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Perspective **Open Access**

Need for standardization and compliance to treatment protocols for COVID-19 within the African Region of the World Health Organization

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Abstract:

COVID-19 pandemic changed the face of global health and brought about new issues in global health security and economy. The World Health Organization published guidelines for clinical management of COVID-19 four months after declaration of COVID-19 as a pandemic. Scholarly reviews and studies from member states within WHO AFRO reveals significant deviation from the WHO published protocols on COVID-19. Assessment of national treatment protocols of 30 of 47 WHO AFRO member states showed widespread inappropriate use of antimicrobial agents for patients, which may worsen the global and concerning threat of antimicrobial resistance. There is need for adopting interventions that optimize antimicrobial use in the context of pre- and post-pandemic preparedness to ensure long-term effectiveness and sustainability for antimicrobials. Treatment guidelines are to be adopted or adapted depending on best clinical evidence available. Non-compliance with guidelines might lead to mismanagement of infectious diseases with attendant negative consequences including antimicrobial resistance and misdirection of critical resources and supplies amongst others.

Keywords: COVID-19, treatment guidelines, azithromycin, hydroxychloroquine, chloroquine, antimicrobial resistance

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Nécessité de normalisation et de respect des protocoles de traitement du COVID-19 dans la région africaine de l'Organisation mondiale de la santé

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Résumé:

La pandémie de COVID-19 a changé le visage de la santé mondiale et a soulevé de nouveaux problèmes en matière de sécurité sanitaire et d'économie mondiale. L'Organisation mondiale de la santé a publié des lignes directrices pour la gestion clinique du COVID-19 quatre mois après la déclaration du COVID-19 comme pandémie. Les revues scientifiques et les études des États membres de l'OMS AFRO révèlent un écart significatif par rapport aux protocoles publiés par l'OMS sur le COVID-19. L'évaluation des protocoles nationaux de traitement de 30 des 47 États membres de l'OMS AFRO a révélé une utilisation inappropriée et généralisée d'agents antimicrobiens chez les patients, ce qui pourrait aggraver la menace mondiale et préoccupante de résistance aux antimicrobiens. Il est nécessaire d'adopter des interventions qui optimisent l'utilisation des antimicrobiens dans le contexte de la préparation pré et post-pandémique afin de garantir l'efficacité et la durabilité à long terme des antimicrobiens. Les directives thérapeutiques doivent être adoptées ou adaptées en fonction des meilleures preuves cliniques disponibles. Le non-respect des directives pourrait conduire à une mauvaise gestion des maladies infectieuses avec des conséquences négatives qui en découlent, notamment la résistance aux antimicrobiens et une mauvaise orientation des ressources et fournitures essentielles, entre autres.

Mots-clés: COVID-19, lignes directrices thérapeutiques, azithromycine, hydroxychloroquine, chloroquine, résistance aux antimicrobiens

Introduction:

A cluster of cases of pneumonia of undetermined aetiology that was later identified as coronavirus disease 2019 (COVID-19), was reported in China on December 31, 2019 (1). As COVID-19 spreads through the global community, it became a pandemic with increasing morbidity and mortality across different regions and countries (2). The COVID-19 pandemic brought up challenges to critical supplies, emergency medication and rationalization for treatment options. Within WHO AFRO, Algeria reported the first case of COVID-19 in late February 2020 (3). By February 18, 2022, there were 418,650,474 COVID-19 cases globally with 5,856,224 deaths (4). Africa had reported 11,095,734 confirmed cases and 246,142 deaths (5).

In response to the pandemic, the global community activated a coordinated system of experts and partners to provide guidance and protocols to be updated and adapted during the life span of the pandemic (3). In January 2020, the World Health Organization (WHO) activated its incident management system to respond to the COVID-19 pandemic in a coordinated fashion for the provision of diagnostics, therapeutics and vaccines towards control measures. Three months later, the WHO launched the COVID-19 Solidarity Therapeutics Trial to curtail the pandemic and give direction for therapy (3).

The challenge of COVID-19 treatment within African Region:

In the WHO AFRO region, with scarce resources, many countries recommended or administered unproven treatments to patients with COVID-19. These include previously known anti-infective drugs/supplements ranging from hydroxychloroquine/chloroquine (HCQ/CQ) combinations, ivermectin, azithromycin, vitamin D, vitamin C, amongst others.

WHO guidance on COVID-19:

To provide practical guidance on COVID-19, the WHO published different guidelines, including that on clinical management by the 6th month of the pandemic (6). The guideline categorizes COVID-19 patients for the purpose of clinical management into four; mild, moderate, severe and critical (6). The guideline was translated into treatment protocols for COVID-19 in some countries of the WHO AFRO region.

Symptomatic therapy was advocated for mild to moderate cases (40% of global cases of COVID-19) by administration of analgesics, antipyretics and other supportive measures without the need for antibiotics except if there is clinical evidence of bacterial infection (6). However, for severe cases (15% of global cases), oxygen therapy, antimicrobial therapy for any possible pathogens, systemic corticosteroids and cautious intravenous fluid were advocated where the indication has been well established at the clinician's judgement (6). Critical cases (5% of global cases) require intensive care support with provision of mechanical ventilation in intensive care units, use of corticosteroids, crystalloids, vasopressors in addition to other measures outlined for severe cases (6). At the time of publication of the WHO guideline on clinical management of COVID-19, several treatment modalities were undergoing clinical trials for the pharmacologic management of COVID-19 without any shown to be safe and effective then.

Practice across WHO AFRO member states:

Six months into WHO guideline for clinical management of COVID-19, an assessment of quality of care was made within WHO AFRO, by conducting comparison of country treatment protocol to the WHO recommendations in 30 of the 47 member states of the region (7). More than half of the 30 countries were recommended HCQ/CQ combinations for major categories of COVID-19 cases (mild, moderate and severe), and a third recommended same drug combination for critical cases (7). Conversely, the protocols from the countries showed convalescent plasma was least recommended for critical and severe cases of COVID-19 (7). One-sixth of the countries recommended the use of lopinavir/ritonavir to replace HCQ/CQ combinations in case of allergy and a quarter of the countries recommended remdesivir for severe and critical diseases. Corticosteroid use was only for severe and critical cases in about a half of the countries while the use of interferon alfa-2 beta was only for mild and moderate cases in less than one-tenth of the countries (7).

The review of the protocols further showed that against the recommendations of WHO guideline, 53% and 80% of the protocols recommended antibiotics for mild and moderate COVID-19 respectively (7). This has grave consequences for the epidemic of AMR, as overuse and misuse of antimicrobials constitute the largest drivers of AMR. Overuse and misuse of antimicrobials increases selection pressure for AMR genetic determinants in microorganisms with consequent widespread difficult-to-treat or completely untreatable AMR infections.

Findings of the assessment raises the question of the quality of care that the COVID-19 patients received during a pandemic in view of the best clinical evidence available. It highlights major deviation(s) of the treatment protocol for COVID-19 across countries in the WHO AFRO region and shows increased drive

for AMR due to widespread use antimicrobials.

The threat of AMR posed by misuse and abuse of antimicrobials was amplified by the unwarranted use of antibiotics during the COVID-19 pandemic (8-12). Although, a known fact that prior to COVID-19 pandemic, AMR was a global health priority, the impact and effect of the changing dynamics of AMR in the context of the pandemic is still evolving and poses an existential threat if not handled properly (13). Further implication to this finding is that the prevalence of AMR may increase across member states within the WHO AFRO region which can then have a spillover effect globally as a consequence to the overuse and misuse of antimicrobials in the treatment of COVID-19 pandemic (13).

The consequences of the COVID-19 pandemic and its direct impact on the AMR threat in the WHO African region maybe far reaching. However, improved access to timely and quality diagnostics, widespread use of non-pharmacologic preventive measures and equitable access to vaccines may mitigate the initial impact seen on the over reliance and inappropriate use of antimicrobials (13,14). Integrated antimicrobial stewardship (AMS) that incorporates not only optimizing antimicrobial use but other health system strengthening components including interventions that address the balance between excess and access to antimicrobial medicines, infection prevention and control (IPC) and water, sanitation and hygiene (WASH), strategic and targeted awareness and education needs to be promoted and implemented across all the member states within the WHO-AFRO region (15,16).

Conclusions/Recommendations:

For COVID-19 pandemic and in anticipation of future infectious diseases outbreak a timely release of standardized protocol by the WHO is highly recommended. Treatment protocols including standard treatment guidelines, National Essential Medicine Lists (NEML) incorporating WHO AWaRe categorization guiding optimization of antibiotic use should be treated as living documents and amended based on the available best clinical evidence in order to provide best quality of care. This is essential to enable access within the context of Sustainable Development Goals (SDGs) and Universal Health Coverage (UHC) and also to maintain the critical balance necessary between access and inappropriate use of antimicrobial medicines.

As the global community moves forward in its COVID-19 response, we must not lose focus on antimicrobial overuse and misuse which drive up resistance and its deleterious consequence to overall health and the economies of member states. It is essential to have in place a systems-based approach that ensures an interdisciplinary crosstalk that will not only address AMR but strengthens/build a resilient health system to tackle future pandemics. While most member states in the AFRO region have developed AMR National Action Plans (NAPs) and are currently implementing same, it is critical to break the 'silos' in line with the 'One-Health' approach. This means effectively linking and mainstreaming at the regional and national levels, supply chain, vaccines, and regulatory strategies. In light of this, we propose WHO-AFRO support member states providing the needed technical support, tools, platforms and convening power for building the necessary advocacy capacity to ensure the breaking of 'silos' and the linking of such critical strategies to boost the overall health security of member states.

Contributions of authors:

WLF was involved in study conceptualization, methodology, original draft preparation, review and editing of the manuscript; AOA was involved in methodology, original draft preparation, review and editing of the manuscript; IM, JBN and YAA were involved in original draft preparation review and editing of the manuscript. All authors read and approved the published version of the manuscript.

Conflict of interest:

Authors declare no conflict of interest.

