equipping medical students with knowledge of various aspects of such initiatives for effective communication and implementation later in clinical practice. This will also enhance accurate patient management and timely notification of unusual infectious disease diagnosis and resistant strains.

As a public health endeavor, medical students should also be equipped with adequate knowledge of infectious disease epidemiology and sensitized on reporting of notifiable infectious agents. This will empower them to capture quality data (such as person time relationship) that can be synthesized to generate vital inferences to trace EIDs and implement timely response decisions to prevent escalation of such unusual events. Essentially this will ultimately contribute to international surveillance of EIDs and epidemic intelligence (49).

Currently, there is extensive automation of microbiology and sophisticated analytic platforms for diagnosis and research on infectious diseases. Therefore, medical students should be kept abreast with the progress so far as new instruments are introduced in routine diagnostics and patient care. Most significantly is the utility of molecular tests in diagnosis and treatment monitoring. One such platform is the GeneXpert, a cartridge based molecular assay which was developed for rapid detection of *Mycobacterium tuberculosis* and resistance to rifampicin (50). This technology has also been explored for the diagnosis of current COVID-19 pandemic and was granted emergency use authorization (EUA) approval by the US Food and Drug Admiration (FDA) on March 22, 2020 (51).

Innovative pedagogical approaches are

also critical in delivery of medical microbiology, which can be adopted for concepts such as EIDs and AMS (46). There have been important developments in current education practice mainly geared towards excellence in teaching and professional development (52). One important concept is the application of a flipped classroom (FC) approach where students are first exposed to the learning materials which may be readily available online or shared by the instructor. Thereafter, time is dedicated where learners are engaged in student-centered activities that encourage active learning. This method has gained acceptance since it promotes the development of life-long skills for self-directed learning among medical students (53). Other approaches include adoption of technology and online teaching platforms which could facilitate sufficient course coverage and are equally engaging and interactive (54,55). Periodic curriculum reviews are also vital to incorporate new developments in medical microbiology relevant to healthcare delivery.

Conclusion:

Emerging infectious diseases remain the greatest threat to international public health and the global economy. The current COVID-19 pandemic is causing untold human suffering with significant fear and mortality. HCWs all over the world are at the greatest risk of infection and the number infected globally is alarming while some have died. There are number of reasons for these which include among others, shortage of PPEs, inadequate training on IPC and extreme work intensity. Consequently, governments of countries have undertaken drastic measures to ensure adequate PPE supplies, training and retraining of HCWs and most importantly prioritization of HCWs in vaccine rollout.

To address these challenges now and in the future, there is need to strengthen foundational microbiology and IPC concepts during college training. Furthermore, health students should be encouraged to develop positive health beliefs to promote adoption and implementation of safe healthcare delivery practices later in their careers. To address the concerns of EIDs, sufficient infectious disease experts should be trained and international collaboration fostered. EIDs are primarily zoonotic and therefore pathogen surveillance in both domestic and wild animals should be heightened. It is now evident that infectious agents can emerge from any region across the world and as such affluent nations should invest and support countries with limited diagnostic and surveillance capacity to be able to detect and control such pathogens early. This will in future serve to avert the widespread damage as witnessed with the current COVID-19 pandemic.

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Review Article **Open Access**

A review of the anti-viral effects of ivermectin

*[1]Adegboro, B., [2]Lawani, O. A., [3]Oriaifo, S. E., and [4]Abayomi, S. A.

Departments of [1]Medical Microbiology & Immunology, and [3]Clinical Pharmacology, Nile University, Abuja, Nigeria
[2]Department of Medical Microbiology and Parasitology, University of Ilorin Teaching Hospital, Ilorin, Nigeria
[4]Department of Medical Microbiology and Parasitology, LAUTECH Teaching Hospital, Ogbomoso, Nigeria
*Correspondence to: boazadegboro@gmail.com

Abstract:

Ivermectin is an avermectin which is a group of pentacyclic sixteen-membered lactone (macrocyclic lactone disaccharide) derived from the soil bacterium *Streptomyces avermitilis*. It is a semi-synthetic broad-spectrum anti-helminthic, anti-viral and anti-cancer agent. It has a wide safety margin with low adverse effects when it is used orally. It has, however, so far only been approved by the Food and Drug Administration (FDA) as a broad spectrum anti-parasitic agent. Because ivermectin also has broad activities as an anti-viral agent, we herein review its pharmacokinetic and pharmacodynamic activities, as well as the *in vitro* and *in vivo* studies conducted on the drug. It is hoped that this work will pave way for ivermectin being seriously considered as an addition to the drugs available for the management of patients with COVID-19.

Keywords: ivermectin; pharmacokinetics; pharmacodynamics; broad-spectrum anti-viral; COVID-19

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Un examen des effets antiviraux de l'ivermectine

*[1]Adegboro, B., [2]Lawani, O. A., [3]Oriaifo, S. E., et [4]Abayomi, S. A.

Départements de [1]Microbiologie médicale et d'immunologie, et [3]Clinique de pharmacologie,
Université du Nil, Abuja, Nigéria
[2]Département de microbiologie médicale et de parasitologie, Hôpital universitaire d'Ilorin, Ilorin, Nigéria
[4]Département de microbiologie médicale et de parasitologie, Hôpital universitaire de LAUTECH, Ogbomoso, Nigéria
*Correspondance à: boazadegboro@gmail.com

Abstrait:

L'ivermectine est une avermectine qui est un groupe de lactone pentacyclique à seize membres (lactone disaccharide macrocyclique) dérivée de la bactérie du sol *Streptomyces avermitilis*. C'est un anthelminthique semi-synthétique à large spectre, antiviral et anticancéreux. Il a une large marge de sécurité avec de faibles effets indésirables lorsqu'il est utilisé par voie orale. Cependant, jusqu'à présent, il n'a été approuvé que par la Administration des aliments et des médicaments (FDA) en tant qu'agent antiparasitaire à large spectre. Étant donné que l'ivermectine a également de vastes activités en tant qu'agent antiviral, nous passons ici en revue ses activités pharmacocinétique et pharmacodynamique, ainsi que les études *in vitro* et *in vivo* menées sur le médicament. On espère que ce travail ouvrira la voie à l'ivermectine qui sera sérieusement considérée comme un complément aux médicaments disponibles pour la prise en charge des patients atteints de COVID-19.

Mots clés: ivermectine; pharmacocinétique; pharmacodynamique; antiviral à large spectre; COVID-19

Introduction:

Ivermectin, an anti-parasitic agent has been used for several years to treat many infectious diseases in mammals due its broad-spectrum antimicrobial activity and high therapeutic efficacy (1). It has a wide safety margin with low adverse effects when it is used orally. Ivermectin is a dihydro derivative of avermectin that was discovered in the late 1970s and this drug was first approved for use in veterinary medicine in 1981 (2). Its potential use in humans was confirmed in 1987 for the treatment of onchocerciasis. Subsequently, William Cecil Campbell, an Irish American scientist and Satoshi Ōmura, a Japanese biochemist both of whom discovered and developed this medication were honored with the 2015 Nobel Prize in Physiology or Medicine (3).

Ivermectin is currently used as an approved drug in several countries for the treatment of filariasis, strongyloidiasis, scabies and onchocerciasis. As a result of mass drug distribution and administration programmes, an estimated 3.7 billion doses of ivermectin have been distributed globally over the past thirty years (4). Studies have suggested the biochemical property of ivermectin as a broad-spectrum drug with high lipid solubility. This characteristic confers on it multipurpose effects on several parasites and viruses through a variety of mechanisms. Apart from the anti-parasitic and antiviral effects, ivermectin also causes immunomodulation in the host cells. Other studies have corroborated the effect of ivermectin on slowing down the proliferation of cancer cells, including the regulation of glucose and cholesterol in animals (5).

It has also been suggested in studies that the inhibitory action of ivermectin on the integrase protein of HIV (6), and non-structural protein 5 in Dengue virus (7) can inhibit their replication. Also, ivermectin has demonstrated inhibition against several RNA viruses such as West Nile virus (8), Venezuelan equine encephalitis virus (9), and influenza virus (10). The antiviral effect of ivermectin has also been demonstrated, both *in vitro* and *in vivo*, against pseudorabies virus (PRV), which is a DNA virus (11). The nuclear inhibitory activity of ivermectin appears to be responsible for its broad-spectrum activity against these viruses (12).

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection, which is officially known as coronavirus disease - 2019 (COVID-19) by the World Health Organization (WHO) was first reported in early December 2019 in Wuhan, China and has subsequently spread globally. Since the outbreak of the virus infection, there has not been any 'gold standard' antiviral drug available for its treatment. However, there are many ongoing clinical trials exploring the potential antiviral activities of a number of drugs. Several of these drugs, which were initially used to eliminate other pathogens, now appear to have shown some inhibitory activities against COVID-19 either by direct action on the viral agent or by modulating the host immune system. Ivermectin is one of those drugs with potential therapeutic activity in COVID-19 as it has demonstrated potential antiviral activity by its action against the nuclear transport of viral proteins (13). The objective of the present study is to review the antiviral activities of ivermectin with the aim of finding an additional drug that may be useful in reducing the morbidity and mortality from the dreaded COVID-19.

Materials and method:

Online literature search was conducted on Google, Google Scholar, JSTOR, and EBSCO for relevant publications on ivermectin use in COVID-19 and for other viral infections. Publications were evaluated based on credibility of the sources, innovative contribution, key concepts and theories, and problem-solving approach, using the Preferred Reporting Items for Systematic Review and Meta-Analysis guide as shown in Fig 1.

Recurring themes were identified, debates, conflicts, contradictions and identified gaps in knowledge were noted, as there were gaps in combinational use of ivermectin with other drugs, as well as about its efficacy. The outline of structure of the review was discussed amongst all authors.

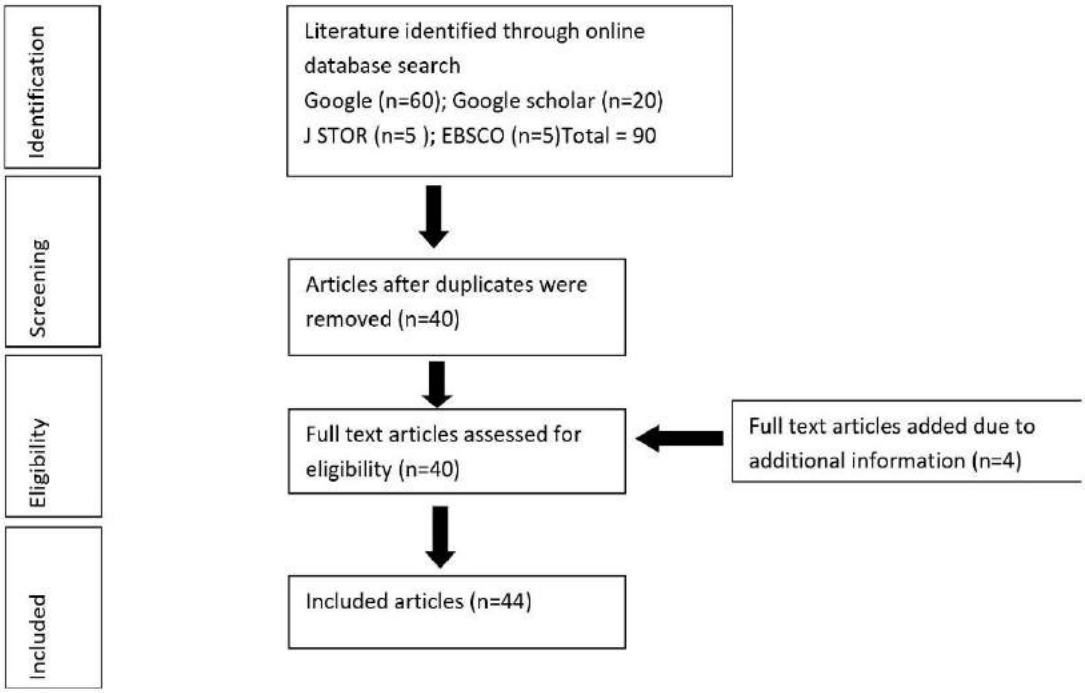

Fig 1: Process of selection of publications (PRISMA guide) used for the review

Chemistry of ivermectin

Ivermectin is a semi-synthetic broad-spectrum anti-helminthic, anti-viral and anti-cancer agent. It is an avermectin, which is a group of pentacyclic sixteen-membered lactone (macrocyclic lactone disaccharide) (Fig 2), derived from the soil bacterium *Streptomyces avermitilis*. The simple derivative of the mixture of the natural compounds, avermectin BIa and BIb, are the most common avermectin.

The peculiar chemistry of ivermectin may make possible for two ivermectin molecules to create a complex to be considered as ionophore. According to this hypothesis (14), this ionophore for cations such as zinc for example, may then affect the hydro-electrolyte balance in the cell to cause early virophagy and even act to inhibit RNA-dependent-RNA polymerase (RdRp), similar to remdesivir (15).

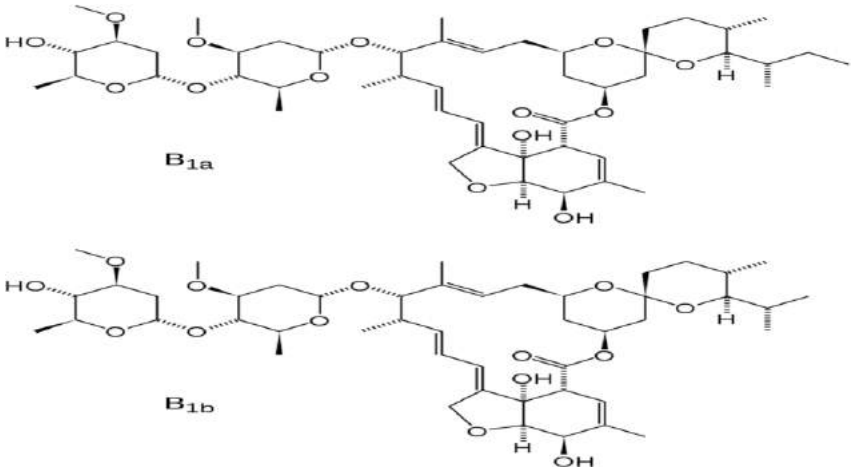

Fig 2: Structural formulas of ivermectin compounds

Pharmacokinetics of ivermectin

Similar to azithromycin, ivermectin is highly lipophilic and usually administered orally and rapidly absorbed. It reaches peak plasma concentration (T_{max}) in 4 hours, and is 93% protein bound with a half-life of 16 hours and terminal half-life of 81-91 hours. Its volume of distribution is 3.5 L/kg and undergoes enterohepatic circulation. After a single oral dose of 150 µg/kg, the C_{max} is 40 ng/ml and T_{max} is 4 hours. These remain unchanged after a dose of 200 µg/kg (16). Plasma concentration achieved with a maximum tested dose of 2 mg/kg was 250 ng/ml. The researchers have speculated that the projected therapeutic level of 2,500 ng/ml for *in vivo* efficacy in COVID-19 may be impractical. Other researchers concluded that the likelihood of clinical treatment using the approved dose of ivermectin is low, since doses 10x higher than the approved dose cannot meet the concentration that will result in 50% inhibition (IC_{50}) (17).

Ivermectin is metabolized by the liver microsomes P450 and converted to mostly hydroxylated and demethylated metabolites which are 90% excreted in faeces, with 1% in urine (18). There is risk of absorption through the blood-brain-barrier (BBB) when taken with other CYP3A4 inhibitors such as statins, HIV-1 protease inhibitors, calcium channel blockers (CCBs), lidocaine, benzodiazepines, and glucocorticoids like dexamethasone which also inhibit p-glycoprotein. This is pertinent because ivermectin is a substrate and an inhibitor of p-glycoprotein (19), thus the neurotoxicity of ivermectin may be marked at high dose.

Doxycycline enhances ivermectin-induced suppression of microfiladermia while ivermectin antagonizes vitamin K to increase prothrombin time. Ivermectin does not readily cross the BBB due to the efflux transporter, p-glycoprotein (20). Large doses may also not be tolerable for humans because of the inhibition of the nuclear transporter, importin or karyopherin α/βI (21,22). Importins are regulatory proteins vital for gametogenesis, embryogenesis and prevention of cancer.

The Food and Drug Administration (FDA) and Pan-American Health Organisation (PAHO) in 2020 have not approved ivermectin for use in COVID-19. Ivermectin is contraindicated in children under 5 years of age or in children with weight less than 15 kilograms (33 pounds) and in individuals with liver or kidney disease (23).

Pharmacodynamics of ivermectin

Ivermectin is an agonist for the gamma aminobutyric acid (GABA)-gated chloride channels, stimulating release of GABA from the presynaptic end of the GABAergic nerves. It thus enhances the post-synaptic GABA binding and amplifies GABA function in the CNS. This may explain its side-effects of dizziness, ataxia and fatigue. The killing of parasites by ivermectin is due to its agonist action at the glutamate-gated chloride channel (24).

Ivermectin has shown *in vivo* effects against RNA viruses such as the West Nile virus and Newcastle disease (NCD) virus at a concentration of 100 µg/ml through inhibition of importin Imp α/βI. This may also explain its action against SARS-COV2 (25). It has also been reported to inhibit *in vivo* DNA viruses such as pseudorabies virus (PRV) and parvovirus by preventing nuclear import of UL42, an accessory subunit of the DNA polymerase.

Ivermectin induction of autophagy may explain its action as an anti-viral agent. Through the induction of autophagy as calorie restriction does, it may directly eliminate intruding viruses (virophagy), enhance immune cell competence, suppress acute inflammation and hyper-inflammation (26,27). Importantly, ivermectin activates AMP-activated protein kinase (AMPK) and upregulates Farnesoid X receptor (FXR) to decrease expression of lipogenesis-related genes (28,29) and this mechanism could help attenuate SARS-COV-2 virus entry. Ivermectin is active against SARS-COV that is homologous to SARS-COV2, by inhibiting host importin protein, Imp α/βI heterodimer, which is the major transporter in nucleocytoplasmic shuttling of SARS-COV nucleocapsid protein. The new hypothetical mechanism to explain anti-COVID-19 effect of ivermectin is that it displays ionophore activity to cause early viral lysis (27).

Clinical uses of ivermectin

Ivermectin is currently used as an approved drug in several countries for the treatment of filariasis, strongyloidiasis, scabies and onchocerciasis (4). It also exhibits action against West Nile virus (8), Venezuelan equine encephalitis virus (9), influenza virus (10) and pseudorabies virus (11,13). Although the drug has not yet gained FDA and PAHO approval for use in COVID-19, clinical trial of ivermectin alone (30) or the combination of zinc, doxy-

cycline and ivermectin in COVID-19, is however recruiting participants (NCT04482686). A previous study (31) showed that a single dose of ivermectin was enough to limit SARS-CoV-2 replication within 24-48 hours; this action was possibly due to the inhibition of the IMPα/β1-mediated nuclear import of viral proteins. The same study also demonstrated that the levels of viral RNA released from the infected cells and cell-associated viral RNA were reduced profoundly by more than 90% and 99% respectively at 24 hours post SARS-CoV-2 infection. In addition to these, the treatment of SARS-CoV-2 infected cells with ivermectin for 48hrs was demonstrated to lead to approximately 5000-fold reduction of viral RNA in comparison with the control group. However, the study reported that no further reduction in the viral RNA was demonstrated at 72 hours (13). Furthermore, it has been proposed that no toxicity of ivermectin was seen in both the test and control groups at any point in time (31).

Benefits of ivermectin in management of viral infections

Ivermectin has antimicrobial, antiviral and anti-cancer properties, which gives it the ability to treat a range of diseases. The drug was approved by the FDA as a broad spectrum anti-parasitic agent (32). Ivermectin has been shown to have antiviral activity against a broad range of viruses both RNA and DNA viruses (33). Examples of RNA viruses include dengue virus, Zika virus, Yellow fever virus, West Nile virus, Hendra viruses, Newcastle virus and SARS-coronaviruses. Examples of DNA viruses include Equine herpes virus type 1, BK polyoma virus, and bovine herpes virus (BHV) (32).

Antiviral effects of ivermectin on RNA viruses

Dengue virus

Dengue virus (DENV) is a positive (+) sense, single stranded RNA virus of genus flavivirus and family Flaviviridae. In an *in vitro* study carried out on human cervical adenocarcinoma cells (HeLa) which was infected with Dengue virus, ivermectin at high concentration (25-50μM) had an inhibitory effect on proliferation of the virus. This action was achieved by inhibition of the transfer of viral proteins between host cell cytoplasm and its nucleus, which is dependent on IMPα/β1 (importin). It was observed that ivermectin inhibited the nuclear aggregation of NS5 protein of Dengue virus, the largest (102 kDa) and the most conserved protein, with approximately 70% sequence identity among the four Dengue sero types expressed during infection by Dengue virus (34).

In a study conducted on Yellow Fever, West Nile and Dengue viruses, ivermectin exerted its inhibitory effect by inhibiting NS3 helicase (which mediate RNA binding and unwinding mechanism). It however did not have any effect on ATPase activity of the helicase domains. Ivermectin showed stronger inhibitory effect on Yellow fever virus, and to a lesser extent, inhibited proliferation of West Nile virus and Dengue virus. It was confirmed that ivermectin exerts its effect against dsRNA unwinding activity by acting on the flavivirus helicase enzyme. It however did not affect the ATPase activity because ATP is a key nucleotide in host cell metabolism (34).

Zika virus

Zika virus is a single stranded (ss) RNA virus of genus flavivirus and family Flaviviridae. It was studied by Barrow et al., (35), who performed the *in vitro* study on Zika-infected Huh-7 cells (ZIKMEX_1_7) and confirmed the antiviral effect of ivermectin on the virus.

SARS-CoV-2

SARS-COV-2 is a novel virus that was discovered in December 2019. In an *in vitro* study conducted on the virus, vero/hSLAM cells infected with the SARS-CoV-2 were exposed to 5μM ivermectin in 48 hours and a 500-fold reduction in viral RNA compared with the control was found (36). The study showed that treatment with ivermectin effectively kills almost all viral particles within 48 hours. It was acknowledged that the drug may have antiviral effects by inhibiting the importin (IMP) α/β receptor, which is responsible for transmitting viral proteins into host cell nucleus (36).

HIV-1

HIV-1 is a single stranded RNA virus belonging to the genus Lentivirus of the family Retroviridae. In an *in vitro* study on the effect of ivermectin as an inhibitor of HIV-1 nuclear protein transfer, it was observed to reduce the nuclear localization signal (NLS)-containing protein binding by IMP α/β and inhibited this interaction at low concentrations. Ivermectin significantly (p=0.003) reduced nuclear accumulation of the green fluorescent protein (GFP-IN) compared to untreated control group, and also significantly reduced (p<0.001) nuclear accumulation of GFP-tagged Op-T-NLS fusion protein. Ivermectin however failed to control nuclear accumulation of telomere repeat factor-1 (GFP-TRF) as IMP β1 is the only way to transfer it to the cell nucleus. The study concluded

that ivermectin is a nuclear transport inhibitor, via IMPβ1 alone, and also, it completely inhibited nuclear import of the active integrase protein of HIV-1 as a critical component of the pre-integration complex (37). Kylie et al., (34) study on HIV-1 infected human cervical adenocarcinoma cells (HeLa) showed that ivermectin at high concentrations (25-50x) had inhibitory effect on proliferation of HIV-1, which was achieved by inhibiting transfer of viral proteins between the host cell cytoplasm and its nucleus, dependent on IMPα/β1. It was also shown that ivermectin inhibited nuclear aggregation of HIV-1 integrase (34).

Newcastle disease virus

Newcastle disease (NCD) virus is a negative sense, single stranded virus of the family Paramyxoviridae. Azeem et al., (38) studied the cytotoxicity of ivermectin and its potential antiviral effect on the NCD virus by using chick primary fibroblast cell line and 9-day old chick embryo. Ivermectin was tested at concentrations of 6.25, 12.5, 25, 50, 100, 200 µg/ml. The results showed that the drug at 100 µg/ml or above had cytotoxic effect but was safe at 50 µg/ml or less. At this dose, drug cytotoxicity was not observed and a moderate to poor antiviral activity was noted.

Antiviral effects of ivermectin on DNA viruses

Equine herpes virus type 1 (EHV-1)

EHV-1 is a double stranded DNA virus. In primary murine neurons infected with 2 different strains of EHV-1, Jan-E and Rac-H, different concentrations of ivermectin had no effect on strain Rac-H proliferation but reduced the proliferation of strain Jan E, suggesting that different strains of EHV-1 use different receptors to enter the nucleus (39).

Pseudorabies virus (PRV)

PRN is an enveloped double stranded DNA based swine virus belonging to α-herpesviridae subfamily, which causes lifelong infection in pigs. Its DNA polymerase enzyme is made up of two subunits UL30 and UL42 (40,41). UL42 subunit has IMP-α/β-mediated bipartriate nuclear localization signals (NLS) which transfer both units into the nucleus (41). Examination of infected baby hamster kidney (BHK-21) cells showed that ivermectin did not produce cytotoxic effects at a concentration <3 µM. Increasing the concentration to 5µM showed drug cytotoxic effect and sharp reduction in cell activity.

Ivermectin inhibited the entry of DNA polymerase accessory subunit UL42 into the nucleus, so that with increasing drug concentration, less UL42 was observed in the nucleus by Western blot analysis method. Ivermectin inhibited transfer of UL42 to the nucleus through the NLS but did not reduce UL42 expression in the cytoplasm (41). In the virus-infected mice model (*in vivo* studies), ivermectin significantly reduced viral loads in the brain and kidney of all animals; the reduction was more significant in the kidneys, the main organ involved in ivermectin metabolism. In addition to the declining virus titers in the organs of the animal, their clinical scores and mortality decreased as the drug concentration increased. It was therefore concluded that ivermectin could be used as a potential antiviral drug against PRV infection (42).

BK polyomavirus (BkPyV)

Bennet et al., (43) studied the effect of ivermectin on BkPyV, a non-enveloped small double stranded (ds) DNA virus belonging to the family Polyomaviridae, in infected epithelial cells of the proximal convoluted tubule (PCT) of the kidney. This was a qualitative study that used reverse transcription-PCR after treating infected cells with 10µM ivermectin. The study showed a reduction in the level of early protein large T antigen mRNA, indicating a reduction of viral gene expression due to the inhibition of nucleus entry. This inhibitory effect of ivermectin indicates that polyomavirus has access to the nucleus through active pore complex transfer (43).

Bovine herpes virus- 1 (BOHV-1)

In one study (44), Madin-darby bovine kidney cells were infected with BOHV-1, a large enveloped double stranded DNA virus from family herpesviridae. Ivermectin decreased UL 42 nuclear transmission by inhibiting IMPα/β dependent nuclear transfer and reduced virus replication in a dose dependent manner. This indicated that UL42 was dependent on IMP α/β for nuclear transfer. Ivermectin at 25µM reduced the virus titer by 4 logs and inhibited virion production by 44%, but had no effect on cell viability at the studied doses. Ivermectin also had no effect on binding and entry of virus into host cell (44).

Discussion:

Ivermectin has been shown to have antiviral activity against a broad range of RNA and DNA viruses (32,33). Examples of such RNA viruses include Dengue virus, Zika virus, Yellow fever virus, West Nile virus, Hendra

viruses, Newcastle virus and SARS viruses, and examples of DNA viruses include Equine herpes type 1, BK polyoma virus and bovine herpes virus (32,33). In *in vitro* studies (34), ivermectin exerted inhibitory effects by inhibiting NS3 helicase (which mediate RNA binding and unwinding mechanism). The drug has also demonstrated *in vitro* activity against other viruses such as HIV-1 (37), equine herpes virus type 1 (39), pseudorabies virus (40,41), BK polyomavirus (43) and bovine herpes virus -1 (44).

Ivermectin is currently used as an approved drug in several countries for the treatment of filariasis, strongyloidiasis, scabies and onchocerciasis. An estimated 3.7 billion doses of ivermectin have been distributed globally over the past thirty years (4). It is an avermectin, which is a group of pentacyclic 16-membered lactone (macrocyclic lactone disaccharide). It is believed that the peculiar chemistry of ivermectin makes it possible for two ivermectin molecules to create a complex to be considered as ionophore (14).

An ionophore for cations such as zinc for example may then affect the hydroelectrolyte balance in the cell to cause early virophagy and even act to inhibit RNA-dependent-RNA polymerase (RdRp) as done by remdesivir (15). It could therefore be expected that when administered with zinc, ivermectin should be able to interfere with the replication process of SARS-CoV-2. The new hypothetical mechanism to explain anti-COVID-19 effect of ivermectin is that it displays ionophore activity to cause early viral lysis (27). Clinical trials of ivermectin alone (30) or the combination of zinc, doxycycline and ivermectin on COVID-19 management are currently recruiting participants (27). It is therefore recommended that more *in vitro* and *in vivo* studies be carried out to determine the clinical efficacy of ivermectin in SARS-COV-2 infections, with the aim of reducing morbidity and mortality from the ongoing COVID-19 pandemic.

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Clinical symptoms and outcomes among hospitalized COVID-19 patients in Ondo State, Southwestern Nigeria

*[1]Usman, S. O., [2]Busari, I. I., [3]Fagbemi, S., [2]Adeniyi, M. M., [2]Irabor, P., [4]Usman, I. N., and [5]Akintayo-Usman, N. O.

[1]APIN Public Health Initiatives, Akure, Ondo State, Nigeria
[2]Infectious Disease Hospital, Akure, Ondo State, Nigeria
[3]Ondo State Ministry of Health, Akure, Ondo State, Nigeria
[4]Department of Public Health, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria
[5]Nurse Tutors Programme, University College Hospital, Ibadan, Oyo State, Nigeria
*Correspondence to: senatorhopsy@yahoo.com

Abstract:

Background: The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a novel strain of coronavirus, which is the cause of the current coronavirus disease 2019 (COVID-19) pandemic, ravaging many countries of the world. The objective of this study is to assess the symptomatology and case management outcome of hospitalized COVID-19 patients in Ondo State, Southwestern Nigeria.
Methodology: This was a longitudinal study carried out on randomly selected patients with COVID-19, confirmed by real-time reverse transcriptase-polymerase chain reaction (rRT-PCR), admitted to the Infectious Disease Hospital, Akure, from March to July 2020. Clinical and outcome data obtained from the patients were analysed using the Statistical Package for the Social Sciences (SPSS) version 24.0 software, and variables were compared using the Chi square (χ^2) test and Odds ratio (OR).
Results: A total of 215 hospitalized COVID-19 patients were randomly recruited, with 103 males and 112 females (M:F ratio of 1:1.1), and mean age of 37.24 ± 16.83 years. The most common symptoms were shortness of breath (22.8%), cough (18.6%), fatigue (17.2%), runny nose (16.7%), fever (16.3%), and sneezing (14.0%). Mortality rate among the patients was 4.7% (10/215). Statistical analysis showed that fever [χ^2 = 8.75, OR 2.17 (95% CI: 0.29-16.63), p=0.003] and sneezing [χ^2=11.35, OR 2.75 (95% CI: 0.34-18.27), p=0.001] were clinical presentations with significant impact on the final outcome of the patients.
Conclusion: This study showed that the most common symptoms in hospitalized COVID-19 patients were shortness of breath, cough, running nose, fever and sneezing, which underscores the importance of monitoring of patients for these symptoms.

Keywords: COVID-19; symptoms; management; hospitalized; outcome; Nigeria

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Symptômes cliniques et résultats chez les patients hospitalisés COVID-19 dans l'État d'Ondo, dans le sud-ouest du Nigéria

*[1]Usman, S. O., [2]Busari, I. I., [3]Fagbemi, S., [2]Adeniyi, M. M., [2]Irabor, P., [4]Usman, I. N., et [5]Akintayo-Usman, N. O.

[1]Initiatives de santé publique 1APIN, Akure, État d'Ondo, Nigéria
[2]Hôpital des maladies infectieuses, Akure, État d'Ondo, Nigéria
[3]Ministère de la Santé de l'État de Ando, Akure, État d'Ondo, Nigéria
[4]Département de la santé publique, Université de technologie Ladoke Akintola, Ogbomoso, État d'Oyo, Nigéria
[5]Programme d'infirmières tuteurs, Hôpital universitaire, Ibadan, État d'Oyo, Nigéria
*Correspondance à: senatorhopsy@yahoo.com

Abstrait:

Contexte: Le coronavirus-2 du syndrome respiratoire aigu sévère (SRAS-CoV-2) est une nouvelle souche de coronavirus, qui est à l'origine de la pandémie actuelle de coronavirus 2019 (COVID-19), ravageant de nombreux pays du monde. L'objectif de cette étude est d'évaluer les résultats de la symptomatologie et de la prise en charge des cas de patients hospitalisés COVID-19 dans l'État d'Ondo, dans le sud-ouest du Nigéria.

Méthodologie: Il s'agissait d'une étude longitudinale réalisée sur des patients sélectionnés au hasard atteints de COVID-19, confirmée par réaction en chaîne par transcriptase-polymérase inverse en temps réel (rRT-PCR), admis à l'hôpital des maladies infectieuses d'Akure de mars à juillet 2020. Les données cliniques et les résultats obtenus des patients ont été analysés à l'aide du logiciel Statistical Package for the Social Sciences (SPSS) version 24.0, et les variables ont été comparées à l'aide du test du Chi carré (χ^2) et du rapport de cotes (OR).
Résultats: Un total de 215 patients hospitalisés COVID-19 ont été recrutés au hasard, avec 103 hommes et 112 femmes (rapport H: F de 1: 1,1), et un âge moyen de 37,24±16,83 ans. Les symptômes les plus courants étaient l'essoufflement (22,8%), la toux (18,6%), la fatigue (17,2%), l'écoulement nasal (16,7%), la fièvre (16,3%) et les éternuements (14,0%). Le taux de mortalité parmi les patients était de 4,7% (10/215). L'analyse statistique a montré que la fièvre [χ^2=8,75, OR 2,17 (IC à 95%: 0,29 à 16,63), p=0,003] et les éternuements [χ^2=11,35, OR 2,75 (IC à 95%: 0,34 à 18,27), p=0,001] étaient présentations cliniques avec un impact significatif sur le résultat final des patients.
Conclusion: Cette étude a montré que les symptômes les plus courants chez les patients hospitalisés sous COVID-19 étaient l'essoufflement, la toux, le nez qui coule, la fièvre et les éternuements, ce qui souligne l'importance de la surveillance des patients pour ces symptômes.

Mots clés: COVID-19; symptômes; la gestion; hospitalisé; résultat; Nigeria

Introduction:

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It was first identified in Wuhan, the capital of Hubei province of China in December 2019 and has gone on to spread across different parts of the world becoming a pandemic in the process (1). Specifically, on the 29th December, 2019, the first four cases of an acute respiratory syndrome of unknown aetiology were reported in Wuhan City, Hubei Province, China among people linked to a local seafood market (2). Studies thus far indicate that the virus origin is connected to a seafood market in Wuhan, but specific animal associations have not been confirmed. Reported symptoms include fever, cough, fatigue, headache, diarrhoea, haemoptysis, and dyspnea. Preventive measures such as masks, hand hygiene practices, avoidance of public contact, and quarantines have been discussed as ways to reduce transmission. To date, no specific antiviral treatment has proven effective, hence, management of infected persons primarily rely on symptomatic treatment and supportive care (3).

On 11th March, 2020, the World Health Organization (WHO) declared COVID-19 a pandemic, having met epidemiological criteria of more than a hundred thousand infected persons in at least 100 countries (4). The virus is primarily spread between people during close contact, most often through small droplets produced by coughing, sneezing, and talking. The droplets usually fall to the ground or onto surfaces rather than travelling through air over long distances (5). While the majority of COVID-19 cases have mild symptoms, severe complications include acute respiratory distress syndrome (ARDs), pneumonia and bronchitis (6). These complications are more pronounced in patients with underlying health conditions such as immuno-compromised individuals, the elderly and those with cardiopulmonary diseases (7,8). The time from exposure to onset of symptoms is typically around 5 days, but may range from 2-14 days (9,10).

The WHO has published several testing protocols for the diagnosis of COVID-19 (11). The standard method of testing is the real-time reverse transcription polymerase chain reaction (rRT-PCR) (9), which is typically done on respiratory samples obtained by a nasopharyngeal swab, however, oropharyngeal swab or sputum sample may also be used. The samples should be collected after 48-72 hours of fever subsisting without treatment (12-15). The treatment of COVID-19 patients remains largely supportive, with a number of cases requiring oxygen supplementation and intensive care (16,17).

The first index case in Nigeria was reported on 27th February 2020, when an Italian citizen in Lagos tested positive for the virus in the wake of increasing cases in Africa (18, 19). A second case of the virus was reported in Ewekoro, Ogun State on 9th March 2020 (20), a Nigerian citizen who had been a contact of the Italian index case. African countries are known to have fragile health systems and this remains a source of concern, especially in the event of increased outbreaks (20). On 23rd March, 2020, Nigeria confirmed the first COVID death, a 67-year-old Engineer and former managing director of Pipelines and Products Marketing Company, who returned from United Kingdom with underlying health conditions (21). A study on the clinical presentation of COVID-19 patients in Nigeria by Bowale et al., (8), revealed that 75% of the patients presented in moderately severe condition while 16% were asymptomatic. The most common presenting symptoms were fever (59%) and dry cough (44%). The study also showed that 97% of the patients were treated with lopinavir-ritonavir with no recorded death while the average duration of hospitalization was 12 days (8).

In a review of 1556 hospitalized patients with COVID-19 (22), 57.5% were male and mean age of the patients was 49.1 years. Pooled data revealed that nasal congestion

was observed in 3.7% of the patients and the most common symptoms were fever (85.6%), cough (68.7%), and fatigue (39.4%). In a South African report on the primary care management of COVID-19 patients by Mash (23), the common clinical symptoms were cough (95%), fever (86%), fatigue (71%), shortness of breath (43%) and diarrhoea (10%). The severities of the COVID-19 among admitted patients in a Chinese study (24) were 62% mild, 19% severe, and 19% critical, according to the Chinese Center for Disease Control (CDC) classification guidelines. The patients in the study reportedly received standard treatments based on the guidelines of Chinese CDC including antiviral remedies (arbidol, lopinavir and ritonavir, interferon-α inhalation) and anti-inflammatory treatments (corticosteroid) among others. Seventeen (81%) patients recovered and were discharged while 4 (19%) died. In a study of 1591 Italian patients with SARS-CoV-2 infection, patients had clinical manifestations of fever (70.1%), cough (45.3%), and expectoration (26.7%) at admission (25).

There has been several and worryingly increasing cases of COVID-19 incidence in the study area of the authors and in Nigeria as a whole. However, dearth of literature exists on the effective management and final outcomes of COVID-19. Hence, the objective of this study is to assess the clinical presentations and outcomes among hospitalized COVID-19 patients in Ondo State, Southwestern Nigeria.

Materials and method

Study setting

The study was conducted in Ondo State, Nigeria, created on February 3, 1976 from the former Western State with GPS coordinates of 7° 6' 0.0180'' N and 4° 50' 30.0984'' E (Fig 1). It originally included the present Ekiti State, which was split off in 1996. Akure is the State capital and according to the 2006 census, the State had a population of 3,460,877 (26).

Study design, population and criteria

This study was longitudinal in design and was conducted on COVID-19 patients admitted to the Infectious Disease Hospital, Akure, Ondo State from March 2020 to July 2020. COVID-19 diagnosis was made on the basis of positive SARS-COV-2 rRT-PCR test (11). Patients whose disease did not require hospitalization or for certain reasons were not admitted into the hospital, were excluded from the study.

A simple random sampling method was used to select the hospitalized COVID-19 patients for the study. Data was collected from each patient medical record using a chart abstraction tool containing biodata, socio-demographic, clinical presentations, underlying health conditions, management and outcome of COVID-19. Ethical approval was obtained from the Ondo State Ministry of Health, Akure, with NHREC/18/08/2016 and OSHREC/17/07/2020/278.

Fig 1: Map of Nigeria showing the location of Ondo State

Specimen collection and laboratory analysis

Both oropharyngeal and nasopharyngeal swabs were obtained from each patient for the diagnosis of COVID-19 by the real-time RT-PCR (rRT-PCR) assay

Statistical analysis

Clinical data were analysed using the Statistical Package for the Social Sciences (SP SS) for windows version 24.0 software (SPSS Inc; Chicago, IL, USA). Frequency distribution was generated for all variables and statistical test of significance was performed with Chi-square test and Odd's ratio (OR). Other data were expressed as mean±standard deviation. Significant association was fixed at $p<0.05$ and highly significant association at $p<0.01$.

Results:

A total of 215 COVID-19 patients were studied; 103 males and 112 females (M:F ratio of 1:1.1). The mean age of the patients was 37.24±16.83 years, and over half (53.0%) were between the ages 25-44 years (Table 1). Tables 2 shows the symptoms exhibited by patients. The most common symptoms were shortness of breath (22.8%), cough (18.6%), fatigue (17.2%), runny nose (16.7%), fever (16.3%) and sneezing (14.0%). Less than 2% of the patients experienced ageusia and anosmia (Tables 2).

The patients were routinely treated with lopinavir-ritonavir, and also received supplements such as vitamin C, zinc and calcium. The mean duration of admission was 9.00±5.20 days, with certain patients admitted for as long as 39 days. Ten (4.7%) of the 215 hospitalized patients died and most death occurred within 1-3 days of admission.

