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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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July 2022; Volume 23: Number 3: Pages 221 - 334

TABLE OF CONTENTS

Musa-Booth et al. *Afr. J. Clin. Exper. Microbiol.* 2022; 23 (3): 221 - 226 https://www.afrjcem.org

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Mini-Review **Open Access**

Evolution of SARS-CoV-2 variants: a mini-review

[1]Musa-Booth, T. O., [*2]Adegboro, B., and [2]Medugu, N.

[1]1928 Woodlawn Drive, Woodlawn, Maryland 21207, USA
[2]Department of Medical Microbiology and Immunology, Nile University of Nigeria, Abuja, Nigeria
[*]Correspondence to: boazadegboro@gmail.com; boaz.adegboro@nileuniversity.edu.ng

Abstract:

SARS-CoV-2 has evolved over time with several mutations, especially on the spike protein, which has led to emergence of various variants. With the evolution of SARS-CoV-2 come new challenges in surveillance, effectiveness of preventive and treatment strategies, and outcome of the disease. Despite the lockdowns, mask mandates and other preventive measures put in place, in addition to over 10 million vaccine doses that have been administered globally as of February 2022, COVID-19 cases have risen to over 435 million and resulted in over 5.9 million deaths, largely as a result of the evolution of SARS-CoV-2 variants. To review the evolution of these variants, we searched different online database sources using keywords such as "source of SARS-CoV-2", "SARS-CoV-2 origin", "evolution of SARS-CoV-2", "SARS-CoV-2 variants", "variants of concern", "variants of interest", and "variants of high consequence". This was to enable us give a good report about the various variants of SARS-CoV-2 that have emerged so far, and the public health challenges posed by them.

Keywords: SARS-CoV-2; COVID-19; variants of concern; variants of interest; variants of high consequence

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Évolution des variantes du SARS-CoV-2: une mini-revue

[1]Musa-Booth, T. O., *[2]Adegboro, B., et [2]Medugu, N.

[1]1928 Promenade Woodlawn, Woodlawn, Maryland 21207, États-Unis
[2]Département de Microbiologie Médicale et d'Immunologie, Université du Nil du Nigéria, Abuja, Nigéria
*Correspondance à: boazadegboro@gmail.com; boaz.adegboro@nileuniversity.edu.ng

Résumé:

Le SRAS-CoV-2 a évolué au fil du temps avec plusieurs mutations, notamment sur la protéine de pointe, ce qui a conduit à l'émergence de diverses variantes. Avec l'évolution du SARS-CoV-2 viennent de nouveaux défis dans la surveillance, l'efficacité des stratégies de prévention et de traitement, et les résultats de la maladie. Malgré les confinements, les masques obligatoires et les autres mesures préventives mises en place, en plus de plus de 10 million de doses de vaccins qui ont été administrées dans le monde en février 2022, les cas de COVID-19 sont passés à plus de 435 million et ont entraîné plus de 5,9 million de décès, en grande partie à la suite de l'évolution des variantes du SRAS-CoV-2. Pour examiner l'évolution de ces variantes, nous avons effectué des recherches dans différentes sources de bases de données en ligne à l'aide de mots clés tels que «source du SARS-CoV-2», «origine du SARS-CoV-2», «évolution du SARS-CoV-2», «SARS- variantes du CoV-2», «variantes préoccupantes», «variantes d'intérêt» et «variantes à conséquence élevée». C'était pour nous permettre de faire un bon rapport sur les différentes variantes du SRAS-CoV-2 qui ont émergé jusqu'à présent, et les défis de santé publique qu'elles posent.

Mots-clés: SARS-CoV-2; COVID-19; variantes préoccupantes; variantes d'intérêt; variantes de grande conséquence

Introduction:

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which caused the current coronavirus disease-2019 (COVID-19) pandemic originated from Wuhan, China (1). The virus is a positive sense RNA virus belonging to the Coronaviridae family, subgroup B Betacoronavirus, and is new to humans (1). It was first reported in December 2019 with many of the early cases (mainly market vendors and market workers) being associated with the Huanan seafoods wholesale market (1,2). SARS-CoV-2 is transmitted between humans by respiratory droplets and is reported to have much higher infectivity than SARS-CoV and Middle East respiratory syndrome-coronavirus (MERS-CoV) (1,3,4).

The virus infects humans by binding with its spike protein to angiotensin converting enzyme 2 (ACE-2) receptors on the inside of the nose. It has a median incubation period of four to five days and symptoms are usually seen within 2 to 14 days of exposure to the virus. COVID-19 symptoms can range from mild, moderate, severe, to critical (death), with risk of severity increasing with age and severe disease usually occurring in persons of all ages with underlying conditions such as respiratory disease, cardiac disease, and diabetes (5). There could also be associated complications in severe cases leading to hospitalization and the need for intensive care which may ultimately lead to death. The symptoms include but are not limited to cough, shortness of breath or difficulty breathing, fever, chills, muscle pain, sore throat, new loss of smell or taste (5). Some other features unique to COVID-19 include its tendency to infect endothelial cell lining, hypercoagulability, multisystem inflammatory syndrome, and long-term sequelae (1).

Despite the lockdowns, mask mandates and other preventive measures put in place, in addition to over 10 million vaccine doses administered globally, there have been over 435 million SARS-CoV-2 cases and 5.9 million deaths from COVID-19, as of February 28, 2022 (6). This has been due largely to the evolution and emergence of different variants of the virus, which has impacted the overall disease outcome, in addition to the lack of adherence to preventive measures, vaccine hesitancy, and inequity in vaccine distribution. Although some studies have reported on the sources and variants of SARS-CoV-2, we seek to bring together in this mini-review what is known about the source of the virus, different variants, and the challenges that each of these variants pose to global health.

Methodology and Results:

In conducting this mini-review, different online databases including PubMed, Google Scholar and Web of Science were searched using keywords such as "source of SARS-CoV-2", "SARS-CoV-2 origins", "evolution of SARS-CoV-2", "SARS-CoV-2 variants", "variants of concern", "variants of interest", and "variants of high consequence". A total of 12,900 articles were identified following the initial search, and after deduplication, 3595 articles were identified. Abstracts of the articles were subsequently reviewed, which reduced the number of articles to 660. Full text review of the articles reduced the number of articles relevant to the study to a total of 35 articles (Fig. 1).

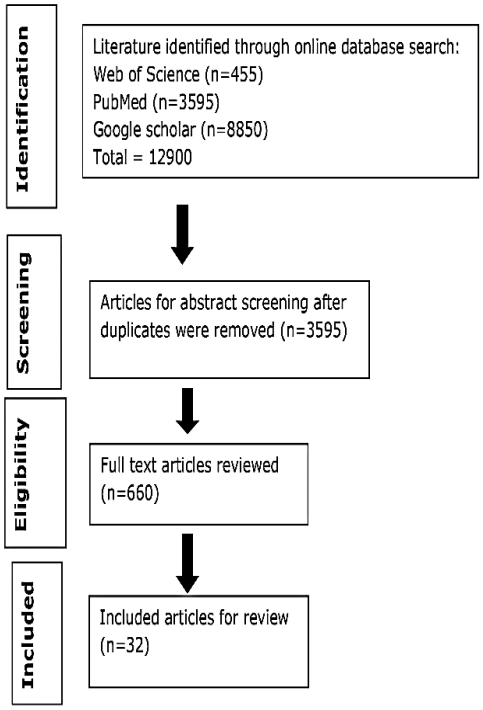

Fig 1: Process of selection of publications (PRISMA guide) used for the mini-review

Discussion:

Origin/source of SARS-CoV-2

Three years into the COVID-19 pandemic, the origin of the causative virus, SARS-CoV-2, remains elusive. SARS-CoV-2 is the seventh coronavirus to infect humans with, SARS-CoV, MERS-CoV and SARS-CoV-2 associated with severe disease while the other four coronaviruses are associated with mild diseases (3,7). SARS-CoV-2 was first reported in Wuhan, Hubei Province, China among individuals who presented with a novel pneumonia (1,2,3), al-

though findings from review of data from different countries suggest that SARS-CoV-2 could have been circulating a few weeks prior to the first reported case in Wuhan (1). Molecular sequence data also suggests that the outbreak occurred sometime in the months before mid-December 2019 (3). This suggests that the virus may have been introduced to humans through direct zoonotic spillover in which the reservoir population with high virus prevalence meets a novel host population, transmitting the virus to the host with or without transmission among the host (3).

The origin of SARS-CoV-2 can also be understood by looking back at the 2002 SARS-CoV outbreak in China which had zoonotic origins linked to live animals (civet cats), spillover events and laboratory accidents (2). A theme common to both SARS and COVID-19 is the trading of viral host animals with studies showing that the coronaviruses most highly related to SARS-CoV-2 are found in bats and pangolins making them possible reservoirs of the COVID-19 virus although neither are direct progenitors and there were no positive results with extensive animal and animal product sampling/testing in the 31 Provinces of China (1,2). Therefore, the intermediate host remains unknown.

The virus has also been found to be linked to the cold chain, persisting on packaging, frozen foods and cold-chain products supplied to China from other countries although initial tests done at the Wuhan market did not reveal any positive results from the cold chain packages (1). This could be a possible introductory route for the virus.

The final source proposed is through introduction into humans by a laboratory accident and this route has been reported as extremely unlikely because prior isolation of a progenitor virus with very high genetic similarity would be required for this to occur, which is not the case (1,3).

Distribution of SARS-CoV-2 variants

A characteristic of viruses which helps them adapt to their environment and evade detection by the immune system is their ability to mutate/change their genetic code leading to new variants of the viruses. This evolution of the virus can make them infective or virulent which may lead to persistence or elimination of the virus. As a result of this viral trait, variants will keep emerging and will require monitoring to enable appropriate actions to be taken. SARS-CoV-2 has shown this trait and evolved over time with several mutations, especially on the spike protein leading to various variants. The mutations have primarily been through purifying selection and to a lesser degree by positive pressure on specific protein coding genes (8,9). Some environmental factors such as temperature and humidity, physical irradiation and biocidal agents, climate change, and air pollution have been shown to affect infectivity, transmission, and spread of SARS-CoV-2 but the environmental impact on mutagenicity remains unclear (10). With the evolution of SARS-CoV-2, come new challenges in surveillance, effectiveness of preventive and treatment strategies, and outcome of the disease. The SARS-CoV-2 variants can be classified into several types as discussed below

Variants to be monitored

These are variants that show potential or confirmed impacts on the medical countermeasures approved for use to mitigate spread of the virus. Variants with low level circulation or that previously caused severe disease or enhanced transmission also fall into this category. Several variants of concern and variants of interest have been downgraded to this class due to sustained significant reduction in the circulating levels of these variants such that they no longer pose a threat to public health. Variants to be monitored include B 1.640 and XD. These variants are under monitoring but could be upgraded if the data, which are being constantly evaluated, suggests an increase in the circulating variant or shows increased risk to public health. This is a challenge with variants to be monitored.

Variants of interest (VOI)

This is a variant that shows specific genetic markers with changes to its ability to bind to receptors leading to certain consequences including; reduction in antibodies previously developed from natural infection or vaccines, reduced ability to neutralize the variants, reduced treatment efficacy, increase in transmission or severity of the disease or effects on diagnosis (11). Appropriate public health actions are required to mitigate the spread of VOI and these include assessment of the virus ease of spread, disease severity, increased surveillance and laboratory characterization, efficacy of prevention and treatment options.

At present, there are no variants in this category but previously considered VOIs include; (i) Epsilon B.1.427, and B.1.429, which were first detected in the USA and designated VOI in March 2021, but re-designated as previous VOI in July 2021; (ii) Zeta P.2 was first detected in

Brazil in April 2020, designated as VOI in March 2021 and redesignated as a previous VOI in July 2021; (iii) Eta B.1.525 was first detected in April 2020 in many different countries, was designated VOI in March 2021, and re-designated a previous VOI in September 2021; (iv) Theta P.3 was first detected in April 2020 in many different countries, was designated VOI in March 2021 and re-designated a previous VOI in July 2021; (v) Iota B.1.526 was first reported in the USA in November 2020, designated VOI in March 2021 and redesignated a previous VOI in September 2021; (vi) Kappa B.1.617.1 was first reported in India in October 2020, designated VOI in April 2021 and redesignated VOI in September 2021; (vii) Lambda was first reported from Peru in June 2021, assigned as VOI in June 2021, and redesignated a previous VOI in March 2022; and (viii) Mu B.1.621 was first reported in Colombia in January 2021, designated a VOI in August 2021 and redesignated a previous VOI in August 2021.

Variants of concern (VOC)

This is a variant with one or more mutations that allow the virus to infect people more easily or spread from person to person more easily, make the virus less responsive to treatments, or affect how well vaccines work against the virus (12).

(i) Omicron - B.1.1.529 and BA lineages

This lineage was first identified in South Africa in November 2021 and shown to be more infective or spreads easily (13,14), but less virulent than other variants thereby causing less severe disease in general (15-19). Omicron has at least 30 mutations in its spike protein, a marked increase from Beta which had 10 and Delta with 9 (20). Omicron is currently the dominant variant globally and the challenge with this variant is that, although it is associated with less severe disease, more people can be infected in a shorter time leading to increased hospitalization, overwhelming the health system and ultimately causing increased mortality (11,20).

Breakthrough infections have also been reported because of this variant among vaccinated individuals, but boosters are effective for preventing severe disease, hospitalization, and deaths (21,22). Infection with this variant can also be treated with monoclonal antibodies. Currently, the subvariant BA.2 is circulating rapidly in Asia and Europe, although the incidence is rising in the United States as well. It is more transmissible than the subvariant BA.1 while BA.1.1 and B.1.1.529 subvariants have higher incidence in the United States.

(ii) Delta - B.1.617.2 and AY lineages 99

This lineage was first identified in India in December 2020 and found to spread more easily than previous variants (11,23), and also causes more severe disease than other variants (11,24,25). Although the authorized vaccines are effective against this variant, breakthrough infections and transmission were seen among fully vaccinated individuals (11,24,26-28).

Previously circulating variants of concern or de-escalated variants

These variants have now been de-escalated either because they have been circulating without serious epidemiological impacts, or because the variants have ceased circulating or scientific evidence does not show any concerning properties.

(i) Alpha (B.1.1.7 and Q lineages)

This lineage was first identified in the United Kingdom with approximately 50% increase in transmissibility than the original SARS-CoV-2 (12). Following the emergence of the Delta variant however, circulation of this strain has mostly ceased.

(ii) Beta (B.1.351 and descendent lineages)

This lineage was initially identified in South Africa in September 2020 with greater transmissibility and more disease severity (29-31) than alpha variant and the original SARS-CoV-2. Beta variant is also less responsive to monoclonal antibodies and vaccines (12,32,33). From the 9th of March 2022, Beta (B.1.351) variant was removed from being a VOC and de-escalated to a variant of previous concern because of limited circulation.

(iii) Gamma (P.1 and descendent lineages)

This was first seen in Brazil in December 2020. This variant had increased transmissibility and disease severity than the original SARS-CoV-2 but less than alpha variant (31,34). It also showed reduced responses to monoclonal antibodies and vaccines as the beta variant (12, 35). The Gamma (P.1) variant was also de-escalated on the 9th of March 2022 to become a variant of previous concern.

Variants of high consequence (VOHC)

Variants with proven evidence of significant reduction in effectiveness of preventive measures or medical countermeasures when compared with previous variants in circulation are variants of high consequence (VOHC) (11, 12). The presence of these variants will require notification of international health regulatory

bodies for preventive and control measures to be updated to contain the variants and maintain the health of the global population.

The challenge with VOHC is that they have the characteristics of VOCs and additional attributes including; demonstrated failure of the diagnostic test targets, evidence to suggest a significant reduction in vaccine effectiveness, a disproportionately high number of infections in vaccinated persons, or very low vaccine-induced protection against severe disease, significantly reduced susceptibility to multiple emergency use authorized (EUA) or approved therapeutics, and more severe clinical disease and increased hospitalizations. There are no variants currently in this category.

Conclusion:

SARS-CoV-2 which caused the COVID-19 pandemic has evolved over time and keeps evolving, thereby creating new variants with varying impact on transmissibility, disease severity and immune response because of mutations usually in the spike protein, that enable the virus to adapt and escape the immune system. The rapidity with which the virus has evolved over time is an indication that more variants will emerge.

Anticipating this evolution should therefore influence genetic sequencing, epidemiological surveillance, environmental factors evaluation, vaccine improvement and uptake, as well as other preventive measures and treatment modalities required to minimize the spread of the virus and optimize the global disease outcome.

Authors contributions:

TOM conducted the literature review, wrote and edited the manuscript. BA designed the review outline, proofread and edited the manuscript. NM edited the review. All authors contributed to the article and approved the submitted version.

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Conflict of interest:

The authors declare that they have no known conflict of interests that could have influenced the work reported in this review.

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Review Article **Open Access**

Overview of human and animal brucellosis in Nigeria and its economic impacts on production

*[1]Ukwueze, C. S., [2]Kalu, E., [3]Odirichukwu, E. O., [4]Ikpegbu, E., and [5]Luka, P. D.

[1]Department of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike, Nigeria
[2]Department of Veterinary Public Health and Preventive Medicine, Michael Okpara University of Agriculture, Umudike, Nigeria
[3]Department of Veterinary Theriogenology, Michael Okpara University of Agriculture, Umudike, Nigeria
[4]Department of Veterinary Anatomy, Michael Okpara University of Agriculture, Umudike, Nigeria
[5]Biotechnology Division, National Veterinary Research Institute, Vom, Nigeria
*Correspondence to**:** ukwueze.chigozie@mouau.edu.ng; +2348030793359

Abstract:

Brucellosis is a wide spread zoonotic bacterial disease of humans and animals**.** In humans the disease is recognized commonly as undulant fever, characterized by headache malaise, and arthritis. Brucellosis can occur in any age group, but mainly found in young men between the ages of 20 and 40 years because of occupational hazards. Domestic animals (cattle, sheep and goat, pigs, dogs etc) are highly susceptible to brucellosis. Generally, brucellosis manifest in female animals as abortion, retained placenta, stillbirth and death of young ones soon after birth. In males, the main features are vesiculitis, orchitis, and epididymitis, which may render infected male infertile for life. The endemicity of brucellosis has remained a threat in low- income countries of sub-Saharan Africa and South Asia and has multiple economic implications across agriculture and public health sectors, and broader implications on economic and social development sectors. Google and Google Scholar were used to retrieve articles used for this review, which included published research articles and local, national and international reports on brucellosis. In this review, we summarised human and animal brucellosis, prevalence of infections in Nigeria, and economic impacts on production. It is believed that this review will guide researchers on the state of brucellosis in developing countries where the disease is still endemic, using Nigeria as a case study.

Keywords: undulant fever; public health; abortion; orchitis; economic impact

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Aperçu de la brucellose humaine et animale au Nigéria et de ses impacts économiques sur la production

*[1]Ukwueze, C. S., [2]Kalu, E., [3]Odirichukwu, E. O., [4]Ikpegbu, E., et [5]Luka, P. D.

[1]Département de Médecine Vétérinaire, Université d'Agriculture Michael Okpara, Umudike, Nigéria
[2]Département de Santé Publique Vétérinaire et de Médecine Préventive, Université d'Agriculture Michael Okpara, Umudike, Nigéria
[3]Département de Thériogénologie Vétérinaire, Université d'Agriculture Michael Okpara, Umudike, Nigéria
[4]Département d'Anatomie Vétérinaire, Université d'Agriculture Michael Okpara, Umudike, Nigéria
[5]Division de la biotechnologie, Institut national de recherche vétérinaire, Vom, Nigéria
*Correspondance à: ukwueze.chigozie@mouau.edu.ng; +2348030793359

Résumé:

La brucellose est une maladie bactérienne zoonotique largement répandue chez les humains et les animaux. Chez l'homme, la maladie est généralement reconnue comme une fièvre ondulante, caractérisée par des maux de tête et de l'arthrite. La brucellose peut survenir dans n'importe quel groupe d'âge, mais principalement chez

les jeunes hommes âgés de 20 à 40 ans en raison des risques professionnels. Les animaux domestiques (bovins, ovins et caprins, porcs, chiens, etc.) sont très sensibles à la brucellose. Généralement, la brucellose se manifeste chez les femelles par l'avortement, la rétention du placenta, la mortinaissance et la mort des jeunes peu après la naissance. Chez les hommes, les principales caractéristiques sont la vésiculite, l'orchite et l'épididymite, qui peuvent rendre l'homme infecté infertile à vie. L'endémicité de la brucellose est restée une menace dans les pays à faible revenu d'Afrique subsaharienne et d'Asie du Sud et a de multiples implications économiques dans les secteurs de l'agriculture et de la santé publique, ainsi que des implications plus larges sur les secteurs du développement économique et social. Google et Google Scholar ont été utilisés pour récupérer les articles utilisés pour cette revue, qui comprenaient des articles de recherche publiés et des rapports locaux, nationaux et internationaux sur la brucellose. Dans cette revue, nous avons résumé la brucellose humaine et animale, la prévalence des infections au Nigeria et les impacts économiques sur la production. On pense que cette revue guidera les chercheurs sur l'état de la brucellose dans les pays en développement où la maladie est encore endémique, en utilisant le Nigeria comme étude de cas.

Mots-clés: fièvre ondulante; santé publique; avortement; orchite; impact économique

Introduction:

Brucellosis is a worldwide bacterial zoonotic disease, caused by several species of Brucella (1,2,3). Brucella is Gram-negative coccobacilli that infect almost all species of domestic animals and man. The domestic animals mainly infected are cattle, swine, goats, sheep and dogs and *Brucella species* known to cause disease in them includes *Brucella abortus, Brucella melitensis, Brucella ovis, Brucella suis, Brucella canis, Brucella microti, Brucella inopinata, Brucella ceti, and Brucella pinnipediali* (4,5,6). In humans, the disease is caused mainly by *B. melitensis* as the most pathogenic species, followed by *B. suis,* whereas *B. abortus* is considered the mildest type of brucellosis (7,8) All ages and sexes, both humans and animals are susceptible to brucellosis, particularly humans with the culture of keeping animals especially the nomads (9,10).

Brucellosis has existed for several years, recent evidence from Egyptian ancient skeletons shows that the disease dates back to 750 BC (6). The disease was first reported in 1887 by Dr. David Bruce who isolated the organisms from patients who were living on the island of Malta and was eventually named after him as brucellosis (11). The disease is also known as Malta fever, intermittent fever, Bang's disease, undulant fever, Gibraltar fever, Mediterranean fever, contagious abortion, Maltese fever, Crimean fever, and rocky fever. Thought to have been eradicated in many developed countries, brucellosis is now a re-emerging neglected zoonosis endemic in several zones, especially in the Mediterranean regions, Europe, Africa, Middle East, South and Central Asia and the Central and South America (2,12,13). The impacts of brucellosis are highly devastating in livestock, and in humans especially among African countries, where the documentation of the disease is very poor (14,15,16).

Brucellosis is often a sub-acute or chronic disease in animals. In cattle, sheep, goats, other ruminants and pigs the initial phase following infection is often not apparent (7). In sexually mature animals the infection localizes in the reproductive system and typically produces placentitis followed by abortion in the pregnant female, usually during the last third of pregnancy, and epididymitis and orchitis in the male (3,7) Human brucellosis is a severe acute or chronic systemic disease, often insidious with symptoms similar to a severe influenza known as undulant fever, which persists for several weeks, months or longer and may get progressively worse if untreated (17). The initial symptoms are fatigue and headaches, followed by high fever, chills, drenching sweats, joint pains, backache, and loss of weight and appetite. Long-term effects can include arthritis, swelling of internal organs, depression, chronic fatigue and recurrent fevers (18). The clinical picture of brucellosis is usually not specific in animals or humans and diagnosis needs to be supported by laboratory tests. A history of recent exposure to a known or probable source of *Brucella species,* occupational exposure or residence in high infection prevalence area is a probable case of brucellosis (19).

Brucellosis is invariably transmitted by direct or indirect contact with infected animals or their products. Animals are the natural hosts of the *Brucella* organisms and are reservoirs for human infections. Humans acquire brucellosis mainly through the consumption of raw and unpasteurized milk or dairy products, inhalation, contaminated environment and tissue (2,19). Brucellosis is considered occupational hazard for veterinarians, abattoir workers and livestock keepers (6,7). Infected animal products; blood, placenta or uterine secretions are capable of gaining access to the body through broken skin and mucosa (6,20). Brucellosis has also been reported in terrestrial and marine mammals as in domestic animals (21). Recent studies have shown that increase in travel to endemic countries and have been associated with some outbreaks of brucellosis in the

human population (22,23,24). About 500,000 new cases of brucellosis are reported annually in human population (25). Animal-to-animal transmission occurs as a result of the large number of organisms shed in the environment.

Human infections are useful indicator of the presence of disease in animal populations, as animals are reservoir of infection and humans are source of information for the disease surveillance (26). The endemicity of brucellosis in Sub-Saharan Africa (15), is largely due to lack of attention and absence of adequate diagnostic facilities (3,10), lack of public awareness, inadequate public-sector animal health services, and low-income communities (2,27). In this review, we examined the previous outbreaks of brucellosis in human and animal populations in different parts of Nigeria with the view to creating awareness of its public health implications and the huge economic impacts on Nigeria economy.

Methodology and Results:

Online databases were searched for publications relevant to human and animal brucellosis in Nigeria using Google and Google Scholar engine during the month of July 2021. Publications used included research articles and local, national and international reports on brucellosis. Each of the 5 authors screened the retrieved publications for eligibility and relevance to the topic, and for adequate and verifiable referencing. Publications not relevant to the topic or that contained unverifiable information, obsolete references, inadequate referencing and duplicate information were excluded. Of the total of 96 articles obtained from the search, 64 were eligible and used for the review.

Discussion:

Distribution of brucellosis in Nigeria

Brucellosis is widespread and the prevalence of brucellosis varies from country to country and region to region in Africa and in various parts of the world (28). Nigeria is the most populous country in Africa with over 170 million as at 2012 (http://esa.un.org/wpp/ascII-data/disk_navigation_ascII.htm) and has an estimated livestock population of 20.49 million cattle, 23.07 million sheep, 28.07 million goats, 6.54 million pigs (http://www.fao.org/ag/againfo/resources/en/glw/GLW_dens.html), 18,200 – 90,000 camels, and 210,000 horses (http://faostat.fao.org/site/573/default.aspx#ancor) (29). Nigeria, India, Ethiopia, and Bangladesh account for 44% of poor livestock keepers globally, with Nigeria ranking second (14). Livestock production has always been important in Nigeria, and the rapidly emerging livestock sector now ranks second among the 20 poorest countries (14).

In Nigeria, brucellosis has been reported in 24.1% of abattoir workers in Abuja, Nigeria with occupational hazard and eating raw meat as risk factors (30). In Bauchi State Nigeria, seroprevalence rate of 33.5% was reported for human brucellosis, with 18.9% of the study population being positive for human IgG, 6.1% for human IgM and 74.15% for both IgG and IgM (19). In the north eastern part of the Nigeria, 5.2% prevalence of brucellosis among 500 occupationally exposed patients was reported (31). In another similar study in north-central part of the country, 43.8% of the 7.8% brucellosis infected hospital patients were reported to be abattoir workers (32). In the southwestern region of Nigeria, over 55% of 7161 people examined for brucellosis in different parts of the region had positive *Brucella abortus* antibodies in their sera, with higher titres found among dairy farmers and slaughter men than the general population (33). In another study in human population, Cadmus et al., (34) reported a high seroprevalence of 66.3% for brucellosis among apparently healthy abattoir workers.

In animals, a prevalence rate of 9.6% was reported in ruminants in Wukari, Taraba State (35). In slaughter cattle, the prevalence rates of 7.8% and 1.9% were reported from Oyo and Lagos (36), 20.0% prevalence in Zamfara State (37), and a herd prevalence of 32.2% in a prison cattle farm in Sokoto State (38). In the three States of Adamawa, Kano, and Kaduna, prevalence rates of 29.2%, 26.7% and 23.3% respectively, were reported (39), while the prevalence of 14.1% was reported in Obudu, Cross River State (40). In Plateau State, the prevalence rates of 37.3%, 2.5% and 3.7% were reported in Bassa, Riyom and Jos South Local Government Areas (LGAs) respectively (40). A more recent study in Kanke Local Government Area, Plateau State, reported a seroprevalence of 38.5% in a herd of cattle (41).

Human brucellosis

Human brucellosis is known by many different names such as Malta fever, Cyprus or Mediterranean fever, intermittent typhoid, Rock fever of Gibraltar, and more commonly, undulant Fever (42). Human brucellosis can occur in any age group, but the majority of cases are found in young men between the ages of 20 and 40 years. This is generally related to occupational hazards in young men (43-47). Brucellosis gained public health imp-

ortance when the bacteria were transmitted to human via unpasteurized milk, meat, and animal by-products of infected animals (48).

In humans, *Brucella* sp infection results in the formation of granulomas consisting of epithelioid cells, polymorphonuclear leukocytes, lymphocytes, and giant cells. The granulomatous response is characteristic of *B. abortus* infections. In *B. melitensis* infections, the granulomas are very small, but there is often association with toxaemia. *Bordetella suis* infection is often accompanied by chronic abscess formation in joints and the spleen (43,49,50,51)

The human disease usually manifests itself as an acute febrile illness which may progress to a chronically incapacitating disease with severe complications. It is nearly always acquired directly or indirectly from animal sources, of which cattle, sheep, goats and pigs are by far the most important. In these natural hosts, the infection usually establishes itself in the reproductive tract, often resulting in abortion. Excretion in genital discharges and milk is common and is a major source of human infection (7). The onset of symptoms is insidious and according to the length or severity of symptoms of the disease is arbitrarily classified as acute (less than 8 weeks), sub-acute (from 8-52 weeks) or chronic (more than 1 year).

The acute form is a typical brucellosis where almost all patients involved have a history of fever accompanied by weakness, malaise, headache, back-ache, anorexia, weight loss, myalgia, and arthralgia with a temperature of over 38.5^{o}C in more than 85% of patients. The sub-acute form, consist of patients who have relapsed due to incomplete or partial antibiotic treatment and patients who have received inappropriate antibiotics because of incorrect diagnosis. The clinical picture is more protean and may be an important cause of fever of unknown origin. The symptoms are generally milder, and localized infection can be seen. Chronic brucellosis is extremely rare in children but more frequent in older people and is similar to chronic fatigue syndrome. Affected patients generally suffer from a psychoneurosis, sweating, and weight loss with ocular manifestations such as episcleritis and uveitis are frequent. Fever is rare and localized infection can be seen (43,49, 51,52).

Localized brucellosis refers to cases in which organisms are not isolated from blood but are localized in specific tissues such as bone, joints, cerebrospinal fluid, liver, kidneys, spleen, or skin. Localization may be the principal manifestation of systemic infection, or may be the only manifestation of a chronic infection. Localized infection is sometimes referred to as complication when it occurs from systemic infection (49). Brucellosis also increase the risk of spontaneous abortion, premature delivery, miscarriage, and intrauterine infection with fetal death in humans as well, and is usually accompanied with malaise, fatigue, and arthritis (53,54). Human-to-human transmission is unusual. However, rare cases due to blood transfusion, bone marrow transplantation and sexual transmission have been reported (55,56).

Brucellosis in domestic animals

Brucellosis is a disease of many animal species, most especially those of food animals (cattle, sheep, goats and pigs) that produce milk, though other species such as camels, buffaloes, yaks and reindeers are also susceptible (26). Recently brucellosis has also been recognized in marine animals, which may also have the capacity to cause human infection (21). The *Brucella* species are host specific but cross species infections has been reported to occur, especially with *B. melitensis.* Infections in many wildlife species have been reported but those that obviously affect population fecundity to result in human infections are quite rare (27).

In cattle, brucellosis is usually caused by *B*. *abortus* which has been identified with seven distinct different biovars, namely 1, 2, 3, 4, 5, 6 and 9. The biovar 1 is the most important and widespread *B. abortus* biovar. Natural infection with other *Brucella* species is quite rare. In areas where *B. melitensis* infection is enzootic in small ruminants, it is rarely seen as abortion in cattle even though some infected animals may become carriers and excrete the bacteria in the urine, milk, and vaginal discharges (57,58). The major clinical sign in pregnant females is abortion in bovine and buffalo cows. Abortion usually occurs from the 5th to the 8th month of gestation. The occurrence of abortion is related to some factors such as the stage of pregnancy, the number of infecting organisms and the animal resistance (57,58) Apart from abortion, premature, stillborn or weak calves may occur. Abortion is often followed by placental retention and metritis, which may cause permanent or transient infertility (59).

In bulls, the disease is characterized by fever, vesiculitis, orchitis, and epididymitis. In severe cases, it can also be the reason for testicular abscesses, metritis or orchitis which can lead to lifetime infertility. In cattle, as well as other animals, brucellosis symptoms can be varied from severe acute to subacute or chronic, depending upon the organ

of infection and the types of animals (60). Brucellosis in sheep and goats are mainly caused by *B. melitensis* and *B*. *ovis*, though other *Brucella* species can infect sheep and goats. Biovars 1 and 3 are most frequently isolated in small ruminants in the Mediterranean, the Middle East and Latin America (61). *B. abortus* has been isolated from eight sheep and from their offspring over a period of 40 months (58,62) and *B. suis* was isolated from the semen of a ram (63). The course of *B. melitensis* infection in small ruminants is similar to *B. abortus* infection in cattle, where the main clinical manifestations of brucellosis in ruminants are abortions, stillbirths and the birth of weak offspring which usually occur in the last third of the pregnancy following infection (64). Sheep and goats usually abort only once, but reinvasion of the uterus and shedding of organisms can occur during subsequent pregnancies (65). Milk yield is significantly reduced in animals that abort, as well as in animals whose udder becomes infected after a normal birth, although mastitis is an uncommon clinical finding (62). Acute orchitis and epididymitis usually occur in males, and may result in infertility. Brucellosis may occasionally result in arthritis in both sexes and many non-pregnant sheep and goats may remain asymptomatic (57). The effect of the disease at flock level is characterized by a general decrease in flock fertility, an increase in lamb/kid mortality with a low weaning percentage, a decrease in milk production and an increased culling of males due to chronic lesion on reproductive organs (58, 66,67,68).

In pigs, *B. suis* is the only known species that causes brucellosis leading to systemic infection and reproductive problems. Pigs can also be infected by other *Brucella* species but the infection is invariably self-limiting (69). Clinical signs of *B. suis* infection in pigs vary considerably, depending on the animal age, previous exposure, and the organ involved (70). Manifestations of swine brucellosis are abortion, birth of weak piglets, infertility, orchitis, epididymitis, spondylitis of especially the lumbar and sacral regions, arthritis, paralysis of hindlimbs, and lameness, but many infected herds may have no signs. There is no pyrexia, and death is rare (69). Abscess of different sizes frequently occur in organs and tissues (59).

Abortions have been observed as early as 17 days following natural insemination by boars disseminating *B. suis* in the semen. Early abortions are usually unnoticed by the owner, and the only evidence of infection is that the sow displays signs of oestrus 30 to 45 days after mating. Little or no vaginal discharge is observed in early abortions. Abortions that occur during mid or late stages of gestation are usually associated with females that acquire infection after 35 to 40 days of pregnancy (58,70). The rate of abortion is higher in sows or gilts exposed to *B. suis* via the genital tract at the time of breeding. Abortions may occur at any time and are influenced more by the time of exposure to the *B. suis* rather than by the stage of gestation (69). Infected boars are unlikely to develop localized genital infection. However, boars that do develop genital infection hardly recover from it. Infertility and lack of sexual activity may occur in infected boars and is frequently associated with testicular abnormality. Most often, boars have infections in their accessory genital glands. Infection of the genital organs lasts for a shorter period of time in the female than in the male (58).

In dogs, *Brucella canis* infection is one of the major causes of reproductive disorders in wild and domestic dogs. The highest prevalence occurs among breeding dogs in commercial kennels (71), where significant reproductive losses can be seen. Up to 75% fewer puppies may be weaned from affected kennels according the hygienic and sanitary conditions (71,72). Clinical signs vary from asymptomatic to mild, despite an ongoing systemic infection. Morbidity is high but mortality is low. Bacteraemia develops within two to three weeks after infection but the incubation period to clinical reproductive signs is variable (73).

The major cardinal sign of canine brucellosis is late abortion, which can occur between 30-57 days of gestation, being more common from 45 to 55 days of gestation in about 75% of the cases. Abortions are followed by mucoid, serosanguineous, brownish or grey vaginal discharge that persists for up to six weeks (73,74). The infected female can produce consecutive abortions and present litters of sick born pups that die a few hours to more than one month after delivery and apparently normal offspring can also develop the disease later in life (58,75). Abortions, premature litters and conception failures are frequently observed in infected kennels. Resorption or early embryonic death within 2 to 3 weeks after breeding can also occur, which usually mistaken for failure to conceive (73,74). Pups are lost as early as 20 days or are carried nearly to term. Infected bitches may deliver a normal litter the next pregnancy or give birth to living, partly autolyzed, stillborn and normal pups that die within hours. The surviving pups are bacteraemic

for a minimum of several months (74). Other congenitally infected pups can be born normal and later develop brucellosis in life (58).

In male dogs, clinical manifestations are severe epididymitis, orchitis and prostatitis. Epididymitis usually begins 5 weeks after infection. Acute inflammation, with pain and swelling, enables physical examination and detection of orchitis and epididymitis. During the acute phase, epididymal swelling increases in size, accompanied by pain and presence of serosanguineous fluids in the tunica. Scrotal dermatitis develops from the constant licking by the male dogs, leading to infective dermatitis from secondary contamination by non-haemolytic staphylococci (74,75).

Economic impacts of brucellosis on production

Brucellosis is consistently ranked among the most economically important zoonoses globally (13,14,76). The economic impact of brucellosis varies by geography and livestock system and could be rightly referred to as multiple burdens with significant economic implications on humans, livestock and wildlife (2). Brucellosis has been successfully controlled or eliminated in livestock populations in many high-income countries persisting only in wildlife populations as sources of reservoirs infection (e. g. Bison and Elk in North America). In emerging middle-income countries, the brucellosis picture is much more variable as they tend to report the greatest number of outbreaks and animal losses with its attendant economic cost whereas, in low-income countries, the disease is endemic and neglected, with large disease and livelihood burdens in animals and people, and almost no effective control (76,77). Not surprisingly, most of these countries have less public investment in veterinary and health services, weaker surveillance and operational capacity. Endemicity of brucellosis in low-income countries of sub-Saharan Africa and South Asia has multiple economic implications across agriculture and public health, and broader implications on economic and social development sectors.

High prevalence of brucellosis associated with increasing intensification of small and medium-sized livestock enterprises and relatively uncontrolled livestock movement in traditional pasture-based systems also reflect in the economic cost. In livestock several studies have shown positive associations between greater productivity losses and higher prevalence as seropositive animals have higher rates of abortion, stillbirth, infertility and calf mortality, as well as reduced growth and longer calving intervals (2,78). Often, infected females will abort only once, although they may remain infected their entire life. Long-standing infections can result in arthritis and hygromas which is a useful marker for brucellosis at herd level.

Production losses are not just limited to pregnancy outcomes but might as well include milk especially in high income countries where it has been documented that aborting cows kept for milking produced 20% to 25% less milk for that season, while seropositive non-aborting cows produced 10% below potential (2). Animal brucellosis caused by *B. melitensis* usually occurs in outbreaks rather than in a more regular endemic pattern and the resulting productivity losses are less well documented in tropical Asia and Africa. The reverse is however the case in some other countries where sheep and goat husbandry substantially contribute to gross domestic product (GDP). For instance, a study in India estimated the annual economic loss at Rs.1180 and Rs.2121.82 (current exchange rate of $1=Rs.56 during this study) per infected sheep and goat respectively (79). Studies on the economic production losses of bovine brucellosis are reasonably consistent across a range of production systems in Africa, with losses estimated at 6% to 10% of the income per animal (77,80,81). In Nigeria, losses were estimated at US $575,605 per year or US $3.16 per bovine (prevalence 7% to 12%) (82), while that of Argentina were estimated at US $60 million per year or US $1.20 per bovine when the prevalence was around 5% (83).

In humans, the main risks for people are occupational (contact with livestock) and consumption of dairy products. Several studies in vulnerable populations reported high seroprevalences an average of 11% among livestock keepers/abattoir workers and 7% among suspect hospital patients. Economic losses caused by the disease in humans are a consequence of the cost of hospital treatment, cost of drugs, patient out of pocket treatment expenses, and loss of work or income due to illness. In Spain, losses from hospital costs and lost pay were estimated at 787.92 pesetas per patient (84), while estimated costs per case in New Zealand were NZ $3,181 (85). Broader human disability adjusted life year (DALY) burdens for brucellosis are yet to be estimated globally or across low-income countries (86). This reflects the fact that human brucellosis is even more under reported than animal brucellosis. It usually presents as an acute febrile illness often mistaken for malaria or typhoid (7,87). There is therefore a great need to introduce

earlier differential diagnosis for brucellosis in high-risk populations (88).

On a general note, estimating the economic impact of brucellosis requires holistic and all-encompassing approach which will put together several of the factors enumerated above. Some of these factors includes but are not limited to; (i) cost of illness in livestock (medication treatment cost, loss of production); (ii) cost of prevention (vaccination, livestock sector treatment, herd slaughter, market loss due to risk of infected meat and milk, mortality, morbidity, lower production, loss of exports increased biosecurity); (iii) opportunity costs (loss of animal genetic resources, loss of opportunities occasioned by spending on disease prevention and cure); and (iv) cost of illness in human (cost of drugs, patient out of pocket treatment expenses, and loss of work or income due to illness). It should however be noted that some possible outcome, for instance, feeling of unwell, emotional cost of infertility following abortion, sterility, still birth etc, and risk of loss of life in humans, may not be accurately quantified although these costs exist

Diagnosis of brucellosis

Human brucellosis has a wide clinical spectrum and presents various diagnostic difficulties because it mimics many other diseases. The diagnosis of brucellosis requires the isolation of *Brucella* from blood or body tissues or the combination of suggestive clinical presentation and positive serology. Proper diagnosis is one of the key obstacles for the complete eradication of brucellosis. Amplification of *Brucella* DNA by polymerase chain reaction (PCR) assay is currently used in the diagnosis of brucellosis. For PCR, peripheral blood or non-blood samples can be used. It was reported that the sensitivity of PCR was 100% and the specificity 98.3% in patients with brucellosis of bacteraemic, non-bacteraemic and focal complications (89,90)

Several serologic tests have been developed to measure antibodies against *Brucella* which includes; tube agglutination test (TAT), Rose Bengal test (RBT), anti-Brucella Coombs test, and enzyme-linked immunosorbent assay (ELISA). The TAT is widely used, and a single titer of $\geq$160 or a fourfold rise in titer is considered significant (91-93). Serology for laboratory workers exposed to *Brucella* is usually performed at 0, 6, 12, 18, and 24 weeks, post exposure. The immune response to *Brucella* is characterized by an initial production of IgM antibodies followed by IgG antibodies. The major antigens that are useful for diagnosis of brucellosis are the smooth (S) lipopolysaccharide (LPS) of *Brucella* outer membrane and internal proteins.

The Centre for Disease Control (CDC) recommends that *Brucella* serology testing only be performed using tests approved by the Food and Drug Administration (FDA) or validated under the Clinical Laboratory Improvement Amendments (CLIA) and shown to reliably detect the presence of *Brucella* antibodies. Results from these tests should be considered supportive evidence for recent infection only and interpreted in the context of a clinically compatible illness and exposure history. Detection of antibodies to *Brucella* cytoplasmic proteins by ELISA and Western blot in cerebrospinal fluid (CSF) is another diagnostic approach in neuro-brucellosis (94). Among the serological methods currently in practice, the serum agglutination test (SAT) is commonly used for the diagnosis of *Brucella* infection in humans (95).

Prevention and control of brucellosis

Despite the huge efforts invested on the control of animal brucellosis, results have not always matched the expectations, particularly in ovine and caprine brucellosis, in which control has proven to be more challenging than that of bovine brucellosis (96, 97). This situation may be the consequence of the combination of several factors, including but not limited to those inherent to the infected host, the aetiological agent, epidemiological situations and environmental factors bordering on human cultural practice (98,99). Many stakeholders have employed various strategies, either in isolation or in combination, which were not without their peculiar differences and challenges.

Prevention of brucellosis is based on surveillance and the prevention of risk factors. The most effective prevention strategy is the elimination of infection in animals. Vaccination of cattle, goats and sheep is recommended in enzootic areas with high prevalence rates. Serological or other testing and culling can also be effective in areas with low prevalence. In countries where eradication in animals through vaccination or elimination of infected animals is not feasible, prevention of human infection is primarily based on raising awareness, food safety measures, occupational hygiene and laboratory safety. Moreover, pasteurization of milk for direct consumption and for creating derivatives such as cheese is an important step to preventing transmission from animals to humans. Education campaigns about avoiding unpasteurized milk products can be effective, as well as government policies on their sale (90). In agricultural work and meat process-

ing, protective barriers and correct handling and disposal of afterbirths, animal carcasses and internal organs are an important prevention strategy. Surveillance using serological tests, as well as tests on milk such as the milk ring test, can be used for screening and could play an important role in campaigns to eliminate the disease. As well, individual animal testing both for trade and for disease control purposes, is practiced (7,100).

In endemic areas, vaccination is often used to reduce the incidence of infection. Several vaccines are available that use modified live viruses as detailed in the OIE Manual of Diagnostic Test and Vaccines for Terrestrial Animals, and as the disease becomes closer to being eliminated, a test and stamping-out program is required to completely eliminate it. Human brucellosis is best prevented by controlling the infection in animal population. Pasteurization of milk from infected animals was an important way to reduce infection in humans (101).

Summarily, despite the nature of this disease and the perceived challenges of its control in animals (the primary host), three major strategies including strict bio-security at the farm level, test and slaughter programs, and immunization of susceptible population, have been demonstrated as effective tools to control brucellosis in domestic animals especially when used in combination. In addition to these strategies, other complementary tools such as epidemiological situation in a given setting, availability of resources, animal identification, animal movement control, economic compensations, and others, should be considered to ensure the success of each instituted program per time (96,102).

Conclusion:

In conclusion, brucellosis can be considered a paradigm of the need for a "One World, One Health" strategy given that the only approach to achieve the control and subsequent eradication of this zoonotic disease is the cooperation between the industry, producers, and public and animal health authorities (103). It is therefore necessary that all hands should be on deck and all necessary arsenals be employed to combat this silent and often neglected zoonosis whose negative impact cannot be denied in several parts of the world, especially in developing countries.

Contribution of authors:

UCS conceptualized the study and contributed to the introduction and distribution of brucellosis in Nigeria; KE contributed to human brucellosis and diagnosis; OEO contributed to economic impacts of brucellosis, prevention and control; IE contributed to brucellosis in domestic animals; and LPD revised the entire manuscript and provided conclusion for the review.

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Original Article **Open Access**

Sero-prevalence of and risk factors associated with *Helicobacter pylori* infections among individuals with peptic ulcer in Owerri, Imo State, Nigeria during 2020-2021

*[1]Okoroiwu, G. I. A., [2]Okoroiwu, I. L., [1]Ubosi, N. I., and [3]Sani, N. M.