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Mini-Review **Open Access**

Prematurity as a secondary immunodeficiency disorder with increased risk of infections: A mini-review

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Abstract:

Prematurity significantly increases neonatal mortality in sub-Saharan Africa. Underdeveloped immune systems and prolonged hospitalization elevate the risk of secondary immunodeficiency leading to heightened vulnerability to healthcare-associated infections, including neonatal sepsis from various sources like intrauterine, intrapartum, and postnatal agents. This review explores the impact of prematurity on infection susceptibility and the role of immature immunity. A literature search using PubMed and Google Scholar identified relevant articles published between January 1980 and December 2022, focusing on terms such as "preterm," "prematurity," "neonatal sepsis," and "secondary immunodeficiency." Despite neonatal susceptibility to sepsis, accurate incidence estimates are lacking in many countries, and preterm infants face higher morbidity and mortality risks compared to full-term babies. Early-onset infections usually manifest within the first 72 hours post-delivery, while late-onset neonatal sepsis occurs after this period. Immaturity affects various immune system components, with gestational age influencing functionality. The compromised innate immune response in preterm infants involves factors such as fragile skin, reduced tear/mucus production, and low antimicrobial peptide levels. Complement deficiencies and impaired neutrophil function increase susceptibility to infections. Macrophages, dendritic cells, and natural killer cells exhibit reduced activity, impacting viral clearance. Preterm infants also have lower immunoglobulin (Ig) G levels, contributing to a weakened adaptive immune response. Hypogammaglobulinaemia heightens susceptibility to infections relying on antibody-mediated protection, while low secretory IgA production and delayed antibody response predispose to gastrointestinal and respiratory infections. The combined effect of immature immunity and medical interventions heightens preterm infants' susceptibility to pathogens. Recommendations for mitigating infection risks include antimicrobial stewardship, prompt initiation of exclusive breastfeeding, and timely administration of routine vaccinations.

Keywords: Secondary immunodeficiency; Preterms; Innate; Adaptive; Neonatal sepsis

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La prématurité en tant que trouble d'immunodéficience secondaire avec risque accru d'infections: une mini-revue

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Résumé:

La prématurité augmente considérablement la mortalité néonatale en Afrique subsaharienne. Un système

immunitaire sous-développé et une hospitalisation prolongée augmentent le risque d'immunodéficience secondaire conduisant à une vulnérabilité accrue aux infections nosocomiales, y compris la septicémie néonatale provenant de diverses sources telles que les agents intra-utérins, intrapartum et postnatals. Cette revue explore l'impact de la prématurité sur la susceptibilité aux infections et le rôle d'une immunité immature. Une recherche documentaire utilisant PubMed et Google Scholar a identifié des articles pertinents publiés entre janvier 1980 et décembre 2022, se concentrant sur des termes tels que "prématuré", "prématurité ", "septicémie néonatale" et "immunodéficience secondaire". Malgré la susceptibilité néonatale au sepsis, des estimations précises de l'incidence font défaut dans de nombreux pays, et les nourrissons prématurés sont confrontés à des risques de morbidité et de mortalité plus élevés que les bébés nés à terme. Les infections précoces se manifestent généralement dans les 72 heures suivant l'accouchement, tandis que les infections néonatales tardives surviennent après cette période. L'immaturité affecte divers composants du système immunitaire, l'âge gestationnel influençant la fonctionnalité. La réponse immunitaire innée compromise chez les nourrissons prématurés implique des facteurs tels qu'une peau fragile, une production réduite de larmes/mucus et de faibles niveaux de peptides antimicrobiens. Les carences en complément et la fonction altérée des neutrophiles augmentent la susceptibilité aux infections. Les macrophages, les cellules dendritiques et les cellules tueuses naturelles présentent une activité réduite, ce qui a un impact sur la clairance virale. Les nourrissons prématurés ont également des taux d'immunoglobuline G plus faibles, ce qui contribue à affaiblir la réponse immunitaire adaptative. L'hypogammaglobulinémie augmente la susceptibilité aux infections reposant sur une protection médiée par les anticorps, tandis qu'une faible production d'IgA sécrétoires et une réponse retardée des anticorps prédisposent aux infections gastro-intestinales et respiratoires. L'effet combiné d'une immunité immature et d'interventions médicales augmente la sensibilité des nourrissons prématurés aux agents pathogènes. Les recommandations pour atténuer les risques d'infection comprennent la gestion des antimicrobiens, le début rapide de l'allaitement maternel exclusif et l'administration en temps opportun des vaccinations de routine.

Mots-clés: Déficit immunitaire secondaire; Prématurés; Innée; Adaptative; Sepsie néonatale

Introduction:

Prematurity is a complex issue with wide-ranging socio-economic implications for individuals, families, and societies. Immaturity of the immune system and other challenges in the management of these babies affect the immediate health of premature infants but also have long-term consequences that can impact their quality of life and place a strain on the healthcare and social systems. Premature/preterm babies refer to babies born before the completion of 37 weeks of gestation (1). There are three groups based on gestational age; extremely preterm (< 28 weeks), very preterm (28-32 weeks), and moderate-to-late preterm (32-36 weeks) (2). The preterm delivery occurs due to maternal- or baby-related factors and may occur spontaneously or by caesarian section (1).

Approximately 15 million infants are born prematurely annually, constituting roughly 11% of all births globally (2). In sub-Saharan Africa, the prevalence of prematurity reportedly ranged from 3.4% to 49.4% (3). In 2019, there was a significant reduction in global newborn deaths, dropping from 5 million in 1990 to 2.4 million (4). Nearly half (47%) of all deaths among under-five children occurred during the neonatal period, with approximately one-third of these deaths occurring on the day of birth and nearly three-quarters occurring within the first week of life, with prematurity as one of the leading causes (2,4).

In 2019, sub-Saharan Africa had the highest neonatal mortality rate, with 27 deaths per 1,000 live births, closely followed by Central and Southern Asia, where the rate was 24 deaths per 1,000 live births. Nigeria ranked as the second country with the highest number of neonatal deaths after India, with 270,000 deaths in 2019 (4).

Despite neonates being particularly susceptible to sepsis, many countries lack accurate incidence estimates for this age group (5). A systematic review, involving 26 studies, identified a pooled neonatal sepsis incidence of 2,824 sepsis cases per 100,000 live births, and a mortality of 17.6% (5). These figures highlight the considerable contribution of neonatal sepsis to morbidity and mortality. Moreover, there were considerable regional differences in incidence, with the low-middle-income-countries (LMICs) having the highest burden, and preterm neonates are particularly affected (5). Indeed, sepsis in preterm infants is reported to be 1000-fold more common than in term infants, and associated with higher mortality and life-long neurodevelopmental handicaps (6,7).

Secondary immunodeficiencies occur due to an external factor affecting the host's immune response, which may be a transient or persistent impairment of the function of cells or tissues of the immune system (8). The secondary immunodeficiency disorder of prematurity is associated with the immaturity of the immune system (8,9). Virtually all immune system compartments are affected, and there is a correlation between the GA and the function of these compartments (10). The younger the GA, the higher the infection susceptibility (9). Furthermore, preterm bab-

ies need intensive care with or without ventilator support, intravenous access, parenteral feeding with attendant prolonged hospital stay, and antibiotic use predispose them to healthcare-associated infections (HCAI). Hence, this study reviewed the burden of prematurity and neonatal sepsis and highlighted the different pathogens responsible for neonatal sepsis and their modes of transmission. Also, the study delves into various aspects of immunity (innate and adaptive), including the anti-infective components of breastmilk, and how each aspect is compromised in preterm babies, thus predisposing them to infections.

Methodology:

A comprehensive literature search was conducted on PubMed and Google Scholar to identify relevant articles on prematurity and infections. The search terms were "preterm", "prematurity", "neonatal sepsis", "infections", and "secondary immunodeficiency". We confined our search to articles published in English language between January 1980 and December 2022 and identified relevant articles through a systematic process that included screening of article titles, abstracts, and full texts. Manual searches of reference lists of relevant articles provided additional articles.

Inclusion criteria for the review were articles that discussed prematurity and infections, neonatal sepsis and immunity. Two reviewers independently performed the data extraction and synthesis, with the resolution of any discrepancies through discussion.

Results and Discussion:

Neonatal sepsis

Neonatal sepsis (NS) refers to a systemic infection affecting a neonate, of bacterial, viral, or fungal (yeast) origin, that is associated with haemodynamic changes and other clinical manifestations, and results in substantial morbidity and mortality (11). Neonatal sepsis has been classified as either early-onset or late-onset depending on the age of onset and timing of the sepsis episode (11,12). Clinical manifestations of early-onset infections usually appear within the first 72 hours of life while those occurring after 72 hours are regarded as late-onset (11). Bacteria are the most common pathogen of NS. Acquisition of these organisms may occur in utero, during delivery or postnatally (11,12). The infection may be systemic or localised. The implicated organism may be a commensal or opportunistic pathogen. The bacterial agents of early and late-onset NS are shown in Table 1.

Intrauterine infections can be caused by pathogens such as rubella, cytomegalovirus (CMV), parvovirus B19, varicella-zoster virus, *Treponema pallidum* (causes syphilis), and *Toxoplasma gondii*. The most common mode of transmission of hepatitis B and hepatitis C virus, human immunodeficiency virus (HIV), and herpes simplex virus (HSV) is intrapartum, although transplacental transmission also occurs (12). The maternal genitourinary and lower gastrointestinal tract flora can cause intrapartum and postpartum infections. The common bacterial agents are enteric Gram-negative organisms, Group B streptococcus (GBS), *Neisseria gonorrhoeae,* and *Chlamydia* spp while the common viruses are HSV, HIV and CMV (12). The bacterial agents that cause healthcare associated infections (HCAIs) in neonates include coagulase-negative staphylococci (CoNS), Gram-negative bacilli, *Staphylococcus aureus,* and *Candida* (Table 1), while viruses commonly causing neonatal HCAIs are enteroviruses, respiratory syncytial virus (RSV), adenovirus, rhinovirus, influenza and parainfluenza viruses.