Table 1: Age and gender distribution of COVID-19 patients in Ondo State, Nigeria (March – July 2020)

Age group (years)	**Gender**		**Total (%)**
	Male (%)	**Female (%)**	
<10	7 (3.3)	7 (3.3)	14 (6.5)
10 – 14	0	2 (0.9)	2 (0.9)
15 – 19	4 (1.9)	4 (1.9)	8 (3.7)
20 - 24	2 (0.9)	11 (5.1)	13 (6.0)
25 – 29	14 (6.5)	19 (8.8)	33 (15.3)
30 – 34	15 (7.0)	14 (6.5)	29 (13.5)
35 – 39	17 (7.9)	14 (6.5)	31 (14.4)
40 – 44	9 (4.2)	12 (5.6)	21 (9.8)
45 – 49	9 (4.2)	8 (3.7)	17 (7.9)
50 – 54	4 (1.9)	6 (2.8)	10 (4.7)
55 – 60	11 (5.1)	6 (2.8)	17 (7.9)
≥ 60	11 (5.1)	9 (4.2)	20 (9.3)
Total	**103 (47.9)**	**112 (52.1)**	**215 (100)**

Table 2: Clinical symptoms in hospitalized COVID-19 patients in Ondo State, Nigeria (March – July 2020)

Clinical symptom	**Frequency (%)**
Fever	35 (16.3)
Cough	40 (18.6)
Shortness of breath	49 (22.8)
Sore throat	14 (6.5)
Runny nose	36 (16.7)
Sneezing	30 (14.0)
Body Ache	13 (6.0)
Fatigue	37 (17.2)
Loss of appetite	14 (6.5)
Muscle/Joint Pain	29 (13.5)
Vomiting	14 (6.5)
Headache	8 (3.7)
Diarrhoea	19 (8.8)
Ageusia	2 (0.9)
Anosmia	3 (1.4)

Table 3: Statistical analysis of impacts of clinical symptoms on outcome of hospitalized COVID-19 patients in Ondo State, Nigeria (March to July 2020)

Clinical symptoms	*χ^2	OR	95% CI	*p* value**
Shortness of breath	0.98	0.14	0.04 – 0.51	0.323
Fever	8.75	2.17	0.29 – 16.63	0.003
Sneezing	11.35	2.75	0.34 – 18.27	0.001
Cough	0.90	0.51	0.13 – 2.08	0.343

*The null hypothesis is rejected when the Chi square test statistic (χ^2) is greater than the critical value of 3.84; **p<0.05 is statistically significant; OR = Odds ratio; CI = Confidence interval

Discussion:

This study revealed that the most common symptoms were shortness of breath (22.8%), cough (18.6%), fatigue (17.2%), runny nose (16.7%), fever (16.3%) and sneezing (14.0%). These symptoms are typical of the epidemiology of the disease and similar to findings of other previous studies in Africa, Europe and Asia (8,27,28) although COVID-19 has been reported to affect different people in different ways, with some asymptomatic for the disease. The most common symptoms across the world have been reported to include cough and fever (29), thus, this study shows that identification of suspected COVID-19 patients may be more difficult than envisaged as over two-thirds of the patients shows neither symptom of cough nor fever. An elevated temperature is one way to identify a person who may have COVID-19 infection, although an infected person may be contagious without an elevated temperature or not detected by the temperature assessment device. Therefore, the efficiency of the use of non-contact temperature assessment devices as part of initial check to identify possible COVID-19 patients may have to be reviewed. This suggests that temperature measurements alone may not be adequate to identify potential positive coronavirus patients.

This study showed that less than 2% of the patients experienced ageusia and anosmia. This is in contrast to a Qatar study which reported prevalence rates of ageusia, anosmia, and ageusia and anosmia to be 8.51%, 11.35%, and 4.96% respectively, with overall prevalence rate 24.8% (30). Loss of smell and taste are common complaints in patients with the COVID-19, which may present alone or with other symptoms. Anosmia has already been reported in the course of COVID-19 worldwide, however, it represents a rare occurrence as shown in this study. It is not uncommon for upper respiratory infections to affect the senses of smell and taste (31), as the sense of taste and sense of smell are closely linked. Infact, experiencing a loss of smell can greatly impact the sense of taste. It is estimated that 95% of the time when there is a loss of taste, it is associated with a reduced sense of smell (31). Therefore, it is not uncommon for both to occur together in patients as indicated in this study.

The Chi square and Odd's ratio (OR) showed that fever and sneezing were the two clinical symptoms that had impact on the outcome of patients. This was evidenced by the fact that of the 35 patients who had fever on presentation at the point of hospital admission, 30 (86%) of them recovered (χ^2=8.75, OR 2.17, p=0.003), while of 30 patients who were sneezing on admission day, 25 (83%) recovered from the virus infection (χ^2=11.35**,** OR 2.75**,** p=0.001). Conversely, cough and shortness of breath did not have significant impact on outcome as 37 (93%) of the 40 patients who presented with cough at the point of hospital admission recovered (χ^2= 0.90, OR 0.51, p=0.343) while 48 of 49 (98%) of the patients who presented with shortness of breath on the day of admission recovered from the disease (χ^2=0.98, OR 0.98, p= 0.323). This observation may be due to the fact that most of the COVID-19 patients who presented with fever and sneezing had some other health conditions (data not shown), which may have contributed to these clinical symptoms that were further exacerbated by the virus infection, thereby impacting the final outcome, with more fatalities in this category of patients compared to others.

Conclusion:

In conclusion, this study showed that the most common clinical presentations in COVID-19 include shortness of breath, cough and fever, however, identification of suspected COVID-19 patients may be more difficult than envisaged as over two-thirds of the patients showed neither symptom of cough nor fever. Temperature measurements alone may not be adequate to identify potential positive COVID-19 patients.

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Original Article **Open Access**

Outbreak of Measles in vaccinated population in Southeastern Nigeria

*[1]Shenge, J. A., [2]Odaibo, G. N., and [2]Olaleye, D. O.

[1]Department of Biological Sciences, Dominican University, Ibadan, Nigeria
[2]Department of Virology, College of Medicine, University of Ibadan, Ibadan, Nigeria
*Correspondence to: jadamma@yahoo.com

Abstract:

Background: Outbreaks of respiratory disease, febrile illness and rash occurred in two adjoining rural communities of Imo State, Southeastern, Nigeria, at different times between 2006 and 2020. Laboratory investigation was carried out to determine the aetiological agent of the outbreak.
Methodology: Oropharyngeal swabs were collected from 6 individuals showing symptoms of disease, within 3-4 days of appearance of rash. Venous blood samples were also collected from a total of 41 symptomatic persons, their contacts and individuals with resolved infections. Swabs were inoculated into Vero, HEp-2c, B95a and MDCK cell lines. Sera were analyzed using enzyme-linked immunosorbent assay (ELISA) for immunoglobulin G and M to rubella and measles viruses, while immunofluorescence assay was used to detect Lassa fever virus immunoglobulins. Descriptive data were analyzed using the Statistical Package for the Social Sciences (SPSS).
Results: Four of the 6 (66.7%) swab samples showed viral activity or cytopathic effect characterized by clumping of cells in Vero cells while 2 (33.3%) in Hep-2c characterized by rounding up of cells. Thirty-nine (95.1%) sera were positive for measles IgG while 13 (31.7%) were positive for IgM. Thirty-six (87.8%) sera were positive for rubella IgG but none was positive for IgM. None of the sera was positive for Lassa fever virus IgG and IgM.
Conclusion: Measles virus was responsible for the outbreak among previously vaccinated population in the communities, while Rubella and Lassa fever viruses were excluded as the etiological agents of the outbreak.

Keywords: Epidemics; IgG and IgM; Cell lines; Vaccination; Measles virus

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Épidémie de rougeole dans la population vaccinée du sud-est du Nigéria

*[1]Shenge, J. A., [2]Odaibo, G. N., et [2]Olaleye, D. O.

[1]Département des sciences biologiques, Université dominicaine, Ibadan, Nigéria
[2]Département de virologie, Collège de médecine, Université d'Ibadan, Ibadan, Nigéria
*Correspondance à: jadamma@yahoo.com

Abstrait:

Contexte: Des flambées de maladies respiratoires, de maladies fébriles et d'éruptions cutanées sont survenues dans deux communautés rurales voisines de l'État d'Imo, dans le sud-est du Nigéria, à des moments différents entre 2006 et 2020. Une enquête en laboratoire a été menée pour déterminer l'agent étiologique de l'épidémie.
Méthodologie: Des écouvillons oropharyngés ont été prélevés sur 6 individus présentant des symptômes de maladie, dans les 3 à 4 jours suivant l'apparition de l'éruption cutanée. Des échantillons de sang veineux ont également été prélevés sur un total de 41 personnes symptomatiques, leurs contacts et des personnes souffrant d'infections résolues. Des écouvillons ont été inoculés dans des lignées cellulaires Vero, HEp-2c, B95a et MDCK. Les sérums ont été analysés en utilisant un test immuno-enzymatique (ELISA) pour les immunoglobulines G et M contre les virus de la rubéole et de la rougeole, tandis que le test d'immunofluorescence a été utilisé pour détecter les immunoglobulines

du virus de la fièvre de Lassa. Les données descriptives ont été analysées à l'aide du progiciel statistique pour les sciences sociales (SPSS).
Résultats: Quatre des 6 échantillons sur écouvillon (66,7%) ont montré une activité virale ou un effet cytopathique caractérisé par l'agglutination des cellules dans les cellules Vero, tandis que 2 (33,3%) dans Hep-2c étaient caractérisés par un arrondissement des cellules. Trente-neuf (95,1%) sérums étaient positifs pour les IgG contre la rougeole tandis que 13 (31,7%) étaient positifs pour les IgM. Trente-six (87,8%) sérums étaient positifs pour les IgG contre la rubéole, mais aucun n'était positif pour les IgM. Aucun des sérums n'était positif pour les IgG et IgM du virus de la fièvre de Lassa.
Conclusion: Le virus de la rougeole était responsable de l'épidémie parmi la population précédemment vaccinée dans les communautés, tandis que les virus de la rubéole et de la fièvre de Lassa ont été exclus comme agents étiologiques de l'épidémie.

Mots clés: épidémies; IgG et IgM; Lignées cellulaires; Vaccination; Virus de la rougeole

Introduction:

Emerging infectious diseases have been the most implicated in recent outbreaks, affecting human and animal lives in very negative ways, globally. Viruses survive only in living cells essentially to create more particles by replication, which involves taking over the host cell protein and genetic material – synthetic processes, to produce virus related nucleic acid and proteins as new viruses (1). If not curtailed, the spread of viral disease in a population may become extensive, and most viruses especially the enteroviruses are known to survive harsh environmental conditions. In endemic areas, viruses are continuously shed into the environment and this can give rise to an outbreak once entrance is gained into a suitable host (2).

Most RNA viruses of the families; *Orthomyxoviridae*, *Paramyxoviridae* and *Corona viridae* have been implicated in viral disease outbreaks, with pandemic potentials. In humans especially, respiratory viruses are one of the most important causative agents of diseases causing acute respiratory infections (ARIs), particularly among children less than five years, often with high morbidity and mortality worldwide (3). The appearance of new diseases and syndromes as well as the resurgence of old ones can be associated to a large extent with ecological changes that favour increased vector densities, coupled with human activities that spread infectious agents and introduce them into areas where they had been absent (4).

Many viral infections and their emergence are brought about by a combination of factors which increase human exposure to viral pathogens (5). Some of these factors include human behaviours such as environmental changes, food production, socio-economic and demographic factors, health care delivery, travel and commerce (6). Ecological factors also play a tremendous role in disease distribution and these changes at one point or the other encourage migration of virus reservoirs (such as wild birds and bats) which transmit viral diseases to both humans and animals in their course of migration (7). Recurrence of epidemics and pandemics of viral origin in a number of cases occur as a result of some other factors which may be genetic changes or mutation (variations) in the genome of viruses or adaptation to new hosts (8).

When a virus succeeds in establishing an infection in the host, a viral disease results through one of the following; destruction of the host cells (cytolytic effects), continuous production of virus by budding in which the virus maintains in a steady state with the host or a chronic and often fatal condition arises due to integration of viral genetic material into the genome of the host, leading to transformation of the host cells (8). An infection is a condition in which viruses, bacteria, fungi or parasites enter the body and cause a state of disease (9). They damage cells of the body by adhering to and damaging the cell walls, releasing toxic substances or causing allergic reactions (10).

Majority of viral disease outbreaks are linked to water as the source. Schuster and colleagues (11) analyzed waterborne outbreaks and impact of drinking water quality on public health disease burden, and found that many factors such as severe weather or climate change, close proximity to animal populations, treatment system malfunctions, poor maintenance and treatment practices were associated with the reported disease outbreaks resulting from drinking water supplies. Transmission of viral diseases is of public health importance. Direct and indirect contacts between humans and other vertebrates have resulted in serious outbreaks (12). It has been estimated that 60% of human infections are caused by viruses (13).

While some viruses show full spectrum of characteristics that identify them as infectious agents in most viral diseases, others do not. Hence, the aim of this outbreak investigation was to identify the etiological agent of the outbreaks in two rural communities of Imo State, Southeastern, Nigeria, with laboratory investigations including serological tests, virus

isolation in cell lines, and immunofluorescent assays.

Materials and method:

Study area

This outbreak investigation was carried out in Obowu Local Government Area of Imo State, Nigeria. The area comprises of fourteen communities. It lies on longitude $7^0$20'E and Latitude $5^0$30'N. It is located about 150 km north of Bonny in Eastern Nigeria, with an estimated population of about 50,000 people as projected from the 2006 census figure (16).

Ethical consideration

Approval to investigate the outbreaks was obtained from the local councils and the traditional rulers of affected communities. Informed consent of all persons from whom samples were collected was obtained.

Data collection and sample processing

Data from individuals including records of vaccination and other useful information regarding the outbreak, were collected. These included information on vaccination history, and symptoms observed such as sore throat, cough, catarrh, coryza, myalgia, fever. Oropharyngeal swab samples were collected from 6 persons presenting with fresh symptoms of rash and respiratory infection while venous samples were collected from a total of 41 persons with symptoms, their contacts and individuals with resolved infections.

The swab specimens were collected on swab sticks into sterile cryovials containing PBS and antibiotics as transport medium. The samples were preserved at -20^0C until transported with ice packs to the laboratory for analysis. In addition, venous blood samples were collected into sterile EDTA bottles containing anticoagulant from all individuals including those with clinical symptoms, resolved cases and contacts of those with active disease and symptoms. The samples were stored at 4^0C until transported to the laboratory.

Both blood and oro-pharyngeal samples were transported to Virology laboratory of the University College Hospital, Ibadan, Nigeria, in insulated boxes containing ice-packs to maintain the cold chain. Blood samples were separated and sera stored in new sterile tubes and stored at -20^0C pending analyses.

Virus isolation in tissue culture

Four cell lines were used for the primary isolation and 'passage' of the suspected viral agent from each swab sample. The cell lines included Vero (from African green monkey kidney), HEp-2c (derived from human epithelial cells), MDCK (Mardin-Darbin Canine Kidney cells) and B95a (from kidney of Marmoset Monkey). Each oro-pharyngeal swab sample was diluted and made up to 2.5 ml with maintenance medium and filtered using a 0.2µl millipore filter into new set of pre-labelled Kahn tubes. After filtration, 0.2ml of each swab sample was inoculated into each cell line after decanting the growth medium. Virus from sample was allowed to adsorb at 37^0C for one hour, and the appropriate maintenance medium was added to each cell line. The medium of cell control tubes was replaced with maintenance medium.

All the tubes were incubated at 37^0C and examined daily for evidence of virus growth (cytopathic effect) for seven days. Tubes showing cytopathic effects (CPE) on or before the seventh day were passed into new culture tubes to rule out toxicity or to increase the virus titre. Similarly, tubes without obvious CPE were also re-passaged. Any culture tubes without CPE after the first blind 'passage' and kept for more days were regarded as negative. The tubes showing cytopathic effects (CPE) were passed up to four times into 25cm^3 culture flasks to increase the virus titre and yield. The supernatant fluid of each flask was collected and stored in aliquots at -80^oC for further analysis.

Detection of Measles immunoglobulin G & M

Measles IgG and M was detected using Orgenics ImmunolisaTM kit. All the reagents and samples were allowed to attain room temperature on the bench. The incubator was set at 37^0C. The microplate R1 was labelled with sample numbers accordingly, including the four control wells. Then, 100µl of serum dilution solution R2 was added into all the wells and 5µl of each sample into appropriate wells. Also, 5µl of positive control R3 and 5µl of cut off serum R5 were added in duplicate, and 5µl of negative control was dispensed. The microplate was covered with sealing sheet and incubated at 37^0C for 45mins. After incubation, 100µL of 1gG conjugate solution R9 was immediately added into each well. The microplate was sealed again and incubated at 37^0C for 30min. This was followed by washing of the plate with 0.3ml of washing solution R13. 100 µl of substrate solution R11 was added into each well. The plate was sealed and incubation done at room temperature for 20mins. The seal was removed and 50µl of stopping solution R12 added into all the wells. Reading of the plate was carried out with a spectrophotometer at 450/620nm.

The mean OD (Optical Density) for each

control (positive, negative and cut off) was calculated as; cut off control = < 0.7 x (OD Positive Control)/>1.5 x (OD Negative Control), and antibody index = sample OD/cut off serum mean OD x 10. For validity of the result, OD for positive control must be > 0.9 while that of negative control must be < 0.55. The antibody index for positive samples was > 11 while that of negative samples was < 9. Samples with IgG and IgM index values of 9 - 11 were equivocal. Hence, samples with index values above 11 are considered as having IgG or IgM specific antibodies against measles, and the final result was based on these values.

Determination of Rubella immunoglobulin G & M by IgM 'capture'.

Using the Dia-Pro Diagnostic Bioprobes serological reagents, samples were diluted in ratio 1:101 with the sample diluent (10µl sample + 1000µl diluent) and dispensed accordingly. Ninety-six well microplate was covered with a sealer and incubated for 60min at 37^0C. Then 100µl of diluted Ag/Ab (immune complex) was added to all well except A1. The plate was incubated at 37^0 C for 60min. After removing the sealer, the microplate was washed five times using the automatic washer. This was followed by addition of 100µl of the chromogen/substrate mixture into the wells, A1 included. The plate was incubated the third time at room temperature for 20min.100µl stop solution was added to all wells, A1 included. The Microplate was read at 450nm and 620-630nm, blanking the A1 well.

Detection of Lassa fever immunoglobulin G & M by immunofluorescence assay (IFA)

The IFA slides and samples were allowed to attain room temperature before use. Samples were diluted in ratio 1:40 by adding 5µl of each serum sample into 195µl of PBS in a pre-labeled microplate. Then, 20µl of 1:40 dilution of specimen was dispensed accordingly into the wells of the slides. Controls were dispensed into the corresponding wells. The IgM slides were placed in a tray and incubated at 37^oC in humidified chamber for 2 hours while IgG slides were incubated for one hour. After incubation, slides were flooded with PBS and rocked for 10min on a rocker. Both slides were air dried and respective Fluorescein isothiocyanate (FITC) conjugated-sheep antihuman Ig (Murex Diagnostic Ltd., Dartford, England) added into all the wells. The second incubation was carried out at 37^0C for 30min.Slides were flooded and washed for another 10min on a rocker. After washing, each slide was covered with a cover slip and slides mounted on the fluorescence microscope for microscopic examination.

Statistical analysis

Descriptive data were analyzed using Statistical Package for Social Sciences (SPSS).

Results:

In the two affected rural communities, 41 persons were serologically investigated, 14 (34.1%) males and 27 (65.9%) females, all aged between 3 months and 50 years. Six (14.6%) had clinical symptoms at the time of the outbreak investigation; 28 (68.3%) had resolved symptoms while 7 (17.1%) contacts reported no symptoms. Forty (97.6%) were previously vaccinated while 1 (2.4%) was yet to be vaccinated.

Of the 6 oropharyngeal swab samples, 4 (66.7%) showed evidence of viral growth (clumping of infected cells) in Vero, 2 (33.3%) samples showed CPE (rounding up of cells) in HEp-2c, and none in B95a and MDCK cell lines. This confirmed that virus was implicated in the outbreak. Serum samples of 39 (95.1%) out of 41 subjects were positive for measles IgG as shown in Fig 1. Fig 2 shows the distribution of measles IgM in which 13 (31.7%) were positive, 12 (92.3%) of whom were positive for both IgG and IgM. Figs 2, 3 and 4 show the number and percentage of positive results within different age groups and gender of the subjects.

Out of the 41 venous blood serum samples, 36 (87.8%) were positive for Rubella IgG. Fig 5 shows the distribution of Rubella IgG result among 13 (36.1%) males and 23 (63.9%) females. All sera were negative for rubella IgM. Furthermore, all samples tested negative for both IgG and IgM to Lassa fever virus. Hence, rubella and Lassa fever viruses were ruled out as the aetiological agents of the outbreak.

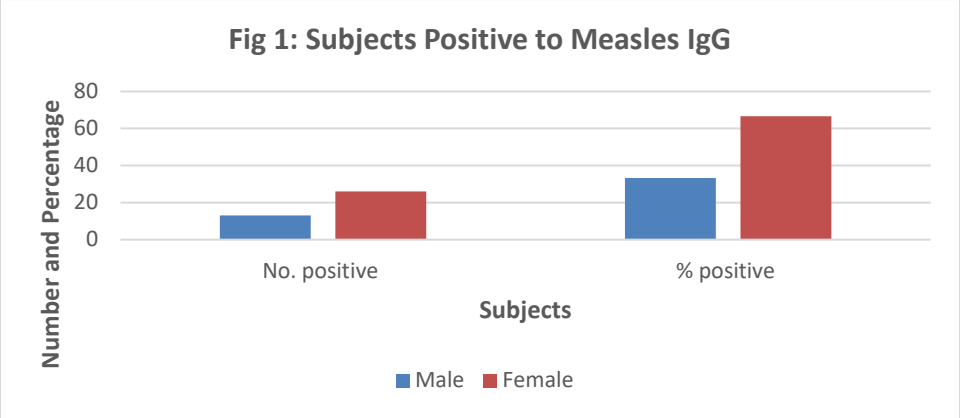


Fig 1: Subjects Positive to Measles IgG
Number and Percentage
80
60
40
20
0
No. positive
% positive
Subjects
Male
Female

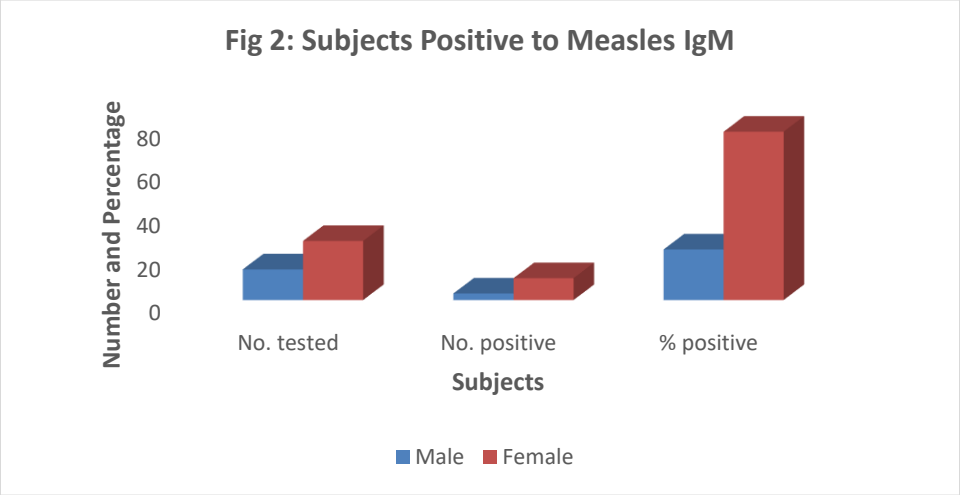


Fig 2: Subjects Positive to Measles IgM
Number and Percentage
80
60
40
20
0
No. tested
No. positive
% positive
Subjects
Male
Female

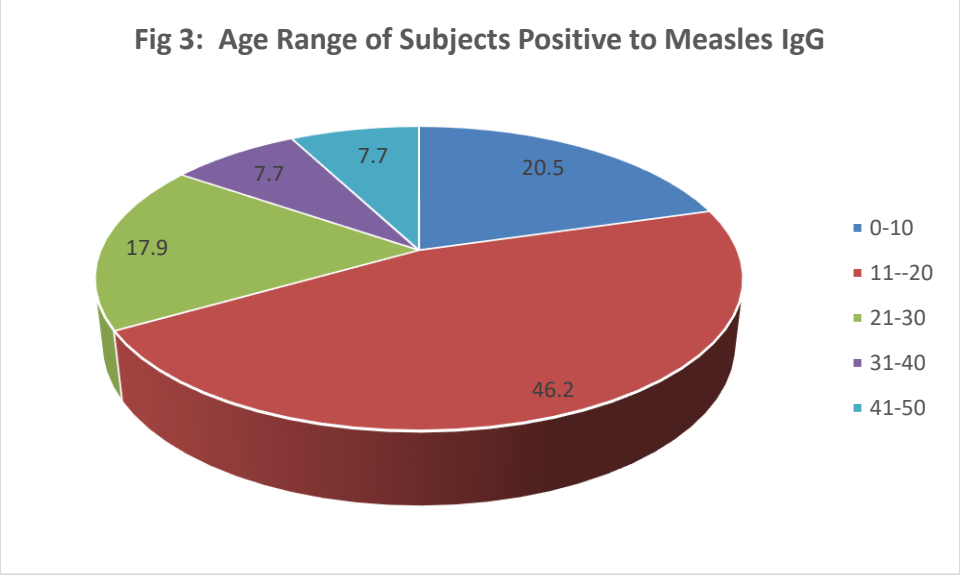
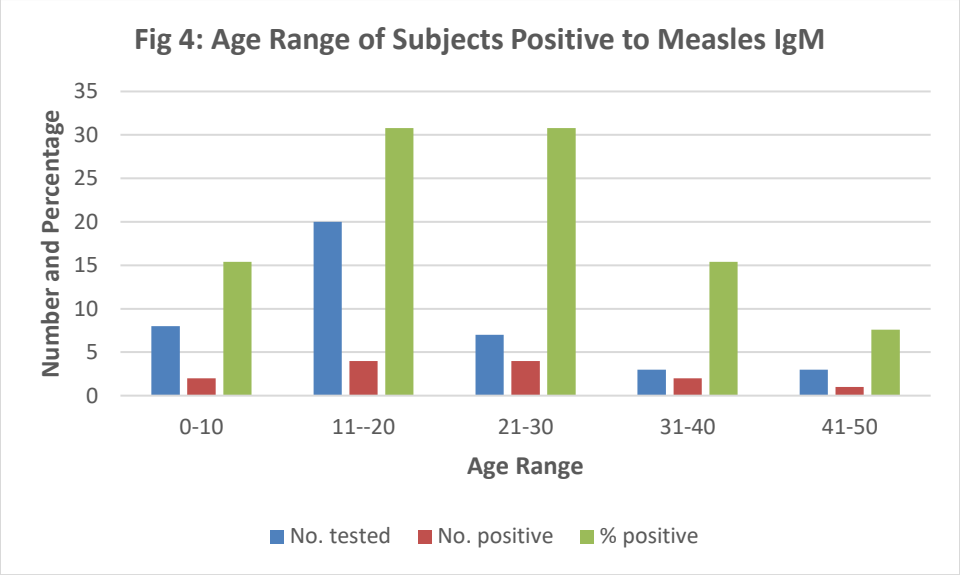
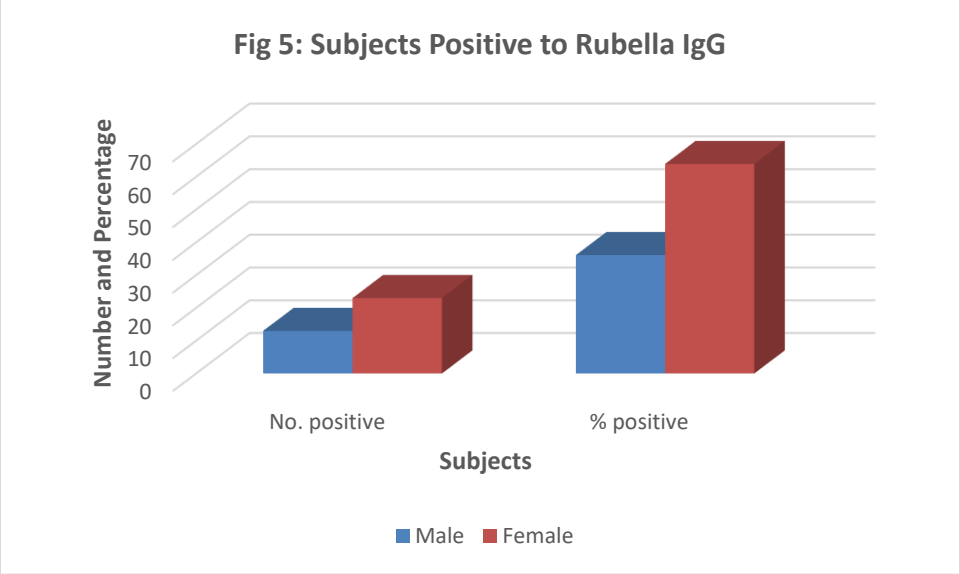


Fig 3: Age Range of Subjects Positive to Measles IgG
20.5
46.2
17.9
7.7
7.7
0-10
11--20
21-30
31-40
41-50





Discussion:

Measles remains a serious global health challenge, which has persisted even with the availability of a vaccine. It is highly contagious, with increased morbidity and mortality, especially among children less than five years of age. Measles virus is an enveloped, ribonucleic acid virus (RNA) of the genus *Morbillivirus* and family *Paramyxoviridae*. There are more than 20 different genotypes that have been isolated in various parts of the world, but with only one serotype (9). In Nigeria, measles, mumps and rubella (MMR) vaccines are given through compulsory routine vaccination exercise to infants at nine months of age (17). It is noteworthy, that this outbreak occurred among vaccinated subjects in those communities as shown by the positive measles IgG results. Measles outbreak among vaccinated children and adults rather poses some questions that require urgent scientific explanation.

Rubella virus was excluded as the causative agent of the outbreak, because none of the samples was positive for rubella IgM, however, 87.8% tested positive for IgG. These individuals (both gender) in the past received MMR vaccine, which may be responsible for rubella IgG antibody response. The same vaccination was supposedly responsible for the high measles IgG antibody level in the

population.

In the outbreak, both male and female subjects were infected with measles virus, although more females (76.9%) than males (32.1%) had active measles (Fig 3) and these subjects fell in the age range 0-50years, with more measles IgG level (46.2%) among those aged 11-20 years (Fig 4), and highest IgM level among age groups 11-20 and 21-30 years respectively (Fig 5). Forty (97.6%) had been vaccinated previously, except 1 (2.4%), a 3-month-old baby, yet to receive MMR routine immunization. Those infected had symptoms including febrile illness (high fever), cough, coryza (runny nose), red watery eyes and maculopapular rash which appeared last in most cases.

Immunity conferred by measles through natural infection and vaccination is apparently long-lasting, but some cases of atypical measles have been reported in adolescents who had been vaccinated as children (14,15). This suggests that immunity in the vaccinated population may have waned and therefore not as long-lasting as that following natural infection. It could also mean circulation of a different strain of the measles virus in the population, to which people have little or no immunity. However, it would be potentially serious, if vaccination in children left some people unprotected in teenage and adult life.

Furthermore, among the subjects who showed evidence of measles infection in this investigation, six presented with clinical acute respiratory symptoms and maculopapular rash and they all had positive measles IgM results. Three individuals who tested positive for measles IgM had resolved symptoms, while 4 were part of the 7 contacts who showed no symptom as at the time of sample collection. This suggests that these exposed individuals (contacts)contracted the virus and could be incubating it at the time of sample collection, owing to the contagious nature of measles virus.

Measles is known as a childhood disease (4) but its occurrence twice or more times in a vaccinated population, as seen in this investigation including adults, requires urgent attention. Measles virus infection remains a serious global health problem. Cases of infection caused by wild strain of measles virus in previously vaccinated subjects have been documented (2). As this trend persists, there is need for increased global campaign and awareness on the importance of booster vaccination especially among teenagers and adults who were vaccinated at infancy. This may be one sure way to prevent future measles outbreaks and ensure its eradication in the globe.

Conclusion:

In conclusion, the cytopathic effects (CPE) on cell cultures implicated virus as the aetiologic agent of the outbreak of an infectious respiratory disease and rashes in the two adjoining communities of Obowu LGA of Imo State, Nigeria. Further serological investigation confirmed measles virus as the cause of the outbreak as indicated by positive IgM antibodies in the infected subjects, which confirmed recent and active infection.

Acknowledgements:

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Conflict of interest:

Authors declared no conflict of interest.

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Original Article **Open Access**

Persistence of cervical human papillomavirus infection among cohort of women in Awka, Nigeria

*[1]Ezebialu, C. U., [2]Ezebialu, I. U., and [2]Ezenyeaku, C. C.

[1]Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Nigeria
[2]Department of Obstetrics and Gynaecology, College of Medicine, Chukwuemeka Odumegwu Ojukwu University, Awka, Nigeria
*Correspondence to: nenyeume@yahoo.com; 08066528090

Abstract:

Background: Many women are known to contract human papilloma virus (HPV) infection in their lifetime but only a few develop cervical cancer. One of the major factors that contribute to development of cervical cancer is HPV persistence. Several other factors including viral load have been implicated in cervical cancer development. This work therefore intends to investigate the persistence of cervical HPV infection among cohort of women in Awka, Nigeria.
Methodology: A cohort of 58 women with normal Papanicolaou (Pap) test but positive HPV DNA selected from a population of 410 women at baseline were followed up over a period of 6 months from April to October 2015. Cervical specimens collected were subjected to HPV DNA test and viral quantification using TaqMan Real Time PCR and cervical cytology. Risk factors were obtained using semi structured interviewer administered questionnaires. Variables were analysed using descriptive statistics and T-test on IBM SPSS statistics version 21.0 and EPI INFO™ 7.0
Results: At the 6-month follow up, cervical HPV infection persisted in 29 women, representing 50% of the women followed up. Among the 29 women, 7 (24.1%) developed abnormal Pap smear (Low grade Squamous Intraepithelial Lesion). Factors significantly associated with persistence at bivariate analysis of HPV include previous sexually transmitted infection (STI) (p=0.005), HIV positivity (p=0.04), HIV positivity but no anti-retroviral drugs (p=0.014), HPV 16 infection (p<0.0001) and age less than 40 years (p<0.0001). At multinomial logistic regression, only age above 17 years at first sexual intercourse (p=0.003, CI=0.012-0.392) and multiple lifetime sexual partners (p=0.021, CI=0.20-0.726) were statistically significant.
Conclusion: High risk HPV infection, in addition to other factors peculiar to an individual may influence HPV persistence

Key words: cervical cancer, human papillomavirus, persistence, cytology, risk factors, infection

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Persistance de l'infection cervicale par le papillomavirus humain parmi une cohorte de femmes à Awka, Nigéria

*[1]Ezebialu, C.U., [2]Ezebialu, I.U., et [2]Ezenyeaku, C. C.

[1]Département de microbiologie appliquée et brassage, Université Nnamdi Azikiwe, Awka, Nigéria
[2]Département d'obstétrique et de gynécologie, Collège de médecine, Université Chukwuemeka Odumegwu Ojukwu, Awka, Nigéria
*Correspondance à: nenyeume@yahoo.com; 08066528090

Abstrait:

Contexte: De nombreuses femmes sont connues pour contracter une infection au virus du papillome humain (VPH) au cours de leur vie, mais seules quelques-unes développent un cancer du col de l'utérus. L'un des principaux facteurs qui contribuent au développement du cancer du col de l'utérus est la persistance du VPH. Plusieurs autres facteurs, y compris la charge virale, ont été impliqués dans le développement du cancer du col de l'utérus. Ce travail vise donc à étudier la persistance de l'infection cervicale au VPH parmi la cohorte de femmes à Awka, au Nigeria.
Méthodologie: Une cohorte de 58 femmes avec un test de Papanicolaou (Pap) normal mais un ADN HPV positif sélectionné parmi une population de 410 femmes au départ ont été suivis sur une période de 6 mois d'avril à octobre 2015. Les échantillons cervicaux collectés ont été soumis à l'ADN HPV. test et quantification virale à l'aide de la PCR en temps réel TaqMan et de la cytologie cervicale. Les facteurs de risque ont été obtenus à l'aide de questionnaires semi-structurés administrés par les intervieweurs. Les variables ont été analysées à l'aide de statistiques descriptives et d'un test T sur IBM SPSS statistics version 21.0 et EPI INFOTM 7.0
Résultats: Au suivi de 6 mois, l'infection cervicale au VPH persistait chez 29 femmes, soit 50% des femmes suivies. Parmi les 29 femmes, 7 (24,1%) ont développé un test Pap anormal (lésion squameuse intraépithéliale de bas grade). Les facteurs significativement associés à la persistance lors de l'analyse bivariée du VPH comprennent les antécédents d'infection sexuellement transmissible (IST) (p=0,005), la positivité au VIH (p=0,04), la positivité au VIH mais pas d'antirétroviraux (p=0,014), l'infection au VPH 16 (p<0,0001) et moins de 40 ans (p<0,0001). Lors de la régression logistique multinomiale, seuls les âges supérieurs à 17 ans lors du premier rapport sexuel (p=0,003, IC=0,012-0,392) et les multiples partenaires sexuels à vie (p=0,021, IC=0,20-0,726) étaient statistiquement significatifs.
Conclusion: Une infection au VPH à haut risque, en plus d'autres facteurs propres à un individu, peut influencer la persistance du VPH

Mots clés: cancer du col de l'utérus, papillomavirus humain, persistance, cytologie, facteurs de risque, infection

Introduction:

Persistence of human papillomavirus (HPV) infection is a known cause of cervical cancer. About 80% of women will acquire an HPV infection in their lifetime (1), and up to 50% of those infections will be with a high-risk type (1,2,3). In majority of the infections, the immune system will suppress the virus and infection is only transient (4) with a clearance rate of about 70% in one year and about 90% in two years (5). In some women however, the infection will become persistence because of poor immune response (6,7) and this may progress and lead to the development of cervical cancer (8). Factors that determine the clearance and persistence of HPV DNA in a person include host factors such as tobacco smoking, prolonged use of oral contraceptives, pregnancy, HIV, parity, health status, and viral factors such as oncogenicity of the HPV type involved and viral load.

High viral load has been suggested to influence HPV persistence though interests were mainly on high-risk types such as HPV type 16 and 18 (9,10). It has been suggested that measurement of viral load could help to identify women who have greater risk of persistent HPV infection and also that women with high viral load but normal cytology could be at risk of HPV persistence (11,12,13,14). This issue of viral load remains controversial and still subject to further verifications

The objectives of this study are to quantify the genome copies in patients with persistent HPV infection in order to determine the threshold copies that may likely trigger the progression to abnormal cervical cytology, and to also determine the factors that influence HPV persistence.

Materials and method:

Study population

This included cohort of 58 women with normal Pap test but positive HPV DNA at the baseline study. This cohort is part of 410 women who were assessed in the initial survey result of which has been published elsewhere (15). These women were recalled after six months for follow up. The participants were not on any treatment for the condition during the follow up period

Ethical consideration

Ethical approval was obtained from the hospital ethics committee. Oral and written consent were obtained from the participants before enrolling them

Collection of data on risk factors for persistence of HPV infection from the study participants

Data collection was carried out using pretested semi structured interviewer administered questionnaires. Detailed socio-demographic information of each patient, including patient's age, and smoking habits, reproductive history, sexual habit of the woman and her partner, previous exposure to STDs, and life

time use of contraceptive were obtained. The questionnaire was administered to each participant in a private room to ensure confidentiality and this preceded sample collection for each participant. The questionnaires were coded with numbers that corresponded with those on the slides and collection bottles of each participant.

Specimen collection

The participants were asked to lie on a couch in the dorsal position. The vulva was cleaned with swab soaked with normal saline. Disposable speculum was inserted into the vagina and opened to expose the cervix for specimen collection. To collect specimen for Pap test, an Ayre spatula was inserted into the external cervical os (opening) and rotated through 360 degrees to take the cervical smear. This was immediately smeared on a glass slide and fixed immediately in 95% alcohol and then transferred to the pathology laboratory for processing.

To collect the specimen for HPV DNA test, a cytobrush was introduced into the external cervical os and rotated through 360 degrees. The cytobrush was then transferred immediately into a collection bottle containing Phosphate Buffered Saline (PBS) and stored at -20^0C until it was taken to the laboratory. The collection bottle was swirled to make sure that the PBS was well mixed with the tip of the cytobrush.

Cervical cytology (Papanicolaou test)

A smear of the cervical exfoliated cells collected using Ayres spatula was made on a grease-free slide, fixed, stained and examined microscopically for the dysplastic cells as characterized by anaplasia, hyperchromatism and large nucleus (16).

Viral DNA isolation

Viral DNA was extracted from GITC lysates using High Pure Viral DNA kit (Roche, UK). The High Pure Kit uses Spin Column method. The Spin Column method of DNA extraction is based on the principle of selective adsorption of viral DNA onto silica membrane and micro-centrifugation to remove impurities.

HPV type detection and quantification by real-time PCR assay

HPV genome copies were amplified by TaqMan real time PCR assay using commercially prepared primer/probe mixes from Life River Technology, China. Type specific probe/ primer (synthesized by Invitrogen UK (Life Technology, UK) mixes were used for typing of HPV (Table 1). The primers were designed based on E1, E6-E7 and L1 regions of the HPV genome. The primer sets were GP168 (for HPV types 11, 16, 18 and any other type); MY313 (for HPV types 31, 33 and others), OLIS35 (for HPV35 and others), and CpG mix were used for the HPV detection.

The samples numbers including the controls were carefully listed on the worksheet. The 2x universal master mix (Applied Biosystem, UK) containing enzyme and primers were prepared according to Manufacturer's instructions. A 40µl of the master mix was pipetted into the wells on the PCR plates and 10µl of the viral DNA samples and standards (Life River Technology, China) were added into appropriate wells to make up 50µl of the reaction volume.

The real time PCR system is OneStep Plus Real Time PCR 96 system (Applied Biosystem, UK). The plates were sealed after all the additions and the thermal profile for real time PCR was set as shown in Table 2. Real time PCR is a software driven analysis therefore, the progress and amplification were monitored on the computer. The standard was used to generate a calibration curve from which all sample viral loads were determined.

Statistical analysis

Relationship between variables was analysed using descriptive statistics on IBM SPSS version 21.0. EPI INFO^{TM} 7.0 was used to analyse 2x2 tables. Independent sample T-test was use to compare the mean of the viral loads and *p* value for significance was set at < 0.05.