[1]Department of Public Health, Faculty of Health Sciences, National Open University of Nigeria, Jabi, Abuja, Nigeria
[2]Department of Medical Laboratory Science, Faculty of Health Sciences, Imo State University Owerri, Imo State, Nigeria
[3]Department of Microbiology, Kano State University of Science and Technology, Wudil, Kano State, Nigeria
*Correspondence to: okoroiwugia@yahoo.com; 08036677539

Abstract:

Background: *Helicobacter pylori*, which is a causative agent of chronic gastritis, duodenal ulcer and gastric cancer, presently affects approximately one half of the world population. This study was conducted to assess the epidemiology and risk factors for acquisition of *H. pylori* among individuals with and without peptic ulcer symptoms in Owerri, Nigeria, in order to provide baseline data and create awareness for effective management and prevention of infection caused by this pathogen.
Methodology: A total of 384 participants, symptomatic and asymptomatic for peptic ulcer, were recruited from the three Local Government Areas (LGAs) of Owerri, Imo State, Nigeria. The symptomatic participants were randomly recruited among patients attending outpatient clinics with symptoms of peptic ulcer disease at the Federal Medical Center Owerri, general hospitals and primary healthcare centers across Owerri LGAs, while asymptomatic patients were recruited from the community. Information on socio-demographic characteristics of each participant and potential risk factors were collected with a pre-tested structured questionnaire. Blood samples were collected for detection of antibodies (IgG) using a one-step *H. pylori* test device while faecal samples were collected for detection of occult blood (from peptic ulceration) using faecal occult blood (FOB) test. Data were analyzed using SPSS version 25.0 and association of risk factors with *H. pylori* sero-prevalence was determined by the Chi-square or Fisher Exact test (with Odds ratio). P value < 0.05 was considered statistically significant.
Results: The sero-prevalence of *H. pylori* infection among the study participants was 72.4% (285/384) while the prevalence of ulcer by FOB test was 71.1% (273/384). A total of 78.4% (214/273) of those with ulcers were sero-positive for *H. pylori* while 64.0% (71/111) of those without ulcers were sero-positive for *H. pylori* (p=0.0045). Factors significantly associated with high sero-prevalence of *H. pylori* were age groups 41-50 (100%), 21-30 (78.4%) and 31-40 (67.6%) years (X^2=66.964, p<0.0001), illiteracy (OR=6.888, p<0.0001), unemployment (OR=2.427, p<0.0001), low social class status (X^2=28.423, p=0.0003), drinking of unclean water (OR=5.702, p<0.0001), living in crowded rooms (OR=2.720, p<0.0001), eating food bought from food vendors (OR=3.563, p<0.0001), family history of ulcer (OR=12.623, p<0.0001), habits of eating raw vegetables and unwashed fruits (OR=6.272, p<0.0001), non-regular hand washing practices before meal (OR=2.666, p<0.0001) and presence of ulcer (OR=2.043, p=0.0045). However, smoking (OR=0.7581, p=0.2449) and gender (OR=0.6538, p=0.0796) were not significantly associated with sero-prevalence of *H. pylori.*
Conclusion: There is need for comprehensive strategy including public health education campaign to create awareness on *H. pylori*, improve personal hygiene and environmental sanitation, provision of safe drinking water by the government to the populace, and discourage indiscriminate and open defecation.

Keywords: *Helicobacter pylori*; peptic ulcer; serology; risk factors; association; Owerri

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Séroprévalence et facteurs de risque associés aux infections à *Helicobacter pylori* chez les personnes atteintes d'ulcère gastro-duodénal à Owerri, État d'Imo, Nigéria en 2020-2021

*[1]Okoroiwu, G. I. A., [2]Okoroiwu, I. L., [1]Ubosi, N. I., et [3]Sani, N. M.

[1]Département de la Santé Publique, Faculté des Sciences de la Santé, Université Nationale Ouverte du Nigéria, Jabi, Abuja, Nigéria
[2]Département des Sciences de Laboratoire Médical, Faculté des Sciences de la Santé, Université d'État d'Imo Owerri, État d'Imo, Nigéria
[3]Département de Microbiologie, Université des Sciences et Technologies de l'État de Kano, Wudil, État de Kano, Nigéria
*Correspondance à: okoroiwugia@yahoo.com; 08036677539

Résumé:

Contexte: *Helicobacter pylori*, qui est un agent causal de la gastrite chronique, de l'ulcère duodénal et du cancer gastrique, affecte actuellement environ la moitié de la population mondiale. Cette étude a été menée pour évaluer l'épidémiologie et les facteurs de risque d'acquisition de H. pylori chez les personnes présentant ou non des symptômes d'ulcère peptique à Owerri, au Nigeria, afin de fournir des données de base et de sensibiliser à la gestion et à la prévention efficaces de l'infection causée par cet agent pathogène.
Méthodologie: Un total de 384 participants, symptomatiques et asymptomatiques de l'ulcère peptique, ont été recrutés dans les trois zones de gouvernement local (LGA) d'Owerri, dans l'État d'Imo, au Nigeria. Les participants symptomatiques ont été recrutés au hasard parmi des patients fréquentant des cliniques externes présentant des symptômes d'ulcère peptique au Federal Medical Center Owerri, des hôpitaux généraux et des centres de soins de santé primaires dans les LGA d'Owerri, tandis que des patients asymptomatiques ont été recrutés dans la communauté. Des informations sur les caractéristiques sociodémographiques de chaque participant et les facteurs de risque potentiels ont été recueillies à l'aide d'un questionnaire structuré pré-testé. Des échantillons de sang ont été prélevés pour la détection d'anticorps (IgG) à l'aide d'un dispositif de test *H. pylori* en une étape, tandis que des échantillons fécaux ont été prélevés pour la détection de sang occulte (provenant d'une ulcération peptique) à l'aide d'un test de sang occulte fécal (FOB). Les données ont été analysées à l'aide de la version SPSS 25.0 et l'association des facteurs de risque avec la séroprévalence de *H. pylori* a été déterminée par le test Chi-carré ou Fisher Exact (avec rapport de cotes). La valeur $p < 0,05$ a été considérée comme statistiquement significative.
Résultats: La séroprévalence de l'infection à *H. pylori* parmi les participants à l'étude était de 72,4% (285/384) tandis que la prévalence de l'ulcère par test FOB était de 71,1% (273/384). Au total, 78,4% (214/273) de ceux qui avaient des ulcères étaient séropositifs pour *H. pylori* tandis que 64,0% (71/111) de ceux qui n'avaient pas d'ulcères étaient séropositifs pour *H. pylori* (p=0,0045). Les facteurs significativement associés à une séroprévalence élevée de *H. pylori* étaient les groupes d'âge 41-50 (100%), 21-30 (78,4%) et 31-40 (67,6%) ans (X^2=66,964, p<0,0001), l'analphabétisme (OR=6,888, p<0,0001), chômage (OR=2,427, p<0,0001), statut social inférieur (X^2=28,423, p=0,0003), consommation d'eau insalubre (OR=5,702, p<0,0001), vie dans des pièces surpeuplées (OR=2,720, p<0,0001), manger des aliments achetés à des vendeurs de nourriture (OR=3,563, p<0,0001), antécédents familiaux d'ulcère (OR=12,623, p<0,0001), habitudes de manger des légumes crus et non lavés fruits (OR=6,272, p<0,0001), pratiques non régulières de lavage des mains avant le repas (OR=2,666, p<0,0001) et présence d'ulcère (OR=2,043, p=0,0045). Cependant, le tabagisme (OR=0,7581, p=0,2449) et le sexe (OR=0,6538, p=0,0796) n'étaient pas significativement associés à la séroprévalence de *H. pylori*.
Conclusion: Il est nécessaire de mettre en place une stratégie globale comprenant une campagne d'éducation à la santé publique pour sensibiliser à *H. pylori*, améliorer l'hygiène personnelle et l'assainissement de l'environnement, fournir de l'eau potable à la population par le gouvernement et décourager la défécation aveugle et à l'air libre.

Mots clés: *Helicobacter pylori*; ulcère peptique; sérologie; facteurs de risque; association; Owerri

Introduction:

Helicobacter pylori (H. pylori) is a spiral-shaped, Gram-negative microaerophilic bacterium, measuring approximately 3–5 µm in length, presently affecting approximately one half of the world population and causing chronic gastritis, the most frequent chronic inflammation worldwide (1). Infection is mostly asymptomatic, but when could cause pathologies such as gastritis, gastric and duodenal ulcers. About 90 - 100% of all duodenal ulcers, and 70 - 80% of all gastric ulcers are caused by *H. pylori* infection (2,3). An International Agency for Research on Cancer (IARC), an arm of the World Health Organization (WHO) classified *H. pylori* as a class I carcinogen for gastric cancer, a definition given to highest cancer-causing microbial agent (4). In Pune, Maras-htra India, Rahul et al., (5), reported 51.0% of *H. pylori* infection among 180 participants diagnosed in their assessment of risk factors of

H. pylori infection and peptic ulcer disease. Beyond its role in several gastroduodenal disorders, *H. pylori* has been involved in many extra-gastroduodenal disorders, such as idiopathic thrombocytopenic purpura, cardiovascular diseases, chronic liver diseases, iron-deficiency anaemia, and diabetes mellitus (6,7,8).

Although, Africa has the highest prevalence of *H. pylori* infections in the world (9), there is paucity of data on *H. pylori* prevalence in the general population across different regions of Africa including Nigeria. The majority of data published on the prevalence of *H. pylori* included patients presenting with symptoms of gastroduodenal diseases. *H. pylori* is reported to infect over 50% of the world population, and its distribution is influenced by factors such as age, gender, geographical location, ethnicity, and socio-economic factors (10,11), with higher prevalence in the developing countries compared to the developed countries especially in younger age groups.

With majority of the countries in Africa classified as developing or underdeveloped, *H. pylori* is therefore largely ubiquitous in this continent. In Rwanda, southern Africa, Walker et al., (12) reported 75% positivity rate to *H. pylori* in patients attending the University Hospital Butare over a period of 12 months. In a study from Kenya (13) among patients who presented with dyspepsia, *H. pylori* prevalence of 73.3% was reported in children and 54.8% in adults, while Awuku et al., (14), using a lateral flow immunochromatographic assay for the qualitative detection of *H. pylori* antigen in faecal specimens, reported 14.2% prevalence among asymptomatic children in a rural setting in Ghana.

In Nigeria, Aje et al., (15) reported a 67.4% prevalence of *H. pylori* among dyspeptic patients in the southwest region of the country, Gide et al., (16) reported 51.95% prevalence among patients with dyspeptic symptoms in Damaturu metropolitan, Yobe State, Jemilohun et al., (17) reported 63.5% prevalence in patients with gastritis in Ibadan, Olokoba et al., (18) reported prevalence rates of 93.6% by serology and 80.0% by histology among Nigerians with dyspepsia, while Ezeigbo and Ezeigbo (19) reported 39.7% rate amongst apparently healthy adults residing in Aba, Abia State, southern Nigeria. Recently, Smith et al., (20) reported *H. pylori* prevalence of 68.4% among type 2 diabetes mellitus patients in southwest and south-south Nigeria, and Nwachukwu et al., (21) reported 52.0% prevalence among patients with gastritis attending the Nnamdi Azikiwe Teaching Hospital (NAUTH), Nnewi, Anambra State, Nigeria. In Kano, northwest Nigeria, Bello et al., (22) reported high prevalence of 81.7% for *H. pylori* infection particularly amongst subjects with low socio-economic status. Factors such as unclean water source, overcrowding and cigarette smoking were significant risk factors noted for high prevalence of *H. pylori* infection in these studies. Nevertheless, in southwest Nigeria, Smith et al., (23), reported that most characteristics studied such as smoking, alcohol consumption, and sources of drinking water were not significantly associated with *H. pylori,* but previous antibiotic use, overcrowding and family history of ulcer/gastritis were significantly associated.

In Imo State, Nigeria, there are no documented evidence in the literature on the prevalence or burden of *H. pylori* infections and risk factors associated with its acquisition. Particularly in Owerri, the burden of the disease continues to mount from observations and history. Based on the lack of awareness and reports in this part of the country, it is apt that this kind of study be conducted to determine the burden and risk factors associated with *H. pylori* infections. Therefore, the objective of our study was to determine the prevalence and associated risk factors for *H. pylori* infections among the populace in Owerri in Imo State, southeast Nigeria.

Materials and method:

Study area:

Owerri is the capital of Imo State in Nigeria, with three Local Government Areas (LGAs); Municipal, North and West, and has an estimated population (predominantly Christian) of about 1,401,873, as of 2016, and is approximately 100 square kilometres (40 sq miles) in area. The city is bordered by the Otamiri river to the east and the Nworie river to the south (24) and has an airport 23 kilometres (14 miles) southeast of the city located in Obiangwu, Ngor Okpala LGA. Owerri sits in the rain forest and produce many agricultural products such as yams, cassava taro, corn, rubber and palm products.

According to the Köppen-Geiger system, Owerri has a tropical wet climate. Rain falls for most months of the year with a brief dry season. The harmattan affects the city in the early periods of the dry season and is noticeably less pronounced than in other cities in Nigeria. The average temperature is 26.4°C. The city is home to many tertiary institutions including Imo State University, Federal University of Technology, Federal Polytechnic and others.

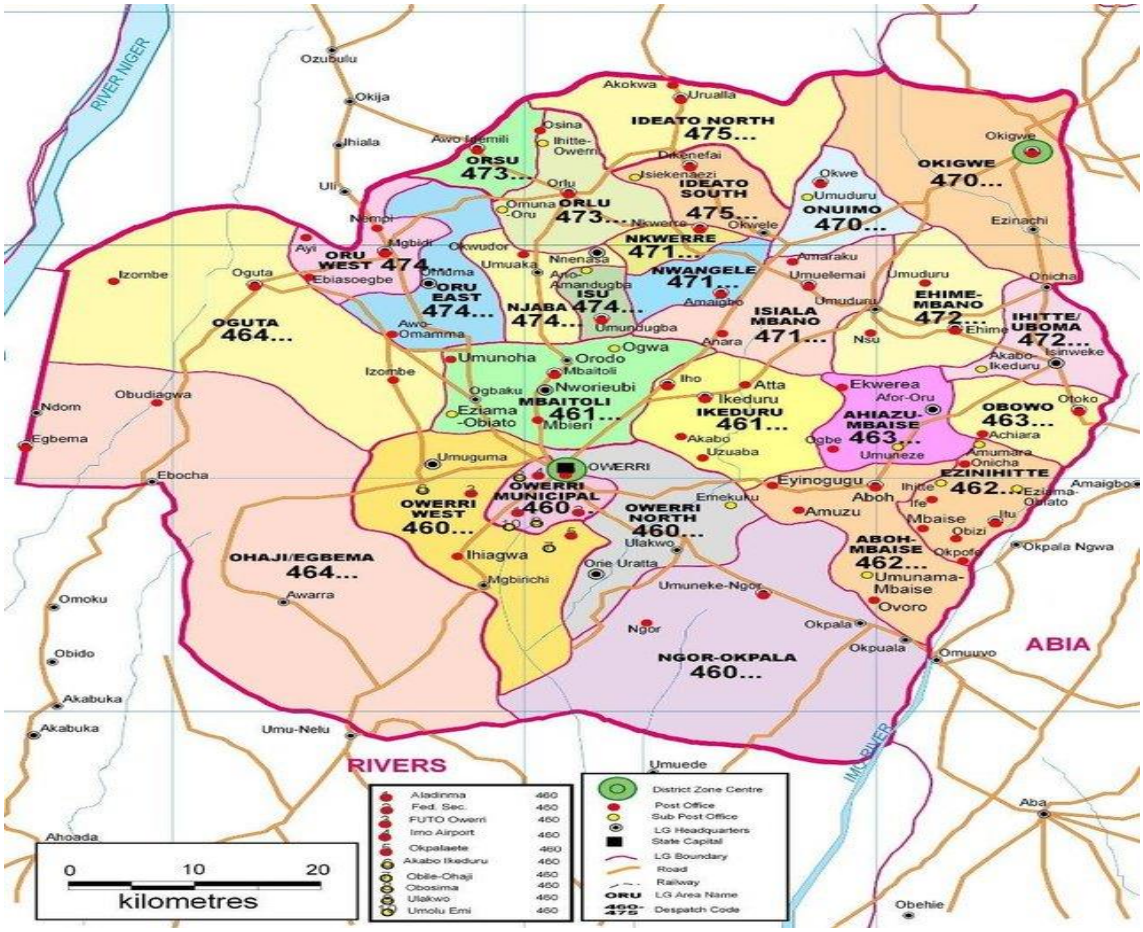

Fig. 1: Map of Imo State, Nigeria showing Owerri

Study design

This is a descriptive cross-sectional study to determine the sero-prevalence and risk factors associated with *H. pylori* infections in symptomatic and asymptomatic populations in Owerri, Imo State, Nigeria.

Study population and subject participants

The subject participants consist of 384 individuals who residents of Owerri, Imo State, Nigeria. The subjects include those with symptoms of dyspepsia such as burning pain in the abdomen, indigestion, bloating, nausea, vomiting, loss of appetite, belching, and weight loss, attending the outpatient clinics of different tiers of healthcare facility across Owerri, and asymptomatic individuals recruited from the Owerri community. The healthcare facilities included Federal Medical Center Owerri, general hospitals, and primary healthcare centers across the city.

Sample size and sampling technique

The sample size of 384 was determined using the sample size formula for simple proportion (25) given as; $n=N^2P(1-P)/d^2$, where n =sample size, N=1.96 (statistical constant), P= prevalence of 52.0% (0.52) adopted because there was no similar study previously conducted in the study area, and d=margin of error of 5.0% (0.05). This gave a calculated sample size of 384.

Participants were eligible for recruitment if they were resident, schooling, working or doing business within the three LGAs of Owerri. Participants below 10 and above 65 yrs of age, non-residence of Owerri, and menstruating females were excluded. The participants were recruited randomly at the healthcare facilities and the community until the sample size of 384 was obtained.

Ethical clearance

Written informed consent was obtained from the study participants. The purpose of the study, procedures, possible risks and benefits, rights and responsibilities of participants including right to withdraw from the study at any time were described.

Data collection

A structured questionnaire was interviewer-administered to each participant to obtain information on socio-demographic characteristics, behavioral and hygiene practices. Information obtained included daily habits, households, potential sources of infection, age, gender, educational level (primary, secondary tertiary), social class status (high, middle, low), source of drinking water (pipe, well, stream), overcrowding, consumption of food from food vendors, and family history of ulcer, cigarette smoking). The questionnaire was pre-tested using 50 people outside the study population.

Sample collection

Participants were provided with clean, dry, disinfectant-free, wide-mouthed plastic container labeled with identifiers such as name, age and date, to collect about 10.0 grams of faecal specimen for faecal occult blood (FOB) test. Written instruction was given on how to avoid contamination of the faecal specimen stool with water and urine.

For blood specimen, 1 ml of venous blood was collected from each participant into test tube and allowed to clot by standing at room temperature for 30 to 45 minutes. Clear serum sample was obtained by centrifugation at 3000 revolutions per minute for 4 to 5 min and this was used for detection of antibodies (IgG) against *H. pylori.*

Laboratory analysis

Faecal occult blood (FOB) was performed by the one-step rapid screen test to infer the presence of peptic ulcer. A small portion of the faecal sample was transferred to a vial with diluents, vigorously agitated for 15 to 30 sec to obtain homogenous solution of faecal matters. Two to three drops of the homogenous solution were squeezed on the test pad. The test result was read within 5 to 10 min in accordance with the manufacturer's instruction, and interpreted based on the appearance of coloured lines on the test pad. Only color-band on the control region of the test pad meant negative for ulcer, two colour bands on the positive and control regions was positive for ulcer while no visible band at meant invalid result.

Serology test for *H. pylori* infection was done using the one-step *H. pylori* test device (whole blood/serum/plasma), which is a rapid immunochromatographic test for the qualitative detection of antibodies (IgG) to *H. pylori* in whole blood, serum or plasma. Briefly, the test device and serum were allowed to equilibrate at room temperature prior to testing. The test device was removed from the foil pouch and placed on a clean working bench. The dropper was held vertically and used to transfer 3 drops of the testing serum to the specimen well of the device, avoiding trapping air-bubble in the process. The timer was started, and result read after 10 mins when the red line was supposed to appear (manufacturer's instructions). The result was interpreted based on the appearance of the red-coloured lines. Two distinct red lines, one in the control and the other in the test region of the device indicate positivity for *H. pylori* while one red line in the control region was interpreted as negative and absence of the red line at both regions was interpreted as an invalid result.

Statistical analysis of data

Data were analyzed using the Statistical Package for Social Sciences (SPSS version 25.0). Association of risk factors with sero-prevalence of *H. pylori* was done using Chi-square or Fisher Exact test (with Odds ratio). The level of significance was set at $p < 0.05$.

Results:

A total of 384 participants comprising 202 symptomatic and 182 asymptomatic, and 211 males and 173 females, were enrolled into the study. Of these, 285 (74.2%) were positive for *H. pylori* antibody (Table 1) while 273 (71.1%) were positive for ulcer by the FOB test (Table 2). One hundred and forty-nine of the 211 (70.6%) male participants were sero-positive for *H. pylori* while 136 of the 173 (78.6%) female participants were sero-positive (p= 0.0796) (Table 1). Also, 149 of 211 (70.6%) males were positive for ulcer by the FOB test while 124 of 173 (71.1%) females were positive (p=0.9100) (Table 2).

One hundred and eighteen (64.8%) of the 182 asymptomatic participants were sero-positive for *H. pylori*, while 167 of 202 (82.7%) symptomatic participants were sero-positive (OR=2.588, p<0.0001) (Table 3). In the age-related sero-prevalence of *H. pylori*, the age-group 41-50 years had the highest prevalence (100.0%), followed by age groups 21-30 (78.4%), 31-40 (67.6%), and 10-20 (42.5%), while age-groups 51-60 (48.0%) and 61-70 years (48%) had the lowest sero-prevalence (X^2=66.964; p<0.0001) (Table 4).

Table 5 is bivariable analysis of factors associated with sero-prevalence of *H. pylori* among the participants, which showed significant association with factors such as age group (X^2=66.964, p<0.0001), educational level (OR =6.888, p<0.0001), occupational status (OR =2.427, p<0.0001), social class status (X^2=

28.423, p=0.0003), water source for drinking (OR=5.702, p<0.0001), living in over-crowded rooms (OR=2.720, p<0.0001), eating from food vendors (OR=3.563, p< 0.0001), family history of ulcer (OR=12.623, p<0.0001), regular habits of eating raw vegetables and unwashed fruits (OR=6.272, p<0.0001), hand washing practices before meal (OR=2.666, p<0.0001) and ulcer disease status (OR= 2.043, p=0.0045). However, smoking (OR= 0.7581, p=0.2449) and gender (OR=0.6538, p=0.0796) were not significantly associated with sero-prevalence of *H. pylori.*

Table 1: Gender-related sero-prevalence of *Helicobacter pylori* among participants in Owerri, Imo State, Nigeria

Gender	No. screened	No. sero-positive (%)	No. sero-negative (%)
Male	211	149 (70.6)	62 (29.4)
Female	173	136 (78.6)	37 (21.4)
Total	384	285 (74.2)	99 (25.8)

OR=0.6538; 95% CI= 0.4091-1.045; p=0.0796

Table 2: Gender-related prevalence of ulcer by faecal occult blood among participants in Owerri, Imo State, Nigeria

Gender	No. screened	No. positive (%)	No. negative (%)
Male	211	149 (70.6)	62 (29.4)
Female	173	124 (71.7)	49 (28.3)
Total	384	273 (71.1)	111 (28.9)

OR=0.9497; 95% CI=0.6091-1.481; p=0.9100

Table 3: Sero-prevalence of *Helicobacter pylori* among asymptomatic and symptomatic participants in Owerri, Imo State, Nigeria

Participants	No. screened	No. positive (%)	No. negative (%)
Asymptomatic	182	118 (64.8)	64 (35.2)
Symptomatic	202	167 (82.7)	35 (17.3)
Total	384	285 (74.2)	99 (25.8)

OR=2.588; 95% CI=1.610-4.161; p<0.0001

Table 4: Age-related sero-prevalence of *H. pylori* among the participants in Owerri, Imo State, Nigeria.

Age-groups	No. screened	No. positive (%)	No. negative (%)
10-20	87	50 (57.5)	37 (42.5)
21-30	111	87 (78.4)	24 (21.6)
31-40	37	25 (67.6)	12 (32.4)
41-50	99	99 (100.0)	0
51-60	25	12 (48.0)	13 (52.0)
61-70	25	12 (48.0)	13 (52,0)
Total	384	285 (74.2)	99 (25.8)

X^2=66.964; p<0.0001

Table 5: Bivariate analysis of risk factors associated with sero-prevalence of *H. pylori* infection among participants in Owerri, Imo State, Nigeria

Variables	No. screened	No. positive (%)	OR	X^2	p-value
Age-group (years)					
10-20	87	50 (57.5)			
21-30	111	87 (78.4)		66.964	<0.0001*
31-40	37	25 (67.6)			
41-50	99	99 (100.0)			
51-60	25	12 (48.0)			
61-70	25	12 (48.0)			
Gender					
Male	211	149 (70.6)	0.6538		0.0796
Female	173	136 (78.6)			
Educational status/Literacy level					
Illiterate	105	98 (93.3)	6.888		<0.0001*
Literate	279	187 (67.0)			
Occupational status/Employment state					
Unemployed	185	153 (82.7)	2.427		0.0003*
Employed	199	132 (66.3)			
Social class status					
Higher social class	110	65 (59.1)			
Middle social class	120	85 (70.8)		28.421	<0.0001*
Lower social class	154	135 (87.7)			
Drinking of pipe/filtered/boiled water					
Yes	214	131 (61.4)	5.702		<0.0001*
No	170	153 (90.0)			
Living in over-crowded room					
Yes	180	151 (83.9)	2.720		<0.0001*
No	204	134 (65.7)			
Consumption of food from food vendors					
Yes	190	163 (85.8)	3.563		<0.0001*
No	194	122 (62.9)			
Family history of ulcer					
Yes	200	187 (93.5)	12.623		<0.0001*
No	184	98 (53.3)			
Smoking					
Yes	182	130 (71.4)	0.7581		0.2449
No	202	155 (76.7)			
Eating raw vegetables and unwashed fruits					
Yes	200	179 (89.5)	6.272		<0.0001*
No	184	106 (57.6)			
Hand washing habit before meal					
Always (regular)	201	132 (65.7)	2.666		<0.0001*
Sometimes (non-regular)	183	153 (83.6)			
Ulcer disease status					
Yes	273	214 (78.4)	2.043		0.0045*
No	111	71 (64.0)			

OR=Odds ratio; X^2=Chi square; * = statistically significant

Discussion:

Helicobacter pylori infection has been a very huge challenge to the world and in particular, African countries. It is of great public health importance, considering the fact that it is associated with serious health issues of chronic gastritis, duodenal and gastric ulcer, gastric cancer, and other extra-gastric pathologies. This infection presently affects approximately half of the world population (1). The result of our study showed that sero-prevalence of *H. pylori* is high (74.2%) among the population studied, and this may constitute a major public health threat to the entire people of Imo State, and by inference to the populace in Nigeria and other countries, especially in Africa.

The high sero-prevalence of *H. pylori* in our study is consistent with reports of previous studies across Nigeria (15-18,20,22). For instance, Aje et al., (15) and Jemilohun et al., (17) reported *H. pylori* prevalence rates 67.4% and 63.5% respectively in southwest Nigeria, Olokoba et al., (18) reported 93.6% prevalence by serology and 80.0% by histology among Nigerians with dyspepsia in northcentral Nigeria, Smith et al., (20) reported 68.4% among patients with type 2-diabetes mellitus in south-south and south-west Nigeria, while Bello et al., (22) reported 81.7% rate amongst subjects with low socio-economic status in north-west Nigeria. For other African countries, Laure et al., (26) reported 64.39% rate in Cameroun, Walker et al., (12) reported 75.0% in Rwanda, while, Kimang'a et al., (13) in Kenya reported 73.3% in children and 54.8% in adults among patients who presented with dyspepsia. Elsewhere, Rahul et al., (5) reported 51.0% in Pune, Marashtra, India. The wide range reported in the different studies including ours, may partly be due to differences in the method used for *H. pylori* detection, geographical locations, which could reflect environmental and personal hygiene levels, ethnicity, socio-economic factors and age of participants studied (10,26).

Nevertheless, *H. pylori* sero-prevalence rate in our study is higher than the rates in some other studies in Nigeria such as Gide et al., (16) who reported 51.9% in Damaturu metropolitan, Yobe State, northeast Nigeria, and Nwachukwu et al., (21) who reported 52.0% in Nnewi, Anambra State, south-south Nigeria, and even much higher than other studies that reported low *H. pylori* sero-prevalence of 14.2% (14), 36.3% (28), 36.8% (27) and 39.7% (19).

The high prevalence rate reported in our study was significantly associated with low social class status, drinking of unclean water, living in crowded rooms, consumption of food from food vendors, family history of ulcer, eating raw vegetables and unwashed fruits, illiteracy, unemployment, age group 41-50 yrs as well as non-regular hand-washing habit before meal. Our findings agree with previous reports (5,16-18,22,26), which underscore the need to improve socio-economic conditions and sanitary standards as well as educational standards of the populace, since low sero-prevalence of *H. pylori* is known to occur in communities with good living conditions and hygienic standards. In Russia for instance, within a period of ten years (1995-2005), it was observed that the prevalence of *H. pylori* infection reduced remarkably due to better standards of living (29). In China, prevalence of *H. pylori* drastically reduced as a result of increase in economic growth and improvement in environmental and hygienic conditions (30).

In our study, *H. pylori* sero-prevalence was high in both the asymptomatic (64.8%, 71/111) and symptomatic (87.7%, 214/273) participants (who had positive faecal occult blood test) but the rate was significantly higher in the symptomatic than asymptomatic participants (OR=2.588, $p<0.0001$). Although *H. pylori* serology has been shown to have sensitivity and specificity in the range of 78-84% and 79-90% respectively (31), the major disadvantage of serology is the inability to distinguish current infection from previous exposure, which can result in misinterpretation of the test (32). The high seroprevalence rate in both groups of participants in our study implies that serology many not be useful in confirming *H. pylori* as the cause of peptic ulcer in many patients. In such cases, other tests such as *H. pylori* antigen test, PCR assays, and *invitro* or *invivo* urea breath test on stool or gastroduodenal samples will be preferred, although many of these tests may be expensive, technically demanding and invasive (32).

The sero-prevalence of *H. pylori* was highest among the age group 41-50 years (100.0%) in our study, but also high in the age groups 21-30 and 31-40 years with 78.4% and 67.6% respectively. This agrees with the study by Gemechu and Dessie (27), who reported highest sero-prevalence of *H. pylori* in the 43-50 years age-group among symptomatic patients attending Jasmin Internal Medicine and Paediatrics Specialized Private clinic in Addis Ababa, Ethiopia. However, Smith et al., (20) reported highest prevalence among age group 50-59 years. It is known that most people are infected with *H. pylori* in their early years and this tends to persist throughout the life time,

nevertheless, the time of infection may not be known (26,27,33). Gender and smoking were factors not significantly associated with *H. pylori* sero-prevalence in our study, which agrees with the finding of Smith et al., (20) and Laure et al., (26), but disagrees with the studies of Rahul et al., (5) and Bello et al., (22), who reported significant association of *H. pylori* with smoking.

Conclusion:

Our study shows that *H. pylori* infection is common in Owerri with high sero-prevalence of 74.2% among the study population, age group 41-50 years being mostly affected, and significantly higher prevalence in population with peptic ulcer disease. Factors such as illiteracy, unemployment, drinking of unclean water, consumption of food from food vendors, living in crowded rooms, family history of ulcer, low social class, eating raw vegetables and unwashed fruits, and non-regular handwashing habits were significantly associated with high sero-prevalence of *H. pylori*. There is need for comprehensive strategy including public health education campaign to create awareness of factors associated with *H. pylori* infections, and government at all levels should enforce policy against filthy environments, discourage indiscriminate and open defaecaction, and provide safe drinking water for the populace.

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Contribution of authors:

GIAO contributed to the concept and design of the study, ILO contributed to the analysis of the samples, while GIAO, ILO, NIU and SNM were involved in the literature review, drafting, revision, editing and final approval of the final version

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No conflict of interest is declared

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Original article **Open Access**

Prevalence of *Salmonella* Typhi, *Staphylococcus aureus* and intestinal parasites among male food handlers in Laghouat Province, Algeria

*[1]Sebaa, S., [2]Baroudi, D., and [1,3]Hakem, A.

[1]Laboratory of Exploration and Valorization of Steppic Ecosystems, Faculty SNV, University of Ziane Achour, 17000 Djelfa, Algeria
[2]National Veterinary School, Issad Abbes Street, El-Alia, Algiers, Algeria
[3]Research Center in Agro-pastoralism, Moudjbara Road, 17000 Djelfa, Algeria
*Correspondence to: soumiasebaa22@gmail.com

Abstract:

Background: Food-borne diseases are a global public health problem, most especially in developing countries. Food handlers with a low level of personal hygiene may be colonized or infected by a wide range of enteric pathogenic micro-organisms including intestinal parasites and bacteria. The aim of this study is to determine the prevalence of *Salmonella* Typhi, *Staphylococcus aureus* and intestinal parasites among male food handlers in Laghouat province, southern Algeria.
Methodology: In this cross-sectional study, stool samples and fingernail samples of both hands were collected from 155 randomly selected male food handlers. Stool specimens were examined by direct wet mount, formalin-ether concentration, xenic *invitro* culture and staining methods for parasitological identification. For bacterial isolation, standard culture media including Hektoen agar, *Salmonella-Shigella* (SS), Mannitol salt, and Blood agar plates were used. Conventional biochemical tests were used for identification of *S.* Typhi and *S. aureus*. Antimicrobial susceptibility test (AST) was performed for bacterial isolates by the Kirby-Bauer disk diffusion method. Data analysis was done using Minitab version 19 software, and Pearson's Chi-square test was used to determine association between categorical variables. P value < 0.05 was considered statistically significant.
Results: The overall prevalence rate of intestinal parasites among the study subjects was 40% (62/155). *Blastocystis* spp was the most frequent parasite isolated (16.8%), followed by *Giardia intestinalis* (8.4%), *Entamoeba histolytica/dispar* (7.7%), *Entamoeba coli* (3.2%), *Trichomonas intestinalis* (2.6%) and *Endolimax nana* (1.3%). Stool cultures revealed 4 (2.6%) positive samples for *S.* Typhi, and *S. aureus* was isolated from fingernail contents of 23 (14.8%) subjects. All *S.* Typhi isolates were sensitive to imipenem and ciprofloxacin while *S. aureus* isolates show high sensitivity to pristinamycin. Hand washing with soap, finger nail status and clinical manifestations were significantly associated with intestinal parasitic infections, while clinical manifestation was the only factor associated with *S. aureus* infection.
Conclusion: The present study indicates a high prevalence of pathogenic micro-organisms among male food handlers which highlight the important role of food handlers in the spread and transmission of foodborne infections, and thus requires more attention.

Keywords: Intestinal parasites, *Salmonella*, *Staphylococcus*, food handlers, Laghouat, Algeria

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Prévalence de *Salmonella* Typhi, *Staphylococcus aureus* et les parasites intestinaux chez les hommes manipulateurs d'aliments dans la province de Laghouat, Algérie

*[1]Sebaa, S., [2]Baroudi, D., et [1,3]Hakem, A.

[1]Laboratoire d'Exploration et de Valorisation des Ecosystèmes Steppiques, Faculté SNV, Université de Ziane Achour, 17000 Djelfa, Algérie
[2]Ecole Nationale Vétérinaire, Rue Issad Abbes, El-Alia, Alger, Algérie
[3]Centre de Recherche en Agropastoralisme, Route de Moudjbara, 17000 Djelfa, Algérie
*Correspondence a: soumiasebaa22@gmail.com

Résumé:

Contexte: Les maladies d'origine alimentaire sont un problème de santé publique mondial, plus particulièrement dans les pays en développement. Les manipulateurs d'aliments avec un faible niveau d'hygiène personnelle peuvent être colonisé ou infectés par un large éventail de micro-organismes entéropathogènes, notamment des parasites intestinaux et des bactéries. L'objectif de cette étude est de déterminer la prévalence de *Salmonella* Typhi, *Staphylococcus aureus* et les parasites intestinaux chez les manipulateurs d'aliments dans la province de Laghouat, sud de l'Algérie.
Méthodologie: Dans cette étude transversale, des échantillons de selles et écouvillonnage des ongles des deux mains ont été prélevés auprès de 155 manipulateurs d'aliments de sexe masculins sélectionnés au hasard. Chaque échantillon de selles a fait l'objet d'un examen microscopique direct, d'un enrichissement, d'une coloration et d'une culture xénique in vitro pour l'identification parasitologique. Pour l'isolement bactérien, des milieux de culture standard comprenant, gélose Hektoen, gélose *Salmonella-Shigella* (SS), sel de mannitol et gélose au sang ont été utilisés. Des tests biochimiques conventionnels ont été utilisés pour l'identification de *S. Typhi* et *S. aureus*. Un test de sensibilité aux antibiotiques (AST) a été réalisé pour les isolats bactériens par la méthode de diffusion sur disque de Kirby-Bauer. L'analyse des données a été effectuée à l'aide du logiciel Minitab version 19 et le test du chi carré de Pearson a été utilisé pour déterminer l'association entre les variables catégorielles. La valeur $p < 0,05$ a été considérée comme statistiquement significative.
Résultats: Le taux de prévalence global des parasites intestinaux parmi les sujets de l'étude était de 40% (62/155). *Blastocystis* spp était le parasite le plus fréquemment isolé (16,8%), suivi de *Giardia intestinalis* (8,4%), *Entamoeba histolytica/dispar* (7,7%), *Entamoeba coli* (3,2%), *Trichomonas intestinalis* (2,6%) et *Endolimax nana* (1,3%). Les cultures de selles ont révélés 4 (2,6%) échantillons positifs pour *S. Typhi*, et *S. aureus* a été isolé du contenu des ongles dans 23 (14,8%) sujets. Tous les isolats de *S. Typhi* étaient sensibles à l'imipénem et à la ciprofloxacine tandis que les isolats de *S. aureus* montrent une sensibilité élevée à la pristinamycine. Lavage des mains au savon, l'état des ongles et les manifestations cliniques étaient significativement associés aux infections parasitaires intestinales, tandis que la manifestation clinique était le seul facteur associé à l'infection à *S. aureus*.
Conclusion: La présente étude indique une prévalence élevée de micro-organismes pathogènes chez les manipulateurs d'aliments, qui soulignent le rôle important des manipulateurs d'aliments dans la propagation et la transmission des infections d'origine alimentaire et nécessite donc plus d'attention.

Mots clés: Parasites intestinaux, *Salmonella, Staphylococcus*, Manipulateurs d'aliments, Laghouat, Algérie.

Introduction:

Foodborne diseases are a public health problem in developed and developing countries, mostly caused by eating food contaminated with bacteria, viruses, parasites or chemical substances such as heavy metals (1). According to the World health organization (WHO), up to 30% of the population suffer from foodborne diseases each year in developed countries, whereas in developing countries up to 2 million deaths are estimated per year (2).

Numerous outbreaks of gastroenteritis have been associated with ingestion and consumption of raw foods or foods obtained from unsafe sources (3,4). Transmission of intestinal parasites and enteropathogenic bacteria is affected directly or indirectly through objects contaminated with faeces, these include food, water, nails, and fingers, indicating the importance of faecal-oral human-to-human transmission (5). Food handlers with a low level of personal hygiene could be potential sources of parasitic worms, protozoa, as well as intestinal pathogenic bacteria, and may contaminate foods from their faeces via their fingers, then to food processing, and finally to healthy individuals (6).

Bacterial food poisoning has been reported to be a result of infection with *Staphylococcus aureus, Salmonella*, *Campylobacter*, *Listeria*, pathogenic *Escherichia coli*, *Yersinia*, *Shigella*, *Enterobacter* and *Citrobacter* (1). These organisms may exist on food handler's hands, and become intoxication agents if these foods are then kept for several hours without refrigeration or stored in containers (3). Although many outbreaks of gastroenteritis caused by protozoan pathogens have occurred, recognized as waterborne parasites, *Giardia intestinalis*, *Cryptosporidium* spp, *Entamoeba histolytica*, *Blastocystis* spp and *Cyclospora* spp have now been associated with several foodborne outbreaks (7-9). In Algeria, food handlers are screened annually for parasitic and bacteriological infections, therefore this study was aimed to determine the prevalence of intestinal parasites and pathogenic bacteria among food handlers in Laghouat Province, southern Algeria.

Materials and method:

Study area

This cross-sectional study was carried out in the province of Laghouat situated in the center of the country at 400 km to the south of the capital Algiers, between latitude 33° 48' north and longitude 02° 53' east. This province is characterized by an agro-pastoral activity and covers about 25,052 km^2 for a population estimated to be 520,188 inhabitants.

Study design and ethical approval

A cross sectional study was conducted from December 2017 to April 2019 to determine the prevalence of bacterial and parasite

infections among 155 randomly selected male food handlers including kitchen workers (n= 61), bakers (n=58) and butchers (n=36). In general, copro-parasitological assessment was requested by the managing physicians after presenting with digestive disorders or following an annual check-up to obtain medical certificate. Written informed consent was obtained from each participant and ethical approval for the study was granted by the Faculty of Science, Nature and Life, Djelfa University, Algeria (Ref: AT04/E.V.E.S/ 2017).

Data and sample collection

A structured questionnaire administered by face-to-face interview was used to collect data on socio-demographic characteristics, age, place of living (rural or urban), personal hygiene practices, risk factors and clinical symptoms. Stool samples were collected from each participant into clean stool cup for bacteriological and copro-parasitological examinations, and the fingernail samples were collected from both hands of each subject using sterile moistened swab.

Intestinal parasite identification

Stool samples were examined microscopically using saline and iodine-stained wet-mount preparations. In addition, specimens were concentrated by formol-ether concentration technique and faecal smears were fixed with methanol and stained by modified Ziehl-Neelsen (ZN) technique. Approximately 50 mg of stool samples were inoculated in 5 ml of Boeck and Drbohlav's Locke-egg-serum medium supplemented with 10% horse serum (10). The cultures were checked for the presence of *Blastocystis* after 48-72 hours of incubation by direct microscopy. The fingernail contents were also examined microscopically by direct wet mount preparations in normal saline and iodine solution for intestinal parasites identification.

Bacterial culture isolation and identification

For bacteriological examination, stool samples were cultured onto Hektoen enteric (HE) agar and enriched in Selenite F broth for 24 hours at 37°C. Inoculum from Selenite F broth was sub-cultured on Hektoen enteric (HE) and *Salmonella-Shigella* (SS) agar plates. After 24 hours incubation at 37°C, the growth of *Salmonella* and *Shigella* was differentiated by their colony characteristic appearance on HE agar (*Salmonella* appear as clear colonies with black center while *Shigella* appear as clear/green colonies) and on SS agar (*Salmonella* as colorless colonies with black center while *Shigella* as colorless colonies without black center). Bacterial species were identified with the API 20E System (bio-Mérieux, France) (11).

Fingernail samples were also cultured onto Mannitol salt agar (MSA) and Blood agar plate (BAP) and incubated for 24 hours at 37°C. *Staphylococcus aureus* colonies were identified by growth characteristics on BAP and MSA, Gram stain reaction and biochemical tests such as catalase and coagulase.

Antimicrobial susceptibility testing

Antimicrobial susceptibility test (AST) was performed for each *S.* Typh*i* and *S. aureus* isolate by Kirby-Bauer disc diffusion method on Mueller Hinton (MH) agar. The antimicrobial agents tested for *S.* Typhi were ampicillin (10µg), amoxicillin/clavulanic acid (20µg/10 µg), imipenem (10µg), cefoxitin (30µg), ciprofloxacin (5µg), cefotaxime (30µg) and gentamicin (10µg). Antimicrobial agents tested for *S. aureus* strain were erythromycin (15µg), ciprofloxacin (10µg), fosfomycine (50µg), pristinamycin (30µg), ofloxacin (5µg), and gentamicin (10µg). The resistance and sensitivity were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (12).

Statistical analysis

Statistical analysis was done using Minitab version 19 software. Pearson's Chi-square test was applied to determine association between variable. Values were considered significant at $p < 0.05$.

Results:

Socio-demographic characteristics and risk factors of food handlers

A total of 155 male food handlers were included in the study. Majority of the study participants were between the age group 18-38 years (68.4%) with a mean age of 33.14± 10.04 years and age range of 19-57 years. Regarding hand washing practice, 58% of food handlers had the habit of washing hands with soap before starting their work and 41.3% of participants did not trim their finger nails. Concerning region of residence, 53.5% of food handlers live in urban area while 46.5% live in rural area. Majority (63.2%) of the participants were not in contact with or rearing animals and 33% reported that they have clinical symptoms. The principal characteristics of the study population of the food handlers are summarized in Table 1.

Table 1: Prevalence of *Staphylococcus aureus* and intestinal parasite infections with socio-demographic characteristics and risk factor of food handlers in Laghouat Province, Algeria

Characteristics	N° examined N (%)	No positive for intestinal parasite N (%)	X^2 (p-value)	No positive for *S. aureus* N (%)	X^2 (p-value)
Food handlers					
Kitchen workers	61 (39.4)	23 (37.7)	1.020 (0.601)	6 (9.8)	2.077 (0.354)
Bakers	58 (37.4)	22 (37.9)		10 (17.2)	
Butchers	36 (23.2)	17 (47.2)		7 (19.4)	
Age group (years)					
18-28	64 (41.3)	26 (40.6)	0.6333 (0.888)	9 (14.0)	0.17038 (0.9822)
29-38	42 (27.1)	18 (42.9)		7 (16.7)	
39-48	36 (23.2)	14 (38.9)		5 (13.9)	
> 49	13 (8.4)	4 (30.8)		2 (15.4)	
Living area					
Urban	83 (53.5)	35 (42.2)	1.03226 (0.310)	12 (14.5)	0.04347 (0.835)
Rural	72 (46.5)	27 (37.5)		11 (15.3)	
Hand washing with soap					
Yes	90 (58.0)	23 (25.6)	4.12903 (0.042*)	15 (16.7)	2.13043 (0.144)
No	65 (42.0)	39 (60.0)		8 (12.3)	
Rearing animals					
Yes	57 (36.8)	25 (43.9)	2.32258 (0.128)	9 (15.8)	1.08696 (0.297)
No	98 (63.2)	37 (37.8)		14 (14.3)	
Finger nail status					
Trimmed	91 (58.7)	20 (21.9)	7.80645 (0.005*)	11 (12.1)	0.04347 (0.835)
Not Trimmed	64 (41.3)	42 (65.6)		12 (18.8)	
Clinical manifestation					
Asymptomatic	104 (67.0)	33 (31.7)	148 (0.0001*)	9 (8.6)	40.0435 (0.0001*)
Symptomatic	51 (33.0)	29 (56.9)		14 (27.5)	
Abdominal pain	27 (17.4)	15 (55.6)		7 (25.9)	
Diarrhea	9 (5.8)	5 (55.6)		3 (33.3)	
Fever	3 (1.9)	1 (33.3)		3 (100)	
Nausea	3 (1.9)	1 (33.3)		0	
Diarrhea+ Abdominal pain	7 (4.5)	5 (71.4)		1 (14.3)	
Diarrhea+ Abdominal pain+ Fever	2 (1.3)	2 (100)		0	

*= significant association; X^2=Chi square

Prevalence of intestinal parasites

The overall prevalence of intestinal parasitic infection among the food handlers was 40% (62/155) with six different parasite species. *Blastocystis* spp was the most frequent parasite identified (16.8%), followed by *Giardia intestinalis* (8.4%), *Entamoeba histolytica/dispar* (7.7%), *Entamoeba coli* (3.2%), *Trichomonas intestinalis* (2.6%) and *Endolimax nana* (1.3%). There was no mixed parasite infections in the study population, and no intestinal parasite was identified in fingernail contents (Table 2).