Defect in innate immune response in neonates

Physical barrier:

Preterm babies have fragile skin and mucosa, easily breachable with consequent bacterial invasion (12). Organisms such as CONS (skin commensals) can gain entry from minute breaches in the skin (13). The enteric Gram-negatives can translocate across the gastrointestinal epithelium to cause infections (13). In preterm babies, there is decreased tear and mucus production and lack of access to the contents such as lysozyme (14,15). Thus, eye infections with discharge due to *S. aureus, N. gonorrhoeae* and *Chlamydia trachomatis* are common in this age group, having come across these organisms during passage through the birth canal.

Table 1: Bacterial causes of infections in neonates

Bacteria	Early-onset	Late-onset (HCAI)	Late-onset (CAI)
Gram-positive	+++	+	
Group B streptococcus	+++	+	+
Other streptococci	++		+
Enterococcus	+	++	
Streptococcus pneumoniae	+		
Listeria monocytogenes	+		
Viridian streptococci	+	++	
Clostridia	+		+
Staphylococcus aureus	+	++	
Coagulase-negative staphylococci	+	+++	
Gram-negative			
Escherichia coli	+++	+	++
Klebsiella spp		++	
Haemophilus influenzae	+	+	
Neisseria gonorrhoeae	+		
Neisseria meningitides	+	+	
Proteus spp		+	
Salmonella spp			+
Pseudomonas spp		+	
Serratia		+	
Enterobacter		+	
Citrobacter		+	+
Bacteroides	+	+	
Others			
Treponema pallidum	+		

+ connotes the relative frequency of occurrence; HCAI=Healthcare associated infection; CAI=Community associated infection; Source: (ref 12)

Soluble factors and complements:

The levels of some antimicrobial peptides (AMPs) such as bactericidal permeability-increasing protein (BPI), defensins and collectins increase with gestational age (10). These substances produced by epithelial cells and phagocytes have direct bactericidal effects. Defensins and BPI can disrupt bacterial cell membranes. BPI is also fungicidal, thus, its deficiency contributes to *Candida* infection (16).

Mannose-binding lectin (MBL) opsonises bacteria, binds to C1q receptors on the macrophage to initiate phagocytosis and is a component of the MBL pathway of complement activation (17). Also, surfactants are crucial in the host defence against infection. The surfactant-associated proteins (SP), SP-A and SP-D, are collectins that enhance bacterial and viral clearance (18). SP-A and SP-D proteins can also bind to fungi to facilitate agglutination and phagocytosis by host cells (18). The low levels of these soluble factors in neonates increase their susceptibility to infections with poor organism clearance.

Breast milk contains a lot of anti-infective substances (Box 1) effective in combating various pathogens involved in respiratory and gastrointestinal infections (19,20). Preterm infant faces challenges in utilizing these substances because of difficulties in breastfeeding and the underdeveloped suck and swallow reflexes. Hence, the risk of preterm babies for sepsis increases.

The levels and functional capacity of the complement system components are decreased in newborns, especially preterms (17). The complements are involved in opsonisation, chemotaxis, and killing of microbes. These deficiencies increase the infection risk from encapsulated organisms such as *S. pneumoniae*, *N. meningitidis*, and Gram-negative bacteria (15).

Neutrophils:

Preterm babies have decreased neutrophil precursor pool and low intracellular killing by reactive oxygen intermediaries (ROIs) compared with term babies (21). Furthermore, neutrophils cannot cast the neutrophil extracellular traps (NETs), which therefore compromise the preterms' innate defensive ability to bind some Gram-positive and Gram-negative bacteria and fungal hyphae (17,22).

Box 1: Anti-infective contents of breast milk

Cells • Granulocytes • Macrophages • Lymphocytes (B and T cells)	**Carbohydrates** • Oligosaccharides and polysaccharides- Prevent bacteria from binding to a mucosal surface • Fibronectin- increase macrophage antimicrobial activity
Enzymes • Lysozymes- bacteriostatic against *Enterobacteriaceae* • Lactoperoxidase- bactericidal • Lipase- disrupts viral envelope • Amylase- digests polysaccharide	**Cytokines and chemokines** • Interferon-γ- antiviral activity • IL-10- anti-inflammatory • TGF-β (transforming growth factor-β- role in intestinal defence • CXC chemokines- chemotactic activity for intraepithelial lymphocytes, crucial for defence against bacterial and viral infection
Proteins • Lactoferrin- binds iron and prevents bacterial growth, especially of *S. aureus* and *E. coli* • Alpha-1-antitrypsin- anti-inflammatory • α-Lactalbumin- has antimicrobial activity against bacteria and fungi • Complements • Bifidus factor- promotes growth of *Lactobacillus bifidus,* deficiency promotes *E. coli* growth • Vitamin B12 binding protein (haptocorrin)- it binds to Vitamin B12 thereby preventing gut bacteria from utilizing it for their growth. • Osteoprotegerin- essential for regulating Th1/Th2 balance in newborn immune system development	**Growth factors** • Examples epidermal growth factor, bifidus bacteria growth factor • Stimulates the maturation of the epithelium, hence reducing its vulnerability to pathogens • Colony-stimulating factors (CSF) are responsible for regulating the proliferation, differentiation, and survival of milk neutrophils and macrophages • e.g. epidermal growth factor, bifidus bacteria growth factor
Antibodies • IgA prevents bacteria adherence in the mucosa • Others are IgM, IgG, IgE, antiviral antibodies	**Lipids** • Free fatty acids (FFAs) and monoglycerides- have lytic effects on various viruses. The FFAs have an antiprotozoal effect, especially against *Giardia lamblia* • Mucins- prevent attachment of bacteria and viruses to the epithelium by binding to organisms • Ganglioside- protects against endotoxin-induced diarrhoea

Source: (ref 19, 20)

Macrophages, dendritic cells and natural killer (NK) cells:

Monocytes are phagocytic cells that differentiate into macrophages and dendritic cells (DCs) in tissues (10,23). Besides being capable of phagocytosis, monocytes and macrophages have bactericidal mechanisms and are potent antigen-presenting cells (APCs) to the T cells (23). Monocytes of preterm infants have reduced ability for cytokine production and a decreased ability to activate the adaptive immune system. This ability is due to the reduced expression of the major histocompatibility complex (MHC) class II molecules on leukocytes in preterm neonates (10,23).

There is also decrease in the quantity of DCs and their antigen-presenting capabilities (10). This decrease correlates with the gestation age although the levels are still low compared to adults (10). Furthermore, the preterms have decreased natural killer (NK) cell activity, which plays a significant role in viral clearance through expression of interferon-gamma (IF-γ) (24). The reduced NK cell activity increases susceptibility to viral infections such as RSV and adenovirus. Also, the poor activity of the alveolar macrophages predisposes to increased susceptibility to viral infections. There is also reduction in the Toll-like receptor (TLR) signaling of the innate immune system, resulting in antigen recognition impairment and decreased cytokine production (25).

Defect in adaptive immune response in neonates

The maturation of the adaptive immunity occurs mainly after term birth. Hence there are deficiencies in T-cell activation and cytokine production, B-cell activation and immunoglobulin production, and interactions between T- and B-cells in newborns, which is worse in preterm compared to term birth (23).

B-cells:

A direct relationship exists between the amount of immunoglobulin (Ig) G transferred across the placenta to the baby and the getational age (26). Maternal transfer of IgG to the fetus commences in the third trimester (26). Thus, early preterms have low IgG compared to late preterms. These IgG are essential in the defence against extracellular organisms; therefore, hypogammaglobulinaemia increases susceptibility to infections such as tetanus and GBS, in which

protection is antibody-mediated (12). The type-specific IgG levels against GBS are 0.3 µg/ml, 0.5 µg/ml and 1.0µg/ml at 28 weeks, 32 weeks and term respectively (10,12). The low levels contribute to GBS susceptibility.

Preterms are predisposed to early Gram-negative enteric infections as these organisms are affected mainly by specific bactericidal and opsonic antibodies of the IgM and IgA classes (12,15). Meanwhile, IgM and IgA are not transferred across the placenta, although the baby produces them in response to in-utero infections such as toxoplasmosis, rubella, CMV, varicella, and syphilis (12). Local secretory IgA production does not start till the first six to eight weeks of life, which, coupled with the lack of breast milk intake (which is rich in IgA content), predisposes preterm babies to respiratory and gastrointestinal infections (15,27).

Neonates develop their antibodies following stimulation by the various organisms after birth. The B lymphocytes are responsible for antibody production (23). In the preterm, there are decreased marginal zone B cells in the secondary lymphoid organs, reducing the B-specific responses generated (9). The secondary lymphoid organs (spleen, lymph nodes, mucosa-associated lymphoid tissue and gut-associated lymphoid tissue) are also immature (9). Furthermore, preterms have decreased ability to produce antibodies against polysaccharide antigens.

T cells:

The T-cells involved in cell-mediated immunity consist of two main types; cytotoxic T-lymphocytes (CTL, $CD8^+$) and helper T-lymphocytes (Th, $CD4^+$). The T-cells recognize pathogens via presentation of peptide segments of the pathogen on MHC of either macrophages or dendritic cells to their T-cell receptors (23). The $CD8^+$ CTLs are involved in eradicating intracellular pathogens, such as viruses, following antigen presentation through MHC class I expression (23). The effector mediators for intracellular killing are perforins and granzymes (26). The $CD4^+$ T-helper (Th) cells, with Th1, Th2 and Th17 subsets, are activated by antigens presented through MHC class II molecules. The Th1 subset produces the major cytokines; interferon-γ (IFN-γ), interleukin (IL)-2, and tumour necrosis factor -alpha (TNF-α), regarded as inflammatory cytokines (23). The Th2 subset is anti-inflammatory, producing IL-4, IL-5, IL-10 and IL-13 cytokines (23).

The Th1 subset play a crucial role in mediating the killing of intracellular organisms while the Th2 subsets are responsible for coordinating the clearance of extracellular organisms including protozoans and helminths (23,26). The Th17 subset is essential for maintaining mucosal homeostasis and activating neutrophils for the clearance of extracellular pathogens (23,26). Additionally, the regulatory T cell (T-reg) subsets are vital for preserving immune homeostasis, maintaining self-tolerance and controlling inflammation (23,26).