Table 1: Primer sequences 5' to 3' for all the HPV types

	Primer Sequences 5' to 3'
HPV-11F	CGC AGA GAT ATA TGC ATA TGC
HPV-11R	AGT TCT AAG CAA CAG GCA CAC
HPV-16 F	TCA AAA GCC ACT GTG TCC TGA
HPV-16 R	CGT GTT CTT GAT GAT CTG CAA
HPV-16 SF	
HPV-16 SR	CCA TCC ATT ACA TCC CGT AC
HPV-18 F	CCG AGC ACG ACA GGA ACG ACT
HPV-18 R	TCG TTT TCT TCC TCT GAG TCG CTT
HPV-31 F	CTA CAG TAA GCA TTG TGC TAT GC
HPV-31 R	ACG TAA TGG AGA GGT TGC AAT AAC CC
HPV- 33 F	AAC GCC ATG AGA GGA CAC AAG
HPV-33 R	ACA CAT AAA CGA ACT GTG GTG
HPV-35 F	CCC GAG GCA ACT GAC CTA TA
HPV-35 R	GGG GCA CAC TAT TCC AAA TG
My09-F	CGT CCM ARR GGA WAC TGA TC
My11-R	GCM CAG GGW CAT AAY AAT GG
Gp5-F	TTT GTT ACT GTG GTA GAT AC
Gp6-R	GAA AAA TAA ACT GTA AAT CA
Gp plus F	TTT GTT ACT GTG GTA GAT ACT AC
Gp plus R	GAA AAA TAA ACT GTA AAT CAT ATT
Cp F	TTA TCW TAT GCC CAY TGT ACC AT
Cp R	ATG TTA ATW SAG CCW CCA AAA TT
Oli F	TGY AAA TAT CCW GAT TAT WT
Oli R	GTA TCI ACI ACA GTA ACA AA
Oli plus F	GCT TCA CCT GGC AGC TGT GT
Oli plus R	GTA TCT ACC ACA GTA ACA AA

Table 2: Real Time PCR Thermal Profile

Step	Temp.	Time	No of cycle
UNG enzyme reaction	50 C	2 min	1
Taq enzyme activation	95 C	5 min	1
Denature	94C	15 sec	45
Anneal, extend and data collected	57 C	30 sec	45

Results

In the baseline study (15), 82 (20.0%) of the 410 participants were HPV positive out of which 75 (91.5%) had normal cervical cytology. These 75 eligible participants were invited for the prospective study but only 58 (77.3%) responded and were follow up for a period of 6 months. At the 6-month follow up, cervical HPV infection persisted in 29 participants representing 50% of the participants. Among these 29 participants, 7 (24.1%) developed abnormal Pap smear (Low grade Squamous Intraepithelial Lesion).

In the bivariate analysis, factors significantly associated with HPV persistence after 6 months follow up include; previous STI (p= 0.005), HIV positivity (p=0.04), HIV positivity but no anti-retroviral drugs (p= 0.014), HPV 16 infection (p<0.0001) and age less than 40 years (p<0.0001) (Table 4). At multinomial logistic regression, only age > 17 years at first sexual intercourse (p=0.003, CI=0.012-0.392) and multiple lifetime sexual partners (p=0.021, CI=0.20-0.726) were statistically significant. The mean viral load was significantly higher among age group < 40 years (p=0.029), parity group ≤ 4 (p=0.015), and HPV type 18 (p= 0.036) (Table 5).

Table 3: Cervical status of the participants with normal cervix at baseline after 6 months follow up

HPV result	Normal n (%)	LSIL n (%)	HSIL n (%)	Cancer n (%)	Total n (%)	*p* value
HPV+	22 (75.9)	7 (24.1)	0	0	29 (50.0)	<0.05
HPV-	29(100%)	0(0.0%)	0	0	29 (50.0)	
Total	51 (87.9)	7 (12.1)	0	0	58 (100)	

LSIL= Low grade Intra epithelial Lesion; HSIL = High grade Intra epithelial Lesion

Table 4: Relationship between persistence of cervical HPV infection after 6months in participants with normal cervix at baseline and some select variables

Factor	Description	Cleared n (%)	Persisted n (%)	*p* value
Previous STI (n=52)	Yes	12 (30)	28 (70.0)	0.005
	No	11 (91.7)	1 (8.3)	
Abnormal discharge (n=45)	Yes	1 (11.1)	8 (88.9)	0.014
	No	23 (63.9)	13 (36.1)	
Vaginal rash (n=55)	Yes	4 (36.4)	7 (63.6)	0.64
	No	22 (50.0)	22 (50.0)	
Method of contraception (n=56)	Hormonal	12 (100)	0	0.0002
	Others	15 (34.09)	29 (65.91)	
HIV status (n=58)	Positive	1 (14.3)	6 (85.7)	0.04
	Negative	28 (56.0)	22 (44.0)	
HIV +ve on ARD (n=7)	Yes	1	0	0.014
	No	0	6	
No. of lifetime sex partner/s (n=56)	Single	28 (68.3)	13 (31.7)	<0.001
	Multiple	0	15 (100)	
Age group (n=58)	<40	15 (34.1)	29 (65.9)	<0.001
	40+	14 (100)	0	
Age at first sex (n=58)	<17	20 (71.4)	8 (28.6)	0.003
	17+	9 (30.0)	21 (70.0)	
No of pregnancies (n=45)	≤4	13 (38.2)	21 (61.8)	0.007
	5+	10 (90.9)	1 (9.1)	
HPV type (n= 58)	HPV 16	6(21.4%)	22(78.6%)	<0.0001
	HPV 18	11(64.7%)	6(35.3%)	
	HPV 33	7(87.5%)	1(12.5%)	
	Others	5(100%)	0(0.0%)	

STI=Sexually Transmitted Infection; HPV = Human Papilloma Virus; HIV = Human Immunodeficiency Virus; ARD = Antiretroviral Drug

Table 5: comparison of the mean viral load of various factors of HPV infection

Factors	Mean Viral Load (IU/ml)	Confidence Interval	*p* value
HPV type			
Type 16	4.90	-1.19506 - 0.18619	0.036
Type 18	5.38		
Cervical status			
Normal LSIL	5.35	-1.534571 - 0.93173	0.6.26
	5.66		
Age group		0.07160 - 1.29895	0.029
<40	5.56		
40+	4.84		
Parity		0.25212 - 2.03242	0.015
≤4	5.51		
5+	4.35		
Baseline viral load	5.24	4.9428 - 5.5424	0.000
Recall viral load	4.60	2.2392 - 6.9508	0.008
Viral load of LSIL at recall	3.48	-9.27757 - 6.30424	0.498

LSIL= Low grade Intra epithelial Lesion

Discussion:

The baseline study of the HPV status of the participants has been published (15). Persistence of cervical HPV infection was seen in 50% of the respondents after the six months follow up. Akaaboune et al., (17) reported persistence of 20.2% after 6 months and 22.4% after 12 months. Persistence of 59.6% (18) over a 24 month has equally been reported. We recognize that there is a possibility of further clearance of the HPV infection if the follow continued for a longer period. The outstanding thing in this follow up is a proportion (24.1%) of those with persistent HPV infection that developed LSIL after 6 months of follow up. Some factors were peculiar with those who developed abnormal cervix after 6months. The factors include being infected with HPV 16, and HIV positivity without antiretroviral drugs. Higher persistence of HPV 16 has equally been reported in other studies (18,19).

First cervical infection with HPV often occurs soon after first sexual intercourse (20). Some authors have suggested that early age at first sexual intercourse is an indicator for early exposure to HPV (21,22). It has also been noted that high levels of circulating oestrogen during puberty may be a major influence in the metaplastic changes in the cervical transformation zone during that period (23). One will therefore expect that early onset of sexual intercourse may be associated with persistent HPV infection. This study, however, showed a lower persistence of HPV infection in the participants with early exposure to sexual intercourse.

There have been differing opinions on the use of hormonal contraceptives and cervical HPV persistence where some authors reported association (13,24) while some others reported no association (25,26). In this study, use of hormonal contraceptive was associated with HPV clearance instead of persistence though this should be interpreted with caution as the duration of hormonal contraceptive use and the type of contraceptives were not specified. Molano et al., (19) equally reported in their work that hormonal contraceptive aided HPV clearance. Persistence was more in participants with abnormal discharge and vaginal rash than those without though that of vaginal rash was not statistically significant. The presence of vaginal rash or abnormal discharge may mean co-infection with other STIs, hence the persistence.

Persistence was significantly higher in HIV patients who were not on ART and some of them had already developed LSIL at 6 months. This persistence is in agreement with some other works (27,28). Anti-retroviral drugs are known to reduce HIV viral load and so improves the immunity of the individual. Therefore, even when ART do not have direct effect on HPV, the improved immunity helps the individual to mount immunological defence against HPV leading to its clearance. A strong association has been found between HPV persistence and an increasing number of full-term pregnancies in studies of pooled data analysis (17,29) but the same was not obtained in this work as higher clearance of HPV infection was seen among the participants with up to 5 deliveries and above which was in line with the work of Kim et al., (30).

The risk of progression to pre-cancers is usually affected by the viral factors, host factors and the behavioural co-factors but the most important determinant of HPV infection to pre-cancers is the viral type (31). Cervical abnormalities persist longer and progress more quickly in women who have carcinogenic HPV infections than in women who have non-carcinogenic infections or no HPV (32). In agreement with above, HPV 16 in our study persisted more than other HPV types

The effect of HPV infection on development of cervical cancer has been said to be influenced by viral load, indicating that estimating load could improve the predictive value of HPV detection; however, the scope of quantification depends on the viral type being detected (33). In this study, HPV 16 viral load was lower than HPV 18, while load for participants with normal cervix was lower than for those with LSIL. The mean viral load at recall was equally lower than the mean viral load at baseline study, which could indicate that the immune systems of the participants are either attempting to clear the HPV infection or that some of the viruses are already integrated into the host's chromosomes (persistent HPV), thereby leading to low viral yield. This calls for further studies.

Kim et al., (30) and Deng et al., (34) equally detected low viral load in cervical cancer patient after treatment with radiotherapy and surgery respectively. In their research, they discovered that that low viral load was significantly associated with poor prognosis in cancer patients (30,34). The above could not be verified in this work as we did not have any participant with HSIL or cancer after the follow up. Higher viral load in the younger age group seen in this study was equally witnessed by Ramanakumar et al., (35), which they attributed to the fact that these women were possibly exposed to HPV

while they were immunologically naïve to HPV. The major limitations of this study are short time follow up and small sample size, and discrepancies encountered may be the result of this short term follow up and small sample size.

Conclusion:

Clearance of HPV infection after a 6-month follow up among a cohort of women with normal cervical cytology was 50%. Clearance of HPV 16 infection was lower than in other HPV types. Persistence of HPV infection is influenced mainly by infection with high-risk HPV types in combination with series of other risk factors peculiar to individuals. No one risk factor is enough for HPV persistence. A multiple of factors peculiar to an individual may have a very strong role to play in HPV progression

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Original Article **Open Access**

Hepatitis B virus infection among pregnant women on antenatal visits: rapid tests or ELISA?

*[1,2]Fowotade, A., [2]Adetunji, S. O., [2]Amadi, E., [2]Ishola, I. O., and [3]Omoruyi, E. C.

[1]Clinical Virology Unit, Department of Medical Microbiology and Parasitology, College of Medicine, University of Ibadan, Nigeria
[2]Department of Medical Microbiology and Parasitology, University College Hospital, Ibadan, Nigeria
[3]Institute of Child and Maternal Health, College of Medicine, University of Ibadan, Nigeria
*Correspondence to: temilabike@gmail.com

Abstract:

Background: Hepatitis B virus (HBV) infection is a global public health challenge with over 360 million people infected worldwide, and is one of the leading causes of death worldwide. The hepatitis B surface antigen (HBSAg) is the most important marker for HBV screening, and HBSAg rapid screening test methods are the most widely used compared with the enzyme-linked immunosorbent assay (ELISA) and nucleic acid testing methods. The objectives of this study are to evaluate the comparative efficacy of rapid test kits and ELISA for HBV screening among pregnant women on antenatal visits and to screen for other HBV serological markers among HBsAg positive patients.
Methodology: This is a cross-sectional study of 172 pregnant women who were recruited consecutively on their first antenatal visit at the University College Hospital, Ibadan, Nigeria between November 2018 and February 2019. All participants were screened for HBsAg using both rapid immunochromatographic test (ICT) and ELISA techniques. HBsAg negative samples were further screened for anti-HBeAg/Ab, anti-HBcAg and anti-HBs by ELISA. Socio-demographic data of the participants were obtained using a semi-structured questionnaire, and data were analyzed using EPI INFO 7.2 statistical software.
Results: The prevalence rate of HBsAg among pregnant women in this study was 10.5% (18/172). The sensitivity, specificity, accuracy, positive predictive value (PPV) and the negative predictive value (NPV) of the rapid ICT kit were 72.2%, 97.4%, 94.8%, 76.5% and 96.8% respectively. Level of education, previous history of sexually transmitted infections (STIs) and previous positive HBV results were significantly associated with HBsAg seropositivity. Majority of the pregnant women (66.9%) tested negative to all the serological markers.
Conclusion: The low efficacy of rapid ICT kits compared to ELISA justifies the need to develop a safer antenatal screening strategy for HBV by combining the use of the less sensitive rapid screening techniques with the more sensitive ELISA method to limit vertical transmission of hepatitis B virus.

Keywords: Hepatitis B virus; Rapid ICT kits; ELISA; pregnant women

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Infection par le virus de l'hépatite B chez les femmes enceintes en consultation prénatale: tests rapides ou ELISA?

*[1,2]Fowotade, A., [2]Adetunji, S. O., [2]Amadi, E., [2]Ishola, I. O., et [3]Omoruyi, E. C.

[1]Unité de virologie clinique, Département de microbiologie médicale et de parasitologie, Collège de médecine, Université d'Ibadan, Nigéria
[2]Département de microbiologie médicale et de parasitologie, Hôpital universitaire, Ibadan, Nigéria
[3]Institut de la santé infantile et maternelle, Collège de médecine, Université d'Ibadan, Nigéria
*Correspondance à: temilabike@gmail.com

Abstrait:

Contexte: L'infection par le virus de l'hépatite B (VHB) est un défi de santé publique mondial avec plus de 360 million de personnes infectées dans le monde et est l'une des principales causes de décès dans le monde. L'antigène de surface de l'hépatite B (HBSAg) est le marqueur le plus important pour le dépistage du VHB, et les méthodes de test de dépistage rapide HBSAg sont les plus largement utilisées par rapport aux méthodes de test immuno-enzymatique (ELISA) et d'acide nucléique. Les objectifs de cette étude sont d'évaluer l'efficacité comparative des kits de tests rapides et de l'ELISA pour le dépistage du VHB chez les femmes enceintes lors de consultations prénatales et de dépister d'autres marqueurs sérologiques du VHB chez les patients AgHBs positifs.
Méthodologie: Il s'agit d'une étude transversale de 172 femmes enceintes qui ont été recrutées consécutivement lors de leur première visite prénatale à l'Hôpital Universitaire, Ibadan, Ibadan, Nigéria entre novembre 2018 et février 2019. Tous les participants ont été dépistés pour l'AgHBs en utilisant les deux tests immuno-chromatographiques rapides (TIC) et techniques ELISA. Les échantillons négatifs à l'AgHBs ont en outre été criblés pour l'anti-HBeAg/Ab, l'anti-HBcAg et l'anti-HBs par ELISA. Les données sociodémographiques des participants ont été obtenues à l'aide d'un questionnaire semi-structuré et les données ont été analysées à l'aide du logiciel statistique EPI INFO 7.2.
Résultats: Le taux de prévalence de l'HBSAg chez les femmes enceintes dans cette étude était de 10,5% (18/172). La sensibilité, la spécificité, la précision, la valeur prédictive positive (VPP) et la valeur prédictive négative (VPN) du kit ICT rapide étaient respectivement de 72,2%, 97,4%, 94,8%, 76,5% et 96,8%. Le niveau d'éducation, les antécédents d'infections sexuellement transmissibles (IST) et les résultats positifs antérieurs pour le VHB étaient significativement associés à la séropositivité de l'AgHBs. La majorité des femmes enceintes (66,9%) ont été testées négatives pour tous les marqueurs sérologiques.
Conclusion: La faible efficacité des kits TIC rapides par rapport à l'ELISA justifie la nécessité de développer une stratégie de dépistage prénatal plus sûre du VHB en combinant l'utilisation des techniques de dépistage rapide moins sensibles avec la méthode ELISA plus sensible pour limiter la transmission verticale du virus de l'hépatite B.

Mots clés: virus de l'hépatite B; Kits TIC rapides; ELISA; femmes enceintes

Introduction:

Chronic hepatitis B virus (HBV) infection is the most common cause of liver cirrhosis and hepatocellular carcinoma (1). Vertical transmission of HBV is the most common cause of chronic HBV infection and is a public health concern in endemic regions, such as the Far East and Africa (2-5). The first detectable antigen in the course of HBV infections is the HBsAg whose presence often predates the appearance of symptoms in clinically ill patients. An identifiable viral core antigen (HBcAg) exists but does not circulate free in serum like HBsAg. A third antigen, the e antigen (HBeAg), is associated primarily with the core antigen in the internal structure of the virus, and can be found circulating in serum, frequently in complexes with immunoglobulin. The three antigens induce the production of equally distinct antibodies; HBsAb, HBcAb, and HBeAb respectively, in the course of non-chronic host infection (6,7).

The risk of vertical transmission of HBV infection is highest at birth and studies have shown that majority of infants born to HBsAg positive mothers are seronegative at birth but seroconvert within the first 3 months postpartum (8-11). HBsAg and HBeAg seropositivity confers the highest risk for virus transmission with 85% to 100% of their offspring becoming infected, while 70% to 90% progress to become chronic carriers. Mothers with lower risk who are HBsAg-positive but HBeAg-negative, presumably still transmit the virus to about 35% of their children in the absence of neonatal prophylaxis (12-15).

There are various laboratory techniques available for the detection of HBsAg including rapid tests, enzyme linked immuno-sorbent assay (ELISA) and nucleic acid amplification tests (NAATs). In lower-middle-income countries, including Nigeria, the rapid test kits are often widely employed for HBV screening. The comparative efficacy of ELISA and the rapid tests among the present cohort of pregnant women would provide a rationale for the use of either test in other high-risk populations.

Materials and method:

Study setting, design and population

This cross-sectional study was performed at the University College Hospital, Ibadan, Nigeria between November 2018 and February 2019. The 900-bedhospital is the first tertiary teaching healthcare facility in Nigeria and provides health care to a wide catchment of patients within the southwest region of Nigeria. One hundred and seventy-two (172) pregnant women, on their first antenatal visit were consecutively recruited into the study after obtaining their written informed consent. Administration of questionnaires was done to obtain the sociodemographic and clinical details of the participants.

Inclusion and exclusion criteria

All pregnant women visiting the hospital antenatal clinic for the first time and who gave informed consent were included into this study while those on subsequent visits together with those who declined consent/or refused sample collection were excluded.

Ethical clearance

Ethical clearance was obtained from the joint Ethical Committee of the University of Ibadan and University College Hospital, Ibadan before the commencement of the study (UI/EC/18/0264).

Specimen collection and storage

Five milliliters of blood were drawn from each patient. Plasma was separated from the blood sample after collection and immediately stored at -20°C in the freezer for hepatitis B virus rapid and ELISA tests.

Rapid HBsAg ICT test and ELISA screening for HBsAg, HBeAg, HBeAb and HBcAb

All the samples were tested for hepatitis B surface antigen (HBSAg) using HBSAg rapid test kit (RTK) (LabACON, Hangzhou Biotest Biotech Company Limited, China). All the samples were further re-tested using enzyme-linked immunosorbent assay (ELISA) kit for HBsAg (Dia.Pro, Milan, Italy) according to the instructions of the manufacturers. ELISA kits for HBeAg, anti-HBeAb, anti-HBcAb and anti-HBsAb (Dia.Pro, Milan, Italy) were used according to the instructions of the manufacturers to determine HBeAg and antibodies to HBeAg, HBcAg and HBsAg.

The cutoff value for the HBsAg kit was determined according to the (mean negative control + mean positive control)/5. The positive and negative controls used were supplied by the manufacturer. Optical density (OD) was measured by an EMax Plus microplate reader (Molecular Devices), and the results were expressed as the cutoff index (COI). The COI was defined as the mean optical density difference between reactive control and non-reactive control 1.0 (RC-NRC=1.0). HBsAg detection was considered positive for a COI value ≥1. The performance of the kits was evaluated in terms of sensitivity, specificity, and overall agreement with the 95% confidence intervals (95% CI) according to the CLSI EP12-A guidelines (16).

Data analysis

Data were collected and entered into Excel spreadsheet and analyzed using EPI-INFO 7.2 statistical software. Association between socio-demographic and clinical features, and HBsAg status of the pregnant women was evaluated using the Chi-square statistic at 95% confidence interval, and statistical significance value of 0.05. Sensitivity, specificity, accuracy, positive and negative predictive values were calculated using the formulae below;

$$Sensitivity = \frac{True\ positive}{(True\ positive + False\ negative)} \times 100$$

$$Specificity = \frac{True\ negative}{(True\ negative + False\ positive)} \times 100$$

$$Accuracy = \frac{(True\ positive + True\ negative)}{(True\ positive + True\ negative + False\ positive + False\ negative)} \times 100$$

$$Positive\ predictive\ value = \frac{True\ positive}{(True\ positive + False\ positive)} \times 100$$

$$Negative\ predictive\ value = \frac{True\ negative}{(True\ negative + False\ negative)} \times 100$$

Results:

A total of 172 pregnant women participated in this study with 44.2% below the age of 30 years while 55.8% were ≥30 years of age. One hundred and sixty-nine (98.3%) were married and three (1.7%) were single. Majority (94.2%) had post secondary school education while the highest educational qualification for others (5.8%) was secondary school certificate examination (SSCE). Majority (87.8%) of the participants were employed while 12.2% were unemployed. Primigravid women constituted a third of the study participants (33.1%) while the other two-third were multigravida women (Table 1).

The prevalence rate of HBsAg among pregnant women in this study was 10.5% by ELISA test (18/172). The distribution of HBV infection (HBSAg positive) varied according to the socio-demographic characteristics as shown in Table 1. Pregnant women below the age of 30 years (11.8%) had higher prevalence rate compared to those aged above 30 years (9.4%) but the difference in the rates was not statistically significant (p=0.560). All the HBSAg positive cases were among the married pregnant women. Educational level and HBV infection had a significant relationship (X^2= 4.324; p=0.038) with those with secondary education having a higher prevalence of 30% compared to 9.3% in those with post secondary education. The unemployed (14.3%) and those pregnant for the first time (12.3%) had a higher rate of HBV infection compared to the employed and multiparous participants respectively although there were no statistically significant differences in the rates (p>0.05).

There was observed a significant relationship between HBV infection and previous HBV results (X^2=15.259; p=0.000) as well as HBV infection with previous history of sexually transmitted infections (STIs) (X^2=6.545; p=

0.011). Meanwhile, there was no statistically significant association between HBV infection and other correlates such as history of HBV vaccination, previous HBV screening, scarification as a child and as an adult, history of contact with HBV patient, history of surgical and dental procedure, circumcision as an adult and HIV status (Table 2).

The total number of HBSAg positive samples using the rapid test kits was 17 (9.9%) while the ELISA method identified 18 (10.5%) positive samples. Five of the 18 ELISA HBsAg positive samples tested negative with the rapid kits while 4 of the 17 RTK positive samples tested negative with the ELISA test as shown in Table 3. The calculated sensitivity was 72.2%, specificity 97.4%, accuracy 94.8%, positive predictive value (PPV) 76.5% and the negative predictive value (NPV) was 96.8%.

All the 154 HBsAg ELISA negative samples were further tested for other HBV serological markers. Thirty-seven (24.0%) tested positive to anti-HBc only, 3 (1.9%) tested positive to anti-HBe only, 11 (7.1%) tested positive to both anti-HBc and anti-HBe. None was positive to HBeAg and HBsAb (Table 4).

Table 1: Frequency of HBsAg distribution with respect to socio-demographic characteristics of pregnant women attending University College Hospital, Ibadan, Nigeria

Variables	Groups	No tested (%) (n=172)	HBsAg		X^2	p-value
			Positive (%)	Negative (%)		
Age (years)	<30	76 (44.2)	9 (11.8)	67 (88.2)	0.276	0.560
	30 and above	96 (55.8)	9 (9.4)	87 (90.6)		
Marital status	Single	3 (1.7)	0 (0)	3 (100)	0.357	0.550
	Married	169 (98.3)	18 (10.7)	151 (89.3)		
Educational level	Secondary	10 (5.8)	3 (30.0)	7 (70.0)	4.324	0.038*
	Post-secondary	162 (94.2)	15 (9.3)	147 (89.7)		
Occupation	Employed	151 (87.8)	15 (9.9)	136 (89.1)	0.373	0.542
	Unemployed	21 (12.2)	3 (14.3)	18 (85.7)		
Parity	Primigravid	57 (33.1)	7 (12.3)	50 (87.7)	0.230	0.584
	Multigravid	115 (66.9)	11 (9.6)	104 (89.4)		

*p-value <0.05- Statistical significance

Table 2: Frequency of HBsAg distribution with respect to clinical characteristics of pregnant women attending University College Hospital, Ibadan, Nigeria

Variables	Groups	Total tested (n=172)	HBsAg		X^2	p-value
			Positive	Negative		
History of vaccination	Yes	29	3	26	0.036	0.850
	No	141	13	128		
	Not sure	3	2	1		
Previous HBV screening	Yes	44	4	40	0.010	0.921
	No	125	12	113		
	Not sure	4	2	2		
Previous HBV result	Positive	4	2	2	15.259	0.000*
	Negative	47	1	46		
	Not sure	122	15	107		
Scarification as child	Yes	53	7	46	0.615	0.433
	No	119	11	108		
Scarification as an adult	Yes	49	8	41	2.512	0.113
	No	123	10	113		
History of contact with HBV patient	Yes	13	3	10	2.387	0.122
	No	159	15	144		
History of STI	Yes	8	3	5	6.545	0.011*
	No	164	15	149		
History of surgical and dental procedure	Yes	56	4	52	0.978	0.323
	No	116	14	102		
Circumcision as an adult	Yes	49	7	42	1.491	0.222
	No	123	10	113		
HIV status	Positive	6	1	5	0.255	0.613
	Negative	166	17	149		

*p-value <0.05- Statistical significance

Table 3: Comparative efficacy of Rapid Test Kits and ELISA in HBsAg screening

Rapid kits	ELISA		Total (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)	X^2	p-value
	Positive (%)	Negative (%)								
Positive	13 (72.2)	4 (2.6)	17 (9.9)	72.2	97.4	94.8	76.5	96.8	87.71	0.00*
Negative	5 (27.8)	150 (97.4)	155 (90.1)							
Total	**18 (100)**	**154 (100)**	**172 (100)**							

PPV – Positive Predictive Value, NPV – Negative Predictive Value; *P-value <0.05- Statistical significance

Table 4: Presence of other HBV markers among HBsAg negative study participants

Profile	Other HBV markers	Frequency (%)	Virological explanation
A1	Anti-HBc only	37 (24.0)	Resolved or occult HBV infection
A2	Anti-HBe only	3 (1.9)	Resolving HBV infection
A3	Anti-HBc and anti-HBe	11 (7.1)	Resolving HBV infection
A4	No additional marker	103 (66.9)	HBV infection susceptible
Total		**154 (100)**	

Discussion:

To achieve the vision of the Global Health Sector Strategy (GHSS) to end HBV infection by 2030, accurate detection of HBV infection is very important (17). This is of greater importance among pregnant women who carry the potential risk of vertical transmission. The seroprevalence of HBSAg among pregnant women reported in this study was 10.5%. The finding from the current study is similar to the reports from a 13-year meta-analytical study which documented 11.5% HBSAg seroprevalence (18). Similar observations among a cohort of pregnant women have also reported HBsAg seroprevalence range of 5.4-12% (19-22). A study among pregnant women from Mali has also reported a seroprevalence of 10.5% (23), while 9.2% was observed in Gambia (24) and 17.3% in Burkina Faso (25). Contrastingly lower levels have been reported from other parts of Africa including; 2.4% in Rwanda, 4.9% in Uganda (26) and 5.6% in Sudan (7).

The level of endemicity of HBV infection in these regions contribute to the prevalence rate observed among the cohort studied. Several studies among other populations within Nigeria have reported HBSAg prevalence ranges that vary between 5 and 18% (28-30). All these studies affirm the hyper-endemicity status of Nigeria and underscore the need for an efficient screening method for HBV infection particularly among pregnant women. The lower HBSAg seroprevalence rate of 2% reported from Western Europe and United States of America might be due to increased access to vaccination services for HBV as well as better infection prevention and control practices towards hepatitis B prevention (31).

The sensitivity of the rapid test used in this study was 72.2% with an accuracy of 94.8% compared with ELISA. This is lower than 90% sensitivity and 99.9% accuracy reported in Bangladesh (32). Previous authors have also reported higher sensitivity for rapid test kits; 97% (33) and 93.4% (34) from two different studies in India. A lower sensitivity of 51.6% sensitivity was reported in South East, Nigeria (35). The rapid test kits are widely used for screening for hepatitis B infection in Nigeria hence the need to assess their efficacy particularly among important risk groups. A false negative HBsAg result among pregnant women pre-empts the need for administration of prophylactic hepatitis B immunoglobulin post-delivery. This increases the risk of development of chronic liver disease among in adulthood (36,37). Although, ELISA offers a more reliable option for the detection of HBsAg, the feasibility of its routine utilization for screening purposes is reduced because of its higher cost, requirement for additional equipment as well as a longer turn-around-time.

There was no significant association between HBV infection and socio-demographic

characteristics such as age, marital status, parity and occupation. There was however a significant association with level of education of the participants (X^2=4.324; p=0.038); those with secondary education had a higher prevalence rate compared with those with post-secondary education. Donbraye et al., (20) reported a similar observation with 59.3% HBSAg seroprevalence rate among those with only secondary education. This suggests that education informs risk taking behavior that might impart on hepatitis B virus infectivity. This is however different from the report of Opaleye et al., (38) where the more educated pregnant women had a higher rate of HBSAg prevalence, thus suggesting possibility of other confounding factors.

Previous positive HBV results and past history of other sexually transmitted infections (STIs) were significantly associated with HBSAg seropositivity. Previous history of STI suggests possible co-infection with HBV due to similarity in mode of transmission. Earlier studies reported similar association as observed in the current study (39,40). Other possible risk factors for HBV infection such as history of HBV vaccination, previous HBV screening, scarification as a child and as an adult, history of contact with HBV patient, history of surgical and dental procedure, circumcision as an adult and HIV status were not significantly associated with HBSAg seropositivity among the pregnant women, which is similar to earlier reports among the same study population (39-41). This finding however contrasts report by Rukunuzzaman and Afroza (43) in their study among a different study population.

Thirty-seven (24%) of the HBsAg negative pregnant women were positive for anti-HBc only, while 3 (1.9%) had anti-HBe only, and 11 (7.1%) had both anti-HBc and anti-HBe. The 24% anti-HBc in this study is far higher than previous findings among pregnant women in other reports; 1.5% reported by Zahn et al., (44) among pregnant women, 5.4% by Adetunji et al., (45) among apparently healthy individuals and 17% by Oluyinka et al., (46) among blood donors in Ile-Ife. HBSAg negative pregnant women with anti-HBc often have low hepatitis B viral load but remain potentially infectious (47-49). The detection of anti-HBe in some pregnant women in this study signifies the resolution of the HBV infection while seropositivity to both anti-HBc and anti-HBe markers connotes convalescent stage.

All the HBsAg seronegative pregnant women also tested negative for HBeAg and HBsAb thus confirming that although none of them was in the active replication phase of HBV infection but all were susceptible to hepatitis B infection. This finding is not surprising as majority of the participants were unvaccinated against hepatitis B virus.

Conclusion:

Although the HBsAg seropositivity is high among the study population, the efficacy of rapid test kits compared to ELISA is low. ELISA is recommended for HBV screening among pregnant women to prevent false-negative results. There is need to develop a safer antenatal screening strategy for HBV possibly by combining the use of the less sensitive rapid screening techniques with the more sensitive ELISA method to limit vertical transmission of hepatitis B virus. The high number of unvaccinated HBV-susceptible pregnant women justifies the need for increased advocacy for HBV vaccination.

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Original Article **Open Access**

Prevalence of occult hepatitis B virus infection among blood donors in Ouagadougou, Burkina Faso

*[1]Ky/Ba, A., [2]Sanou, M., [3]Ouédraogo, A. S., [2]Sourabié, I. B., [4]Ky, A. Y., [5]Sanou, I., [2]Ouédraogo/Traoré, R., and [6]Sangaré, L.

[1]Centre Hospitalier Universitaire de Bogodogo, 14 BP 371, Ouagadougou, Burkina Faso
[2]Centre Hospitalier Universitaire Charles De Gaule, Ouagadougou, Burkina Faso
[3]Centre Hospitalier Universitaire Souro Sanou, Bobo-Dioulasso, Burkina Faso
[4]Intrahealth International, Burkina Faso
[5]Centre Hospitalier Universitaire Blaise Compaoré, Ouagadougou, Burkina Faso
[6]Centre Hospitalier Universitaire Yalgado Ouedraogo, Ouagadougou, Burkina Faso
*Correspondence to: absetou@yahoo.fr

Abstract:

Background: In Burkina Faso, the polymerase chain reaction (PCR) assay is not routinely used in the biological qualification of blood donations and this constitutes a risk factor for the transmission of occult hepatitis B virus (HBV) infection during blood transfusion. The objective of this study is to determine the prevalence of occult B infection (OBI) among blood donors for the purposes of improved blood safety in Burkina Faso.
Methodology: A descriptive cross-sectional study of 300 HBsAg negative blood donors was conducted in the city of Ouagadougou, Burkina Faso from April to October 2020. Anti-HBc antibody was determined using the BOSON® brand rapid tests. HBV DNA was detected in 75 selected donors by real-time PCR (rt PCR) using the 7500 Fast Real Time PCR assay technique.
Results: Of the 300 HBsAg negative donors, 208 (69.3%) were males while 92 (30.7%) were females, with average age of 30.18 years. Anti-HBc antibody was detected in 39 cases (13%). Of the 75 donor samples tested by rt PCR, 3 (4%) were positive for HBV DNA (occult B infection); 2 of which were anti-HBc antibody positive (seropositive OBI) while 1 was anti-HBc antibody negative (seronegative OBI).
Conclusion: Given the prevalence of OBI of 4% in this study and its consequences in blood recipients, it appears necessary that in addition to the classic serological markers of hepatitis B, to test for the presence of HBV DNA among blood donors in order to improve transfusion safety.

Keywords: Prevalence, Occult B infection; Blood donors, Ouagadougou.

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Prévalence de l'infection occulte par le virus de l'hépatite B chez les donneurs de sang à Ouagadougou, Burkina Faso

*[1]Ky/Ba, A., [2]Sanou, M., [3]Ouédraogo, A. S., [2]Sourabié, I. B., [4]Ky, A. Y., [5]Sanou, I., [2]Ouédraogo/Traoré, R., and [6]Sangaré, L.

[1]Centre Hospitalier Universitaire de Bogodogo, 14 BP 371, Ouagadougou, Burkina Faso
[2]Centre Hospitalier Universitaire Charles De Gaule, Ouagadougou, Burkina Faso
[3]Centre Hospitalier Universitaire Souro Sanou, Bobo-Dioulasso, Burkina Faso
[4]Intrasanté International, Burkina Faso
[5]Centre Hospitalier Universitaire Blaise Compaoré, Ouagadougou, Burkina Faso
[6]Centre Hospitalier Universitaire Yalgado Ouedraogo, Ouagadougou, Burkina Faso
*Correspondance à: absetou@yahoo.fr

Résume:

Contexte: Au Burkina Faso, la polymérase chain réaction (PCR) n'est pas utilisée lors de la qualification biologique des dons et cela constitue un facteur de risque de transmission de l'Infection Occulte du virus B

(VHB) lors des transfusions sanguines. L'objectif de cette étude était de déterminer la prévalence de l'infection occulte B chez les donneurs de sang en vue d'une meilleure sécurité transfusionnelle.
Méthodologie: Une étude transversale prospective, réalisée d'avril à octobre 2020 dans la ville de Ouagadougou incluant 300 donneurs de sang AgHBs négatif. L'anticorps anti HBc a été déterminé par les tests rapides de marque BOSON®. L'ADN du VHB a été recherché chez 75 donneurs par PCR en temps réel (rt PCR) avec le 7500 Fast Real Time PCR.
Résultats: Parmi les 300 donneurs AgHBs négatifs, 208 (69,3%) étaient des hommes et 92 (30,7%) des femmes. L'âge moyen était de 30,18 ans. La recherche de l'Ac anti-HBc était positive dans 39 cas (13 %). Parmi les 75 échantillons passés à la rt PCR, 3 (4%) étaient positifs pour l'ADN du VHB. Sur les 3 cas d'ADN VHB positifs, 2 (66,67%) étaient positifs Ac anti HBc et 1 (33,33%) Ac anti HBc négatif.
Conclusion: Compte tenu de la prévalence de l'infection occulte B et ses conséquences chez les donneurs de sang et chez les receveurs, il devient nécessaire de rechercher, en plus des marqueurs sérologiques classiques de l'hépatite B, l'ADN VHB pour une meilleure sécurité transfusionnelle.

Mots clés: Prévalence, Infection occulte de l'HBV, Donneurs de sang, Ouagadougou

Introduction:

Hepatitis B virus (HBV) infection is a global public health challenge. According to the World Health Organization (WHO) in 2017 there were nearly two billion people infected with HBV worldwide, including 360 million chronic carriers who are at risk of cirrhosis and hepatocellular carcinoma (HCC) (1). In Africa, chronic hepatitis is the second leading cause of death after tuberculosis, with more than 60 million people affected. Injection with non-sterile needles and sharps in health facilities, tattoos, scarifications, and to a lesser extent, blood transfusion are the various means of HBV transmission (2). In Burkina Faso, HBV infection is the major cause of hepatic cirrhosis and liver cancer (3). The country is among those with high endemicity, where the carriage of hepatitis B virus surface antigen (HBsAg) is high in the general population, with a prevalence varying from 9% to 15% in 2018 (4–7), and seroprevalence rate of 7.28% among blood donors in 2017 (8).

In some people, there is persistence of HBV DNA in liver tissue and/or serum of individuals in whom the HBsAg is not detectable in the blood, with or without the presence of anti-HBc antibodies (9), which is referred to as occult B infection (OBI). This OBI occurs all over the world, but its frequency is linked to the prevalence of HBV infection in a specific geographical area. Occult HBV infection is transmissible through blood transfusion in the event of biological qualification of donations without the use of the polymerase chain reaction (PCR) assay, and through organ transplants. The prevalence of occult HBV infection in Burkina Faso varies according to studies; Birama et al., (10) reported a prevalence rate of 7.3% in 2018 in the population of Ouagadougou while Somda et al., (11) reported a seroprevalence rate of 32.8% among blood donors in 2016.

The molecular mechanisms underlying the occurrence of OBI are diverse and play an important role in the development of hepatocellular cancer (12). Chronic carriage of hepatitis C virus (HCV) and human immunodeficiency virus (HIV) are associated with a high prevalence of OBI (13). In Burkina Faso, PCR assay is not used in the biological qualification of donations, and this constitutes a risk factor for transmission of OBI during blood transfusions. It is in order to determine the prevalence of OBI in blood donors in the city of Ouagadougou that this study was carried out, with the aim of ensuring better transfusion safety.

Materials and method:

Study setting and design

This was a descriptive cross-sectional study of blood donors declared suitable for blood donation and tested negative for HBsAg in Ouagadougou Regional Blood Transfusion Center of (CRTS/O) and the Pietro Annigoni Biomolecular Research Center (CERBA) from April to October 2020. The CRTS/O served as the site for collecting study samples as well as for testing HBsAg and anti-HBcAb in blood donors. The CERBA served as the site for the detection of HBV DNA by the real-time PCR assay.

Sampling

All blood donors declared suitable for donation and tested negative for HBsAg were included in this study. A total of 300 randomly selected donors were divided into 6 groups of 50, and one group consisted of samples collected and tested on the same day for anti-HBc antibody. In each group of 50, 15 samples were randomly selected for the quantitative detection of HBV DNA, but included all anti-HBc antibody positive samples, with a total of 75 HBsAg negative samples (made of 39 anti-HBc positive and 36 anti-HBc negative samples) as shown in the workflow diagram (Fig 1).

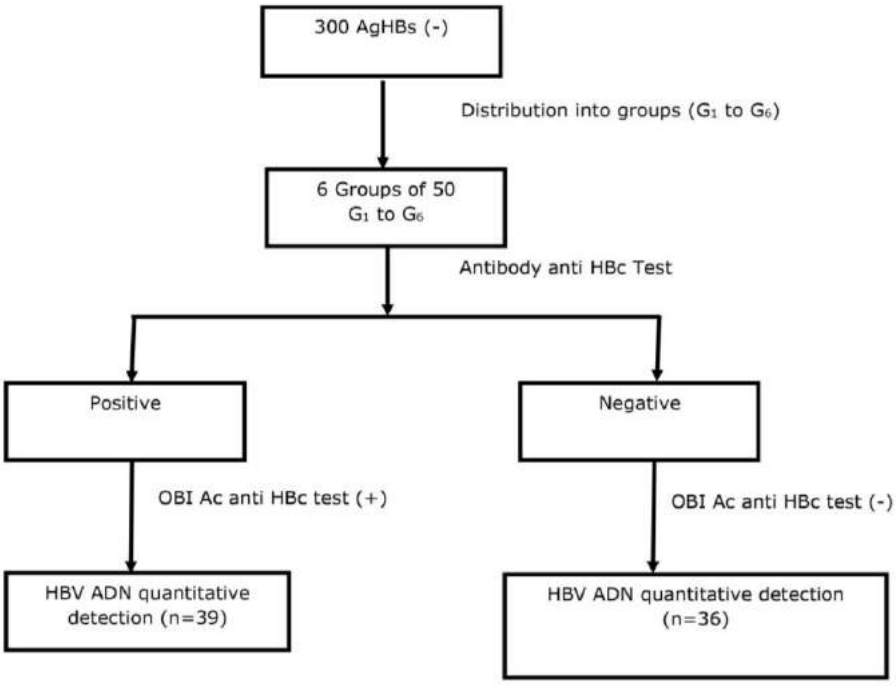

Fig 1: The workflow of the study

Detection of HBsAg and anti-HBc antibody

The Cobas e 601 automaton was used for the detection of HBsAg at CRTS/O. This test is an immunoassay that uses electro-chemiluminescence for the qualitative detection of hepatitis B surface antigen (HBsAg) in human serum and plasma. This automaton has a sensitivity of 99.9% and a specificity of 100%. The method for the detection of anti-HBc antibody was carried out using the rapid test 1H27C2 HBcAb IgG brand 'Boson'. The sensitivity of this test method is 97.2% and the specificity is 98.4%.