Prevalence of bacterial pathogens

Of the 155 participants, stool cultures were positive for *S.* Typhi in 4 (2.6%) (Table 2). All 4 participants had co-infecting pathogens; 3 with intestinal parasites (*Blastocystis* spp., *G. intestinalis* and *E. histolytica/dispar*) and 1 with *S. aureus* (Table 3). No *Shigella* species or other enteropathogenic bacteria were isolated from the stool samples.

For the fingernails culture result, *S. aureus* was isolated in 23 (14.8%) of the 155 samples (Table 2). Other bacterial species recovered from the fingernail cultures include coagulase negative staphylococci (37.4%), *Escherichia coli* (1.9%) and *Klebsiella* spp (0.7%), while no bacteria was isolated from 45.2% of the fingernail contents. However, co-infection was observed in 11 participants positive for *S. aureus*; 1 (7.1%) with *S.* Typhi and 10 (21.4%) with intestinal parasites, which include *Blastocystis* spp 3 (21.4%), *G. intestinalis* 2 (14.3%), *E. histolytica/dispa* 2 (14.3 %), *E. coli* 2 (14.3%) and *T. intestinalis* 1 (7.1%) (Table 3).

Table 2: Prevalence of pathogenic bacterial and intestinal parasites among 155 male food handlers

Pathogens	Frequency (%)
Pathogenic bacteria	
Salmonella Typhi	4 (2.6)
Staphylococcus aureus	23 (14.8)
Parasites species	
Blastocystis spp	26 (16.8)
Giardia intestinalis	13 (8.4)
Entamoeba histolytica/dispar	12 (7.7)
Entamoeba coli	5 (3.2)
Trichomonas intestinalis	4 (2.6)
Endolimax nana	2 (1.3)
Total	**89 (57.4)**

Table 3: Co-infections of bacterial and intestinal pathogens

Pathogens	Frequency (%)
Blastocystis spp + *Staphylococcus aureus*	3 (21.4)
Giardia intestinalis + *Staphylococcus aureus*	2 (14.3)
Entamoeba coli + *Staphylococcus aureus*	2 (14.3)
Entamoeba histolytica/dispar + *Staphylococcus aureus*	2 (14.3)
Trichomonas intestinalis + *Staphylococcus aureus*	1 (7.1)
Blastocystis spp + *Salmonella* Typhi	1 (7.1)
Giardia intestinalis + *Salmonella* Typhi	1 (7.1)
Entamoeba coli + *Salmonella* Typhi	1 (7.1)
Staphylococcus aureus + *Salmonella* Typhi	1 (7.1)
Total	**14 (100)**

Antimicrobial susceptibility test (AST) results

Concerning the AST result, all *S.* Typhi isolates were sensitive to imipenem and ciprofloxacin, while all were resistant to ampicillin and cefotaxime, 75% to amoxicillin/clavulanic acid, 50% to cefoxitin, and 25% to gentamicin (Table 4). AST result of *S. aureus* isolates showed that they were highly resistant to fosfomycine (82.6%), 11 (47.8%) were resistant to erythromycin, 10 (43.5%) to ofloxacin and 9 (39.1%) to ciprofloxacin and gentamicin each, while the *S. aureus* isolates showed high sensitivity to pristinamycin (Table 4).

Risk factors associated with bacterial and intestinal parasites infection

Significant associations were observed between prevalence of intestinal parasites and socio-demographic characteristics and risk factors among the food handlers (Table 1). Of the food handlers positive for intestinal parasites, the highest prevalence was seen in 29-38 years age group (42.9%) which decreased gradually with advancing age of participants, but this was not significant (p=0.888) (Fig.1).

Twenty five percent of the participants with intestinal parasites had the habit of washing hand with soap before starting their work while 60% do not usually wash their hands with soap before starting their work, and this difference was statistically significant (p=0.042).

Table 4. Antimicrobial resistance patterns of *Salmonella* Typhi and *Staphylococcus aureus* isolates from male food handlers in Laghouat Province, Algeria

Antimicrobial agents	**No of resistant *S.* Typhi strain (%)**	**Antimicrobial agents**	**No of resistant *S. aureus* strain (%)**
Ampicillin (10µg)	4 (100)	Erythromycin (15µg)	11 (47.8)
Amoxicillin/Clavulanic acid (20µg/10µg)	3 (75)	Fosfomycine (50µg)	19 (82.6)
Imipenem (10µg)	0 (00)	Pristinamycin (30µg)	3 (13)
Cefoxitin (30µg)	2 (50)	Ciprofloxacin (10µg)	9 (39.1)
Ciprofloxacin (5µg)	0 (00)	Ofloxacin (5µg)	10 (43.5)
Cefotaxime (30µg)	4(100)	Gentamicin (10µg)	9 (39.1)
Gentamicin (10µg)	1 (25)	/	/

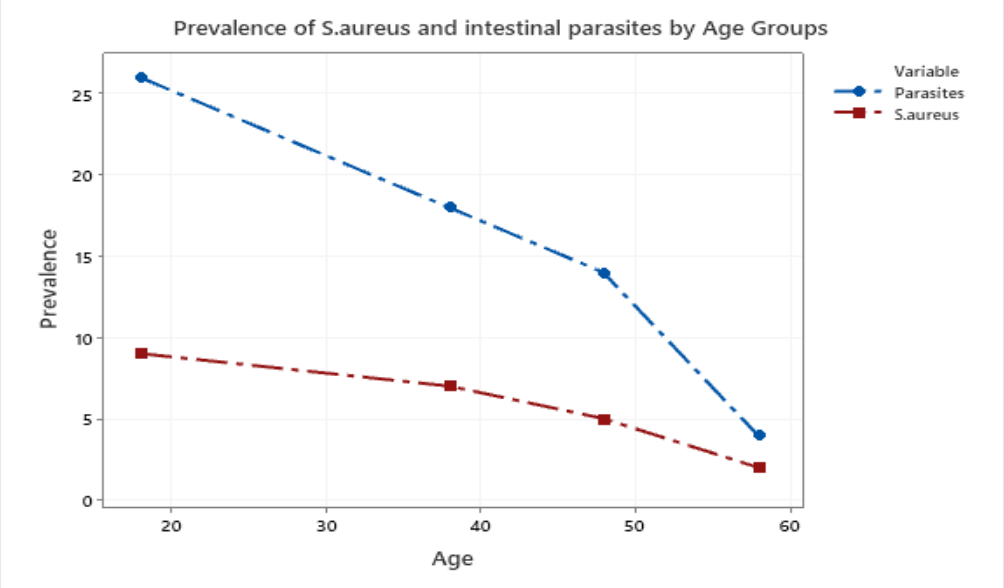

Fig 1. Prevalence of *Staphylococcus aureus* and intestinal parasites by age groups of male food handlers

Univariate analysis of the risk factors for intestinal parasite infection in the study showed significant association between fingernail status and prevalence of intestinal parasites, with prevalence of 65.6% among food handlers who do not trim their fingernails, compared to 21.9% in those who trimmed their fingernails (X^2=7.8064, p=0.005). Additionally, the prevalence of intestinal parasites was significantly higher in the symptomatic participants compared to asymptomatic ones (56.9% vs 31.7%; X^2=148; p=0.0001). With respect to clinical features observed among the food handlers with intestinal parasites (as summarized in Table 1), there was no significant association between prevalence of intestinal parasitic infections and categories of food handlers, living area of participants and rearing of animals by the participants (p>0.05)

The prevalence of *S. aureus* was high in the age group 29-38 years (16.7%) but this was not significantly different from the prevalence in other age groups (p=0.9822) (Fig 1). Also, prevalence of *S. aureus* was higher in the participants who did not trim their nails (18.8%), but there was no significant association with respect to nail trimming (p=0.835). However, there was significant association between prevalence of *S. aureus* and clinical manifestations, with 27.5% of symptomatic participants having *S. aureus* isolated from them compared to 8.6% of asymptomatic participants (p=0.0001). The most frequent clinical features were fever (100%), diarrhea (33.3%) and abdominal pain (25.9%) (Table 1), but there was no significant association between prevalence of *S. aureus* and socio-demographic and risk factors among the food handlers.

Also, there was no significant association between socio-demographic and risk factors among the food handlers and carriage of *S.* Typhi (p=0.276), but all 4 food handlers infected with *S.* Typhi were symptomatic, 2 had diarrhea, abdominal pain and fever, 1 had abdominal pain, and 1 had fever.

Table 5: Distribution of different pathogenic species among male food handlers

Food handlers	Pathogenic species (%)					Total
	S. aureus	***S.* Typhi**	***Blastocystis* spp**	***G. intestinalis***	***E. histolytica/dispar***	
Kitchen workers	6 (26.1)	4 (100)	12 (46.2)	4 (30.8)	3 (25)	29 (37.2)
Bakers	10 (43.5)	0	9 (34.6)	4 (30.8)	5 (41.7)	28 (35.9)
Butchers	7 (30.4)	0	5 (19.2)	5 (38.4)	4 (33.3)	21 (26.9)
Total	**23 (100)**	**4 (100)**	**26 (100)**	**13 (100)**	**12 (100)**	**78 (100)**

S. aureus = *Staphylococcus aureus*; *S.* Typhi = *Salmonella* Typhi; *B.* spp = *Blastocystis* spp; *G. intestinalis* = *Giardia intestinalis*; *E. histolytica/dispar* = *Entamoeba histolytica/dispar*

The distribution of different pathogenic species according to the types of food handlers showed that *S. aureus* was most frequently isolated in bakers (43.5%) followed by butchers (30.4%) and kitchen workers (26.1%), while *S.* Typhi was isolated only in kitchen workers. *Blastocystis* spp was more frequently identified among kitchen workers (46.2%) than in bakers (34.6%) and butchers (19.2%), *G. intestinalis* was more frequently identified in butchers (38.4%) followed by kitchen workers (30.8%) and bakers (30.8%), while *E. histolytica/dispar* was more frequently identified in bakers (41.7%) than in butchers (33.3%) and kitchen workers (25%) (Table 5).

Discussion:

This study was undertaken to determine the prevalence of bacterial and intestinal parasites among food handlers and antibiotic susceptibility profile of the isolated bacteria in Laghouat Province of southern Algeria. The study reported 40% (62/155) prevalence rate of intestinal parasitosis among food handlers, which were mainly protozoan parasites with no helminthes. Our findings are consistent with studies conducted in Qatar (13), Nigeria (14), Yebu, southwest Ethiopia (15) and Bahir Dar, Ethiopia (16), with comparative prevalence of 33.9%, 41.2%, 44.1% and 41.1% respectively, but relatively higher than the rates reported in studies from Tunisia 13.5% (17), Libya 8.26% (18) and Sudan 6.9% (3). Our rate is however much lower than the rates of 97% from Nigeria (19), 50.15% from Jeddah, Saudi Arabia (20), and 52.2% from southeastern Anatolia, Turkey (21). The differences in prevalence rates of intestinal parasitosis in various studies may be related to differences in socioeconomic status, geographical location, personal hygienic practice, lack of supply of safe water and environmental sanitation.

In the current study, *Blastocystis* spp (16.8%) was the predominant parasite identified followed by *G. intestinalis* (8.4%) and *E. histolytica/dispar* (7.7%). Similar results have been reported in previous studies conducted in Jeddah-Saudi Arabia (20) and Sirte-Libya (22) which identified *Blastocystis* spp as the predominant parasite with prevalence of 23.9% and 35.5% respectively. Also, previous studies conducted in other countries revealed that the leading parasites were *G. intestinalis*, *Blastocystis* spp and *E. histolytica/dispar* among food handlers (23-25).

It should be noted that these parasites are recognized as water-food borne pathogens (7-9). However, the prevalence of intestinal parasitosis was higher in the age groups 29-38 years (42.9%) and 18-28 years (40.6%). The higher prevalence in these younger age groups (though the difference was not statistically significant, X^2=0.633, p=0.888), is consistent with the result of the study conducted in Bahir Dar, Ethiopia (16), with the highest infection rate (18.5%) reported in age group 20-40 years, and in Addis Ababa, Ethiopia with 56.4% in the age group 17-34 years (26), but with no significant associations. Furthermore, the finding of our study showed that the prevalence of parasitic infection was significantly higher among food handlers who did not trim their fingernails (65.6%, p= 0.005)) and those who did not wash their hands with soap before starting work (60%, p=0.042). This indicates that most of the intestinal parasites in the present study must have been transmitted through improper hand washing, as a result of ignorance of the food handlers about the importance of personal and hand hygiene.

In the present study, *S.* Typhi was isolated from stool samples of 4 (2.6%) food handlers, which is similar to the low prevalence (2.3%) of *Salmonella* among food handlers reported in Kumasi, Ghana (27), 3.5% in Addis Ababa, Ethiopia (26), 3.8% in Omdurman, Sudan (3) and 1.6% in Bahir Dar, Ethiopia (16). However, these *S.* Typhi were exclusively isolated from the kitchen workers, which is an alarming finding that requires taking into consideration the importance of bacteriological analysis as a routine checkup of food handlers. There was no statistical significance with respect to *S.* Typhi infection and socio-demographic and risk factors among the food handlers. Also, the *S.* Typhi isolates were highly resistant to ampicillin (100%), cefotaxime (100%) and amoxicillin/clavulanic acid (75%), whereas they were 100% sensitive to imipenem and ciprofloxacin, which is compar-

able to the results of the studies conducted in Addis Ababa, Ethiopia where *S.* Typhi also showed 100% resistance to ampicillin, and 100% sensitivity to ciprofloxacin (26), and in Bahir Dar, Ethiopia where *S.* Typhi showed 100% resistance to ampicillin and 33.3% resistance to gentamicin (16), indicating that antimicrobial resistance of *S.* Typhi is an increasing concern. The high resistance to antimicrobial agents in the current study may be due to the easy access to antimicrobial drugs in Algeria.

Several species of bacteria were isolated from the fingernail contents of the food handlers including *S. aureus* (14.8%), coagulase negative staphylococci (37.4%), *Escherichia coli* (1.9%) and *Klebsiella* spp (0.7%). This is similar to bacterial isolates from fingernails of food handlers in other countries like Saudi Arabia (23), Nigeria (28), Ethiopia (29-31) and Iran (32). Coagulase-negative staphylococci are normal flora of the skin, which explains why they are the predominant bacteria isolate from finger nails in the present study. The presence of Gram-negative bacterial species in fingernails indicates faecal contamination due to inadequate hand washing of the food handlers, supporting the notion of poor personnel hygiene.

The prevalence of *S. aureus* reported in the present study (14.8%) is lower than the rates in studies conducted in Gondar, Ethiopia with 16-16.5% (29,30), Saudi Arabia with 17.5% (23) and Iran with 46% (32), but is higher than the rates reported from studies from Nigeria with 7.1% (28) and northwest Ethiopia with 5% (31). Many studies have reported high prevalence of *S. aureus* from nasal and throat swabs including 21.6% in Sudan (3), 20.5% in Gondar, Ethiopia (33), 31% in Egypt (34) and 23.1% in Anatolia, Turkey (21). The detection of *S. aureus* in fingernails of food handlers may pose significant risk for the consumers and public health because certain strains of *S. aureus* may produce enterotoxin on contaminated food substances, which can cause outbreak of staphylococcal food poisoning.

The *S. aureus* isolates showed multiple resistance to antibiotics and were resistant to fosfomycine (82.6%), erythromycin (47.8%), ofloxacin (43.5%), ciprofloxacin (43.5%), and gentamicin (39.1%), but highly sensitive to pristinamycin (87%). Although, we did not test for methicillin resistance among the *S. aureus* isolates in our study, other studies had reported high resistance of *S. aureus* to methicillin and emphasized the importance of the emergence and dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA), which has complicated the therapeutic management of staphylococcal infections (30,31,33).

In the present study, no intestinal parasites or *Salmonella-Shigella* species were detected from fingernails content, which is consistent with the results obtained from studies done in Ethiopia (29-31) and Saudi Arabia (23). However, a study done in Jimma, Ethiopia showed the presence of ova, larvae, and cysts of intestinal parasites under fingernails of the study participants (35), another study done in India showed the presence of *Salmonella* in 65.7% from fingernails and 79% from nail cuts among hotel workers (36).

Conclusion:

The present study reported a high prevalence of intestinal parasitosis, *S.* Typhi and *S. aureus* among stool and fingernails contents of symptomatic and asymptomatic male food handlers in Laghouat Province, Algeria, with high antibiotic resistance among the bacterial isolates. This finding indicate that food handlers may be potential source of food borne disease, for which local health authorities in Algeria should implement strict medical checkup, training on food safety and good-hygiene practices for food handlers.

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Original Article **Open Access**

Assessment of microbial quality and detection of extended spectrum β-lactamase genes in Gram-negative bacterial isolates of herbal mixtures commonly hawked in Sagamu metropolis, Ogun State, Nigeria

*[1]Olaniran, O. B., [1]Ajayi, S. E., [2]Oluwatobi, O. B., and [2]Adeleke, O. E.

[1]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria
[2]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria
*Correspondence to: oluwatoyinolaniran@gmail.com

Abstract:

Background: The use of herbal mixtures in the treatment of diseases is on the rise. Many of these herbal drugs are not produced under hygienic conditions and safety issues associated with herbal medicines may have an exacerbated impact in immunocompromised and elderly individuals. This study aimed to determine the microbial loads of locally prepared herbal mixtures and detect extended spectrum beta-lactamase (ESBL) genes in any isolated Gram-negative bacteria pathogen.
Methodology: Fifty local herbal mixtures were purchased randomly from three locations in Sagamu town (Sagamu market, Ita-Oba Road and Isale Oko) in Ogun State, Nigeria. The mean total viable bacterial (MTVB), mean total coliform (MTC), and mean total fungal (MTF) counts were determined by the plate count method. The bacterial isolates were streaked on differential bacteriological media while the fungi isolates were grown on potato dextrose agar. The isolates were identified upon growth on culture media using conventional biochemical tests. Antibiotic susceptibility pattern of the isolates was determined using Kirby-Bauer disk diffusion technique. Phenotypic detection of ESBL was done by the modified double disc synergy test followed by amplification detection of bla_{TEM}, bla_{CTX-M} and bla_{SHV} genes with polymerase chain reaction (PCR) assay.
Results: Bacteria and fungi were isolated from 38 (76.0%) and 25 (50.0%) of the herbal samples respectively. Ten (20.0%) and 14 (28.0%) of the samples had mean bacterial and fungal load that exceeded 10^5CFU/mL or g, respectively. Nineteen (38.0%) of the herbal samples analyzed had total coliforms. Fifty-one isolates belonging to eight bacterial genera and 28 fungi isolates belonging to four fungal genera were obtained. Thirty-two (62.7%) of the bacterial isolates were Gram-negative while 19 (37.3%) isolates were Gram-positive. *Staphylococcus aureus* was the most common bacterial isolate (33.3%) while *Aspergillus* species was the most prevalent fungus (60.7%). Sixteen (84.2%) *S. aureus* and 26 (81.3%) Gram-negative isolates were multidrug resistant, and 6 (18.8%) of 32 Gram-negative isolates were ESBL producers. ESBL-encoding genes were detected in 7 (27%) of the 26 multidrug resistant Gram-negative bacteria with TEM and SHV being the most prevalent 4 (14.8%) while CTX-M was identified in only one isolate.
Conclusion: This study reported the presence of microbial contaminants which exceeded the safety limits of 10^5 CFU/g according to World Health Organization. The use of locally prepared herbal medicines poses a major health risk due to the lack of microbial quality standards.

Keywords: Herbal medicines, Gram-negative bacteria, extended spectrum beta-lactamase, microbial contaminant

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Évaluation de la qualité microbienne et détection de gènes de β-lactamase à spectre étendu dans des isolats bactériens à Gram

négatif de mélanges d'herbes couramment vendus dans la métropole de Sagamu, dans l'État d'Ogun, au Nigeria

*[1]Olaniran, O. B., [1]Ajayi, S. E., [2]Oluwatobi, O. B., et [2]Adeleke, O. E.

[1]Département de Microbiologie Pharmaceutique, Faculté de Pharmacie, Université Olabisi Onabanjo, Sagamu, État d'Ogun, Nigéria
[2]Département de Microbiologie Pharmaceutique, Faculté de Pharmacie, Université d'Ibadan, Ibadan, Nigéria
*Correspondance à : oluwatoyinolaniran@gmail.com

Résumé:

Contexte: L'utilisation de mélanges à base de plantes dans le traitement des maladies est en augmentation. Bon nombre de ces médicaments à base de plantes ne sont pas produits dans des conditions d'hygiène et les problèmes de sécurité associés aux médicaments à base de plantes peuvent avoir un impact exacerbé chez les personnes immunodéprimées et les personnes âgées. Cette étude visait à déterminer les charges microbiennes de mélanges à base de plantes préparés localement et à détecter les gènes de bêta-lactamase à spectre étendu (BLSE) dans n'importe quel agent pathogène bactérien à Gram négatif isolé.
Méthodologie: Cinquante mélanges d'herbes locales ont été achetés au hasard dans trois endroits de la ville de Sagamu (marché de Sagamu, Ita-Oba Road et Isale Oko) dans l'État d'Ogun, au Nigeria. Les nombres moyens de bactéries viables totales (MTVB), de coliformes totaux moyens (MTC) et de champignons totaux moyens (MTF) ont été déterminés par la méthode de comptage sur plaque. Les isolats bactériens ont été striés sur des milieux bactériologiques différentiels tandis que les isolats de champignons ont été cultivés sur gélose au dextrose de pomme de terre. Les isolats ont été identifiés lors de la croissance sur des milieux de culture à l'aide de tests biochimiques conventionnels. Le profil de sensibilité aux antibiotiques des isolats a été déterminé à l'aide de la technique de diffusion sur disque de Kirby-Bauer. La détection phénotypique des BLSE a été effectuée par le test de synergie à double disque modifié suivi de la détection par amplification des gènes bla_{TEM}, bla_{CTX-M} et bla_{SHV} avec un test de réaction en chaîne par polymérase (PCR).
Résultats: Des bactéries et des champignons ont été isolés de 38 (76,0%) et 25 (50,0%) des échantillons d'herbes, respectivement. Dix (20,0%) et 14 (28,0%) des échantillons avaient une charge bactérienne et fongique moyenne supérieure à 10^5 UFC/ml ou g, respectivement. Dix-neuf (38,0%) des échantillons de plantes analysés contenaient des coliformes totaux. Cinquante et un isolats appartenant à huit genres bactériens et 28 isolats de champignons appartenant à quatre genres fongiques ont été obtenus. Trente-deux (62,7%) des isolats bactériens étaient Gram-négatifs tandis que 19 (37,3%) isolats étaient Gram-positifs. *Staphylococcus aureus* était l'isolat bactérien le plus courant (33,3%) tandis que l'espèce *Aspergillus* était le champignon le plus répandu (60,7%). Seize (84,2%) isolats de *S. aureus* et 26 (81,3%) isolats à Gram négatif étaient multirésistants, et 6 (18,8%) des 32 isolats à Gram négatif étaient producteurs de BLSE. Des gènes codant pour ESBL ont été détectés dans 7 (27,0%) des 26 bactéries Gram-négatives multirésistantes, TEM et SHV étant les 4 les plus répandues (14,8%) tandis que CTX-M n'a été identifié que dans un seul isolat.
Conclusion: Cette étude a rapporté la présence de contaminants microbiens qui dépassaient les limites de sécurité de 10^5 UFC/g selon l'Organisation mondiale de la santé. L'utilisation de médicaments à base de plantes préparés localement pose un risque majeur pour la santé en raison de l'absence de normes de qualité microbienne.

Mots-clés: Médicaments à base de plantes, bactéries Gram-négatives, bêta-lactamase à spectre étendu, contaminant microbien

Introduction:

Herbal medicine is becoming more popular around the world because of the easy availability of raw materials and low cost compared to synthetic industrial preparations (1,2). Herbal drugs are used as a primary mode of treatment by up to 80% of the population in Africa and are still used to treat 70–80% of the population in many industrialized economies (3,4). Herbal materials may contain microbial contaminants due to their origin. Microorganisms of different types are able to adhere to the leaves, stems, floral, seeds, and root systems from which herbal medicine can be prepared, and potential pathogens may be introduced during harvesting and processing (5,6). Consequently, the safety of medicinal herbs has become a serious public health problem (7), as consumers may become ill as a result of ingesting herbs contaminated with pathogenic microorganisms.

Microbial contamination of traditional medicinal herbs has long been recognized as a source of infections, which can result in gastroenteritis, sepsis, blindness, and even death (6, 8). The microbiological limit determination of herbal preparations is therefore required to ensure that the final product is free of health-related risks (9). Herbal medicines in Africa harbor bacterial contaminants which are highly resistant to antibiotics (10). Several studies on

medicinal herbs have revealed the presence of pathogenic microbial strains that are resistant to multiple antibiotics which can be transferred to consumers if present in medicinal herbs (6, 11).

The extensive use of herbal preparations or medicines necessitates the assurance of long-term availability of high-quality, safe preparations of these herbs, particularly for rural or low-income populations, without jeopardizing patients' health (7). A higher standard of hygienic practices during production is required to minimize contamination (9,12). However, most African countries are finding it difficult to implement the World Health Organization (WHO) safety policies regarding herbal medicines since herbalists continue to disregard safety concerns about herbal drug preparations (12,13). The majority of herbal producers in Nigeria lack the necessary skills to perform quality control and resolve safety concerns with the products they produce (9).

Despite the growing number of herbal products in the market in Nigeria, there is still scarcity of information on microbiological quality of herbal drugs in some regions and the genetic basis of resistance in these bacteria (9,10). Therefore, the goal of this study was to determine the level of microbiological contamination and the prevalence of extended spectrum beta-lactamase genes in Gram negative bacterial contaminants of herbal preparations sold in Sagamu, Ogun state, Nigeria.

Materials and method:

Study site, design and sampling process

This cross-sectional study of herb producers was conducted in Sagamu town, Ogun State, Nigeria, between August and September 2021. A total of 50 oral and local preparations (38 liquid and 12 solid indigenous herbal mixtures) were purchased at random from 15 voluntarily consented herb sellers at three locations (Sagamu market, Ita Oba Road, Isale Oko). About 10ml of liquid and 5g of solid herbal medicines were collected into sterile screw-capped bottles and transported to the Pharmaceutical Microbiology Laboratory, Faculty of Pharmacy, Olabisi Onabanjo University, Sagamu within one hour for processing.

Inclusion and exclusion criteria

The herb preparations included in the study were powder and liquid preparations meant to be taken orally and topically without further processing. Herbal medicinal products (in liquid and powder forms) that had undergone additional processing or were administered through other routes were excluded.

Informed consent

Each study participant gave informed consent. All of the information obtained during the study was kept private and confidentially.

Determination of bacterial and fungi load

The determination of bacteria and fungi counts was done using the pour plate method. Ten-fold serial dilution of the samples was performed by placing 1 ml of liquid samples (or 1 g of powdered samples) into 9 ml of physiological saline and allowed to soak for 1 hour. From the suspension, 1 ml was transferred to another tube containing 9 ml of physiological saline and thoroughly mixed. This dilution procedure was further repeated so that there were series of five tubes, giving serial dilutions of 10^{-1} to 10^{-5}. An aliquot of 0.5 ml was pipetted from 10^{-5} dilution for each sample into the sterile Petri dishes. Thereafter, 20 ml of molten Nutrient agar, MacConkey agar and Potato Dextrose agar (PDA) was introduced into each of the Petri dish plates and swirled. The plates were allowed to solidify and then incubated at 37°C for 24 hours for bacteriological analyses and for 5 days for fungal analysis. The study experiment was performed in duplicate.

After incubation, visible colonies on duplicate agar plates were enumerated and recorded as mean colony forming units/ml (CFU/ml). The mean total colony counts were calculated using the formula; Mean total colony counts (CFU/ml) = Mean number of colony formed (x dilution factor) / Volume plated.

Identification of bacterial isolates

A discrete colony of the bacteria to be identified was collected from cultured plates using sterile wire loop and sub-cultured on Mannitol salt agar, MacConkey agar, Eosin Methylene Blue agar and Salmonella-Shigella agar. The plates were incubated aerobically at 37°C for 24 hours. After incubation, the isolates were transferred to Nutrient agar slants for further test. The conventional biochemical tests used to identify different bacterial species were sugar fermentation on triple sugar iron (TSI) agar, hydrogen sulphide production as well as citrate, methyl red, Voges-Proskauer, oxidase, indole and urease tests for Gram-negative, and catalase and coagulase tests for Gram-positive bacteria (14).

Identification of fungal isolates

The fungal morphology was studied macroscopically by observing the colony features such as size, shape, color and hyphae. Microscopic examination of fungi was done by mounting a small portion of the colonies from

culture plate on a slide and staining with Lactophenol-in-cotton blue. The slides were observed under the compound light microscope for characteristic conidia, conidiophores and arrangement of spores (15,16).

Antimicrobial susceptibility testing of isolates

The antibiotic susceptibility patterns of the bacterial isolates were determined using Kirby-Bauer disk diffusion technique (17). Standard conventional antibiotic discs such as levofloxacin (5µg), imipenem (10µg), azithromycin (15µg), gentamicin (10µg), cefuroxime (30µg), carbenicillin (100µg), cefotaxime (30 µg), amoxicillin/clavulanic acid (30µg), cefepime (30µg), cephalexin (30µg) and ceftazidime (30µg) were used. All the isolates were first sub-cultured on nutrient agar and incubated overnight. Then, 3-4 colonies of each isolate were picked and suspended in sterile distilled water, to give turbidity equivalent to 0.5 McFarland standards. The isolate suspension was inoculated on sterile Mueller-Hinton agar plates using a sterile swab stick. Antibiotic discs were placed on the surface of the inoculated agar plates and gently pressed down onto the agar with the aid of a sterile pair of forceps to ensure complete contact with the agar surface.

The plates were left on the bench for 30 minutes to give time for the antibiotics to diffuse into the agar, and then incubated aerobically at 37°C for 24 hours. The diameters of zone of growth inhibition were measured in millimeters, reported and interpreted as sensitive, intermediate or resistant using the Clinical and Laboratory Standards Institute performance standards for antimicrobial susceptibility testing (17).

Phenotypic detection of ESBL

ESBL detection was done by using the modified double-disc synergy test (MDDST). The bacterial colonies obtained were sub-cultured into Nutrient broth, and following incubation for 24 hours at 37°C, the broth cultures were further streaked on Nutrient agar and incubated overnight, 3-4 colonies of each isolate were picked and suspended in sterile distilled water and serially diluted to give turbidity equivalent to McFarland standards ($1x10^8$ cells/ml). The isolate suspension was inoculated on Mueller-Hinton agar plates using sterile swab stick. Amoxicillin-clavulanic acid (30µg) disc was placed at center of the plate, and discs containing cefotaxime (30µg), cefepime (30µg), ceftazidime (30µg**)** and cefixime (30µg**)** were placed 2 cm (center to center) from amoxicillin-clavulanic acid disc using a pair of sterile forceps. The plates were then incubated at 37°C for 24 hours. A clear extension of the edge of the zone of growth inhibition of the cephalosporin discs towards amoxicillin-clavulanic acid disc was interpreted as positive for ESβL production (17).

Extraction of DNA from Gram-negative isolates

The bacterial chromosomal DNA was extracted by a boiling method (18). Briefly, 18-24 hours culture from tryptic soy agar (TSA) was inoculated in 2 ml Luria Bertani broth (LB) and incubated for 18-24 hours. The LB broth was centrifuged (10 000 rpm/min for 10 min) and bacterial cells were suspended in 500 µl of phosphate buffer (100 mM, pH 7) to weaken the membranes and immersed in a boiling water-bath at 100°C for 15 min to release the genetic materials. The DNA was precipitated with 250 µl of absolute alcohol, washed twice in 1000 µl of 70% alcohol, and re-suspended in 100 µl of sterile water (18).

ESBL genes detection in Gram-negative isolates

The ESBL genes (*bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$) were detected by PCR in a thermal cycler (Applied Biosystems, USA). The sequences of the different primers are presented in Table 1. The PCR mix consisted of 1 µl of DNA, 12.5 µl of WizPure™ PCR 2x Master (Wizbiosolutions, South Korea), 1 µl of each primer (0.2 pmol/µl) (Inqaba Biotechnical Industries, South Africa) and molecular grade water to make 25 µl final volume. Two PCR types were performed; a duplex PCR for *bla*$_{TEM}$ and *bla*$_{SHV}$, and a simplex PCR for *bla*$_{CTX-M}$ (19,20). The amplification condition for *bla*$_{TEM}$ and *bla*$_{SHV}$ consists of initial denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 40s, annealing at 50°C for 40s, and elongation 72°C for 1 min, with a final elongation step at 72°C for 7 min. The amplification condition for *bla*$_{CTX-M}$ consists of initial denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 40s, annealing at 60°C for 40s and elongation 72°C for 1 min with a final elongation step at 72°C for 7 min.

Table 1: Primer sequences for amplification of ESBL genes

ESBL genes	Sequence (5'-3')	Amplicon size (bp)	References
TEM	F-GAGTATTCAACATTTTCGT R-ACCAATGCTTAATCAGTGA	857	Maynard et al., (20)
SHV	F-TCGCCTGTGTATTATCTCCC R-CGCAGATAAATCACCACAATG	768	Maynard et al., (20)
CTX-M	F-TTTGCGATGTGCAGTACCAGT AA R-CGATACGTTGGTGGTGCCATA	544	Edelstein et al., (19)

Separation of the amplicons in an electrophoretic tank with migration at 100 volts for 1 hour was performed on 2% agarose gel stained with ethidium bromide (0.5µg/ml) to visualize the PCR products. A 100bp DNA marker was used as reference to determine the molecular size of each amplicon. After migration, the various bands were observed under UV transillumination and photographed using a gel documentation and analysis system (Geno-Sens 1560).

Statistical analysis:

The quantitative data were analyzed statistically using SPSS (version 20) statistical software. Variables were described using standard descriptive statistics such as means, frequencies and percentages.

Results:

Microbial load of herbal mixtures

Twenty-three (46.0%) of the 50 herbal samples were alcohol based, 15 (30.0%) were water based, while 12 (24.0%) were in powdered form. Of the 50 samples, 43 (86.0%) had bacterial and fungal growth while 7 (14.0%) had no bacterial or fungi growth. The microbial loads of the herbal mixtures are presented in Table 2. Bacterial and fungal growths were observed in 38 (76.0%) and 25 (50.0%) of the herbal samples respectively. Ten (20.0%) of the samples had bacterial load that exceeded the safety limit ($\leq 10^5$ CFU/ml or g) set by the World Health Organization (WHO), while 14 (28.0%) samples exceeded the safety limits for fungal growth. Of the herbal samples analyzed, 19 (38.0%) were positive for total coliforms.

The highest total mean bacterial load was $2.6x10^7$ CFU/ml (sample O) while the least total mean bacterial load was $1.0x10^4$ CFU/ml (sample TA). The highest total coliform count and total fungal count were $9.0x10^6$ CFU/g and $4.4x10^7$ CFU/g respectively (sample F), while the least total coliform count was 1.3×10^4 CFU/ml (sample AA) and the least total fungal load was 2.0×10^4 CFU/ml (sample DA).

Bacterial isolates

Fifty-one bacterial isolates were obtained from the 38 herbal samples consisting of seven bacterial genera. Thirty-two (62.7%) of the isolates were Gram-negative while 19 (37. 3%) were Gram-positive. *Staphylococcus aureus* was the most frequent bacterial pathogen 17 (33.3%), followed by *Pseudomonas aeruginosa* 9 (17.6%), and *Acinetobacter baumannii* was the least frequent species (Table 3).

Fungal isolates

Twenty-eight fungal isolates belonging to four genera (*Aspergillus*, *Rhizopus*, *Penicillium* and *Alternaria*) were identified in 23 herbal mixtures. *Aspergillus* species was the most frequently isolated species (60.7%), followed by *Rhizopus* species (25%) (Table 4).

Antibiotic resistance profile of bacterial isolates from herbal mixtures

Fig 1 shows the antibiotic resistance profile of bacterial isolates. *Acinetobacter* species was the most resistant isolate with 100% resistance to cefuroxime, cefotaxime, cefepime, cephalexin and ceftazidime, followed by *Shigella* species with 100% resistance to cefuroxime, cefotaxime, cephalexin and ceftazidime.

Antibiotic resistance pattern of multi-drug resistant (MDR) isolates

Sixteen (94.1%) of the 17 *S. aureus* were multidrug resistant (resistant to three or more classes of antibiotics) while 26 (81.3%) of the 32 Gram-negative isolates were multidrug resistant. All *Escherichia coli* (n=6, 100%) and *Klebsiella oxytoca* (n=6, 100%) isolates were multidrug resistant, while 7 (77.7%), 6 (85.7%), 4 (80%) and (50%) of *K. pneumoniae*, *P. aeruginosa*, *Salmonella* and *Shigella* species respectively were MDR (Table 5).

Table 2: Bacterial and fungal load of herbal mixtures

S/N	Sample	Mean total viable bacterial count (CFU/ml or g)	Mean total coliform count (CFU/ml or g)	Mean total fungal count (CFU/ml or g)
1	A	2.4×10^5	-	1.4×10^6
2	B	3.0×10^5	-	4.0×10^5
3	C	1.0×10^5	2.0×10^5	1.0×10^5
4	D	3.3×10^6	-	-
5	E	-	1.2×10^6	1.4×10^7
6	F	1.52×10^7	9.0×10^6	4.4×10^7
7	G	1.24×10^7	8.0×10^5	1.2×10^6
8	H	-	2.0×10^5	1.6×10^6
9	I	1.6×10^7	4.0×10^5	1.22×10^7
10	J	6.0×10^5	2.0×10^5	2.0×10^7
11	K	1.4×10^5	1.0×10^6	1.2×10^6
12	L	8.0×10^5	-	6.0×10^5
13	M	2.0×10^5	-	4.0×10^5
14	N	8.0×10^5	-	1.48×10^7
15	O	2.6×10^7	2.2×10^6	3.32×10^7
16	P	-	-	2.0×10^7
17	Q	-	-	1.76×10^7
18	R	-	-	2.4×10^6
19	S	3.6×10^5	-	-
20	T	1.7×10^5	-	-
21	U	4.4×10^5	1.8×10^5	-
22	V	2.7×10^5	-	-
23	W	2.3×10^5	-	-
24	X	5.2×10^5	-	-
25	Y	-	-	2.0×10^4
26	AA	3.9×10^5	1.3×10^4	-
27	BA	-	-	-
28	CA	2.6×10^5	-	-
29	DA	1.6×10^5	-	2.0×10^4
30	EA	1.4×10^5	1.6×10^5	7.0×10^4
31	FA	1.3×10^4	-	-
32	GA	-	-	-
33	HA	9.0×10^4	-	-
34	IA	2.1×10^5	1.7×10^5	-
35	JA	1.5×10^6	1.1×10^6	-
36	KA	2.4×10^5	1.3×10^5	1.5×10^7
37	LA	-	-	-
38	MA	2.0×10^4	-	-
39	NA	1.1×10^5	0.9×10^5	-
40	OA	1.7×10^5	0.4×10^5	-
41	PA	1.2×10^7	-	1.2×10^5
42	QA	1.8×10^6	-	6.0×10^4
43	RA	1.0×10^5	-	-
44	SA	-	-	-
45	TA	1.0×10^4	-	1.4×10^5
46	UA	-	-	-
47	VA	-	-	2.7×10^5
48	WA	3.2×10^7	2.8×10^6	-
49	XA	2.2×10^4	-	-
50	YA	1.9×10^6	2.5×10^4	-

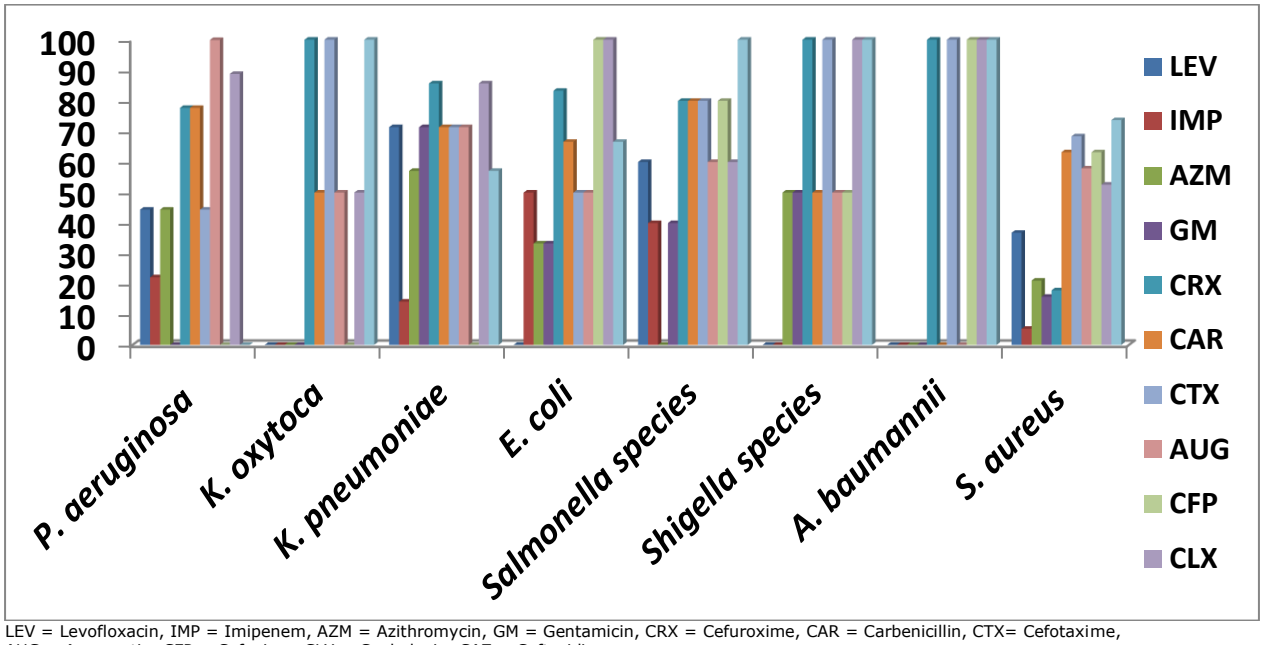
CFU/ml or g = Colony forming unit per ml or gram; - = No Growth

Table 3: Bacteria isolates recovered from the herbal mixtures

S/N	Isolated bacterial species	No of isolated bacteria	Percentage of isolated bacteria
1	*Pseudomonas aeruginosa*	9	17.6
2	*Klebsiella oxytoca*	2	3.9
3	*Klebsiella pneumoniae*	7	13.7
4	*Escherichia coli*	6	11.7
5	*Salmonella* species	5	9.8
6	*Shigella* species	2	3.9
7	*Acinetobacter baumannii*	1	1.9
8	*Staphylococcus aureus*	17	33.3
9	*Staphylococcus saprophyticus*	2	3.9
	Total	**51**	**100**

Table 4: Fungi isolates recovered from the herbal mixtures

S/N	Isolated fungal species	No of isolated fungi	Percentage of isolated fungi
1	*Aspergillus* species	17	60.7
2	*Rhizopus* species	7	25.0
3	*Penicillium* species	3	10.7
4	*Alternaria* species	1	3.6
	Total	**28**	**100**



LEV = Levofloxacin, IMP = Imipenem, AZM = Azithromycin, GM = Gentamicin, CRX = Cefuroxime, CAR = Carbenicillin, CTX= Cefotaxime, AUG = Augmentin, CFP = Cefepime, CLX = Cephalexin, CAZ = Ceftazidime

Fig 1: Percentage antibiotic resistance profile of isolates from herbal mixture

Prevalence of ESBL in Gram-negative bacterial isolates

Six (18.8%) of the 32 Gram-negative isolates were positive for ESBL production by the phenotypic detection method. The isolates were *Pseudomonas aeruginosa* (n=1), *A. baumannii* (n=1), *Salmonella* species (n=1), *K. pneumoniae* (n=1), and *E. coli* (n=2) as shown in Table 6. Figs 2 and 3 showed PCR amplification of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes. ESBL-encoding genes were detected in 7 (27.0%) of the 32 multidrug resistant Gram-negative bacteria, with bla_{TEM} and bla_{SHV} being the most frequent 4 (14.8%), while bla_{CTX-M} was identified in only one isolate. The bla_{TEM} gene was detected in *P. aeruginosa*, *K. pneumoniae* and *E. coli*, bla_{SHV} was detected in *P. aeruginosa, A. baumannii*, *Salmonella* species

and *E. coli*, while *bla*$_{CTX-M}$ was detected only in *K. pneumoniae.* Co-occurrence of *bla*$_{TEM}$ and *bla*$_{SHV}$ was detected in one *E. coli* isolate while co-occurrence of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ was detected in one *K. pneumoniae.* All the 6 isolates positive for phenotypic ESBL production carried ESBL-encoding genes while one isolate that was negative for phenotypic ESBL production carried *bla*$_{SHV}$ gene as shown in Table 6.