Without maternal infections, the baby is not exposed to antigens in-utero, therefore, it lacks "immunological memory" (27). This lack prohibits their ability to mount an adequate T-cell response soon after exposure to a pathogen (27). Also, preterms have an exaggerated expression of the genes controlling the negative regulation of IFN-γ production, T cell proliferation, and IL-10 secretion (26). Indeed, when there is an imbalance of $CD4^+$ T cells, γδ T-cells, Th17, and T-reg subsets and their associated cytokine production in the gut of preterms, necrotising enterocolitis (NEC) tends to occur. In a healthy intestine, the production of IL-17 by the γδ T-cells in the epithelial layer protects the intestinal layer and prevents bacterial translocation. However, γδ T and T-reg cells are lost in babies with NEC, while Th17 and $CD4^+$ T cells increases (26).

Vaccination response:

Studies have found that the absolute primary antibody responses to antigens in the routine vaccination schedule are lower in preterm infants vaccinated according to chronological age than in term infants (28). However, majority achieve concentrations generally accepted to correlate with protection (28). Indeed, the responses to scheduled immunisations are adequate, except for the hepatitis B vaccine, where there is a need for a repeat of the full schedule in infants who received their first dose when they weighed less than 2000 g (29).

Conclusion:

The combination of immune system immaturity and the various interventions to improve the survival of the preterm baby increases their susceptibility to pathogens. Prompt human breast milk feedings and appropriate antimicrobial use through antimicrobial stewardship program, are crucial to promote the maturation of the preterm immune system and healthy microbiome. Commencement of routine vaccination in preterms without any delay is vital.

Contributions of authors:

RMI conceived the review idea, designed the outline, and wrote the initial draft of the manuscript. AI wrote the abstract, introduction and conclusion segment of the manuscript. Both authors approved the final manuscript.

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Original Article **Open Access**

COVID-19 in children aged 0-15 years seen at Amirou Boubacar Diallo National Hospital in Niamey, Niger, 2020-2021

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Abstract:

Background: In 2020, the COVID-19 pandemic affected all age groups. Although COVID-19 is generally benign in children, a diagnostic problem may arise due to clinical similarities with certain pathologies such as malaria, dengue fever and influenza. The objective of this study is to describe the epidemiological profile of COVID 19 in children seen at consultation and to determine the prevalence of influenza, malaria and dengue fever as differential diagnoses.
Methodology: We conducted a prospective cohort analytical study from October 1, 2020 to February 28, 2021 in COVID-19 suspected children aged 0 to 15 years admitted to the pediatrics department at the hospital. We used EPI INFO 7.2.4. software for data entry and analysis. Frequencies and proportions were calculated.
Results: A total of 570 suspected cases of COVID-19 were enrolled. Of the suspected cases, 53.2% were males and 46.9% were females, with a M/F ratio of 1.13. The median age was 2 years (IQR: 1- 3 years), with age range of 0 to 15 years, and 68,8% in the age range 1 to 5 years. Exposure factors were travel (3.7%), contact with a suspected case of COVID-19 (1.0%), while only 2.6 % (15/570) of suspected cases were confirmed positive for COVID-19. The median age of COVID-19 confirmed children was 2.7 years (IQR 0.33-5). There were more male positive cases, with a M/F ratio of 2. Fever (100%) and cough (53.3%) were the predominant symptoms. The prevalence of malaria, Dengue fever and influenza among suspected COVID-19 cases were 16.8%, 0% and 54.7% respectively, while the respective prevalence in COVID-19 confirmed cases were 66.7%, 0% and 33.3%
Conclusion: COVID-19 should be investigated in children presenting with symptoms and signs of malaria, influenza or Dengue fever.

Keywords: COVID-19; malaria; Dengue; flu; children; Niger

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COVID-19 chez les enfants de 0 à 15 ans vus à l'Hôpital National Amirou Boubacar Diallo de Niamey, 2020-2021

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Résumé:

Contexte: en 2020, la pandémie de COVID-19 a touché toutes les tranches d'âge. Bien que le COVID-19 soit généralement bénin chez l'enfant, un problème de diagnostic peut surgir en raison de similitudes cliniques avec certaines pathologies comme le paludisme, la dengue et la grippe. L'objectif de cette étude est de décrire le profil

épidémiologique du COVID 19 chez les enfants vus en consultation et de déterminer la prévalence de la grippe, du paludisme et de la dengue comme diagnostics différentiels.
Méthodologie: Nous avons mené une étude prospective descriptive de cohorte du 1er octobre 2020 au 28 février 2021, chez des enfants suspects de COVID-19 âgés de 0 à 15 ans admis au service de pédiatrie de l'hôpital. Nous avons utilisé EPI INFO 7.2.4. Logiciel de saisie et d'analyse de données. Les fréquences et les proportions ont été calculées.
Résultats: Au total, 570 cas suspects de COVID-19 ont été recrutés. Parmi les cas suspects, 53,2% étaient des hommes et 46,9% des femmes, avec un ratio H/F de 1,13. L'âge médian était de 2 ans (IQR: 1-3 ans), avec une tranche d'âge de 0 à 15 ans, et 68,8% dans la tranche d'âge de 1 à 5 ans. Les facteurs d'exposition étaient les voyages (3,7%), le contact avec un cas suspect de COVID-19 (1,0%), tandis que seulement 2,6% (15/570) des cas suspects ont été confirmés positifs à la COVID-19. L'âge médian des enfants confirmés par le COVID-19 était de 2,7 ans (IQR 0,33-5). Il y a eu davantage de cas positifs chez les hommes, avec un ratio H/F de 2. La fièvre (100%) et la toux (53,3%) étaient les symptômes prédominants. La prévalence du paludisme, de la dengue et de la grippe parmi les cas suspects de COVID-19 était respectivement de 16,8%, 0% et 54,7%, tandis que la prévalence respective des cas confirmés de COVID-19 était de 66,7%, 0% et 33,3%.
Conclusion: Le COVID-19 doit être recherché chez les enfants présentant des symptômes et des signes de paludisme, de grippe ou de Dengue.

Mots-clés: COVID-19; paludisme; Dengue; grippe; enfants; Niger

Introduction:

On January 9, 2020, the discovery of a new coronavirus, severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), that causes coronavirus disease-2019 (COVID-19) was officially announced by the Chinese health authorities and the World Health Organization (1). By mid-November 2022, COVID-19 had caused over 660 million cases and 6.6 million deaths worldwide (2). Current data show that children affected by SARS-CoV-2 generally belong to clustered familial cases (3). Infected children may appear asymptomatic or present with mild symptoms such as cough, fever and nasal congestion. In addition, gastrointestinal symptoms such as diarrhea, nausea, vomiting and abdominal discomfort are more frequent in children than in adults (3,4). Infected children accounted for 2.0% of COVID-19 cases diagnosed in China, 1.2% of cases in Italy and 1.7% of cases in the USA (3). Unlike the majority of respiratory viruses, children appear less susceptible to SARS-CoV-2 and generally develop a mild form, with low mortality (5). The reported mortality rate among severely ill children was 2.0% (6).

Africa was largely spared of COVID-19 compared to China, US and Europe (7), and it accounted for about 4.0% of all cases reported worldwide in 2020 (8). In tropical countries however, there are concerns about the similarities of COVID-19 with other infectious diseases, due to the same chief complaint of fever. The differential diagnosis must be sufficiently broad and always include COVID-19 when a person presents to the emergency department with a chief complaint of fever. COVID-19 is difficult to distinguish from malaria, influenza and dengue fever, as they share similar clinical features in addition to fever, headache, cough, myalgias, and gastrointestinal disturbances such as diarrhea, vomiting, nausea, and abdominal pain (9-11).

In Niger, the first wave of the COVID-19 epidemic began in March 2020, and as of August 9, 2020, the prevalence of confirmed cases in the under-16 age group was 4.7%, with a case-fatality rate of 0.01% (12). Children are a source of contamination that can exacerbate the spread of the disease (13). Niger, like other African countries, was not sufficiently prepared to contain an epidemic of COVID-19, and no paediatric studies exist. The aim of this study is to describe the epidemiology of COVID-19 in children seen at our hospital, Amirou Boubacar Diallo National Hospital, Niamey, and to investigate differential diagnoses in order to contribute to a better understanding of the disease in Niger.

Materials and method:

Study design and period:

This was a prospective cohort analytical study of COVID-19 suspected children aged 0-15 years conducted from October 1, 2020 to February 28, 2021 in the pediatrics department of Amirou Boubacar Diallo National Hospital in Niamey, Niger.

Study participants, inclusion and exclusion criteria:

The study participants were sick children admitted to the hospital pediatric ward during the study period. The inclusion criteria were; (i) all children with fever or any notion of fever and at least one of the following signs; mainly dry cough (then wet cough) and fatigue, angina, nasal congestion and gastrointestinal symptoms including nausea, vomiting, abdominal pain and diarrhea; and (ii) children whose parents have given their free and informed consent to participate in the study. Children who did not meet the case definition and/or whose parents did not give their consent were excluded. The sample size included all suspected cases of COVID-19 during the study period.

Ethical approval:

The study protocol was approved by National Ethics Committee of Niger (N°069/

2020/CNERS). Written informed consent was obtained from the parents/legal guardians of the children for publication. All procedures were compatible with the declaration of Helsinki.

Laboratory analysis of samples:

Two samples were taken for all children included in the study, including nasopharyngeal swab and blood sample of approximately 1 to 2 ml into a dry specimen bottle. SARS-CoV2, influenza and dengue viral RNA were detected in the samples by real-time RT-PCR using specific primers and probes. IgM and IgG serology for SARS-CoV2 was also carried out using a commercial kit from SD Biosensor "Standard Q COVID-19 IgM/IgG Duo" which presents acceptable sensitivity and specificity performances. Malaria parasite was detected in children using thin blood smear and thick film techniques at the Amirou Boubacar Diallo National Hospital laboratory.

Data collection:

Questionnaires were interviewer-administered to collect information from the caregivers of the children at the hospital, which include socio-demographic characteristics of the children (age, sex, origin, nutritional status, mode of admission, sorting, travel history, antecedents), socio-demographic factors of parents (education level, socio-economic status), clinical features of children (signs and symptoms, diagnosis) and biological variables of children (results of COVID-19, influenza and Dengue PCR, malaria blood smear microscopy and C-reactive protein).