Detection of HBV DNA by real time PCR

HBV DNA was extracted from 200µl of plasma using the PureLink® Genomic DNA Kits (Life Technologies, Van Alleen Way Carlsbad, CA, USA) according to the manufacturer's protocol. This is a column extraction method based on the principle of nucleic acid molecules retention on a silicate membrane (filter). Quantification of HBV DNA was performed on the 7500 Fast Real Time PCR machine, in a reaction volume of 20µl containing 10µl of oasigTM 2 x qPCR Master Mix, 1µl of HBV Primer/Probe mix, 4µl of nuclease-free water and 5µl of extracted DNA. The amount of viral load in the samples is related to the standard curve obtained by making a cascade dilution to 1/10 of the positive control provided by the manufacturer (5 times). The concentration of control was $2x10^8$ copies/ml.

Statistical analysis

Data were analyzed using EPI INFO version 7.2 software. Chi-square was used to measure association between the variables, and the level of significance (*p* value) used was 0.05

Ethical considerations

The study received approval from the CNTS Internal Scientific Review Committee (CIRS). The anonymity and confidentiality of serological results from blood donors were ensured.

Results:

A total of 300 HBsAg negative blood donors, including 208 males (69.3%) and 92 females (30.7%) participated in the study. The male to female ratio was 2.3 and the mean age was 30.18 years with a range of 18-58 years. Majority of the donors were in the age groups 20-34 (68.0%) and 35-50 (24.3%) years. Of the 300 donations, 201 were collected at fixed sites and 99 at mobile sites. Table 1 shows the frequency distribution of the donors by age group and gender. The hepatitis B virus anti HBc antibody was detected in 39 donors representing a prevalence of 13%. Table 2 shows the frequency distribution of anti-HBc antibody with respect to gender and age group of donors.

Of the 75 donors with undetectable HBsAg screened by real time PCR, HBV DNA was detected in 3, representing a rate of 4%. Of the 3 positive samples, 2 (2.7%) were anti HBc positive (seropositive OBI) and 1 (1.3%) was anti HBc antibody negative (seronegative

OBI). The viral load of samples with HBV DNA varied from 335 IU/ml - 26454 IU/ml. Table 3 presents the prevalence of OBI with respect to gender and age groups. Table 4 presents the summary of the results of the three OBI cases with respect to the type of donor and the viral load.

Table1: Gender and age group distribution of HBsAg negative blood donors at Ouagadougou Regional Blood Transfusion Center of (CRTS / O), Burkina Faso

Socio-demographic characteristics		Number (n)	Percentage (%)
Gender			
	Male	208	69.3
	Female	92	30.7
Age group (years			
	<20	10	3.3
	20-34	204	68.0
	35-50	73	24.3
	> 50	13	4.3

Table 2: Frequency of anti HBc antibodies with respect to gender and age group of HBsAg negative blood donors at Ouagadougou Regional Blood Transfusion Center of (CRTS / O), Burkina Faso

Socio-demographic characteristics	Anti-HBc negative n (%)	Anti-HBc positive n (%)	X^2	*p* value
Gender				
Male	178 (85.6)	30 (14.4)	1.2145	0.2705*
Female	83 (90.2)	9 (9.8)		
Total	261 (87.0)	39 (13.0)		
Age group (years)				
< 20	10 (100)	0	9.8331	0.0073**
20 – 34	184 (90.2)	20 (9.8)		
35 – 50	58 (79.5)	15 (20.5)		
> 50	9 (69.2)	4 (30.8)		
Total	261 (87.0)	39 (13.0)		

* = not statistically significant; ** = statistically significant; X^2 = Chi square

Table 3: Prevalence of occult B infection (OBI)with respect to gender and age groups of donors

Socio-demographic characteristics	HBV DNA negative n (%)	HBV DNA positive (%)	n
Gender			
Male	56 (96.6)	2 (3.4)	
Female	16 (94.1)	1 (5.9)	
Total	**72 (96.0)**	**3 (4.0)**	
Age group (years)			
< 20	1 (100)	0	
20 – 34	38 (95.0)	2 (5.0)	
35 – 50	27 (96.4)	1 (3.6)	
> 50	6 (100)	0	
Total	**72 (96.0)**	**3 (4.0)**	

Table 4: Summary of the results of the three cases of occult hepatitis B infections

Number sample	Gender	Age (years)	Donor type	Viral load (UI/mL)
11190226100	Male	35	Occasional	8134
11190228800	Male	38	Occasional	26454
11190225140	Female	26	Occasional	337

Discussion:

Among the 300 donors who made up the study population, males represented 69.3% compared to 30.7% females, with a male to female ratio of 2.3, similar to that reported by Yooda et al (8) of 2.47 in 2017 among blood donors in Ouagadougou. This can be explained by the much greater willingness of males to donate blood, unlike females where there are situations in which blood donation may be relatively contraindicated, such as pregnancy, menstruation, childbirth, and breastfeeding. The age group 20-34 year constituted the majority in the study, similar to the studies of Doumbia et al., (14) in 2015 in Burkina Faso and Oluyinka et al., (15) in 2015 among blood donors in Nigeria. This finding could probably be linked to the dynamism and ability of the young population to donate blood. It is all fairer to say that the best blood donors in Burkina Faso are pupils and students (8).

The prevalence of anti-HBc antibody of 13% in this study is lower than the rate of 18.4% reported by Yooda et al., (8) in 2017 and 44.0% by Somda et al., (11) in 2016 among blood donors in Burkina Faso. However, it is higher than the 7.8% rate reported among blood donors in Egypt by Antar et al., (16) in 2010. The difference in rates reported may be due to the variations in sample size and study population, type of methods used, countries of study and the levels of HBV endemicity. Carriage of the anti HBc antibody is associated with the age of the study population. The age group over 50 years were most positive (30.8%) for anti-HBC antibody in our study. Burkina Faso is one of the areas of high endemicity of the hepatitis B virus, and very often, the infection is contracted early in childhood and develops in a latent fashion. Chronic active HBV infection usually occurs late and may progress to cirrhosis or primary liver cancer.

In the present study, we recorded a prevalence of 4% for occult HBV infection, which may be linked to the endemicity of HBV in Burkina Faso. This rate is lower than 7.3% reported in Ouagadougou by Birama et al., (10) in 2018 and 32.8% reported among blood donors in Burkina Faso by Somda et al., in 2016 (11). On the other hand, this prevalence rate is closely related to 4.72% reported among blood donors in Ouagadougou by Yooda et al., (8) in 2017 but higher

than 0.56% and 2.86% rates reported among blood donors respectively in Cameroon by Fopa et al., in 2019 (17) and in China by Ye et al., in 2017 (18). Although, several studies have shown that OBI depends on the endemicity of HBV, it is not limited to only countries with high HBV endemicity (10). The quality of the sample tested (blood or liver tissue) as well as the risks of contamination can also affect the detection of OBI (19).

In our study, donors with HBV DNA had viral load greater than 200 IU/ml (337-26,454 IU/ml). In 2008, the expert meeting clarified the definition of OBI by establishing cut-off value for serum HBV DNA <200 IU/ml (20). In addition, it also clarified the confusion between overt HBV infection and "false" OBI. Thus, cases where viral loads are comparable to stages of overt HBV infection are usually due to infection with escape mutants and should be labeled as "false" OBIs (20), because these cases are in fact overt chronic hepatitis B. This is attributed to escape mutations that could have altered the target epitope of the HBsAg test (21). We believe that disregarding these definitions may contribute to overestimation of the prevalence of OBI.

In the present study, the viral loads being greater than 200 IU/ml, is in favor of "false" OBIs. Of the 3 donors positive for HBV DNA, two were HBV anti-HBc antibody positive while one was negative. The two donors with positive anti-HBc antibody are considered as cases of occult B seropositive OBI while the one negative donor is considered a seronegative OBI, which is an indication of a recent HBV infection not detectable by the test method used.

Conclusion:

Occult HBV infection remains a big concern in Burkina Faso, as demonstrated by our current study and several other studies. Given the prevalence of OBI of 4% in this study and the consequences in blood recipients, it appears necessary that in addition to the detection of classic serological markers of hepatitis B, the presence of HBV DNA among blood donors should be tested, in order to ensure better transfusion safety.

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Original Article **Open Access**

Molecular detection and characterization of bacteria from CSF samples of patients with suspected cerebrospinal meningitis in parts of northern Nigeria using metagenomic DNA extracts

*[1,2]Peletiri, I. C., [1]Ikeh, E. I., [1]Ayanbimpe, G. M., and [3]Nna, E.

[1]Department of Medical Microbiology, Faculty of Clinical Sciences, College of Health Sciences, University of Jos, Nigeria
[2]Medical Microbiology & Parasitology Laboratories, National Hospital, Abuja, FCT, Nigeria
[3]Safety Molecular Pathology Laboratory, The Molecular Pathology Institute, Enugu, Enugu State, Nigeria
*Correspondence to: kumochris@hotmail.com

Abstract:

Background: The most commonly used approaches for detection and characterization of bacterial pathogens of meningitis in developing countries include culture, Gram stain, and latex agglutination. The positivity rate of culture is relatively low due to suboptimal storage and transportation conditions, culture practice, and/or antibiotic treatment administered before specimens are collected. Specimens that yield no growth in culture can still be analyzed using molecular methods, and metagenomic DNA (mDNA) extracted directly from clinical samples (CSF) can be used. We aimed to detect and characterize three major bacterial causes of cerebrospinal meningitis (CSM); *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* using mDNA extracted directly from CSF samples.
Methodology: Metagenomic DNA templates were prepared directly from CSF specimens collected from 210 patients with suspected CSM. A multiplex Real Time PCR (mRT-PCR) using the ABI StepOne Plus Machine and Taqman Probe chemistry was used in the molecular detection, while serogroup/serotype-specific singleplex RT-PCR was used to characterize all positives samples.
Results: Eighty-eight (41.9%) of the 210 samples were positive with the mRT-PCR assay for one or a combination of two of the three bacteria. Of these, 59 (67.1%) were *N. meningitidis,* 2 (2.3%) were *H. influenzae,* 3 (3.4%) were *S. pneumoniae*, 15 (17 %) had co-infections of *N. meningitidis* with *H. influenzae*, and 9 (10.2%) had co-infections of *H. influenzae* and *S. pneumoniae*. The serogroups of *N. meningitidis* encountered were A (13.5%), B (23%), C (8.1%), W135 (8.1%), X (5.4%), Y (32.4%), and non-groupable (9.5%). The serotypes of *H. influenzae* were Hia (3.8%), Hib (57.7%), Hic (3.85%), Hie (11.5%) and Hif (23.1%). The serotypes of *S. pneumoniae* were *Wxy1* (8.3%), *Wxy4* (33.3%), *Wxy5* (50.0%), and *Wxy9* (8.3%).
Conclusion: Multiplex RT-PCR is a fast and accurate method for detecting and characterizing serogroups/serotypes of major bacteria implicated in CSM. Isolating DNA directly from CSF improves turnaround time, which will speed up patient care and management.

Keywords: Cerebrospinal meningitis, metagenomic DNA, multiplex Real Time PCR, Northern Nigeria

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Détection moléculaire et caractérisation de bactéries à partir d'échantillons de LCR de patients suspectés de méningite cérébrospinale dans certaines parties du nord du Nigéria à l'aide d'extraits d'ADN métagénomique

*[1,2]Peletiri, I. C., [1]Ikeh, E. I., [1]Ayanbimpe, G. M., et [3]Nna, E.

[1]Département de microbiologie médicale, Faculté des sciences cliniques, Collège des sciences de la santé, Université de Jos, Nigéria

[2]Laboratoires de microbiologie médicale et de parasitologie, Hôpital national, Abuja, FCT, Nigéria
[3]Laboratoire de pathologie moléculaire de sécurité, Institut de pathologie moléculaire, Enugu, État d'Enugu, Nigéria
*Correspondance à: kumochris@hotmail.com

Abstrait:

Contexte: Les approches les plus couramment utilisées pour la détection et la caractérisation des agents pathogènes bactériens de la méningite dans les pays en développement comprennent la culture, la coloration de Gram et l'agglutination au latex. Le taux de positivité de la culture est relativement faible en raison des conditions de stockage et de transport sous-optimales, des pratiques de culture et/ou du traitement antibiotique administré avant le prélèvement des échantillons. Les échantillons qui ne donnent pas de croissance en culture peuvent toujours être analysés à l'aide de méthodes moléculaires, et l'ADN métagénomique (ADNm) extrait directement d'échantillons cliniques (LCR) peut être utilisé. Nous visions à détecter et à caractériser trois causes bactériennes majeures de la méningite cérébrospinale (CSM); *Neisseria meningitidis, Haemophilus influenzae* et *Streptococcus pneumoniae* à l'aide d'ADNm extrait directement d'échantillons de LCR.
Méthodologie: Des matrices d'ADN métagénomique ont été préparées directement à partir d'échantillons de LCR prélevés sur 210 patients suspects de CSM. Une PCR multiplex en temps réel (mRT-PCR) utilisant la chimie de la machine ABI StepOne Plus et de la sonde Taqman a été utilisée pour la détection moléculaire, tandis que la RT-PCR monoplex spécifique au sérogroupe/sérotype a été utilisée pour caractériser tous les échantillons positifs.
Résultats: Quatre-vingt-huit (41,9%) des 210 échantillons étaient positifs avec le test mRT-PCR pour une ou une combinaison de deux des trois bactéries. Parmi ceux-ci, 59 (67,1%) étaient *N. meningitidis*, 2 (2,3%) étaient *H. influenzae,* 3 (3,4%) étaient *S. pneumoniae*, 15 (17%) avaient des co-infections de *N. meningitidis* avec *H. influenzae* et 9 (10,2%) avaient des co-infections à *H. influenzae* et *S. pneumoniae*. Les sérogroupes de *N. meningitidis* rencontrés étaient A (13,5%), B (23%), C (8,1%), W135 (8,1%), X (5,4%), Y (32,4%) et non groupables (9,5%). Les sérotypes de *H. influenzae* étaient Hia (3,8%), Hib (57,7%), Hic (3,85%), Hie (11,5%) et Hif (23,1%). Les sérotypes de *S. pneumoniae* étaient *Wxy1* (8,3%), *Wxy4* (33,3%), *Wxy5* (50,0%) et *Wxy9* (8,3%).
Conclusion: La RT-PCR multiplex est une méthode rapide et précise de détection et de caractérisation des sérogroupes/sérotypes des principales bactéries impliquées dans le CSM. Isoler l'ADN directement du LCR améliore le temps de traitement, ce qui accélérera les soins et la gestion des patients.

Mots clés: méningite cérébro-spinale, ADN métagénomique, PCR multiplex en temps réel, nord du Nigéria

Introduction:

Over the years, laboratory results confirming cases of cerebrospinal meningitis (CSM) in Nigeria have been under reported due to the methodology used in processing cerebrospinal fluid (CSF) samples for the diagnosis of meningitis. The most commonly used approaches for detection and characterization of bacterial meningitis pathogens in developing countries include culture, Gram stain, and latex agglutination. Although culture is considered as the gold standard for case confirmation in hospitals/clinics, the positivity rate is relatively low due to suboptimal storage and transportation conditions, cultural practice, and/or antibiotic treatment administered before specimens are collected (1).

Bacterial meningitis remains a serious global health problem and a life-threatening condition that requires prompt recognition and treatment. Beyond the newborn period, the most common causes of bacterial meningitis are *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* (1). Specimens that do not yield any culture can still be analyzed by molecular methods that can be applied on DNA extracted from clinical samples, typically, blood and CSF (1).

Molecular detection is by way of the PCR technology. In PCR, the method does not require live or intact cells, and is a valuable tool for detecting bacterial pathogens from clinical specimens where bacteria die or lyse easily due to inappropriate storage conditions or prior antibiotic treatment (2). Real Time PCR (RT-PCR) combines amplification and detection in one step through the use of fluorescent dyes. The PCR strategy typically employed to detect the causative agent(s) in a suspected case of bacterial meningitis is to first run each of the species-specific assays concurrently on the metagenomic DNA (mDNA) extracted from the clinical specimens. The appropriate serogroup/serotype specific assays should then be run on any positive specimen.

Multiplex RT-PCR (mRT-qPCR) refers to the simultaneous amplification of multiple target regions in a sample using different pairs of primers (2). It allows for development of multiplex assays for detection of several genes in the same reaction mix by using specific probes with different fluorescent dye labels, and mRT-qPCR assays are available for detection of *N. meningitidis, H. influenzae,* and *S. pneumoniae* in a single reaction (1).

Species-specific RT-PCR assays have been developed for use on DNA extracted from clinical specimens, typically, blood and CSF and from bacterial isolates. A RT-PCR assay which targets the Cu and Zn superoxide dismutase gene, *sodC,* to detect all meningococci, regard-

less of encapsulation status, has been developed and validated. The *sodC* assay detects encapsulated meningococci but it is also useful for detecting non-groupable meningococci that do not contain an insert *ctrA* (3). Serogroup-specific RT-PCR assay for *N. meningitidis* captures the major disease-causing serogroups which include A, B, C, Y, and W135 (4). The gene *syn* for capsule biosynthesis (5) is used for genotyping for serogroups B (*synD*), C (*synE*), Y (*synF*) and W135 (*synG*). The *sacB* gene is targeted for serogroup A and the *xcbA* gene, which most likely encodes the capsule polymerase, is targeted for serogroup X (6,7).

The *hpd* gene encodes protein D, a highly conserved, surface-exposed lipoprotein that is present in all encapsulated and non-encapsulated *H. influenzae* (8,9). Validated *hpd* RT-PCR assay is capable of detecting all six serotypes (a-f) and non-typeable (HiNT) *H. influenzae* with high sensitivity and specificity (10). For *H. influenzae* serotype-specific RT-PCR assay, the genes are named *acs, bcs*, etc, for "a capsule synthesis", "b capsule synthesis" etc. The genes targeted for RT-PCR assays specific to each serotype are as follows: *acsB* (Hia), *bcsB* (Hib), *ccsD* (Hic), *dcsE* (Hid), *ecsH* (Hie), and *bexD* (Hif).

The RT-PCR assay for detection of *S. pneumoniae* using a specific segment of the autolysin gene (*lytA*), is highly conserved with the species and has been shown that this assay best separates *S. pneumoniae* from the genotypically similar *S. mitis*, *S. oralis*, and *S. pseudopneumoniae* (11). A number of RT-PCR assays for serotyping *S. pneumoniae* are recommended for determining serotypes from clinical specimens when DNA may be present in low amounts and insufficient for conventional multiplex PCR serotyping (1).

The objective of this research was to utilize RT-PCR techniques to detect and characterize three major bacterial causes of cerebrospinal meningitis (CSM); *N. meningitidis, H. influenzae,* and *S. pneumoniae*, in parts of northern Nigeria using metagenomic DNA (mDNA) extracted directly from CSF.

Materials and method:

Study settings

The study sites were Federal Capital Territory (FCT): National Hospital, Abuja, and all District/General Hospitals in the FCT (Asokoro, Wuse, Maitama, Garki, Gwarinpa, Bwari, Kubwa, Kuje, and Nyanya); and some States in Northern Nigeria (Kebbi, Plateau, Sokoto and Zamfara States) during outbreak seasons of February – May 2017 and January – April, 2018.

Study design:

This was a cross-sectional study of hospitalized patients with suspected cerebrospinal meningitis. The study work flow is shown in Fig 1.

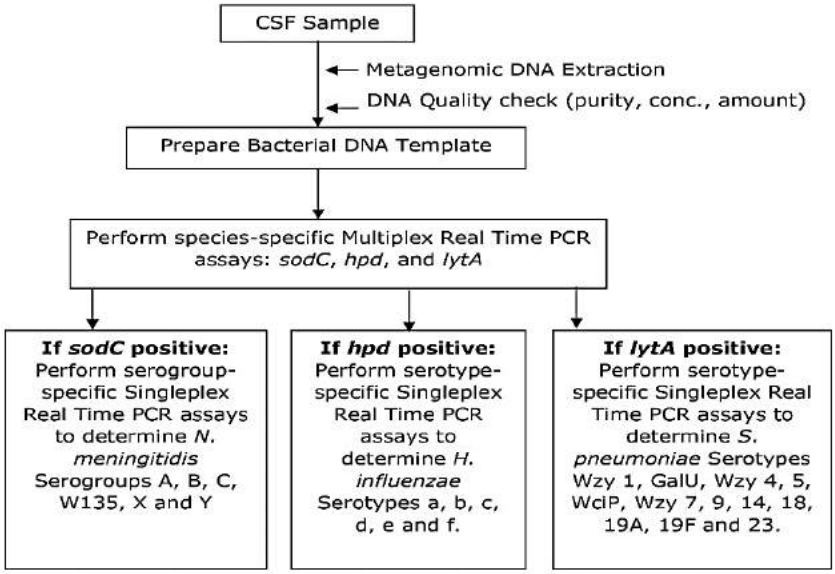

Fig 1: Study workflow

Subjects

All hospitalized patients (all ages and gender) with clinical symptoms of meningitis as reviewed by the attending physician who gave informed consent 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 (12) 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% (13), '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.

Ethical consideration

Ethical approvals were obtained from the Health Research 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 /No101.3/2015), 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 (ZSH REC/ 02/03/2017). A letter of introduction from the Nigeria Centre for Disease Control (NCDC), 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 sample, and sample material and data transfer agreement were also obtained.

Laboratory analysis

Collection and transportation of CSF specimens, metagenomic DNA extraction, metagenomic DNA extract quality check as well as amount of mDNA present in CSF specimens were done as previously reported by Peletiri et al., (14).

i. Metagenomic DNA (sample requirement)

Bacterial mDNA was extracted directly from CSF samples using spin column method as previously described (14) and mDNA was stored at -20^{O}C until time for testing. DNA quantity and quality were checked fluorometrically using Qubit 3.0 Fluorometer. DNA concentration in ng/µL (as measured by Qubit Fluorometer 3.0) as DNA yield = DNA concentration x eluted volume (60µL) per 200µL of CSF and amount of DNA = DNA concentration x 5µL per qPCR reaction.

ii. Multiplex RT-PCR for *N. meningitidis, H. influenzae,* and *S. pneumoniae:*

A multiplex RT-PCR using Taqman probe chemistry that detects *sodC* gene for *N. meningitidis, hpd* gene for *H. influenzae,* and *lytA* gene for *S. pneumoniae* simultaneously in a single reaction tube using three different fluorescent dyes; FAM, HEX and Cy3 reporter dyes and Blackhole Quencher (BHQ), was developed using published primer and probe sequences (1,38). The American Type Culture Collection (ATCC) bacterial control strains (CultiControl™ Freeze-Dried Bacterial Strains) produced by Liofilchem® S.R.L., via scozia, Zona Industriale, 64026 Roseto Degli Abruzzi (TE), Italy, were procured through a supplier, Hospitest Laboratory Ltd, Lagos, Nigeria. The assay format used is as shown in Table 1.

Table 1: Target genes for preparing NHS primer probe mix

Target gene	Oligonucleotide name	5' to 3' nucleotide sequence	Amplicon size (bp)
sodC	F351	GCA CAC TTA GGT GAT TTA CCT GCA T	127
	R478	CCA CCC GTG TGG ATC ATA ATA GA	
	Pb387	(FAM)-CAT GAT GGC ACA GCA ACA AAT CCT GTT T–BHQ1	
hpd	F729	AGA TTG GAA AGA AAC ACA AGA AAA AGA	113
	R819	CAC CAT CGGCAT ATT TAA CCA CT	
	Pbr762ibb	(HEX)-AAA CAT CCA ATC GTA ATT ATA G-(BHQ1)	
lytA	F373	ACG CAA TCT AGC AGA TGA AGC A	51
	R424	TCG TGC GTT TTA ATT CCA GCT	
	Pb400	(Cy3)-TGC CGA AAA CGC TTG ATA CAG GGA G-(BHQ1)	

NHS = *Neisseria/Haemophilus/Streptococcus*

All primers and probes were synthesized by Eurofins, Germany, and supplied in lyophilized form. Primers and probes were first reconstituted to 100 µM following the manufacturer's instructions and working concentrations of 10µM prepared using 1xTE buffer as diluent. The 10µM primers and probes are used in preparing the mRT-qPCR primer probe mix.

The mRT-qPCR primer probe mix was aliquoted into 3 tubes and stored at –80^{O}C until use. When required, each aliquot was thawed in a heating block set at 37^{O}C and mixed well by gentle vortex before use. Other reagents and materials include probe master mix (Promega, UK); ABI OneStep Plus RT-PCR System (Thermo Fischer, UK); P10, P100 and P1000 pipettes and tips; ABI 96 well qPCR plate; Quality Control DNA samples [DNA from *N*. *meningitidis* (ATCC® No. 13090™), *H*. *influenzae* (ATCC® No. 10211™), *S*. *pneumoniae* (ATCC® No. 49619)]; nuclease free water; 4-point serially diluted pooled DNA standards (A, B, C, and D); Thermal seal for PCR plate; and refrigerated centrifuge with plate holder (Heraeus, UK) were used in setting up reactions.

Multiplex reaction set up and real time amplification

The worksheet was created according to the number of samples to be tested. The ABI 96 well plate placed into a plate holder on ice pack rack. Into each well, 12.5µL of probe Master Mix was dispensed and 7.5µL of the mRT-qPCR primer–probe mix was added. 5µL of DNA sample or ATCC bacterial strains or QC or NTC was then added into appropriate well. The No Template Control (NTC) was the DNA sample of *Escherichia coli*. Elution buffer was used as Negative Control. The plates were sealed with thermal seal and centrifuged at 1000 rpm for 1 minute in refrigerated centrifuge at 5^{O}C. The plates were then placed into the plate holder in the ABI RT-PCR machine. The manufacturer's instruction was followed for setting up template run for mRT-qPCR.

TaqMan chemistry and standard mode, with absolute quantification were selected. The channels for FAM, Hex, and Cy3 as reporters and BHQ as non-fluorescent quencher were equally selected. The standard values for A to D: 10^6 - 10^2 copies/mL entered appropriately. The thermal profile used was TaqMan chemistry at 50^{O}C for 2 min, 1 cycle; 95^{O}C for 10 min, 1 cycle; 95^{O}C for 15 sec, 45 cycles; 60^{O}C for 1 min. Run was started and saved accordingly.

Result analysis, recoding and interpretation

After the run, the slope of the calibration curve, the PCR efficiency, y-interval and plateau were checked. The slope of the reaction ranged from -3.30 to -3.60; the y-intercept should be < 40. Cycle threshold (Ct) value < 36 was positive for the species; Ct value undetected or > 40 was negative; Ct value between 36–40 was equivocal and was retested with higher concentration of DNA.

iii. Singleplex RT-PCR for *N*. *meningitidis, H. influenzae,* and *S. pneumoniae*

All positive samples at mRT-qPCR run were further analyzed appropriately using singleplex PCR for *N*. *meningitidis, H. influenzae, or S. pneumoniae* accordingly. All the samples positive for *N*. *meningitidis* at mRT-qPCR level were subjected to singleplex RT-PCR for typing of sero-markers of *N*. *meningitidis* based on FAM dye as a reporter and Blackhole Quenchers (BHQ). The assay detects six sero-markers as shown in Table 2 (primers, probes and targets). All the samples positive for *H. influenzae* at mRT-qPCR level were subjected to singleplex RT-PCR for typing of sero-markers of *H. influenzae* based on SYBR chemistry (intercalating dye). The assay detects six sero-markers as shown in Table 3 (primers and targets). All samples positive for *S. pneumoniae* at mRT-qPCR level were subjected to singleplex RT-PCR for typing of sero-markers of *S. pneumoniae* based on SYBR chemistry (intercalating dye). The assay detects twelve sero-markers as shown in Table 4 (primers and targets).

Singleplex reaction set up and real time amplification

The worksheet was created according to the number of samples to be tested. The ABI 96 well plate placed into a plate holder on ice pack rack. For *N. meningitidis,* into each well, 10µL of 2x Probe Master Mix was dispensed and 7µL of appropriate primer-probe mix was added. 3µL of DNA sample or ATCC bacterial strains or QC or NTC was then added into appropriate well. For *H. influenzae* and *S. pneumoniae,* 10µL of 2x Ampigene Green Master mix was dispensed and 7µL of appropriate primer was added. 3µL of DNA sample or ATCC bacterial strains or QC or NTC was then added into appropriate well. All other steps were as stated above under multiple reaction set up, based on specific standard operating procedures (SOPs).

Table 2: Target genes for preparing six sero-markers of *Neisseria meningitidis*

Target gene	Primer/probe name	Sequence (5' to 3')
Nm A	F2531	AAAATTCAATGGGTATATCACGAAGA
sacB	R2624	ATATGGTGCAAGCTGGTTTCAATAG
	Pb2591i	FAM-CTAAAAG''T"AGGAAGGGCACTTTGTGGCATAAT-BHQ
Nm B	F737	GCTACCCCATTTCAGATGATTTGT
synD	R882	ACCAGCCGAGGGTTTATTTCTAC
	Pb839i	FAM-AAGAGATGGGYAACAAC" T"ATGTAATGCTTTATTT-BHQ
Nm C	F478	CCCTGAGTATGCGAAAAAAATT
synE	R551	TGCTAATCCCGCCTGAATG
	Pb495i	FAM-TTTCAATGC"T"AATGAATACCACCGTTTTTTTGC-BHQ
Nm W135	F857	TATTTATGGAAGGCATGGTGTATG
synG	R964	TTGCCATTCCAGAAATATCACC
	Pb907i	FAM-AAATATGGAGCGAATGATTACAGTAACTATAATGAA-BHQ
Nm X	F173	TGTCCCCAACCGTTTATTGG
xcbB	R237	TGCTGCTATCATAGCCGCC
	Pb196	FAM-TGTTGCCCACATGAATGGCGG-BHQ
Nm Y	F787	TCCGAGCAGGAAATTTATGAGAATAC
synF	R929	TTGCTAAAATCATTCGCTCCATAT
	Pb10099i	FAM-TATGGTG" T"ACGATATCCCTATCCTTGCCTATAAT-BHQ

Table 3: Target genes for preparing six sero-markers of *Haemophilus influenzae*

Target gene	Primer name	Sequence (5' to 3')
Hi a	F261	GGT CTG CGG TGT CCT GTG T
acsB	R427	CCG GTC ATC TTT TAT GCT CCA A
Hi b	F192	TGA TGC ATT GAA AGA AGG TGT AAT TT
bcsB	R359	TGA TGC ATT GAA AGA AGG TGT AAT TT
Hi C	F7667	CAT TGG TGA TGG TTC AGT TAT TGG
ccsD	R7784	TAC AGC ATT CAG CAA TAA TGG G
Hi D	F2211	CCT AAA ATA CGG ACC TAG TGC AC
dcsE	R2255	CCG ATG AGA CCA AGT ATG GTT A
Hi e	F1523	ACT AAA ATA TGG CCC AAA CCC AC
ecsH	R1589	CCG ATG AGC CCA AGT ATG ATG A
Hi f	F7164	CCC TGA AAA GCG TTG ACT TTG
bexD	R7313	CCA ACT TCA GGA CCA AGT CAT TC

Results:

Of the 210 subjects, 129 (61.4%) were males comprising 104 (49.5%) children (<15 years of age) and 25 (11.9%) adults while the females were 81 (38.5%) comprising 66 (31.4%) children (<15 years of age) and 15 (7.1%) adults. Of the 210 CSF samples analyzed bacteriologically, 94 (44.8%) were Gram stain positive, while 17 (8.1%) were culture positive. Multiplex RT-PCR confirmed 45 (21.4%) of the 94 Gram stain positive samples as shown in Table 5.

Of the 17 culture positive samples, multiplex/singleplex RT-PCR confirmed 14 cases (82.4%). Of these 17 bacterial isolates, 5 (29.4%) showed same bacterial serogroup or serotype, 9 (52.9%) showed different bacterial serogroups including 3 (17.6%) that showed co-infection at PCR as against the single bacteria reported in culture, while 3 (17.6%) were PCR negative, as shown in Table 6.

Table 4: Target genes for preparing twelve sero-markers of *Streptococcus pneumoniae*

Target gene	Primer/probe name	Sequence (5' to 3')
Wzy 1	1-F	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA
	1-R	CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C
GalU	3-F	ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G
	3-R	CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G
Wzy 4	4-F	CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G
	4-R	GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G
Wzy 5	5-F	ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG
	5-R	GCT CGA TAA ACA TAA TCA ATA TTT GAA AAA GTA TG
WciP	6-F	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG
	6-R	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA
Wzy 7	7-F	TCC AAA CTA TTA CAG TGG GAA TTA CGG
	7-R	ATA GGA ATT GAG ATT GCC AAA GCG AC
Wzy 9	9-F	GGG TTC AAA GTC AGA CAG TGA ATC TTA A
	9-R	CCA TGA ATG AAA TCA ACA TTG TCA GTA GC
Wzy 14	14-F	GAA ATG TTA CTT GGC GCA GGT GTC AGA ATT
	14-R	GCC AAT ACT TCT TAG TCT CTC AGA TGA AT
Wzy 18	18-F	CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC
	18-R	TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC
Wzy 19A	19A-F	GAG AGA TTC ATA ATC TTG CAC TTA GCC A
	19A-R	CAT AAT AGC TAC AAA TGA CTC ATC GCC
Wzy 19F	19F-F	GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C
	19F-R	GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG
Wzy 23	23-F	GTA ACA GTT GTC GTA GAG GGA ATT GGC TTT TC
	23-R	CAC AAC ACC TAA CAC TCG ATG GCT ATA TGATTC

Table 5: Comparison of Gram stain and multiplex real-time PCR

Diagnostic test		**Multiplex RT-PCR**		Total (%)
		Positive (%)	Negative (%)	
Gram reaction	Positive (%)	45 (21.4)	49 (23.3)	94 (44.8)
	Negative (%)	43 (20.5)	73 (34.8)	116 (55.2)
		88 (41.9)	122 (58.1)	210 (100)

One hundred and eighty (85.7%) of the 210 CSF samples from which mDNA was extracted had concentrations of $\geq$ 0.005 ng/µL. The amounts of mDNA present in the 180 samples were; DNA concentration range of 0.03-50.5 ng/µL, DNA yield of 1.8 - 3030 µg and DNA amount of 0.15-252.5 ng/µL; while 30/210 (14.3%) had DNA concentrations less than 0.005 ng/µL (Table 7).

Multiplex RT-PCR was positive in 88 (41.9%) of the 210 CSF samples analyzed with mDNA concentration between 0.005-50.5ng/µL, for either one or co-infection of any two of the three bacteria; *N*. *meningitidis, H. influenzae,* and *S. pneumoniae*. Of these 88, 59 (67.1%) were *N*. *meningitidis,* 2 (2.3%) were *H*. *influenzae,* 3 (3.4%) were *S*. *pneumoniae,* 15 (17%) had co-infection of *N*. *meningitidis* with *H*. *influenzae* and 9 (10.2%) had co-infection of *H. influenzae* and *S*. *pneumoniae*, as shown in Table 8.

Table 6: Comparison of culture results and RT-PCR results from isolated bacterial DNA and CSF metagenomic DNA by multiplex and singleplex PCR assay

S/N	Culture results (bacteria isolated)	RT-PCR results			
		Bacterial DNA (from culture isolates)		Metagenomic DNA (from CSF samples)	
		Multiplex RT-PCR	Species-specific singleplex RT-PCR	Multiplex RT-PCR	Species-specific singleplex RT-PCR
1	Nm A	Nm	Nm B	Nm	Nm B
2	Nm Y	Nm	Nm Y	Nm	Nm Y
3	Nm C	Nm	Nm C	Nm	Nm C
4	Nm C	Nm	Nm C	Nm	Nm C
5	Nm C	Nm	Nm B	Nm	Nm B
6	Nm C	Nm	Nm B	Nm	Nm B
7	Nm C	Negative	Negative	Negative	Negative
8	Nm C	Negative	Negative	Negative	Negative
9	Nm C	Nm	Nm Y	Nm	Nm Y
10	Nm C	Nm	Nm C	Nm	Nm C
11	Nm C	Nm	Nm B	Nm	Nm B
12	Nm C	Nm	Nm Y	Nm	Nm Y
13	Hi B	Hi	Hi B	Hi	Hi B
14	Nm C	Nm	Nm Y	Nm, Hi	Nm Y, Hi f
15	Nm C	Nm	Nm Y	Nm, Hi	Nm Y, Hi b
16	Nm C	Hi	Hi f	Hi, Sp	Hi f, Sp
17	Nm C	Negative	Negative	Negative	Negative

Nm = *Neisseria meningitidis;* Hi = *Haemophilus influenzae*; Sp = *Streptococcus pneumoniae;* Negative = No bacteria detected. Of the 17 culture positives, mRT-PCR confirmed 14 cases; 5 were same bacteria while 9 had varied results. Culture confirmed by PCR is 14 (6.7%) of the 210 CSF samples analyzed

Table 7: Amount of mDNA present in extracted CSF samples for molecular analysis

No of samples	DNA concentration (ng/µL)	DNA yield (µg)	Amount of DNA (ng)
180	0.03 – 50.5	1.8 – 3030	0.15 – 252.5
30	< 0.005	--------	----------

Table 8: Detection rate of *Neisseria meningitidis, Haemophilus influenzae,* and *Streptococcus pneumoniae* by mRT-PCR

Bacteria detected	Number	Percentage
Neisseria meningitidis (only)	59	67.1
Haemophilus influenzae (only)	2	2.3
Streptococcus pneumoniae (only)	3	3.4
N. meningitidis + *H. influenzae* (co-infection)	15	17.0
H. influenzae + *S. pneumoniae* (co-infection)	9	10.2
Total	88	100

Table 9: Singleplex real time PCR result for *Neisseria meningitidis* serogroups

Serogroup detected	Gene	Number	Percentage
A	*sacB*	10	13.5
B	*synD*	17	23.0
C	*synE*	6	8.1
W135	*synG*	6	8.1
X	*xcbB*	4	5.4
Y	*synF*	24	32.4
*Negative (non-groupable)		7	9.5
Total		74	100

*All seven samples were *N. meningitidis* serogroup negative (non-groupable) after repeated PCR run with concentrated DNA extract

Table 10: Singleplex real time PCR result for *Haemophilus influenzae* serotypes

Serotype detected	Gene	Number	Percentage
a	*acsB*	1	3.8
b	*bcsB*	15	57.7
c	*ccsD*	1	3.8
e	*ecsH*	3	11.5
f	*bexD*	6	23.1
Total		26	100

Table 11: Singleplex real time PCR result for *streptococcus pneumoniae* serotypes

Serotype detected	Gene	Number	Percentage
1	*Wzy1*	1	8.3
4	*Wzy4*	4	33.3
5	*Wzy5*	6	50.0
9	*Wzy9*	1	8.3
Total		12	100

Of the 74 *N. meningitidis* detected by mRT-PCR (59 single infections and 15 from mixed infection with *H. influenzae),* the sero-groups are as shown in Table 9. Of the 26 *H. influenzae* detected by PCR (2 single infection, 15 co-infection with *N. meningitidis* and 9 co-infection with *S. pneumoniae),* the serotypes are as shown in Table 10. Of the 12 *S. pneumoniae* detected by PCR (3 single infections and 9 co-infection with *H. influenzae),* the serotypes are as shown in Table 11.

Discussion:

Our CSF culture results as confirmed by mRT-PCR showed 6.7% recovery rate. Previous reports from Nigeria using culture of CSF samples had shown bacterial isolation rates ranging from 1.7% to 16.7% (15-20). Results from culture with latex agglutination had rate of 2.9-15.3% (21,22) while results from latex agglutination only was between 9.1-20.4% (23). Therefore, our findings are in tune with

earlier reports. The detection rate of mRT-PCR using metagenomic DNA extract from CSF samples was 88 (41.9%) including 15 (17.0%) that had mixed infections (co-infection) of *N. meningitidis* with *H. influenzae*, and 9 (10.2%) with co-infection of *H. influenzae* and *S. pneumoniae*. No co-infection of *N. meningitidis* and *S. pneumoniae* was encountered. Though, reports on mixed bacterial meningitis in Nigeria are unavailable, literature search revealed such reports from elsewhere such as Kansas City Missouri USA, and Taiwan, with varying prevalence rates ranging from 1% to 11.7% (24-28). The mixed infection rate of 17% for *N. meningitidis* and *H. influenzae*, and 10.2% for *H. influenzae* and *S. pneumoniae* in our study agrees with reports elsewhere (27,28), though, our findings are a little higher for the *N. meningitidis* and *H. influenzae* association.

Mixed infections are reported to be rare but can occur with certain predisposing conditions (26,29) such as trauma, tumours or infections like acute paranasal sinusitis that may extend directly to the meninges. Mixed infections can also arise by direct entry of organisms via fistulae or as a result of a ruptured brain abscess (30). However, we do not know if these factors were present in our study population. This being that, apart from the fact that we least expected mixed infections in the course of this research, we did not follow up by visiting the various sites to have access to patients' hospital folders and identify other underlying issues including ascertaining whether they had these predisposing factors.