Table 5: Antibiotic resistance profile of multidrug resistant bacterial isolates

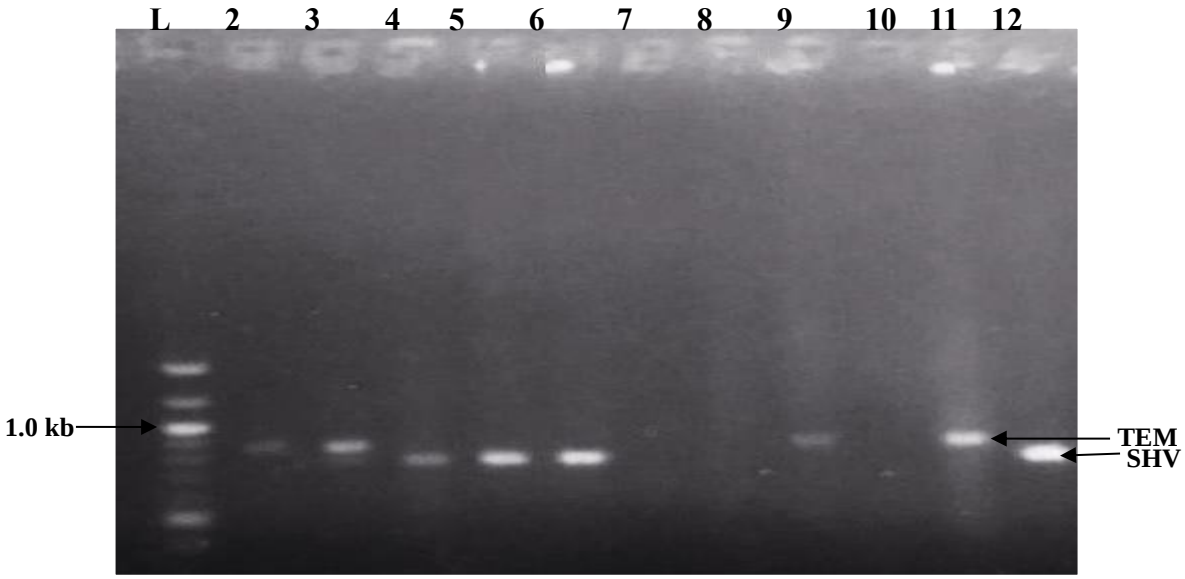
Isolate code	Isolates	Resistant profile
A1	*Staphylococcus aureus*	CRX, AUG, CLX
D1	*Staphylococcus aureus*	LEV, AZM GM, CRX, CAR, CTX, CAZ
F1	*Staphylococcus aureus*	IMP, CRX, CAR, AUG, CLX, CAZ
G1	*Staphylococcus aureus*	LEV, GM, CRX, CAR, CTX, AUG, CFP, CLX, CAZ
H1	*Staphylococcus aureus*	AZM, CRX, CTX, CLX, CFP, CAZ
S	*Staphylococcus aureus*	LEV, AZM, CRX, CTX, AUG, CFP, CLX, CAZ
T	*Staphylococcus aureus*	LEV, CRX, CTX, AUG, CFP, CLX
X	*Staphylococcus aureus*	LEV, CRX, CAR, CTX, CFP, CLX, CAZ
CA2	*Staphylococcus aureus*	CAR, AUG, CLX
FA	*Staphylococcus aureus*	LEV, CRX, CAR, CFP, CAZ
XA	*Staphylococcus aureus*	CRX, CAR, CTX, AUG, CFP, CLX, CAZ
K2	*Staphylococcus aureus*	CRX, CAR, CTX, AUG, CFP, CAZ
M1	*Staphylococcus aureus*	AZM, GM, CRX, CAR, CTX, AUG, CLX, CFP, CAZ
MA	*Staphylococcus aureus*	CRX, CAR, CTX, AUG, CLX
QA	*Staphylococcus aureus*	CRX, CAR, CTX, AUG, CFP
TA	*Staphylococcus aureus*	LEV, CRX, CAR, CTX, CFP, CAZ
B2	*Pseudomonas aeruginosa*	CRX, CAR, AUG, CFP, CLX
E3	*Pseudomonas aeruginosa*	CRX, CAR, AUG, CLX
DA	*Pseudomonas aeruginosa*	LEV, IMP, AZM, GM, CRX, CAR, CTX, AUG, CLX
U2	*Pseudomonas aeruginosa*	AZM, CRX, AUG, CLX
AA1	*Pseudomonas aeruginosa*	LEV, AZM, CRX, CAR, CTX, AUG, CLX
CA1	*Pseudomonas aeruginosa*	LEV, AZM, GM, CRX, CAR, CTX, AUG, CLX
HA	*Pseudomonas aeruginosa*	LEV, IMP, GM, CRX, CAR, CTX, AUG, CLX
AA2	*Klebsiella oxytoca*	CRX, CAR, CTX, AUG, CLX, CFP, CAZ
L	*Klebsiella oxytoca*	CRX, CTX, CLX, CFP, CAR
O2	*Klebsiella pneumoniae*	AZM, GM, CRX, CTX, AUG, CFP, CLX, CAZ
JA	*Klebsiella pneumoniae*	IMP, GM, CRX, CAR, CTX, AUG, CLX, CFP
KA	*Klebsiella pneumoniae*	AZM, GM, CRX, CAR, CTX, AUG, CLX, CFP, CAZ
W	*Klebsiella pneumoniae*	LEV, AZM, GM, CRX, CAR, CTX, AUG, CLX, CAZ
WA	*Klebsiella pneumoniae*	LEV, CRX, CAR, CLX
NA	*Klebsiella pneumoniae*	LEV, AZM, GM, CRX, CAR, CTX, AUG, CFP, CLX
A2	*Escherichia coli*	IMP, GM, CRX, CAR, AUG, CLX, CFP
M2	*Escherichia coli*	LEV, IMP, AZM, GM, CRX, CAR, CTX, AUG, CLX, CFP, CAZ
EA	*Escherichia coli*	CRX, CTX, CLX, CAZ, CFP
U1	*Escherichia coli*	CAR, CTX, CLX, CAZ, CFP
OA	*Escherichia coli*	AZM, CRX, CTX, AUG, CLX, CFP, CAZ
YA	*Escherichia coli*	LEV, CRX, CAR, AUG, CFP, CLX, CAZ
I3	*Salmonella* species	CRX, CAR, CTX, AUG, CFP, CAZ
IA	*Salmonella* species	CRX, CAR, CFP, CLX, CAZ
V	*Salmonella* species	LEV, IMP, GM, CRX, CAR, CTX, AUG, CLX, CAZ
RA	*Salmonella* species	LEV, IMP, GM, CRX, CAR, CTX, AUG, CLX, CAZ
J	*Shigella* species	AZM, GM, CRX, CAR, CTX, AUG, CLX, CFP, CAZ

LEV = Levofloxacin, IMP = Imipenem, AZM = Azithromycin, GM = Gentamicin, CRX = Cefuroxime, CAR = Carbenicillin, CTX= Cefotaxime, AUG = Augmentin, CFP = Cefepime, CLX = Cephalexin, CAZ = Ceftazidime

Table 6: Detection of extended spectrum beta-lactamase (ESBL) genes in Gram negative isolates

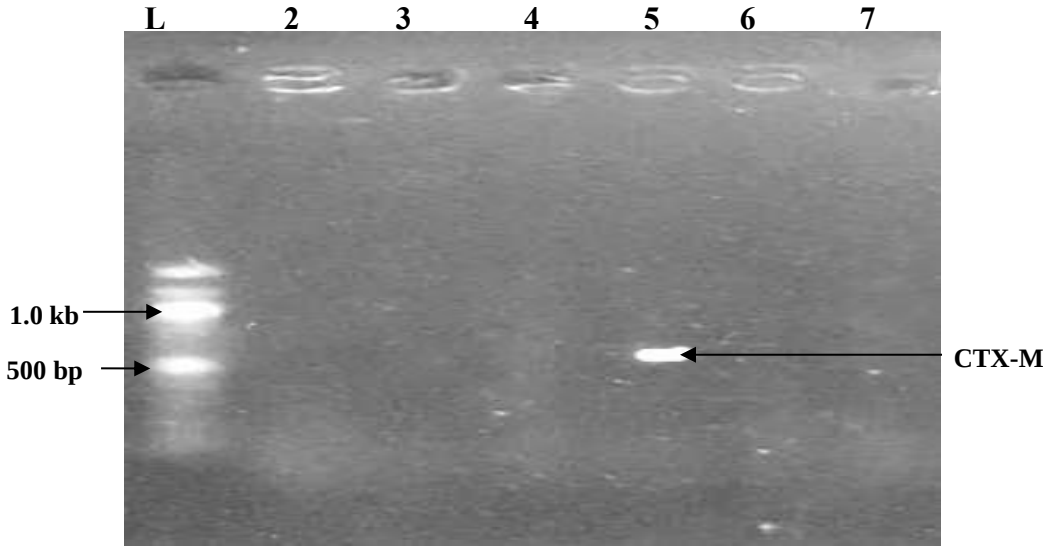
S/N	Isolate code	Isolates	ESBL genes			MDDST
			CTX-M	SHV	TEM	
1.	B2	*Pseudomonas aeruginosa*	-	-	+	+
2.	G2	*Acinetobacter* species	-	+	-	+
3.	IA	*Salmonella* species	-	+	-	+
4.	JA	*Klebsiella pneumoniae*	+	-	+	+
5.	OA	*Escherichia coli*	-	-	+	+
6.	YA	*Escherichia coli*	-	+	+	+
7.	CA1	*Pseudomonas aeruginosa*	-	+	-	-

MDDST- Modified double-disc synergy test



L = Molecular weight marker; 2 = *Acinetobacter* species, 3 = *K. pneumoniae*, 4 = *Acinetobacter* species, 5 = *Salmonella* species, 6 = *E. coli*, 7 = *P. aeruginosa*, 8 = *K. oxytoca*, 9 = *E. coli*, 10 = *K. pneumoniae*, 11 = *E. coli*, 12 = *P. aeruginosa*

Fig 2: PCR amplification of *bla*$_{SHV}$ (768 bp) and *bla*$_{TEM}$ (857 bp) genes



L = Molecular weight marker; 2-7 = 2 = *P. aeruginosa*, 3 = *Acinetobacter* species, 4 = *Acinetobacter* species, 5 = *K. pneumoniae*, 6 = *E. coli*, 7 = *E. coli*

Fig 3: PCR amplification of *bla*$_{CTX-M}$ gene (543 bp)

Discussion:

Both traditional and modern medicine use medicinal plants as a source of raw materials. Plant materials have been in use as home remedies, over-the-counter drugs, and biopharmaceutical ingredients in both the developed and developing worlds (4). Medicinal herbs are the major source of primary health care for most rural populations, particularly in developing countries (3,4). Plant-based medicines are increasingly being integrated into primary health care systems in developing countries but safety concerns are being overlooked (21,22). The production of herbal medicines by unlicensed vendors with no or limited educational backgrounds, as well as a lack of food hygiene knowledge, have contributed significantly to

the high rate of bacterial contamination of herbal drugs (23). Furthermore, most medicinal plants are prepared in open environment under unsanitary conditions, which leads to contamination with pathogens that are harmful to the public (24,25). The risk of microorganisms such as coliform and others being present in pharmaceutical products, including herbal medicines, is determined by the nature of the product, the intended use, and the possibilities for end user harm (26,27). The inability to prevent moisture levels in herbal medicines during transportation and storage, as well as the temperatures of liquid forms and finished herbal products, may have resulted in the proliferation of microorganisms (22).

Several studies have reported bacterial counts in herbal materials and herbal medicines (28,29). Bacterial and fungal contaminations are common, particularly in locally produced herbal medicines, with CFU/g levels exceeding the recommended WHO standards of $\leq 10^5$ CFU/ml (22), demonstrating increased risks in the intake of these products (22). In this study, 20.0% and 28.0% of herbal medicine preparations exceeded the safety limits for bacterial and fungal growths respectively, indicating that consuming these products poses a risk. Herbal medicines in liquid pharmaceutical form for oral use had the highest microbial contamination and were the most commonly consumed products among the elderly (25).

The locally sold herbal mixtures in our study showed a wide variety of potential pathogens such as *S. aureus, P. aeruginosa, K. pneumoniae* and *E. coli* that have been implicated in many diseases. The presence of *E. coli, Salmonella* and *Shigella* species in liquid herbal preparations makes them unfit for human consumption according to the WHO guidelines (12). Previous studies from Nigeria also identified the presence of *S. aureus, P. aeruginosa*, *Salmonella* species and other coliforms in herbal mixtures from Abuja, Nigeria (9,30). The mean total viable bacterial counts (range of $1.0x10^4$-$3.2x10^7$ CFU/ml) and mean total fungi count (range of $2.0x10^4$-$4.4x10^7$ CFU/ml or g) obtained in this study are higher than the study of Ya'aba et al., (9) who reported a range of $2.0x10^1$-$6.7x10^3$ CFU/ml for bacteria and $1.0x10^1$-$3.0x10^1$ CFU/ml for fungi. However, in a study from Jos, Nigeria, Dashen et al., (30) reported a higher mean total viable bacterial count of $1.0x10^6$–$1.4x10^7$ CFU/ml. *Aspergillus* species was the most predominant fungal isolate from this study. Previous studies have reported the presence of fungi in herbal medicines (29,31,32). The contamination of herbal preparations by fungi has been attributed to contamination by dust following storage in moist conditions (33).

According to a meta-analysis on antibiotic-resistance in medically important bacteria isolated from commercial herbal medicines in Africa from 2000 to 2021, *E. coli* was the most frequently reported MDR species, followed by *S. aureus* (10). All the bacterial isolates from herbal mixtures by Ayansina and Akinsola (29) were multidrug resistant. In our study however, 84.2% of Gram-positive isolates and 81.3% of Gram-negative isolates were multidrug resistant. Yesuf et al., (28) reported that all *E. coli* from herbal preparations were sensitive to gentamicin and ciprofloxacin, whereas our study found that all *E. coli* were sensitive to levofloxacin but only 66.7% were sensitive to gentamicin. Resistance to ceftazidime was found in all *K. oxytoca, A. baumannii* and *Salmonella* species in our study, which agrees with the findings of Ayansina and Akinsola (29), who reported all *P. aeruginosa, E. coli, Klebsiella*, and *Salmonella* isolates in their study to be ceftazidime resistant (29). Resistance to cefuroxime was seen in all *P. aeruginosa, E. coli, Klebsiella,* and *Salmonella* species (29) in our study, with resistance ranging from 78 to 86%.

The detection of bla_{TEM}, bla_{SHV}, and bla_{CTX-M} among bacterial isolates recovered from herbal mixtures in our study agrees with the findings of Ayansina and Akinsola (29), who also detected bla_{TEM} and bla_{CTX-M} genes in bacterial isolates recovered from Nigerian herbal mixtures. The presence of multidrug-resistant Gram-negative bacteria isolates carrying ESBL-genes in herbal mixtures is a major source of concern, because of the potential risk of more difficult-to-treat ESBL infections in consumers of these herbal preparations, as well as the risk of transfer of ESBL genes, that can be carried on mobile genetic elements such as plasmids, transposons and integrons, from one isolate to another (34). Furthermore, high proportion of elderly people in Nigeria who use herbal medicines to treat a variety of ailments may be at increased risk of infections by ESBL-producing and multi-drug resistant bacterial pathogens, with dire consequences.

Conclusion:

From the finding of our study, local herbal preparations are not sufficiently safe to consume, because of high degree of contamination with pathogenic microorganisms that are resistant to most commonly used antibiotics in our environment. As a result, herbal mixtures prepared locally in an unsanitary env-

ironment or sold without regulatory approval are not safe for human consumption. It is recommended that proper hygienic conditions be maintained throughout the entire preparation process.

Authors' contributions:

OBO* conceived and designed the study; OBO* and OEA coordinated the experiment; OBO*, SEA and OBO carried out the study experiments; OBO* drafted the manuscript and analyzed the data; and OEA did necessary editing of the manuscript. All authors read and approved the manuscript.

Source of funding:

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Conflict of interest:

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Original Article **Open Access**

Investigation of rodent reservoirs of emerging pathogens in Côte d'Ivoire, West Africa

*[1,2]Meite, S., [1,2]Koffi, K. S., [1]Kouassi, K. S., [1]Coulibaly, K. J., [1]Koffi, K. E., [1]Sylla, A., [1]Sylla, Y., [1,2]Faye-Ketté, H., and [1,2]Dosso, M.

[1]Molecular Biology Platform and Environnement and Health Department, Pasteur Institute, Cote d'Ivoire
[2]Medical Sciences, Microbiology department, Felix Houphouet Boigny University, Cocody, Abidjan
*Correspondence to: meitesynd@yahoo.fr

Abstract:

Background: One of the main health problems in West Africa remains upsurge of emerging pathogens. Ebola virus disease outbreak occurred in 2014 in Liberia, Guinea and Sierra Leone, Monkeypox virus in Nigeria in 2017 and most recently Lassa virus in Nigeria, Togo and Benin in 2018. These pathogens have animal reservoirs as vectors for transmission. Proper investigation of the pathogens in their rodent vectors could help reduce and manage their emergence and spread.
Methodology: This study was conducted with an approval from the Côte d'Ivoire Bioethics Community. Small mammal trappings were carried out in 9 sites within three zones namely, peri-urban, peri-rural and protected areas. Liver, lung and kidney tissues from trapped small mammals were sampled in accordance with the recommended conditions of biosafety and bioethics. The organs were transported in liquid nitrogen to the laboratory. Molecular tests were used to detect pathogens. Orthopoxviruses and Monkeypox virus were detected in the organs by PCR using consensus primers targeting the virus surface membrane haemagglutinin (HA) genes, while *Leptospira* species were detected by PCR using primers targeting the *rrs* and *lfb1* genes.
Results: Out of 4930 night-traps, 256 (5.19%) small mammals were trapped including *Crocidura*, *Rattus, Lophuromys, Praomys, Mus* and *Mastomys*. *Leptospira* species were detected in 6 genera from 7 study sites and the infected small mammals accounted for 13.3%. *Leptospira* sp was detected mainly in the rodent vector genera *Rattus* (32.3%), *Lophuromys* (29.0%), and *Praomys* (16.1%). Three species of *Leptospira* were detected and *Leptospira interrogans* was the most common frequent species (74.2%). Monkeypox virus was not detected from studied small mammals.
Conclusion: The initial data from our investigation indicates the presence of *Leptospira* sp in rodent vectors, *Rattus, Lophuromys* and *Praomys*, which are the potential small mammalian reservoirs of this pathogen in Cote d'Ivoire.

Keywords: Environment; reservoir; emerging pathogen; Côte d'Ivoire; West Africa

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Investigation des rongeurs réservoirs d'agents pathogènes émergents en Côte d'Ivoire, Afrique de l'ouest

*[1,2]Meite, S., [1,2]Koffi, K. S., [1]Kouassi, K. S., [1]Coulibaly, K. J., [1]Koffi, K. E., [1]Sylla, A., [1]Sylla, Y., [1,2]Faye-Ketté, H., et [1,2]Dosso, M

[1]Plateforme de Biologie Moléculaire et Département Environnement et Santé, Institut Pasteur, Côte d'Ivoire
[2]Sciences Médicales, Département de Microbiologie, Université Félix Houphouët Boigny, Cocody, Abidjan
*Correspondance à: meitesynd@yahoo.fr

Résumé:

Contexte: L'un des principaux problèmes de santé en Afrique de l'Ouest reste la recrudescence des pathogènes émergents. En 2014, une épidémie de maladie à virus Ebola s'est produite au Libéria, en Guinée et en Sierra Leone, Monkeypox virus au Nigeria en 2017 et plus récemment Lassa virus au Nigeria, au Togo et au Bénin en 2018. Ces agents pathogènes ont des réservoirs animaux dont les rongeurs impliqués dans la transmission à l'homme. Une

investigation appropriée de ces réservoirs pourrait aider à réduire et à gérer la propagation de ces agents pathogènes émergents.
Méthodologie: Cette étude a été menée avec l'approbation du comité bioéthique de Côte d'Ivoire. Des piégeages de petits mammifères ont été effectués sur 9 sites répartis en trois zones, à savoir les zones périurbaines, périrurales et protégées. Des tissus du foie, du poumon et des reins de petits mammifères capturés ont été prélevés conformément aux recommandations en biosécurité et de bioéthique. Les tissus prélevés ont été transportés dans de l'azote liquide jusqu'au laboratoire. Des tests moléculaires ont été effectués pour détecter les agents pathogènes. Monkeypox virus a été recherché dans les tissus par PCR à l'aide d'amorces consensus *Orthopoxvirus* ciblant les gènes de l'hémagglutinine et puis à l'aide d'amorces spécifiques ciblant des gènes de la membrane de surface du Monkeypox virus. *Leptospira* a été recherché par PCR à l'aide d'amorces ciblant les gènes rrs et lfb1.
Résultats: Pour 4930 nuit piège, 256 petits mammifères ont été capturés. Le taux de succès de capture était de 5,19%. Les rongeurs capturés étaient du genre *Crocidura, Rattus, Lophuromys, Praomys, Mus et Mastomys*. *Leptospira* a été détecté chez 13,3% des rongeurs capturés. Cette bactérie a été isolée sur 7 sites sur 9. *Leptospira* sp a été détecté principalement chez les rongeurs du genre *Rattus* (32,3%), *Lophuromys* (29,0%) et *Praomys* (16,1%). Trois espèces de *Leptospira* ont été détectées dont *Leptospira interrogans*, l'espèce la plus fréquente (74,2%) isolée. Monkeypoxvirus n'a pas été détecté chez les petits mammifères capturés.
Conclusion: Les premières données de notre investigation indiquent la présence de *Leptospira sp*, chez les rongeurs *Rattus, Lophuromys et Praomys*, qui sont les potentiels réservoirs de ce pathogène en Côte d'Ivoire.

Mots clés: Environnement; réservoir; agent pathogène émergent; Côte d'Ivoire; Afrique de l'Ouest

Introduction:

The new challenge of West Africa region on health was the fight against emerging pathogens. The recent events are worrying. In 2014, Ebola virus disease occurred in Guinea, Liberia and Sierra Leone for up to 20,000 cases (1,2). After the isolation of Monkeypox virus in a dead primate in the Tai-forest of Cote d'Ivoire in 2012 (3), Monkeypox virus epidemic disease went up to 80 cases in 2017 in Nigeria (4). Lassa fever outbreak, caused by a haemorrhagic fever virus, also occurred in Benin, Togo and Nigeria in 2018 (5,6). In addition to these viruses, the threat of *Leptospira* bacteria is real. Studies have shown the presence of these bacteria in humans suspected with viral fever (7,8). These pathogens have animal reservoirs as vectors for transmission (9-13).

The contact among people and animals is frequent for various reasons including urbanization, agriculture or hunting. This contact promotes pathogen transmission and spread in the human population. Cote d'Ivoire, a West Africa country with more than 20 million inhabitants remain under the threat of these pathogens. The largest population movements in the region are towards Cote d'Ivoire. It is paramount to develop the different strategies taking into account the reservoir animal vectors to cope with threats. Proper investigation of the pathogens in their animal vectors could help reduce and prevent their emergence and spread. Therefore, the local investigation scientist group for emerging pathogens, composed of physicians, veterinarians, microbiologists and epidemiologists, was created, with the aim of identifying the main animal reservoir vectors.

Materials and method:

Study sites

The investigated sites were chosen according to bibliography data (14-16). Several emerging pathogens such as *Leptospira*, Monkeypox virus, and haemorrhagic fever viruses were investigated. For biosafety level, *Leptospira* and Monkeypox virus were investigated in the first phase. Human serology test results for *Leptospira* and Orthopoxviruses in Cote d'Ivoire were obtained (8,17). Small mammal trappings were carried out at 9 sites within three zones namely; peri-urban, perirural and protected areas (Fig. 1).

Bioethics approval

This study was conducted with an approval from the Côte d'Ivoire Bioethics community (Number of bioethics agreement 024/ MSLS/CNER-dkn in 2015). Biodiversity protection, ecology protection, biosafety and bioethics remain the preoccupation of the investigating group.

Small mammals trapping and autopsy:

Rodents trapping were made by Sherman trap, Chauvancy trap, locally made trap and Pitfalls trap. Traps used in this study were all livetraps. The laying of traps was done at 5 pm and checked in the morning at 6 am. The traps were arranged in line of 20 separated by 5 meters for 3 successive days. The traps containing rodents were retrieved and replaced with a new one. All trapped small mammals were transported to the autopsy sites under biosafety conditions.

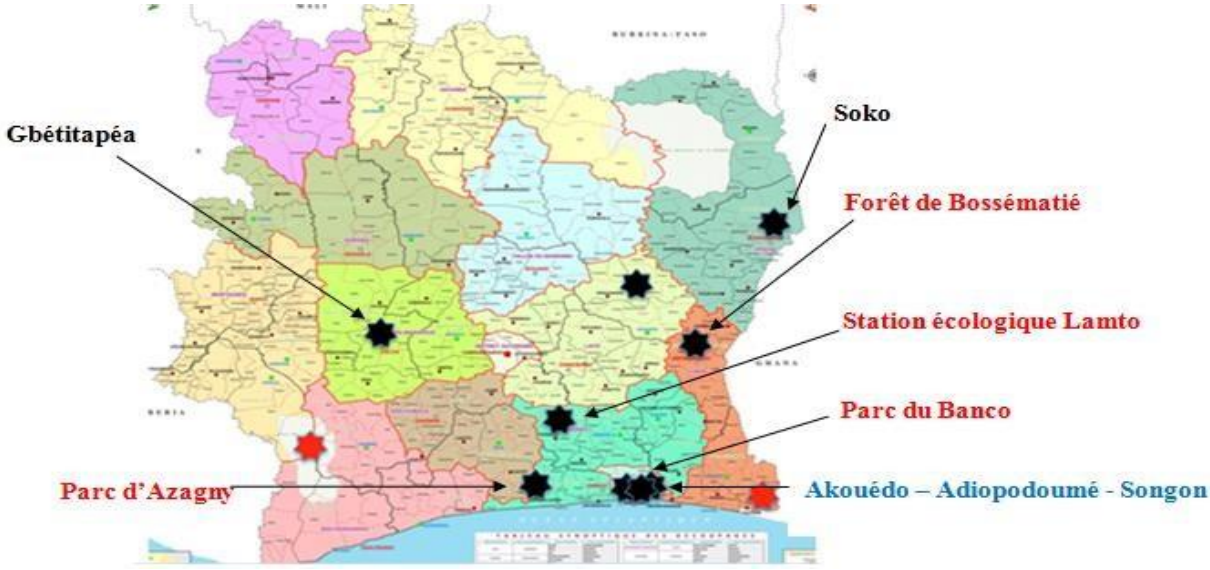

Fig 1: Study sites investigated for rodent reservoirs of emerging pathogens in Cote d'Ivoire
(The black stars are the different sites, the sites written in blue are peri-urban, in black are peri-rural areas and in red are protected areas)

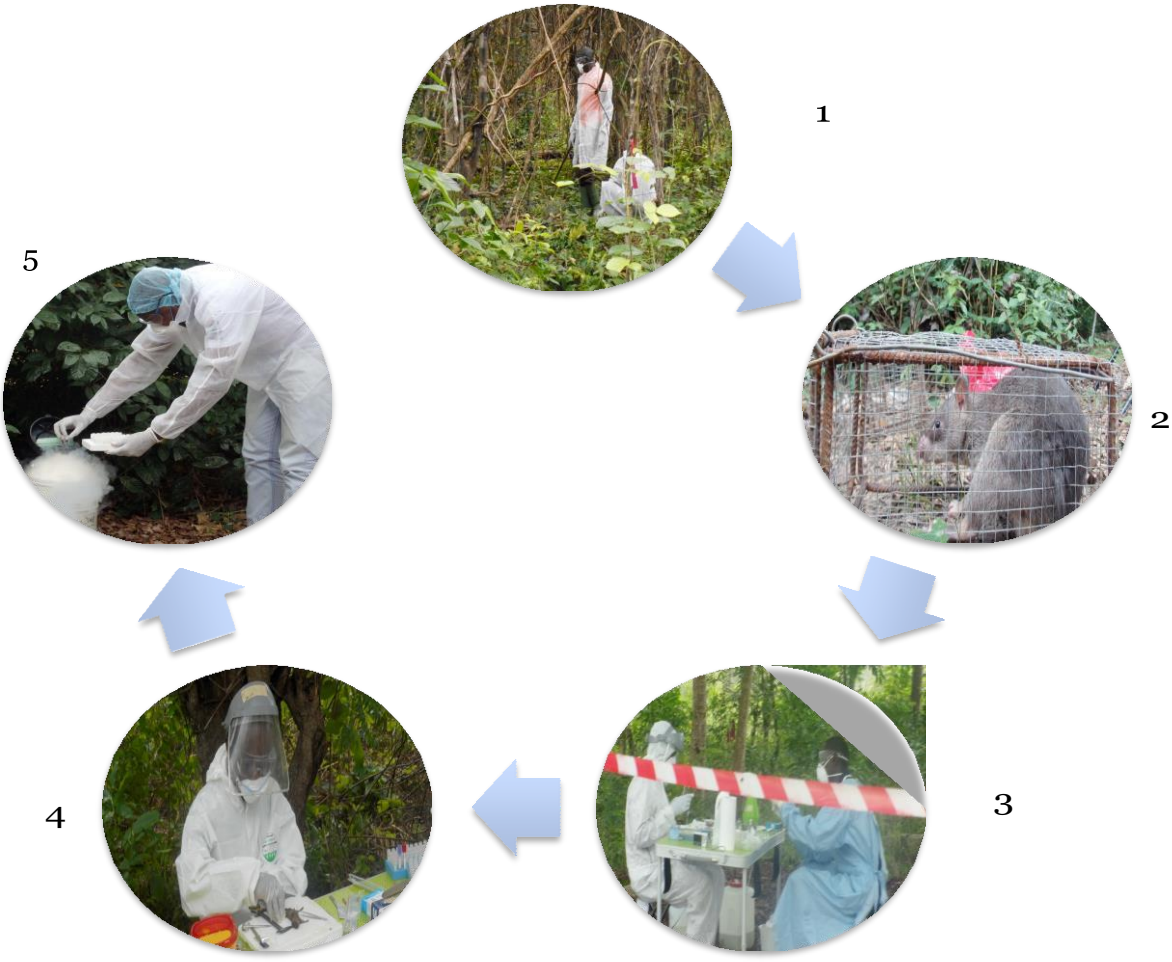

Fig 2: Workflow process of trapping and autopsy of rodents

1 = Inspection of trapping sites, 2= specimen trapping (here a local trap), 3 = Autopsy delimited zone, 4 = equipment wearing for autopsy, 5 = liquid nitrogen for transport sample.

The rodents were anaesthetized with isofloran. After measurements and phenotypic identifications were made, autopsy was performed, and the liver, lung, kidney and other organs were harvested in accordance with the recommended conditions of biosafety and bioethics. Three to four tissue samples were removed per organ. Samples were transported in liquid nitrogen to the laboratory. Carcasses were preserved in 2% formalin. All infectious wastes produced during autopsies were incinerated. Fig 2 shows the workflow for sample processing.

Rodent identification:

Identification of rodents was based on morphological characteristics. The measurements (general length, body/tail percentage, head-to-body length, tail length, posterior leg length (soles), length of the ears) were determined as previously described by Herbreteau (18). The weight and coat inspection were taken into account.

Tissues preparation

About 25g of kidney, liver or lung were ground in a sterile mortar with a sterile pestle. The ground material obtained was dissolved in 1 ml of 1 x PBS. This mashed potato-like material obtained was used for the extraction of DNA

DNA extraction:

The DNeasy Blood and Tissue Kit (Qiagen® Cat No./ID: 69506) was used for DNA extraction from the tissues. To 200 µl broth of tissue, 180 µl of ATL buffer and then 20 µl of proteinase K, were added. The whole solution was incubated at 56°C for 1 hour in a thermomixer, then 200 µl of buffer AL and 200 µl of absolute ethanol were added for the precipitation of the DNA. DNA was collected on a silicate spin column and then washed with AW1 and AW2 solution. A volume of 200 µl of eluate buffer AE was used to collect the DNA

Molecular detection of Orthopoxvirus and Monkeypox virus by PCR assay

Molecular detection of Orthopoxvirus and Monkeypox virus was done by PCR using consensus primers of Orthopoxviruses designed from the virus surface membrane HA gene (forward: EACP1: 5' ATG ACA CGA TTG CCA ATA C 3', and reverse: EACP2: 5' CTA GAC TTT GTT TTC TG 3') with expected PCR product band size of 942 bp (19). For Monkeypox virus, the primers used were forward: MPV1: 5' CTG ATA ATG TAG AAG AC 3' and reverse: MPV2: 5' TTG TAT TTA CGT GGG TG 3' with expected PCR product of 406 bp size (19).

The GoTaq G2 Flexi DNA polymerase kit (Promega Corporation, USA) was used for the PCR mixes, containing 0.2 µM of each primer, 1.5 µM, $MgCl_2$, 0.1 µM dNTPs, 1unit Taq polymerase, 1 x of buffer and 5 µl of DNA template for a final volume of 50 µl. Amplification conditions for the detection of Orthopoxviruses were; 94°C for 5 min (1 cycle), 94°C for 30 sec, 48°C for 1 min, 72°C for 1 min (36 cycles), and then 72°C for 8 min (1 cycle). The PCR products were viewed on a GelDoc Bioanalyzer (BioRad®) after electro- phoresis on 1.5% agarose gel.

Molecular detection of *Leptospira* by PCR

Molecular detection of *Leptospira* was performed by PCR targeting the *rrs* and *lfb1* genes. PCR targeting *rrs* gene used primers previously published by Merien (20) (LA:5'-GGCGGCGCGTCTTAAACATG-3' and LB:5'-TTC CCCCCATTGAG CAA GATT-3'), which amplify a 331 bp PCR product. The Taq DNA Polymerase kit (New England BioLabs®) was used for the PCR mixes containing 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 1 µM of each oligo, 200 µM of each dNTPs and 1 UI of Taq DNA polymerase. Five microlitres of DNA template was used for a final volume of 50µl. Amplification conditions were as follows; 94°C 3 min (1 cycle), 94°C 1 min, 63°C 1 min 30 sec, 72°C 2 min (30 cycles), and 72°C 10 min (1 cycle). The PCR products were viewed on a GelDoc Bioanalyzer (BioRad®) after electro- phoresis on 1.5% agarose gel stained by SYBR safe (ThermoScientific®). Bench Top 100 bp DNA Ladder (Promega®) was used as the size marker.

Real time PCR targeting the *lfb1* gene used primers previously published by Merien (21) (LFB1-F: 5'-CATTCATGTTTCGAATCATTT CAAA-3' and LFB1-R: 5'-GGCCCAAGTTCCTTC TAAAAG-3'. The Maxima SYBR Green qPCR Master Mix (Sigma®) was used for the PCR mixes with 0.8 µM of each oligos, 0.5 µL of 1/10 dilution of Rox dye and 5 µL of DNA template for a final volume of 25 µL. Amplification conditions were as follows; 95°C 10 min (1 cycle), 95°C 15 sec, and 57°C 1 min (49 cycles). This stage was followed by Tm analysis with 0.5°C increase each 30 seconds between 75 to 90°C.

Data analysis

Statistical analysis of data was performed using EPI INFO 7.2 software and the Chi-square test was used to compare the variables with significance level of *p* fixed below 5%.

Results

The number of night-trap was 4930 for a total catch of 256 specimens, with trapping success or yield of 5.19% (range of 1.35% to 8.75% depending on the trapping area). Overall, the yield was 2.9% in protected areas, 4.5% in peri-rural areas and 8.2% in peri-urban areas (protected areas vs peri-urban areas X^2=57,96, p<0.00001, peri-rural vs peri-urban areas X^2=11,48, p<0.001, protec- ted areas vs peri-rural areas X^2=4.7, p<0.01) (Table 1). Success rate was higher in the residential of peri-urban areas, and diversity was more in forest and bush of protected area.

Table 1: Small rodents trapping success by area and sites

Capture site	Night-trap number (n = 4930)	Specimen captured number (n = 256)	Trapping success efficiency (%) (5.19%)	X^2	*p* value
Protected areas					
Azagny	590	8	1.32		
Banco	840	27	3.2		
Bossématié	600	17	2.83		
Lamto	240	14	5.8	4.18 (a)	<0.04*(a)
Sub-total	2,270	66	2.90	11.48 (b)	<0.001*(b)
Peri-rural areas				57.96.33 (c)	<0.00001*(c)
Soko	500	25	5.0		
Gbetitapea	300	11	3.6		
Sub-total	800	36	4.5		
Peri-urban areas					
Adiopodoumé	840	70	8.33		
Akouédo	720	63	8.75		
Songon	300	21	7.0		
Sub-total	1,860	154	8.2		

(a): comparison of trapping success-protected areas and peri-rural areas; (b): comparison of trapping success-peri-urban areas and peri-rural area; (c): comparison of trapping success-protected areas and peri-urban area; X^2= Chi square; * = statistically significant

Table 2: Micromammals captured

Order	Family	Genus	Number (n = 256)	Percentage (%)
Insectivora (Eulipotyphia) (n= 60)	Soricidae	*Crocidura*	60	23.4
Rodentia (n=195)	Scuiridae	*Funisciurus*	5	2.0
	Gerbillidae	*Tatera*	3	1.1
	Cricetomydae	*Cricetomys*	3	1.1
	Muridae	*Rattus*	70	27.34
		Lophuromys	28	10.9
		Praomys	25	9.7
		Mus	20	7.8
		Mastomys	20	7.8
		Lemniscomys	5	2.0
		Uranomys	4	1.5
		Myomys	3	1.1
		Hylomuscus	2	0.7
		Hybomys	1	0.4
		Nanomys	1	0.4
		Malacomys	1	0.4
		Not identified	5	2.0

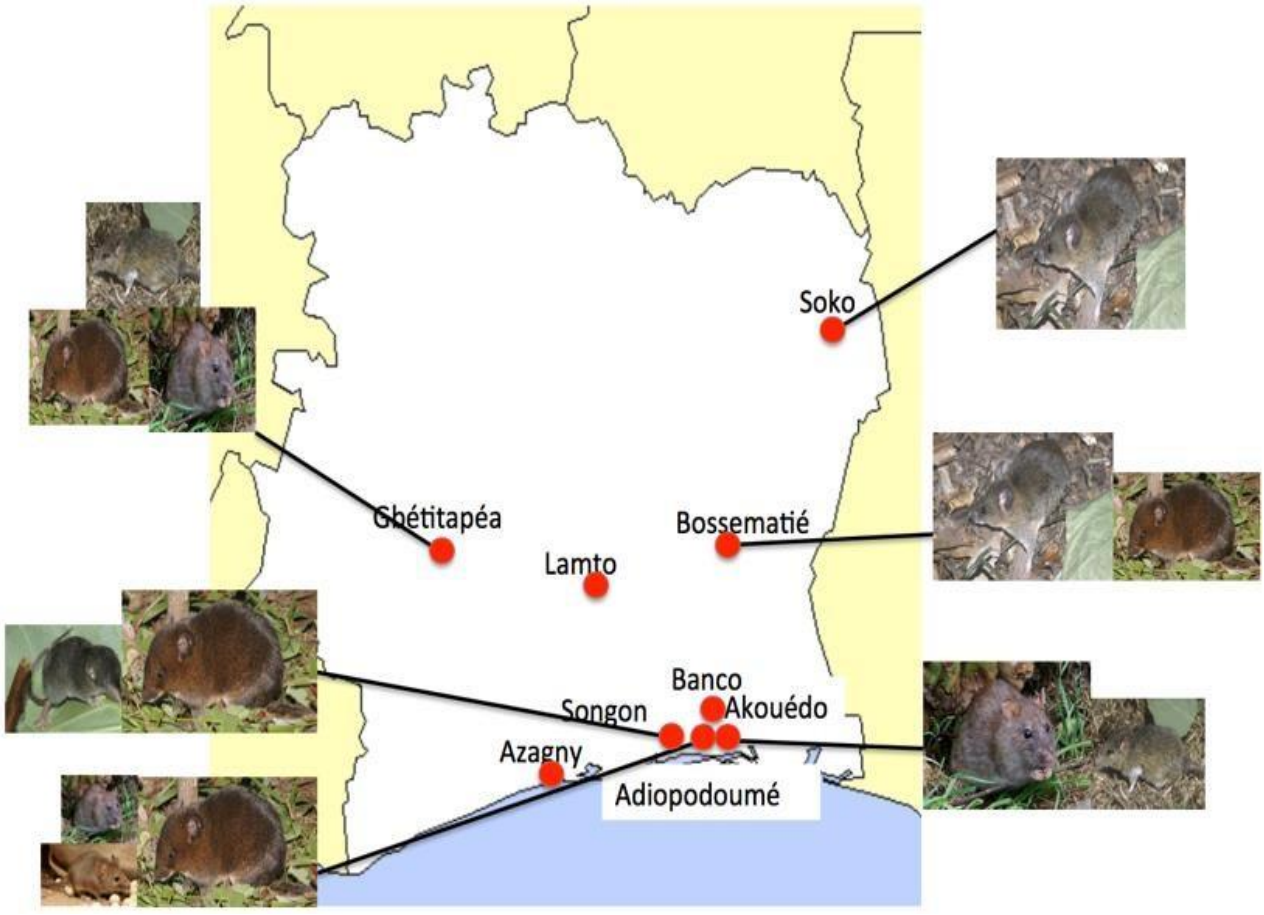

Fig 3: Distribution of infected small mammals by trapping sites

A total of 256 small mammals were trapped including 76.2% (n=195) rodents and 23.8% (n=60) insectivora. The insectivora consists mainly of the genus *Crocidura*, with species such as *Crocidura olivieri* (90.2%), *Crocidura occidentalis* (1.6%), *Crocidura obscuruor* (3.3%) and Crocidura sp (3.0%). Concerning rodents, four families were trapped including *Muridae, Sciuridae, Gerbillidae* and *Cricetomydae* with 94.4% being *Muridae* with mainly genera; *Rattus, Lophuromys, Praomys, Mus* and *Mastomys* (Table 2). Diversity of trapped animals was seen in protected areas but abundance was more in urban areas (Table 3)

Liver, kidney and lung were analysed by molecular test to detect Monkeypox virus and *Leptospira* bacteria. One (0.39%) *Crocidura olivieri* liver was positive for Orthopoxvirus but Monkeypox virus negative, and was a rodent of urban area. *Leptospira* DNA was detected in 31 (12.1%) small mammals, which included 6 genera; *Rattus* sp (32.3%, n=10), *Lophuromys* sp (29.0%, n= 9), *Praomys* sp (16.1%, n=5), *Mastomys* sp (9.7%, n=3), *Mus* sp (6.5%, n=2) and *Crocidura* sp (6.5%, n=2).

Of the *Leptospira* infected small mammals, *Lophuromys* sp was the most infected (36.0%), followed by *Praomys* sp (20.8%), *Rattus* sp (16.7%), *Mastomys* sp (12.0%), *Mus* sp (9.5%) and *Crocidura* sp (3.8%). Three *Leptospira* species were detected, *Leptospira interrogans* was commonest (74.2%), followed by *Leptospira borgpetersenii* (16.1%) and *Leptospira kirshneri* (9.7%). Fig 3 shows distribution of infected small mammals in the 9 trapping sites.

Table 3: Micromammal captured by area and sites

Species of micromammal	**Peri-rural area Abundance = 36 Diversity = 11**		**Protected area Abundance = 66 Diversity = 14**				**Peri-urban area Abundance = 154 Diversity = 11**		
	Soko	**Gbetitapea**	**Azagny**	**Lamto**	**Bossématié**	**Banco**	**Adiopodoumé**	**Songon**	**Akouédo**
Crocidura olivieri (n=55)	2	3		2		5	23	7	13
Crocidura occidentalis (n=1)	1								
Crocidura obscuruor (n=2)			2						
Crocidura sp (n=2)			1		1				
Mastomys natalensis (n=15)	9	3					3		
Mastomys erythrolocus (n=5)	2		2				1		
Mus mucuslus (n=20)	3						16		1
Myomys daltoni (n=3)	3								
Praomys sp (n=25)	4		3		12	5	1		
Hylomuscus sp (n=2)			2						
Cricetomys sp (n=3)			1	2					
Lophuromys sp (n=27)		1	1		1	7	10	7	
Lophuromys sicapusis (n=1)			1						
Rattus rattus (n=16)		1	5				3		7
Rattus norvegicus (n=24)			4				10	2	8
Rattus sp (n=31)							31		
Hybomys sp (n=1)			1						
Malacomys longipes (n=1)			1						
Tatera sp (n=3)		3							
Lemniscomys sp (n=5)			3				2		
Uranomys ruddi (n=4)			4						
Funisciurus carruthesis (n=5)							5		
Nanomys sp (n=1)							1		
Not identified (n=4)	1						3		
Abundance	25	11	8	14	17	27	70	21	63
Species wealth	8	5	5	5	4	5	7	5	4

Discussion:

Success rate of trapping in our study was similar to Duplantier in Madagascar during an investigation of a plague epidemic (22) and he had linked this rate to a significant death of the murine population due to the plague epidemic. He also noted a high rate of success capture in homes, which was due to the fact that they are food storage areas attracting rodents. It is an important factor of contact between humans and rodents. On the other hand, Le Quilliecet (23) reported a lower rate 1%, which was attributed to the low rodent density in the trapping area and the model of trap used. Thus, several factors could influ- ence the success rate including rodent density in the area and the type of trap used. Diversity of rodents captured was important in protec- ted area. This is due to the absence or reduc- tion of the human impact on protected areas.

Several genus including *Crocidura, Rattus, Mastomys, Mus, Myomys, Praomys, Hylomuscus, Hybomys, Lophuromys, Malacomys, Terara, Lemniscomys, Uranomys*, and *Nanomys* were captured in this study. In Côte d'Ivoire, several studies had already underlined this diversity (24-28) and this was also confirmed in other West African regions (28). The involvement of rodents captured in the transmission of certain diseases is well known (9-13).

However, for pathogen like Monkeypox virus, doubts still exist for the true reservoir. The most suspected reservoir vector would be *Funisciurus anerythrus* (30,31). In the first phase of this study, Monkeypox virus was not isolated, however, the presence of Orthopoxvirus or anti-Orthopoxvirus antibodies in different studies in Africa (11,32,33) indicate the need to maintain this surveillance in order to prevent epidemic as occurred in Nigeria in 2017 (4).

In this study, *Leptospira* were detected in 6 genera from 7 trapping sites. These finding suggest a large distribution of these bacteria in small mammals in Côte d'Ivoire. *Leptospira* is able to infect a wide range of mammals and the large distribution of *Leptospira* in small mammals is now well known (34). Regarding infection rate of the small mammal, *Lophuromys* sp, *Praomys* sp and *Rattus* sp were the most infected. With respect to geographic distribution of these infected small mammals, the most infected are *Lophuromys* sp, *Praomys* sp and *Rattus* sp in the southern peri-urban sites, eastern peri-urban sites and Abidjan urban sites respectively. These small mammals could play major role in maintenance of bacteria and their spread in the environment at their local level. Three species of *Leptospira* were found in our study, with *L. interrogans* being the most frequently detected, followed by *L. borgpetersenii* and *L. kirshneri*. These 3 species are the ones reported in published studies from Africa, and *L. interrogans* is the most frequent specie reported (35, 36,37).

Conclusion:

The initial data from our investigation indicated the presence of *Leptospira* sp in the rodent vectors, *Rattus, Lophuromys* and *Prao mys* as the potential small mammalian reservoirs in Cote d'Ivoire. Further exploration of the tissue samples taken from these reservoirs will continue, in our bid to search for other emerging pathogens.

Contribution of authors:

MS was responsible for conceptualization, investigation, methodology and writing of original draft; KofKS was involved in investigation and writing of the original draft; KouKS was involved in investigation and writing of original draft; CKJ, KKE, SA and SY were involved in resources and visualization; FH and DM were involved in supervision of the study and validation of the manuscript

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Conflict of interest:

No conflict of interest is declared

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Original Article **Open Access**

Chemical composition analysis of essential oils of four plants from Aurès region of Algeria and their antibacterial and antibiofilm activities against coagulase-negative staphylococci

*[1]Zatout, A., [1]Djibaoui, R., [2]Flamini, G., [2]Ascrizzi, R., [3]Benbrahim, C., [4]Mazari, H. E., [5]Benkredda, F., [6]Mechaala, S., and [7,8]Kassah-Laouar, A.