Data analysis:

Data were analysed using EPI INFO 7.2.4. Frequencies, proportions, median and inter-quantile range (IQR) of variables were calculated and presented in the form of tables and graphs. Association of dependent variables (COVID-19 status) with independent variables (sociodemographic, clinical and biological) was determined using Pearson Chi square, with calculations of Odds ratio (and 95% confidence interval), and p value < 0.05 was considered to be statistically significant value.

Results:

Socio-demographic and clinical characteristics of COVID-19 children and their parents:

A total of 570 COVID-19 suspected cases were enrolled in the study. Of these cases, 53.2% were males and 46.9% were females, with a M/F ratio of 1.13. The median age was 2 years (IQR: 1-3 years), age range of 0 to 15 years, with 1-5 years age group being the most represented at 68.8%. Most of the children (96.8%) lived in urban areas. Exposure factors were mainly travel by the suspected case (3.7%) and contact with a suspected or probable case of COVID-19 (1.0%). Less than a quarter (18.0%) of the children were malnourished, and 4.2% had a medical history (Table 1).

Children with suspected COVID-19 were admitted directly to the pediatric ward without referral in 87.5% of cases. About 60% of cases were classified as a priority state for systematic triage for all pathologies combined on admission. The average duration between onset of illness and admission was 3.37 ± 2.74 days. The course of the disease was marked by a hospital stay of 1.23 ± 2.90 days, with a case-fatality rate of 0.9% (Table 1). Fever was one of the inclusion criteria and was present in all (100.0%) the recruited children. The most common symptoms and signs aside this, were cough (54.4%), gastrointestinal disorders such as vomiting, diarrhoea and abdominal pain (44.5%), nasal congestion (23.7%) and angina (20.9%) (Table 2).

The age of the parents is marked by the predominance of 25-34 years age group for mothers (49.0%) and 35-44 for fathers (43.5%). The level of education is low among both fathers and mothers, with 30.5% and 29.5% respectively having received no education, compared to 22.0% and 17.2% with a higher education level. Socio-economic status is generally low among families, with 54.2% in low socio-economic group.

Characteristics of the children with confirmed COVID-19:

Of the 570 samples tested, only 2.6 % (n=15) were confirmed positive for COVID-19. The median age of confirmed children was 2.70 years (IQR: 0.33-5), with more males (67.0%, 10/15) than females (33.0%, 5/15), and M/F ratio of 2. However, the prevalence of COVID-19 in the males (3.3%, 10/303) compared to the females (1.9%, 5/267) among the study participants was not significantly different (OR=1.79, 95% CI=0.603-5.30, p= 0.310) (Table 3).

Majority of COVID-19-positive children were in the 1-5 age group (86.7%) and mostly from urban areas (86.7%). In confirmed cases, dry cough was present in 53.3%, nasal congestion, angina and vomiting were reported in 26.7% of cases each, and headache and abdominal pain occurred in 6.7% and 13.3% of cases respectively (Table 2).

Table 1: Socio-demographic and clinical characteristics of children with suspected COVID-19

Characteristics	Number	Percentage
Age group (years)		
< 1	133	23.3
1 - 5	392	68.8
6 - 11	30	5.3
11 - 15	15	2.6
Total	570	100.0
Median age (IQR) in years	2 (1-3)	
Gender		
Male	303	53.0
Female	267	47.0
Total	570	100.0
Residence		
Rural	24	4.2
Urban	546	95.8
Total	570	100.0
Malnutrition		
Yes	102	18.0
No	468	82.0
Total	570	100.0
Admission mode		
Referral	71	12.5
Direct	499	87.5
Total	570	100.0
Medical record		
Yes	155	27.0
No	415	73.0
Total	570	100.0
Triage sorting		
Priority	343	60.0
Ordinary	227	40.0
Total	570	100.0
Travel history (14 days in advance)		
Yes	21	3.7
No	549	96.7
Total	570	100.0
Visit to a healthcare facility		
Yes	82	14.5
No	488	85.5
Total	570	100.0
Participation in a mass event		
Yes	27	5.0
No	543	95.0
Total	560	100.0
Travel history in entourage 14 days prior		
Yes	8	1.5
No	562	98.5
Total	570	100.0
History of contact with a suspect case		
Yes	6	1.0
No	564	99.0
Total	570	100,0
Pathological history		
Yes	3	0.5
No	567	99.5
Total	570	100.0
Outcome of disease		
Survived	565	99.1
Deceased	5	0.9
Total	570	100.0
Time between BD* and admission (days)	3.37 ± 2.74	
Length of hospital stay (days)	1.23 ± 2.90	

*BD= Beginning of disease

Table 2: Clinical signs and symptoms in suspected and confirmed cases of COVID-19

Clinical signs and symptoms	COVID-19 PCR Result		Total (%)
	Negative (%)	Positive (%)	
Fever	555 (100.0)	15 (100.0)	570 (100.0)
Dry cough	278 (50.1)	8 (53.3)	286 (50.2)
Nasal congestion	131 (23.6)	4 (26.7)	135 (23.7)
Angina	115 (20.7)	4 (26.7)	119 (20.9)
Vomiting	112 (20.2)	4 (26.7)	116 (20.4)
Diarrhoea	75 (13.5)	4 (26.7)	79 (13.9)
Abdominal pain	51 (9.2)	2 (13.3)	53 (9.3)
Respiratory distress	25 (4.5)	0	25 (4.4)
Fatty cough	23 (4.1)	1 (6.7)	24 (4.2)
Head ache	21 (3.8)	1 (6.7)	22 (3.9)
Fatigue	11 (2.0)	0	11 (1.9)
Nausea	5 (0.9)	0	5 (0.9)
Arthralgia	4 (0.7)	0	4 (0.7)
Myalgia	2 (0.4)	0	2 (0.4)
Others	255 (45.9)	6 (40.0)	261 (45.8)
Total	**555 (97.4)**	**15 (2.6)**	**570 (100.0)**

Table 3: Bivariate analysis of characteristics of children with suspected and confirmed COVID-19

Characteristics	COVID-19 PCR Test		OR (95% CI)	*p* value
	Negative (%)	Positive (%)		
Gender				
Male (n=303)	293 (96.7)	10 (3.3)	1	
Female (n=267)	262 (98.1)	5 (1.9)	1.79 (0.603-5.30)	0.31
Total (n=570)	555 (97.4)	15 (2.6)		
Outcome of COVID-19				
Survived (n=565)	550 (97.3)	15 (2.7)	1	
Deceased (n=5)	5 (100.0)	0	3.23 (0.17-61.04)	1.000
Total (n=570)	555 (97.4)	15 (2.6)		

OR=Odds ratio; CI=Confidence interval; PCR=Polymerase chain reaction

No positive child was hospitalized or received specific treatment for COVID-19 and all were followed up at home. No death (0%) was reported in confirmed COVID-19 cases. The influenza PCR, dengue PCR and thick film microscopy tests were positive in 16.8% (96/570), 0% and 54.7% (312/570) of suspected COVID-19 cases respectively, whereas in confirmed COVID-19 cases, these tests were positive in 33.3% (5/15), 0% and 66.7% (10/15) cases respectively (Table 4).

Discussion:

In our study, the median age of children with suspected COVID-19 was 2 years (IQR: 1-3 years), with age range of 0 to 15 years. The 1-5 years age group was the most represented at 68.8% and most of the children (96.8%) lived in urban areas. This is comparable to a study in Guinea, where the 0-4 years age group was the most represented (38.6%), with age range of 0-16 years, with a median age of 7.19 years (which is higher than the median age in our study), and majority of the children (70.4%) in the study lived in urban center, Conakry (14). The predominance of children aged 0 to 5 years can be explained by their susceptibility to various infections and the free health care available to this age group in Niger (15).

Table 4: Bivariate analysis of biological characteristics of children with suspected and confirmed COVID-19

Biological characteristics	COVID-19 PCR Result		Total (%)	OR (95% CI)	*p* value
	Negative (%)	Positive (%)			
Influenza PCR test					
Positive	91 (16.4)	5 (33.3)	96 (16.8)	2.549 (0.851; 7.634)	0.084
Negative	464 (83.6)	10 (66.7)	474 (83.2)		
Total	555 (100.0)	15 (100.0)	570 (100.0)		
Thick blood film test for malaria					
Positive	302 (54.4)	10 (66.7)	312 (54.7)	1.675 (0.565; 4.966)	0.347
Negative	253 (45.6)	5 (33.3)	258 (45.3)		
Total	555 (100.0)	15 (100.0)	570 (100.0)		
CRP					
Positive	380 (70.0)	14 (93.3)	394 (70.6)	6.005 (0.789; 46.048)	0.037
Negative	163 (30.0)	1 (6.7)	164 (29.4)		
Total	543 (100.0)	15 (100.0)	558 (100.0)		
Dengue PCR test					
Positive	0	0	0	-	-
Negative	555 (100.0)	15 (100.0)	570 (100.0)		
Total	555 (100.0)	15 (100.0)	570 (100.0)		

OR=Odds ratio; CI=Confidence interval; PCR=Polymerase chain reaction

Exposure factors were travel by the suspected case (3.7%) and contact with a suspected or probable case of COVID-19 (1.0%). We found much higher figures in the literature, where 28.0% of children had a history of travel (16) and 68.0% had been in contact with confirmed infected adults (17). Children are often infected by their families, and at the very start of the epidemic, the notion of travel was a risk factor (3).

The level of education was low among both fathers and mothers, with 30.5% and 29.5% respectively having received no education at all. According to Camara et al., (14) 28.6% of mothers were merchants and 39.7% were contact persons, compared with 37.6% of fathers who were civil servants. In the literature, we found no link between the parents' intellectual and economic level and COVID-19 in children.