The use of PCR assay has been reported by several authors to be more sensitive than phenotypic methods. Favaro and colleagues (31) had previously reported a higher detection rate with PCR. In their study that analyzed 296 samples, 59 (19.9%) were positive by CSF culture and/or molecular assays, 46 (15.5%) by both CSF culture and PCR assay, while 13 (4.4%) by RT-PCR, but negative for the traditional assays, implying that only 46 (15.5%) of the samples were culture positive while 59 (19.9%) were PCR positive (31). In another study (32) of 451 CSF specimens analyzed, 80 (17.7%) had culture isolation of one of the three pathogens (40 *S. pneumoniae,* 36 *N. meningitidis,* and 4 *H. influenzae),* and 113 (25.1%) were positive by RT-PCR assay (51 *S. pneumoniae,* 57 *N. meningitidis,* and 5 *H. influenzae*). Our findings corroborated these previous reports that showed higher detection rate with RT-PCR method.

While comparing multiplex PCR and other traditional methods for diagnosis of acute bacterial meningitis, Yahia and Balach (33) reported that of 110 CSF samples analyzed, Gram stain for any bacteria was positive in 32 cases (29.1%) including five pathogens (*S. pneumoniae, H. influenzae* type b*, N. meningitidis,* Group B streptococcus and *Listeria monocytogenes)* in 11 cases (10%). Bacteria culture was positive in 38 cases (34.5%) including the five pathogens in 8 cases (7.2%). Multiplex PCR was positive in 60 cases (54.5%); 50 cases of acute bacterial meningitis were diagnosed by multiplex PCR while both Gram stain and bacterial culture missed the diagnosis. In another study, Kwambana-Adams and colleagues (19) reported PCR detection of pathogens in 95 (46%) of 208 CSF samples analyzed as against 9% culture recovery rate. Our finding of 41.9% detection rate by mRT-PCR method and 6.7% culture recovery are in agreement with these previous reports.

We were also able to identify the circulating serogroups and serotypes alike of the three bacteria under study using the metagenomic approach. Of the 74 *N. meningitidis* positive on PCR*,* 10 (13.5%) were serogroup A (*sacB*), 17 (23%) serogroup B (*synD*), 6 (8.1%) serogroup C (*synE*), 6 (8.1%) serogroup W135 (*synG*), 4 (5.4%) serogroup X (*xcbB*), 24 (32.4%) serogroup Y (*synF*), and 7 (9.5%) were non-groupable (serogroup negative). Over the years (2008 to 2018), several authors (a total of twelve) have implicated *N. meningitidis* serogroups A, B, C, and W135 as responsible for CSM in Northern Nigeria. Seven of these authors (19, 39-44) reported *N. meningitidis* serogroup C as the only offending pathogen. Only one author, Mado and colleagues (13), identified *N. meningitidis* serogroup A. Two authors (45,46) implicated both *N. meningitidis* serogroups A and C; Bassey and colleagues (23) reported *N. meningitidis* serogroups A, C, and W135 while Ujah et al., (47) reported four serogroups (*N. meningitidis* serogroups A, B, C, and W135) as the circulating serogroups in Jigawa State, Northern Nigeria.

None of the twelve authors reported ever encountering *N. meningitidis* serogroups X and Y in their research studies. Therefore, our results which identified *N. meningitidis* serogroup X (5.4%) and *N. meningitidis* serogroup Y (32.4%) as invasive serogroups are baseline data for reference. We reason that the recovery of these two serogroups (Nm X and Nm Y) which hitherto had not been reported in Northern Nigeria could be attributable to the use of metagenomic protocol in this research. It is noteworthy to mention here that previous reports implicating non-groupable (serogroup negative) strains of *N. meningitidis* in CSM cases in our study area are not available in the

literature. Therefore, our finding in the course of this research of 9.5% non-groupable or serogroup negative strains is another baseline data for reference. Hence, we ascribe that non-groupable (serogroup negative) strains of *N. meningitidis* should also be considered in outbreaks in Nigeria.

Of the 26 *H. influenzae* serotypes positive for PCR (2 single infections, 15 co-infection with *N. meningitidis* and 9 co-infection with *S. pneumoniae)*, serotype a *(acsB*) was 1 (3.8%), serotype b (*bcsB*) was 15 (57.7%), serotype c (*ccsD*) was 1 (3.8%), serotype e (*ecsH*) was 3 (11.5%) and serotype f (*bexD*) was 6 (23.1%). A previous report (48) implicated Hib as the only serotype encountered. Therefore, our finding in this current study is well revealing because of the six serotypes tested, only serotype d (*dcsE*) was not encountered. Of the 12 *S. pneumoniae* serotypes identified by PCR (3 single infections and 9 co-infection with *H. influenzae),* serotype 1 (*Wxy1*) was 1 (8.3%), serotype 4 (*Wxy4*) was 4 (33.3%), serotype 5 (*Wxy5*) was 6 (50%), and serotype 9 (*Wxy9*) was 1 (8.3%). Previous reports had shown varying results, while Kwambana-Adams and colleagues (19) implicated *S. pneumoniae* serotypes 1, 5 and 19F, Suleiman et al., (49) implicated *S. pneumoniae* serotypes 6, 19, and 20. Of the twelve serotypes tested in our study, only four positive serotypes were encountered.

Serotyping is of great importance for the development of vaccination strategies (34). The identification of the serogroup or serotype responsible for an outbreak is crucial for its containment (35). The serogroup or serotype data can impact policy decisions regarding selection of appropriate vaccination programs and can improve epidemiological data (36). Some types of meningitis such as those associated with *N. meningitidis, H. influenzae,* and *S. pneumoniae* can be prevented by immunization (37).

Conclusion:

Our results showed that multiplex RT-PCR using mDNA extracted directly from CSF samples offered a higher detection rate than culture for major bacterial pathogens involved in meningitis in northern parts of Nigeria. Multiplex RT-PCR is a highly sensitive, specific, fast and reliable method of detecting bacterial pathogens implicated in CSM. Utilizing both mRT-PCR and singleplex RT-PCR enabled serogrouping/serotyping of positive cases with a shorter turn-around time. These outcomes will speed patient care and management when applied in national responses to meningitis outbreaks.

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Authors' contributions:

PIC and IEI conceived, and led the design and writing of the manuscript. PIC and NE were responsible in the performance of molecular diagnostic (PCR) activities. PIC, IEI, AGM, and NE were responsible for the final editing of the manuscript.

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Original Article **Open Access**

A retrospective study of antibiotic resistance patterns of bacterial pathogens isolated from patients in two Lebanese hospitals for two consecutive years (2018 and 2019)

[1]Sakr, S., [2]Abboud, M., [3]Tawbeh, K., [1]Hamam, B., and *[1]Sheet, I.

[1]Department of Biological and Chemical Sciences, School of Arts and Sciences,
Lebanese International University, Lebanon
[2]Haroun Hospital, Beirut, Lebanon
[3]Department of Mathematics and Physics, School of Arts and Sciences,
Lebanese International University, Lebanon
*Correspondence to: imtithal.sheet@liu.edu.lb

Abstract:

Background: Misuse of antibiotics is the leading factor promoting emergence of bacterial resistance, a situation that has become a serious public health challenge. Among the leading bacteria that have developed resistance to antibiotics are *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, which have caused infections in patients, resulting in considerable mortality. The objective of this retrospective study was to assess antibiotic resistance rates of bacterial pathogens isolated from clinical specimens in two Lebanese hospitals between the years 2018 and 2019.
Methodology: Bacteria isolated from routine clinical specimens collected from hospitalized patients in two hospitals, Haroun and Bekaa, in Lebanon for 2018 and 2019, were analyzed. Bacteria isolation and identification were carried out at the laboratory of each hospital using conventional microbiological methods. Antimicrobial susceptibility testings (AST) of each bacterial isolate to antibiotics were performed by the disc diffusion test and interpreted using EUCAST, CLSI or WHO/AST guidelines. Comparisons of the mean resistance rates of each isolate to individual antibiotics by year of isolation were done using the Z-test and $p< 0.05$ was considered statistically significant.
Results: There were a total of 1698 bacteria isolates recovered from hospitalized patients in the two hospitals for 2018 and 2019, of which 87.5% were Gram-negative and 12.5% were Gram-positive bacteria. The most frequent among the Gram-negative isolates was *E. coli* (66.1%) followed by *P. aeruginosa* (13.3%), *K. pneumoniae* (7.7%), *Proteus mirabilis* (6.7%) and *Enterobacter* spp (6.3%), while coagulase positive staphylococci CoPS (68.4%) and *E. faecalis* (31.6%) were the two Gram positive isolates. Of the Gram-negative isolates over the two-year period, 72.2% of *E. coli* and 76.3% of *K. pneumoniae* were resistant to ceftazidime, 93% of *P. mirabilis* to colistin, and 98% of *Enterobacter* to cefoxitin, but low resistance rates were demonstrated by *E. coli* to imipenem (1%), *K. pneumoniae* to tigecycline and amikacin (0.9%), *P. mirabilis* to imipinem (2%), and *Enterobacter* to amikacin, ertapenem and tigecycline (3%). Resistance of *P. aeruginosa* varied between 2% to colistin and 24% to levofloxacin. For the Gram-positive bacteria, 79.1% of *E. faecalis* were resistant to erythromycin while 70% of CoPS were resistant to cefoxitin, but no isolate was resistant (0%) to linezolid, and only 1% to teicoplanin. Except for *Enterobacter* spp that showed significant increase in resistance rates (by 250%) to piperacillin/tazobactam in 2019 over 2018, resistance rates of other Gram-negative isolates significantly decreased in 2019 compared to 2018 ($p<0.05$). For the Gram-positive isolates, resistance rates to many antibiotics tested significantly increased (by a factor of 36.5 - 2569%) in 2019 compared to 2018 among *E. faecalis* isolates in contrast to the rates for CoPS which significantly decreased by 16.7 - 65.7%, except for penicillin G which increased by a factor of 123%.
Conclusion: Overuse and misuse of antibiotics, which is possible because of the easy access of the populace to these drugs, is a leading factor contributing to the high antibiotic resistance rates in this study. There is need to promote awareness of antimicrobial resistance in Lebanon among students especially in non-health related majors and enactment of govermental policy that will limit access to antibiotics.

Keywords: antibiotic resistance; changing pattern; hospitalized patients; retrospective

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Une étude rétrospective des profils de résistance aux antibiotiques de pathogènes bactériens isolés de patients dans deux hôpitaux libanais pendant deux années consécutives (2018 et 2019)

[1]Sakr, S., [2]Abboud, M., [3]Tawbeh, K., [1]Hamam, B., et *[1]Sheet, I.

[1]Département des Sciences Biologiques et Chimiques, École des Arts et des Sciences, Université internationale Libanaise, Liban
[2]Haroun Hospital, Beyrouth, Liban
[3]Département de Mathématiques et de Physique, École des Arts et des Sciences, Université Internationale Libanaise, Liban
*Correspondance à: imtithal.sheet@liu.edu.lb

Abstrait:

Contexte: La mauvaise utilisation des antibiotiques est le principal facteur favorisant l'émergence de la résistance bactérienne, une situation qui est devenue un sérieux défi de santé publique. Parmi les principales bactéries qui ont développé une résistance aux antibiotiques figurent *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae* et *Pseudomonas aeruginosa*, qui ont provoqué des infections chez les patients, entraînant une mortalité considérable. L'objectif de cette étude rétrospective est d'évaluer les taux de résistance aux antibiotiques des pathogènes bactériens isolés à partir d'échantillons cliniques dans deux hôpitaux Libanais entre les années 2018 et 2019.
Méthodologie: Les isolats bactériens prélevés sur des patients hospitalisés dans deux hôpitaux, Haroun et Bekaa, au Liban pour 2018 et 2019, ont été analysés. L'isolement et l'identification des bactéries ont été réalisés au laboratoire de chaque hôpital en utilisant des méthodes microbiologiques conventionnelles. Les tests de sensibilité aux antimicrobiens (AST) de chaque isolat bactérien aux antibiotiques ont été réalisés par le test de diffusion sur disque et interprétés selon les directives EUCAST, CLSI ou WHO/AST. Des comparaisons des taux moyens de résistance de chaque isolat à des antibiotiques individuels par année d'isolement ont été effectuées à l'aide du test Z et $p<0,05$ a été considéré comme statistiquement significatif.
Résultats: Il y a eu un total de 1698 isolats de bactéries récupérés de patients hospitalisés dans les deux hôpitaux durant 2018 et 2019, dont 87,5% étaient à Gram négatif et 12,5% étaient des bactéries à Gram positif. Les isolats à Gram négatif les plus fréquents étaient *E. coli* (66,1%), suivis de *P. aeruginosa* (13,3%), *K. pneumoniae* (7,7%), *Proteus mirabilis* (6,7%) et *Enterobacter* spp (6,3%), tandis que les staphylocoques à coagulase positive CoPS (68,4%) et *E. faecalis* (31,6%) étaient les deux isolats Gram positifs. Parmi les isolats à Gram négatif sur la période de deux ans, 72,2% d'*E. coli* et 76,3% de *K. pneumoniae* étaient résistants à la ceftazidime, 93% de *P. mirabilis* à la colistine et 98% d'*Enterobacter* à la céfoxitine, mais faible les taux de résistance ont été démontrés par *E. coli* à l'imipénem (1%), *K. pneumoniae* à la tigécycline et à l'amikacine (0,9%), *P. mirabilis* à l'imipinem (2%) et *Enterobacter* à l'amikacine, à l'ertapénem et à la tigécycline (3%). La résistance de *P. aeruginosa* variait entre 2% à la colistine et 24% à la lévofloxacine. Pour les bactéries Gram positif, 79,1% des *E. faecalis* étaient résistantes à l'érythromycine tandis que 70% des CoPS étaient résistantes au céfoxitin, mais aucun isolat n'était résistant (0%) au linézolide et seulement 1% à la teicoplanine. À l'exception d'*Enterobacter* spp qui ont montré une augmentation significative des taux de résistance (de 250%) à la pipéracilline/tazobactam en 2019 par rapport à 2018, les taux de résistance des autres isolats à Gram négatif ont considérablement diminué en 2019 par rapport à 2018 ($p<0,05$). Pour les isolats Gram-positifs, les taux de résistance à de nombreux antibiotiques testés ont augmenté de manière significative (d'un facteur de 36,5 à 2569%) en 2019 par rapport à 2018 parmi les isolats d'*E. faecalis* contrairement aux taux de CoPS qui ont significativement diminué de 16,7 à 65,7%, à l'exception de la pénicilline G qui a augmenté d'un facteur de 123%.
Conclusion: la surutilisation et la mauvaise utilisation des antibiotiques, ce qui est possible en raison de l'accès facile de la population à ces médicaments, est l'un des principaux facteurs contribuant aux taux élevés de résistance aux antibiotiques dans cette étude. Il est nécessaire de promouvoir la sensibilisation à la résistance aux antimicrobiens au Liban parmi les étudiants, en particulier dans les spécialisations non liées à la santé, et la promulgation d'une politique gouvernementale qui limitera l'accès non contrôlé aux antibiotiques.

Mots clés: résistance aux antibiotiques; changement de modèle; patients hospitalisés; rétrospective

Introduction:

Antimicrobial resistance (AMR) occurs when the drug loses its ability to effectively inhibit bacterial growth (1). In the developing countries, almost all antibiotics are available and can be purchased without medical prescription, which is one of the main factor underlying emergence of antimicrobial resistance (1). AMR is creating a serious global public health threat (2). In 2017, 12 bacterial species were identified by the World Health Organisation (WHO) to represent a threat to human health with majority being Gram negative bacteria (GNB), such as *Acinetobacter, Pseudomonas aeruginosa,* Enterobacteriaceae*, Helicobacter pylori, Salmonella* spp.*, Neisseria gonorrhoeae* and *Shigella* spp (3). The Enterobacteriaceae are

now globally reported to be resistant to carbapenems, third generation cephalosporins, and colistin (3).

Recently, the European Center for Disease Prevention and Control (ECDC) reported that GNBs are responsible for more than 500,000 infections and more than 24,600 deaths in Europe in just one year (4). The WHO press release highlights the real threat of GNBs which have developed remarkable mechanisms enabling them to resist antibiotic actions (5). In the United States and European countries, the number of deaths due to antibiotic resistance in *Staphylococcus aureus, Escherichia coli, Enterococcus faecium, Klebsiella pneumoniae* and *P. aeruginosa* was estimated to be 50,000 yearly (6). Laws et al., (7) reported that between 2011 and 2014, the percentage of *K. pneumoniae* and *E. coli* infections resistant to fluoroquinolones, third-generation cephalosporins or aminoglycosides, as well as combined resistance to all three antibiotic groups, significantly increased in Europe.

The WHO global report on antimicrobial resistance indicates that resistance of common bacteria has reached alarming levels in many African countries, as over 50% of *E. coli* and *Klebseilla* isolates were reported to be resistant to the third generation cephalosporins and carbapenems (5). Mouiche et al., (8) also reported in Cameroon in 2019 high levels of resistance of *E. coli, Klebsiella* sp, *S. aureus, P. aeroginosa, Enterobacter* spp*,* and *Proteus* spp to tetracycline, amoxicillin, nalidixic acid, ampicillin and trimethoprim but low levels of resistance to gentamicin, ceftriaxone and ciprofloxacin. Ahmed et al., (9) reported in 2019 that the prevalence of *E. coli* resistance to most antibiotics such as amoxicillin, amoxicillin/clavulanic acid, ampicillin and ciprofloxacin was very high in Bangladesh. Resistance patterns of microbes vary from country to country, large hospital to small hospital, and from hospital to the community (10). Among these increasingly antimicrobial resistance infections are methicillin resistant *S. aureus* (MRSA), which have become prevalent in many countries, including the USA, European countries, South America and Asia (11). Another growing threat worldwide is *E. coli* and *K. pneumoniae* harboring extended-spectrum beta lactamases (ESBL) (12).

In the past few years, in the Middle East, the frequency of drug-resistant bacteria isolates has been increasing in different hospitals as confirmed by the WHO (13). Over 700,000 deaths are reported yearly due to AMR, and in the absence of appropriate control and preventive measures, AMR is projected to become one of the main causes of death among hospitalized and non-hospitalized patients in the developed countries (14). In the Mediterranean countries, many studies have reported emergence of bacterial resistance mechanisms such as ESBL, AmpC and carbapenemases in Gram negative bacteria, methicillin and vancomycin resistance in Gram positive organisms, and penicillin resistance in *Streptococcus pneumoniae* (15).

In Lebanon, as in other developing countries, AMR is responsible for significant morbidity and mortality in different hospitals (16). The most important factor leading to the emergence of AMR is the uncontrolled or inappropriate use (misuse and overuse) of antimicrobial drugs (17). This is mainly due to incorrect diagnosis and missuse of antimicrobials due either to an inappropriate prescription or poor compliance (18). Sakr et al., (19) reported in 2020 that in order to control the growing problem of antibiotic resistance in Lebanon, there is an urgent need for awareness campaigns on appropriate use of antibiotics in parallel with rigorous surveillance system for antimicrobial use and resistance.

In a cross-sectional study conducted by Moghnieh et al., (20) on antimicrobial susceptibility pattern of bacterial isolates from hospitalized patients in 13 Lebanese hospitals over two consecutive years (2015 - 2016), an overall decrease in susceptibility of bacterial isolates to different antibiotics among clinical GNB and GPB involved in various infections was reported. To the best our knowledge, there are no reports of antibiotic resistance rates of isolated strains in Lebanese hospitals during the last two years (2018 and 2019). The objective of this current study therefore is to investigate the changing pattern of antibiotic resistance of bacteria pathogens isolated from hospitalized patients with clinical infections in two Lebanese hospitals over this period.

Materials and method:

Study setting and population

This retrospective study was conducted in two hospitals (in Haroun and Bekaa) in Lebanon with about 70% of bacterial isolates from hospitalized patients in Internal Medicine department and 30% from patients in Surgical and Outpatient deparments, Intensive Care Unit (ICU) and intubated patients in Coronary Care Unit.

Specimen types

Bacterial isolates were recovered from routine clinical specimens such as urine, sputum, tracheal aspirates, pus, abscess, blood, wounds and rectal specimens, which were

collected from patients of different age groups and gender.

Culture isolation and identification of bacterial isolates from specimens

All clinical samples were routinely cultured in the laboratory of the two hospitals on standard agar media appropriate for each specimen, and these included Blood agar, Salmonella Shigella (SS) agar, MacConkey agar, Columbia agar, Chocolate agar, Schaedler agar, and Thiosulfate Citrate Bile salt Sucrose (TCBS) agar, using standard microbiological methods.

Antibiotic susceptibility testing

The antibiotic susceptibility testings (AST) of the isolates were routinely performed against anti-bacterial agents (as shown in Table 1) by the disc diffusion method, and zone diameters of inhibition interpreted according to the EUCAST/2019, CLSI/2018 or WHO/MOH/ SOP susceptibility testing/2018 guidelines. The ASTs were performed using Muller-Hinton (MH) agar except for streptococcal (enterococcal) isolates which were performed on MH media supplemented with blood.

The discs contents used for the AST were; trimetropim/sulfamethoxazole 1.25/23.75 µg, ciprofloxacin 5µg, ofloxacin 5µg, pefloxacin 5µg, penicillin G 10µg, imipenem 10µg, gentamicin 10µg, colistin 10µg, tigecycline 15µg, amoxicillin /clavulinic acid 20/10µg, piperacillin/tazo-bactam 100/10µg, cefepime 30µg, cefoxitin 30 µg, cefotaxime 30µg, cefuroxime 30µg, cefotaxime 30µg, ceftazidime 30µg, amikacin 30 µg, clindamycin 30µg, erythromycin 30µg, vancomycin 30µg, teicoplanin 30µg, fosfomycin 300µg, and nitrofurantoin 300µg.

Statistical analysis of data

The bacterial identification and susceptibility data were tabulated in Excel spreadsheets. The resistance rates to individual antibiotic were calculated for every bacterial isolate by year of isolation. The mean percentage resistance of each isolate to all tested antibiotics were also calculated. Yearly comparisons were performed using Z-test after checking the applicability conditions. When comparing results from the two different years, $p<0.05$ was considered statistically significant if at least one value was different from the other.

Table 1: Antibacterial agents with their respective classes used in this study

Antibacterial drug class	Antibacterial drug	Disc content (µg)
Aminoglycoside	Gentamicin	10
	Amikacin	30
Beta-lactam	Amoxicillin/clavulanic acid	20/10
	Cefepime	30
	Cefotaxime	30
	Cefoxitin	30
	Ceftazidime	30
	Ceftriaxone	30
	Cefuroxime	30
	Imipinem	10
	Piperacillin/tazobactam	100/10
	Penicillin G	10
Glycopeptide	Vancomycin	30
	Teicoplanin	30
Fluoroquinolone	Ciprofloxacin	5
	Ofloxacin	5
	Pefloxacin	5
Fosfomycin	Fosfomycin	300
Inhibitor of folate pathway	Sulfamethoxazole/trimethoprim	1.25/23.75
Lincosomide	Clindamycin	30
Macrolide	Erythromycin	30
Nitrofuran	Nitrofurantion	300
Oxazolidinone	Linezolid	30
Polymyxin E	Colistin	10
Glycycyline	Tigecycline	15

Ethical consideration

The study was reviewed and approved by the Lebanese International University Institutional Review Board (IRB) ethical committee (Reference LIUIRB-200305-SS2). All collected data were purely based on micro-organisms, and there was no need for a written informed consent as all the patients were anonymous and no personal information was used in the study.

Results:

A total of 1698 bacterial isolates from patients with clinical infections in the two hospitals were recovered in the year 2018 and 2019. As shown in Table 2, there were 1486 (87.7%) Gram-negative bacterial isolates and 212 (12.3%) Gram-positive isolates (12.3%). The Gram-negative isolates are distributed as follows; 982 *E. coli* (66.1%), 114 *K. pneumoniae* (7.7%), 99 *P. mirabilis* (6.7%), 198 *P. aeruginosa* (13.3%) and 93 *Enterobacter* sp. (6.3%). Among the Gram-positive isolates; 67 (31.6%) were *E. faecalis* and 145 (67.4%) were coagulase positive staphylococci (mainly *S. aureus*).

As presented in Table 3a, a total of 982 *E. coli* isolates were recovered; 465 isolates in 2018 and 517 isolates in 2019. Their resistance pattern to 18 antibiotics showed that between 44% and 68% isolates were resistant to amoxicillin/clavulanic acid, cefotaxime, ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, colistin, pefloxacin and sulfamethoxazole/trimethoprim. However, compared to 2018, the resistance rate of the isolates in 2019 to cefoxitin, ceftazidime, colistin, fosfomycin,

Table 2: Frequency of bacterial pathogens isolated from patients in two Lebanese hospitals (2018-2019)

Bacterial isolates	Number (%)
Gram-negative isolates	**1486 (87.5)**
Escherichia coli	982 (66.1)
Klebsiella pneumoniae	114 (7.7)
Proteus mirabilis	99 (6.7)
Pseudomonas aeruginosa	198 (13.3)
Enterobacter sp.	93 (6.3)
Gram positive isolates	**212 (12.5)**
Enterococcus faecalis	67 (31.6)
Coagulase positive staphylococci	145 (68.4)

Table 3a: Resistance rates of Gram-negative isolates in 2018 and 2019: *Escherichia coli*

Antibacterial agent	*Escherichia coli*			*p*-value
	Percentage of resistance to antibacterial drugs			
	2018 (n=465)	2019 (n=517)	Both years (n=1037)	
Amikacin	3.2	1.6	2.2	0.0745
Amoxicillin/clavulanic acid	58.7	63.3	57.9	0.1502
Cefepime	36.1	41.6	36.9	0.0768
Cefotaxime	45.4	49.5	45.0	0.1929
Cefoxitin	45.4	29.8	35.2	**0.0000[a]**
Ceftazidime	78.3	66.7	68.4	**0.0001[a]**
Ceftriaxone	46.2	50.9	46.1	0.1458
Cefuroxime	56.5	58.2	54.4	0.6017
Ciprofloxacin	50.8	45.6	45.5	0.1087
Colistin	61.1	35.0	44.8	**0.0000[a]**
Fosfomycin	4.9	2.1	3.3	**0.0132[a]**
Gentamicin	23.6	21.9	21.5	0.4932
Imipinem	1.3	0.8	1.0	0.4081
Nitrofurantion	7.5	9.5	8.1	0.2632
Pefloxacin	76.8	62.1	65.4	**0.0000[a]**
Piperacillin/tazobactam	17.0	9.9	12.5	**0.0007[a]**
Sulfamethoxazole/trimethoprim	53.8	53.4	50.7	0.9056
Tigecycline	1.9	1.2	1.4	0.3098

n=number, a = *p* value < 0.05 respresent significant difference in % between 2018 and 2019

Table 3b: Resistance rate of Gram-negative isolates in 2018 and 2019: *Klebsiella pneumoniae*

Antibiotic	***Klebsiella pneumoniae***			***p*-value**
	Percentage of resistance to antibacterial drugs			
	2018 (n=74)	2019 (n=40)	Both years (n=114)	
Amikacin	1.4	0.0	0.9	0.4602
Amoxicillin/clavulanic acid	50.0	50.0	50.0	1
Cefepime	24.3	27.5	25.4	0. 7102
Cefotaxime	37.8	32.5	36.0	0.5708
Cefoxitin	44.6	20.0	36.0	**0.0090[a]**
Ceftazidim	83.8	62.5	76.3	**0.0107[a]**
Ceftriaxone	39.20	35.0	37.7	0.6596
Cefuroxime	47.3	40.0	44.7	0.4545
Ciprofloxacin	23.0	27.5	24.6	0.5920
Colistin	71.6	42.5	61.4	**0.0023[a]**
Fosfomycin	14.9	2.5	10.5	**0.0400[a]**
Gentamicin	16.2	12.5	15.0	0.5949
Imipinem	4.1	0.0	2.6	0.1968
Nitrofurantion	33.8	42.5	36.8	0.3571
Pefloxacin	82.4	55.0	72.8	**0.0016[a]**
Piperacillin/tazobactam	17.6	7.5	14.0	0.1397
Sulfamethoxazole/trimethoprim	51.3	35.0	45.6	0.0943
Tigecycline	1.3	0.0	0.9	0.4602

n=number, a = *p* value < 0.05 respresent significant difference in % between 2018 and 2019

pefloxacin and piperacillin/tazobactam decreased by 34.4%, 14.8%, 42.7%, 56.9%, 19.1% and 41.9% respectively. Also, *E. coli* isolates over the 2 years exhibited considerable susceptibility to amikacin, imipenem, and tigecycline. Indeed, less than 3% of *E. coli* isolates were resistant to each one of these antibiotics, while 8.6% of the isolates in 2018/19 were resistant to nitrofurantoin.

The results of resistance of 114 *K. pneumoniae* isolates to 18 different antibiotics assessed for 2018 and 2019 are presented in Table 3b, which shows a significant decrease in 2019 of *K. pneumoniae* resistance to 5 antibiotics; cefoxitin, ceftazidime, colistin, fosfomycin and pefloxacin by 55.1%, 25.4%, 49.3%, 83.2% and 33.3% respectively. Fifty percent of the 114 isolates were resistant to amoxicillin/clavulanic acid, 76.3% to ceftazidime, 44.7% to cefuroxime, 61.4% to colistin and 72.8% to pefloxacin. The resistance of the 114 isolates to amikacin, imipenem and tigecycline did not exceed 3% while between 10 to 14% were resistant to fosfomycin, gentamicin and piperacillin/tazobactam.

The data on the 99 *P. mirabilis* isolates in the study as shown Table 3c, showed that 2% were resistant to imipenem, 5% to amikacin and piperacillin/tazobactam, and 46% to amoxicillin/clavulanic acid. Between 60 and 93% of these isolates were resistant to ceftazidime, colistin, nitrofurantoin, pefloxacin, sulfamethoxazole/trimethoprim and tigecycline. In 2019, there was a significant decrease in resistance of *P. mirabilis* isolates by 54.6%, 18%, 65.1%, 46.7%, 34% and 35.9% respectively to ceftazidime, colistin, fosfomycin, nitrofurantoin, pefloxacin and tigecycline, compared to 2018.

Regarding the resistance of the 93 *Enterobacter* isolates, as shown in Table 3d, 3% of the isolates were resistant to amikacin, ertapenem, imipenem and tigecycline. Between 11 and 16% were resistant to ciprofloxacin, cefepime, gentamicin and piperacillin/tazobactam. Most of the isolates were resistant to amoxicillin/clavulanic acid, cefoxitin, ceftazidime, colistin and pefloxacin, with resistance rate varying between 84 and 98% of the total isolates. About 250% more isolates were resistant to piperacillin/tazobactam in 2019 compared to 2018, while in 2019, the resistance of the isolates to colistin and pefloxacin decreased by 26.6% and 23.4% respectively, compared to the year 2018.

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Table 3c: Resistance rate of Gram-negative isolates in 2018 and 2019: *Proteus mirabilis*

Antibiotic	***Proteus mirabilis***			***p*-value**
	Percentage of resistance to antibacterial drugs			
	2018 (n=60)%	2019 (n=39)%	All years (n=99)%	
Amikacin	7	3	5	0.3623
Amoxicillin/clavulanic acid	52	38	46	0.1980
Cefepime	10	10	10	0.9670
Cefotaxime	12	13	12	0.8635
Cefoxitin	28	23	26	0.5614
Ceftazidim	97	44	76	**0.0000[a]**
Ceftriaxone	13	13	13	0.9411
Cefuroxime	27	28	27	0.8666
Ciprofloxacin	37	31	34	0.5459
Colistin	100	82	93	**0.0006[a]**
Fosfomycin	43	15	32	**0.0036 [a]**
Gentamicin	30	46	36	0.1025
Imipinem	3	0	2	0.2493
Nitrofurantion	92	49	75	**0.0000[a]**
Pefloxacin	97	64	84	**0.0000[a]**
Piperacillin/tazobactam	7	3	5	0.3623
Sulfamethoxazole/trimethoprim	60	59	60	0.9190
Tigecycline	92	59	79	**0.0001[a]**

n=number, a = *p* value < 0.05 respresent significant difference in % between 2018 and 2019

Table 3d: Resistance rate of Gram-negative isolates in 2018 and 2019: *Enterobacter* spp

Antibiotic	***Enterobacter* spp**			***p*-value**
	Percentage of resistance to antibacterial drugs			
	2018 (n=54)	2019 (n=39)	Both years (n=93)	
Amikacin	4	3	3	0.7588
Amoxicillin/clavulanic acid	93	92	92	0.9590
Cefepime	15	18	16	0.6851
Cefotaxime	22	26	24	0.7018
Cefoxitin	98	97	98	0.8152
Ceftazidime	94	82	89	0.0569
Ceftriaxone	22	28	25	0.5093
Cefuroxime	63	49	57	0.1709
Ciprofloxacin	15	5	11	0.1367
Colistin	94	69	84	**0.0011[a]**
Ertapenem	2	5	3	0.3775
Gentamicin	19	8	14	0.1373
Imipinem	2	5	3	0.3775
Pefloxacin	94	72	85	**0.0025[a]**
Piperacillin/tazobactam	6	21	12	**0.0275[a]**
Sulfamethoxazole/trimethoprim	24	28	26	0.6532
Tigecycline	6	0	3	0.1345

n=number, a = *p* value < 0.05 respresent significant difference in % between 2018 and 2019

Among the 198 *P. aeroginosa* isolates (Table 3e), only 2% were resistant to colistin and 11% to amikacin. Between 19% and 24% were resistant to cefepime, ceftazidime, ciprofloxacin, gentamicin, imipenem, levofloxacin and piperacillin/tazobactam. In 2019, the decrease of the resistance to amikacin and imipenem was respectively 66.7% and 65.5%.

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Table 3e: Resistance rates of Gram-negative isolates in 2018 and 2019: *Pseudomonas aeruginosa*

Antibiotic	***Pseudomonas aeroginosa***			***p*-value**
	Percentage of resistance to antibacterial drugs			
	2018 (n=105)	2019 (n=93)	Both years (n=198)	
Amikacin	15	5	11	**0.0245[a]**
Cefepime	21	23	22	0.7815
Ceftazidime	22	16	19	0.3030
Ciprofloxacin	25	20	23	0.4678
Colistin	1	2	2	0.4909
Gentamicin	19	23	21	0.5403
Imipinem	29	10	20	**0.0008[a]**
Levofloxacin	26	22	24	0.4872
Piperacillin/tazobactam	20	20	20	0.9400

n=number, a = *p* value < 0.05 respresent significant difference in % between 2018 and 2019

Table 4a: Resistance rate of Gram-positive bacteria isolates in 2018 and 2019: *Enterococcus faecalis*

Antibiotic	***Enterococcus faecalis***			*p*-value
	Percentage of resistance to antibacterial drugs			
	2018 (n=29)	2019 (n=38)	Both years (n=67)	
Ciprofloxacin	6. 9	94.7	56. 7	**0.0000[a]**
Erythromycin	65. 5	89.5	79. 1	**0.0168[a]**
Gentamicin	3. 5	92.1	53. 7	**0.0000[a]**
Linezolid	0	0	0	1
Teicoplanin	13.8	34.2	25.4	0.0570
Tigecycline	0	31.6	17. 9	**0.0008[a]**
Vancomycin	24. 1	36. 8	31. 3	0.2667

n=number, a = *p* value < 0.05 respresent significant difference in % between 2018 and 2019

Table 4b: Resistance rate of Gram-positive bacteria isolates in 2018 and 2019: Coagulase Positive Staphylococci

Antibiotic	**Coagulase positive staphylococci**			***p*-value**
	Percentage of resistance to antibacterial drugs			
	2018 (n=82)	2019 (n=63)	Both years (n=145)	
Amoxicillin/clavulanic acid	30.5	25. 4	28	0.4998
Cefoxitin	87. 8	46. 0	70	**0.0000[a]**
Ceftriaxone	54.9	34. 9	46	**0.0168[a]**
Cefuroxime	87. 8	50.8	72	**0.0000[a]**
Ciprofloxacin	9. 8	19.1	14	0.1077
Clindamycin	24. 4	15.9	21	0.2094
Erythromycin	18.3	22. 2	20	0.5576
Linezolid	0	0	0	1
Ofloxacin	12. 2	19.1	15	0.2542
Penicillin G	26. 8	58. 7	41	**0.0001[a]**
Sulfamethoxazole/trimethoprim	9.8	6.4	8	0.4604
Teicoplanin	0	1.6	1	0.2522
Vancomycin	23.2	7.9	17	**0.0144[a]**

n: number, a: *p* value <0.05 represents significant difference in % between 2018 and 2019.

Analysis of the resistance of 67 *E. faecalis* isolates to 7 different antibiotics (as shown in Table 4a) clearly revealed a major increase of the resistance of these isolates between 2018 and 2019 to four antibiotics. Compared to the 29 isolates in 2018, the 38 isolates in 2019 showed an increase of their resistance by 1273% to ciprofloxacin, by 36.6% to erythromycin, and by 2569.8% to gentamicin (Table 5). This was added to the fact that in 2018, none of the isolates was resistant to the tigecycline, compared to 31% of isolates in 2019 showing resistance to the same antibiotics. None of the isolates in 2018 and 2019 was resistant to linezolid.

The antibiotic resistance patterns of the 145 coagulase positive staphylococci (mainly *S. aureus*) are summarized in Table 4b, with 82 isolates in 2018 and 63 isolates in 2019. Over these two years, no resistance to linezolid was detected, and only 1% of patients developed resistance to teicoplanin. Between 8% and 21% were resistant to ciprofloxacin, clindamycin, erythromycin, ofloxacin, and sulfamethoxazole/ trimethoprim. Approximately 70%, 46% and 41% were respectively resistant to cefoxitin, ceftriaxone and penicillin G. The resistant rates in 2019 to amoxicillin/clavulanic acid, cefoxitin, ceftriaxone, cefuroxime and vancomycin decreased by 16.7%, 47.6%, 36.4%, 42.2% and 65.7% respectively, compared to 2018. The resistant rate to penicillin G however increased by 123% in 2019, compared to 2018 (Table 5).

Table 5: Antibiotic resistance rates and trends among bacterial isolates in two Lebanese hospital (2018 and 2019)

Isolate	Bacterial species	Lowest and highest resistance rate of total isolates		Trends in resistance to antibiotics between 2018- 2019			
		≤5% of resistance	≥60% of resistance	Significant decrease of the resistance to	Decrease by:	Significant increase of the resistance to	Increase by:
GNB isolates	*E. coli*	Amikacin Fosfomycin Imipinem Tigecycline	Amoxicillin/clavulanic acid Ceftazidime Pefloxacin	Cefoxitin Ceftazidime Colistin Fosfomycin Pefloxacin Piperacillin/tazobactam	14.7 - 56.9%	None	NA
	K. pneumoniae	Amikacin Imipinem Tigecycline	Ceftazidime Colistin Pefloxacin	Cefoxitin Ceftazidime Colistin Fosfomycin Pefloxacin	25.4 - 83.2%	None	NA
	P. mirabilis	Amikacin Imipinem Piperacillin/tazobactam	Ceftazidime Colistin Nitrofurantion Pefloxacin Sulfamethoxazole/trimethoprim Tigecycline	Ceftazidime Colistin Fosfomycin Nitrofurantion Pefloxacin Tigecycline	18 - 65.1%	None	NA
	Enterobacter spp	Amikacin Imipinem Ertapenem Tigecycline	Amoxicillin/clavulanic acid Cefoxitin Ceftazidime Colistin Pefloxacin	Colistin Pefloxacin	23.3 - 26.6%	Piperacillin/tazobactam	250%
	P. aeruginosa	Colistin	None	Amikacin Imipinem	65.5 - 66.7%	None	NA
GPB isolates	*E. faecalis*	Linezolid	Erythromycin	None	NA	Ciprofloxacin Erythromycin Gentamycin Tigecycline	36.5-2569%
	CoPS	Linezolid Teicoplanin	Cefuroxime	Amoxicillin/clavulanate Cefoxitin Ceftriaxone Cefuroxime Vancomycin	16.7 - 65.7%	Penicillin G	123%

NA= Not applicable; GNB = Gram negative bacteria; GPB = Gram positive bacteria; CoPS = Coagulase Positive Staphylococci; *E. coli* = *Escherrichia coli*; *K. pneumoniae* = *Klebsiella pneumoniae*; *P. mirabilis* = Proteus mirabilis; *P. aeruginosa* = *Pseudomonas aeruginosa*; *E. faecalis* = *Enterococcus faecalis*

Discussion:

In this study, we aimed to compare our findings on antibiotic resistance pattern of *E. coli*, *K. pneumoniae*, *P. mirabilis*, *Enterobacter* sp, *P. aeruginosa*, *E. faecalis,* and *S. aureus* isolates from hospitalised patients in Lebanon in 2018 and 2019 with national and international data. The irrational use of antibiotics, easy access, low cost of many antibiotics and absence of an efficient national antimicrobial resistance surveillance system have all contributed to the increase of the resistance of many pathogenic bacterial species to different antibiotics. Our findings however showed a significant decrease (14.8 - 56.9%) of *E. coli* resistance in 2019 compared to 2018 against cefoxitin, ceftazidime, colistin, fosfomycin, pefloxacin, and piperacillin/tazobactam, while resistance rate was lowest (less than 3.5%) against imipinem, amikacin, and tigecycline. This pattern is similar to what was reported between 2011 and 2013 (18), but more than 60% of our isolates were resistant to amoxicillin/clavulanic acid and ceftazidime, which is significantly higher compared to 2011 and 2013 (which reported 39 and 29% respectively). In 2015/2016, resistance rate of *E. coli* compared to 2011/2013 in Lebanon increased to most of the tested antibiotics (20). This increase could have been caused by selective pressure of irrational use, promoted by easy access to these antibiotics, which are available over-the-counter in many Lebanese community. This highlights the urgent need for awareness and national educational campaigns to increase the knowledge and ameliorate the attitude and practice towards antibiotic use.