[1]Laboratory of Microbiology and Plant Biology, Department of Biological Sciences, Faculty of Natural Sciences and Life, University of Abdlhamid Ibn Badis, Mostaganem, Algeria
[2]Dipartimento di Farmacia, Via Bonanno 6, 56126 Pisa, Italy
[3]Laboratory of Microbiology Applied to the Agroalimentary Biomedical and the Environment, Department of Biology, Faculty of Natural Sciences and Life, University Abou BekrBelkaid, Tlemcen, Algeria
[4] Geo-environment and Space Development (LGEDE), University of Mustapha Stambouli, Mascara, Algeria
[5]Laboratory of Applied Microbiology, University of Oran 1 Ahmed Ben Bella, Algeria
[6]Laboratory of Genetics, Biotechnology and Valorization of Bio-resources, Department of Natural Sciences and Life, Faculty of Exact Sciences and Sciences of Nature and Life, Mohamed Khider University, Biskra, Algeria
[7]Central Laboratory of Medical Biology, Anti-Cancer Center, Batna, Algeria
[8]Faculty of Medicine, University of Batna 2, Batna, Algeria
*Correspondence to: asma.zatout@univ-mosta.dz

Abstract:

Background: The altitudinal and geographical variability of the Aurès mountains of Algeria favored the existence of some endemic and rare varieties of medicinal plants. The aim of the present work is to determine the chemical composition, antimicrobial and antibiofilm properties of the essential oils (EOs) from aerial parts of four medicinal plants from Aurès region of Algeria; *Juniperus thurifera* L., *Juniperus oxycedrus* L., *Salvia officinalis* L. and *Thymus ciliatus* ssp. *munbyanus* (Boiss. & Reut.) Batt. on coagulase negative staphylococci (CoNS) isolates.
Methodology: Extraction of EOs from the four plant materials was carried out by hydro-distillation, and the EO yield expressed in gram of the distillate per 100 grams of dry matter. The chemical composition of the EOs was analyzed by gas chromatography-mass spectrometry (GC-MS) method. *In vitro* antibacterial and antibiofilm activities of the EOs were evaluated against CoNS previously isolated at the Anti-Cancer Center of Batna, Algeria using the agar disc diffusion assay and biofilm inhibition study, respectively. Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of the EOs of *S. officinalis* L. and *T. ciliatus* ssp. *munbyanus* were determined by the dilution method.
Results: Twenty-seven and 41 compounds rich in monoterpene hydrocarbons were identified from *J. oxycedrus* and *J. thurifera* plants respectively, while 45 and 32 compounds, constituted mainly by oxygenated mono-terpenes, were identified from *S. officinalis* L. and *T. ciliatus* ssp. *munbyanus*, respectively. The EOs of *T. ciliatus* ssp. *munbyanus* showed the most inhibitory activity of all the four plants on CoNS isolates (n=66) with mean inhibition zone diameter of 24.99±6.29mm, and mean MIC and MBC values of 2.65±3.77mg/ml and 5.31±7.41mg/ml respectively, followed by *S. officinalis* L., with mean inhibition zone diameter of 13.38± 6.52mm, and mean MIC and MBC values of 27.53±28.2 mg/ml and 31.97±33.19 mg/ml respectively (p<0.0001 by one-way ANOVA). Also, percentage biofilm inhibition of CoNS isolates (n=59) was high for EOs of *T. ciliatus* ssp. *munbyanus* (65.63±10.71%) and *S. officinalis* L. (53.13±5.83%), although was significantly higher for *T. ciliatus* ssp. *munbyanus* compared to *S. officinalis* L. (p<0.0001, t=7.874).
Conclusion: Essential oils from *T. ciliatus* ssp. *munbyanus* and *S. officinalis* L. could represent an alternative to classical antibiotics against planktonic cells and biofilms of CoNS.

Keywords: coagulase-negative staphylococci; chemical composition; essential oils; antibacterial activity; antibiofilm activity

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Analyse de la composition chimique des huiles essentielles de quatre plantes de la région des Aurès en Algérie et leurs

activités antibactériennes et anti biofilm contre les staphylocoques à coagulase négative

*[1]Zatout, A., [1]Djibaoui, R., [2]Flamini, G., [2]Ascrizzi, R., [3]Benbrahim, C., [4]Mazari, H. E., [5]Benkredda, F., [6]Mechaala, S., et [7,8]Kassah-Laouar, A.

[1]Laboratoire de Microbiologie et Biologie Végétale, Département des Sciences Biologiques, Faculté des Sciences Naturelles et de la Vie, Université Abdlhamid Ibn Badis, Mostaganem, Algérie
[2]Dipartimento di Farmacia, Via Bonanno 6, 56126 Pise, Italie
[3]Laboratoire de Microbiologie Appliquée à l'Agroalimentaire Biomédical et à l'Environnement, Département de Biologie, Faculté des Sciences Naturelles et de la Vie, Université Abou BekrBelkaid, Tlemcen, Algérie
[4]Géo-environnement et Développement Spatial (LGEDE), Université Mustapha Stambouli, Mascara, Algérie
[5]Laboratoire de Microbiologie Appliquée, Université d'Oran 1 Ahmed Ben Bella, Algérie
[6]Laboratoire de Génétique, Biotechnologie et Valorisation des Bio-ressources, Département des Sciences Naturelles et de la Vie, Faculté des Sciences Exactes et des Sciences de la Nature et de la Vie, Université Mohamed Khider, Biskra, Algérie
[7]Laboratoire Central de Biologie Médicale, Centre Anti-Cancéreux, Batna, Algérie
[8]Faculté de Médecine, Université de Batna 2, Batna, Algérie
*Correspondance à: asma.zatout@univ-mosta.dz

Résumé:

Contexte: La variabilité altitudinale et géographique des montagnes des Aurès en Algérie a favorisé l'existence de certaines variétés endémiques et rares de plantes médicinales. L'objectif de ce travail est de déterminer la composition chimique, les propriétés antimicrobiennes et antibiofilm des huiles essentielles (HE) des parties aériennes de quatre plantes médicinales de la région des Aurès en Algérie; *Juniperus thurifera* L.*, Juniperus oxycedrus* L.*, Salvia officinalis* L. et *Thymus ciliatus* ssp. *Munbyanus* (Boiss. & Reut.) Batt. sur des isolats de staphylocoques à coagulase négative (SCN).
Méthodologie: L'extraction des HE des quatre matières végétales a été réalisée par hydro-distillation, et le rendement en HE exprimé en gramme de distillat pour 100 grammes de matière sèche. La composition chimique des HE a été analysée par la méthode de chromatographie en phase gazeuse-spectrométrie de masse (GC-MS). Les activités antibactériennes et antibiofilm *in vitro* des HE ont été évaluées par rapport à la SCN précédemment isolée au Centre anticancéreux de Batna, en Algérie, en utilisant respectivement le test de diffusion sur disque d'agar et l'étude d'inhibition du biofilm. Concentration minimale inhibitrice (CMI) et concentration minimale bactérienne (CMB) des HE de *S. officinalis* L. et *T. ciliatus* ssp. *munbyanus* ont été déterminés par la méthode de dilution.
Résultats: Vingt-sept et 41 composés riches en hydrocarbures monoterpéniques ont été identifiés chez les plantes *J. oxycedrus* et *J. thurifera* respectivement, tandis que 45 et 32 composés, constitués principalement de monoterpènes oxygénés, ont été identifiés chez *S. officinalis* L. et *T. ciliatus* ssp. *munbyanus*, respectivement. Leuile de *T. ciliatus* ssp. *munbyanus* a montré l'activité la plus inhibitrice des quatre plantes sur les isolats SCN (n=66) avec un diamètre moyen de la zone d'inhibition de 24,99±6,29 mm et des valeurs moyennes de CMI et CMB de 2,65±3,77 mg/ml et 5,31±7,41 mg/ml respectivement, suivi de *S. officinalis* L., avec un diamètre moyen de la zone d'inhibition de 13,38±6,52 mm, et des valeurs moyennes de CMI et CMB de 27,53 ±28,2 mg/ml et 31,97±33,19 mg/ml respectivement (p<0,0001 par ANOVA à un facteur). De plus, le pourcentage d'inhibition du biofilm des isolats de SCN (n=59) était élevé pour les HE de *T. ciliatus* ssp. *munbyanus* (65,63±10,71%) et *S. officinalis* L. (53,13±5,83%), bien qu'il soit significativement plus élevé pour *T. ciliatus* ssp. *munbyanus* par rapport à *S. officinalis* L. (p<0,0001, t=7,874).
Conclusion: Les huiles essentielles de *T. ciliatus* ssp. *munbyanus* et *S. officinalis* L. pourraient représenter une alternative aux antibiotiques classiques contre les cellules planctoniques et les biofilms de SCN.

Mots clés: staphylocoques à coagulase négative; composition chimique; huiles essentielles; activité antibactérienne; activité antibiofilm

Introduction:

Coagulase-negative staphylococci (Co NS) are very widespread in nature (air, soil, water), and also part of the normal flora of the skin and mucous membranes of mammals and birds. This flora plays an important role in the physicochemical balance of the skin and constitutes a barrier against bacteria of the transient flora (1). The emergence of CoNS as pathogens for various infections may be the result of the increasing use of invasive procedures such as catheters and intravascular prostheses, intensive care unit (ICU) treatment of patients with cancers, transplant recipients, immunocompromised states and premature children (2). In addition, the production of biofilm has been considered as an important factor in the pathogenesis of CoNS, protecting against antibiotics and the immune system (3). This situation has forced scientists to look for new alternative strategies to eliminate these bacteria that are resistant to antibiotics and producing biofilms (4). A possible approach is the use of medicinal plants, which are good sources of new antimicrobial chemotherapeutic agents, in particular, essential oils (EOs).

Four plant species from the Aurès mountains of Algeria are widely used for many therapeutic properties; *Juniperus oxycedrus* L. and *Juniperus thurifera* L. from the Cupressaceae family, and *Salvia officinalis* L. and *Thymus ciliatus* ssp. *munbyanus* (Boiss. & Reut.) Batt. from the Lamiaceae family. *Juniperus oxycedrus* L., juniper (also known as cade tree) is a tree that can reach up to 8m in height and is native to the Mediterranean region (5). The trunk has a coat of grey to reddish-brown fibrous bark in longitudinal stripes, and has many branches, spreading or ascending. The leaves are similar to needles and alternate in three turns. The needles are 1 to 2.5 cm long and 1 to 2.5 mm wide, with two furrows of white, waxy stomata above, an edge below and a thorny tip. This tree is used in traditional medicine for the treatment of various diseases such as hyperglycaemia, obesity, tuberculosis, bronchitis and pneumonia (6).

The Thuriferous Juniper (*J. thurifera* L.) known as Aiwal or Hazenzna in Berber (7) is a tree or shrub of the Cupressaceae family which grows only in isolated parts of the western Mediterranean basin; France, Italy and Spain in Europe, and Algeria and Morocco in North Africa (8-9). In Algeria, this species can be found associated with cedar and its areal is strictly limited to the Aures mountains with a number of scattered and often very large trees, which are probably the remains of more extensive juniper stands (8). It is a dioecious tree or shrub, with scale leaves and bluish black berries at maturity (9). Different species of Juniperus have been used in traditional medicine for centuries as incense, diuretics, remedies for indigestion (10), cough suppressants, anti-fertility, antituberculosis, colds, dysentery, leukorrhea, rheumatic arthritis and fever (11).

Salvia officinalis L. (sage, garden sage, or common sage) is a perennial, evergreen shrub (12), grey-green and with wrinkles on the upper surface and in the lower surface are almost white with much shorter soft fluff (13), woody stems, and blue flowers with purplish colour (12-14). *S. officinalis* is part of the Lamiaceae family (14), and this species is generally cultivated, but also grows spontaneously in the wild in different geographical areas. It is encountered in the glades, forests, scrub, grasslands, steppes, plains, highlands and mountains up to 2500m altitude. Sage is characterized by a very widespread distribution, and is mainly found in Yugoslavia, Bulgaria, France, Italy, USA, India, Spain, United Kingdom, Turkey, Morocco, Greece, South Africa, South America, and South East Asia (15). *S. officinalis* has various uses, essentially as herbal remedy for a wide range of disorders and diseases by applying it internally or externally. It is used as a diuretic, tonic, pain relief styptic, antiseptic, anti-inflammatory, anti-fungal and as anti-spasmodic. It is also used as a treatment for dysentery, cough, ulcers, varicose veins, insect bites (16), obesity, diabetes, depression and cancers in ancient times (14).

Thymus (*Thymus* L.) is a large genus of the Lamiaceae family encompassing about 215 medicinal and aromatic species, and 20 species have been reported in Algeria. *T. ciliatus* ssp. *munbyanus* locally known as "Zaatar", is a fragrant subshrub, with flowers 16 to 20 mm long, pooled in false whorls; leaves are more or less contracted, with their accompanying flowers being morphologically different from those inserted on the stem, which are generally wider at the base (17). Thymus is largely used in traditional Algerian medicine for its expectorant, antitussive, anti-bronchiolitic, anti-spasmodic, anti-helminthic, carminative and diuretic properties (18). This species has been used in the Aurès region (Eastern Algeria) as a traditional remedy for bronchitis, lung infections, influenza, cough and certain gastrointestinal disorders (19).

The objective of the present study is to determine the chemical composition, antibacterial and antibiofilm activities of essential oils (EOs) extracted from *J. oxycedrus* L., *J. thurifera* L., *S. officinalis* L. and *T. ciliatus* ssp. *munbyanus* (Boiss. & Reut.) Batt. on coagulase negative staphylococci (CoNS) previously isolated at the Anti-Cancer Center of Batna, Algeria (20).

Materials and Method:

Bacterial isolates

Sixty-six previously identified clinical CoNS isolates by Zatout et al., (20) were used to test for antibacterial activity of the EO extracts of the plants, and among them, those showing biofilm production ability (59 isolates) were used to test for anti-biofilm activity of the EOs (Table 1).

Table 1: CoNS isolates used to evaluate antibacterial and antibiofilm activities of essential oils

CoNS species	Number	
	Antibacterial activity	**Antibiofilm activity**
S. epidermidis	29	27
S. haemolyticus	15	13
S. hominis	8	6
S. chromogenes	6	6
S. xylosus	4	3
S. capitis	1	1
S. saprophyticus	1	1
S. cohnii	1	1
S. simulans	1	1
Total	**66**	**59**

CoNS = coagulase negative staphylococci; S = *Staphylococcus*

Plant materials

The plant materials consist of leaves of *S. officinalis* L. and *T. ciliatus* ssp. *munby-*

anus (Boiss. & Reut.) Batt. from the Lamiaceae family, and *J. thurifera* L. and *J. oxycedrus* L. from the Cupressaceae family. The botanical identification of the four plants was carried out by Pr. Oudjehih B., Botanical Laboratory, Department of Agronomy of the University of Batna 1. The leaves of *J. oxycedrus* and *J. thurifera* were collected from the region of El Mahmel, Batna in October 2016 while the leaves of *S. officinalis* and *T. ciliatus* ssp. *munbyanus* were harvested in April 2017 from the region of El Madher and Tazoult respectively. The leaves of the four plants were washed, dried in the shade and then grinded using an electric grinder, and the powders obtained are kept away from light and moisture (21).

Extraction of essential oils

The extraction of essential oils was carried out by hydro-distillation of the plant materials in a Clevenger type device (22). During each test, 100 g of raw materials were processed. The distillation lasts approximately 180 minutes after the appearance of the first drop of the distillate at the outlet of the steam condensation tube. The recovered essential oils were treated with a few crystals of anhydrous magnesium sulfate and then stored at +4°C in the dark. Three distillations were carried out for each plant.

Determination of essential oil yield

The essential oil yield was determined as described by Bourkhiss et al., (23), expressed in gram of the distillate per 100g of dry matter, by the following relationship; EOY=[(M/Ms)x100] ± [(ΔM/Ms)x100], where EOY is essential oil yield, M is the mass of collected essential oils (g), ΔM is error on reading, and Ms is dry plant mass (g).

Gas chromatography-mass spectrometry (GC-MS) analysis

Gas chromatography – mass spectrometry (GC-MS) analyses of the EOs were conducted using an Agilent 7890B gas chromatograph equipped with an Agilent HP-5MS, capillary column (30m×0.25 mm; coating thickness 0.25µm) and an Agilent 5977B mass, using the following working conditions; (i) injector and transfer line temperatures 220 and 240°C, respectively; (ii) oven temperature programmed from 60 to 240°C at 3°C/min; (iii) carrier gas helium at 1 ml/min; (iv) injection of 1 µl (5% HPLC grade n-hexane solution); split ratio 1:25

Identification of the constituents was based on a comparison of the retention times with those of the authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons and computer matching against commercial (NIST 14 and ADAMS) and laboratory-developed mass spectra library (24).

Antibacterial activity of essential oils of the four plants by agar disc diffusion assay

For determination of the antibacterial activity of the essential oils, the disc diffusion technique described by Rajouani et al., (25) was used. A Whatman paper (N° 03) was cut into disc sizes of 6 mm in diameter and sterilized by autoclaving in tubes. The discs were then impregnated with 10 µl of each pure EO. Pure 24-hour cultures of CoNS isolates were suspended in Mueller-Hinton (MH) broth and adjusted to 0.5 McFarland standards. The suspension was then streaked with a sterile swab on MH agar plates. The EO discs and 30µg vancomycin disc (used as positive control) were placed on the inoculated MH agar plates, which were then incubated aerobically at 37°C for 24 hours. Antibacterial activity was assessed by measuring the diameter of the inhibition zone (mm) around the discs.

Determination of MIC and MBC of essential oils of *S. officinalis* and *T. ciliatus* ssp. *munbyanus*

The determination of the minimum inhibitory concentrations (MICs) of EOs of *S. officinalis* L. and *T. ciliatus* ssp. *munbyanus* was carried out according to the technique of dilution in a liquid medium described by Bazargani and Rohloff (26), with some modifications. The CoNS isolates were cultured on MH agar plates and incubated aerobically at 37°C for 12 hours. After incubation, 5 to 7 isolated colonies were inoculated in tubes containing 5ml of MH broth and incubated at 37°C for 8 to 12 hours to ensure that the bacteria were in the log phase. Each bacterial suspension was then diluted 1:100 in MH broth. The EOs were dissolved in MH broth + 0.5% Tween 80 to obtain solutions at concentrations of 400 mg/ml for *S. officinalis* L. and 200 mg/ml of *T. ciliatus* ssp. *munbyanus.*

For each microplate well, 100 µl of MH broth was added, then 100 µl of each concentration of EOs were placed in the first microplate well and diluted in the sterile MH broth. Finally, 100 µl of the diluted CoNS isolates suspension were added to obtain a final concentration in the range of 100 to 0.39 mg/ml and 50 to 0.195 mg/ml of *S. officinalis* L. and *T. ciliatus* ssp. *munbyanus* respectively. Approximately 200 µl of the MH broth + 0.5% Tween 80 served as negative control.

The plates were prepared in three replicates, and incubated aerobically for 18 to 24 hours at 37°C. The MIC was defined as the lowest concentration of EOs that produced no macroscopically visible growth of CoNS isolates. The minimum bactericidal con-

centration (MBC) represents the lowest concentration of EOs at which 99.9% of CoNS bacteria were killed.

Biofilm inhibition study (inhibition of bacterial cell fixation)

The EOs of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* were evaluated at their MIC concentrations for their inhibition potential with regard to cell attachments as described by Marino et al., (27) with some modifications. 100 µl of each essential oil solubilized in the TSB+1% Glu+0.5% Tween 80 (at the MIC x 2 value) were added to each well of a 96-well microplate. The negative control contains 100 µl of TSB + 1% Glu. Finally, 100 µl of each bacterial culture suspension of CoNS (~10^6 CFU/ml) was introduced into each well (the final volume was 200 µl in each well). 200 µl of TSB + 1% Glu + 0.5% Tween 80 was included and 200 µl of EO was added in blank wells.

The microplates were incubated at 37°C for 24 hours. After incubation, the medium was aspirated, rinsed twice with phosphate-buffered saline (PBS), and rinsed once with ethanol (20%) to remove traces of EOs, then were fixed by drying at 60°C for 30 minutes and 200 ml of 1% crystal violet staining was added to the wells for 30 minutes. The contents of the wells were then aspirated, and after rinsing with distil water, 200 mL of ethanol (95%v/v) was added to the wells for spectrophotometric analysis (at OD 550 nm). The mean (average) absorbance of the samples was determined, the absorbance in the blank well was subtracted from the reading of the absorbance of each sample (experimental OD) and the percentage inhibition and efficiency were determined according to the following formula described by Bazargani and Rohloff (26) as; Inhibition percentage = (OD of negative control – OD of experimental)/OD of negative control × 100.

Statistical analysis

The SPSS version 22.0 software was used for statistical analysis. A single factor (one-way) ANOVA (for comparison of ≥ 3 means) and unpaired *t* tests, were carried out to determine the effect of diameters of inhibition zones, MICs, MBCs and biofilm inhibition of the EO extracts of the plants on CoNS isolates, and $p < 0.05$ was considered statistically significant.

Results:

Essential oils yield from the plants

The yields of the EOs were expressed relative to the weight of the plant dry matter. The extraction of the EOs by hydro-distillation showed greater extraction in *S. officinalis* L. (1.41±0.05%) and *T. ciliatus* ssp. *munbyanus* (Boiss. & Reut.) Batt. (1.25±0.03%) than in *J. oxycedrus* L. (0.81±0.04%) and *J. thurifera* L. (0.36±0.04%) ($p<0.0001$ by one way ANOVA test) (Table 2).

Chemical composition of essential oils

The complete identification by GC-MS of the components of the EOs is shown in Table 3. In this study, 27 compounds were identified in the EO of *J. oxycedrus*, with α-pinene (56.1%), β-phellandrene (17.9%), α-phellandrene (4.4%) and myrcene (3.3 %) being the main ones. In *J. thurifera* EO, 41 compounds were identified, and sabinene (24.2%), 4-terpineol (12.5%), methyl eugenol (8.9%), safrole (7.5%) and α-pinene (6.5%) characterized its composition. In the EO of *S. officinalis*, 45 compounds were identified, with α-thujone (16.7%) as the main constituent, followed by camphor (14.9%), 1,8-cineole (11.8%) and viridiflorol (10.1%). In *T. ciliatus* ssp. *munbyanus* EO, 32 compounds were identified, among which thymol (69.0%) was the main component, followed by γ-terpinene (5.1%), p-cymene (3.7%), carvacrol (3.7%) and β-caryophyllene (3%).

Table 2. Percentage yield of essential oils from four medicinal plant species

Medicinal plants	Yield of essential oils (%) (Mean ± SD)	*p* value (ANOVA)
Thymus ciliatus ssp. *munbyanus* (Boiss. et Reut.) Batt.	1.41 ± 0.05	<0.0001*
Salvia officinalis L.	1.25 ± 0.03	
Juniperus thurifera L.	0.81 ± 0.04	
Juniperus oxycedrus L.	0.36 ± 0.04	

SD = Standard deviation; ANOVA = Analysis of variance; * = statistically significant

Table 3: Chemical composition of the essential oil extracts of four medicinal plants from Aurès region of Algeria

Chemical compound	**LRI**	***J. oxycedrus***	***J. thurifera***	***S. officinalis***	***T. ciliatus* ssp. *munbyanus***
Tricyclene	928	0.1			
α-thujene	933		3.9	0.6	0.1
α-pinene	941	56.1	6.5	2.2	1.0
Camphene	955	0.3	0.1	3.1	0.3
Sabinene	977	0.4	24.2	2.9	
β-pinene	982	1.5	0.6	2.3	0.3
Myrcene	992	3.3	2.3	1.3	0.5
δ-2-carene	1002	0.4			
α-phellandrene	1006	4.4	0.1		0.1
α-terpinene	1020		3.9	0.7	0.9
p-cymene	1028	1.5	0.8	0.9	3.7
Limonene	1032		1.2	2.2	0.5
β-phellandrene	1033	17.9			0.8
1,8-cineole	1034			11.8	
(E)-β-ocimene	1051		0.1		
γ-terpinene	1063	0.1	6.5	1.3	5.1
Cis-sabinene hydrate	1070		0.5	0.2	
Terpinolene	1090	0.8	1.7	0.5	0.1
Trans-sabinene hydrate	1099		0.5		
Linalool	1101		3.3	0.6	1.1
α-thujone	1105			16.7	0.7
β-thujone	1116			3.5	
Cis-p-menth-2-en-1-ol	1123		0.5		
α-campholenal	1126	0.3			
Trans-pinocarveol	1141			0.2	
Trans-p-menth-2-en-1-ol	1142		0.3		
Camphor	1144		0.1	14.9	1.1
Borneol	1168			1.5	0.1
4-terpineol	1179		12.5	2.7	0.3
α-terpineol	1191	1.1	1.3	0.5	
Methyl chavicole (=estragole)	1197		0.6		
Methyl thymol	1234	0.2			0.9
Linalyl acetate	1259		2.4	0.5	
Bornyl acetate	1287			0.5	
Safrole	1287		7.5	1.6	
Thymol	1291		2.5	0.6	69.0
Carvacrol	1301				3.7
α-terpinyl acetate	1352	0.2	0.7	0.2	
Piperitenone oxide	1365	1.4			
Neryl acetate	1366		0.1		
β-bourbonene	1385	0.1			
Geranyl acetate	1386		0.2		

Methyl eugenol	1403		8.9	2.1	
β-caryophyllene	1419	0.3	0.2	2.0	3.0
α-humulene	1455			1.4	0.2
Cis-muurola-4(14),5-diene	1463		0.1		
γ-muurolene	1478			0.2	0.4
Germacrene D	1482	0.8	0.2	0.4	0.2
Valencene	1492				0.2
2-tridecanone	1497	0.3			
α-muurolene	1499				0.1
β-bisabolene	1508				1.2
Trans-γ-cadinene	1515		0.4	0.3	0.4
δ-cadinene	1524	0.2	1.1	0.7	1.0
α-calacorene	1543				0.1
Elemol	1550		0.9	0.5	
Germacrene B	1556		0.2		
Elemicin	1557		0.8	0.3	
Caryophyllene oxide	1582			1.3	0.4
Viridiflorol	1591			10.1	1.6
Humulene epoxide II	1607			0.9	
1-epi-cubenol	1629		0.4	0.3	
γ-eudesmol	1630		0.2	0.2	
T-cadinol	1641			0.2	
β-eudesmol	1650		0.4	0.3	
α-eudesmol	1651		0.4	0.3	
α-cadinol	1652			0.3	
(Z)-9-tetradecen-1-ol	1665	2.8			
(E, E)-farnesol	1732	1.2			
Manoyl oxide	1993	0.7			
Abietatriene	2054	0.6			
Manool	2056			4.0	
Abietadiene	2081	2.5			
Monoterpene hydrocarbons		86.8	51.9	18.0	13.4
Oxygenated monoterpenes		3.2	24.9	54.4	76.9
Sesquiterpene hydrocarbons		1.4	2.2	5.0	6.8
Oxygenated sesquiterpenes		1.2	2.3	14.4	2.0
Diterpenes		3.8	0.0	4.0	0.0
Phenylpropanoids		0.0	17.8	4.0	0.0
Non-terpene derivatives		3.1	0.0	0.0	0.0
Total identified		**99.5**	**99.1**	**99.8**	**99.1**

LRI = Linear Retention Index

Antibacterial activity of the essential oils on CoNS by the disc diffusion assay

The antibacterial activity of the EOs by the disc diffusion test showed that growth inhibition varies depending on the species of CoNS, concentrations and type of EOs (Table 4). The results as presented in the table shows that EOs of *T. ciliatus* ssp. *munbyanus* had the highest inhibitory activity on CoNS isolates (n=66), with mean inhibition zone diameter of 24.99±6.29 mm, followed by *S. officinalis* with mean inhibition zone diameter

of 13.38±6.52 mm. On the contrary, low inhibitory activity was observed with the EOs of *J. oxycedrus* and *J. thurifera,* having mean inhibition zone diameters of 6.67±1.36mm and 6.40±0.82mm, respectively. The inhibitory activity of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* on the CoNS isolates was significantly higher than the inhibitory activity of *J. thurifera* and *J. oxycedrus* on the CoNS isolates and for each species (p<0.0001 by one-way ANOVA test). However, the inhibitory effects of the EOs were not significantly different among the CoNS species for *T. ciliatus* ssp. *munbyanus* (p=0.836) and *S. officinalis* (p=0.080), *J. thurifera* (p=0.989), and *J. oxycedrus* (p=0.170).

MICs and MBCs of essential oils of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* L.

The MIC and MBC results showed that bactericidal activity of the EOs varies depending on the species of CoNS, the concentration and type of EOs tested (Table 5). As shown in the table, EOs of *T. ciliatus* ssp. *munbyanus*, had high bactericidal activity against the CoNS isolates (n=66), with mean MIC and MBC values of 2.65±3.77 and 5.31± 7.41 mg/ml respectively, which is significantly higher than the bactericidal activity of the EOs of *S. officinalis*, with mean MIC and MBC values of 27.53±18.2mg/ml (p<0.0001, t = 7.104) and 31.97 ± 33.19 mg/ml (p< 0.0001, t = 6.369) respectively. The mean MIC and MBC values of *T. ciliatus* ssp. *munbyanus* EOs are also significantly higher than those of *S. officinalis* across the different CoNS species except for *S. hominis*, which was not significantly different for MIC (p=0.1624) and MBC (p=0.1250). While the mean MIC values of *T. ciliatus* ssp. *munbyanus* EOs were not significantly different across the CoNS species (p=0.838), the mean MIC values of *S. officinalis* were significantly different across the CoNS species (p=0.002), with low mean MIC value for *S. hominis* (8.20±7.97mg/ml) and high MICs for *S. chromogenes* (50.0±51.44mg/ml) and *S. epidermidis* (37.01±36.58 mg/ml).

Antibiofilm activity of the essential oils on CoNS isolates

The evaluation results of the EOs of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* on biofilm producing CoNS isolates (n=59) indicated that the percentage biofilm inhibition was significantly higher for *T. ciliatus* ssp. *munbyanus* (65.63±10.71%) than *S. officinalis* (53.13%±5.83) EOs (p<0.0001, t=7.874) (Table 6). The percentage biofilm inhibition by the EOs of *T. ciliatus* ssp. *munbyanus* was also significantly higher than that of *S. officinalis* for the different CoNS species except for *S. hominis* (p=0.9691) and other CoNS species (p=0.2112), which were not significantly different. While the percentage biofilm inhibition by the EOs of *T. ciliatus* ssp. *munbyanus* was not significantly different for each CoNS species (p=0.997 by one-way ANOVA test), that of *S. officinalis* was significantly different (p=0.000133 by one-way ANOVA), with high percentage inhibition for *S. hominis* (65.00±4.03%) and low percentage inhibition for *S. chromogens* (43.79±3.50%).

Table 4: Mean (Average) inhibition zone diameters of essential oil extracts of four medicinal plants on CoNS species

CoNS isolates	*T. ciliatus* ssp. *munbyanus*	*S. officinalis* L.	*J. thurifera*	*J. oxycedrus*	*p* value (one way ANOVA)
S. epidermidis (n=29)	26.35± 6.37	14.56±6.30	6.43±0.76	6.32±0.98	<0.0001*
S. haemolyticus (n=15)	24.67±5.19	14.23±6.28	6.32±0.84	6.97±1.67	<0.0001*
S. hominis (n=8)	25.14±5.82	17.58±7.10	6.52±1.07	7.39±1.97	<0.0001*
S. chromogenes (n=6)	23.36±6.14	7.91±1.76	6.33±0.76	6.00±0.00	<0.0001*
Other species of CoNS (n=8)	25.43±8.02	12.62±6.51	6.41±0.76	6.70±1.33	<0.0001*
***p* value (one way ANOVA)**	0.836	0.080	0.989	0.170	
Total (n=66)	**24.99±6.29**	**13.38±6.52**	**6.40±0.82**	**6.67±1.36**	**<0.0001***

CoNS = coagulase negative staphylococci; n= number; ANOVA=analysis of variance; * = statistically significant

Table 5: MICs and MBCs of essential oils of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* L. on CoNS species

CoNS isolates	MIC (mg/ml)		*p* value (unpaired *t* test)	MBC (mg/ml)		*p* value (unpaired *t* test)
	T. ciliatus* ssp. *munbyanus	***S. officinalis***		***T. ciliatus* ssp. *munbyanus***	***S. officinalis***	
S. epidermidis (n=29)	3.31±4.43	37.01±36.58	<0.0001* (t=4.925)	6.84±8.96	37.36±42.09	0.0003* (t=3.819)
S. haemolyticus (n=15)	2.55±3.21	26.33±27.69	0.0026* (t=3.304)	4.61±6.12	43.15±41.43	0.0013* (t=3.564)
S. hominis (n=8)	3.49±4.25	8.20±7.97	0.1624 (t=1.475)	6.02±7.42	15.36±14.39	0.1250 (t=1.632)
S. chromogenes (n=6)	1.59±0.87	50.00±51.44	0.9821 (t=0.2305)	4.40±2.30	-	
Other CoNS species (n=8)	2.34±2.45	16.14±17.35	0.0428* (t=2.228)	4.71±4.87	32.03±34.88	0.0456* (t=2.194)
***p* value (one way ANOVA)**	0.838	0.002*		0.674	0.215	
Total CoNS (n=66)	**2.65±3.77**	**27.53±28.2**	**<0.0001* (t=7.104)**	**5.31±7.41**	**31.97±33.19**	**<0.0001* (t=6.369)**

CoNS=coagulase negative staphylococci; n= number; MIC = minimum inhibitory concentration; MBC=minimum bactericidal concentration; ANOVA = analysis of variance; - = no inhibition; * = statistically significant

Table 6: Antibiofilm activity of the essential oils of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* L. on CoNS isolates

CoNS isolates	Percentage inhibition of biofilm formation		*p* value (unpaired *t* test)
	***T. ciliatus* ssp. *munbyanus* Mean (Average) (%)**	***S. officinalis* L. Mean (Average) (%)**	
S. epidermidis (n=27)	65.78±10.98	48.22±8.51	<0.0001* (t=6.568)
S. haemolyticus (n=13)	66.77±10.63	49.22±4.93	<0.0001* (t=5.400)
S. hominis (n=6)	64.78±12.94	65.00±4.03	0.9691 (t=0.03976)
S. chromogenes (n=6)	65.47±10.44	43.79±3.50	0.0007* (t=4.823)
Other CoNS species (n=7)	65.36±8.54	59.45±8.20	0.2112 (t=1.321)
***p* value (one way ANOVA)**	0.997	0.000133*	
Total (n=59)	**65.63±10.71**	**53.13±5.83**	**<0.0001* (t=7.874)**

CoNS = coagulase negative staphylococci; n= number; ANOVA=analysis of variance; * = statistically significant

Discussion:

Previous studies on Thymus reported variable EO yield values; Kabouche et al., (19) reported 2.1%, Heni et al., (28) 2.5%, Amarti et al., (29) 1.2% and Ouknin et al., (30) 1.7%. For *S. officinalis*, reported EO yields also varies; Soković et al., (31) 2.2%, Meziou-Chebouti et al., (32) 1.06% and Golparvar et al., (33) 2.4%. For *J. thurifera*, Bahri et al., (34) reported 1.03% yield, while for *J. oxycedrus*, Angioni et al., (35) and Marongiu et al., (5) reported 0.04% and 0.20% yield, respectively. The difference in percentage yield reported in the literature in comparison with our results could be attributed to different factors such as the nature of the soil, the genetic variation of the plant, the mode of extraction of the oil (36), climate, collection period, age (28-36), altitude, as well as by the interaction of various factors (37), the part of the plant extracted, and the specific geographic location of the plants (28).

Different chemical composition of EOs have also been reported from GC-MS of *J. oxycedrus*, Angioni et al., (35) mainly found α-pinene (85.95%), δ−3-carene (2.81%) and myrcene (1.20%), and Boudjedjou et al., (38) reported the main components of EOs of *J. thurifera* to be m-mentha-6,8-diene (15. 43%), β-pinene (10.59%), elemol (8.31%) and 4-terpineol (7.44%). Other researchers have reported different compositions for *S. officinalis* EOs, Nikolić et al., (39) identified cis-thujone (32.7%), camphor (17.2%), 1,8-cineole (10.1%), α-pinene (8.6%), trans-thujone (7.7%) and camphene (7.3%), while Golparvar et al., (33) identified α-thujone (37.18%), 1,8-cineole (12.71%), β-thujone (9.10%), camphene (5.54%) and viridiflorol (5.33%). Ouknin et al., (30) described a different composition for the EO of *T. ciliatus* ssp. *munbyanus*, with carvacrol (31.8%), γ-

terpinene (21.9%), p-cymene (14.7%), thymol (7.6%), linalool (4.3%), borneol (3.9%) and α-terpinene (2.1%) as principal constituents. The differences in the chemical compositions reported in our current study and those of other studies may be due to different growth stages, ecological conditions, method of extraction (16), variation in population, organs of the plant and stress conditions (40).

The antibacterial activity of EOs depends on their chemical composition. The most active ones are often characterized by two or three main components at fairly high concentrations (up to 80%) together with other minor compounds (41). Khadir et al., (42) reported similar results using *T. ciliatus* ssp. *munbyanus* on 19 MRSA isolates, with a diameter of 25.8mm. Different results were obtained from *S. officinalis* by Meziou-Chebouti et al., (32) on clinical isolates of *S. aureus*, with an inhibition diameter of 35mm. Also, Bahri et al., (34) obtained different results (inhibition diameter of 27.0mm) with EOs of *J. thurifera* on *S. aureus* (ATCC 33862). Zeraib et al., (9) testing the EOs of male and female leaves of this plant on MRSA reported diameters of 12.66±1.15mm and 13.33±1.15 mm, respectively. The results obtained by Bousmaha-Marroki et al., (43) from the EOs of *T. ciliatus* ssp. *euciliatus* on clinical *S. aureus* were also different with a MIC of 920 µg/mL (0.92mg/mL).

The main mode of antibacterial action of thymol is not completely understood, but researchers have thought that it involves a disturbance of internal and external membranes and interaction with membrane proteins and intracellular targets (44) or disruption of bacterial enzyme systems (45). Another component which is carvacrol is a terpene known for its antimicrobial activity against a wide range of bacteria (43-46). It is also considered as biocide, with its precursor, p-cymene weakly antibacterial, but probably acts in synergy by the expansion of the membrane, causing its destabilization (46). Soković et al., (31) reported a strong activity of *S. officinalis* on *S. epidermidis* (ATCC 12228) with MIC and MBC values of 6.0 and 6.0 µg/ml, respectively. Pierozan et al., (47), testing on *S. aureus* the EOs obtained from two different plants, *S. officinalis* 1 and *S. officinalis* 2, obtained MICs of 3.42 and 2.87 mg/ml respectively. The antimicrobial activity of *S. officinalis* has been recognized for several decades and has been attributed to the presence of 1,8-cineole, α-thujone, camphor (48), as well as to β-thujone, borneol, p-cymene, and others (47). Infact, a synergistic effect may be observed between major and minor constituents (25).

Several studies have described that thymol and carvacrol are able to inhibit the growth of preformed biofilm and interfere with biofilm formation during planktonic growth. Memar et al., (49) reported that carvacrol and thymol attenuated biofilm formation in *S. aureus* and *S. epidermidis* on polystyrene microplates. Thymol can prevent the early stages of biofilm formation and interfere with the formation of mature biofilms due to metabolic activity in biofilms. All of these events can lead to a major membrane and block the production of filamentous forms at the start of biofilm formation. Biofilms formation, being a multifactorial event, can be affected by thymol with various mechanisms at different stages of their development. Furthermore, Karpanen et al., (50) demonstrated increased susceptibility of staphylococci in a biofilm mode of growth to an EO-based formulation, compared with planktonic cells. Thymol and carvacrol are phenolic compounds having both hydrophilic and hydrophobic properties, which may enhance their diffusion in a biofilm and allow their access to bacterial cells where they can alter the permeability of the plasma membranes.

Conclusion:

Chemical analyses by GC-MS allowed the identification of 99.1 to 99.8% of the EO composition in our study. The major constituents were α-pinene (56.1%), sabinene (24.2%), α-thujone (16.7%) and thymol (69.0%) for *J. oxycedrus*, *J. thurifera*, *S. officinalis* and *T. ciliatus* ssp. *munbyanus*, respectively. From this study, EOs of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* clearly showed high antibacterial and antibiofilm activities, but the activities of *T. ciliatus* ssp. *munbyanus* were significantly higher than those of *S. officinalis*. EOs of these two plants could serve as alternatives to classical antibiotics against planktonic and biofilm forms of CoNS isolates in view of their high antibacterial and antibiofilm formation capabilities.

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Contributions of authors:

AZ designed the study and contributed to specimen collection, laboratory isolation of bacterial pathogens, extraction of EOs, antibacterial and antibiofilm detection, and manuscript writing. RD, FG, AR, BC, MHE, BF, MS and KA contributed to the study protocol and manuscript revision. All authors read the final manuscript.

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Original Article **Open Access**

Characterization of bacteria isolates colonizing the throat of hospitalized patients at Sobi Specialist Hospital, Ilorin, Nigeria and *in vitro* antimicrobial effects of *Citrus aurantifolia* and Alum on the isolates

[1]Olajide, O. A., [*1]Kolawole, O. M., [1]Bada-Siyede, I. B., [1]Ayanda, O. O., and [1,2]Suleiman, M. M.

[1]Infectious Disease and Environmental Health Research Group, Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Nigeria
[2]Department of Biological Sciences, College of Natural and Applied Sciences, Summit University, Offa, Nigeria
*Correspondence to: tomak7475@gmail.com; omk@unilorin.edu.ng; +234-8060088495

Abstract:

Background: Antibiotic resistance in microorganisms implicated in nosocomial respiratory infections is a major reason for prolonged hospital stay and increased cost of therapeutic treatment of hospital acquired pneumonia (HAP). This study was designed to isolate bacterial pathogens colonizing the throat of hospitalized patients at the Sobi Specialist Hospital, Ilorin, and to evaluate antibacterial effects of extracts of *Citrus aurantifolia* peel and Alum against these bacterial isolates.
Methodology: This was a cross sectional study of 100 randomly recruited hospitalized patients at the Sobi Specialist Hospital, Ilorin, Nigeria. Throat samples collected from consenting participants were cultured on selective agar media (MacConkey, Eosin-Methylene blue and Mannitol salt) for isolation of bacteria. Identification of isolates from culture plates was done by Gram reaction and conventional biochemical tests while confirmation of the isolates was done by the polymerase chain reaction (PCR) assay. Antibiotic susceptibility test for each isolate to selected antibiotics (ampicillin, amoxicillin-clavulanate, cefuroxime, ceftazidime, gentamicin, nitrofuran, ofloxacin and ciprofloxacin) was done by the Kirby Buer disc diffusion method. Aqueous extract of Alum ($[KAl(SO_4).12H_2O]$) was done to produce concentrations of 10, 20, 30, 40 and 50% (w/v) at pH 3.6 and tested on the bacterial isolates using agar diffusion method. *Citrus aurantifolia* peel was extracted using methanol and hexane solvents to produce extract concentrations of 500mg/ml, 250mg/ml and 150mg/ml, and tested on the isolates by agar diffusion, and by the broth dilution method to obtain minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of *C. aurantifolia*.
Results: A total of 14 bacterial isolates were recovered from throat samples of 100 hospitalized patients with *Staphylococcus aureus* (43%, n=6) being the most frequent while *Escherichia coli* (14.5%, n=2) was the least frequent. The isolates were generally resistant to penicillin, aminoglycoside and fluoroquinolone groups of antibiotics tested. The zone of inhibition for hexane and methanol extracts of *C. aurantifolia* and aqueous extract of alum on the bacterial isolates ranged from 11.5-19.2mm, 9.8-15.8mm, and 9.3-21.2mm respectively while those of selected antibiotics ranged from 7.0-25.0mm. The MICs of hexane and methanol extracts of *C. aurantifolia* against *S. aureus* were 10mg/ml and 25mg/ml, while the MBCs were 50 and 100mg/ml respectively.
Conclusion: Findings from this study showed the presence of resistant pathogenic bacteria colonizing the throat of hospitalized patients receiving care at the Sobi Specialist Hospital, Ilorin, Nigeria. The crude extracts of *C. aurantifolia* and Alum in this study showed inhibitory effects (albeit at higher concentrations) on the bacterial isolates comparable to the standard antibiotics. We posit that based on the inhibition capacity, further studies to characterize, purify and isolate the active anti-bacterial components in the extracts should be considered for novelty.

Keywords: Antibacterial; antibiotic resistance; *Citrus aurantifolia*; MBC; MIC.

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Caractérisation des isolats de bactéries colonisant la gorge des patients hospitalisés à l'hôpital spécialisé Sobi, Ilorin, Nigeria et effets antimicrobiens *in vitro* de *Citrus aurantifolia* et Alum sur les isolats

[1]Olajide, O. A., *[1]Kolawole, O. M., [1]Bada-Siyede, I. B., [1]Ayanda, O. O., et [1,2]Suleiman, M. M.

[1]Groupe de recherche sur les maladies infectieuses et la santé environnementale, Département de microbiologie, Faculté des sciences de la vie, Université d'Ilorin, Nigeria
[2]Département des sciences biologiques, Collège des sciences naturelles et appliquées, Université du Sommet, Offa, Nigeria
*Correspondance à: tomak7475@gmail.com; omk@unilorin.edu.ng; +234-8060088495

Résumé:

Contexte: La résistance aux antibiotiques chez les micro-organismes impliqués dans les infections respiratoires nosocomiales est une cause majeure de séjour prolongé à l'hôpital et d'augmentation du coût du traitement thérapeutique de la pneumonie nosocomiale. Cette étude a été conçue pour isoler les agents pathogènes bactériens colonisant la gorge des patients hospitalisés à l'hôpital spécialisé Sobi, Ilorin, et pour évaluer les effets antibactériens des extraits de zeste de *Citrus aurantifolia* et d'alun contre ces isolats bactériens.
Méthodologie: Il s'agissait d'une étude transversale de 100 patients hospitalisés recrutés au hasard à l'hôpital spécialisé de Sobi, à Ilorin, au Nigeria. Des échantillons de gorge prélevés sur des participants consentants ont été cultivés sur des milieux gélosés sélectifs (MacConkey, éosine-bleu de méthylène et sel de mannitol) pour l'isolement des bactéries. L'identification des isolats à partir de plaques de culture a été effectuée par la réaction de Gram et des tests biochimiques conventionnels tandis que la confirmation des isolats a été effectuée par le test de réaction en chaîne par polymérase (PCR). Le test de sensibilité aux antibiotiques de chaque isolat aux antibiotiques sélectionnés (ampicilline, amoxicilline-acide clavulanique, céfuroxime, ceftazidime, gentamicine, nitrofurane, ofloxacine et ciprofloxacine) a été effectué par la méthode de diffusion sur disque de Kirby Buer. Un extrait aqueux d'alun ([KAl(SO4).12H2O]) a été réalisé pour produire des concentrations de 10, 20, 30, 40 et 50 % (p/v) à pH 3,6 et testé sur les isolats bactériens en utilisant la méthode de diffusion sur gélose. La peau de *C. aurantifolia* a été extraite à l'aide de solvants méthanol et hexane pour produire des concentrations d'extrait de 500mg/ml, 250mg/ml et 150mg/ml, et testée sur les isolats par diffusion sur gélose, et par la méthode de dilution en bouillon pour obtenir un minimum inhibiteur (MIC) et concentrations minimales bactéricides (CMB) de *C. aurantifolia*.
Résultats: Un total de 14 isolats bactériens ont été récupérés à partir d'échantillons de gorge de 100 patients hospitalisés, *Staphylococcus aureus* (43.0%, n=6) étant le plus fréquent tandis qu'*Escherichia coli* (14,5%, n=2) était le moins fréquent. Les isolats étaient généralement résistants aux groupes d'antibiotiques testés pénicilline, aminoglycoside et fluoroquinolone. La zone d'inhibition pour les extraits à l'hexane et au méthanol de *C. aurantifolia* et l'extrait aqueux d'alun sur les isolats bactériens variait de 11,5 à 19,2mm, 9,8 à 15,8mm et 9,3 à 21,2mm respectivement, tandis que celles des antibiotiques sélectionnés variaient de 7,0 à 25,0mm. Les CMI des extraits à l'hexane et au méthanol de *C. aurantifolia* contre *S. aureus* étaient de 10 mg/ml et 25mg/ml, tandis que les MBC étaient respectivement de 50 et 100mg/ml.
Conclusion: Les résultats de cette étude ont montré la présence de bactéries pathogènes résistantes colonisant la gorge des patients hospitalisés recevant des soins à l'hôpital spécialisé de Sobi, à Ilorin, au Nigeria. Les extraits bruts de *C. aurantifolia* et d'alun dans cette étude ont montré des effets inhibiteurs (bien qu'à des concentrations plus élevées) sur les isolats bactériens comparables aux antibiotiques standard. Nous postulons que sur la base de la capacité d'inhibition, d'autres études pour caractériser, purifier et isoler les composants antibactériens actifs dans les extraits devraient être envisagées pour la nouveauté.