Among the suspected children, 2.6% were positive for COVID-19. Our result is similar to those of Farfan et al., (2) where COVID-19 PCR positivity of 3.2% and 2.5% were reported but higher than those of Tagarro et al., (18) who were 1.0% positive. In our study, COVID-19-positive children were mainly found in the 1-5 years age group (86.7%). In other studies, children aged 1-5 years (25.2%) and 6-10 years (33.9%) were the most predominant (19,20). The median age of infected children was 2.70 years (IQR: 0.33-5). Median ages ranging from 3-12 years were reported in some studies (17-20).

There were more males (67.0%, 10/15) than females (33.3%, 5/15) COVID-19 positive cases, with male to female ratio of 2 in our study, and prevalence of 3.3% (10/303) for males and 1.9% (5/267) for females among the study participants, albeit, the prevalence difference was not statistically significant (OR=1.79, 95% CI=0.603-5.3, p=0.31). Nevertheless, male predominance has been reported in the literature (17,19,21-24). In Niger, girls, unlike boys, tend to be kept at home, which exposes the boys more to the disease.

In the COVID-19 positive cases, all (100.0%) the children selected had fever, and dry cough was found in 53.3% of cases, which were the most predominant symptoms. Our results are in line with the findings of other studies (22,25-28). Thick blood film test for malaria in COVID-19 confirmed cases were positive in 66.7% (10/15). Malaria and COVID-19 are strikingly similar in their symptoms (29). People living in malaria-endemic areas who are infected with SARS-CoV-2 may be at increased risk of severe COVID-19 or adverse disease outcomes if they are unaware of their malaria status (30). Results from literatures show malaria seroprevalence ranging from 3.1% to 40% (2,24,30,31).

SARS-CoV-2 and alpha influenza virus are RNA viruses that cause COVID-19 and influenza respectively. Both viruses present similar symptoms and use surface proteins to infect the host (32). We found influenza in

33.3% (5/15) of confirmed COVID-19 cases in our study compared to influenza in 5.0% of COVID-19 confirmed children in the study by Tagarro et al., (18). Overlapping clinical and pathological similarities can lead to missing cases, which in turn can be fatal (33). In our study, we found no co-infection between COVID-19 and Dengue fever. However, co-infection of Dengue and COVID-19 has been reported in some studies and is associated with significant morbidity and mortality (34,35).

Treatment is marked by the absence of specific anti-COVID-19 therapy, but in our study, no COVID-19 positive children were hospitalized and no deaths were reported in the COVID-19 confirmed cases. Other studies (18,24) agree with the findings of our study.

Conclusion:

Our study highlights the need to strengthen the capacity of African health centers, particularly those in Niger, to manage moderate to severe paediatric cases of COVID-19. We noted that many cases of COVID-19 escape surveillance because the similarity of their symptoms and signs to malaria, influenza and Dengue fever can lead to confusion in endemic areas. Health care providers need to redouble their efforts to diagnose and manage co-infected individuals rapidly, in order to prevent serious consequences.

Conflicts of interest:

Authors declare no conflict of interest

Contributions of authors:

HIA developed the study protocol and was involved in data collection, processing and analysis, manuscript preparation, and proofreading; SA was involved in data collection; AO and MMY were involved in data processing, analysis and manuscript revision; OBT, SM and MG were involved in manuscript revision; and BD was involved in manuscript proofreading. All authors reviewed and approved the final version of the manuscript.

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Predominant amino acid substitutions in NS5B gene of hepatitis C virus in blood donors and treatment-naïve hepatitis and HIV patients in Nigeria

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Abstract:

Background: Hepatitis C virus (HCV) genome undergoes high rate of mutation, which results in generation of genetically diverse HCV isolates. There is paucity of data on mutations in the nonstructural 5b (NS5b) gene of circulating HCV and their implications in the Nigerian population. Here, we identified clinically-important mutations in HCV isolates, which may influence response to therapy and disease prognosis.
Methodology: HCV RNA was extracted from a total of 301 blood samples collected from 99 symptomatic treatment-naïve hepatitis patients, 125 HIV-infected individuals and 77 asymptomatic blood donors in Ibadan, Nigeria. The RNA was reverse–transcribed to complimentary DNA and HCV NS5B gene amplified by nested PCR. The amplified products of 42 HCV were sequenced and sequences were aligned with those from GenBank and HCV databases in MEGA 7.0. Nucleotide sequences were translated to amino acids while substitutions in the amino acids were analyzed with reference to H77 prototype strain of HCV.
Results: A total of 10 amino acid polymorphisms were observed from the 42 sequenced NS5B gene, with the major clinically-important amino acid mutations being S15G in 28 (66.7%) participants, T7N (24, 57.1%), G61R (23, 54.8%), S54L (22, 52.4%), G89E (14, 33.3%), T79M (12, 28.6%), and T711 (11, 26.2%). Others were Q67R (7, 16.7%), Q47H (7, 16.7%) and S84F (2, 4.8%). S15G/A/V mutations were more predominant in patients with HIV (76.9%, 10/13) followed by patients with clinical hepatitis (75.0%, 12/16) and blood donors (46.1%, 6/13). Q67R and T71I mutations were not predominant in patients with clinical hepatitis as they were detected in only 31.3% (5/16) and 43.8% (7/16) participants respectively, compared to S15G (75.0%, 12/16), S54L (68.8%, 11/16), G61R/E (68.8%, 11/16) and T7N/S (56.3%, 9/16). There was no statistically significant difference in the distribution of each of the 10 amino acid polymorphisms detected within patients with symptomatic clinical hepatitis (x^2=9.311, p=0.409), HIV-infected patients (x^2=13.431, p=0.1440) and asymptomatic blood donors (x^2=3.775, p=0.9256). Similarly, there was no significant difference in the distribution between the 3 categories of the study participants except for T79M mutation, which was significantly higher in HIV-infected patients (61.5%, 8/13) compared to patients with clinical hepatitis (18.8%, 3/16) and asymptomatic blood donors (7.7%, 1/13) (x^2=10.456, p=0.0054).
Conclusion: Mutations in the NS5B gene could be associated with worse prognosis of the disease or antiviral failure due to viral resistance in patients undergoing therapy. The absence of Q47H mutations in majority of the study participants in our study implies that they will not respond well to daprevir and mericitabine. Screening of patients for pre-existing resistant mutations before commencement of therapy and monitoring during and after therapy are recommended.

Keywords: Hepatitis C virus; symptomatic patients; blood donors, NS5B gene; mutation

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Substitutions prédominantes d'acides aminés dans le gène NS5B du virus de l'hépatite C chez les donneurs de sang et les patients atteints d'hépatite et de VIH naïfs de traitement au Nigeria

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Résumé:

Contexte: Le génome du virus de l'hépatite C (VHC) subit un taux élevé de mutation, ce qui entraîne la génération d'isolats de VHC génétiquement divers. Il existe peu de données sur les mutations du gène non structurel 5b (NS5b) du VHC en circulation et leurs implications dans la population nigériane. Ici, nous avons identifié des mutations cliniquement importantes dans les isolats du VHC, qui peuvent influencer la réponse au traitement et le pronostic de la maladie.
Méthodologie: L'ARN du VHC a été extrait d'un total de 301 échantillons de sang prélevés auprès de 99 patients symptomatiques naïfs d'hépatite, 125 personnes infectées par le VIH et 77 donneurs de sang asymptomatiques à Ibadan, au Nigeria. L'ARN a été transcrit de manière inverse en ADN complémentaire et en gène NS5B du VHC amplifié par PCR nichée. Les produits amplifiés de 42 VHC ont été séquencés et les séquences ont été alignées sur celles des bases de données GenBank et VHC dans MEGA 7.0. Les séquences nucléotidiques ont été traduites en acides aminés tandis que les substitutions dans les acides aminés ont été analysées en référence à la souche prototype H77 du VHC.
Résultats: Un total de 10 polymorphismes d'acides aminés ont été observés à partir des 42 gènes NS5B séquencés, les principales mutations d'acides aminés cliniquement importantes étant S15G chez 28 (66,7%) participants, T7N (24, 57,1%), G61R (23, 54,8%), S54L (22, 52,4%), G89E (14, 33,3%), T79M (12, 28,6%) et T711 (11, 26,2%). Les autres étaient Q67R (7, 16,7%), Q47H (7, 16,7%) et S84F (2, 4,8%). Les mutations S15G/A/V étaient plus prédominantes chez les patients atteints du VIH (76,9%, 10/13), suivis des patients atteints d'hépatite clinique (75,0%, 12/16) et des donneurs de sang (46,1%, 6/13). Les mutations Q67R et T71I n'étaient pas prédominantes chez les patients atteints d'hépatite clinique puisqu'elles ont été détectées respectivement chez seulement 31,3% (5/16) et 43,8% (7/16) des participants, par rapport aux patients S15G (75,0%, 12/16), S54L (68,8%, 11/16), G61R/E (68,8%, 11/16) et T7N/S (56,3%, 9/16). Il n'y avait pas de différence statistiquement significative dans la distribution de chacun des 10 polymorphismes d'acides aminés détectés chez les patients présentant une hépatite clinique symptomatique (x^2=9,311, p=0,409), les patients infectés par le VIH (x^2=13,431, p=0,1440) et les patients sanguins asymptomatiques donneurs (x^2=3,775, p=0,9256). De même, il n'y avait pas de différence significative dans la répartition entre les 3 catégories de participants à l'étude, à l'exception de la mutation T79M, qui était significativement plus élevée chez les patients infectés par le VIH (61,5%, 8/13) par rapport aux patients atteints d'hépatite clinique (18,8%, 3/16) et donneurs de sang asymptomatiques (7,7%, 1/13) (x^2=10,456, p=0,0054).
Conclusion: Les mutations du gène NS5B pourraient être associées à un plus mauvais pronostic de la maladie ou à un échec antiviral dû à une résistance virale chez les patients sous traitement. L'absence de mutations Q47H chez la majorité des participants à notre étude implique qu'ils ne répondront pas bien au daprévir et à la méricitabine. Le dépistage des mutations résistantes préexistantes chez les patients avant le début du traitement et une surveillance pendant et après le traitement sont recommandés**.**

Mots-clés: Virus de l'hépatite C; patients symptomatiques; donneurs de sang; gène NS5B; mutation

Introduction:

Hepatitis C virus (HCV) exhibits great genetic variation which is responsible for the enormous diversity associated with its lifecycle. The degree of this variation can determine to a large extent the functionality of most antivirals such as NS3/4A protease inhibitors which have successfully been used to treat patients with infected HCV genotype 1, but has lower potency against genotype 3 (1). With respect to heterogeneous population of HCV, substitutions associated with resistance in diverse HCV genotypes can result in drug resistance which may lead to treatment failure (2). Viral hepatitis including HCV infection continues to pose global public health challenge that affects more than 185 million people (3).