In Gabon, *E. coli* isolates recovered between 2009 and 2012 were susceptible to ceftriaxone, ciprofloxacin, and gentamicin (21). However, a similar study conducted in Ethiopia between 1990 and 2013 showed that most Gram-negative isolates were relatively resistant to most of the tested antibiotics (22). Between 2012 and 2016 in Nepal (23), 75% of *E. coli* isolates from children with urinary tract infection were resistant to cefotaxime, which is higher compared to the 37.2% reported in our study, but the resistance rates of *E. coli* to gentamicin and amikacin were similar. Compared to a study conducted in Zambia between 2016 and 2018 (24), our *E. coli* isolates demonstrated higher resistance to ceftazidime but lower resistance to ciprofloxacin. In Iran, *E. coli* isolates between 2015 and 2016 (25), demonstrated higher resistance to ceftriaxone, ciprofloxacin gentamicin, and imipinem but lower resistance to ceftazidime and amikacin compared to the findings in our study. Smilarly in Yucatan city, in Mexico, between 2016 and 2018 (26), higher resistance rates were seen in *E. coli* isolates compared to the rates reported in our current study.

The *K. pneumoniae* isolates in our study showed low resistance (less than 5%) to amikacin, fosfomycin, imipinem, and tigecycline and high resistance (more than 60%) to amoxicillin/clavulanic acid, ceftazidime and pefloxacin. When compared to the year 2018, the resistance rate decreased in 2019 by 25.4 - 83.2% to cefoxitin, ceftazidime, colistin, fosfomycin and pefloxacin. *K. pneumoniae* isolates in our study showed significantly less resistance to ciprofloxacin, ceftriaxone, and gentamicin compared to *K. pneumoniae* isolates reported between 2009 and 2012 in Gabon (21). In Ethiopia, the resistance rate of *K. pneumoniae* isolates between 1990 and 2013 was considerably lower against cefoxitin compared to the isolates in our current study, but higher resistance to ceftriaxone, amoxicillin/clavulanic acid, ciprofloxacin, gentamicin and trimethoprim/sulfamethoxazole (22). In Iran between the year 2015 and 2016, *K. pneumoniae* isolates compared to our study showed a lower resistance rate to all the antibiotics tested by Hasani et al., (25) except for ceftazidime. Compared to the findings of Uc-Cachon et al., (26) in Mexico between 2016 and 2018, our isolates showed a significantly lower resistant rates to cefepime, cefuroxime, gentamicin, imipenem, piperacillin/tazobactam and trimethoprim/sulfamethoxazole, but similar resistance rate to ciprofloxacin. According to the WHO and based on the tests performed on 30 isolates per nation, Prestinaci et al., (11) reported that 17.4% of *K. pneumoniae* isolates Eastern Mediterranean countries were resistant to carbapenems and third generation cephalosporins, while 36% of isolates from South East Asia and regions of America were resistant, and almost 60% for the European region.

Our study revealed that amikacin, imipinem, and piperacillin/tazobactam are the antibiotics to which *P. mirabilis* isolates were less resistant to compared to ceftazidime, colistin, nitrofurantion, pefloxacin, sulfamethoxazole/trimethoprim, and tigecycline to which these isolates were most resistant to. A

significant decrease (18 - 65.1%) in *P. mirabilis* resistance to ceftazidime, colistin, nitrofurantoin, pefloxacin, and tigecycline was reported in our study. This significant decrease could be due to the fact that most of these antibiotics are not accessible in pharmacies but are administrated only in hospitals. Our data showed significantly lower resistance rate to amoxicillin/clavulanic acid, ceftriaxone, ciprofloxacin, gentamicin, and sulfamethoxazole/trimethoprim compared to *P. mirabilis* isolates from the study in Ethiopia (22).

Regarding the resistance patterns of *Enterobacter* isolates in our study, less than 5% were resistant to amikacin, imipinem, ertapenem, and tigecycline, but more than 60% were resistant to amoxicillin/clavulanic acid, cefoxitin, ceftazidime, colistin and pefloxacin. The isolates in 2019 showed a significant decrease (23.3% - 26.6%) in resistance rates to colistin and pefloxacin, but significant increase (250%) to piperacillin/tazobactam. Clearly, *Enterobacter* isolates in the current study showed a lower resistance rate to ciprofloxacin, gentamicin and trimethoprim/sulfamethoxazole but higher resistance rate to ceftriaxone and amoxicillin/clavulanic acid, compared to the isolates in Ethiopian study (22), which could also be explained by the easy access and relative low cost of these two antibiotics in Lebanaon. In Romania, Golli et al., (27) in 2017 reported that *Enterobacter* isolates showed considerably high resistance rate to cefepime, ceftriaxone, ciprofloxacin, imipenem, but low resistance to ceftazidime.

Our data confirmed that less than 5% of *P. aeruginosa* isolates were resistant to colistin, and resistance to amikacin, cefepime, ceftazidime, ciprofloxacin, gentamicin, imipinem, levofloxacin, and piperacillin/tazobactam did not exceed 24% (varied between 11% to amikacin and 24% to levofloxacin). There was a decrease in 2019 in the resistance rate to amikacin and imipenem by 65.5% and 66.7% compared to 2018. The low incidence of infections caused by *P. aeroginosa* in the Lebanese community could support the significantly lower resistance of this pathogenic bacteria to the tested antibiotics. No significant difference in the pattern of antibiotic resistance of *P. aeruginosa* in our study was observed while comparing the results of the two studies done in Lebanon by Chamoun et al., (18) between 2011 and 2013, and by Moghnieh and colleagues (20) between 2015 and 2016. A five-year study conducted in Saudi Arabia between 2013 and 2017 (28) reported high resistance rate in *P. aeruginosa* isolates to β-lactams, fluoroquinolones and aminoglycosides. The study conducted in India between the 2013 and 2015 reported a similar resistance rate of *P. aeruginosa* to imipenem, comparable to our study. In the study conducted in Iran in 2015 and 2016 (25), *P. aeruginosa* isolates were more resistant to amikacin, ceftazidime, ciprofloxacin, gentamicin, and levofloxacin, compared to the isolates in our study. As reported by Reta et al., (22) in their retrospective study in Ethiopia between the year 1990 and 2013, *P. aeruginosa* isolates were significantly more resistant to ciprofloxacin than our isolates, but similar resistance rates were reported to gentamicin. The German study (29) conducted between 2013 and 2018 reported similar resistance rates of *P. aeruginosa* to imipenem, ciprofloxacin and piperacillin/tazobactaman as the isolates in our study, but the German *Pseudomonas* isolates were less resistant to ceftazidime. In Romania (27), *P. aeruginosa* isolates in 2017 were more resistant to piperacillin/tazobactaman, ciprofloxacin and ceftazidime compared to our isolates. In Mexico, the resistance rates of *P. aeruginosa* isolates reported between 2016 and 2018 (26) were higher to amikacin, ceftazidime, cefepime, ciprofloxacin, imipenem, gentamicin, levofloxacin, and piperacillin/tazobactam, compared to our isolates.

While analyzing the *E. faecalis* isolates, our results showed a significant increase (36.6% - 2569.8%) in the resistance rates of isolates in 2019 to ciprofloxacin, erythromycin, gentamicin, and tigecycline, compared to 2018**,** which could be explained by the overuse due to wide availability and low cost of macrolides and β-lactam antibiotics in Lebanon. In Ethiopia, the study by Reta et al., (22) showed lower rates of resistance of *E. faecalis* isolates compared to our isolates while similar resistance rate to erythromycin was reported. In Germany, as shown by Rothe et al., (29), *E. faecalis* isolates recovered between the year 2013 and 2018 showed no resistance (0%) to vancomycin and linezolid while 31.3% of our tested isolates were resistant to vancomycin though none to linezolid.

Our study also showed that coagulase positive staphylococci CoPS (mostly *S. aureus*) showed no resistance (0%) to linezolid and only 1% was resistant to teicoplanin. The highest resistance rates were to cefoxitin (70%) and cefuroxime (72%). This discrepancy in resistance rates could be correlated with overuse of the widely available β-lactam drugs for treatment of *S. aureus* infections whereas linezolid

and teicoplanin use is very limited and restricted to hospitalised patients. Cefoxitin is a surrogate phenotypic marker of methicillin resistance in *S. aureus*, which implies that the phenotypic MRSA rate in our study is 70%, a relatively high rate for this pathogen in Lebanon. Approximately 14%, 15% and 17% of the CoPS isolates were resistant to ciprofloxacin, ofloxacin and vancomycin respectively, 20% to erythromycin, 21% to clindamycin, 41% to penicillin G, and 46% to ceftriaxone. Between the year 2011 to 2019, the resistance of *S. aureus* isolates to clindamycin, erythromycin and trimethoprim/sulfamethoxazole was relatively stable whereas the rate increased for vancomycin (18,19,20). However, it should be noted that by the CLSI guideline, vancomycin disc diffusion is no longer used to assess phenotypic resistance of clinical *S. aureus* isolates to vancomycin, haven been replaced with vancomycin MIC determination by broth dilution or E-test methods. In Ethiopia, between 1990 and 2013, Reta et al., (22) reported that resistance rate of *S. aureus* isolates was lower for cefoxitin, but higher for amoxicillin/clavulanic acid, erythromycin, trimethoprim/sulfamethoxazole, ceftriaxone and ciprofloxacin. In Iran, between 2015 and 2016 (25), *S. aureus* isolates showed lower resistance to ceftriaxone compared to the isolates in our study but similar pattern of resistance to ciprofloxacin and amikacin. In the German study by Rothe et al., (29) between 2013 and 2018, 3.3% of *S. aureus* isolates were resistant to cefuroxime and ceftriaxone compared to 72% and 46% in our isolates, and fewer isolates were also resistant to ciprofloxacin compared to our isolates. All *S. aureus* isolates in the German study were susceptible to linezolid as in our study. However, the resistance rate to vancomycin in the German study was 0% while the rate was 17% in our study. In comparing the resistance pattern of *S. aureus* isolates reported by the Greek study conducted between 2010 and 2015 (30) to our current data, it was clear that our isolates were more resistant to cefoxitin, trimethoprim/sulfamethoxazole and vancomycin, while they were less resistant to erythromycin and penicillin G, but similar resistance pattern to teicoplanin and clindamycin.

It has been reported that more than 2.8 million infections and 35,000 deaths caused by antibiotic resistant bacteria and fungi occurs yearly in the United States (31). If no action is taken, drug-resistant infections could cause 10 million deaths each year by 2050 (32). Such infections have serious burden on the economy. Indeed, the cost of AMR across the globe is extremely high though differs between countries (33). Resistance rates are generally higher in low-income countries compared to middle and upper income countries (34).

In Lebanon, the use of antibacterial guidelines should be among the priorities in line with the practice in advanced countries (35). Effective research directed at not only human health sector, but also across animal health and environment sectors, should be prioritised since they are inter-connected (36). This is similar to the action plan adopted by the United States since the year 2015 (37). Global partnerships between industry, researchers and academia are needed to develop new antibiotics such as the European program, New Drugs 4 Bad Bugs (ND4BB) (38), and the Combating Antibiotic-Resistant Bacteria (CARB-X) program, which since its establishment in 2016, is investing in 75 projects around the world and accelerating the global antibacterial innovations (39). A reason to hope of a better future is the fact that approximately 41 new antibiotics (as of March 2017) are in different phases of clinical development aimed at antimicrobial therapy of serious bacterial infections (40).

The present study has some limitations. First, only two hospitals were included in the study, which may not represent the total picture of bacterial AMR in Lebanon. Another limitation is the lack of descriptive details related to the source of the isolates, but we are aiming to assess the multi-drug resistance details among the bacterial isolates in the future. Also, being a retrospective study, we have to rely on the conventional phenotypic methods used for routine bacteria identification and susceptibility tests by the laboratories, which are are not as accurate as genotypic methods. However, our study could motivate the implementation of a national surveillance for antimicrobial resistance in Lebanon.

Conclusion:

Overuse of antibiotics, which is possible because of easy access to these drugs, is among the major factors underlying emergence and increasing antibiotic resistance in Lebanon. We agree with the 2020 recommendation of Sakr et al., (19) on the need to promote awareness among students, especially in the non-health related majors, and to enact governmental policy that will limit access to antibiotics.

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Conflict of interest:

Authors declared no conflict of interest

Contributions of authors:

AM and TK contributed equally to the study

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Original Article **Open Access**

Bioinformatic analysis of multi-drug resistant class 1 integron-coded protein of *Citrobacter freundii*

Popoola, O. D., and *Thomas, B. T.

Department of Microbiology, Olabisi Onabanjo University, Ago Iwoye, Ogun State, Nigeria
*Correspondence to: benthoa2013@gmail.com; +2348064011412; ORCID: http://orcid.org/0000-0003-0675-5749

Abstract:

Background: The understanding of the secondary structure of the class 1 integron coded protein is necessary to decipher potential drug target and also to infer evolutionary ancestry at the proteomic level. This study was therefore aimed at determining the secondary structure of class 1 integron-coded protein and also to provide information on their evolutionary ancestry.
Methodology: Five different sequences of *Citrobacter freundii* with the following accession numbers; KP902625.1, KP902624.1, KP902623.1, KP901093.1 and KP902609.1 were obtained using nucleotide BLAST (http://blast. ncbi.nlm.nih.gov/Blast.cgi) and subjected to evolutionary analysis, pairwise distance calculation, secondary structure and neutrality test using MEGA explorer, Kimura 2 parameter, SOPMA tool and Tajima's test respectively.
Results: Results of the NCBI queries revealed significant identity with class 1 integron of the studied *Citrobacter freundii*. The nucleotide sequence alignment depicted several conserved regions with varying degree of transitions, transversions, insertions, and deletions while the amino acid sequences of the nucleotides showed 42 conserved sites among all the sequences. The secondary structure of the class 1 integron coded protein depicted significant representation of the random coil (43.74±3.24), alpha helix (25.69±6.29) and the extended strands (22.42±2.41) than the beta turns (8.15±1.12). The Tajima's Neutrality test of five nucleotide sequences of *Citrobacter freundii* analyzed by considering the first, second and third codons as well as the non-coding regions revealed a total of 127 positions in the final datasets while the Tajima's Neutrality test was estimated to be -0.1038.
Conclusion: The study confirmed common evolutionary ancestor for the class 1 integron coded protein found in *Citrobacter freundii.* Our study also documents the higher representation of random coil, alpha helix and extended strands than the beta turns. The negative value of the Tajima's neutrality test suggests higher levels of both low and high frequency polymorphisms thus indicating a decrease in the class 1 integron population size and balancing selection

Keywords: Evolutionary, Protein structure, Class 1 integrons, *Citrobacter freundii*

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Analyse bioinformatique de la protéine de *Citrobacter freundii* codée en intégron de classe 1 multi-résistante aux médicaments

Popoola, O. D., et *Thomas, B. T.

Département de microbiologie, Université Olabisi Onabanjo, Ago Iwoye, État d'Ogun, Nigéria
*Correspondance à: benthoa2013@gmail.com; +2348064011412; ORCID: http://orcid.org/0000-0003-0675-5749

Abstrait:

Contexte: La compréhension de la structure secondaire de la protéine codée par intégron de classe 1 est nécessaire pour déchiffrer la cible potentielle du médicament et également pour déduire une ascendance évolutive au niveau protéomique. Cette étude visait donc à déterminer la structure secondaire de la protéine codée en intégron de classe 1 et aussi pour fournir des informations sur leur ascendance évolutionnaire.
Méthodologie: Cinq séquences différentes de *Citrobacter freundii* avec les numéros d'accession suivants; KP902625.1, KP902624.1, KP902623.1, KP901093.1 et KP902609.1 ont été obtenus à l'aide du nucléotide BLAST (http://blast. ncbi.nlm.nih.gov/Blast.cgi) et soumis à une analyse évolutive, en paire, calcul de la distance, test de la structure secondaire et de la neutralité avec MEGA explorer, le paramètre Kimura 2, l'outil SOPMA et le test de Tajima, respectivement
Résultats: Les résultats des requêtes NCBI ont révélé une identité significative avec l'intégon de classe 1 de *Citrobacter freundii* étudié. L'alignement de la séquence nucléotidique décrit plusieurs régions conservées avec un degré variable de transitions, transversions, insertions et délétions, tandis que les séquences d'acides aminés des nucléotides présentent 42 sites conservés parmi toutes les séquences. La structure secondaire de la protéine codée en intégron de classe 1 représentait une représentation significative de la bobine aléatoire (43,74±3,24), de l'hélice alpha (25,69±6,29) et des brins étendus (22,42±2,41) par rapport aux tours bêta (8,15±1,12). Le test de neutralité de Tajima de cinq séquences de nucléotides de *Citrobacter freundii* analysé en considérant les premier, deuxième et troisième codons ainsi que les régions non codantes a révélé un total de 127 positions dans les jeux de données finaux, tandis que le test de neutralité de Tajima était estimé à -0,1038.
Conclusion: L'étude a confirmé l'ancêtre commun de l'évolution pour la protéine codée en intégron de classe 1 trouvée dans *Citrobacter freundii*. Notre étude documente également la représentation plus élevée de la bobine aléatoire, de l'hélice alpha et des brins étendus que les tours bêta. La valeur négative du test de neutralité de Tajima suggère des taux plus élevés de polymorphismes à basse et à haute fréquence, indiquant ainsi une diminution de la taille de la population d'intégrons de classe 1 et de la sélection d'équilibrage.

Mots-clés: évolutionnaire, structure protéique, intégrons de classe 1, *Citrobacter freundii*

Introduction:

Integrons are genetic elements that contain the component of a site-specific recombination system which recognizes and captures mobile gene cassettes (1). These genetic elements have genes for an integrase (*intI*), a recombination site (*attI*) and a promoter (P_C) that allows the expression of the gene cassettes incorporated in the variable region (2) and also reshuffling of the gene cassettes and so they are called genetic construction kit for bacteria (3). These integrons usually harbor antibiotic resistant genes that play vital roles in the emergence of new multidrug resistant bacteria (4). This is because they equip bacteria with the ability to scavenge foreign genes, especially antibiotic resistance genes.

The subjection of antibiotic resistant gene cassettes to episodic selection leads to removal of unnecessary genes through excision events catalyzed by integrase which subsequently results in reduction of genetic burden (5). Integrons by themselves are not mobile (6,7) but they may be part of mobile elements such as transposons and plasmids (8,9) which further enhance the spread of antibiotic resistant genes. Antimicrobial resistance on its own, is a large and growing problem in infection control due to increase in the proportion of death as well as the cost of effective antimicrobial agents (10,11).

Citrobacter freundii, which is the microorganism of interest in this study is an important food-borne and hospital-acquired pathogen, which causes diarrhea, urinary tract infection, peritonitis, bacteremia, brain abscess and meningitis (12-16). This organism is an opportunistic pathogen in aquaculture and has been reported to be associated with a wide spectrum of infectious diseases in aquaculture animals including gastroenteritis of one-year rainbow trout *Oncorhynchus mykiss* (17), red-leg syndrome of *Rana catesbeiana* (18), septicaemia of *Garra rufa obtusa* and *Ziphius carvirostris* (18, 19), systemic infection with no typical syndrome of *Carcharhinus melanopterus* (20), hepatopancreas necrosis and rotted gill of *Cherax quadricarinatus, Portunus trituberculatus* and *Procambarus clarkii* (21,22).

In Gram-negative bacteria, different classes of integrons have been identified in several clinical isolates, where they contribute significantly to the prevalence and dissemination of antibiotic resistant genes (23,24). This study therefore examined evolutionary relationship and the secondary structure of class 1 integron coded protein of *Citrobacter freundii*.

Materials and method:

The sequences KP902625.1, KP902624.1, KP902623.1, KP901093.1 and KP902609.1 were obtained using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (25) and subjected

to evolutionary analysis using the MEGA explorer (26). Pair-wise distances were calculated using Kimura 2 parameter. In MEGA explorer, translate option was used for converting the gene sequence into amino acid sequence. Sequence similarity search with BLASTP and best homologous protein was found using the multiple sequence alignment. The secondary structures of the protein sequences were predicted using SOPMA tool (27).

The analysis of the Tajima's test of Neutrality involved five nucleotide sequences. Codon positions included were the first, second, third and the non coding regions. All positions containing gaps and missing data were eliminated prior to the estimation of the Tajima's test.

Results:

The phylogenetic analysis of the class one integron coded protein of *Citrobacter freundii* retrieved from NCBI GenBank revealed significant evolutionary relationships. As shown in Fig 1, all the retrieved class 1 nucleotide sequences shared several conserved regions. In terms of relatedness of their class 1 integrons, organisms in cluster 1 (KP902625.1, KP902624.1, KP902623.1) are closer than those in cluster 2 (KP901093.1 and KP902609.1). Although, organisms in cluster 2 shared higher level of homology in their nucleotide sequence, the secondary structure of all the class 1 integron coded protein analyzed depicted significant representation of the random coil (43.74±3.24), alpha helix (25.69±6.29) and the extended strands (22.42±2.41) than the beta turns (8.15±1.12).

Fig 2 represents the chromatograph of one of the *Citrobacter freundii* coded protein. The data in table 1 connotes the results from the Tajima's Neutrality test of five nucleotide sequences of *Citrobacter freundii* analyzed by considering the first, second and third codons and the non-coding regions. All positions containing gaps and missing data were eliminated. There were total of 127 positions in the final datasets while the Tajima's Neutrality test was calculated to be -0.1038.

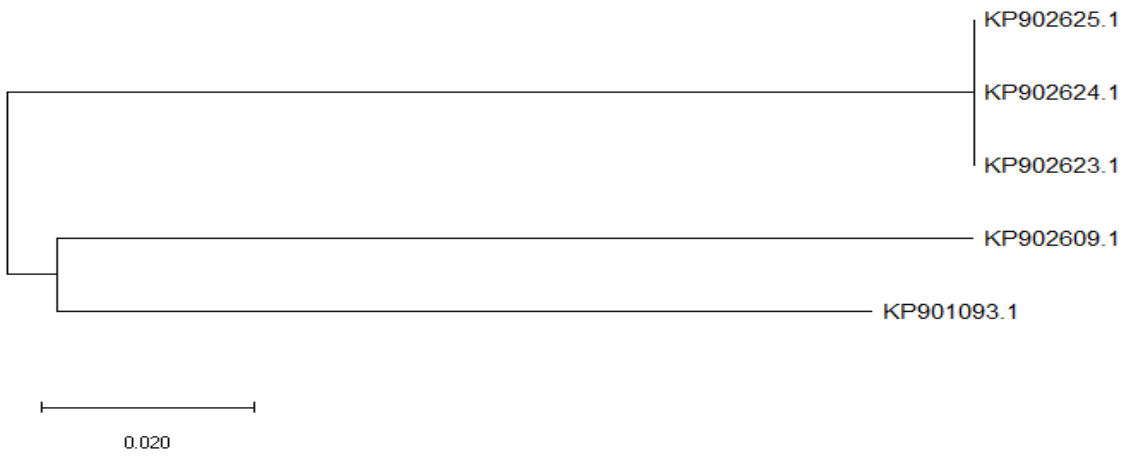
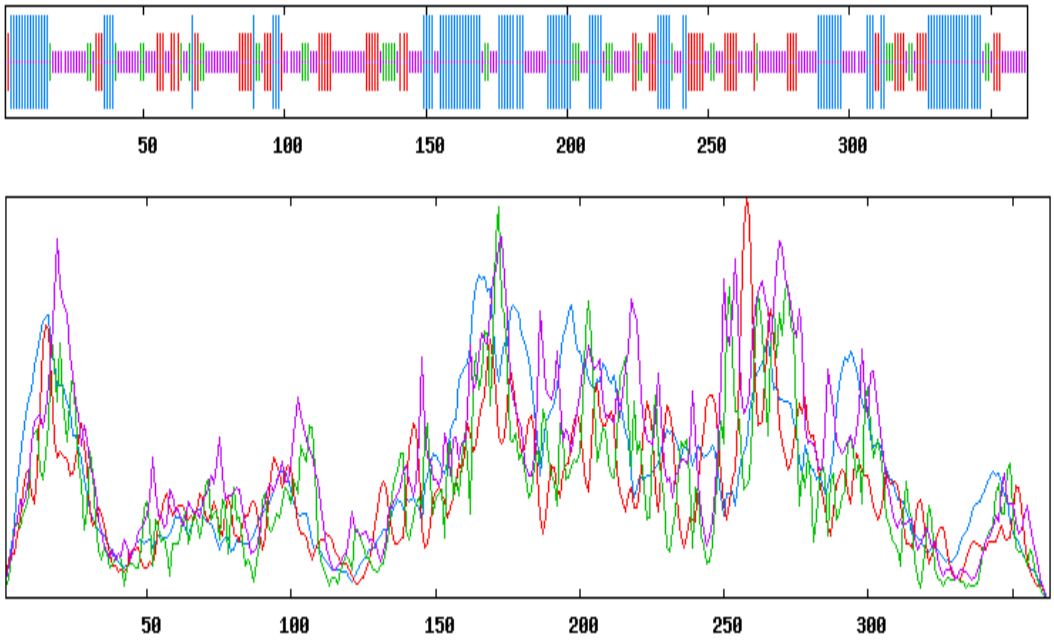


Fig 1: Phylogenetic relationship of class 1 integron-coded protein of *Citrobacter freundii*



Parameters:

Window width: 17
Similarity threshold: 8
Number of states: 4

Fig. 2: Chromatographical representation of the secondary structure of JN108891

Table 1: Results of the Tajima's neutrality test

m	s	ps	Θ	π	D
5	30	0.236	0.113386	0.11	-0.1038

m = number of sequences, n= total number of sites, S= number of segregating sites, Ps= S/n, Θ = Ps/a1, π = nucleotide diversity, D = Tajima's test of statistic

Discussion:

The use of protein structure and nucleotide sequences to understand evolution of cell and the function of genes cannot be overemphasized. According to Zuckerkandl and Pauling (28), evolutionary relationships between organisms can be studied by comparing their DNA sequences. In this study, a very high level of similarities was observed in the DNA sequences of the studied class 1 integron coded protein. This is an indication that class I integron may have common ancestors, with most of them probably being transferred as an insert in plasmid and/or transposons (6,7).

The fact that some of the *Citrobacter freundii* connoted themselves together as a simplicifolious clade is an indication that the distribution of their nucleotide sequences is significantly related than those on other clades. However, such observation may be connected to the fact that such acquired class I integron have been rearranged due to different levels of mutation resulting from substitution in form of transition, transversion, deletion and even insertions.

The BLAST analysis revealed homology with the available class 1 integrons of *Citrobacter freundii* present in the GenBank. The secondary structure analysis revealed comparatively higher random coil, alpha helix and extended strands than the beta turns. This knowledge is important as it helps in designing low-molecular-weight synthetic agents that reproduce their essential features (29) by using synthetic agents to mimic the helices. This in particular, has immense interest in drug dis-

covery because of the central role the α-helical regions play in many biological processes.

Conclusion:

This study confirmed common evolutionary ancestor for the class 1 integron coded protein found in *Citrobacter freundii.* Our study also documents the higher representation of random coil, alpha helix and extended strands than the beta turns. Again, information about the amino acid residues in the class 1 integron coded protein is needed to study the binding of target drugs in these regions. This is because changes in these residues result in the modification of drug binding sites.

Authors' contributions:

ODP conceptualized the study and drafted the first manuscript. BTT carried out the bioinformatic analysis as well as data interpretation. All the authors approved the final version of the manuscript.

Conflict of interest:

Authors declared no conflict of interest.

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Original Article **Open Access**

Antibacterial activity and time kill kinetics of Amlodipine, Thioridazine and Promethazine against pathogenic clinical bacterial isolates

*[1]Akinjogunla, O. J., [2]Umo, A. N., [3]Alozie, M. F., [2]Oshosanya, G. O., and [1]Saturday, G. I.

[1]Department of Microbiology, University of Uyo, P.M.B. 1017, Uyo, Akwa Ibom State, Nigeria
[2]Department of Medical Microbiology and Parasitology, Faculty of Clinical Sciences, University of Uyo, Uyo, Akwa Ibom State, Nigeria
[3]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, University of Uyo, Uyo, Akwa Ibom State, Nigeria
*Correspondence to: papajyde2000@yahoo.com

Abstract:

Background: The emergence of multi-drug resistant bacterial strains worldwide has necessitated the scientific search for novel, potent, and affordable antimicrobial agents including medicinal plants and non-antibiotic drugs for therapy of infectious diseases. The objective of this study is to assess *in vitro* antibacterial activities and time kill kinetics of some non-antibiotic drugs against pathogenic clinical bacterial isolates.
Methodology: *In vitro* antibacterial activities including minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time kill kinetics of Amlodipine (AML), Thioridazine (THI) and Promethazine (PRO) against *Staphylococcus aureus*, coagulase negative staphylococci (CoNS), *Streptococcus* spp, *Escherichia coli*, *Enterobacter* spp, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* clinical isolates were determined using disc diffusion, broth microdilution and plate count techniques.
Results: The mean growth inhibition zones by the disc diffusion assay of AML, THI and PRO against the isolates were ≤15.1±1.0 mm with MIC and MBC values ranging from 12.5 to 50µg/ml and 25 to 100µg/ml respectively. The time-kill assay revealed bactericidal effect of AML, THI and PRO on Gram positive bacteria evidenced by mean log reductions in viable bacterial cell counts ranging from 0.13 Log_{10} to 2.41 Log_{10} CFU/ml for *S. aureus*, 0.88 Log_{10} to 2.08 Log_{10} CFU/ml for *Streptococcus* spp, and 0.26 Log_{10} to 2.34 Log_{10} CFU/ml for CoNS after ≤30hrs post inoculation at 1xMIC. The range of log reduction in viable cell counts of Gram-negative bacteria exposed to AML, THI and PRO were *E. coli* (0.11 to 3.23 Log_{10} CFU/ml), *P. aeruginosa* (0.52 to 2.56 Log_{10} CFU/ml), *K. pneumoniae* (0.85 to 3.0 Log_{10} CFU/ml) and *Enterobacter* spp (0.38 to 2.08 Log_{10} CFU/ml) after ≤30 hrs post inoculation at 1x MIC.
Conclusion: These findings demonstrate *in vitro* antibacterial efficacies and time kill kinetics of AML, THI and PRO against pathogenic clinical bacterial isolates, which indicate that these non-antibiotic drugs may be useful therapeutic alternatives in the bid to reduce the burden of infectious diseases associated with antibiotic resistant pathogens.

Keywords: Amlodipine, Thioridazine, Promethazine, Time-Kill, Kinetics, MIC, MBC, bacteria

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Activité antibactérienne et cinétique de destruction du temps de l'amlodipine, de la thioridazine et de la prométhazine contre les isolats bactériens cliniques pathogènes

*[1]Akinjogunla, O. J., [2]Umo, A. N., [3]Alozie, M. F., [2]Oshosanya, G. O., et [1]Saturday, G. I.

[1]Département de microbiologie, Université d'Uyo, P.M.B. 1017, Uyo, Akwa Ibom State, Nigéria
[2]Département de microbiologie médicale et de parasitologie, Faculté des sciences cliniques, Université d'Uyo, Uyo, État d'Akwa Ibom, Nigéria

[3]Département de microbiologie et biotechnologie pharmaceutiques, Faculté de pharmacie,
Université d'Uyo, Uyo, État d'Akwa Ibom, Nigéria
*Correspondance à: papajyde2000@yahoo.com

Abstrait:

Contexte: L'émergence de souches bactériennes multirésistantes dans le monde a rendu nécessaire la recherche scientifique d'agents antimicrobiens nouveaux, puissants et abordables, notamment des plantes médicinales et des médicaments non antibiotiques pour le traitement des maladies infectieuses. L'objectif de cette étude est d'évaluer les activités antibactériennes in vitro et la cinétique de destruction temporelle de certains médicaments non antibiotiques contre les isolats bactériens cliniques pathogènes.
Méthodologie: activités antibactériennes *in vitro*, y compris la concentration minimale inhibitrice (CMI), la concentration bactéricide minimale (MBC) et la cinétique de destruction du temps de l'amlodipine (AML), de la thioridazine (THI) et de la prométhazine (PRO) contre *Staphylococcus aureus*, les staphylocoques à coagulase négative (CoNS), *Streptococcus* spp, *Escherichia coli*, *Enterobacter* spp, *Klebsiella pneumoniae* et *Pseudomonas aeruginosa* ont été déterminés en utilisant des techniques de diffusion sur disque, de microdilution en bouillon et de numération sur plaque.
Résultats: Les zones moyennes d'inhibition de la croissance par le test de diffusion de disque d'AML, THI et PRO contre les isolats étaient ≤15,1±1,0mm avec des valeurs MIC et MBC allant de 12,5 à 50µg/ml et de 25 à 100µg/ml respectivement. Le dosage temporel a révélé un effet bactéricide de la LMA, du THI et du PRO sur les bactéries Gram positives, mis en évidence par des réductions logarithmiques moyennes du nombre de cellules bactériennes viables allant de 0,13 Log10 à 2,41 Log10 CFU/ml pour *S. aureus*, 0,88 Log10 à 2,08 Log10 CFU/ml pour *Streptococcus* spp et 0,26 Log10 à 2,34 Log10 CFU/ml pour CoNS après ≤ 30 heures après l'inoculation à 1 x MIC. La plage de réduction logarithmique du nombre de cellules viables de bactéries à Gram négatif exposées à la LMA, au THI et au PRO était *E. coli* (0,11 à 3,23 Log10 CFU/ml), *P. aeruginosa* (0,52 à 2,56 Log10 CFU/ml), *K. pneumoniae* (0,85 à 3,0 Log10 CFU/ml) et *Enterobacter* spp (0,38 à 2,08 Log10 CFU/ml) après ≤ 30 heures après l'inoculation à 1 x MIC.
Conclusion: Ces résultats démontrent une efficacité antibactérienne *in vitro* et une cinétique de destruction du temps des LMA, THI et PRO contre les isolats bactériens cliniques pathogènes, ce qui indique que ces médicaments non antibiotiques peuvent être des alternatives thérapeutiques utiles dans le but de réduire le fardeau des maladies infectieuses associées aux antibiotiques pathogènes résistants.

Mots-clés: Amlodipine, Thioridazine, Prométhazine, Time-Kill, Cinétique, MIC, MBC, bactéries

Introduction:

Although the pharmaceutical industries are still in the business of producing new antibiotics for treatment of infectious diseases, the numbers of infections caused by resistant microorganisms continue to increase owing to combinations of microbial characteristics and selective pressure created by antimicrobial use (1,2). The mechanisms of microbial resistance to antimicrobial agents include the production of structure-inactivating enzymes, alteration of cell wall, cell membrane and enzyme (e.g. DNA gyrase) target sites and ribosomal modification (3). The emergence of multi-drug resistant (MDR) microbial strains has necessitated the scientific search for potent, novel and affordable antimicrobial agents including plants (4,5) and non-antibiotic drugs for the treatment of infectious diseases.

Scores of non-antibiotic drugs, pharmaceutical preparations belonging to different pharmacological classes, currently used in the treatment of non-infectious diseases have been reported to exhibit both *in vitro* and *in vivo* antimicrobial activities (6). The non-antibiotic drugs, which inhibit microbial metabolism (7), with either biocidal or biostatic activity include anti-psychotics (thioridazine, clozapine), anti-hypertensives (nifedipine and amlodipine) (8), non-steroidal anti-inflammatory drugs (ibuprofen, diclofenac sodium) (9), pump inhibitors (esomeprazole, omeprazole), anti-histamines (promethazine, trimeprazine) (10), and anti-depressants (sertraline and paroxetine) (11).

Promethazine is usually administered to attenuate inflammatory process in disease conditions such as allergic rhinitis, conjunctivitis, and urticaria (12,13). The use of anti-histamines, especially the first-generation phenothiazine H1-inhibitors such as promethazine for patients with microbial infection may become inevitable and this has prompted the evaluation of their antimicrobial properties.

The antibacterial activity of amlodipine, a cardiovascular drug, was reported by Kumar et al., (8) on several clinical bacterial isolates including *Staphylococcus aureus*, *Escherichia coli*, *Vibrio* spp, *Salmonella* spp, *Bacillus* spp and *Pseudomonas aeruginosa.* Similarly, in addition to the antipsychotic properties of thioridazine, significant antimicrobial activities against intracellular microorganisms have been reported (14). The objective of this study is to

determine the antibacterial activities and time kill kinetics of amlodipine, thioridazine and promethazine against selected pathogenic clinical bacterial isolates.

Materials and method:

Identification of bacterial isolates

Fourteen (14) clinical bacterial isolates comprising *Staphylococcus aureus* (n=2), coagulase negative staphylococci (n=2), *Streptococcus* spp (n=2), *Escherichia coli* (n=2), *Klebsiella pneumoniae* (n=2), *Enterobacter* spp (n=2), and *Pseudomonas aeruginosa* (n=2) were obtained from the Department of Microbiology, University of Uyo, Akwa Ibom State, Nigeria. These bacteria were isolated from clinical specimens and identified using conventional morphological and biochemical tests (15).

Source of the non-antibiotic drugs (Amlodipine, Thioridazine and Promethazine)

Amlodipine (AML, MAF India Pharmaceutical, India), Thioridazine (THI, Indian Generic Company, India) and Promethazine (PRO, CSC Pharmaceuticals International, India) were purchased in tablet forms from registered Pharmacy stores in Uyo. Stock solution of each drug was prepared by dissolving 1g of each in 100ml of sterile distilled water to give a concentration of 10mg/ml. The stock solution was further diluted to produce 5mg/ml and 2.5 mg/ml concentrations for each of AML, THI and PRO.

Determination of antibacterial activity of the non-antibiotic drugs by disc diffusion assay

The antibacterial activities of AML, THI and PRO on the clinical bacterial isolates were determined by the disc diffusion method (16). Paper discs of AML, THI and PRO were prepared by punching sterile Whatman filter paper (No.1) with a 6 mm disc puncher and sterilizing them at 160°C for one hour. Each disc was then impregnated with 10µL of 2.5mg/ml and 5.0mg/ml concentrations of AML, THI and PRO to give disc contents of 25µg and 50µg respectively.

Mueller-Hinton agar (MHA) plates were aseptically prepared and 0.1ml of each bacterial inoculum, prepared directly from an overnight nutrient agar plate and adjusted to 0.5 McFarland standards, was inoculated using sterile pipette onto each of the MHA plate. The impregnated discs were carefully placed on the MHA plates and incubated aerobically at 37^{o}C for 24 hours followed by measurement of the diameters of zone of inhibition (in millimetres) using a calibrated ruler. The disc diffusion assays were performed in triplicates and the mean diameters of zones of inhibition were recorded.

Determination of minimum inhibitory (MIC) and minimum bactericidal concentration (MBC)

The MICs of AML, THI and PRO against the bacterial isolates were determined using macrobroth dilution technique in test tubes (5). One ml of the stock solution (10mg/ml) of AML, THI and PRO was serially diluted in test tubes to obtain concentrations of 5, 2.5, 1.25 and 0.625mg/ml for each of the non-antibiotic solution. To 0.1ml (100µL) of each of these concentrations was added 9.9ml nutrient broth (1 in 100 dilution) to give the final concentrations of AML, THI and PRO of 100, 50, 25, 12.5 and 6.25µg/ml for the MIC testing. A loopful of prepared inoculum of each bacterium was added to each of the non-antibiotic solution. A tube containing only nutrient broth was inoculated with bacterial isolate to serve as control. All culture tubes were incubated aerobically at 37^{o}C for 24 hours, after which the tubes were examined for microbial growth by observing for turbidity. The MIC was read as the least concentration of the non-antibiotic solution that visibly inhibited the growth of the test bacterial isolate after 24 hours incubation.

For the MBC, an aliquot of 1ml from each of the MIC broth tubes that showed no visible growth was streak-inoculated onto sterile nutrient agar plates using a sterile pipette. The inoculated plates were inverted and incubated at 37°C aerobically for 24 hours. The MBC values of AML, THI and PRO were considered as the concentration of each non-antibiotic solution that resulted in killing of the bacterial isolates, which showed as no visible colonies on the agar plates.

Time-kill analysis

The time-kill analysis was carried out using macrobroth dilution and pour plate techniques. An overnight nutrient broth culture of each bacterial isolate in test tube was adjusted to 0.5 McFarland turbidity standard to obtain a starting inoculum of between 10^5 and 10^6 CFU/ml (confirmed by quantitative plate counts). The tubes were incubated at 37^{o}C with shaken at 150 rpm for 90 mins to ensure that microbial growths were in the logarithmic (exponential) phase. One (1) millilitre of this exponentially growing bacterial cultures was added to 9ml of MHB containing MIC concentration (1ml) of AML, THI and PRO. Bacterial growth was quantified at time '0' hour and at

6, 12, 18, 24, and 30 hours of incubation by aseptically taking 1ml of the aliquot, diluting serially (10-fold dilutions) in sterile normal saline and plating out 1ml of the final dilution onto nutrient agar plates. The plates were incubated aerobically for 24 hours at 37°C after which the colonies on each plate were enumerated and viable cells expressed as CFU/ml. Inoculated medium without AML, THI and PRO was also set up and plated on nutrient agar as control. All experiments were performed in triplicates.

The percentage and logarithm reductions of the bacterial cells exposed to AML, THI and PRO were calculated for each of the time intervals. The Log_{10} CFU/ml of survived bacterial cells against exposure time (in hours) were plotted on a semi-logarithm graph for each bacterial pathogen to obtain the time–kill curve. Activity of the non-antibiotic drugs was considered bactericidal at the lowest concentration that reduced the initial inoculum by $>3Log_{10}$ CFU/ml (99.9% killing). The percentage and logarithm reductions of the bacterial cells exposed to AML, THI and PRO were respectively calculated as; percentage reduction = (initial counts – counts at 'x' interval)/ initial counts (multiply by 100), while the logarithm reduction = Log_{10} (initial counts) – Log_{10} (counts at 'x' interval).

Statistical analysis

All experiments were performed in triplicates and data analysis was done with the Statistical Package for the Social Sciences (SPSS) version 20.0. Data were presented as mean ± SD and comparison between mean values was done using the Duncan multiple range test, with significance level at $p<0.05$.