Mots clés: Antibactérien; résistance aux antibiotiques; *Citrus aurantifolia*; CMB; MIC

Introduction:

Hospital acquired pneumonia (HAP) also known as nosocomial pneumonia accounts for the high mortality and morbidity reported amongst hospitalized patients. Generally, pneumonia accounts for about 15% of all children death according to World Health Organizations (1). Hospital acquired pneumonia (HAP) is a pulmonary infection developing during hospitalization, 48 hours or more after admission, and not present or incubating at the time of admission. Ventilator-associated pneumonia (VAP) is a type of HAP that arises more than 48–72hours after endotracheal intubation. The spectrum of pathogens involved in HAP and VAP is certainly different from that of community acquired pneumonia and is influenced by the severity of illness, presence of risk for specific pathogens and time of onset of the pneumonia (2). The pathogens that are most frequently involved in HAP are aerobic Gram-negative bacilli such as *Pseudo-*

monas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Acinetobacter sp. and *Staphylococcus aureus*. These bacteria can be considered the "core" pathogens in HAP. The role of a polymicrobial etiology of HAP has been proposed in 50% of cases (1,3).

According to World Health Organization, medicinal plants are used for therapeutic purpose and used as a pioneer in the synthesis of semi-synthetic and chemical drugs (4). About 80% of the world population use herbal medicine to treat ailment (5) because medicinal plants contain many chemical compounds such as alkaloids, flavonoids, glycosides, saponins, resins, oleoresins, sesquiterpene, phenolic compounds, fats and oils amongst others that possess therapeutic or ameliorating ability (6). The plants are easily available and cheaper than the conventional drugs and one of such plants is *Citrus aurantifolia* (lime fruit). Lime in its natural state is widely used in West Africa, particularly in the southwestern part of Nigeria.

The peel of lime fruit is commonly used in the treatment of pneumonia together with aluminum potassium sulfate (KAl (SO_4).$12H_2O$) commonly known as Alum. When added to sugar and palm oil or to honey, the juice has been found to be an excellent cough relieving mixture. The rind is burnt in some homes to act as insecticides against mosquitoes while the mesocarp is also used as a very good facial scrub that helps in prevention of pimples due to its cleansing action on the skin (7). Due to the increasing rate of antibiotic resistance, there is need to explore medicinal plants or agents with reported ameliorating effects for the provision of less costly, relatively available and effective alternatives to combat the burden of antibiotic resistance

Materials and method:

Study area

This research was carried out at Sobi Specialist hospital in Ilorin, Kwara State Nigeria. The hospital is located in the eastern part of Ilorin on N 8°31' 29.0784" and E 4°33' 13. 3236".https://www.findlatitudeandlongitude.com/l/Sobi+Road%2C+Ilorin%2C+Nigeria/2962723/)

Study design

This was a cross sectional study using simple random sampling method for recruitment of consenting participants. The study further employed an *invitro* study for the antibacterial efficacy testing of isolates against commercially available antibiotics, alum and *Citrus aurantifolia* (lime fruit).

Study population and subject participants

The population of study included consenting participants/patients (age ≥ 10 years) from surgical, accident and emergency and maternity wards of Sobi specialist hospital. The population size was estimated to be <500 and thus, using the Conroy (8) table for sample size determination which required a minimum of 81 participants, a sample size of 100 was drawn over a period of three (3) months (Dec, 2019 to March, 2020).

A total of 100 hundred throat swab samples and structured questionnaires (closed ended) were utilized for the study. Inclusion criteria included patients with >48 hours on hospital admission, productive cough, breathing difficulty, chest pain and previous use of antibiotics. Approval for the study was obtained at the Kwara State Ministry of Health with approval number: MOH/KS/EU/777/341.

Data collection

The structured questionnaires was used to obtain information about the socio-demographic characteristics (age, gender) and socio-economic status (level of education, occupation) and other related factors such as duration of hospital stay, use of antibiotics and patients' exposure to risk factors (such as medical history, smoking, cough) associated with hospital acquired pneumonia.

Clinical sample collection

Throat swab samples were obtained from patients who meet the inclusion criteria through the laboratory technologist of the hospital. The swab stick was carefully labeled corresponding to the patients' identity number. The sealed container containing the swab sample was then transported to the Public Health and Infectious Laboratory, Department of Microbiology, University of Ilorin for analysis.

Citrus and Alum sample collection/preparation

Citrus aurantifolia (identification number; TSN852203) and Alum was purchased from Maraba Park in Ilorin, Kwara State, Nigeria. The peel of *Citrus aurantifolia* was extracted from the fruit, dried under the shade and blended to powdery form using an electric blender. The powdery form was weighed using electric weighing balance and Aluminum foil. The powdery form was divided into two different places, weighed and stored in separate conical flask for the respective solvents i. e. methanol and hexane.

Five (5), 2.5 and 1.5 gram of the powder samples were weighed into conical flask contain-

ing 100 ml of hexane and methanol separately to give 500mg/ml, 250mg/ml and 150mg/ml concentration respectively. The mixture in the conical flask was placed in flask shaker for 72 hours to ensure homogeneity of the sample in the solvent. Each of the solvent mixture was then filtered using a filter paper (Whatman No. 4) after 72 hours of incubation (9). Aqueous extraction of alum was done by dissolving crystals of alum [$KAl(SO_4).12H_2O$] completely in 100 ml hot distilled water at 92ºC to prepare concentrations of 10, 20, 30, 40 and 50 w/v % at pH 3.6 respectively.

Laboratory analysis of throat samples

Selective and general-purpose media was used and they include MacConkey, Eosin-Methylene blue agar, Mannitol salt agar, Mueller Hinton agar and Nutrient agar. The preparation of the respective media was with adherence to manufacturer protocol as well as use of laboratory manual of Fawole and Osho (10) and Anibijuwon et al., (9). The throat swab samples were cultured on the prepared selective media for isolation and screening of bacteria isolates (11). Identification was achieved through Gram staining technique and conventional biochemical tests while isolate confirmation was by polymerase chain reaction (PCR) assay.

DNA extraction and PCR procedure

The DNA of the isolates was extracted by suspending 4 to 5 pure colonies from culture media in 300 µl of 1 x TBE buffer in appropriately labelled Eppendorf tubes. The cells were boiled at 100ºC for 10 minutes and were cooled rapidly on ice for 30 minutes. Three (3) µl of proteinase K was added to the lysed cells and the mixture was incubated for 15 minutes at 60ºC. The enzyme was denatured by boiling at 100ºC for 10 minutes and was centrifuged at 13,400 rpm for 3 minutes. The supernatant containing the DNA was transferred into a fresh sterile Eppendorf tube and stored at -20ºC until required for PCR.

PCR was performed in a 20µl reaction containing 4µl of master mix, 0.5µl of forward primer, 0.5µl of reverse primer, 13µl of nuclease free water and 2µl of DNA lysate. Amplification conditions were initial denaturation at 95ºC for 5min, followed by 35 cycles of denaturation at 95ºC for 1 min, annealing at 58ºC, extension at 72ºC for 1 min, and final extension at 72ºC for 10min. Table 1 shows the details of the primers for the target genes of respective isolates.

Gel electrophoresis

At the completion of the amplification, PCR products were resolved on l.5% agarose gel prepared by dissolving 1.5g of agarose powder in 100 ml of 1 x Tris-Borate-EDTA (TBE) buffer solution inside a clean conical flask and into which 0.5 µl of ethidium bromide has been added. The agarose plate was placed inside the electrophoresis tank containing 1 x TBE solution. Five (5) µl of the amplicon was mixed with 1µl of loading buffer and the mixture was loaded to the wells of the agarose gel. The power supply was adjusted to 100 volts for 25 minutes. For each run, a 100 base-pair molecular weight DNA standard (size marker) was used to determine the size of each PCR product. The DNA bands were then visualized with a short wave ultra-violet trans-illuminator and photographed using gene gel bio-imaging system.

Table 1: Primer sequences used for amplification confirmation of the bacteria isolates

Bacteria	Genes	Sequence	Base pair	Annealing temp	Reference
Acinetobacter baumannii	OXA-51-F OXA-51-R	TAA TGC TTT GAT CGG CCT TG TGG ATT GCA CTT CAT CTT GG	353bp	58ºC	Woodford et al. (27)
Staphylococcus aureus	Staph 756F Staph 756R	AACTCTGTTATTAGGGAAGAACA CCACCTTCCTCCGGTTTGTCACC	756bp	55ºC	Zhang et al. (28)
Escherichia coli	TEcol553 TEcol754	TGGGAAGCGAAAATCCTG CAGTACAGGTAGACTTCTG	258bp	58ºC	Maheux et al. (29)
Klebsiella pneumoniae	Pf Pr1	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130bp	58ºC	Einas et al. (30)

Antibacterial susceptibility of isolates against selected antibiotics

Antibiotic susceptibility testing of isolated organisms was carried out using the disc diffusion method. The isolates were spread on solidified Mueller Hinton agar and an antibiotic disc was aseptically placed on the surface of the media using sterile forceps. Plates was incubated at 37°C for 18-24 hours, after which plates was observed for zones of inhibition and characterized according to the most recent Clinical and Laboratory Standard Institutes (CLSI) guidelines (9,12,13,14). The following commercially available antibiotics were used against the isolates; ciprofloxacin (CPR), ofloxacin (OFL), augmentin (AUG), nitrofuran (NIT), ampicillin (AMP), ceftazidime (CAZ), cefuroxime (CRX), and gentamicin (GEN).

Antibacterial screening of *Citrus aurantifolia* peel against bacteria isolates

Suspension of the microorganisms were made in sterile normal saline and adjusted to 0.5 McFarland standards, which contains approximately 1 x 10^8 CFU/ml. A sterile swab stick was dipped into the peptone water containing the standardized organisms and then streaked on the solidified sterile Mueller-Hinton agar. Wells of 6 mm in diameter and about 2 cm apart were punched in the culture media with a sterile cork borer. The respective plant extract from the solvents were dropped into each well to fullness (approximately 100 µl) before incubating at 37°C for 24 hours. The zones of inhibition around the wells, measured in millimeters, were used as positive anti-bacterial activity.

Antibacterial screening of Alum against bacteria isolates

The agar well diffusion method was also used for the determination of antibacterial activity of aluminum potassium sulphate (Alum) aqueous extracts against the bacterial isolates. Loop-full growth from bacterial isolate was inoculated into peptone water, incubated at 37°C for 18 hours. The bacterial suspensions were diluted with normal saline, and the turbidity adjusted to 0.5 McFarland standards to yield a uniform suspension containing 1×10^8 CFU/ml. Muller Hinton agar was inoculated with bacterial inoculum, and a sterile cork borer was used to make wells of 6 mm diameter on the inoculated media. Then, 0.1ml of aqueous extracts of each alum concentration was added to the wells and incubated at 37°C for 24 hrs. The activity of alum aqueous extract was determined by measuring the diameter of inhibition zone in millimeter.

Determination of minimum inhibitory concentration (MIC) of *C. aurantifolia*

The MICs of the *Citrus* lime peel (methanol and hexane) extracts were determined for selected isolates using tube dilution susceptibility test. Two-fold serial dilutions of each extract i. e. 500 mg/ml was carried out in four tubes containing sterile 2 ml Mueller Hinton broth to give the following extract concentrations; 300, 200, 100 and 50 mg/ml. A loopful of the standardized test organisms was inoculated aseptically into the tubes containing the serially diluted extracts and incubated at 37°C for 24 hours. The concentration that showed no visible growth was taken as the MIC of the extract for each isolate (15).

Determination of minimum bactericidal concentration (MBC) of *C. aurantifolia*

Samples from the tubes used in MIC determination that showed no microbial growth were sub-cultured on Mueller Hinton agar and incubated at 37°C for 24 hours. The MIC tube with least concentration that showed no visible growth on the plate was taken as the MBC.

Data analysis

The data were analyzed to determine association and significance using the Statistical Package for the Social Sciences (IBM SPSS version 26.0). Informative and relevant data and result were presented in the best self-explanatory form either as figures, charts and/ or tables.

Results:

Demographic characteristics and predisposing factors of subject participants

Of the 100 participants recruited for the study, 50% (n=50) had spent less than 48 hours on admission at the hospital while 50% (n=50) have spent beyond 48 hours on admission. There are 30% males represented while 70% are females (Table 2). Table 3 shows the predisposing factors and medical history amongst the participants. About 28% have a previous history of chronic cough, 10% had history of smoking, and 22% exhibited symptoms of chest pain. A total of 31% participants were admitted due to severe underlying medical conditions while 68% were on antibiotics treatment at the time of study.

Table 2: Demographic characteristics of hospitalized participants at Sobi Specialist Hospital, Ilorin, Nigeria

Demographic characteristics	No of participants (%)
Gender	
Male	30 (30.0)
Female	70 (70.0)
Age group (years)	
11-20	17 (17.0)
21-30	34 (34.0)
31-40	6 (6.0)
41-50	18 (18.0)
51-60	8 (8.0)
61-70	10 (10.0)
>70	7 (7.0)
Educational status	
Non-educated	12 (12.0)
Primary	18 (18.0)
Secondary	36 (36.0)
Tertiary	34 (34.0)
Religion	
Islam	26 (26.0)
Christianity	74 (74.0)
Traditional	0
Others	0
Marital status	
Single	19 (19.0)
Married	77 (77.0)
Divorced	0
Widowed	4 (4.0)
Occupation	
Student	19 (19.0)
Civil servant	8 (8.0)
Business	55 (55.0)
Artisan	10 (10.0)
Unemployed	19 (19.0)

Table 3: Clinical characteristics and medical history of hospitalized patients at Sobi Specialist Hospital, Ilorin

Factors	Frequency (%)
Underlying illnesses	
Severe	21 (21.0)
Non-severe	79 (79.0)
Smoking history	
Yes	10 (10.0)
No	90 (90.0)
Symptom of cough	
Yes	28 (28.0)
No	72 (72.0)
Chest pain	
Yes	22 (22.0)
No	78 (78.0)
Administration of antibiotics	
Yes	68 (68.0)
No	32 (32.0)

Table 4: Bacterial pathogens colonizing the throat of hospitalized patients at Sobi Specialist Hospital, Ilorin

Bacterial isolates	Number (%)
Staphylococcus aureus	6 (42.8)
Acinetobacter baumannii	4 (28.6)
Escherichia coli	2 (14.3)
Klebsiella pneumoniae	2 (14.3)
Total	**14 (100.0)**

Bacteria isolates

A total of 14 bacterial isolates were recovered from throat swab samples collected from the 100 patients (Table 4). The most frequent bacterium was *Staphylococcus aureus* 6 (43%), followed by *Acinetobacter baumannii* 4 (28%) while *Klebsiella pneumoniae* and *Escherichia coli* had 2 (14.5%) each.

PCR assay confirmed the identity of the bacterial isolates as shown in the plates. Plate 1 (lane 2 is positive control and lane 4 is test organism positive for *E. coli*), plate 2 (lane 1 and 7 are positive controls, lane 6 is test organism positive for *K. pneumoniae*), plate 3 (lane 1 is positive control and lane 5 and 6 are organisms positive for *A. baumannii*) and plate 4 is *S. aureus.* Ten bacterial isolates were recovered from the throat samples of female patients (33.3%, 10/70) while 4 were recovered from those of the male patients (13.3%, 4/30) (OR=0.01582, 95% CI=0.3111-3.773, *p*=1.00)

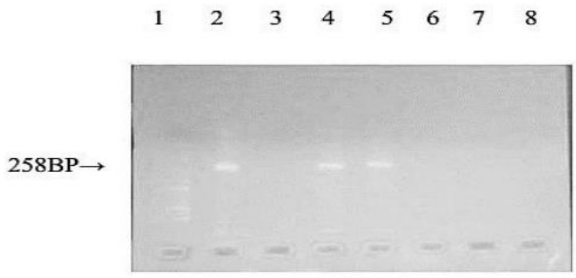

Plate 1: PCR product of *E. coli* on agarose gel (258bp)

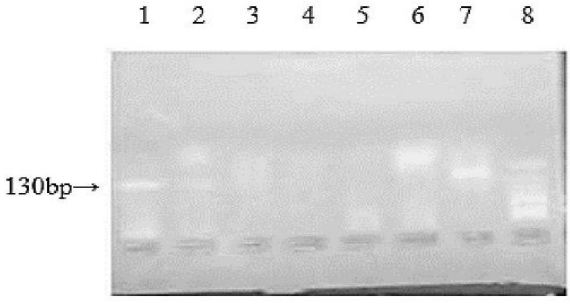

Plate 2: PCR product of *K. pneumoniae* on agarose gel (130bp)

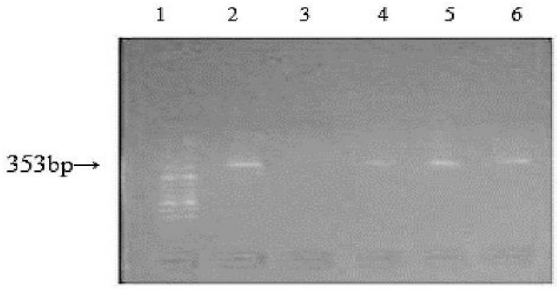

Plate 3: PCR product of *A. baumannii* on agarose gel (353bp)

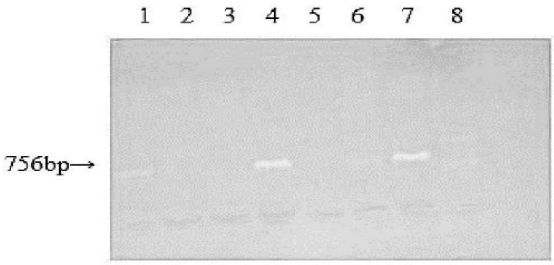

Plate 4: PCR product of *S. aureus* on agarose gel (756bp)

Antibacterial profile of isolates against selected antibiotics

Table 6 reveals the profile of the isolates in relation to the antibiotics. Of the 8 standard antibiotics tested, 3 isolates (*A. baumannii* TSS3*, K. pneumoniae* TSS6 and *A. baumannii* TSS37) were resistant to all while 3 others (*K. pneumoniae* TSS33*, S. aureus* TSS42 and *E. coli* TSS65) were resistant to at least five antibiotics. The inhibitory activity of hexane extract of *C. aurantifolia* compares favorably with the commercial antibiotics used in the study. The highest inhibition zone against *A. baumannii* TSS3 was 12 mm with nitrofuran (NIT) while hexane extract of *C. aurantifolia* gave highest inhibition zone of 19.2mm.

Although, the concentrations of the crude plant extracts were higher than the concentrations of the standard antibiotics tested, amoxicillin-clavulanate (augmentin AUG) was most active against *K. pneumoniae* TSS33 with zone diameter of inhibition of 24.5mm while the crude plant extract gave the highest inhibition zone diameter of 17.4mm. With exception of amoxicillin-clavulanate (AUG) for *K. pneumoniae* TSS 33, ciprofloxacin (CPR) and ofloxacin (OFL) for *S. aureus* TSS42 and ofloxacin for *E. coli* which had higher inhibitory effects, the plant crude extracts had comparative inhibitory effect or more active than the standard antibiotics, with diameters of inhibition zones ranging from 11.5-19.2mm (for hexane extract) and 9.8-15.8mm (for methanol extract).

Minimum inhibitory and bactericidal concentrations of *Citrus aurantifolia* peel

The MIC was carried out on isolates that were sensitive to the extract. Table 7 shows that the MIC of hexane extracts of *C. aurantifolia* for the isolates to range from 5-10 mg/ml while the MIC for methanol extracts range from 10-25 mg/ml. The MBC of hexane extracts for the isolates ranged from 25-50 mg/ml while the MBC of methanol extracts ranged from 50-100 mg/ml.

Table 5: Association of throat colonization by bacterial pathogens in relation to gender and age of hospitalized patients at Sobi Specialist Hospital, Ilorin, Nigeria

Characteristics	No of participants	No positive for enteropathogens (%)	X^2	OR (95% CI)	*p* value
Gender					
Male	30	4 (13.3)	0.016	1.083	1.000
Female	70	10 (33.3))		(0.3111-3.773)	
Total	100	14 (14.0)			
Age group (years)					
11-20	17	1	15.290	0.852	0.018
21-30	34	3		(0.737-0.967)	
31-40	6	1			
41-50	18	0			
51-60	8	3			
61-70	10	3			
>70	7	3			
Total	100	14 (14.0)			

X^2=Chi square; OR = Odd ratio; CI = Confidence interval

Table 6: Comparative inhibitory effects (inhibition zone diameters) of extracts of *Citrus aurantifolia* peel and standard antibiotics on bacterial isolates colonizing throats of hospitalized patients in Sobi Specialist Hospital, Ilorin, Nigeria

Bacterial isolates	**Antibiotic disc zone of inhibition (mm)**								**Hexane extract zone of inhibition (mm)**		**Methanol extract zone of inhibition (mm)**	
	CPR (5µg)	OFL (5µg)	AUG (30µg)	NIT (30µg)	AMP (30µg)	CAZ (30µg)	CRX (30µg)	GEN (10µg)	H (50mg/ml)	H (33mg/ml)	M (50mg/ml)	M (33mg/ml)
A. baumannii TSS3	6	11	8	12	9	7	10	9	19.2	16.20	13.3	10.0
K. pneumoniae TSS33	18	14	24.5	11.9	13	20	9	9	17.4	16.0	12.5	-
K. pneumoniae TSS6	9	10	8	6	7.7	8	9.2	8.3	13.7	11.5	15.8	-
A. baumannii TSS37	11	9.5	13	10	8	7	11	9.7	18.2	16.0	12.1	-
S. aureus TSS42	21	20	15	11	9	12	7	9	15.0	17.1	14.0	9.8
E. coli TSS65	14	25	8	9.3	13	12	15	17	15.1	13.7	10.1	-

CPR: Ciprofloxacin 5µg, OFL: Ofloxacin 5µg, AUG: Augmentin 30µg, NIT: Nitrofuran 300µg, AMP: Ampicillin 5µg, CAZ: Ceftazidime 30µg, CRX: Cefuroxime 30µg, GEN: Gentamicin 10µg. S: Sensitive, R: Resistant. H= Hexane and M= Methanol; - = no inhibition.

Table 7: Minimum inhibitory and minimum bactericidal concentrations of *Citrus aurantifolia* peel extracts against selected bacterial isolates colonizing the throats of hospitalized patients in Sobi Specialist Hospital, Ilorin, Nigeria

Isolates	**MIC (mg/ml)**										**MBC (mg/ml)**	
	Hexane extract					**Methanol extract**					**Hexane**	**Methanol**
	5	**10**	**25**	**50**	**100**	**5**	**10**	**25**	**50**	**100**		
A. baumannii TSS3	+	-	-	-	-	+	+	+	-	-	25	100
K. pneumoniae TSS33	+	+	-	-	-	-	+	-	-	-	50	50
K. pneumoniae TSS6	+	-	-	-	-	+	+	-	-	-	25	50
A. baumannii TSS37	+	-	-	-	-	+	+	-	-	-	25	50
S. aureus TSS 42	+	+	-	-	-	+	+	+	-	-	25	50
E. coli TSS65	+	-	-	-	-	+	+	-	-	-	25	50

'+' represents turbid (growth). '-' represents not turbid (no growth)

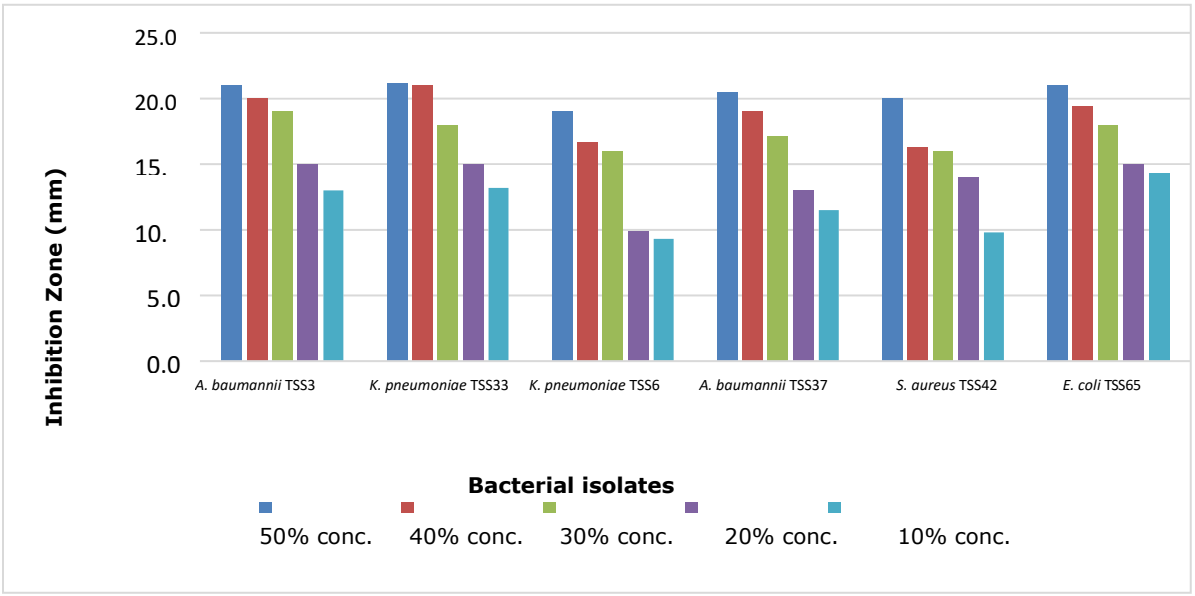

Fig 1: Antibacterial activity of aqueous extract of Alum on bacterial isolates colonizing the throats of hospitalized patients in Sobi Specialist Hospital, Ilorin, Nigeria

Antibacterial activity of Alum against isolates

Aqueous extracts of Alum [$KAl(SO_4).12H_2O$] at different concentrations (w/v%), produced zone diameters of inhibition for the isolates, ranging from 9.3 - 21.2 mm, which was concentration dependent, as shown in Fig 1.

Discussion:

The overall prevalence of bacteria pathogens colonizing the throat of hospitalized patients in this study was 14%. This is significant because presence of such pathogens can predispose to development of pneumonia as a result of immune compromise. The recorded prevalence is higher than 6.9% reported in Eastern Ethiopia in a nosocomial infection study (16) but lower than the reported 67% from patient in a referral hospital, Sikkim (17). The location as well as prevalence of predisposing factors such as underlying illness, hygiene and smoking history which could compromise the immunity of participants could account for the discrepancy of prevalence.

The outcome of antibiotic activity testing showed that isolates were multi-drug resistant bacteria with the isolates resistant to three or more classes of antibiotics, including aminoglycosides, fluoroquinolones, penicillin and cephalosporins. In Africa, higher rates of antibiotics resistant bacteria (10-100%) are reported among isolates from hospital-acquired infections (18), and multidrug resistant pathogens have continued to create critical health challenging issues especially in our environment with little or non-existent functional health infrastructure to cater for increasing population of the citizens.

In this study, both strains of *A. baumannii* and a strain of *K. pneumoniae* were completely resistant to antibiotics while others show 75% resistance to antibiotics and this agreed with the work of Levin et al. (19) but not in compliant with the work of Lim et al., (2) that reported HAP that were highly susceptible to antibiotics which could have resulted from geographical locations and practices such as controlled antibiotic usage and administration that could affect the emergence and distribution of resistant strains. However, it may possibly be thought that some of the reasons for the geometric rise of multi drug resistance cases might be connected to poor prescriptions expertise of antibiotic regimen and non-adherence to drug intake pattern recommendation by the subjects as suggested by the physician, even as it seems to be a verification of lack of health awareness education on the subject matter among the subjects, in our remote communities.

Increase in sensitivity of the test organisms to *C. aurantifolia* (lime) and alum solution was noticed to improve as the concentration increases and this corresponds to a previous report (20). Antibiotic susceptibility of test organisms was found to be relatively low compared to their susceptibility to lime peel extract and alum. This could have resulted from the difference in measurement where the crude extract had higher concentration compared to the standard antibiotics that was maintained at the standard quantity. The pattern of antibiotic activity could also be attributed to the general increase in antibiotic resistivity of bacteria isolates that is gradually on rise. Alum was however, most effective against test organisms having highest inhibition zone at 50% concentration. Out of the four organisms isolated from throat swab samples in this research, *S. aureus* was the only Gram-positive organism and it was observed to show the least sensitivity to the test agent. This finding is in accordance to the work of Nwankwo et al., (20) which posits that due to the nature of their cell wall, Gram-negative bacteria are more sensitive to antimicrobial agents.

Some compounds in citrus fruit can provide additional protection for the body against chronic disease and basic nutrition. Citrus fruits contain lots of phytochemicals, including essential oils, alkaloids, flavonoids, ouramin, psoralens, and carotenoids. Previous pharmacological studies revealed that citrus fruits have antimicrobial, anti-helminthic, insect repellent, antioxidant, anticancer, cardiovascular, anti-inflammatory, analgesic, anti- diabetic, reproductive, gastrointestinal, immunological, respiratory and many other pharmacological effects (21). Flavonoid compounds have properties that are effective in inhibiting the growth of bacteria, fungi, and viruses because these compounds include groups of phenol that can denaturize bacterial cell proteins and damage bacterial cell membranes (22).

Doughari et al., (23) stated that the anti-microbial effect of the plant could be due to the bioactive compounds such as the phytochemicals constituent present in the plant. The finding of this study showed that the efficacy of *C. aurantifolia* leaves extracts on the test isolates had different hierarchy of susceptibility among the organisms. The findings of this study indicated that higher concentration of the extract shows a larger zone of inhibition. This result agrees with that of Bisno and Stevens (24) which reported that the higher the concentration of antibacterial substance, the higher the zone of inhibition. However, this is most significant

for crude substances where higher quantity may be required to capture the active components. Thus, it is expected that with purified extract of concentrated bioactive agents, a lower quantity would yield required antibiotic activity.

The result of the antibacterial activity of *C. aurantifolia* leaves extracts in the present study was in conformity with studies that reported its antibacterial potentials (25,26). The molecular report of the target genes in the respective bacteria was confirmatory because they conform to the expected band size as reported by Woodford et al., (27), Zhang et al., (28) Maheux et al., (29) and Einas et al., (30).

Conclusion:

In conclusion, findings from this study showed the presence of resistant pathogenic bacteria colonizing the throat of hospitalized patients receiving care at the Sobi Specialist hospital, Ilorin. The isolates were resistant to aminoglycoside, fluoroquinolone and penicillin group of antimicrobials which are among the commercially available antibiotics. Although, the extract utilized were crude and of higher concentration, it can be concluded based on the recorded inhibition capacity that further studies to characterize, purify and isolate the active antibacterial components in the extracts should be considered. This can be harnessed as novel compounds for the production of antimicrobial drugs. There is need for proper health education to enlighten the public on the increasing trend of multi-drug resistant pathogen, transmission, prevention and control, which is not limited to eradication of indiscriminate use of antibiotics and self-medication.

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Authors contributions:

OAO, OMK, and MMS were involved in result computation, interpretation and manuscript preparation; OMK and OOA were involved in the study design while OAO, IBB, and OOA implemented the field work. All authors read the manuscript.

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Conflict of interest:

Authors declare no conflict of interest

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Original Article **Open Access**

In vivo assessment of antibacterial activity of *Cassia sieberiana* stem bark extracts on enterohaemorrhagic *Escherichia coli* infection in Wister rats

[1]Usanga, V. U., [1]Ukwah, B. N., [2]William, O., *[1]Kalu, M. E., [3]Akpan, J. L., [1]Azi, O. S., and [1]Ude, U. A.

[1]Department of Medical Laboratory Science, Faculty of Health Science and Technology, Ebonyi State University, Abakaliki, Nigeria
[2]Department of Laboratory Services, Alex Ekwueme Federal University Teaching Hospital, Abakaliki, Nigeria
[3]Department of Pharmacology, University of Calabar, Cross River State, Nigeria
*Correspondence to: kalu.erem@ebsu.edu.ng; +234806 402 8592

Abstract:

Background: The acceptance of traditional medicine as an alternative form of health care has led researchers to further investigate the antimicrobial and other health benefits of medicinal plants including *Cassia sieberiana*. The objective of this study is to assess the *in vivo* antibacterial effects of *C. sieberiana* stem bark extracts on infections caused by human and animal isolates of enterohaemorrhagic *Escherichia coli* (EHEC) in Wister rats.
Methodology: This *in vivo* study was designed for 21 days in 3 phases of 7 days each; adaptation, infection and treatment. *Escherichia coli* were isolated from aerobic cultures of human and cattle faecal samples and EHEC 0157 identified by serological typing using latex agglutination method. Aqueous and ethanol extracts of authenticated *C. sieberiana* stem bark, were prepared using standard method. Forty-five Wister rats were randomly divided into 9 groups (A-I) of 5 rats each. Rats in group A (uninfected with human or animal EHEC isolate and untreated) served as negative control while rats in group B (infected with EHEC animal isolate and untreated) and group C (infected with EHEC human isolate and untreated) served as positive controls. Rats in group C through group I were experimental groups that were either infected with human or animal EHEC isolate and treated, or uninfected but treated with ethanol and aqueous extracts of *C. sieberiana*. During each of the study phase, faecal samples were collected from the rats and processed for evaluation of EHEC count and to determine faecal occult blood. Data were analyzed using the Statistical Package for Social Sciences, version 20.0 and categorical variables were compared with Pearson's Chi-square, with significant value taken as $p<0.05$
Results: Three EHEC isolates (2 from cattle and 1 from human) were identified from 22 *E. coli* isolates cultured from cattle and human faecal samples. Rats in group A (negative control) and those in group G (infected with EHEC human isolate and treated with ethanol extract), group H (not infected but treated with aqueous extract) and group I (not infected but treated with ethanol extract) were faecal occult blood negative throughout the study period. Rats in group B (infected with animal isolate of EHEC without treatment) were occult blood negative after infection on day 14 but positive on day 21, while rats in group C (infected with human isolate of EHEC without treatment) were occult blood positive on day 14 but negative on day 21. Rats in groups D, E and F infected with human and cattle EHEC isolates and treated, were faecal occult blood positive on day 14 but negative on day 21, with high colony counts recorded, cleared within 7 days of treatment by both aqueous and ethanolic extracts of *C. sieberiana*.
Conclusion: The findings of this study confirmed the antibacterial potentials of *C. sieberiana* stem bark against EHEC. The beneficial effects of this plant extract should be exploited for commercial medicinal purposes.

Keywords: *Cassia sieberiana*; Enterohemorrhagic *Escherichia coli*; Wister rats; Antibacterial; *In vivo* assessment

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Évaluation *in vivo* de l'activité antibactérienne d'extraits d'écorce de tige de *Cassia sieberiana* sur l'infection entérohémorragique à *Escherichia coli* chez des rats Wister

[1]Usanga, V. U., [1]Ukwah, B. N., [2]William, O., *[1]Kalu, M. E., [3]Akpan, J. L., [1]Azi, O. S., et [1]Ude, U. A.

[1]Département des Sciences de Laboratoire Médical, Faculté des Sciences et Technologies de la Santé, Université d'État d'Ebonyi, Abakaliki, Nigéria
[2]Département des Services de Laboratoire, Hôpital Universitaire Fédéral Alex Ekwueme, Abakaliki, Nigéria
[3]Département de Pharmacologie, Université de Calabar, État de Cross River, Nigéria
*Correspondance à: kalu.erem@ebsu.edu.ng; +234806 402 8592

Résumé:

Contexte: L'acceptation de la médecine traditionnelle comme une forme alternative de soins de santé a conduit les chercheurs à étudier plus avant les avantages antimicrobiens et autres pour la santé des plantes médicinales, y compris *Cassia sieberiana*. L'objectif de cette étude est d'évaluer les effets antibactériens *in vivo* des extraits d'écorce de tige de *C. sieberiana* sur les infections causées par des isolats humains et animaux d'*Escherichia coli* entérohémorragique (EHEC) chez des rats Wister.
Méthodologie: Cette étude *in vivo* a été conçue sur 21 jours en 3 phases de 7 jours chacune ; adaptation, infection et traitement. *Escherichia coli* a été isolé à partir de cultures aérobies d'échantillons fécaux humains et bovins et EHEC 0157 identifié par typage sérologique à l'aide de la méthode d'agglutination au latex. Des extraits aqueux et à l'éthanol d'écorce de tige de *C. sieberiana* authentifiée ont été préparés en utilisant la méthode standard. Quarante-cinq rats Wister ont été répartis au hasard en 9 groupes (A-I) de 5 rats chacun. Les rats du groupe A (non infectés par l'isolat EHEC humain ou animal et non traités) ont servi de contrôle négatif tandis que les rats du groupe B (infectés par l'isolat animal EHEC et non traité) et du groupe C (infectés par l'isolat humain EHEC et non traité) ont servi de contrôle positif. Les rats du groupe C au groupe I étaient des groupes expérimentaux qui étaient soit infectés avec un isolat EHEC humain ou animal et traités, soit non infectés mais traités avec de l'éthanol et des extraits aqueux de *C. sieberiana*. Au cours de chacune des phases de l'étude, des échantillons fécaux ont été prélevés sur les rats et traités pour l'évaluation du nombre d'EHEC et pour déterminer le sang occulte fécal. Les données ont été analysées à l'aide du package statistique pour les sciences sociales, version 20.0 et les variables catégorielles ont été comparées au chi carré de Pearson, avec une valeur significative prise comme $p<0,05$
Résultats: Trois isolats d'EHEC (2 bovins et 1 humain) ont été identifiés à partir de 22 isolats d'*E. coli* cultivés à partir d'échantillons fécaux bovins et humains. Les rats du groupe A (témoin négatif) et ceux du groupe G (infectés avec l'isolat humain EHEC et traités avec de l'extrait à l'éthanol), du groupe H (non infectés mais traités avec de l'extrait aqueux) et du groupe I (non infectés mais traités avec de l'extrait à l'éthanol) ont été sang occulte fécal négatif pendant toute la période d'étude. Les rats du groupe B (infectés par l'isolat animal d'EHEC sans traitement) étaient négatifs au sang occulte après l'infection au jour 14 mais positifs au jour 21, tandis que les rats du groupe C (infectés par l'isolat humain d'EHEC sans traitement) étaient positifs au sang occulte le jour 14 mais négatif au jour 21. Les rats des groupes D, E et F infectés par des isolats EHEC humains et bovins et traités, étaient positifs au sang occulte fécal au jour 14 mais négatifs au jour 21, avec un nombre élevé de colonies enregistrées éliminées dans les 7 jours suivant le traitement par des extraits aqueux et éthanoliques de *C. sieberiana*.
Conclusion: Les résultats de cette étude ont confirmé les potentiels antibactériens de l'écorce de tige de *C. sieberiana* contre l'EHEC. Les effets bénéfiques de cet extrait de plante devraient être exploités à des fins médicales commerciales.

Mots clés: *Cassia sieberiana*; *Escherichia coli* entérohémorragique; Wister rats; Antibactérien; Évaluation *in vivo*

Introduction:

The genus "*Cassia*" is a member of the Fabaceae family (Leguminosae family) in the major group angiosperms (flowering plants), commonly known as the legume, pea or bean family, and is a large and economically important family of flowering plants (1). The name "*Cassia*" means Cinnamon-like bark (2). Plants of this family are found throughout the world, growing in many different environments and climates (3). The plants ranged from giant trees to small annual herbs with the majority being herbaceous perennials, and have indeterminate inflorescences, which are sometimes reduced to single flower. The flowers have short hypanthium and single carpel with short gynophores, and after fertilization produce fruits that that are legumes (1). The leaves are usually alternate compounds and are even- or odd-

pinnate.

The entire plants have numerous food, medicinal and non-medicinal uses (4). The medicinal value of this plant lies in some chemical substances that produce a definite physiological effect and these substances are alkaloids, flavonoids, glycosides, tannin oils, phenols and many others (5). Many plants in the genus are used extensively in traditional medicine in tropical and warm sub-tropical countries (6). It is believed to possess laxative effect and its extract is reported to be beneficial in treating many skin diseases such as eczema, rashes and ringworms. These plants have also been reported to be effective in the treatment of constipation, common colds, fevers, intestinal disorders, and for wound healing (7).

Alkaloids are very important in medicine and constitute most of the valuable drugs used locally. They have marked physiological effects on animals and show considerable pharmacological activity (8). Alkaloids are stimulants that act by prolonging actions of several hormones which require phosphodiesterase (7, 8), though are poisonous to cattle (9). Tannins are useful in medicine because of their astringent properties. Tannins and alkaloids are also known to have anti-herbivore defense functions in plants (5,9). Thus, the presence of tannins and alkaloids in this medicinal plant could be serving as a deterrent to grazers (8). Herbs that contain tannins are recommended for a wide range of treatments including inflammation, liver injury, kidney problems, arteriosclerosis, hypertension, stomach problems and inhibition of active oxygen, and are commonly recommended as diuretics, anti-diarrheal and haemostatics (10).

The recognition of enterohaemorrhagic *Escherichia coli* (EHEC) as an aetiologic agent of diarrhea with life-threatening complications has made this type of infection a public health challenge of serious concern. The pioneering work leading to the discovery of *E. coli* verocytotoxins (VTs) was carried out by Konowalchuk et al., (11) and O'Brien and LaVeck (12) in the late 1970s. They soon purified it and found that it had similar structure and biological activity to the shiga toxin produced by *Shigella dysenteriae* type1 (11,12). The verotoxigenic *E. coli* belonged to a previously rare serogroup 0157: H7 (13) that has been most commonly associated with large outbreaks (14).

The justification for continued interest in EHEC infections from a clinical perspective is the high rate of serious complications associated with this infection especially in children. Haemolytic uraemic syndrome (HUS) is defined by a triad of features; acute renal failure, thrombocytopenia and microangiopathic haemolytic anaemia, which occurs in 2-15% of cases of EHEC infections (15-17). Mortality from HUS is high between 3% and 17% (18) and a significant number (approximately 30%) suffer a range of permanent disabilities, including chronic renal insufficiency, hypertension and neurological deficits (19,20).

The pathogenesis of EHEC infection involves the establishment of the organism in the gut where it has to compete for space and nutrients with other microorganisms of the normal intestinal microflora (21,22). Local intestinal effects cause the development of bloody diarrhea as elaborated toxin internalizes in the cells of the gut where it blocks cellular protein synthesis, and may lead to apoptosis. HUS results from microvascular disease when the toxins enter the bloodstream and bind to receptors on endothelial cells that are abundant in kidneys and brain (22). The capacity to control EHEC infection in humans and to the scale off outbreaks is dependent upon rapid and accurate diagnosis and identification of the source of infection. Molecular biological techniques have been tested and shown to be very useful in this regard.

According to Anon (4), there is a need to evaluate local herbs for phytochemicals so as to determine the potential of these indigenous sources of medicines. The acceptance of traditional medicine as an alternative form of healthcare has led researchers to further investigate the antimicrobial and other health benefits of these medicinal plants. Medicinal plants are the richest bio-resources of drugs of traditional systems of modern medicine, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Extracting the relatively complex mixtures of metabolites is achieved by using selective solvents. During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity (23).

Stem barks are very important to nutrition (4). *Cassia sieberiana* nuts are edible and the majority of human calories come from stem barks, especially from cereals, legumes and nuts. Seeds of *C. sieberiana* provide most cooking oils, many beverages and spices and some important food additives (3,5). These stem barks range in colors from greenish-brown to dark brown with smooth surfaces, and may have small bright-colored bands on the outer surface. It is used for multiple medical purpo-

ses in Africa (4). The objective of this study is to assess the *in vivo* antibacterial efficacy of *C. sieberiana* stem bark extracts on experimental EHEC infection in Wister rats.

Materials and method:

Test plants

Cassia sieberiana was obtained from Uyo, Akwa Ibom State, Nigeria, and authenticated by a botanist in the herbarium section of the Department of Botany, University of Nigeria, Nsukka. The stem bark of *C. sieberiana* was used for the study

Collection and preparation of plant material

The stem bark of *C. sieberiana* was rinsed in clean water, cut into smaller pieces and air-dried at room temperature. The dried stem bark was pulverized to homogeneous powder using mortar and pestle.

Extraction of plant material and preparation of extracts for administration

One hundred grams (100 g) each of the powder was soaked in 1 litre of hot water and ethanol separately for 24 hours with intermittent stirring. A 2-mm pore size mesh filter paper was used to filter the extract and the filtrate was air-dried into concentrate. For the aqueous extract, 1g of the concentrate was dissolved in 4 ml of distilled water, while for ethanol extract, 1g of the concentrate was dissolved in 10% v/v of Tween 20 in distilled water.

Source of EHEC

Faecal samples were collected from cattle and humans at a slaughter house located in Gariki, Ebonyi State, Nigeria for isolation of *Escherichia coli* on MacConkey agar plates following aerobic incubation of the culture plates at 37^{o}C for 24 hours. Identification of *E. coli* isolates on MacConkey agar plates was done by conventional microbiological methods including colony morphology (lactose fermentation), Gram stain reaction, and biochemical tests such as indole, citrate utilization and urease tests (24). Presumptive EHEC isolates were inoculated on blood agar and incubated for 24 hours at 37^{0}C to observe for β-haemolytic colonies. The colonies were also tested for 0157 antigen by the latex bead agglutination assay (Oxoid, UK). Confirmed EHEC isolates were used for the *in vivo* study

Test animals and ethical issues

A total of 45 healthy Wister rats, weighing between 150 and 170 grams were supplied by an animal breeder in Abakaliki, Nigeria. The rats were housed in standard cages for 7 days for acclimatization (adaptation) during which they were confirmed not to be pre-infected by EHEC. All experimental procedures were performed with strict adherence to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised in 1996).

Phases of the experimental study

The study was carried out in three phases of 7 days each, over a period of 21 days. The first 7 days was acclimatization (adaptation) period during which the Wister rats were observed, and faecal samples collected for screening to ensure that the rats had no EHEC. In the second phase (infection period), rats were infected with human and animal EHEC isolates, and faecal samples were collected and processed for identification and enumeration of EHEC as well as for occult blood, as evidence of successful infection of the rats. In the third phase (treatment period), the extracts (aqueous and ethanol) were administered orally to the Wister rats, and faecal samples were collected and cultured for identification and enumeration of EHEC, and for detection of faecal occult blood.

Experimental design

After the acclimatization (adaptation) period, rats were randomly divided into 9 groups of 5 rats each; group A contains healthy Wister rats not infected with human or animal EHEC isolates, and not treated with the extracts of *C. sieberiana* (negative control); group B contains Wister rats infected with animal EHEC isolate without treatment with *C. sieberiana* extract (positive control 1); group C contains Wister rats infected with EHEC human isolate without treatment with *C. sieberiana* extract (positive control 2); group D contains Wister rats infected with EHEC animal isolate and treated with aqueous extract of *C. sieberiana*; group E contains Wister rats infected with EHEC animal isolate and treated with ethanol extract of *C. sieberiana*; group F contains Wister rats infected with EHEC human isolate and treated with aqueous extract of *C. sieberiana*; group G contains Wister rats infected with EHEC human isolate and treated with ethanol extract of *C. sieberiana*; group H contains Wister rats not infected with EHEC but treated with aqueous extract of *C. sieberiana*, while group I contains Wister rats not infected

with EHEC but treated with ethanol extract of *C. sieberiana*.

Infection of the Wister rats

The Wister rats in groups B, C, D, and E, were infected on day 8 by oral gavage with 1.0 x 10^8 CFU/ml of each of EHEC human and animal isolates, which were prepared from inoculum of each isolate on nutrient broth and standardized by comparing with 0.5 McFarland standards.

Administration of the extract

The Wister rats in groups D, E, F, and G, were treated with 0.25 ml of the extracts per kg body weight (250 mg/kg body weight) by oral gavage daily for 7 days from day 15. Extract of *C. sieberiana* has previously been reported to be safe at a dosage of 500 mg/kg body weight (4).