In most low-income-countries, HCV is presenting a more serious challenge, and direct acting antivirals (DAAs) are not accessible to many, even though it is a major breakthrough in the treatment of HCV infections (4). In those settings, combination of pegylated alpha-interferon (PEG-IFN-γ) and ribavirin, two protease inhibitors, is still the 'gold standard' of therapy and in most cases. However, treatment of patients does not take into consideration the circulating genotypes and their genetic characteristics. These antivirals are known for their potency and sustained viral response against HCV genotype 1 but possess substantial side effects as well as low genetic barrier and cross-resistance (5).

The NS5B genes encode viral RNA-dependent RNA polymerase (RdRp), an enzyme responsible for viral RNA replication and a target for most antiviral cocktails mostly polymerase inhibitors (6,7). As a result of the low proof-reading ability of RdRp, errors occur during viral replication coupled with high viral replication rate, resulting in HCV extreme variability and several isolates that are distinctly distributed in different regions of the world (8). Accumulation of these amino acid substitutions in the isolates due to high genetic variability of HCV overtime, results in minor or major resistant variants with important clinical implications (9). These implications include but not limited to poor response to therapy, inability to develop effective vaccines, and broad spectrum or pan-genotypic antiviral agents against HCV (10). In most cases, resistance-associated mutations may occur as natural polymorphisms and could affect response to existing NS5B inhibitors that target the viral gene (11).

Previous studies on HCV RNA samples from chronically HCV-infected drug-naïve patients have linked mutations in HCV genome with resistance to interferon, ribavirin, nucleoside inhibitors (NIs) and non-nucleoside inhibitors (NNIs) (12), and even worse prognosis of the disease in HCV-infected patients (13). Mutations in form of natural polymerphisms that occur overtime in diverse strains of HCV may affect the functionality of the virus in terms of response to therapy, chronicity, transmissibility, virulence and/or pathogenicity. Moreover, those HCV genotypes differ in their response to antiviral drugs (14).

Most of the approved DAAs such as sofosbuvir and other NS5B polymerase inhibitors that suppress NS5B replication are out of reach of many in sub-Saharan Africa due to high costs. As such, the degree of HCV escape mutants or drug-resistant variants in circulation is not known in the region, and this information is critical for proper and effective treatment of patients. In the absence of DAA treatment protocols however, identification of pre-existing mutations in circulating variants is important for successful treatment and control of HCV infection now and in the future. The diversity exhibited by the virus has been previously reported to associate with antiviral failure, obstacle to design of a universal vaccine against HCV infection, as well as influence on viral persistence and disease progression in infected individuals. These have been attributed to presence of natural polymorphisms in HCV isolates (2).

In sub-Saharan Africa, where DAAs are grossly inaccessible to most patients, studies are required to identify pre-existing amino acid substitutions responsible for HCV diversity, which may influence therapy, especially with polymerase inhibitors, and contribute to effective control and eradication of the virus by 2030. The present study therefore aims to identify important pre-existing mutations in the different HCV genotypes circulating in treatment-naïve blood donors, HIV & HCV co-infected patients and patients with clinical hepatitis in Nigeria.

Materials and methods:

Study setting/period/ethical consideration:

The study was carried out in Ibadan, southwest Nigeria, between the period 2015 and 2018, and was approved by University of Ibadan/University College Hospital Ethical Committee under the assigned number UI/EC/14/0019.

Study participants:

The study involved a total of 301 HCV-seropositive participants; 99 symptomatic hepatitis patients or those with clinical hepatitis, 125 HIV-infected individuals referred to the laboratory for anti-HCV screening, and 77 asymptomatic or apparently healthy blood donors screened at the Blood Bank, University College Hospital. The anti-HCV positive blood samples of the participants, stored at -80^{o}C in the HIV laboratory of the Department of Virology, College of Medicine, University of Ibadan, were retrieved for this study.

HCV RNA extraction and reverse transcription

A total of 42 anti-HCV positive samples were selected for HCV mutation analysis. Aliquots of plasma were made from each blood sample and from which 5ml blood was used for RNA extraction according to the manufacturer's instructions of the commercially available extraction kit used (Jena Bioscience total RNA Purification kit, Germany). Then reverse-transcription (first strand cDNA synthesis) of the extracted RNA was performed with random hexamer using Script cDNA synthesis kit (Jena Bioscience, Germany), in a final volume of 20 µl assay. The thermal cycling and time conditions of the sequences were 42^{o}C for 10min, followed by incubation at 50°C for 45min (15).

PCR amplification of NS5B gene:

Amplification of the NS5B gene segment of the virus located at positions 8275–8616 was carried out using a nested PCR protocol with 5µl of gene specific primers and 2.5µl of the RT-PCR product as the template previously described by Shenge et al., (16). Amplified fragments were viewed using gel electrophoresis in 1.5% agarose gel concentration.

Sequence of primers and the cycling conditions for the nested PCR are as follows: First round of PCR, the primers used were; NS5B-k1 for forward reaction; 5′-TGGGGATC CCGTATGATACCCGCTGCTTTGA-3′ and NS5B-k2 reverse reaction; 5′-GGCGGAATTCCTGGT CATAGCCTCCGTGAA-3′ as designed by the authors. The cycling condition was 95°C for 5min, 94°C for 30sec, 50°C for 30sec, 72°C for 45sec, 72°C for 10min, for 30cycles. The expected size of amplification product for the first round PCR was 400bp.

Five microlitres of the first round PCR product was used as the template for the second round PCR. The nested PCR was performed in a 25µl reaction mixture with 2.5µl forward primer-NS5B-122; 5′-CTCAACCGTCA CTGAGAGAGACAT-3′ and reverse primers NS 5B-R1; 5′-GCTCTCAGGCTCGCCGCGTCCTC-3′. The thermal cycling condition was same as the first-round reaction but for 45 cycles. The PCR product with the expected band size of 301bp was detected by electrophoresis in 1.5% agarose gel and visualized using Bio-Rad Gel Doc XR+ System.

DNA sequencing and alignment:

The PCR products were purified with ExoSAP Amplicon Purification kit (Applied Biosystems, Foster city, CA,) according to the manufacturer's instructions and sequenced with ABI V3.1 Big dye terminator (Applied Biosystems, Foster city, CA). The second-round PCR products and the same inner primers used for the nested PCR were used for sequencing. Sequencing reactions were commercially carried out by Inqaba Biotec, South Africa.

Phylogenies of the HCV include subtypes 1a, 1b, 2b, 2c, 3a and 5a in 42 sequenced NS5B genes as previously reported by Shenge et al., (16) and Shenge et al., (17). Nucleotide sequences of the partial HCV NS5B gene obtained from the samples are available at the Figshare repository (doi:10.6084/m9.figshare.7471454). Sequences were deposited at the GenBank under accession numbers LC 484047-LC506601 and LC538234-LC538262.

Mutation profiling and statistical analysis:

The amino acid substitutions generated were aligned with prototype H77 strain (GenBank accession number NC.004102.1). Nucleotide variations observed were compared with the H77 strain and within the isolates. Finally, the differences in the observed mutations were compared among the study participants using SPSS version 20.0. Chi-square test was used to compare the variations and to determine significant difference between the observed mutations (dependent variable) and the three categories of study participants (independent variable). Statistical significance was considered at $p < 0.05$.

Results:

The HCV NS5b gene of the 42 participants' blood samples sequenced were distributed as follows; 16 from symptomatic hepatitis patients, 13 from HIV-infected patients and 13 from asymptomatic blood donors. A total of 10 amino acid polymorphisms were observed from the 42 sequenced NS5B gene, with the major clinically-important amino acid mutations being S15G in 28 participants (66.7%), T7N (24 participants, 57.1%), S54L (22 participants, 52.4%), G61R (23 participants, 54.8%), G89E (14 participants, 33.3%), T79M (12 participants, 28.6%) and T711 (11 participants, 26.2%). Others were Q67R (7 participants, 16.7%), Q47H (7 participants, 16.7%) and S84F (2 participants, 4.8%).

The S15G/A/V mutations were more predominant in patients with HIV (76.9%, 10/13), followed by patients with clinical hepatitis (75.0%, 12/16) and asymptomatic blood donors (46.1%, 6/13). The Q67R and T71I mutations are not predominant in patients with clinical hepatitis as they were detected in only 31.3% (5/16) and 43.8% (7/16) participants respectively, compared to S15G (75.0%, 12/16), S54L (68.8%, 11/16), G61R /E (68.8%, 11/16) and T7N/S (56.3%, 9/16). In order of dominance in HIV patients, S15G (76.9%, 10/13) was the most predominant, followed by T7N/S (69.2%, 9/13), T79M (61.5%, 8/13) and G61R (61.5%, 8/16). The Q47H mutation was largely absent among the study participants in 83.3% (35/42) of those whose samples were sequenced.

There was no statistically significant difference in the distribution of each of the 10 amino acid polymorphisms detected within patients with symptomatic clinical hepatitis (x^2=9.311, p=0.409), HIV-infected patients (x^2=13.431, p=0.1440) and asymptomatic blood donors (x^2=3.775, p=0.9256). Similarly, there was no significant difference in the distribution of each of the 10 amino acid polymorphisms between the 3 categories of study participants except for T79M mutation, which was significantly higher in HIV-infected patients (61.5%, 8/13) compared to patients with clinical hepatitis (18.8%, 3/16) and asymptomatic blood donors (7.7%, 1/13) (x^2= 10.456, p=0.0054) (Table 1).