Results:

The mean growth inhibitory zone of AML against Gram-positive bacteria (GPB) ranged from 10.1±0.2mm (25µg disk) to 13.6 ±0.5mm (50µg disk), and against Gram-negative bacteria (GNB), from 10.8±0.2mm (25µg disk) to 14.5±0.5mm (50µg disk). The mean growth inhibitory zone of THI against GPB ranged from 8.8±0.1mm (25µg disk) to 14.1 ±0.5mm (50µg disk) and against GNB, from 10.2±0.2mm (25µg disk) to 14.7±1.0mm (50 µg disk). For PRO, the mean growth inhibitory zone ranged from 9.3±0.1mm (25µg disk) to 14.5±0.3mm (50µg disk) against GPB, and 8.3 ±0.1 mm (25 µg disk) to 15.1±1.0mm (50µg) against GNB. The mean growth inhibitory zone of the levofloxacin control disk (30µg) was

Table 1: Antibacterial activities of non-antibiotic drugs on bacterial pathogens with disk diffusion method

Bacterial Isolates	Isolates Code	Zone of Inhibition in mm (mean±SD)						Control (Levofloxacin) (30µg disk)
		Amlodipine		Thioridazine		Promethazine		
		2.5mg/ml (25µg disk)	5mg/ml (50µg disk)	2.5mg/ml (25µg disk)	5mg/ml (50µg disk)	2.5mg/ml (25µg disk)	5mg/ml (50µg disk)	
Staphylococcus aureus	SA01	11.2±0.1^a	13.6±0.5^b	11.6±0.1^a	14.1±0.5^b	12.3±0.2^b	14.5±0.3^b	13.6±0.3^a
Staphylococcus aureus	SA02	NZ	9.8±0.2^a	NZ	9.3±0.1^a	NZ	NZ	NZ
CoNS	CS02	NZ	10.2±0.1^a	9.0±0.1^a	13.6±0.4^b	9.3±0.1^a	13.0±0.2^b	14.5±0.5^a
CoNS	CS01	NZ	12.4±0.3^b	11.2±0.3^a	14.0±0.2^b	NZ	11.6±0.2^a	14.0±0.1^a
Streptococcus spp	SS01	10.1±0.2^a	13.0±0.5^b	10.0±0.1^a	12.7±0.2^b	10.4±0.2^a	12.1±0.5^b	15.6±0.3^b
Streptococcus spp	SS02	NZ	9.1±0.1^a	8.8±0.1^a	10.4±0.1^a	9.6±0.1^a	11.2±0.1^a	14.3±0.2^a
Escherichia coli	EC01	12.9±0.3^b	14.5±0.5^c	10.5±0.2^b	13.1±0.3^b	11.3±0.3^a	13.9±0.5^b	15.1±0.5^b
Escherichia coli	EC02	12.2±0.1^b	14.0±0.3^b	11.9 ±0.1^a	13.8±0.3^b	11.5±0.2^a	13.0±0.5^b	NZ
Klebsiella pneumoniae	KP01	NZ	10.9±0.5^a	10.2±0.2^a	11.5±0.1^a	NZ	12.4±0.2^b	NZ
Klebsiella pneumoniae	KP02	11.5±0.5^a	13.7±1.0^b	12.1±0.5^b	14.7±1.0^c	11.0±0.5^a	12.9±0.5^b	16.0±1.0^b
Enterobacter spp	ES02	12.0±0.2^b	14.4±1.0^b	12.6±0.5^b	14.7±0.5^c	12.6±0.2^b	15.1±1.0^c	16.5±0.5^b
Enterobacter spp	ES01	10.8±0.2^a	12.9±0.2^b	10.8±0.1^a	13.3±0.2^b	NZ	11.3±0.2^a	NZ
Pseudomonas aeruginosa	PA01	12.4±0.5^b	13.6±0.5^b	11.6±0.2^a	12.9±0.3^b	12.1±0.2^b	13.7±0.5^b	14.8±0.5^a
Pseudomonas aeruginosa	PA02	NZ	8.6±0.1^a	NZ	9.5±0.1^a	8.3±0.1^a	10.9±0.2^a	12.0±0.3^a

CONS = Coagulase negative staphylococci; SD = Standard deviation; NZ = No inhibitory zone. Each value represents the mean plus standard deviation of three replicates. Mean within the column followed by different superscript letters are significant by Duncan's multiple range test ($p<0.05$)

Table 2: Minimum inhibitory and minimum bactericidal concentrations of non-antibiotic drugs against bacterial pathogens

Bacterial Isolates	Isolate Codes	MIC/MBC (µg/ml)		
		AML	THI	PRO
Staphylococcus aureus	SA01	12.5/25	12.5/25	12.5/25
Staphylococcus aureus	SA02	50/> 50	50/> 50	> 50/100
CoNS	CS02	50/> 50	25/50	25/50
CoNS	CS01	50/50	12.5/25	50/50
Streptococcus spp	SS01	25/50	25/50	12.5/50
Streptococcus spp	SS02	50/> 50	25/> 50	25/> 50
Escherichia coli	EC01	12.5/50	25/> 50	12.5/50
Escherichia coli	EC02	12.5/25	12.5/25	12.5/25
Klebsiella pneumoniae	KP01	50/50	25/50	50/> 50
Klebsiella pneumoniae	KP02	25/> 50	12.5/25	25/50
Enterobacter spp	ES02	12.5/25	12.5/25	12.5/50
Enterobacter spp	ES01	25/> 50	25/50	50/> 50
Pseudomonas aeruginosa	PA01	12.5/> 50	12.5/50	12.5/25
Pseudomonas aeruginosa	PA02	50/> 50	50/> 50	25/50

CoNS = Coagulase negative staphylococci; MIC = Minimum inhibitory concentration; MBC = Minimum bactericidal concentration; AML = Amlodipine; THI = Thioridazine; PRO = Promethazine

13.6±0.3 to 15.6±0.3 mm against GPB, and 12.0±0.3 to 16.5±0.5 mm against GNB (Table 1).

The AML, THI and PRO inhibited the growth of all the 14 isolates tested with MIC values in the range of 12.5 – 50 µg/ml, 12.5 - 50µg/ml and 12.5 - >50µg/ml respectively. The MIC values of THI were lowest for *S. aureus*, *E. coli*, *K. pneumoniae*, *Enterobacter* spp, *P. aeruginosa* and CoNS. The MBC values of AML, THI and PRO were in the range of 25 - 100 µg/ml. The MBC of AML for 78.6% of the bacterial isolates tested was 50 µg/ml, the MBC of THI for 35.7% of bacterial isolates tested was 25 µg/ml while the MBC of PRO for 7.1% of the bacterial isolates tested was 100 µg/ml (Table 2). The results also indicated that both MIC and MBC end points obtained by visual reading for AML on CoNS and *K. pneumoniae* were equal.

The bactericidal activity was deemed to be present if there was a ≥ 99.9% reduction in survival from the original inoculum (≥99.9% killing). Table 3 showed the percentage and log reductions in GPB cells exposed to AML, THI and PRO at 6 hours intervals after incubation. The percentage and log reduction in viable cell counts of *S. aureus* exposed to AML ranged from 33.3 to ≥ 99.9% and 0.18 to 2.41 Log_{10} CFU/ml after 30 hrs of interaction respectively while the percentage and log reduction in viable cell counts of *Streptococcus* spp exposed to AML ranged from 86.96 to ≥ 99.9% and 0.88 to 2.08 Log_{10} CFU/ml after 30 hours of interaction respectively.

The lowest percentage and logarithm

Table 3: Percentage and logarithm reductions in Gram positive bacterial cells exposed to non-antibiotic drugs

Isolate Codes	Exposed Time (hr)	Amlodipine			Thioridazine			Promethazine		
		PC (CFU/ml)	Log_{10} CFU/ml	% / Log Reduction	PC (CFU/ml)	Log_{10} CFU/ml	% / Log Reduction	PC (CFU/ml)	Log_{10} CFU/ml	% / Log Reduction
SA01	0	3.2×10^5	5.51	NA / NA	3.6×10^5	5.56	NA /NA	3.2×10^5	5.51	NA / NA
	6	4.5×10^4	4.65	33.33 / 0.18	2.4×10^4	4.38	93.33 /1.18	4.5×10^4	4.65	85.90 / 0.86
	12	1.3×10^3	3.11	91.67 /1/08	2.2×10^3	3.34	90.83 / 1.04	1.3×10^3	3.11	97.11 / 1.54
	18	2.7×10^2	2.43	83.50 / 0/78	1.7×10^2	2.23	92.27 / 1.11	2.7×10^2	2.43	79.23 / 0.68
	24	2.0×10^2	2.30	92.12 /1.10	NG	0.0	≥99.9 / 2.23	2.0×10^2	2.30	25.93 / 0.13
	30	1.2×10^2	2.08	≥99.9/ 2.41	NG	0.0	≥99.9 / 0.0	1.2×10^2	2.08	40.00 /0.22
SS02	0	3.6×10^5	5.56	NA / NA	4.8×10^5	5.68	NA / NA	5.5×10^5	5.74	NA / NA
	6	2.3×10^4	4.36	93.61 /1.20	2.4×10^4	4.38	95.0 /1.30	2.4×10^4	4.38	95.64 /1.36
	12	3.0×10^3	3.48	86.96 / 0.88	1.0×10^3	3.00	95.83 / 1.38	3.3×10^3	3.52	86.25 / 0.86
	18	1.2×10^2	2.08	96.00 / 1.40	1.2×10^2	2.08	88.00 / 0.92	2.2×10^2	2.34	93.33 / 1.18
	24	NG	0.0	≥99.9 /2.08	NG	0.0	≥99.9 /2.08	1.2×10^2	2.08	45.50 / 0.26
	30	NG	0.0	≥99.9 / 0.0	NG	0.0	≥99.9 /0.0	NG	0.0	≥99.9 / 2.08
CS02	0	4.8×10^5	5.68	NA / NA	3.6×10^5	5.56	NA / NA	3.6×10^5	5.56	NA / NA
	6	3.6×10^4	4.56	92.50 / 1.12	7.2×10^4	4.86	80.0 /0.70	2.0×10^5	5.30	44.44 / 0.26
	12	2.9×10^3	3.46	91.94 /1.10	5.3×10^3	3.72	92.64 / 1.14	3.6×10^4	4.56	82.00 / 0.74
	18	1.2×10^3	3.08	58.62 / 0.38	3.0×10^3	3.48	43.40 / 0.24	2.0×10^3	3.30	94.44 / 1.26
	24	2.0×10^2	2.30	83.33 / 0.78	2.2×10^2	2.34	92.67 / 1.14	1.6×10^2	2.20	92.00 / 1.10
	30	NG	0.0	≥99.9 /2.30	NG	0.0	≥99.9 / 2.34	NG	0.0	≥99.9 / 2.20

SA01 = *Staphylococcus aureus*; SS02 = *Streptococcus* spp; CS02 = Coagulase negative staphylococcus; PC = Plate Counts; CFU = Colony Forming Units; ml = Millilitre; NG = No Growth; NA = Not Available

Table 4: Percentage and logarithm reductions in Gram negative bacterial cells exposed to non-antibiotic drugs

Isolate Codes	Exposed Time (hr)	Amlodipine			Thioridazine			Promethazine		
		PC (CFU/ml)	Log_{10} CFU/ml	% / Log Reduction	PC (CFU/ml)	Log_{10} CFU/ml	% / Log Reduction	PC (CFU/ml)	Log_{10} CFU/ml	% / Log Reduction
EC01	0	3.6×10^5	5.56	NA / NA	6.9×10^5	5.84	NA / NA	3.9×10^5	5.59	NA / NA
	6	7.2×10^4	4.86	80.00 / 0.70	7.0×10^4	4.85	89.85 / 0.99	2.4×10^4	4.38	93.85 / 1.21
	12	6.3×10^3	3.80	91.25 / 1.06	2.4×10^3	3.38	96.57 / 1.47	2.2×10^3	3.34	90.83 / 1.04
	18	2.0×10^2	2.30	96.83 / 1.50	2.3×10^2	2.36	90.42 / 1.02	1.7×10^2	3.23	92.27 /0.11
	24	NG	0.0	≥99.9 /2.30	NG	0.0	≥99.9 / 2.36	NG	0.0	≥99.9 / 3.23
	30	NG	0.0	≥99.9 / 0.0	NG	0.0	≥99.9 / 0.0	NG	0.0	≥99.9 / 0.0
ES02	0	1.9×10^5	5.28	NA / NA	2.4×10^5	5.38	NA / NA	2.0×10^5	5.30	NA / NA
	6	1.7×10^4	4.23	91.05 / 1.05	1.0×10^5	5.00	58.33 / 0.38	2.4×10^4	4.38	88.00 / 0.92
	12	1.2×10^3	3.08	92.94 / 1.15	1.5×10^4	4.18	85.00 / 0.82	1.2×10^4	4.08	50.00 / 0.30
	18	2.4×10^2	2.38	80.00 / 0.70	2.4×10^3	3.38	84.00 /0.80	3.6×10^3	3.56	70.00 / 0.52
	24	1.0×10^2	2.00	58.33 / 0.38	1.2×10^2	2.08	95.00 /1.30	2.2×10^2	2.34	93.89 / 1.22
	30	NG	0.0	≥99.9 /2.00	NG	0.0	≥99.9 /2.08	1.0×10^2	2.00	54.55 / 0.34
KP02	0	3.9×10^5	5.59	NA / NA	3.9×10^5	5.59	NA / NA	3.3×10^5	5.52	NA / NA
	6	2.4×10^4	4.38	93.85 /1.21	2.4×10^4	4.38	93.85 / 1.21	3.0×10^4	4.48	90.91 / 1.04
	12	1.2×10^3	3.08	95.00 /1.30	2.2×10^3	3.34	90.83 / 1.04	1.0×10^3	3.00	96.67 / 1.48
	18	1.7×10^2	2.23	85.83 /0.85	1.7×10^2	2.23	92.27 / 1.02	NG	0.0	≥99.9 / 3.00
	24	NG	0.0	≥99.9 /2.23	NG	0.0	≥99.9 / 2.23	NG	0.0	≥99.9 / 0.0
	30	NG	0.0	≥99.9/ 0.0	NG	0.0	≥99.9 / 0.0	NG	0.0	≥99.9 / 0.0
PA01	0	7.7×10^5	5.89	NA / NA	2.0×10^5	5.30	NA / NA	4.8×10^5	5.68	NA / NA
	6	2.0×10^5	5.30	74.03 / 0.59	1.4×10^4	4.15	93.00 / 1.15	2.4×10^4	4.38	95.00 / 1.30
	12	1.2×10^4	4.08	94.00 /1.22	1.2×10^3	3.08	91.43 / 1.07	1.0×10^3	3.00	95.83 / 1.38
	18	3.3×10^3	3.52	72.50 / 0.56	3.6×10^2	2.56	70.00 / 0.52	1.2×10^2	2.08	88.00 / 0.92
	24	2.0×10^2	2.30	93.94 /1.22	NG	0.0	≥99.9 / 2.56	NG	0.0	≥99.9 / 2.08
	30	NG	0.0	≥99.9 /2.30	NG	0.0	≥99.9 / 0.0	NG	0.0	≥99.9 / 0.0

EC01 = *Escherichia coli*; ES02 = *Enterobacter* spp; KP02 = *Klebsiella pneumoniae*; PA01 = *Pseudomonas aeruginosa*; PC = Plate Counts; CFU = Colony Forming Units; ml = Millilitre; NG = No Growth; NA = Not Available

reduction in viable cell count of CoNS exposed to AML was ≥ 58.6% and 0.38 Log_{10} CFU/ml respectively. The log reduction in viable cell counts of *S. aureus, Streptococcus* spp and CoNS exposed to PRO for 30 hrs ranged from 0.13 to 1.54 Log_{10} CFU/ml, 0.26 to 2.08 Log_{10} CFU/ml and 0.26 to 2.20 Log_{10} CFU/ml respectively (Table 3). At 1xMIC, THI achieved bactericidal effects on *S. aureus, Streptococcus* spp, *E. coli, K. pneumoniae* and *P. aeruginosa* at 24 hours post inoculation while ≥ 99.9% reduction in survival from the original inoculum was achieved for CoNS and *Enterobacter* spp at 30 hours post inoculation (Tables 3 and 4).

The ranges of log reduction in viable cell counts of GNB exposed to AML for 30 hrs were *E. coli* (0.7 to 2.30 Log_{10} CFU/ml), *Enterobacter* spp (0.38 to 2.0 Log_{10} CFU/ml), *K. pneumoniae* (0.85 to 2.23 Log_{10} CFU/ml) and *P. aeruginosa* 0.56 to 2.3 Log_{10} CFU/ml) (Table 4). The bactericidal activity of PRO on *K. pneumoniae* and *P. aeruginosa* was achieved at 18- and 30-hours post inoculation respectively whereas 1.0 x 10^2 CFU/ml *Enterobacter* spp were still viable at 30 hours post inoculation (Table 4). The time kill kinetics curves of AML, THI and PRO (1.0 x MIC) against the bacterial isolates are shown in Figs 1 and 2.

The increase in viable cell counts of the GPB and GNB not exposed to AML, THI and PRO within the 30 hours of incubation were observed and presented in Tables 5 and 6. The viable cell count of *S. aureus* increased from 5.57 to 7.79 Log_{10} CFU/ml, those of S*trepto-coccus* spp cells increased from 5.61 to 7.85 Log_{10} CFU/ml while those of CoNS increased from 5.61 to 7.85 Log_{10} CFU/ml (Table 5). The increase in the viable cell count of GNB within the 30 hours incubation period was 5.60 to 7.84 Log_{10} CFU/ml for *E coli,* 5.41 to 7.60 Log_{10} CFU/ml for *Enterobacter* spp, 5.58 to 7.72 Log_{10} CFU/ml for *K. pneumoniae* and 5.76 to 7.86 Log_{10} CFU/ml for *P. aeruginosa* (Table 6).

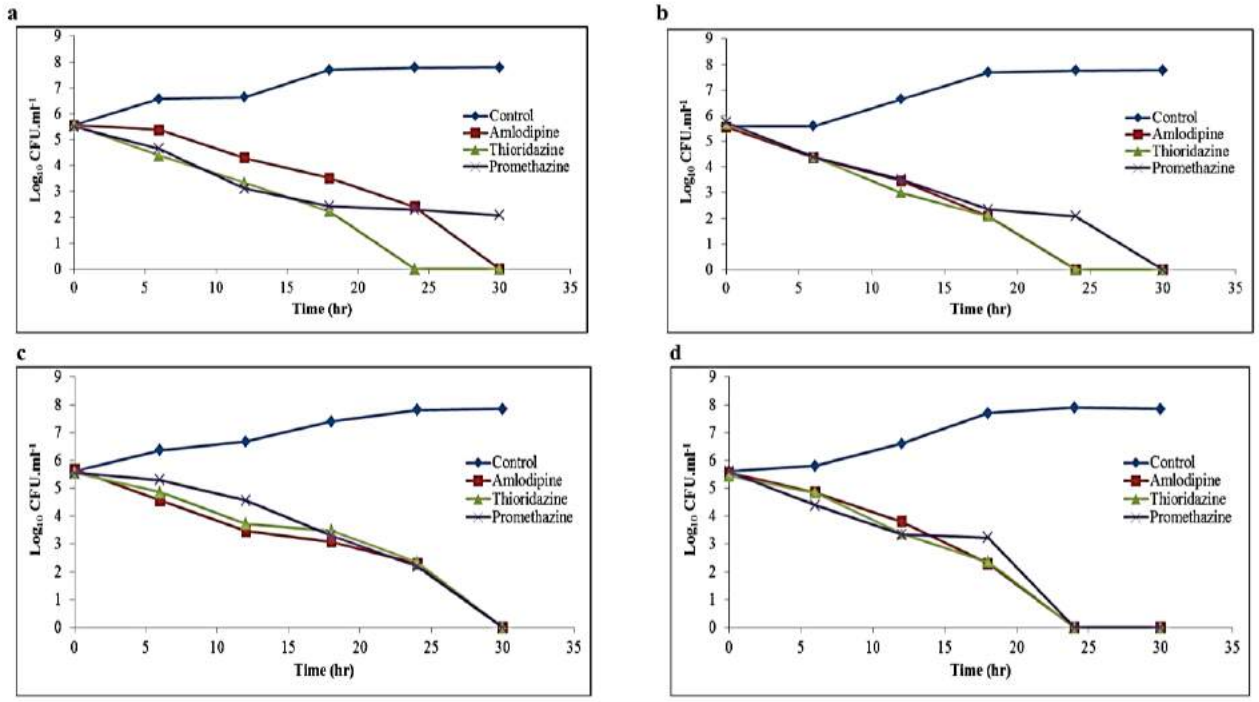

Fig 1: Time Kill Kinetics Curve of Amlodipine, Thioridazine and Promethazine (1 x MIC) and control against (a) *S. aureus,* (b) *Streptococcus* spp., (c) Coagulase negative staphylococci, (d) *Escherichia coli*

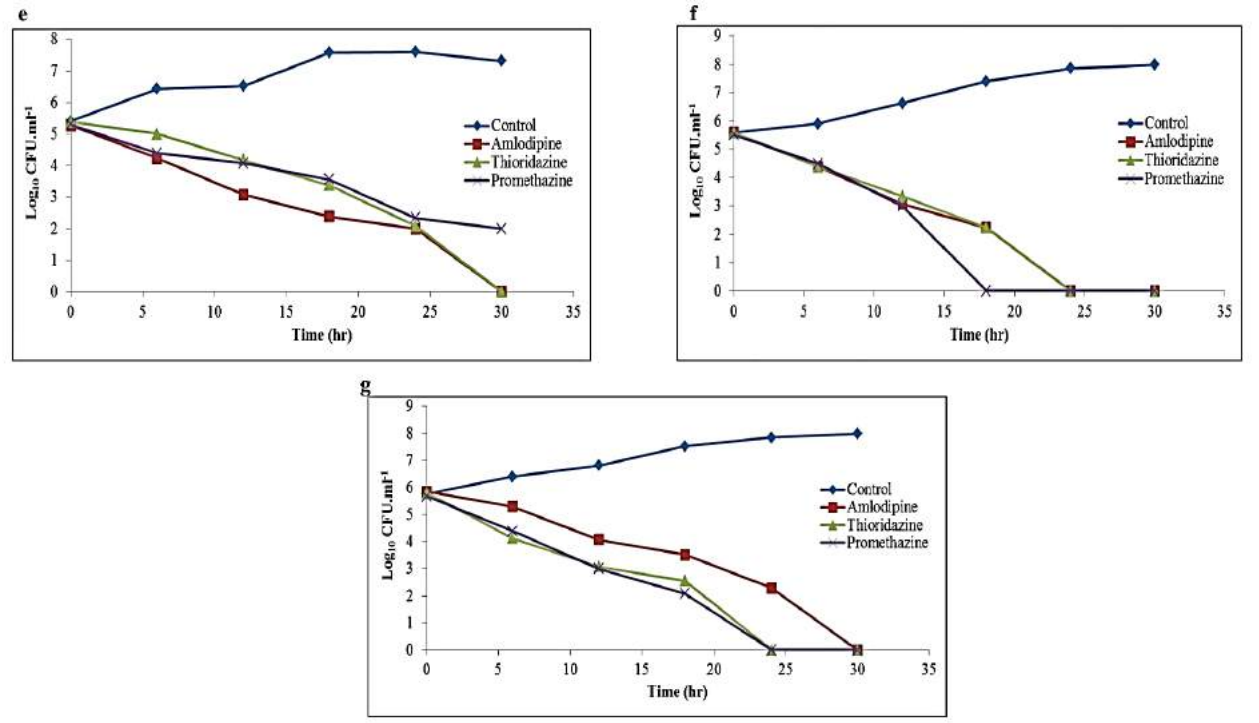

Fig 2: Time Kill Kinetics Curve of Amlodipine, Thioridazine, Promethazine (1 x MIC) and control against (e) *Enterobacter* spp., (f) *Klebsiella pneumoniae*, (g) *Pseudomonas aeruginosa*

Table 5: Growth of Gram-positive bacterial cells unexposed to non-antibiotic drugs

Bacterial Isolates	Codes	Time Interval (Hr)	Plate Counts (CFU/ml)	Log $_{10}$ CFU/ml
Staphylococcus aureus	SA01	0	3.7×10^5	5.57
		6	3.8×10^6	6.58
		12	4.4×10^6	6.64
		18	5.0×10^7	7.70
		24	6.1×10^7	7.78
		30	6.2×10^7	7.79
Streptococcus spp	SS02	0	3.9×10^5	5.59
		6	3.9×10^5	5.59
		12	4.4×10^6	6.64
		18	4.8×10^7	7.68
		24	5.7×10^7	7.76
		30	5.9×10^7	7.77
Coagulase negative *Staphylococcus*	CS02	0	4.1×10^5	5.61
		6	2.3×10^6	6.36
		12	4.7×10^6	6.67
		18	3.0×10^7	7.48
		24	6.4×10^5	7.81
		30	7.0×10^5	7.85

Table 6: Growth of Gram-negative bacterial cells unexposed to non-antibiotic drugs

Bacterial Isolates	Codes	Time Interval (Hr)	Plate Counts (CFU/ml)	Log $_{10}$ CFU/ml
Escherichia coli	EC01	0	4.0×10^5	5.60
		6	6.6×10^5	5.82
		12	4.1×10^6	6.61
		18	5.5×10^7	7.74
		24	7.9×10^7	7.90
		30	6.9×10^7	7.84
Enterobacter spp	ES02	0	2.6×10^5	5.41
		6	2.7×10^6	6.43
		12	3.3×10^6	6.52
		18	3.8×10^6	6.58
		24	4.3×10^7	7.63
		30	4.0×10^7	7.60
Klebsiella pneumoniae	KP02	0	3.8×10^5	5.58
		6	1.0×10^6	6.00
		12	4.3×10^6	6.63
		18	4.9×10^7	7.69
		24	5.1×10^7	7.71
		30	5.3×10^7	7.72
Pseudomonas aeruginosa	PA01	0	5.8×10^5	5.76
		6	6.0×10^6	6.78
		12	6.4×10^6	6.81
		18	6.9×10^7	7.82
		24	7.1×10^7	7.85
		30	7.3×10^7	7.86

Discussion:

The global emergence of multi-drug resistant pathogens as well as the continuing challenge of infectious diseases have necessitated the scientific exploration of novel, potent and affordable antimicrobial agents such as medicinal plants (5) and non-antibiotic drugs (6) in the management of infectious diseases. In this study, amlodipine (AML) demonstrated higher growth inhibitory activities at 5mg/ml concentration (50µg disk) against the GPB (*S. aureus,* CoNS, *Streptococcus* spp) and GNB (*E. coli, Enterobacter* spp, *K. pneumoniae* and *P. aeruginosa*) than at 2.5mg/ml concentration (25µg), indicating a concentration-dependent inhibition of bacterial growth.

The antibacterial activity of AML in this study substantiates the findings of Kumar et al., (8) that AML, a calcium channel blocker and cardiovascular drug, exhibited antibacterial activities against *S. aureus, E. coli* and *P. aeruginosa.* The antibacterial activities of AML on both GPB and GNB indicated its broad-spectrum activity and these findings concurs with the reports of Mazumdar et al., (17) and Pereira et al., (18), which reported broad-spectrum antibacterial activities of AML. The pattern of time-kill kinetics, investigated by the microbroth kinetic growth assay, against the GPB and GNB showed that AML exhibited bactericidal activities (≥ 99.9% reduction in

survival from the original inoculum) within 30 hours of exposure against the bacteria. These bactericidal activities of AML against the isolates in this study confirmed the findings previously reported by Kumar et al., (8) and Mazumdar et al., (17).

The MBC values of THI for the isolates ranged between 25 and 100µg/ml with bactericidal effects on *S. aureus, Streptococcus* spp, *E. coli, K. pneumoniae* and *P. aeruginosa* at ≤ 24 hours post inoculation. The antibacterial activities of THI on GPB and GNB in this study corroborates the findings of Radhakrishnan et al., (19) on the potentiality of THI as an effective antibacterial agent. Our findings on antibacterial efficacy of THI on *P. aeruginosa* was also in conformity with a previous study by Mukherjee et al., (20), who reported that THI, an anti-psychotic drug, was a highly promising agent in the treatment of acute infections caused by *P. aeruginosa.* Studies have shown that THI exerts a bactericidal effect on bacteria by damaging the cell wall and causing major changes in expression of many genes involved in peptidoglycan biosynthesis (21). THI has also been reported to reverse antibiotic resistance by facilitating the elimination of resistance (R) plasmid from MDR bacteria (14,19).

In this study, PRO also exhibited antibacterial activities on both GPB and GNB, with log reduction in viable cell counts of GPB exposed for 30 hours ranging from 0.13 to 2.20 Log_{10} CFU/ml. Our finding substantiates the reports of Dasgupta et al., (22) on antibacterial activity of PRO against antibiotic resistant bacterial isolates. Bactericidal activity of PRO against *S. aureus* in our study also agrees with the findings of Dastidar et al., (23). PRO, a phenothiazine, acts by inhibiting the efflux pump that protects bacterial cell against harmful chemical substances (24), and also affects energy sources, adenosine triphosphatase (ATPase), and genes that regulate permeability in bacteria (25). The increased permeability ensures that PRO molecules get to the DNA sites, intercalate between the bases, and consequently hinder the DNA transcription and translation processes (26). It has also been reported that PRO can reverse the phenotypes of MDR bacteria (14).

Conclusion:

Our study demonstrated *in vitro* antimicrobial efficacies and time kill kinetics of amlodipine, thioridazine and promethazine against pathogenic clinical bacterial isolates, which indicates that these non-antibiotic drugs may be useful therapeutic alternatives in the bid to reduce the burden of infectious diseases associated with antibiotic resistant pathogens. Consequent upon these findings, *in vivo* antibacterial studies of these non-antibiotic drugs are required.

Authors' contributions:

AOJ and SGI designed the study, wrote the protocol and first draft of the manuscript. UAN and AMF wrote part of the manuscript and managed the analyses of the study. AOJ and OGO managed the literature searches and performed the statistical analysis. All authors read and approved the final manuscript.

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Original Article **Open Access**

Differences in haematological parameters and haemoglobin phenotypes in symptomatic and asymptomatic subjects with *Plasmodium falciparum* infection in parts of Kaduna metropolis, Nigeria

*[1]Dikwa, K. B., [2]Maikaje, D. B., [1]Yahaya, U. A., and [3]Suleiman, A. B.

[1]Department of Biological Sciences, Nigerian Defence Academy, Kaduna, Nigeria
[2]Department of Microbiology, Kaduna State University, Kaduna, Nigeria
[3]Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria
*Correspondence to: kbdikwa@nda.edu.ng

Abstract

Background: *Plasmodium falciparum* is the leading cause of malaria morbidity and mortality in Nigeria with varied symptoms and haematological consequences. The objective of this study is to assess the differences in haematological parameters and haemoglobin phenotypes in symptomatic *P. falciparum* infected and apparently healthy asymptomatic individuals in parts of Kaduna metropolis.
Methodology: A total of 1000 subjects; 500 symptomatic and 500 apparently healthy subjects asymptomatic for malaria, were recruited from selected hospitals and National Blood Bank in Kaduna metropolis. Blood samples were collected for thick and thin film microscopy to determine malaria parasitaemia and parasite species identification respectively. Haematological parameters were determined using automated blood analyser (KX-21N, Sysmex, Japan) and haemoglobin phenotypes by alkaline cellulose acetate electrophoresis.
Results: Of the 1000 subjects recruited, 347 (34.7%) were positive for *P. falciparum* on blood film, which included 226 (45.2%) of 500 symptomatic and 121 (24.2%) of 500 asymptomatic subjects ($p<0.00001$). Of the 347 *P. falciparum* infected subjects, 275 (79.3%) had HbAA, 61 (17.6%) had HbAS, 1 (0.3%) had HbAC, 8 (2.3%) had HbSS, and 2 (0.6%) had HbSSf phenotypes. One hundred and sixty-three (72.1%) of the 226 symptomatic subjects had HbAA while 112 (92.6%) of the 121 asymptomatic subjects had HbAA, which indicated a significantly higher frequency of asymptomatic malaria in subjects with HbAA ($p<0.00001$). Conversely, 53 (23.5%) of the 226 symptomatic subjects had HbAS while 8 (6.6%) of 121 asymptomatic subjects had HbAS, indicating a significantly higher frequency of symptomatic malaria in subjects with HbAS ($p=0.000086$). The frequency of parasitaemia > 3,000 parasites/µL of blood was 100% for HbSSf, 25% for HbSS, 8.2% for HbAS and 2.2% for HbAA, which showed significantly higher frequency in subjects with HbSS ($X^2=7.5989$, $p=0.0054$) and HbAS ($X^2=3.9627$, $p=0.046519$) compared to HbAA. In symptomatic subjects, only MCHC value was significantly higher in subjects with HbAS (33.21±2.430) compared to those with HbAA (32.09 ±2.315) ($p=0.003$), while all other haematological parameters were not significantly different ($p>0.05$). In asymptomatic subjects, none of the haematological parameters was significantly different between subjects with HbAS and HbAA ($p>0.05$).
Conclusion: Although the frequency of *P. falciparum* infection in this study is generally higher in subjects with HbAA, symptomatic infection and higher parasite density are associated with HbAS, HbSS and HbSSf. Effective utilisation of personal preventive measures by inhabitants, in addition to current malaria control and intervention strategies should be adequately implemented in Kaduna metropolis.

Keywords: Haematological parameters, haemoglobin, electrophoresis, *Plasmodium falciparum*, malaria

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Différences dans les paramètres hématologiques et les phénotypes d'hémoglobine chez les sujets symptomatiques et asymptomatiques

atteints d'une infection à *Plasmodium falciparum* dans certaines parties de la métropole de Kaduna, Nigéria

*[1]Dikwa, K. B., [2]Maikaje, D. B., [1]Yahaya, U. A., et [3]Suleiman, A. B.

[1]Département des sciences biologiques, Académie nigériane de défense, Kaduna, Nigéria
[2]Département de microbiologie, Université d'État de Kaduna, Kaduna, Nigéria
[3]Département de microbiologie, Université Ahmadu Bello, Zaria, Nigéria
*Correspondance à: kbdikwa@nda.edu.ng

Abstrait:

Contexte: *Plasmodium falciparum* est la principale cause de morbidité et de mortalité liées au paludisme au Nigéria avec des symptômes et des conséquences hématologiques variés. L'objectif de cette étude est d'évaluer les différences de paramètres hématologiques et de phénotypes d'hémoglobine chez des individus symptomatiques infectés par P. falciparum et asymptomatiques apparemment en bonne santé dans certaines parties de la métropole de Kaduna.
Méthodologie: Un total de 1000 sujets; 500 sujets symptomatiques et 500 sujets apparemment sains asymptomatiques pour le paludisme ont été recrutés dans certains hôpitaux et dans la Banque nationale du sang de la métropole de Kaduna. Des échantillons de sang ont été prélevés pour la microscopie à couche épaisse et mince afin de déterminer respectivement la parasitémie du paludisme et l'identification des espèces de parasites. Les paramètres hématologiques ont été déterminés à l'aide d'un analyseur sanguin automatisé (KX-21N, Sysmex, Japon) et des phénotypes d'hémoglobine par électrophorèse sur acétate de cellulose alcaline.
Résultats: Sur les 1000 sujets recrutés, 347 (34,7%) étaient positifs pour P. falciparum sur frottis sanguin, qui comprenait 226 (45,2%) de 500 sujets symptomatiques et 121 (24,2%) de 500 sujets asymptomatiques (p<0,00001). Sur les 347 sujets infectés par P. falciparum, 275 (79,3%) avaient HbAA, 61 (17,6%) avaient HbAS, 1 (0,3%) avaient HbAC, 8 (2,3%) avaient HbSS et 2 (0,6%) avaient des phénotypes HbSSf. Cent soixante-trois (72,1%) des 226 sujets symptomatiques avaient une HbAA tandis que 112 (92,6%) des 121 sujets asymptomatiques avaient une HbAA, ce qui indiquait une fréquence significativement plus élevée de paludisme asymptomatique chez les sujets avec HbAA (p<0,00001). À l'inverse, 53 (23,5%) des 226 sujets symptomatiques avaient une HbAS tandis que 8 (6,6%) des 121 sujets asymptomatiques avaient une HbAS, indiquant une fréquence significativement plus élevée de paludisme symptomatique chez les sujets avec HbAS (p=0,000086). La fréquence de parasitémie> 3000 parasites / µL de sang était de 100% pour l'HbSSf, 25% pour l'HbSS, 8,2% pour l'HbAS et 2,2% pour l'HbAA, ce qui a montré une fréquence significativement plus élevée chez les sujets atteints d'HbSS (X^2=7,5989, p=0,0054) et HbAS (X^2=3,9627, p=0,046519) par rapport à l'HbAA. Chez les sujets symptomatiques, seule la valeur MCHC était significativement plus élevée chez les sujets avec HbAS (33,21±2,430) par rapport à ceux avec HbAA (32,09±2,315) (p=0,003), tandis que tous les autres paramètres hématologiques n'étaient pas significativement différents (p>0,05). Chez les sujets asymptomatiques, aucun des paramètres hématologiques n'était significativement différent entre les sujets avec HbAS et HbAA (p>0,05).
Conclusion: Bien que la fréquence des infections à *P. falciparum* dans cette étude soit généralement plus élevée chez les sujets atteints d'HbAA, une infection symptomatique et une densité parasitaire plus élevée sont associées à l'HbAS, l'HbSS et l'HbSSf. Une utilisation efficace des mesures de prévention personnelle par les habitants, en plus des stratégies actuelles de lutte antipaludique et d'intervention, devrait être mise en œuvre de manière adéquate dans la métropole de Kaduna.

Mots clés: Paramètres hématologiques, hémoglobine, électrophorèse, *Plasmodium falciparum*, paludisme

Introduction:

Although, global malaria burden has reduced, the burden is still high in the African region. According to the World Health Organization malaria report (1), the African region still accounts for the high global malaria burden in 2018, with an estimated 93% malaria cases and 94% deaths. *Plasmodium falciparum* accounted for 99.7% of estimated malaria cases in the region. Of the six countries that accounted for more than half of all malaria cases worldwide, Nigeria accounted for 25% of such cases and *P. falciparum* remained the dominant species (1). Several factors are responsible for the transmission and spread of the malaria. These include climatic conditions such as rainfall pattern, temperature and humidity. These factors have been responsible for the seasonal transmission of malaria, with peak prevalence occurring during and immediately after rainy season (2,3,4,5).

Genetic factors have been shown to offer protection against malaria. These includes the possession of high concentration of haemoglobin F (HbF) in the red blood cells of new born infants (neonates) as well as individuals with sickled red cells containing abnormal haemoglobin (HbS). Sickle cell trait (HbAS) is also known to confers protection against severe falciparum malaria (2,6). The malaria-protective effect of HbAS or HbAC has been

hypothesized to include several innate immune mechanisms. Parasite growth and replication in the erythrocytes that contains HbAS or HbAC may be impeded in relative normal red cells when subjected to low oxygen tension. In addition, the proteins are the targets of specific antibodies may be more rapidly exposed in HbAS containing red blood cells resulting in an enhanced immune response to infection. There is also the possibility of unknown innate protection processes, which may up-regulate malaria-specific immune response and enhance non-specific immunity to malaria, thus, the optimal development of plasmodium in the deep organs where oxygen pressure is reduced may not be allowed in abnormal haemoglobin (7).

Malaria parasites grow and multiply in red blood cells with varied haematological consequences, resulting in changes in haematological parameters of the infected individuals (8). In previous studies, the effect of haemoglobin variants in *P. falciparum* infected individuals using haematological parameters were assessed with focus mainly on malaria infected individuals with clinical symptoms (7). In endemic regions, prolonged exposure confer immunity over time among adults, a condition in which resistance is acquired that is associated with continued asymptomatic parasitic infection (premunition).

There is need to examine individuals of different haemoglobin genotypes (variants) and determine the effect of *P. falciparum* infection on haematological parameters of both infected symptomatic and asymptomatic individuals. This research was carried out with aim of assessing the differences in haematological parameters and haemoglobin phenotypes in *P. falciparum* infected symptomatic and asymptomatic individuals in parts of Kaduna metropolis.

Materials and method:

Study area

This study was carried out in Kaduna metropolis, the capital of Kaduna State, Nigeria between March and November 2011. The state is located in North-western geopolitical zone and lies geographically within latitude 10^0 $21^1 23^{11}$N and longitude,$7^0 26^1 2^{11}$E, and is 608 meters above sea level. The state experiences both dry and rainy seasons. Dry season commences in the months of November to March and a rainy season usually from April through October and last between 4-5 months in the far and northern parts of the state and 5-6 months in the southern parts of the state, with vegetation typically of guinea savannah type (9). Temperatures are high during the dry season, with annual average high temperature of 31.6˚C, while relatively lower temperatures occur during the rainy season with annual low temperatures of 18.5ºC (10).

The hospitals studied in the area were; Yusuf Dantsoho Memorial Hospital, Tudun Wada, Kaduna; Nigerian Army Reference Hospital (44), Kaduna; Gwamna Awang Hospital, Nassarawa, Kaduna; and St. Gerald Hospital, Kakuri, in Kaduna South LGA; and Barau Dikko Specialist Hospital, Kaduna; Barau Dikko Children Hospital; Kaduna; Nigerian Defence Academy Medical Centre, Ribadu Cantonment, Kaduna; and General Hospital, Kawo, Kaduna, in Kaduna North LGA.

Study population

The study population comprised of two subject categories; symptomatic malaria and asymptomatic apparently healthy individuals. Symptomatic subjects were persons manifesting aggregate of symptoms of falciparum malaria, which could either be mild uncomplicated or severe complicated malaria (11).