Faecal sample collection and determination of EHEC counts

Fresh stool samples were collected from the rats at an interval of 3 days (2 times a week) through the entire study period using sterile containers. All stool samples were processed within 3 hours of collection. Briefly, 0.5 g of faecal sample was homogenized in 5 ml of phosphate buffered saline (PBS) containing 0.8% NaCl; 0.2% KH_2PO_4; and 0.115% Na_2HPO_4 at pH 7.4, which had been sterilized by autoclaving at 121^0C for 15 minutes and 15 psi pressure. The emulsified faecal samples were serially diluted 10-folds in the sterile PBS, then, 0.1 ml of the 10^5 dilution was inoculated onto two sets of replicate MacConkey agar plates, which had been prepared according to manufacturer's instructions. The agar plates were incubated aerobically at 37^{o}C for 24 hours, and the mean EHEC count was determined on the agar plates (24).

Evaluation of faecal occult blood

Faecal occult blood test kit (QUICK VUE CLIA, Waive Inc. Ohio, USA) was used to determine the presence of blood in the faecal samples.

Statistical analysis:

The data generated were analyzed using the Statistical Package for the Social Sciences version 20.0 (SPSS Inc. Chicago, Il). Results were presented in percentages. Categorical variables were compared with Pearson's chi-square, and significant value was taken as p <0.05.

Results:

As shown in Table 1, 22 *Escherichia coli* isolates were recovered from 25 faecal samples collected from cattle and humans in the slaughter slabs, 3 of which were identified by serotyping as EHEC pathotype, while the remaining 19 were other (non-EHEC) pathotypes. Two EHEC pathotypes were from cattle, while 1 was from human.

Table 2 shows that Wistar rats in group A (not infected and not treated, negative control), group G (infected with EHEC human isolate and treated with ethanol extract of *C. sieberiana*), group H (not infected but treated with aqueous extract of *C. sieberiana*), and group I (not infected but treated with ethanol extract of *C. sieberiana*) were faecal occult blood negative throughout the study period.

Wistar rats in group B (infected with EHEC animal isolate without treatment, positive control 1) were faecal occult blood negative on day 14 but became positive on day 21, while rats in group C (infected with EHEC human isolate without treatment, positive control 2) were occult positive on day 14 but became negative on day 21.

Rats in group D (infected with EHEC animal isolate and treated with aqueous extract of *C. sieberiana*), group E (infected with EHEC animal isolate and treated with ethanol extract of *C. sieberiana*), and group F (infected with EHEC human isolate and treated with aqueous extract), were occult blood positive on day 14, but became negative on day 21.

Table 1: Source of Enterohaemorrhagic *Escherichia coli* (EHEC) used for the *in vivo* experiment

Source of faecal sample	Number examined	Number (%) positive of other strains of *E. coli*	Number (%) positive for EHEC
Human	8	7 (87.5)	1 (12.5)
Cattle	17	15 (88.2)	2 (11.8)
Total	**25**	**22 (88)**	**3 (12)**

Table 2: Detection of faecal occult blood in Wister rats during the study period

Wistar rat group	Day 8-14 (Infection period)	Day 15-21 (Treatment period)
A (Not infected, not treated, negative control)	Negative	Negative
B (Infected with EHEC animal isolate, not treated, positive control 1)	Negative	Positive
C (Infected with EHEC human isolate, not treated, positive control 2)	Positive	Positive
D (Infected with EHEC animal isolate, treated with aqueous extract)	Positive	Negative
E (Infected with EHEC animal isolate, treated with ethanol extract)	Positive	Negative
F (Infected with EHEC human isolate, treated with aqueous extract)	Positive	Negative
G (Infected with EHEC human isolate, treated with ethanol extract)	Negative	Negative
H (Not infected, treated with aqueous extract)	Negative	Negative
I (Not infected, treated with ethanol extract)	Negative	Negative

Fig 1 shows the effects of *C. sieberiana* extracts on the EHEC animal isolate counts. Wistar rats in group B (infected with EHEC animal isolate without treatment, positive control 1) had the highest EHEC count (10,000 CFU/ml) on day 14, which remained at < 5,000 CFU/ml on day 21, whereas rats in groups D and E (infected with EHEC animal isolate but treated with *C.* extracts) had EHEC count of > 5,000 CFU/ml each on day 14, but these reduced to 0 CFU/ml on day 21.

Fig 2 shows the effects of *C. sieberiana* extracts on the EHEC human isolate counts. Wistar rats in group C (infected with EHEC human isolate without treatment, positive control 2) had the highest EHEC count (10,000 CFU/ml) on day 14, which remained at >5,000 CFU/ml on day 21, while rats in groups F and G (infected with EHEC human isolate and treated with *C. sieberiana* extracts), had EHEC count of > 5,000 CFU/ml on day 14 but these reduced to 0 CFU/ml on day 21.

Fig 3 shows the comparative effects of *C. sieberiana* aqueous extracts on both human and animal EHEC isolates. The EHEC counts for both isolates were > 5,000 CFU/ml on day 14 but these reduced to 0 CFU/ml on day 21 following the 7-day treatment with *C. sieberiana* aqueous extracts, while the EHEC count for the positive control 1 rats (infected with EHEC animal isolate without treatment) was > 5,000 CFU/ml on day 14 and <5,000 CFU/ml on day 21, and the count for the positive control 2 rats (infected with EHEC human isolate without treatment) was > 5000 CFU/ml on day 14, and was still > 5000 CFU/ml on day 21.

Fig 4 shows the comparative effects of *C. sieberiana* ethanolic extracts on both human and animal EHEC isolates. The EHEC counts for both isolates were > 5,000 CFU/ml on day 14 but reduced to 0 CFU/ml on day 21 following the 7-day treatment with *C. sieberiana* ethanol extracts, while the EHEC count for the positive control 1 rats (infected with EHEC animal isolate without treatment) was >5,000 CFU/ml on day 14 and < 5,000 CFU/ml on day 21, and the count for the positive control 2 rats (infected with EHEC human isolate without treatment) was > 5,000 CFU/ml on day 14, which was still > 5,000 CFU/ml on day 21.

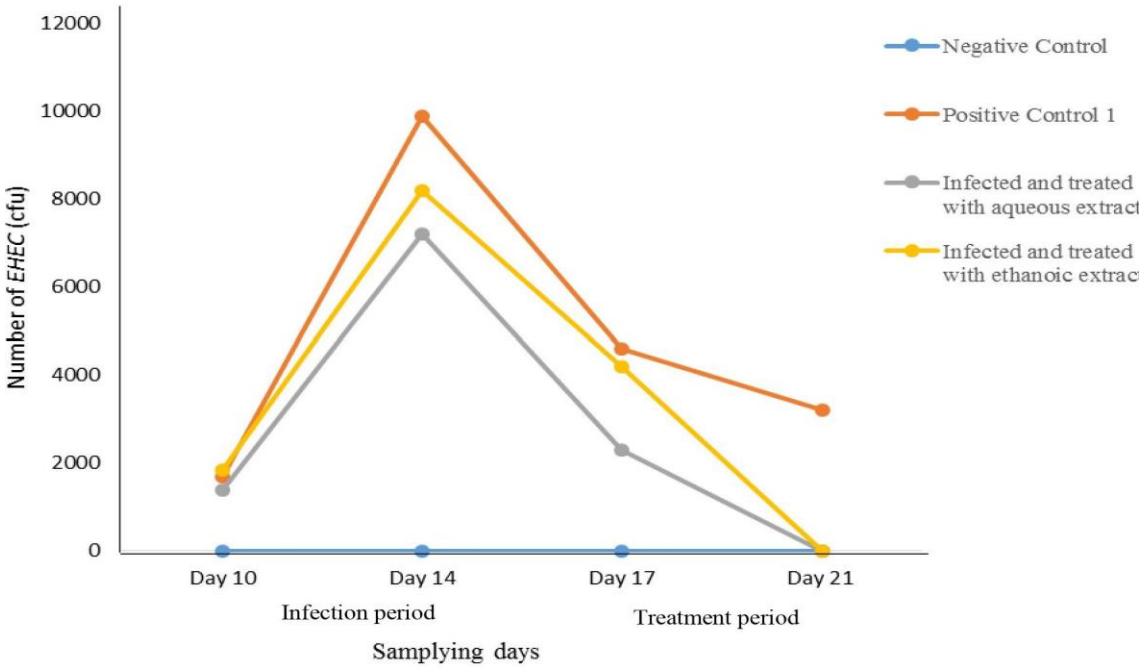

Fig 1: Antibacterial effects of *Cassia sieberiana* extracts on the EHEC animal isolate counts

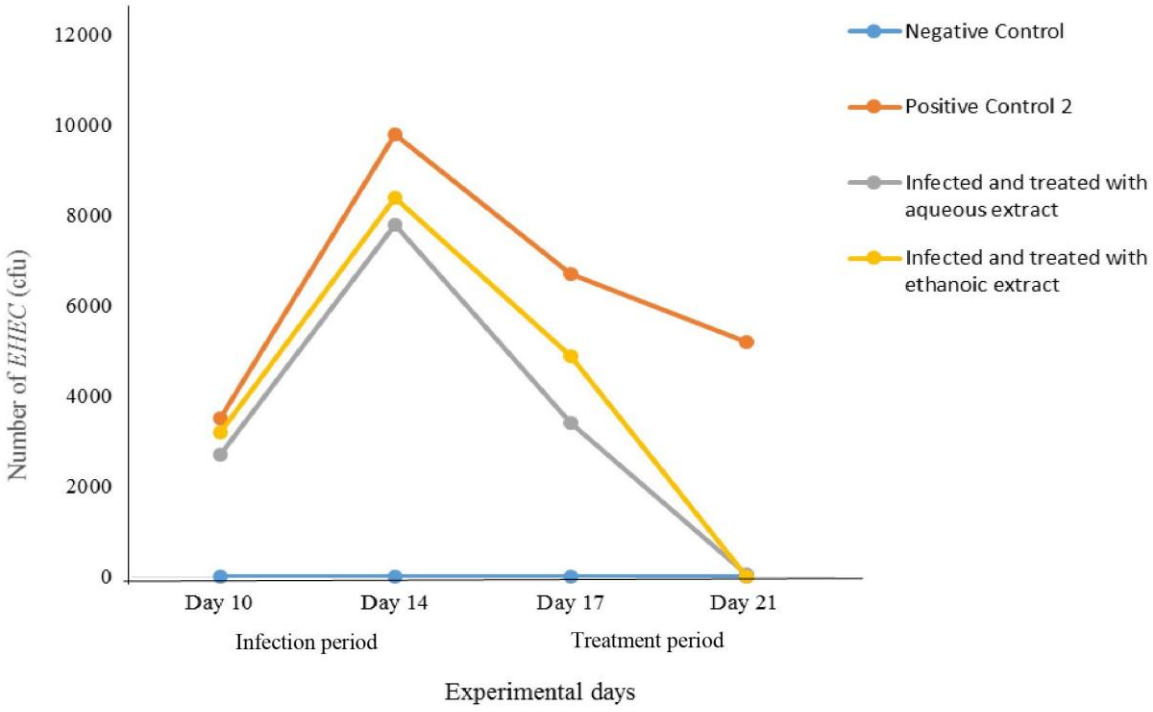

Fig 2: Antibacterial effects of *Cassia sieberiana* extracts on the EHEC human isolate counts

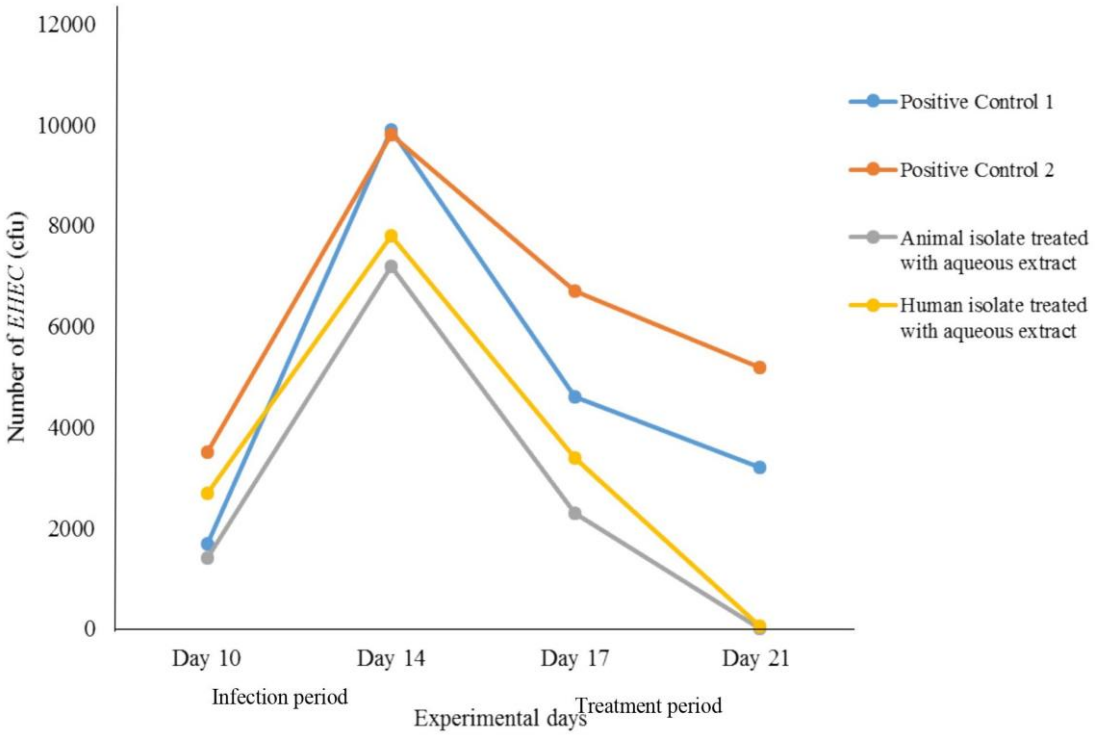

Fig 3: Comparative antibacterial effects of *Cassia sieberiana* aqueous extracts on human and animal EHEC isolates

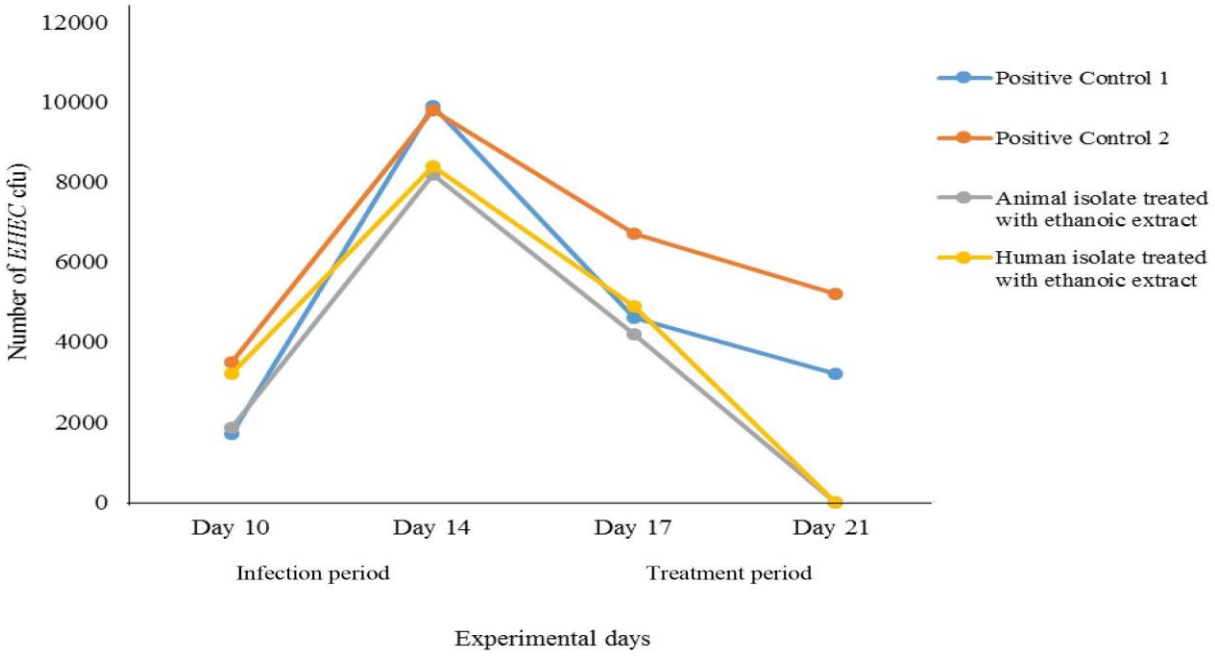

Fig 4: Comparative antibacterial effects of *Cassia sieberiana* ethanolic extracts on human and animal EHEC isolates

Discussion:

The findings of this study confirmed the presence of EHEC in humans and cattle. This is in conformity with previous studies conducted by Cornick and Vukhac (25) and Okere et al., (26), who reported that cattle are the major reservoir hosts of EHEC. They tend to be infected asymptomatically and shed the bacteria in faeces. Other animals, including rabbits and pigs, have also been reported to be carrier of EHEC (26). Humans acquire EHEC O157:H7 by direct contact with animal carriers, their faeces and contaminated soil or water, or via ingestion of undercooked beef, other animal products, and contaminated vegetables and fruits (26). Although other pathotypes of *E. coli* were isolated in our study, EHEC was the pathotype of utmost priority but we confirmed that there are other pathotypes of *E. coli* in animals as reported by O' Brien and LaVeck (12).

The current study demonstrated *in vivo* efficacy of *C. sieberiana* in treatment of experimentally induced EHEC infections in Wistar rats that manifested as haemorrhagic colitis, as all the rats which tested positive to faecal occult blood before treatment, became negative after 7 days treatment with *C. sieberiana* stem bark extracts. The gastro-protective and anti-ulcer property of *C. sieberiana* could be attributed to the high content of alkaloids (27), which is in line with similar ethnopharmacological studies conducted by other researchers in Nigeria (28-32).

Our study showed that positive control rats (group B rats infected with EHEC animal isolate without treatment and group C rats infected with EHEC human isolate without treatment) had the highest EHEC counts (~10,000 CFU/ml) as expected, but infected rats treated with aqueous extract of *C. sieberiana* showed higher CFU/ml on day 17 than those treated with ethanol extract, indicating a slightly higher anti-bacterial activity of ethanol over aqueous extract. However, in all the infected rats, treatment with either aqueous or ethanolic extract reduced EHEC counts to zero level after 7 days of extract administration. Our findings agree with reports of previous studies on similar plant extracts (27,33).

Apart from the documented antimicrobial activity of alkaloids, a major phytochemical constituent of *C. sieberiana* stem bark (27), against bacterial infections, flavonoids, tannins and saponins present in the stem are also known to possess antimicrobial potential by either altering the bacterial cell membrane or inhibiting the synthesis of nuclei acid and proteins (33). *Cassia sieberiana* has been shown to possess both bacteriostatic and bactericidal effects against *E. coli, Staphylococcus aureus, Pseudomonas* spp. and other pathogens (34, 35). In another study, Ulanowska and co (36) attributed the antimicrobial activity of *C. sieberiana* to the action of the phytochemical compounds on the RNA, DNA and protein synthesis apparatus of the bacteria.

Conclusion:

The findings of this study confirmed *in vivo* anti-EHEC potential of *C. sieberiana*, which provide evidence for its use in traditional medicines for treatment of infectious diseases. This could serve as source of readily available and less expensive raw materials for production of novel antimicrobial drugs that are useful for treatment of infections caused by *E. coli* and other infective agents.

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Short Communication **Open Access**

Prevalence of bacterial vaginosis in pregnant women attending Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria using the complete Amsel's diagnostic criteria

*[1]Udeogu, C. V., [1]Agbakoba, N. R., [1]Chukwuma, L. N., [2]Okwelogu, S. I., and [3]Oguejiofor, C. B.

[1]Department of Medical Laboratory Science, Medical Microbiology Unit, Nnamdi Azikiwe University, Nnewi, Anambra State, Nigeria
[2]Department of Parasitology and Entomology, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria
[3]Department of Obstetrics and Gynaecology, Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria
*Correspondence to: chidozie.udeogu@yahoo.com

Abstract:

Background: Bacterial vaginosis (BV) in pregnant women remains a cause for clinical concern among clinicians and health care professionals. BV has been linked to prenatal, antenatal and postnatal challenges in pregnant women. Information on prevalence of BV across trimesters of pregnancy is expected to give better clinical insight into the pathophysiology of this polymicrobial disorder. This study was conducted to determine the prevalence of BV in pregnant women attending the Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi, Nigeria.
Methodology: This was a cross-sectional study of 120 pregnant women (40 in each trimester of pregnancy) who had symptoms suggestive of BV, selected by systematic random sampling from among the women attending the Obstetrics and Gynaecology (O & G) clinic of NAUTH, Nnewi. Each subject participant was examined by the attending clinician, and high vaginal swab (HVS) sample was collected for diagnostic analysis of BV using with complete Amsel's clinical criteria, which consists of three of the four criteria; (i) adherent and homogenous vaginal discharge, (ii) vaginal pH > 4.5, (iii) detection of clue cells on saline wet mount, and (iv) amine odor after the addition of potassium hydroxide (positive Whiff test).
Results: The mean age of the 120 selected participants was 27.25±6.09 years. The age groups 25-29 (36.7%) and 20-24 years (33.3%) constituted the largest proportion, while age groups <20 (5.0%) and 40-45 years (5.0%) constituted the least. Of the 120 participants, 26 (21.7%) were positive for BV by the Amsel's criteria. Pregnant women in age group <20 years had the highest prevalence of BV (100%, 6/6), followed by those in the age groups 20-24 (27.5%), 40-45 (16.7%), 25-29 (15.9%), 30-34 (9.1%) and 35-39 years (0%) (X^2=28.063, p=0.0001). Prevalence of BV was significantly higher in single (unmarried) pregnant women (45.5%, X^2=4.038, p=0.045), women with primary school education level (66.7%, X^2=14.530, p=0.001), unemployed women (36.1%, X^2=13.278, p=0.0013), and nulliparous women [36.4%, X^2 (for trend) = 4.805, p=0.0274), while there was no significant difference in the prevalence of BV with relation to trimester of pregnancy (X^2=2.750, p=0.253).
Conclusion: This study reveals a relatively high prevalence of BV and significant association with factors such as age group, education and occupational status among pregnant women attending NAUTH Nnewi. Regular screening of women for BV prenatally may enable appropriate interventions to prevent adverse pregnancy outcomes.

Keywords: Bacterial vaginosis, pregnancy, trimesters, Amsel's criteria, polymicrobial, Nnewi, Nigeria

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Prévalence de la vaginose bactérienne chez les femmes enceintes fréquentant l'hôpital universitaire Nnamdi Azikiwe, Nnewi, Nigeria en utilisant les critères de diagnostic complets d'Amsel

*[1]Udeogu, C. V., [1]Agbakoba, N. R., [1]Chukwuma, L. N., [2]Okwelogu, S. I., et [3]Oguejiofor, C. B.

[1]Département des Sciences de Laboratoire Médical, Unité de Microbiologie Médicale, Université Nnamdi Azikiwe, Nnewi, État d'Anambra, Nigéria
[2]Département de Parasitologie et d'Entomologie, Université Nnamdi Azikiwe, Awka, État d'Anambra, Nigéria
[3]Département d'Obstétrique et de Gynécologie, Hôpital Universitaire Nnamdi Azikiwe, Nnewi, Nigéria
*Correspondance à: chidozie.udeogu@yahoo.com

Résumé:

Contexte: La vaginose bactérienne (VB) chez les femmes enceintes demeure une cause de préoccupation clinique chez les cliniciens et les professionnels de la santé. La VB a été associée à des problèmes prénatals, prénatals et postnatals chez les femmes enceintes. Les informations sur la prévalence de la VB au cours des trimestres de la grossesse devraient donner un meilleur aperçu clinique de la physiopathologie de ce trouble polymicrobien. Cette étude a été menée pour déterminer la prévalence de la VB chez les femmes enceintes fréquentant le Université Nnamdi Azikiwe, Nnewi, État d'Anambra, Nigéria
Méthodologie: Il s'agissait d'une étude transversale de 120 femmes enceintes (40 à chaque trimestre de grossesse) qui présentaient des symptômes évocateurs de VB, sélectionnées par échantillonnage aléatoire systématique parmi les femmes fréquentant la clinique d'obstétrique et de gynécologie (O & G) de NAUTH, Nnewi. Chaque sujet participant a été examiné par le clinicien traitant et un échantillon d'écouvillonnage vaginal élevé (HVS) a été prélevé pour une analyse diagnostique de la VB en utilisant les critères cliniques complets d'Amsel, qui se composent de trois des quatre critères ; (i) pertes vaginales adhérentes et homogènes, (ii) pH vaginal > 4,5, (iii) détection de cellules indices sur montage humide salin et (iv) odeur d'amine après l'ajout d'hydroxyde de potassium (test Whiff positif).
Résultats: L'âge moyen des 120 participants sélectionnés était de 27,25±6,09 ans. Les tranches d'âge 25-29 (36,7%) et 20-24 ans (33,3%) constituaient la plus grande proportion, tandis que les tranches d'âge <20 (5,0%) et 40-45 ans (5,0%) constituaient la plus faible. Sur les 120 participants, 26 (21,7%) étaient positifs pour la VB selon les critères d'Amsel. Les femmes enceintes du groupe d'âge < 20 ans présentaient la prévalence la plus élevée de VB (100%, 6/6), suivies de celles du groupe d'âge 20-24 (27,5%), 40-45 (16,7%), 25-29 (15,9%), 30-34 (9,1%) et 35-39 ans (0%) (X^2=28,063, p=0,0001). La prévalence de la VB était significativement plus élevée chez les femmes enceintes célibataires (45,5%, X^2=4,038, p=0,045), les femmes ayant un niveau d'études primaires (66,7%, X^2=14,530, p=0,001), les femmes sans emploi (36,1%, X^2=13,278, p=0,0013), et femmes nullipares [36,4%, X^2 (pour tendance) = 4,805, p=0,0274), alors qu'il n'y avait pas de différence significative dans la prévalence de la VB en fonction du trimestre de grossesse (X^2=2,750, p=0,253).
Conclusion: Cette étude révèle une prévalence relativement élevée de VB et une association significative avec des facteurs tels que le groupe d'âge, l'éducation et le statut professionnel chez les femmes enceintes fréquentant NAUTH Nnewi. Le dépistage régulier de la VB chez les femmes avant la naissance peut permettre des interventions appropriées pour prévenir les issues défavorables de la grossesse.

Mots-clés: Vaginose bactérienne, grossesse, trimestres, critères d'Amsel, polymicrobien, Nnewi, Nigeria

Introduction:

The vaginal microbial milieu undergoes significant structural changes at various stages during the life of woman that may be linked to several factors such as normal flora colonization, vaginal PH, hormonal modulation among other factors (1). The vaginal milieu is a dynamic ecosystem usually inhabited by bacteria which may be aerobic or anaerobic, with *Lactobacillus* species making up a significant population of the vaginal microbiota (2). Bacterial vaginosis (BV) is a polymicrobial disorder with accession of various fastidious bacteria, concurrent with a decrease of Lactobacilli, the dominant bacteria in the normal vaginal flora of women of reproductive age (3). It is also characterized by the overgrowth of anaerobes such as *Gardnerella*, *Bacteroides* and *Prevotella* species, which may counteract the growth of Lactobacilli and other commensals (4). Among other factors, genital mycoplasmas have also been implicated in various adverse conditions concerning pregnant women and neonates (5).

Although, a previous Nigerian study (6) did not demonstrate significant adverse effects of BV on pregnancy outcome, subsequent longitudinal study by Afolabi et al., (7) showed significant association between BV in pregnant women and preterm delivery, low birth weight (LBW) and preterm rupture of membranes (PROM) in pregnant women resident in southwestern Nigeria. In East Africa, findings by Mengistie et al., (8) also inferred that prevalence of BV is high among pregnant women, which was linked with adverse situations such as preterm birth. Similar findings have also been established in Europe by Donati et al., (9). The Amsel's criteria have been shown to have a positive predictive value of 90% and proven to be convenient (10), reliable and inexpensive for clinical diagnosis (4,11), especially in developing countries with weak health system.

There is a dearth of recent and similar studies examining association between sociodemographics and BV in pregnant women resident in southeastern Nigeria and specifically in

Anambra State. The objective of this study therefore is to determine the prevalence of BV in pregnant women attending the Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi, Nigeria

Materials and method:

Study setting and design

The study setting was the Obstetrics and Gynaecology (O & G) of Nnamdi Azikiwe University Teaching (NAUTH), Nnewi, Anambra State, Nigeria. NAUTH is a tertiary health institution established in 1991 primarily to provide specialized clinical service, teaching and research. This was a cross-sectional study of pregnant women with symptoms suggestive of BV attending the ANC clinic of the hospital between June and September, 2021.

Study population, sample size calculation and subject selection/recruitment

The study population comprise pregnant women with complaints of vaginal symptoms suggestive of BV attending the O & G clinic of the hospital. The sample size was determined using Daniel's sample size determination formula (12); $N= Z^2PQ/ D2$, where N = minimum sample size, Z=level of statistical significance of the expected result (in this case 1.96 for 95% confidence interval), P = proportion in the target population, Q = 1-P, and D= maximum allowable error, which is normally put at 0.05. Using the prevalence rate of BV in pregnant women of 7.5% from a previous study (13), the sample size was calculated as 114, which was adjusted to 120 participants.

Eligible participants were pregnant women at any trimester, who had symptoms suggestive of BV or complained of symptoms resembling BV. Women with vaginal bleeding, previously diagnosed with human immunodeficiency virus (HIV) infection or were on antibiotics were excluded from the study. Written informed consent was obtained from each partici pant.

Ethical approval

This study is a part of a wider research conducted in NAUTH, with ethical approval granted by Nnamdi Azikiwe University Teaching Hospital Health Research Ethics Committee (NAUTHHREC) with reference number NAUTH/ CS/66/VOL.14/VER 3/121/2021/039.

Sampling technique

Participants were recruited using systematic random sampling until the sample size was obtained. Participation of subjects in the study was voluntary. The subjects were properly informed on the nature, merits and aim of the study before sample collection. At recruitment, subjects were enrolled only after signing the informed consent form.

Clinical examination and specimen collection

Each subject was clinically examined by the attending clinicians in the O & G clinic for the presence or absence of discharge. The consistency, homogeneity, color and odor of the discharge were recorded. Sterile cotton-tipped swabs were labelled with the participants' study identification numbers, and were used to obtain vaginal samples from each consenting subject with the aid of a Cusco speculum for the purpose of diagnostic analysis with complete Amsel's clinical criteria and laboratory analysis

Amsel's diagnostic criteria:

Clinical diagnosis for bacterial vaginosis requires that at least three of the following four criteria are met; an adherent and homogenous vaginal discharge, vaginal pH greater than 4.5, detection of clue cells (vaginal epithelial cells with such a heavy coating of bacteria that the peripheral borders are obscured) on saline wet mount, and an amine odor after the addition of potassium hydroxide (positive whiff test) (11).

Demographic and clinical data collection

Data collection was done with aid of a well-structured questionnaire which took into consideration demographic information and factors that could influence prevalence of BV intended for evaluation. Essentially, information on age, marital status, trimester, educational status, occupational status, parity, use of antibiotics and poly-herbals were collected. Participants of antibiotics and poly-herbals were not excluded from the study.

Laboratory analysis of specimens

Laboratory analysis of specimen was done in the medical microbiology unit by of the laboratory complex of NAUTH. Test for vaginal pH, wet preparations and Whiff's tests were conducted as recommended

Test for pH

Vagina pH was measured by rolling the swab over a pH strip immediately after swabbing and matched with the provided color range to record the pH of the subject.

Wet film preparation for clue cell

Normal saline was added into one of the swabs containers and mixed gently several times. A drop was then be placed on a glass slide and gently covered with a cover slip. The

wet mount was examined using the 40x objective of a compound light microscope for the presence of clue cells (11).

Whiff's test

A drop of 10% potassium hydroxide (KOH) was added on a glass slide, followed by rolling of the swab on it. It was then whiffed for the liberation of a fishy odor indicative of the presence of volatile amines such as trimethylamine. The whiff result as recorded as either positive or negative on the Amsel record sheet.

Data analysis

Data analysis was conducted using SPSS version 20. Data obtained from the subjects were presented in tables, percentages and mean ± SD.

Results:

A total of 120 pregnant women (40 each in first, second and third trimester) were recruited as participants in this study. The mean age of the participants was 27.25±6.09 years. The age groups 25-29 (36.7%) and 20-24 years (33.3%) constituted the largest percentage of the pregnant women, while age groups <20 (5.0%) and 40-45 years (5.0%) constituted the least. Majority of the pregnant women were married (90.8%), multiparous (48.3%), self-employed (50.8%) and had secondary level education (74.2%). (Table 1).

Of the 120 participants, 26 (21.7%) were positive for BV by the Amsel's criteria, while 94 (78.3%) were negative. Pregnant women in the age group <20 years had the highest prevalence of BV (100%, 6/6), followed by those in the age group 20-24 (27.5%), 40-45 (16.7%), 25-29 (15.9%), 30-34 (9.1%) and 35-39 years (0%), which shows significant association (X^2=28.063, p=0.0001) (Table 2). Prevalence of BV was significantly higher in single (unmarried) pregnant women (45.5%, X^2=4.038, p=0.045), women with primary school education level (66.7%, X^2=14.530, p=0.001), unemployed women (36.1%, X^2=13.278, p= 0.0013), and nulliparous women [36.4%, X^2 (for trend) = 4.805, p=0.0274), while there was no significant difference in the prevalence of BV with relation to trimester of pregnancy (X^2=2.750, p=0.253).

Discussion:

Demographic data of pregnant women in our study such as age group, marital, educational and occupational status are similar to those reported in other research studies, and the mean age of 28.06±4.91 years among women of reproductive age group attending NAUTH Nnewi, reported by Okoli et al., (4) is similar to the mean age of 27.25±6.09 years among the women in our current study. Our study reported a BV prevalence of 21.7% for pregnant women, which is similar to the rate reported by Afolabi et al., (7), and also falls within the range of 20.0-49.0% reported in African countries among women of reproductive age (14,15). The cumulative relationship between a positive whiff test, presence of clue cells and a vaginal pH >4.5 in the evaluation of BV have been shown to be statistically significant and reliable as a diagnostic test (16), and these are implemented in the complete Amsel's criteria for diagnosis of BV and vaginitis (11).

Findings from this study show significant association between prevalence of BV and age groups of pregnant women, with high prevalence in those in age groups < 20 (100%) and 20-24 years (27.5%). A probable explanation for this may be that younger women are more sexually adventurous and are prone to multiple sexual relationships, compared to older women, as previously reported by Ranjit et al., (17). Another explanation may be that older women are more experienced with regards to pregnancy and are more likely to pay attention to antenatal care and personal health during pregnancy compared to younger women. Our study also reported a significant association between prevalence of BV and educational levels, with high prevalence in women who had primary school education level. It is generally understood that education play a vital role in behavioral modification and this may have a direct bearing on health and hygiene habits of participants in our study. Our finding agrees with Ness et al., (15), who reported a significant association between prevalence of BV and low levels of education. There was also a significant association between occupational status and BV prevalence in our study, with the unemployed pregnant participants having the

Table 1: Socio-demographic characteristics of pregnant women recruited for the study at NAUTH, Nnewi, Nigeria

Characteristics	Frequency (%)
Age group (years)	
<20	6 (5.0)
20-24	40 (33.3)
25-29	44 (36.7)
30-34	11 (9.2)
35-39	13 (10.8)
40-45	6 (5.0)
Mean age (mean ± SD)	27.25±6.09
Marital status	
Married	109 (90.8)
Single	11 (9.2)
Trimester of pregnancy	
First	40 (33.3)
Second	40 (33.3)
Third	40 (33.3)
Educational status	
Primary	9 (7.5)
Secondary	89 (74.2)
Tertiary	22 (18.3)
Occupational status	
Government employed	23 (19.2)
Self-employed	61 (50.8)
Unemployed	36 (30.0)
Parity	
Nulliparous	33 (27.5)
Primiparous	29 (24.2)
Multiparous	58 (48.3)

Table 2: Association of socio-demographic characteristics and other factors with prevalence of BV in the participants

Variables	Bacterial vaginosis		Total (n=120)	X^2	*p* value
	Positive (%) (n=26)	Negative (%) (n=94)			
Age group (years)					
<20	6 (100)	0	6	28.063	<0.0001*
20-24	11 (27.5)	29 (72.5)	40		
25-29	7 (15.9)	37 (84.1)	44		
30-34	1 (9.1)	10 (90.9)	11		
35-39	0	13 (100)	13		
40-45	1 (16.7)	5 (83.3)	6		
Marital status					
Married	21 (19.3)	88 (80.7)	109	4.038	0.045*
Single	5 (45.5)	6 (54.5)	11		
Trimester of pregnancy					
First	12 (30)	28 (70)	40	2.750	0.253
Second	8 (20)	32 (80)	40		
Third	6 (15)	34 (85)	40		
Educational status					
Primary	6 (66.7)	3 (33.3)	9	14.530	0.001*
Secondary	19 (21.3)	70 (78.7)	89		
Tertiary	1 (4.5)	21 (95.5)	22		
Occupational status					
Government employed	8 (34.8)	15 (65.2)	23	13.278	0.001*
Self-employed	5 (8.2)	56 (91.8)	61		
Unemployed	13 (36.1)	23 (63.9)	36		
Parity					
Nulliparous	12 (36.4)	21 (63.6)	33	4.805^{+}	0.0274*
Primiparous	5 (17.2)	24 (82.8)	29		
Multiparous	9 (15.5)	49 (84.5)	58		

* = statistically significant; X^2 = Chi square; $^{+}$ = Chi square for trend

highest prevalence, and self-employed women with lowest prevalence. This is similar to the findings of Ranjit et al., (17), who reported a lower BV prevalence in women who were engaged in business ventures, compared to unemployed housewives and farmers. Furthermore, single (unmarried) women had significantly higher prevalence of BV (45.5%) than married women (19.3%) (p=0.045) in our study, which may be attributed to the tendency of single women to have multiple sexual relationships, that may enhance sexual transmission of BV. Similarly, nulliparous women in our study had significantly higher prevalence of BV (36.4%) compared to multiparous (15.5%) and primiparous women (17.2%) (X^2 for trend 4.0805, p=0.0274). A previous study (6) showed that BV was significantly commoner (p= 0.0341) in primigravida than multigravida woman but the significance of these findings is not known. Although, Nelson et al., (18) postulated that BV in the first trimester may proceed to cause preterm complications in pregnant women in the second trimester, there was no significant association between prevalence of BV and trimesters of pregnancy in our study.

Although adverse pregnancy outcomes have not been conclusively linked to BV in pregnancy (6), the possibility of negative pregnancy outcomes was noted in a longitudinal study conducted by Afolabi et al., (7), and therefore, significant association between BV, and education and socio-economic factors reported in our study should not be de-emphasized. It is understood that the more educated and economically empowered a woman is, the more open she is to health and education interventions and willingness to engage in antenatal care during pregnancy, which promotes positive pregnancy outcomes (19). Conversely, less educated and economically challenged women may perhaps be open to patronizing cheaper means of alternative medical and antenatal care provided by untrained persons (quacks and charlatans), who may sometimes administer unregistered polyherbal regimens, some of which have been proven to be unsafe for human consumption (20). To prevent the possibility of negative pregnancy outcomes arising from BV, propagation of health jingles and sensitization programs in local languages are recommended, and subsidized antenatal care is also advocated.

Limitations of study

The study design is hospital-based and therefore exclude pregnant women in the community who do not visit the teaching hospital but traditional birth attendants for antenatal care. This will limit the generalization of our findings in this study.

Contributions of authors:

CVU and NRA conceived and designed the study, CVU wrote the first draft of the manuscript (MS) and reviewed literature along with NRA, LNC and SIO. CBO collected clinical samples from subjects. CVU performed laboratory analysis on the samples collected, analyzed data, performed statistical analysis and revised updated latest version of the manuscript (MS). All authors read and approved the final manuscript.

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Authors declare no conflict of interest

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Anueyiagu et al. *Afr. J. Clin. Exper. Microbiol.* 2022; 23 (3): 318 - 322 https://www.afrjcem.org

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Short Communication **Open Access**

Prevalence of antibiotic residues in body organs of pigs slaughtered in Jos, Nigeria

*[1]Anueyiagu, K. N., [1]Nandi, S., [1]Uzochukwu, I. A., and [2]Sule, S. O.

[1]Federal College of Animal Health and Production Technology, NVRI Vom, Nigeria
[2]Federal College of Veterinary and Medical Laboratory Technology, NVRI Vom, Nigeria
*Correspondence to: anueyiagunnamdi@yahoo.com; +2348035841582;
ORCiD: https://orcid.org/0000-0002-2566-7982

Abstract:

Background: Drug residue is a serious issue for the food chain when antimicrobial substances are inappropriately used or when the withdrawal times before slaughtering the treated animals are not respected. The aim of this study is to determine the prevalence of antibiotic residues in pork slaughtered for human consumption in Jos South Local Government Area (LGA), Plateau State, Nigeria.
Methodology: A total of 96 samples which included muscles (n=32), liver (n=32) and kidneys (n=32), were randomly collected from Gyel, Kuru, Du, and Vwang slaughter slabs in the LGA. The three-plate-test (TPT) technique was conducted where three batches of nutrient agar plates were aseptically prepared and adjusted to pH 6.0, 7.2 and 8.0. Each plate was seeded with isolated *Bacillus subtilis* and 5 holes were made on the plate with a sterile cork borer. The holes were inoculated with 80 µl of each organ extract, 10 µg/ml gentamicin (positive control) and distill water (negative control), and incubated aerobically at 37°C for 18-24 hours. Positive antibiotic residue was shown by a clear zone of inhibition (annular diameter of ≥ 2 mm) around the holes.
Results: The result showed that 55.2% (53/96) of the organs tested positive for antibiotic residues while 44.8% (43/96) tested negative. The liver had the highest prevalence of antibiotic residues (68.8%, 22/32), followed by the kidneys (56.3%, 18/32) and the muscles (40.6%, 13/32). The difference in the prevalence of antibiotic residues between the organs was not statistically significant (χ^2=5.1391, p=0.0765). Detection of tetracycline at pH 6.0 was highest in the organs while detection of sulphonamides was lowest at pH 7.2.
Conclusion: The determination of antibiotic residues in pork slaughtered for consumption in Jos South LGA of Plateau State, Nigeria posed potential public health risks. This situation indicates a wide spread uncontrolled use of antimicrobials in pig production, and requires urgent attention of government to enforce regulations guiding antibiotic use in veterinary settings in Nigeria.

Keywords: Antibiotic residues, antimicrobials, pigs, three plate test, Jos, Nigeria

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Prévalence des résidus d'antibiotiques dans les organes corporels des porcs abattus à Jos, Nigeria

*[1]Anueyiagu, K. N., [1]Nandi, S., [1]Uzochukwu, I. A., et [2]Sule, S. O.

[1]Collège fédéral de santé animale et de technologie de production, NVRI Vom, Nigeria
[2]Collège Fédéral de Technologie de Laboratoire Vétérinaire et Médical, NVRI Vom, Nigéria
*Correspondance à: anueyiagunnamdi@yahoo.com; +2348035841582;
ORCiD: https://orcid.org/0000-0002-2566-7982

Résumé:

Contexte: Les résidus de médicaments constituent un grave problème pour la chaîne alimentaire lorsque les substances antimicrobiennes sont utilisées de manière inappropriée ou lorsque les délais d'attente avant l'abattage des animaux traités ne sont pas respectés. Le but de cette étude est de déterminer la prévalence des résidus d'antibiotiques dans le porc abattu pour la consommation humaine dans la zone de gouvernement local (LGA) de Jos South, dans l'État du Plateau, au Nigeria.
Méthodologie: Un total de 96 échantillons comprenant des muscles (n=32), du foie (n=32) et des reins (n=32) ont été prélevés au hasard dans les dalles d'abattage de Gyel, Kuru, Du et Vwang dans la LGA. La technique du test à trois plaques (TPT) a été réalisée dans laquelle trois lots de plaques de gélose nutritive ont été préparés de manière aseptique et ajustés à pH 6,0, 7,2 et 8,0. Chaque plaque a été ensemencée avec *Bacillus subtilis* isolé

et 5 trous ont été faits sur la plaque avec un perce-bouchon stérile. Les trous ont été inoculés avec 80 µl de chaque extrait d'organe, 10 µg/ml de gentamicine (témoin positif) et de l'eau distillée (témoin négatif), et incubés en aérobiose à 37°C pendant 18 à 24 heures. Un résidu d'antibiotique positif a été mis en évidence par une zone claire d'inhibition (diamètre annulaire ≥ 2 mm) autour des trous.
Résultats: Le résultat a montré que 55,2% (53/96) des organes étaient positifs pour les résidus d'antibiotiques tandis que 44,8 % (43/96) étaient négatifs. Le foie présentait la plus forte prévalence de résidus d'antibiotiques (68,8%, 22/32), suivi des reins (56,3%, 18/32) et des muscles (40,6%, 13/32). La différence de prévalence des résidus d'antibiotiques entre les organes n'était pas statistiquement significative ($\chi 2=5,1391$, $p=0,0765$). La détection de la tétracycline à pH 6,0 était la plus élevée dans les organes tandis que la détection des sulfamides était la plus faible à pH 7,2.
Conclusion: La détermination des résidus d'antibiotiques dans la viande de porc abattue pour la consommation à Jos South LGA de l'État du Plateau, au Nigeria, pose des risques potentiels pour la santé publique. Cette situation indique une large utilisation incontrôlée des antimicrobiens dans la production porcine et nécessite une attention urgente du gouvernement pour faire appliquer les réglementations régissant l'utilisation des antibiotiques dans les établissements vétérinaires au Nigeria.

Mots-clés: Résidus d'antibiotiques, antimicrobiens, porcs, test à trois plaques, Jos, Nigeria

Introduction:

Antibiotics have been known to be incorporated in animal feeds to boost growth or to prevent and treat animal diseases. Antimicrobial residues are metabolites of antibiotics found in trace quantities found in edible portion of livestock products after antibiotics administration. When these residues are in excess of the acceptable limit they may lead to the development of antibiotic resistance in man or animals. The antimicrobial molecules are metabolized into other metabolites in blood, liver, and muscles and partially eliminated through kidneys or other routes (1). Consequently, these drugs and metabolites are accumulated in body cells and are known as drug residue. Drug residues may be detected in meat (2), eggs (3), milk or swine and poultry slurries (4). Drug residues are serious issues for the food chain when the antimicrobial substances are inappropriately used or when the withdrawal times before slaughtering the treated animals are not respected (5).

Antibiotic residues in meat and other foods are suspected to be responsible for drug allergy and other public health issues (6). Although there are scarce epidemiological data on the adverse effect of antibiotics residues, research studies indicate that animal food products may be a significant vehicle for the development and distribution of antimicrobial resistant bacteria (7,8). Many developed countries have banned the use of toxic antibiotics like chloramphenicol or avoparcin in animal food production, but they are still being used in developing countries (9).

Food safety legislation in Europe is known to be given a distinct attention with regards to checking for antibiotic residues in animal food products (10,11), and developing countries targeting to export products to Europe are mandated to keep strictly to specified guideline (12). Furthermore, consumers and retailers in non-industrialized nations are progressively mindful of the ill health conditions caused by drug residues in food of animal origin (13,14). Therefore, misuse of antimicrobial drugs in farms and residues in animal products becomes a major issue for the veterinary sector and public health services in Africa (2,15). The aim of this study is to determine the antibiotic residues in pork slaugh- tered for human consumption in Jos.

Materials and method:

Study area

Jos South is a Local Government Area (LGA) in Plateau State, Nigeria, located between latitude 9° 48'00N and Longitude 8° 52'00'' E, with an area of 510 km^2 and a population of 306,716 at the 2006 census.

Sample collection

A total of 96 samples which included muscles (n=32), liver (n=32) and kidneys (n=32), were randomly collected from Gyel, Kuru, Du, and Vwang slaughter slabs located in Jos South LGA. Each slab was visited for 4 weeks and 32 samples were collected from each slab.

Sample preparation

Five grams of each organ were macerated using sterile pistol and mortar, and emulsified with 5mls of distilled water and then centrifuged at 5000rpm for 10 minutes. The supernatant was decanted into dark colored Eppendorf tubes and stored for analysis.

Isolation of *Bacillus subtilis* from soil sample

Five grams (5g) of the soil sample from waste dump site was weighed into 45 ml of Ringer's solution to form a stock mix and heated at 90°C for one hour to encourage the formation of spores of *Bacillus subtilis* if present as well as eliminate other unwanted microorganisms in the sample. One-tenth of the mixture was inoculated into already prepared 0.4% dextrose nutrient agar and incubated at 37°C for 24 hours. Identification of *Bacillus subtilis* was done according to method described by Cheesebrough (16).

Three-plate-test for detection of antibiotic residues in organ samples.

The 3-plate test (TPT) technique (17)

was used to detect residual antibiotics in the pork samples. Three batches of nutrient agar plates were aseptically prepared in Petri dishes and the pH adjusted to 6.0, 7.2 and 8.0. Each plate was seeded with *B. subtilis*. On each agar plate, 5 holes were made with a sterile cork borer and 80 µl of each organ extract (muscle, liver and kidney) was placed in the holes, while the remaining 2 holes were inoculated with 80 µl each of distill water and gentamicin (10µg/ml) serving as negative and positive control respectively. The plates were inoculated aerobically at 37°C for 18-24 hours, after which a positive antibiotic residue was shown by a clear zone of inhibition around the holes with an annular diameter of ≥ 2 mm (17).

Data analysis

The Chi-square test of independence, using R commander (18) software, was performed to test for the association between the prevalence of antibiotic residue and the type of organs sampled, and between the prevalence of antibiotic residues and slaughter slabs sampled.

Results:

Out of 96 organ samples, 53 (55.2%) tested positive while 43 (44.8%) tested negative for antibiotic residues (Table 1). The proportion of organs positive for antimicrobial residues in descending order of frequency was liver (68.8%, 22/32), kidneys (56.3%, 18/32) and muscles (40.6%, 13/32). Test statistic showed that there was no association between the type of organs and the prevalence of antibiotic residues (χ^2=5.1391, p=0.0765).

From Table 2, distribution of antibiotic residues by the slaughter slabs showed that of the 96 samples, 12 (22.6%) from Du slab were positive, 14 (26.4%) from Kuru slab were positive, 12 (22.6%) from Vwang slab were positive, and 14 (26.4%) from Gyel slab were positive. Test statistics showed that there was no association between antibiotic resi- dues and the slaughter slabs (χ^2=3.699, p= 0.7173).

Of the 53 positive organ samples, 6 (11.3%) were detected at pH 6.0 (best detects oxytetracyclines and β-lactams), 4 (7.5%) were detected at pH 7.2 (best detects sulphonamides), 6 (11.3%) were detected at pH 8.0 (best detects aminoglycosides), 37 (69.8%) were detected at multiple pH i. e. 12 (22.6%) at pH 6.0 and 7.2; 7 (13.2%) at pH 6.0 and 8.0; 7 (13.2%) at pH 7.2 and 8.0, and 11 (20.8%) at the three levels of pH. There was no statistical association between antibiotic residues detection in the organs at a single pH level (χ^2=2, p=0.7358) or at multiple pH levels (χ^2=1.5401, p=0.9568).

Table 1: Prevalence of antimicrobial residues in organ samples of pig

Organs	No examined	No positive (%)	χ^2	p
Liver	32	22 (68.8)	5.1391	0.0765
Kidney	32	18 (56.3)		
Muscle	32	13 (40.6)		
Total	**96**	**53 (55.2)**		

χ^2 = Chi square

Table 2: Prevalence of antimicrobial residue in organ samples of pig according to location

Organs	No examined	No positive (%)				χ^2	p
		Du	Kuru	Vwang	Gyel		
Muscle	32	3 (7.4)	2 (4.9)	3 (7.4)	5 (12.3)	3.699	0.7173
Liver	32	6 (27.3)	5 (22.7)	6 (27.3)	5 (22.7)		
Kidney	32	3 (16.7)	7 (38.9)	3 (16.7)	4 (22.2)		
Total	**96**	**12 (22.6)**	**14 (26.4)**	**12 (22.6)**	**14 (26.4)**		

χ^2 = Chi square

Table 3: Organ distribution of antibiotic residues according to pH

Organ	No Positive	pH						
		Single			Multiple			
		6.0	7.2	8.0	6.0/7.2	6.0/8.0	7.2/8.0	6.0/7.2/8.0
Muscle	13	1	1	0	3	2	2	4
Liver	22	3	2	2	5	2	3	5
Kidney	18	3	1	3	4	3	2	2
Total	**53**	**7**	**4**	**5**	**12**	**7**	**7**	**11**
χ^2			2.0				1.5401	
p			0.7358				0.9568	

χ^2 = Chi square

Discussion:

In this present study, the results showed that out of 96 organ samples examined for antibiotic residues using the three-plate-test (TPT) method, 55.2% tested positive for the presence of antimicrobial residues. This high prevalence of antimicrobial residues in the meat samples may be a reflection of excessive antimicrobial prescription, overuse, and non-observance of the withdrawal period in veterinary practice that commonly occur in developing countries. However, it is believed that treatment of pigs with antimicrobial drugs is an important source of antibacterial residue in pigs (19). This problem in turn is due to unrestricted availability of antimicrobial drugs and the practice of self-medication by pig farmers. This unauthorized and unprofessional exposure of pigs to veterinary drugs without adherence to recommended dosages promotes accumulation of violative residues in tissues.

Despite the detection of antibiotic residues from all the organs, the liver stood out as the organ with the highest detection level of 68.75%, while the kidney level was 56.23% and muscle 40.62%. This could be due to the role the liver plays in humans and animals, which include protein synthesis, metabolism and detoxification processes. Most of the toxic substance and residues are metabolized in the liver. Our findings agree with that of Muriuki (20) in Nairobi, Kenya who found tetracycline residues in animal tissues with 24% in liver, 14% in kidney, and 7.6% in muscle samples. Additionally, in Nigeria, Olatoye and Ehinmowo (21) also reported that liver had the highest residue levels of 80% compared to the kidney and muscle with 55.0% and 28.3% respectively. However, the finding of our study contradicted the report of Hind (22) who reported higher antibiotic residues in the muscle (29.0%) than in the liver (28.0%) and kidney (21.4%). This contrasted situation was reported for pork meat in Nigeria, where 30.0% of sampled pork carcasses tested positive for anti microbial residues (3), whereas in Vietnam, a study reported antimicrobial residues prevalence of 41.24% for pork meat samples analyzed (23).

Detection of antimicrobials at different pH in the same organ implies that different classes of antimicrobials are being administered to pigs at the same time. Sekyere (24) confirmed in their study, that farmers use different drugs at the same time to either treat or prevent diseases in their pigs. The farmers' attempts to control the problem of concurrent infections on their fields may be linked to the usage of various antibiotic classes, as was also reported by Riviere and Sundlof (25). *Bacillus subtilis* at pH 6.0 is said to best detect tetracycline (OTC) which is the most widely used antibiotic in pig production in Nigeria (26). The detection of tetracycline was highest (n=7) in this study compared to sulphonamide (n=4) detected at pH 7.2 and aminoglycosides (n=5) detected at pH 8.0. The presence of antibiotic residues in some of the meat samples calls for concern, as this could pose serious public health risks to humans and animals, such as toxicity and resistance development.

The indiscriminate use of antimicrobial drugs as well as the failure to observe the withdrawal time, have been identified as the leading cause of violative residues in animal feeding items (25,27), which have led to availability of unsafe pork meats. The absence of enforcement of regulations of veterinary drug usage in Nigeria has resulted in drug abuse and misuse in food animals. The result of this study and those of others that detected residues in food animals in Nigeria should create awareness to the stakeholders (government and consumers) that should necessitate the enforcement of adherence to withdrawal periods, effective monitoring and surveillance of drug residues in meat animals and control of drug use in food animals, to prevent the occurrence of violative drug residues.

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Contribution of authors:

KNA was the principal investigator who conceived and designed the study. KNA and SN carried out sampling and bacteriological assays. KNA, SN, IAU and SOS wrote the manuscript and were responsible for the final editing of the manuscript.

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Case Report **Open Access**

Clinical neglect of aspergillosis in pulmonary tuberculosis co-infection: a case report of avoidable mortality in a resource-constrained setting

*[1]Adeyemo, A. T., [1]Obadare, T. O., [2]Edward, S. S., [1]Ibrahim, A. O., [1]Irek, E. O., [1]Amupitan, A. A., [2]Olorunsogo, O. A., [1]Anuforo, A. C., [2,3]Obiajunwa, P. O., and [1,4]Aboderin, A. O.

[1]Department of Medical Microbiology and Parasitology, Obafemi Awolowo University Teaching Hospitals Complex, Ile-Ife, Nigeria
[2]Department of Paediatrics, Obafemi Awolowo University Teaching Hospitals Complex, Ile-Ife, Nigeria
[3]Department of Paediatrics, Obafemi Awolowo University, Ile-Ife, Nigeria
[4]Department of Medical Microbiology and Parasitology, Obafemi Awolowo University, Ile-Ife, Nigeria
*Correspondence to: adeyemiadeyemo3@gmail.com; ORCiD: https://orcid.org/0000-0003-3544-2530

Abstract:

Background: Pulmonary aspergillosis (PA) is common among patients with tuberculosis (TB). With both infections presenting with similar clinical and radiologic features, diagnosis of PA is often made too late or missed completely due to lack of clinical suspicion and poor diagnostic laboratory capacity for mycotic infections prevalent in our settings. We present a case of preventable mortality caused by delayed diagnosis and treatment of PA in a patient with pulmonary TB (PTB).
Case presentation: A 13-year-old female was diagnosed and treated for PTB, having received anti-TB regimen for 8 months in a mission hospital from where she was referred due to worsening cough, chest pain and progressive breathlessness. The patient was re-assessed and investigated, with GeneXpert detecting *Mycobacterium tuberculosis*, susceptible to rifampicin. Diagnosis of pulmonary tuberculosis complicated by right pneumothorax was made indicating an emergency thoracotomy and chest tube insertion and continuation of the first line anti-TB regimen. At about 2 weeks into admission, patients had features of superimposed acute bacterial sepsis with fever becoming high grade, marked neutrophilia with toxic granulation and elevated sepsis biomarker, and this necessitated empiric antibiotic treatment with parenteral meropenem and vancomycin. However, the patient only had mild clinical improvement following which there was progressively worsening respiratory symptoms and massive haemoptysis. Result of sputum fungal study was available on admission day 20 and revealed a growth of *Aspergillus flavus*. Treatment with intravenous voriconazole was however commenced rather late when the fungal respiratory disease could no longer be remedied. The patient died on admission day 23.
Conclusion: Diagnosis of PA in patients with background TB is often made too late to guarantee timely and effective antifungal treatment with negative consequences on patients' outcomes. Improving clinical and laboratory capacities is essential to reducing mortality from PA in healthcare facilities.

Keywords: pulmonary aspergillosis; tuberculosis; co-infection; voriconazole

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Négligence clinique de l'aspergillose dans la co-infection tuberculeuse pulmonaire: à propos d'un cas de mortalité évitable dans un contexte de ressources limitées

*[1]Adeyemo, A. T., [1]Obadare, T.O., [2]Edward, S. S., [1]Ibrahim, A. O., [1]Irek, E. O., [1]Amupitan, A. A., [2]Olorunsogo, O. A., [1]Anuforo, A. C., [2,3]Obiajunwa, P. O., et [1,4]Aboderin, A. O.

[1]Département de Microbiologie Médicale et de Parasitologie, Complexe Hospitalier Universitaire Obafemi Awolowo, Ile-Ife, Nigéria
[2]Département de Pédiatrie, Complexe Hospitalier Universitaire Obafemi Awolowo, Ile-Ife, Nigéria
[3]Département de pédiatrie, Complexe Hospitalier Universitaire Obafemi Awolowo, Ile-Ife, Nigéria

[4]Département de Microbiologie Médicale et Parasitologie, Université Obafemi Awolowo, Ile-Ife, Nigéria
*Correspondance à: adeyemiadeyemo3@gmail.com; ORCiD: https://orcid.org/0000-0003-3544-2530

Résumé:

Contexte: L'aspergillose pulmonaire (AP) est fréquente chez les patients atteints de tuberculose (TB). Les deux infections présentant des caractéristiques cliniques et radiologiques similaires, le diagnostic d'AP est souvent posé trop tard ou complètement manqué en raison d'un manque de suspicion clinique et d'une faible capacité de diagnostic en laboratoire pour les infections mycotiques prévalentes dans nos contextes. Nous présentons un cas de mortalité évitable causée par un diagnostic et un traitement tardifs de l'AP chez un patient atteint de tuberculose pulmonaire (TBP).
Présentation de cas: Une femme de 13 ans a été diagnostiquée et traitée pour une TBP, après avoir reçu un traitement antituberculeux pendant 8 mois dans un hôpital de mission d'où elle a été référée en raison d'une aggravation de la toux, de douleurs thoraciques et d'un essoufflement progressif. Le patient a été réévalué et investigué, GeneXpert détectant *Mycobacterium tuberculosis*, sensible à la rifampicine. Le diagnostic de tuberculose pulmonaire compliquée d'un pneumothorax droit a été posé indiquant une thoracotomie d'urgence et l'insertion d'un drain thoracique et la poursuite du traitement antituberculeux de première intention. À environ 2 semaines après l'admission, les patients présentaient des caractéristiques de septicémie bactérienne aiguë superposée avec une fièvre devenant de haut grade, une neutrophilie marquée avec une granulation toxique et un biomarqueur de septicémie élevé, ce qui a nécessité un traitement antibiotique empirique avec du méropénème parentéral et de la vancomycine. Cependant, le patient n'a présenté qu'une légère amélioration clinique, suivie d'une aggravation progressive des symptômes respiratoires et d'une hémoptysie massive. Le résultat de l'étude fongique des expectorations était disponible au jour 20 de l'admission et a révélé une croissance d'*Aspergillus flavus*. Le traitement par voriconazole par voie intraveineuse a cependant été commencé assez tardivement lorsque la maladie respiratoire fongique ne pouvait plus être soignée. Le patient est décédé le jour 23 de l'admission.
Conclusion: Le diagnostic d'AP chez les patients atteints de tuberculose de fond est souvent posé trop tard pour garantir un traitement antifongique rapide et efficace avec des conséquences négatives sur les résultats des patients. L'amélioration des capacités cliniques et de laboratoire est essentielle pour réduire la mortalité due à l'AP dans les établissements de santé.

Mots clés: aspergillose pulmonaire; tuberculose; co-infection; voriconazole

Introduction:

Aspergillus is a saprophytic ubiquitous mould, acquired and spread by inhalation of aerosolised spores with conidia invading and destroying host tissues. It causes a spectrum of pulmonary infections which is principally determined by virulence of the pathogen, underlying lung disease and host immunity (1). As an opportunistic pathogen, it commonly affects immunocompromised patients or immunocompetent patients with underlying respiratory tract diseases (2). Patients with background pulmonary tuberculosis (PTB) are also particularly prone to pulmonary aspergillosis (PA) resulting from associated generalised debility from impaired immunity and reduced macrophage functions (3). PA commonly complicates but often co-infects with tuberculosis (TB) especially in low socioeconomic environment with inherent insufficient capacities for diagnosis and treatment (4,5).

Global epidemiological data revealed that of the approximately 3 million people suffering from chronic pulmonary aspergillosis (CPA), 1.2 million cases are associated with PTB (2). Annual prevalence of co-infection varies but is pronounced globally, estimated at 145,372 in South-East Asia and 98,551 in Africa (6). PA lacks definite pathognomonic clinical syndrome and co-infection with PTB often reduces tendencies for detection because of similarities in clinical features especially in settings where diagnosis of fungal pathogens is still rudimentary (7). About 2 million new cases of non-bacteriologically diagnosed PTB were reported by WHO in 2015 and a proportion of these patients probably had undiagnosed chronic pulmonary aspergillosis (CPA) (8). Five-year estimated prevalence rates of chronic PA among patients with PTB vary globally ranging from 0.4 to 51 per 100,000 population; developing countries are more affected with Nigeria having a high rate of 42.9 per 100,000 population (6).

Information about fungal infections is sparse in Africa; available data from 15 of 57 African countries revealed that estimated 1.7 million people suffer from serious and life-threatening fungal diseases including PA with key factors influencing management outcomes being availability of appropriate diagnosis and promptness of treatment. This duo has been greatly challenged in Africa and other resource-limited countries which do not only have high burden of infections by these opportunistic fungi but also basically deficient in advanced fungal diagnostics and mycologic experts (9,10). Missed clinical diagnoses of PA in patients with background PTB coupled with unavailability of rapid, sensitive and reliable laboratory capacity for fungal infections is a great challenge to early commencement of appropriate antifungal drug therapy, access to which is rather poor in Nigeria and most of other countries in sub-Saharan Africa, and all

these have led to high mortality (11-13). We report a case of avoidable mortality resulting from late diagnosis of PA in a patient with TB co-infection.

Case presentation:

A 13-year-old female referred from a faith-based specialist hospital where she had presented with a low-grade fever and chronic productive cough. Patient had positive sputum GeneXpert (Xpert 2110 polymerase chain reaction) results as well as chest radiographic features of PTB for which she had been on treatment for 8 months at the referral hospital. The patient was commenced on another course of anti-tuberculosis drugs (still first line regimen) having completed an initial 6-month course with no improvements in clinical symptoms and signs with persistence of radiologic features of PTB.

Patient was referred to the Obafemi Awolowo University Teaching Hospital, Ile-Ife, Nigeria on the 3rd January 2020 as a result of progressively worsening cough associated with dull aching non radiating chest pain, and difficulty with breathing. Initial examination revealed chronically ill-looking patient, febrile (temperature of 37.9^0C), mildly pale, stage III finger clubbing, tachypnoeic (respiratory rate of 84 cycles per minute), in respiratory distress with intercostal and sub-costal recessions and pectus escavatum, reduced chest expansion in the left hemithorax. Percussion notes were dull in the left middle and lower lung zones but hyper-resonant in the right lung fields with increased tactile fremitus and vocal resonance in the left middle and lower lung zones, reduced air entry in the right lung zones as well as left middle and lower lung zones, There were widespread bilateral crepitations and oxygen saturation of 78% (at room air), The patient was tachycardic (pulse rate of 148 beats per minute), blood pressure was normal and jugular venous pressure was not raised, apex beat was however displaced to the 6th left intercostal space in the anterior axillary line (non-heaving, non-tapping) and heart sounds were normal with no murmur. There was mild tender hepatomegaly (liver was 4 centimetres below the right costal margin).

Complete blood count showed packed cell volume of 33%, white cell count of 9200/mm^3 with neutrophil and lymphocyte proportion of 54% and 46% respectively, and platelet count of 316,000/mm^3. Chest radiograph showed features of PTB including cavitatory lesions within both upper lung zones with adjacent reticular and patchy opacities (Fig. 1). Sputum by GeneXpert detected *Mycobacterium tuberculosis*, not rifampicin-resistant. Erythrocyte sedimentation rate (ESR) was elevated at 117mm/hour (Westergreen method). Further investigations revealed negative results for retroviral screening, hepatitis B surface antigen and anti-hepatitis C antibody, and blood culture yielded no growth of bacteria.

Abdominal and chest ultrasonography revealed no abnormality. Plasma blood glucose, serum electrolytes, and renal and liver function tests gave parameter values that were within normal range. In addition to features of TB, the patient also had radiographic features of right sided pneumothorax and was consequently placed on intranasal oxygen (2-3 litres per minute) with first line anti-tuberculosis drugs. She immediately had right-sided thoracotomy and chest tube insertion with post intubation chest radiograph showing satisfactory lung re-expansion, and subsequently had right pleurodesis post extubation.

The clinical condition mildly improved until 2 weeks into admission when patient developed high-grade continuous fever with worsening cough, productive of copious blood-stained sputum and an associated worsening respiratory distress. A new set of laboratory investigations ordered at this time included another set of blood culture which yielded no bacterial growth, sputum bacteriological culture which yielded no growth of pathogen, packed cell volume of 35%, leukocytosis (total white cell count of 21,600/mm^3) with neutrophilia (neutrophil count of 19,872/mm^3 with toxic granulations) and normal platelet count (335,000/mm^3), and serum procalcitonin level >10 ng/ml (confirming bacterial sepsis). Additional diagnosis of bacterial sepsis was made and the antibiotic treatment was escalated to intravenous meropenem and vancomycin.

Twenty days into admission, result of sputum fungal study revealed fungal hyphae on direct microscopy (Fig. 2) and *Aspergillus flavus* on culture (Figs 3 and 4). A new diagnosis of pulmonary aspergillosis (possibly chronic pulmonary aspergillosis) was made necessitating request for computerized tomographic (CT) scan of the chest and commencement of intravenous voriconazole. At that time, the patient's clinical conditions had deteriorated, with cough and respiratory distress becoming very severe, and the patient had haemoptysis lasting for about 2 hours. The clinical condition continued to take a turn for the worse despite aggressive resuscitation. There was cessation of breathing and the patient was confirmed dead on admission day 23.

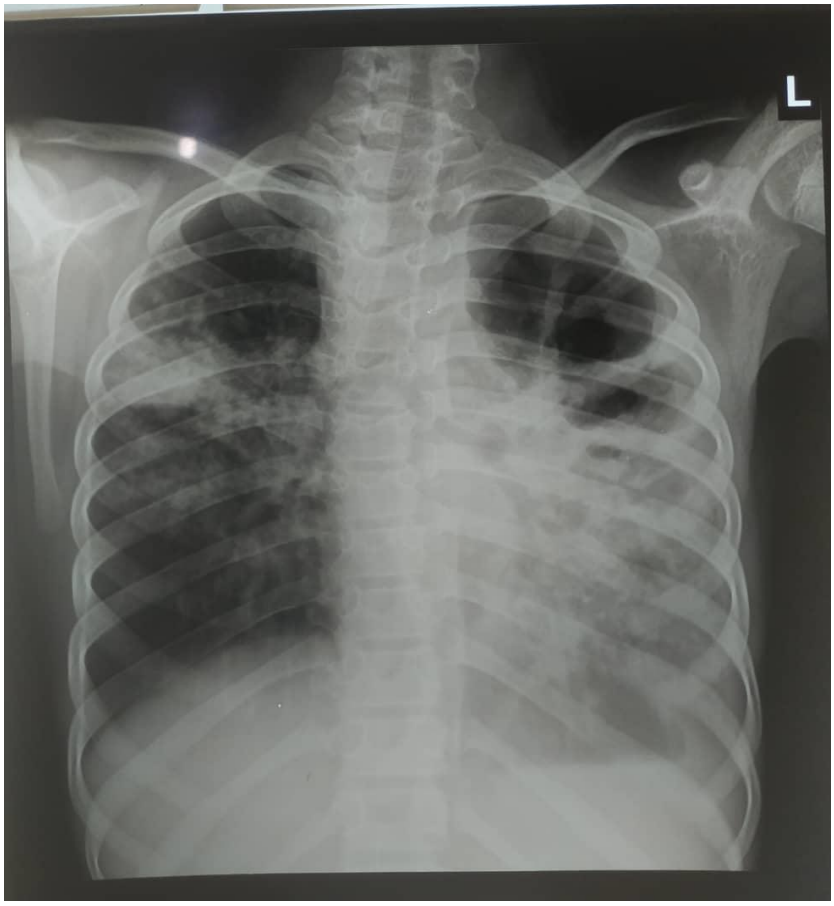

Fig. 1: Chest radiograph showing cavitatory lesions within both upper lung zones with adjacent reticular and patchy opacities It also shows homogeneous opacification of the left middle and lower lung zones with obliteration of the left cardiac silhouette, diaphragmatic outline and costophrenic angle in keeping with pleural effusion with ipsilateral mediastinal shift.

Fig. 2: Direct microscopy (wet mount) with lactophenol cotton blue stain showing septate fungal hyphae

Fig 3: Darkish green cottony colonies of *Aspergillus flavus* on Saboraoud dextrose agar

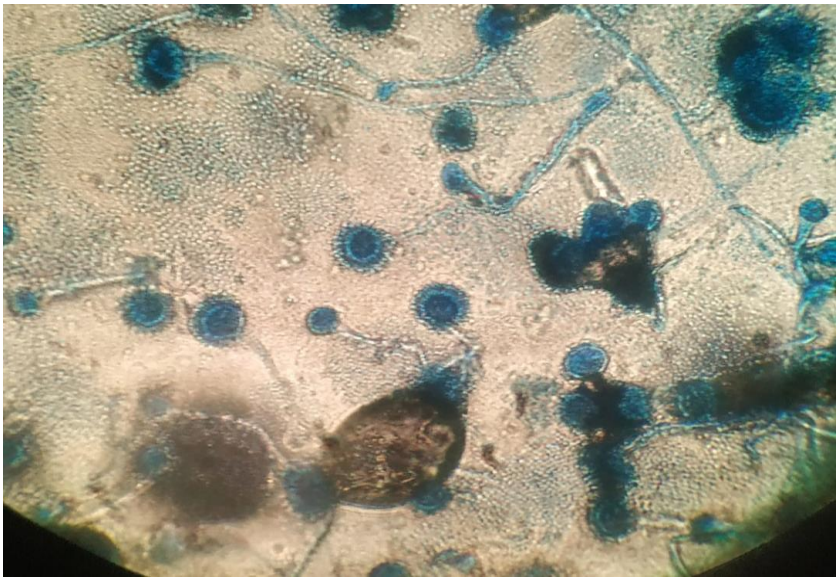

Fig 4: Lactophenol cotton blue preparation of colony showing microscopic morphology of *Aspergillus flavus* with conidia heads which are radiate and split to form loose columns, biseriate but some heads have phialides borne directly on the vesicles. Conidiophores are hyaline and coarsely roughened and more noticeable near the vesicles. Conidia are globose and conspicuously echinulated. Note that each metula covers three-quarter of circumference (25)

Discussion:

We report a case of PA co-existing with rifampicin-susceptible PTB in a 13-year-old female who initially presented in a health-care facility with low grade fever and productive cough, confirmed to be PTB by positive GeneXpert assay on sputum sample. She had first line anti-tuberculous drugs for six months without improvement in the presenting symptoms. A repeat sputum examination by GeneXpert was positive for rifampicin susceptible *Mycobacterium tuberculosis* that necessitated commencement of another course of first line anti-tuberculous drugs. Patient was referred to our facility after two months into the second cycle of anti-tuberculosis drugs when there was worsening clinical condition due to the primary chest infection which was complicated by right sided pneumothorax. We made a diagnosis of co-infection of PTB and PA 20 days into admission in our facility, late enough for the patient's clinical condition to have become worse with marked respiratory distress and haemoptysis. For the patient, presence of pulmonary cavitatory TB lesion with persistence of symptoms of chronic lung infection for many weeks and positive sputum culture for *Aspergillus*, coupled with absence of pronounced angio-invasive symptoms (i. e haemoptysis lasing for few hours) suggests a possibility of CPA (14).

Co-existing PTB and pulmonary fungal infections is common yet usually not looked for in patients (5). The burden of co-infection is substantial particularly in low-resourced countries. According to a systematic review and meta-analysis of studies in Asia, prevalence of co-infections varied between 12.3-68.8% (combined prevalence of 17.4%) with *Aspergillus* being among the major fungal isolates and *Candida* the most predominant (15). A separate report from Africa showed that pooled prevalence of *Aspergillus* co-infection among patients with PTB was 17.7% with individual country rates varying from 5.2% in Kenya, to 24% in Egypt and 25.4% in Cameroun. According to the report, co-infection occurs at all ages but particularly higher above 40 years, and cases in those above age 50 are largely due to recurrent TB, immunosuppression and prolong anti-tuberculosis therapy in a background of active PTB (16). A more recent multicentre study gave an overall prevalence of aspergillosis to be 8.7% among TB cases in Nigeria. It further revealed that PA is a neglected disease found among both HIV positive and negative patients, and among TB smear negative individuals (17). Neglect of PA among TB patients is also underscored in a French study in which 35.7% (50/140) of cases of TB had concomitant positive culture for *Aspergillus* among which only 6% received treatment for PA (18).

In sub-Saharan Africa, the burden of TB is enormous, however, clinical and radiological criteria for diagnosis of other chronic chest infections such as chronic PA are non-discriminatory and often identical with those of PTB. Diagnosis of PA is mostly challenging and often made late especially in settings of low index of suspicion like in this case precluding timely laboratory testing and appropriate antifungal treatment. This problem is made worse by decoy from clinical symptoms of the co-infecting chronic lung infection like

TB. Defining cases of PA therefore requires specific testing for *Aspergillus* (19). However, it is often impossible to identify pulmonary fungal infection objectively without a good clinical awareness and laboratory diagnostic capacity which are not optimal in our environment (9). Recent revolution in non-culture diagnostic techniques across the world is yet to be popular in African countries and there is near total reliance on the less efficient, laborious and time-consuming microscopy and culture methods with unfavourable consequences on patient outcomes (9).

Serologic testing for PA is simple but are still largely unavailable in our hospitals, and this is responsible for under-diagnosis of chronic PA both at initial presentation and during treatment and follow-up for TB (6,15). Serum galactomannan assay has been widely used for rapid detection of *Aspergillus* in body fluid, especially in bronchioalveolar lavage to diagnose CPA with high sensitivity and specificity (20). In addition, specific radiologic imaging including CT scan and magnetic resonance imaging (MRI) is essential for precise diagnosis and determining the spectrum of PA, however these are not accessible to patients in the low- and middle-income countries (21). Furthermore, shortage of experts in mycology is also a major impediment to prompt review and diagnosis of cases (10). All these setbacks make Africa not to have in place a robust network for providing the required platform for increasing consciousness on mycological infections (9).

PA is an under-rated and often neglected disease of global health impact (2), especially in more chronic forms, it is associated with slow but progressive lung destruction leading to increased morbidity and mortality (22). Apart from failure to make definitive diagnosis early enough to really be useful in patients management, promptness of determination of extent of tissue invasion by *Aspergillus* is another major problem in patients. Performing invasive procedures to obtain biopsy specimens for microbiological and histological examination is a difficult decision in majority of patients who have often been overwhelmed by the co-existing diseases and whose clinical conditions are too critical to cope with such procedures. For this patient, diagnostic biopsy was requested but her clinical condition was not good enough for the procedure to be carried out. Lateness of diagnosis of PA has been reportedly linked to unprecedented mortalities; even upon treatment, short-term mortality of chronic PA could be as high as 33% (6). It is worthy of note that lateness in the definitive diagnosis and treatment of the pulmonary fungal infection in the index case gave room for major complications such as pneumothorax with severe respiratory distress, lung destruction with cavitations and haemoptysis which were responsible for the poor treatment outcome. A review of some recent publications highlighted pulmonary factors such as shortness of breath, cavitary lung disease, and pleural involvement as predictors of mortality from chronic PA (2). In another systematic review of 50 studies involving 1941 patients, an overall PA case fatality rate of 58% was reported a proportion of which were due to pulmonary complications from PTB co-infection (23).

In this case, persistence of clinical features attributable to PTB coupled with a repeat GeneXpert positivity gave an impression of persistent infection that further obscured the possibility of pulmonary fungal infection in the patient. Although patients with persistent infection have repeat sputum nucleic acid amplification testing coming out positive, it is well known that the sputum test can remain positive even after successful treatment of PTB especially when there are lung cavities, and this is attributable to presence of residual mycobacterium nucleic acid as well as non-viable bacilli in the lower respiratory tract (24).

Conclusion:

PA is common among patients with TB but it is often missed due to poor clinical awareness and suboptimal laboratory capacity for diagnosing mycologic infections. The consequences of these deficiencies are much more pronounced in our environment which is already faced with problems of access to highly sensitive and specific radiologic imaging. There is therefore a crucial need for improved human and infrastructural capacities for diagnosing PA in our environment to enhance timely interventions and good outcomes in patients.

Authors' contributions:

ATA, TOO, SSE, AOI, EOI, AAA, ACA and AOA conceptualised and conducted the microbiological analyses; ATA, TOO, SEE, AOI, EOI, AAA, OAO, ACA, POO and AOA managed the patient; ATA, TOO and AOA prepared initial draft; ATA, TOO and AOA revised the manuscript and prepare the final draft.

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Case Report **Open Access**

'Metastatic seed' of cholera in Edo State, Nigeria: a case report

*[1,3]Adewuyi, G. M., [1,3]Samuel, O. S., [1]Unuane, A. E., [2]Iraoyah, K. O., [1]Onuha, G. O., [1]Otumu, O. T., and [1]Ogbue, J. I.

Departments of [1]Medical Microbiology/Parasitology and [2]Internal Medicine, Irrua Specialist Teaching Hospital, Irrua, Edo State, Nigeria
[3]Department of Medical Microbiology and Parasitology, Ambrose Alli University, Ekpoma, Edo State, Nigeria
*Correspondence to: gbolawuyi@yahoo.com; ORCiD: 0000-0002-8976-5565

Abstract:

There were reported cholera epidemics in some States in Nigeria. Cholera is an acute diarrhea disease with marked epidemic propensity, caused by colonization of the small intestine by *Vibrio cholerae* serogroup 01 or 0139. Cholera, like other infectious diseases epidemics, has propensity for sending metastatic seed to any susceptible remote community. If the metastatic seed can be promptly diagnosed and managed appropriately, the spread and development of new epicenter can be aborted. This report is a case of metastatic cholera who presented in a tertiary hospital in Edo State, Nigeria. The case was promptly detected and effectively managed using good surveillance system, inter-departmental collaboration, swift responses, good laboratory practices, patient isolation and infection prevention and control measures, coupled with appropriate fluid and antimicrobial treatments. This prevented cholera epidemic in the hospital and Edo State in general.

Keywords: Cholera; epidemics; surveillance; control

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«Graine métastatique» de choléra dans l'État d'Edo, au Nigeria: à propos d'un cas

*[1,3]Adewuyi, G. M., [1,3]Samuel, O. S., [1]Unuane, A. E., [2]Iraoyah, K. O., [1]Onuha, G. O., [1]Otumu, O. T., et [1]Ogbue, J. I.

Départements de [1]Microbiologie Médicale/Parasitologie et [2]Médecine Interne, Hôpital Universitaire Spécialisé d'Irrua, Irrua, État d'Edo, Nigéria
[3]Département de Microbiologie Médicale et de Parasitologie, Université Ambrose Alli, Ekpoma, État d'Edo, Nigéria
*Correspondance à: gbolawuyi@yahoo.com; ORCiD: 0000-0002-8976-5565

Résumé:

Des épidémies de choléra ont été signalées dans certains États du Nigéria. Le choléra est une maladie diarrhéique aiguë à propension épidémique marquée, causée par la colonisation de l'intestin grêle par Vibrio cholerae sérogroupe 01 ou 0139. Le choléra, comme d'autres épidémies de maladies infectieuses, a tendance à envoyer des semences métastatiques à toute communauté éloignée sensible. Si la semence métastatique peut être rapidement diagnostiquée et gérée de manière appropriée, la propagation et le développement d'un nouvel épicentre peuvent être interrompus. Ce rapport est un cas de choléra métastatique qui s'est présenté dans un hôpital tertiaire de l'État d'Edo, au Nigeria. Le cas a été rapidement détecté et géré efficacement grâce à un bon système de surveillance, une collaboration interdépartementale, des réponses rapides, de bonnes pratiques de laboratoire, l'isolement du patient et des mesures de prévention et de contrôle des infections, associées à des traitements liquidiens et antimicrobiens appropriés. Cela a empêché une épidémie de choléra à l'hôpital et dans l'État d'Edo en général.

Mots clés: Choléra; épidémies; surveillance; contrôler

Introduction:

There were reported cholera epidemics in some States in Nigeria (1). The worst affected states are Bauchi, Bayelsa, Gombe, Kaduna, Kano, Plateau and Zamfara (1). In 2021, as at June 21, 10,833 suspected cases of cholera have been reported with 112 cases confirmed, and 289 deaths (1). The Nigeria Center for Disease Control (NCDC) urges members of the public to be aware of the risk of the disease and adhere to precautionary measures to ensure safety (1).

Cholera is an acute diarrhoea disease with marked epidemic propensity, caused by colonization of the small intestine with *Vibrio cholerae* serogroup 01 or 0139 (2). The disease is due to the enterotoxin produced by the pathogen which causes mild to fulminant diarrhoea (2) and leads in some cases to rapid dehydration, acidosis, circulatory collapse and death within hours (3). Cholera is associated with poor sanitation, and direct contact with contaminated food or surface water for drinking, bathing, cooking, and irrigation, as major risk factors (3). Infection occurs through the ingestion of water contaminated by faeces and vomitus or food contaminated by faeces, dirty water, soiled hands, or rarely by flies (3). Raw or undercooked food from polluted areas can be responsible for outbreaks. Some outbreaks are explosive, caused by contamination of a common source, while in some outbreaks, the modes of transmission may reflect person-to-person spread (4,5).

Cholera, like other infectious disease epidemics, has propensity for sending metastatic seed to any susceptible remote community, especially in this era of rapid human movements across geographical locations (5). This results in propagated source pattern of epidemics spread (4,5). If the metastatic seed can be promptly identified and managed appropriately, the spread and development of new epicenter can be aborted (4). We present here the case of a cholera patient admitted with pathognomonic features of the disease and was promptly diagnosed, treated and thus breaking the chain of possible propagated source cholera in Edo State of Nigeria.

Case report:

A 30-year-old Hausa male apprentice to a truck driver enroute from Kaduna to Benin-city presented to the accident and emergency (A&E) unit of the Irrua Specialist Teaching Hospital, Irrua, Edo State, Nigeria on June 2, 2021 with history of multiple episodes of watery, non-mucoid, non-bloody stooling of a few hours' duration, several bouts of non-bilious, non-projectile vomiting, body weakness and inability to move the left leg. He had no history of fever, but there was abdominal pain and reduced urine output. The onset of the illness was rapid, shortly after eating 'okpa' (a traditional Nigerian delicacy) (6) on transit. He was severely dehydrated with altered sensorium at presentation (Glasgow coma score was 10). Pulse was fast and thready (pulse rate of 118 beats per minute), blood pressure was not recordable at presentation. Patient was not anaemic, and was anicteric and acyanosed. The stool was rice water in appearance.

Patient was admitted with the working diagnosis of acute gastroenteritis complicated with dyselectrolytaemia. The medical microbiology team was promptly informed to investigate the case. Stool specimen of the patient was collected into a bottle containing alkaline peptone water and immediately transported to the microbiology laboratory of the hospital for analysis. Specimens for full blood count (FBC), electrolytes, urea and creatinine, HIV screening and blood culture were also collected. Intensive intravenous rehydration using Ringer's lactate alternated with normal saline was immediately instituted.

In the laboratory, wet film microscopy revealed densely populated and highly motile bacteria while Gram stain smear showed Gram-negative curved bacilli (Fig 1). The preliminary test result was communicated to the managing team in the ward. The stool specimen was inoculated on thiosulphate citrate bile salt sucrose (TCBS) agar medium and plate incubated aerobically for 18 hours, which grew bright-yellow pure discrete colonies (Fig 2). The isolate was biochemically identified as oxidase and catalase positive, producing yellow butt and yellow slant on triple sugar iron (TSI) agar. Furthermore, a rapid immunochromatographic cassette (Standard Diagnostics, Inc, Republic of Korea) test to differentiate between *V. cholera*e 01 and 0139 confirmed the aetiologic agent to be *V. cholerae* 01 serotype (Fig 3).

The patient was isolated and other infection prevention and control measures such as hand hygiene, use of personal protective equipment, waste and linen management were enforced. Doxycyline therapy was commenced and rehydration was continued. Stooling and vomiting stopped after about 36 hours of hospitalization. Patient was discharged on day 4 of admission, after proper health education on the need for good food, water, personal and environmental hygiene.

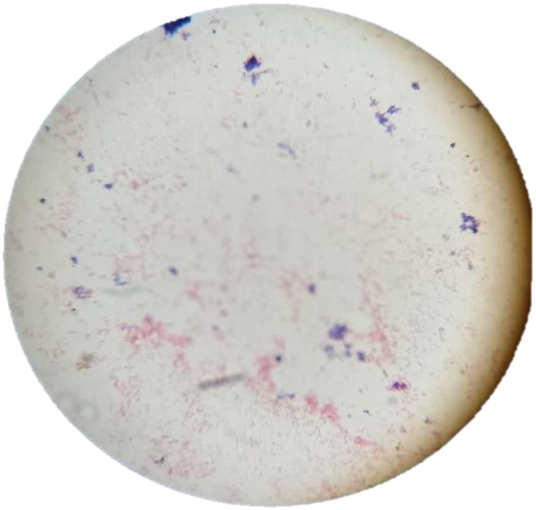

Fig 1: Gram stain slide of *Vibrio cholerae* shows Gram-negative curved rods (x1000 magnification)

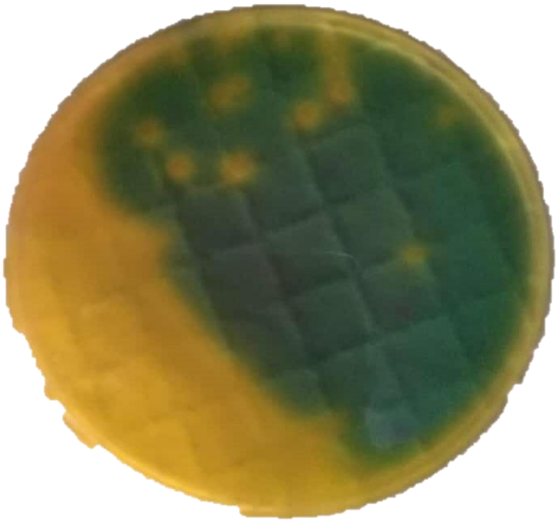

Fig 2: Golden yellow colonies of *Vibrio cholerae* on thiosulphate citrate bile salt agar plate

Fig 3: Confirmation of *Vibrio cholerae* serotype 01 on immunochromatographic cassette

Discussion:

An infectious disease epidemic, just like malignant tumor of the body, has capacity to send metastatic seed to another site away from the epicenter to start a new propagated outbreak (7,8). This was the experience in this case, a traveler from Kaduna (cholera epicenter at the time) came to Edo State, to serve as a seed to start up cholera epidemic in the State. If not for prompt detection and effective case management, Edo State would have joined the league of cholera outbreak States for the period. In keeping cognizance of this, emergency preparedness team, especially the surveillance team, must watch out for any possible seed from a neighboring or distant epicenter whenever there is an outbreak.

Cholera control aiming at reduced morbidity and mortality from outbreaks required a multifaceted approach (3). This includes surveillance, adequate good water supply, sanitation and hygiene, social mobilization, treatment and oral vaccines (3). Effective implementation of this World Health Organization recommended approach was seen in the sporadic case above. The first medical contact to the patient had high index of suspicion for cholera in the patient based on his good working knowledge of case definition for cholera, the travel history from cholera infected State and the clinical presentation of the case, vis-a-vis several bouts of 'rice-water' stooling and vomiting with severe dehydration. High index of suspicion coupled with working knowledge of case definition are pivotal to effective disease surveillance (9). In view of this, as part of emergency preparedness, healthcare workers should be periodically updated on the epidemiology of diseases under surveillance, including the case definitions and clinical features of the disease.

A good surveillance system aiming at epidemic and case detection with confirmation is dependent on availability of competent and reliable laboratory where aetiologic cause of the disease can be ascertained (10). The laboratory team at the centre of this report is efficient as evident in the promptness and accuracy of the diagnosis. Good laboratory system as the hub of infectious disease surveillance is

exemplified in the creation of Pulsenet International, a network of national and regional laboratory networks dedicated to tracking foodborne infections world-wide. Each laboratory utilizes standardized genotyping methods, sharing information in real-time. The resulting surveillance provides early warning of food and waterborne disease outbreaks, emerging pathogens, and acts of bioterrorism.

Furthermore, good intersectoral and interdepartmental collaborations are expedient in epidemic preparedness and containment. A disjunction between the clinicians and the laboratory can leads to missed cases and escalation of epidemic. The clinicians and the laboratory synergistically contained the intending cholera epidemic in the Edo State of Nigeria by prompt consult to the medical microbiology team and immediate response by the latter in collecting stool specimen and analyzing it in good time. The specimen culture isolate was confirmed to be *V. cholerae* serotype 01, and the laboratory communicated their findings to the clinician without delay. These measures strengthened the zeal of the teams to aggressively combat the case and observe all necessary standard precautions. Quality assurance in laboratory practice is not only on generating reliable results, but include timely and appropriate communication of the result to the clinician for proper case management. Hence, it is not adequate for the laboratorians to generate good results, but the results must be communicated rapidly to the field officers/clinicians for evidence-based definitive management of the affected people.

In addition, with good IPC practices including patient isolation, prompt notification, effective treatment, excellent use of personal protective equipment, hand hygiene, good waste management and environmental sanitation, the cholera case was cured without transmission to other patients, healthcare workers or the community. It is worthy of note that, outbreak containment is impossible without implementation of IPC measures (11). According to WHO, IPC is a practical evidence-based approach, preventing patients and healthcare workers from being harmed by avoidable infections. Defective IPC causes harm and can kill (12). Effective IPC requires constant action at all levels of the heath system, including policymakers, facility managers, health workers and those who access health services. IPC affects all aspects of healthcare, including hand hygiene, injection safety, antimicrobial resistance and how hospitals operate during and outside emergencies (12). In rural setting of low- and middle-income countries, such as Irrua, support for IPC is very important because secondary infections may negatively affect healthcare delivery and medical hygiene standards (12).

Effective management of cholera includes intravenous rehydration in severe cases, where oral rehydration will be inadequate and slow (3). Antimicrobial treatment based on microbial sensitive profile is also very important. This patient was adequately rehydrated and was treated with doxycycline, the drug of choice for cholera. Doxycycline therapy in cholera shortens duration of diarrhea, reduce the volume of fluid needed for rehydration and more importantly can stop excretion of *V. cholerae* in stool within 48 hours (3,5). No wonder, the patient was fit for discharge within a very short period having been well managed.

In conclusion, good emergency preparedness comprising disease surveillance system, competent manpower (health workers), good laboratory services, case management, and IPC are undisputedly the best measures for halting infectious diseases outbreak. All sectors saddle with containment of disease outbreaks must work concertedly to make it work.

Contributions of authors:

AGM coordinated the resident doctors' laboratory testing of the patients' samples, co-managed the patient with the internal physician, was involved in specimen analysis, and initiated and wrote the case report. SOS was involved in specimen analysis, provided the immunochromatographic test kit for typing the isolate and reviewed and made corrections in the draft case report. UAE collected and analyzed the patient's specimens, participated in patients' clinical management, and contributed to writing the report. IKO was the consultant involved in patient management, invited the medical microbiology team and contributed to report writing. OGO, OTO and OJI participated in the laboratory analysis of the patient's specimen, were involved clinical management of the patient and ensured compliance with infection prevention and control measures.

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doi: 10.4314/ajtcam.v4i2.31207

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

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