Ethical approval

Ethical approvals were obtained from Kaduna State Ministry of Health (MOH/ ADM/744/T/9), the Federal Ministry of Health (NBTS/HQ/058/04), Nigerian Army Reference Hospital (44) (44/NARHK/GI/300/60) Kaduna, and confirmation was obtained from Nigerian Defence Academy Medical Centre and Saint Gerald Hospital.

Subject selection and recruitment

A sample size of 382 was obtained using a previous prevalence of 46.5% (12) in Kaduna State, and the formula described by Rothman et al., (13). However, a total of 1000 subjects comprising of 500 symptomatic and 500 asymptomatic subjects were randomly selected and recruited for the study.

Sample and data collection

During sample collection, biodata of all the subjects were collected with the aid of a questionnaire to obtain information on age, gender, occupation, socio-economic status, and daily recreational activities. In addition, information on malaria symptoms (mild or severe), type of anti-malaria drug intake, as well as the habitual use of mosquito net and insecticides by symptomatic individuals were recorded. Five millilitres of venous blood from each subject were collected into labelled tubes (vacutainer) containing Ethylene diamine tetra acetic acid (EDTA, sequestrene) anticoagulant (6). Medical personnel from selected hospitals

and the National Blood Transfusion Service (NBTS) assisted in sample collection.

Blood film microscopy for *P. falciparum*

Malaria parasite detection and parasitaemia level determination were carried out on thick blood films while parasite species were identified on thin blood film using the procedures described by Cheesbrough (6). Parasite density (parasitaemia) was estimated by counting number of parasites per 200 white blood cells (WBCs) assuming 8000 WBCs per µL of blood (5).

Haemoglobin electrophoresis (phenotypes)

The haemoglobin phenotypes of all subjects were determined by alkaline cellulose acetate electrophoresis (14), which was used to separate and identify the different haemoglobin types based on their migration within an electric field. Haemoglobin variants separate at different rates due to differences in their surface electrical charge as determined by their amino acids structure.

In performing the test, 100 ml of Tris-EDTA borate buffer was poured into each of the outer sections of an electrophoresis chamber. Cellulose acetate paper was impregnated with Tris-EDTA borate buffer and known control HbAA (normal adult haemoglobin phenotype), HbAS (sickle cell trait), HbAC, HbSS (sickle cell anaemia) and HbSSf (sickle cell anaemia with fetal haemoglobin) were placed at both ends of the cellulose acetate paper. Lysed blood samples were then placed between the controls using a Hb comb. The cellulose acetate paper was placed in the Hb electrophoresis tank containing Tris-EDTA buffer and run at 150 voltage for 15 min. Hb phenotypes were read according to their separation (14,15).

Haematological parameters

Haematological parameters were determined using automated blood analyser (Model KX-21N, Sysmex, Japan) on the blood samples. The following blood indices were determined; white blood cell (WBC) count and differentials (neutrophil, monocytes and lymphocytes), platelets count, packed cell volume (PCV) or haematocrit, red cell counts, and red cell indices, which include mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), and mean corpuscular volume (MCV) (14).

Statistical analysis

Data generated in this study were subjected to statistical analysis (SPSS version 17 statistical package). Association between *P. falciparum* infection in relation to different haemoglobin phenotypes of symptomatic and asymptomatic subjects was determined using Chi-square test. Means (±SD) of haematological parameters of *P. falciparum* infected symptomatic and asymptomatic subjects were compared using one way analysis of variance (ANOVA). Difference was considered significant at 95% confidence interval ($p<0.05$).

Results:

Of the total 1000 subjects recruited for the study, 347 (34.7%) were positive for *P. falciparum* on blood film microscopy, which included 226 (45.2%) of 500 symptomatic and 121 (24.2%) of 500 asymptomatic individuals ($p<0.00001$). Of the 347 *P. falciparum* infected subjects, 275 (79.3%) had HbAA, 61 (17.6%) had HbAS, 1 (0.3%) had HbAC, 8 (2.3%) had HbSS, and 2 (0.6%) had HbSSf phenotypes. Of the 226 symptomatic *P. falciparum* infected subjects, 163 (72.1%) had HbAA phenotype, while 112 (92.6%) of the 121 asymptomatic *P. falciparum* infected subjects had HbAA, which indicated a significantly higher frequency of asymptomatic malaria in subjects with HbAA ($p<0.00001$). Conversely, 53 (23.5%) of the 226 symptomatic *P. falciparum* infected subjects had HbAS while 8 (6.6%) of 121 asymptomatic *P. falciparum* infected subjects had HbAS, indicating a significantly higher frequency of symptomatic malaria in subjects with HbAS ($p=0.000086$) (Table 1).

Table 1: Prevalence of *Plasmodium falciparum* infection in relation to phenotypes of infected symptomatic and asymptomatic individuals

Plasmodium falciparum infection	No of subjects examined	No of subjects infected (%)	Haemoglobin phenotypes				
			HbAA (%)	HbAS (%)	HbAC (%)	HbSS (%)	HbSSf (%)
Symptomatic	500	226 (45.2)	163 (72.1)	53 (23.5)	0	8 (3.5)	2 (0.9)
Asymptomatic	500	121 (24.2)	112 (92.6)	8 (6.6)	1 (0.8)	0	0
Total	1000	347 (34.7)	275 (79.3)	61 (17.6)	1 (0.3)	8 (2.3)	2 (0.6)
p value		<0.00001	<0.00001	<0.000086	NA	NA	NA

NA = Not Applicable

Table 2: Distribution of *Plasmodium falciparum* parasitaemia level in relation to haemoglobin phenotypes of symptomatic and asymptomatic subjects

Haemoglobin phenotypes	No of subjects infected	Parasitaemia level (parasites/µL of blood)		
		<1000 (%)	1000-3000 (%)	> 3000 (%)
HbAA	275	199 (72.4)	70 (25.5)	6 (2.2)
HbAS	61	41 (67.2)	15 (24.6)	5 (8.2)
HbAC	1	0	1 (100)	0
HbSS	8	4 (50.0)	2 (25.0)	2 (25.5)
HbSSf	2	0	0	2 (100)
Total	347	244 (70.3)	88 (25.4)	15 (4.3)

The frequency distribution of *P. falciparum* parasitaemia among individuals of different haemoglobin phenotypes is presented in Table 2. Out of the 275 *P. falciparum* infected subjects with HbAA phenotype, 199 (72.4%) had parasitaemia level of < 1000 parasites/µL, 70 (25.45%) had parasitaemia level of <1000-3000 parasites/µL and 6 (2.2%) had parasitaemia level of >3000 parasites/µL. Of the 61 *P. falciparum* infected subjects with HbAS phenotype, 41 (67.2%) had parasitaemia level of <1000 parasites/µL, 15 (24.6%) had parasitaemia level of 1000–3000 parasites/µL, and 5 (8.2%) had parasitaemia level of >3,000 parasites/µL. Of the 8 *P. falciparum* infected subjects with HbSS phenotype, 4 (50.0%) had parasitaemia level of < 1000 parasites/µL, 2 (50.0%) had parasitaemia level of 1000–3000 parasites/µL, and 2 (25.0%) had parasitaemia level of > 3,000 parasites/µL. The frequency of parasitaemia > 3,000 parasites/µL of blood was 100% for HbSSf, 25% for HbSS, 8.2% for HbAS and 2.2% for HbAA, which showed significantly higher frequency in subjects with HbSS (X^2=7.5989, p=0.0054) and HbAS (X^2= 3.9627, p=0.046519) compared to HbAA phenotype. The only subjects with HbAC had parasitaemia level of < 3,000 parasites/µL but all the 2 subjects (100%) with HbSSf phenotype had parasitaemia level > 3,000 parasites/µL (Table 2).

The mean (±SD) haematological values of symptomatic *P. falciparum* infected subjects with HbAA and HbAS phenotypes are presented in Table 3. Significantly higher mean (±SD) value of MCHC was seen in HbAS (33.21± 2.430) compared to HbAA subjects (32.09± 2.315) (p=0.003). However, there were no statistically significant differences (p>0.05) in the mean (±SD) values of other haematological parameters between symptomatic *P. falciparum* infected subjects with HbAS and HbAA phenotypes (Table 3). The mean (±SD) haema tological values of asymptomatic *P. falciparum* infected subjects with HbAA and HbAS phenotypes are presented in Table 4. There were no significant differences in the values of all the parameters between asymptomatic *P. falciparum* infected subjects with HbAS and HbAA phenotypes (p>0.05).

Table 3: Mean (±SD) haematological values of HbAA and HbAS phenotypes of infected symptomatic subjects

Haematological parameter	Haemoglobin phenotypes		*p* value
	HbAA (n=163) Mean (±SD)	HbAS (n=53) Mean (±SD)	
WBC x10^9/L	6.22 (±2.987)	7.16 (±4.228)	0.076
RBC x10^{12}/L	4.39 (±0.807)	4.63 (±0.869)	0.064
HB (g/dl)	12.11 (±8.411)	12.45 (±2.424)	0.772
PCV (%)	35.52 (±6.492)	37.43 (±6.904)	0.068
MCHC (g/l)	32.09 (±2.315)	33.21 (±2.430)	*0.003
MCH (pg)	26.17 (±3.539)	27.00 (±3.065)	0.128
MCV (fl)	81.73 (±9.570)	81.23 (±6.845)	0.727
LYM (%)	42.39 (±15.383)	44.39 (±16.514)	0.420
MONO (%)	10.51 (±5.422)	9.67 (±5.485)	0.333
NEUT (%)	46.71 (±15.79)	45.93 (±16.430)	0.756
PLATELETS (x 10^9/L)	231.40 (±128.844)	233.60 (±97.855)	0.909

*Difference is significant (p<0.05); WBC-White Blood Cells; RBC-Red Blood Cells; HB-Haemoglobin; PCV-Packed Cell Volume; MCHC-Mean Corpuscular Haemoglobin Concentration; MCH-Mean Corpuscular Haemoglobin; MCV-Mean Corpuscular Volume; LYM- Lymphocytes; MONO- Monocytes, NEUT-Neutrophils.

Table 4: Mean (±SD) haematological values of asymptomatic *Plasmodium falciparum* infected subjects with HbAA and HbAS phenotypes

Haematological parameters	Haemoglobin phenotypes		*p* value
	HbAA (n=112) Mean (±SD)	HbAS (n=8) Mean (±SD)	
WBC (x 10^9/L)	5.12 (± 1.405)	5.50(±2.014)	0.480
RBC (x 10^{12}/L)	4.72(± 0.631)	4.68(±0.680)	0.870
HB (g/dl)	14.04(±0.811)	14.50(±0.978)	0.131
PCV (%)	41.87(±2.522)	43.18(±4.302)	0.182
MCHC (g/l)	33.25(±1.039)	32.89(±1.066)	0.347
MCH (pg)	28.56(±1.822)	28.59(±0.985)	0.968
MCV (fl)	87.34(±5.059)	86.95(±3.513)	0.832
LYM (%)	36.76(±8.851)	39.10(±8.966)	0.472
MONO (%)	8.92(±3.271)	10.83(±6.387)	0.144
NEUT (%)	54.30(±9.888)	50.08(±12.864)	0.255
PLATELETS (x 10^9/L)	213.19(±62.359)	225.25(±65.798)	0.599

WBC-White Blood Cells; RBC-Red Blood Cells; HB-Haemoglobin; PCV-Packed Cell Volume; MCHC-Mean Corpuscular Haemoglobin Concentration; MCH-Mean Corpuscular Haemoglobin; MCV-Mean Corpuscular Volume; LYM- Lymphocytes; MONO- Monocytes, NEUT-Neutrophils.

Discussion:

In this study, the prevalence of *P. falciparum* infection in both symptomatic and asymptomatic subjects examined was highest in those with HbAA phenotype, followed by those with HbAS phenotype. In a related study in Zaria, similar trend was also observed by Benjamin et al., (16) in which participants with HbAA phenotype had the highest percentage (76.9%) of malaria followed by those with HbAS (18.5%). The study by Onaiwu et al., (17) also revealed highest distribution of malaria infection among HbAA (66.7%) followed by HbAS (23.8%) among the study participants. This observation is similar to that of Albiti and Nsiah (18) who reported higher prevalence of *P. falciparum* among patients with HbAA phenotype than those with HbAS phenotype in Yemen. In another study in Nigeria, Esan (7), reported high frequency of malaria attacks in persons with HbAA phenotype.

The susceptibility of individuals with haemoglobin phenotype AA to malaria infection is due to the low red cell membrane resistance to the invading parasite (19). Red cells are conducive for the growth and development of the parasite (17). However, the mechanism that cause reduction in the level of *Plasmodium* infection in heterozygous and homozygous sickle cell alleles (AS and SS), and confer resistance to *Plasmodium* infection was predicted to be due to distortion in the membrane of the cells as a result of which the morphology of the binding receptors on the surface of the red cell membrane cannot be recognized by the *P. falci parum* binding ligand (pfbl) (20).

The high parasitaemia level (>3,000 parasites/µL of blood) reported in subjects with HbSS (25%) and HbSSf (100%) phenotypes in this study is similar to the findings of Otajevwo

and Enabulele (19) who reported highest malaria infection rates of 71.4% among HbSS phenotype compared to others. This may be due to the non-protective nature of the HbS against severe falciparum malaria, and could be responsible for fatalities, especially in young infected children with sickle cell anaemia (2,6). The report of Daskum and Ahmed (21) showed that HbAS and HbSS do not impair parasite invasion of RBCs because high parasite densities are seen in *P. falciparum* infected RBCs of HbAS and HbSS *in vivo*. Despite their inability to impair parasite invasion, parasite growth was however proven to be impaired. According to Luoni et al., (22), phenotypes other than HbAC and HbCC are associated with reduced risk of clinical malaria and limited pathology, compared to severity of the dis-advantaged HbSS and HbSC.

Significantly higher mean (±SD) value of mean corpuscular haemoglobin concentration (MCHC) was seen in HbAS compared to HbAA subjects in this study. This finding is in agreement with the study of Kosiyo et al., (23), who observed reduced MCHC among children with HbSS. MCHC is an index of red blood cells, derived from haemoglobin concentration and haematocrit, which are primary red cell measurements, and any factor affecting these parameters in either sickle cell or malaria patients would virtually affect MCHC (23).

Conclusion:

Although the frequency of *P. falciparum* infection in this study is generally higher in subjects with HbAA phenotype, symptomatic infection and higher parasite density are associated with HbAS, HbSS and HbSSf phenotypes. Effective utilisation of personal preventive measures by inhabitants, in addition to current malaria control and intervention strategies should be adequately implemented in Kaduna metropolis.

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Contributions of authors

KBD conceived the study, carried out sample collection, sample analysis, data analysis and manuscript writing. DBM (deceased), YAU and ABS supervised and provided guidance on research conduct, manuscript review and production. All authors read and approved the final version of the manuscript.

Conflicts of interest:

Authors declared no conflicts of interest

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Short Communication **Open Access**

Evidence of virological failure in patients on second-line anti-retroviral therapy in Southwestern Nigeria: an indication for HIV drug resistance testing

*Usman, S. O., Ajayi, O. M., Ebiekura, O., Egbonrelu, N., Ebhojie, G., and Ariyo, A. O

APIN Public Health Initiatives, Abuja, Nigeria
*Correspondence to: senatorhopsy@yahoo.com

Abstract:

Background: In sub-Saharan Africa where genotypic anti-retroviral (ARV) drug resistance testing is rarely performed and poor adherence is blamed for the inability to achieve viral suppression and treatment failure, programmatic approaches to preventing and handling these are essential. This study was aimed at assessing the virological outcomes among HIV patients receiving second-line anti-retroviral therapy (ART) in Southwestern Nigeria.
Methodology: This was a 5-year observational retrospective study of randomly selected people living with HIV (PLWHIV) who have been switched to second-line ART for at least six months before the commencement of the study in multiple comprehensive ART sites across the three levels of care, in Ondo and Ekiti States, Southwestern Nigeria, from January 2015 to December 2019. Quantitative viral load analysis was done using polymerase chain reaction (PCR) assay. Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 24.0.
Results: A total of 249 (71 males and 178 females) subjects eligible for the study were recruited using simple random sampling technique. The mean age (± SD) of the subjects was 44.21 ± 11.45 years. The mean number of years the patients have been on ART regimen was 7.92 ± 2.68 years. The mean number of years the patients were on first line ART regimen before being switched to second line was 4.27 ± 2.63 years. Patients with viral load <1000 RNA copies/ml (suppressed viral load) were 216 (86.7%) out of which 113 (45.4%) had viral load <20 RNA copies/ml while 33 (13.3%) had viral load >1000 RNA copies/ml (unsuppressed viral load or virological failure).
Conclusion: About 13% of the patients on second line ART had unsuppressed viral load of more than 1000 RNA copies/ml indicating virological failure. Thus, critical factors such as poor adherence to ART and drug resistance chiefly contributing to virological failure have to be routinely checked.

Keywords: suppression, ART, resistance, virological, failure, Nigeria

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Preuve d'échec virologique chez les patients sous traitement antirétroviral de deuxième intention dans le sud-ouest du Nigeria: une indication pour le test de résistance aux médicaments contre le VIH

*Usman, S.O., Ajayi, O.M., Ebiekura, O., Egbonrelu, N., Ebhojie, G., et Ariyo, A.O.

Initiatives de santé publique de l'APIN, Abuja, Nigéria
*Correspondance à: senatorhopsy@yahoo.com

Abstrait:

Contexte: En Afrique subsaharienne, où les tests génotypiques de résistance aux antirétroviraux (ARV) sont rarement effectués et où une mauvaise observance est imputée à l'incapacité d'obtenir la suppression virale et l'échec du traitement, des approches programmatiques pour les prévenir et les gérer sont essentielles. Cette étude visait à évaluer les résultats virologiques chez les patients VIH recevant un traitement antirétroviral (TAR) de deuxième intention dans le sud-ouest du Nigeria.
Méthodologie: Il s'agissait d'une étude rétrospective d'observation de 5 ans portant sur des personnes vivant

avec le VIH (PVVIH) sélectionnées au hasard et passées à un TAR de deuxième intention pendant au moins six mois avant le début de l'étude dans plusieurs sites de TAR complets aux trois niveaux. de soins, dans les États d'Ondo et d'Ekiti, dans le sud-ouest du Nigéria, de janvier 2015 à décembre 2019. L'analyse quantitative de la charge virale a été effectuée à l'aide d'un test de réaction en chaîne par polymérase (PCR). Les données ont été analysées à l'aide du logiciel Paquet statistique pour les sciences sociales (SPSS) version 24.0.
Résultats: Un total de 249 (71 hommes et 178 femmes) sujets éligibles à l'étude ont été recrutés à l'aide d'une technique d'échantillonnage aléatoire simple. L'âge moyen (± ET) des sujets était de 44,21±11,45 ans. Le nombre moyen d'années pendant lesquelles les patients ont été sous traitement antirétroviral était de 7,92±2,68 ans. Le nombre moyen d'années pendant lesquelles les patients étaient sous traitement antirétroviral de première ligne avant de passer en deuxième ligne était de 4,27 ± 2,63 ans. Les patients avec une charge virale <1000 copies d'ARN/ml (charge virale supprimée) étaient 216 (86,7%) dont 113 (45,4%) avaient une charge virale <20 copies d'ARN/ml tandis que 33 (13,3%) avaient une charge virale >1000 ARN copies/ml (charge virale non supprimée ou échec virologique).
Conclusion: Environ 13 % des patients sous TAR de deuxième ligne avaient une charge virale non supprimée de plus de 1000 copies d'ARN/ml indiquant un échec virologique. Ainsi, les facteurs critiques tels qu'une mauvaise adhésion au TARV et la résistance aux médicaments contribuant principalement à l'échec virologique doivent être systématiquement vérifiés.

Mots clés: suppression, TAR, résistance, virologique, échec, Nigeria

Introduction:

In many countries of the sub-Saharan Africa, hundreds of thousands of people living with HIV (PLHIV) have initiated antiretroviral therapy (ART), which has led to a reduction in the death toll related to HIV. While a majority of these patients have achieved treatment success on first-line therapy, the number of those requiring second-line protease inhibitor-based drugs is increasing due to failure of the first-line therapy (1,2). The challenge with patients on second-line ART in resource-limited settings, where genotypic drug resistance testing is rarely performed in programs for patients before start of treatment (3), has been in the event of treatment failure, due to limited availability and financial implication of third-line therapy (4,5).

In Nigeria, the government in partnership with international collaborators, has since responded to the epidemic scourge of the HIV infection through different intervention programs, including the establishment and scale-up of national ART (6,7) as well as domesticated policy documents that are in line with international best standards. Although the "National Guidelines for HIV Prevention Treatment and Care" of 2016 has provided a guide on management of patients failing the second-line ART, the challenge of unavailability of drug sensitivity testing has also been highlighted (8).

Available studies assessing virological failure among patients on second-line ART in resource-limited settings have had different definitions for virological failure (4). In the Nigerian ART program, viral load of <1000 RNA copies/ml is regarded as evidence of viral suppression while a persistently detectable viral load of ≥ 1000 copies/ml on two consecutive viral load measurements within a 3-month interval, with Enhance Adherence Counselling (EAC) between measurements, after at least six months on ART, is considered virological failure (8). To reap maximum benefits from the second-line ART as well as plan for better management of patients requiring this line of ART, including drug sensitivity testing, there is need for extensive study of patients on this therapy, which appears to be limited in the Nigerian context.

During a median follow up period of 29 months of PLHIV in Southern Vietnam, 18.4% of the patients experienced treatment failure with four WHO stage IV AIDS events and 13.5% death recorded during the period (9). In Myanmar, where long-term outcomes of second-line ART of 824 patients was followed up, it was reported that of 52 patients who received viral load testing, 19 had virological failure and at the end of a seven year follow up, 88 (11%) patients died with 680 (83%) still under care (10).

The objective of this study is to assess the virological outcomes among HIV patients receiving second-line ART in Southwestern Nigeria, with the hypothesis that opportunistic infection and ARV adherence level do not have significant impact on viral load outcome.

Materials and method:

Study setting and design

This was a 5-year retrospective study of paediatric, adolescent and adult HIV-infected patients who have been switched to second-line ART for at least six months as at the commencement of the study in multiple comprehensive ART sites across the three levels of care in Ondo and Ekiti States, Southwestern Nigeria, from January 2015 to December 2019.

Sample size and method of sampling

The sample size for the study was calculated using the formula, $n = Z^2PQ/d^2$ for population more than 10,000 (11) at 95% confidence interval (Z=1.96), 0.05 precision, and prevalence rate of Nigerians currently living with HIV/AIDS in Southwestern Nigeria of 1.2% (12). A total of 249 eligible HIV-

infected patients were selected by simple random sampling technique.

The inclusion criteria were patients living with HIV of all age groups and gender who have been on second-line anti-retroviral therapy (ART) at least six months as at the time of the study, and have at least one documented viral load result after the commencement of second-line ART.

Data collection

Relevant demographic ad clinical data such as age, gender, functional status, WHO clinical staging, ART regimen (at start and current) and ART adherence level were obtained from the patients' electronic medical record (EMR) with triangulation from patients' case folders. Confidentiality was strictly maintained throughout the study period in a computerized form through adequate security provision regarding data storage on the computer system.

Statistical analysis

The data analysis was done using the Statistical Package for the Social Sciences (SPSS) for windows version 24.0 software (SPSS Inc; Chicago, IL, USA). Frequency counts was generated for all variables and statistical test of significance for categorical data was performed with Chi-square test. Other data were expressed as mean ± standard deviation and analysed using the analysis of variance (ANOVA) with multiple comparisons done by Post Hoc Bonferroni test. Statistical significance was fixed at $p < 0.05$ and highly significance at $p < 0.01$.

Results:

Socio-demographic and clinical data

A total of 249 subjects (71 males and 178 females) eligible for the study were recruited. Majority of the subjects (54.7%) are in the age range 35-49 years with mean age (± SD) of 44.21 ± 11.45 years (Table 1). The mean number of years the patients have been on ART regimen is 7.92±2.68 years. The mean number of years the patients were on first line ART regimen before being switched to second line is 4.27±2.63 years. All the subjects were active on antiretroviral treatment.

Using the WHO strategy, those with viral load <1000 RNA copies/ml (suppressed viral load) were 216 (86.7%), out of which 113 (45.4%) had viral load <20 RNA copies/ml, while 33 (13.3%) had viral load >1000 RNA copies/ml (unsuppressed viral load). At the commencement of ART, 151 (60.6%) patients were on zidovudine, lamivudine and nevirapine regimen, 91 (36.5%) on tenofovir, lamivudine and efavirenz, while 7 (2.8%) were on zidovudine, lamivudine and efavirenz.

Discussion:

This study outcome shows that 86.7% of the patients had suppressed viral load based on the viral load outcome using the WHO strategy for surveillance and monitoring of HIV drug resistance in low-and-middle-income-countries (LMICs), which indicated that viral load of <1000 RNA copies/ml should be taken as evidence of viral suppression (13). This outcome differs from that of Myanmar study which reported 36.5% virological failure among HIV patients on second line ART (10) as against 13.3% in our study. This finding implies generally that more patients in our study demonstrated treatment improvement with impressive suppression rate (86.7%) that can be alluded to the use of the protease inhibitors, and better drug adherence with 91.2% having good adherence (≥ 95%) than when they were on first line regimen where the adherence level was 82.3% and suppression rate was 83.1%. Our finding from monthly dose assessment carried out revealed that missed doses in patients on ARV drugs taken twice daily was ≤ 3 in a month while missed doses for ARV drugs taken once daily was only once a month. This finding may be due to reported general minimal side effects of the protease inhibitors in the combination, especially atazanavir/ritonavir (14).

The Chi-square analysis and odd ratio (OR) showed that the presence of opportunistic infections did not have significant impact on viral load outcome in our study [χ^2 =1.15, OR: 1.05 (95% CI: 0.11 –1.76), p= 0.283], which may be occasioned by the fact that only about 5% of the patients had reported mild cases of opportunistic infections. Generally, opportunistic infections are caused by non-pathogenic micro-organisms which become pathogenic when the immune system is impaired by an unrelated disease. Their prevention or effective treatment when detected has always formed a core part of the HIV treatment and management program, with the use of antibiotics and antifungal medications in order to significantly reduce morbidity and mortality thus prolonging the lives of the patients (15).

However, antiretroviral (ARV) adherence level significantly affected the viral load outcome of the patients, as patients on second line ART having 91.2% good adherence (> 95% adherence) had suppressed viral load of 86.7% compared to patients on first line ART having 82.3% good adherence with suppressed viral load of 83.1% (χ^2=16.03, OR: 2.15 (95% CI 0.37-7.31), p<0.001]. This indicates that patients on second-line ART tend to miss fewer doses, which may be due to the reported general minimal side effects of the

Table 1: Socio-demographic and clinical data of patients living with HIV on second line anti-retroviral therapy in Ondo and Ekiti States, Nigeria

Variables	Frequency (%)
Age group (years)	
15 – 19	5 (2.0)
20 - 24	4 (1.6)
25 – 29	6 (2.4)
30 – 34	25 (10.0)
35 – 39	47 (18.9)
40 – 44	52 (20.9)
45 – 49	37 (14.9)
50 – 54	31 (12.4)
55 – 60	20 (8.0)
≥ 60	22 (8.8)
Gender	
Male	71 (28.5)
Female	178 (71.5)
Current WHO clinical staging	
Stage I	179 (71.9)
Stage II	37 (14.9)
Stage III	31 (12.4)
Stage IV	2 (0.8)
First line antiretroviral therapy (ART)	
Tenofovir, Lamivudine & Efavirenz	91 (36.5)
Zidovudine, Lamivudine & Efavirenz	7 (2.8)
Zidovudine, Lamivudine & Nevirapine	151 (60.6)
Second line antiretroviral therapy (ART)	
Zidovudine, Lamivudine & Lopinavir/Ritonavir	18 (7.2)
Abacavir, Lamivudine & Atazanavir/Ritonavir	4 (1.6)
Abacavir, Lamivudine & Lopinavir/Ritonavir	2 (0.8)
Tenofovir, Lamivudine & Atazanavir/Ritonavir	104 (41.8)
Tenofovir, Lamivudine & Lopinavir/Ritonavir	121 (48.6)
Total number of years active on ART	
3	20 (8.0)
4	6 (2.4)
5	12 (4.8)
6	35 (14.1)
7	49 (19.7)
8	28 (11.2)
9	30 (12.0)
10	20 (8.0)
11	25 (10.0)
12	14 (5.6)
13	5 (2.0)
14	3 (1.2)
15	2 (0.8)
Number of years on first line ART regimen before switching	
1	44 (17.7)
2	27 (10.8)
3	47 (18.9)
4	27 (10.8)
5	29 (11.6)
6	23 (9.2)
7	15 (6.0)
8	14 (5.6)
9	17 (6.8)
10	4 (1.6)
11	0 (0.0)
12	2 (0.8)
Adherence on first line ART	
Good (≥ 95%)	205 (82.3)
Fair (85 – 94%)	31 (12.5)
Poor (< 85%)	13 (5.2)
Adherence on second line ART	
Good (≥ 95%)	227 (91.2)
Fair (85 – 94%)	16 (6.4)
Poor (< 85%)	6 (2.4)
Viral load outcome on first line ART	
Suppressed viral load	207 (83.1)
Unsuppressed viral load	42 (16.9)
Viral load outcome on second line ART	
Suppressed viral load	216 (86.7)
Unsuppressed viral load	33 (13.3)
Current tuberculosis status	
No signs of tuberculosis	230 (92.4)
Presumptive tuberculosis with signs	19 (7.6)
Opportunistic infections (OIs)	
Present	13 (5.2)
Absent	236 (94.8)

Table 2: Impact of opportunistic infections and anti-retroviral therapy adherence on viral load outcome

Variables	X^{2*}	OR	95% CI	*p* value
Impact of opportunistic infection on viral load outcome	1.15	1.05	0.11-1.76	0.283
Impact of ARV drugs adherence level on viral load outcome	16.03	2.15	0.37-7.31	<0.001

*Null hypothesis is rejected when the test statistic (χ^2) is greater than the critical value of 3.84; X^2 = Chi square; OR = Odd ratio; CI = Confidence interval

protease inhibitors in the combination, especially atazanavir/ritonavir (14), resulting in higher compliance and therefore higher suppression rates. Good adherence (> 95%) has always been associated with the beliefs regarding the positive impact of the medications on the patient's quality of life.

Conclusion:

This study shows that about 13% of the HIV-infected patients on second line ART had unsuppressed viral load of more than 1,000 RNA copies/ml, indicating virological failure. With critical factors such as poor adherence to ART and drug resistance chiefly contributing to virological failure, routine adherence and viral load monitoring as well as the availability of HIV drug resistance testing to determine the resistance pattern of a specific HIV strain to ARV drugs in use will ensure that more patients comply with their regimen.

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Correspondence **Open Access**

Non-enteric adenovirus among children with gastroenteritis in Warri, Southern Nigeria

*[1,2]Osazuwa, F., [2]Johnson, W., and [2]Grobler, H. S.

[1]MDS Molecular Services, Sub-Saharan African Office, Jabi, Abuja, Nigeria
[2]MDS Molecular Services, Johannesburg, South Africa
*Correspondence to: favourdesires@gmail.com; +2348100792725

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Adénovirus non entérique chez les enfants atteints de gastro-entérite à Warri, dans le sud du Nigéria

*[1,2]Osazuwa, F., [2]Johnson, W., et [2]Grobler, H. S.

[1]MDS Molecular Services, Bureau de l'Afrique subsaharienne, Jabi, Abuja, Nigéria
[2]MDS Molecular Services, Johannesburg, Afrique du Sud
*Correspondance à: favourdesires@gmail.com; +2348100792725

Dear Editor,

Gastroenteritis is a major cause of mortality among children in Sub-Saharan Africa and in different parts of the world affected by poverty, malnutrition and poor health standards. Human adenovirus most especially the species belonging to HAdV-F types 40, 41, and HAdV-G type 52 are majorly implicated in childhood gastroenteritis (1), while other species are associated with respiratory disease, obesity and conjunctivitis (2). We here present an unusual finding of the detection of an adenovirus species potentially known to be associated with respiratory disease in the stool specimen of a child with gastroenteritis in Warri, South-South geopolitical region of Nigeria.

A cross-sectional study was conducted from March, 2018 to February, 2019. A total of 75 children with diarrhea from a pool of 356, attending paediatric outpatient clinic of Central Hospital, Warri, South-South region, Nigeria, were randomly selected. The protocol for this study was approved by the Ethics and Research Committee of the Ministry of Health of Delta with reference numbers CHW/VOL14/130. Consent to participate in this study was obtained from each parent or guardian on behalf of the participants. Children with at least 3 episodes of diarrhea within 1 to 7 days, whose parents or guardians consented for their ward/children to participate were included in the study.

Stool specimens were collected into clean universal containers. The supernatants obtained from stool suspension of 50% in 1 ml sterile phosphate buffered saline were stored at -20°C for PCR assay of adenovirus. The stool specimens were initially screened for adenovirus using a rapid lateral flow immunochromatographic (LF-ICT) assay kit (Gastro Vir-Strip, Coris Bioconcept, Belgium). The result of this preliminary screening has been published (3).

DNA was extracted from all stool specimens of children positive for adenovirus by the LF-ICT assay (11/75) using QIAamp DNA stool kit (Qiagen, Germany) according to the manufacturer instructions, and stored at −20 °C till time of amplification. The primer pairs used for real-time PCR of the partial gene regions of the viral capsid genes, were Ad-1 (5′-TTCCCCA TGG CICAYAACAC-3′) and Ad-2 (5′-CCCTGGTA KCCR ATRTTGTA-3′). The PCR cycling program was as follows; 94°C for 4 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min and a final extension cycle at 72°C for 7 min. The amplicons were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). Nucleotide sequencing was done using Big Dye Terminator v 3.1 Cycle sequencer

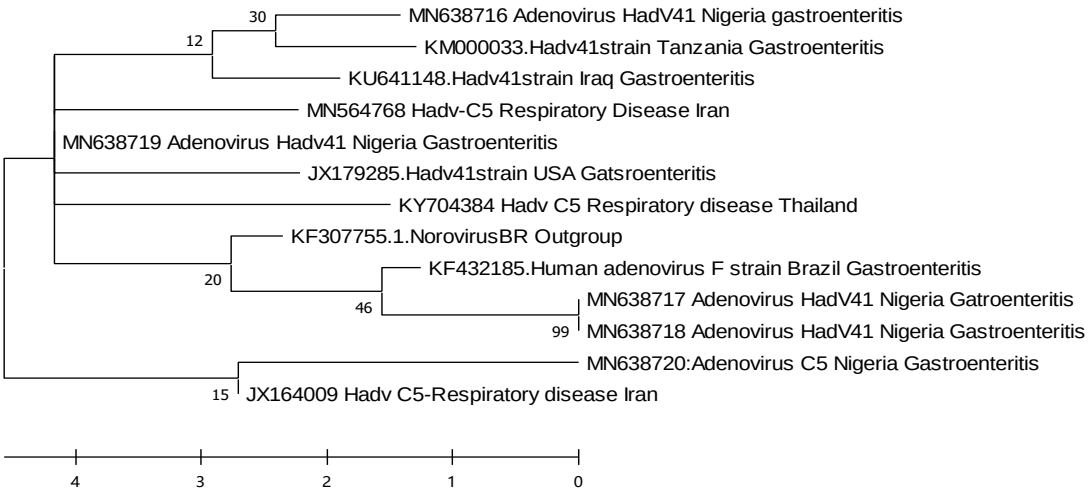

Fig 1: Phylogenetic tree of human adenovirus detected among children in Warri, Southern-Nigeria

(Applied Biosystems, Carlsbad, CA) on 3130 DNA genetic analyzer (Applied Biosystems, Carlsbad, CA), and sequences were edited using sequencher® version 5.4.6 DNA sequence analysis software (Gene codes Corporation, Ann Arbor, MI, USA). Phylogenetic analysis of the detected human adenovirus was conducted using the MEGA X. All HAdv nucleotide sequences obtained in this study were deposited into National Centre for Biotechnology Information (GenBank: http://www.ncbi.nlm.nih.gov/) under the accession number MN638716-MN638720.

Five of 11 (45.0%) children were positive for HAdv on PCR. The report of phylogenetic analysis shows that 4 of the HAdv (MN638716-MN638719) clustered with the well-known HAdv genotype responsible for majority of HAdv associated gastroenteritis, HAdv F 41 (Fig 1). It was observed that one of the HAdv detected (MN638720) clustered with HAdv C 5, an HAdv genotype usually detected in patients with respiratory disease.

The finding of non-enteric HAdv species among children with diarrhea has been reported in previous studies in other countries (4,5,6). This study shows that non-enteric HAdv may also play a potential role in childhood gastroenteritis in our study area. A major limitation of this study was the small sample size. Large sample size would have help establish the frequency of detection of non-enteric genotypes of HAdv among children with gastroenteritis in our study area. Further studies involving a large sample pool is advocated.

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Authors' contribution:

This study is part of the first author's doctoral thesis in Medical Molecular Biology, University of Benin, Benin City.

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CORRIGENDUM

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Corrigendum

Antimicrobial Stewardship Implementation in Nigerian Hospitals: Gaps and Challenges

*[1]Iregbu, K. C., [1]Nwajiobi-Princewill, P. I., [1]Medugu, N., [2]Umeokonkwo, C. D., [3]Uwaezuoke, N. S., [4]Peter, Y. J., [5]Nwafia, I. N., [6]Elikwu, C., [7]Shettima, S. A., [8]Suleiman, M. R., [9]Awopeju, A. T. O., [10]Udoh, U., [11]Adedosu, N., [12]Mohammed, Y., [13]Oshun, P., [14]Ekuma, A., [15]Manga, M. M., [16]Osaigbovo, I. I., [17]Ejembi, C. J., [18]Akujobi, C. N., [19]Samuel, S. O., [20]Taiwo, S. S., and [13]Oduyebo, O. O.

[1]National Hospital Abuja, Nigeria; [2]Alex Ekwueme Federal Teaching Hospital, Abakaliki; [3]Federal Medical Centre, Jabi, Abuja, Nigeria; [4]University of Abuja Teaching Hospital, Gwagwalada; [5]University of Nigeria Teaching Hospital, Enugu; [6]Babcock University Teaching Hospital, Ilishan-Remo, Nigeria; [7]Federal Medical Centre, Yola; [8]Federal Medical Centre, Katsina; [9]University of Port Harcourt Teaching Hospital, Port Harcourt; [10]University of Calabar Teaching Hospital, Calabar; [11]Federal Medical Centre, Owo; [12]Usmanu Danfodiyo University Teaching Hospital, Sokoto; [13]Lagos University Teaching Hospital, Lagos; [14]University of Uyo Teaching Hospital, Uyo; [15]Federal Teaching Hospital, Gombe; [16]University of Benin Teaching Hospital, Benin-City; [17]Ahmadu Bello University Teaching Hospital, Zaria; [18]Nnamdi Azikiwe University Teaching Hospital, Nnewi; [19]Irrua Specialist Teaching Hospital, Irrua; [20]Ladoke Akintola University of Technology Teaching Hospital, Ogbomoso, Nigeria.
*Correspondence to: keniregbu@yahoo.co.uk

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In the published article, there was an error in the initial of the author, "Mohammed A", instead of "Mohammed Y". This error has been corrected as appropriate.

Mise en œuvre de la gestion des antimicrobiens dans les hôpitaux Nigérians: lacunes et défis

*[1]Iregbu, K. C., [1]Nwajiobi-Princewill, P. I., [1]Medugu, N., [2]Umeokonkwo, C. D., [3]Uwaezuoke, N. S., [4]Peter, Y. J., [5]Nwafia, I. N., [6]Elikwu, C., [7]Shettima, S. A., [8]Suleiman, M. R., [9]Awopeju, A. T. O., [10]Udoh, U., [11]Adedosu, N., [12]Mohammed, Y., [13]Oshun, P., [14]Ekuma, A., [15]Manga, M. M., [16]Osaigbovo, I. I., [17]Ejembi, C. J., [18]Akujobi, C. N., [19]Samuel, S. O., [20]Taiwo, S. S., et [13]Oduyebo, O. O.

*[1]Hôpital national d'Abuja, Nigéria; [2]Hôpital universitaire fédéral Alex Ekwueme, Abakaliki; [3]Centre médical fédéral, Jabi, Abuja, Nigéria; [4]Hôpital d'enseignement de l'Université d'Abuja, Gwagwalada; [5]Hôpital d'enseignement de l'Université du Nigéria, Enugu; [6]Hôpital universitaire de Babcock, Ilishan-Remo, Nigéria; [7]Centre médical fédéral, Yola; [8]Centre médical fédéral, Katsina; [9]Hôpital d'enseignement de l'Université de Port Harcourt, Port Harcourt; [10]Hôpital d'enseignement de l'Université de Calabar, Calabar; [11]Centre médical fédéral, Owo; [12]Hôpital universitaire Usmanu Danfodiyo, Sokoto; [13]Hôpital universitaire de Lagos, Lagos; [14]Université de l'hôpital d'enseignement d'Uyo, Uyo; [15]Hôpital universitaire fédéral de Gombe; [16]Hôpital d'enseignement de l'Université du Bénin, Benin-City; [17]Hôpital universitaire d'Ahmadu Bello, Zaria; [18]Hôpital universitaire Nnamdi Azikiwe, Nnewi; [19]Hôpital universitaire spécialisé d'Irrua, Irrua; [20]Hôpital d'enseignement de l'Université de technologie Ladoke Akintola, Ogbomoso, Nigéria
*Correspondance à: keniregbu@yahoo.co.uk

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Dans l'article publié, il y avait une erreur dans l'initiale de l'auteur, «Mohammed A», au lieu de «Mohammed Y». Cette erreur a été corrigée le cas échéant.

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3. Bakare, R. A., Oni, A. A., Okesola, A. A., et al. Efficacy of pefloxacin on acute uncomplicated gonococcal urethritis. Nig Qt J Hosp Med. 1996; 6: 335

For books:

1. Arya, O. P., Osoba, A. O., and Bennett, P. Tropical Venereology, Churchill Livingstone, Edinburgh, 1980 OR when referring to a chapter in a book and where the names of authors are also given, the reference should be as follows:

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