Discussion:

This study demonstrated that no significant difference exists among the pre-existing amino acid substitutions or mutations in HCV genome in the different participants studied. The major clinically-important mutation associated with NS5B gene observed in our study was S15G (66.7%) where amino acid serine was substituted by glycine. In addition, some important mutations such as T7N/S (57.1%), G61R/E (54.8%), S54L (52.4%), G89E (33.3%), T79M (28.6%), T71I (26.2%), Q67R (16.7%), Q47H (16.7%), and S84F (4.8%) were detected. The Q47H mutation was largely absent among the study participants in 83.3% (35/42) of those whose samples were sequenced.

Table 1: Comparison of distribution of amino acid polymorphisms among and within the three study populations

Participants with sequenced NS5B gene	**Frequency of observed mutations and amino acid substitutions in HCV NS5B gene (%)**										***x*²**	***p* value**
	S15G/A/V	**Q47H (absent)**	**T7N/S**	**S54L**	**G61R/E**	**T71I**	**Q67R**	**T79M**	**S84F**	**G89E**		
Patients with HIV (n=13)	10 (76.9)	11 (84.6)	9 (69.2)	6 (46.1) deletions	8 (61.5)	1 (7.7)	0	8 (61.5)	1 (7.7)	5 (38.5)	13.43	0.14
Blood donors (n=13)	6 (46.1)	10 (76.9)	6 (46.1)	5 (38.5)	4 (30.8)	3 (23.1)	2 (15.4)	1 (7.7)	1 (7.7)	4 (30.8)	3.78	0.93
Patients with clinical hepatitis (n=16)	12 (75.0)	14 (87.5)	9 (56.3)	11 (68.8)	11 (68.8)	7 (43.8)	5 (31.3)	3 (18.8)	0	5 (31.3)	9.31	0.41
Total (n=42)	28 (66.7)	35 (83.3)	24 (57.1)	22 (52.4)	23 (54.8)	11 (26.2)	7 (16.7)	12 (28.6)	2 (4.8)	14 (33.3)		
***x*²**	3.577	0.600	1.422	2.931	4.525	4.918	5.065	10.456	1.292	0.2236		
***p* value**	0.1672	0.7408	0.4912	0.2310	0.1041	0.0855	0.0794	0.0054*	0.5241	0.8942		

The absence of Q47H mutation in the NS5B gene in any population has implication for anti-HCV therapy in such population. This important NS5B gene mutation was largely absent in the genome of our study participants, which has great implication for response to daprevir and disease prognosis. In a study by Tong et al., (18), HCV-infected persons who possessed Q47H mutation in their viral NS5B gene had improved prognosis and response to daprevir and mericitabine, while those who did not possess the mutation never improved during therapy. The implication of the absence of Q47H in the majority of the study participants in the present study is that they may likely not respond to treatment with daprevir and mericitabine (a prodrug of NS5B polymerase inhibitor, PSI-6130) as reported in previous studies.

NS5B polymorphisms may explain differences in treatment outcomes among patients and mutations at the NS5B region could be associated with poor prognosis of the disease in HCV-infected patients as such mutations alter the polymerase activity of NS5B. According to Castilho et al., (3), changes in the amino acid position 109 of NS5B is notably associated with resistance to antiviral therapy and infectivity of the virus. Resistance to antiviral drugs may also be the result of uncharacterized mutations and interactions among mutations. The L159/L320F mutations in NS5B polymerase confer a low resistance to most HCV polymerase inhibitors especially mericitabine and sofosbuvir (18). In our study, this mutation was not observed but at other positions along the NS5B gene, leucine (L) was substituted by different amino acids other than phenylalanine (F). These polymorphisms may be linked to more dysfunctionality in the gene and not only resistance.

In the previous study by Tong et al., (18) and others, it was observed that S15G mutation affects the replication capacity of HCV NS5B polymerase which reduces the replication fitness of the RNA template (19, 20). The S15G mutation was detected in 66.7% of our study participants, and this is a major mutation at amino acid position 15 in which serine (S) was substituted by glycine (G), alanine or valine. This mutation occurs more in HCV NSB5a infected persons and might be under positive selection in this group, hence may determine the outcome of infection in the groups.

Another factor that affects replication fitness is co-infection. Our earlier study on co-infection inferred that HCV co-infection with HIV has been linked with reduced treatment response (16), and same may equally go for other co-infections including HIV/HBV. HIV can affect NS5B variability, suggesting that an already compromised immune structure (by HIV) can actually affect genetic diversity of HCV by pathogenically influencing the viral replication fitness (16).

Substitution of asparagine (N) with thymine (T) at position 142 of the finger domain is selected for resistance to NS5B nucleotide inhibitor, sofosbuvir (SOF). Sofosbuvir has demonstrated high efficacy in HCV-infected patients in combination therapy (20). Other mutations include A56V, Q67R and T71I, in which the participants had no or few substitutions at those amino acid sites 56, 67 and 71 respectively. Viral infectivity, virulence, disease progression and prognosis in infected individuals as well as antiviral resistance are seen to be affected by several substitutions of amino acids in the NS5B polymerase. The mechanism of antiviral resistance is mainly achieved through change in the NS5B conformation that affects the hydrophobic binding of the residues (21).

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Contributions of authors:

JAS was involved with study design, analysis, securing funding and writing of the manuscript; GNO was involved with study design and writing of the manuscript; DOO was involved in securing funding and editing of the manuscript. All authors approved the final manuscript submitted.

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Conflict of interests:

Authors declare no conflict of interest

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Original Article **Open Access**

Serological and molecular detection of hepatitis C virus among students in a tertiary educational institution in Calabar, Nigeria

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Abstract:

Background: Hepatitis C virus (HCV) infection is a global health problem and continues to be a major disease burden in the world, associated with serious health challenges including liver cirrhosis, cancer, lymphomas and death. This study was carried out to determine the prevalence of HCV infection among students of the University of Calabar.
Methodology: In a cross-sectional study, 200 students were tested for the presence of anti-HCV antibodies using a rapid immunochromatographic (ICT) assay (CTK Biotech, Inc. USA). Seropositive samples were confirmed using reverse transcriptase-polymerase chain reaction (RT-PCR) assay for detection of HCV RNA. Structured questionnaires were used to collect subjects' socio-demographic data and risk factors of infection. Data were analyzed using SPSS version 16.0, with the level of significance set at $p<0.05$.
Results: Of the 200 students screened, the seroprevalence of HCV was 15.0% (n=30) and 9.5% (n=19) was positive for HCV RNA by RT-PCR assay. The prevalence of anti-HCV antibody was significantly higher in females (18.8%, 12/64) than males (13.2%, 18/136) ($x^2=3.84$, $p=0.036$). Alcohol consumption (OR=4.67, 95% CI=2.04-10.67, $p=0.002$), skin piercing (OR=32.99, 95% CI=5.95-72.37, $p<0.0001$), multiple sexual partners (OR=4.03, 95% CI=1.7-9.6, $p=0.0018$), and history of blood transfusion (OR=8.00, 95% CI=2.97-21.58, $p<0.001$) were risk factors significantly associated with HCV infection in the study participants.
Conclusion: The findings of 15.0% and 9.5% prevalence of HCV infection by anti-HCV antibody and HCV RNA, respectively in this study, showed that there is relatively high prevalence of HCV infection among the students' population in University of Calabar, Nigeria. Hence, routine medical screening of students for HCV infection using rapid ICT and RT-PCR techniques is hereby recommended.

Keywords: Hepatitis C virus; Prevalence; ELISA; RT-PCR; Calabar

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Détection sérologique et moléculaire du virus de l'hépatite C chez les étudiants d'un établissement d'enseignement supérieur à Calabar, Nigeria

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Résumé:

Contexte: L'infection par le virus de l'hépatite C (VHC) est un problème de santé mondial et continue de représenter un fardeau de morbidité majeur dans le monde, associé à de graves problèmes de santé, notamment la cirrhose du foie, le cancer, les lymphomes et la mort. Cette étude a été réalisée pour déterminer la prévalence de l'infection par le VHC parmi les étudiants de l'Université de Calabar.

Méthodologie: Dans une étude transversale, 200 étudiants ont été testés pour la présence d'anticorps anti-VHC à l'aide d'un test immunochromatographique rapide (ICT) (CTK Biotech, Inc., USA). Les échantillons séropositifs ont été confirmés à l'aide d'un test de réaction en chaîne par transcriptase inverse-polymérase (RT-PCR) pour la détection de l'ARN du VHC. Des questionnaires structurés ont été utilisés pour collecter les données sociodémographiques des sujets et les facteurs de risque d'infection. Les données ont été analysées à l'aide de SPSS version 16.0, avec le niveau de signification fixé à $p<0,05$.
Résultats: Parmi les 200 étudiants dépistés, la séroprévalence du VHC était de 15,0% (n=30) et 9,5% (n=19) étaient positifs à l'ARN du VHC par test RT-PCR. La prévalence des anticorps anti-VHC était significativement plus élevée chez les femmes (18,8%, 12/64) que chez les hommes (13,2%, 18/136) ($x^2=3,84$, $p=0,036$). Consommation d'alcool (OR=4,67, IC 95%=2,04-10,67, $p=0,002$), perçage cutané (OR=32,99, IC 95%=5,95-72,37, $p<0,0001$), partenaires sexuels multiples (OR=4,03, IC 95%=1,7-9,6, $p=0,0018$) et les antécédents de transfusion sanguine (OR=8,00, IC à 95 %=2,97-21,58, $p<0,001$) étaient des facteurs de risque significativement associés à l'infection par le VHC chez les participants à l'étude.
Conclusion: Les résultats de 15,0 % et 9,5 % de prévalence de l'infection par le VHC par les anticorps anti-VHC et l'ARN du VHC, respectivement dans cette étude, ont montré qu'il existe une prévalence relativement élevée de l'infection par le VHC parmi la population étudiante de l'Université de Calabar, au Nigéria. Par conséquent, un dépistage médical de routine des étudiants pour l'infection par le VHC à l'aide de techniques rapides de TIC et de RT-PCR est recommandé.

Mots-clés: Virus de l'hépatite C; Prévalence; ELISA; RT-PCR; Calabar

Introduction:

Hepatitis C virus (HCV) infection is a serious and major health threat viral disease of the liver that is of global issue and concern (1). Historically, HCV was identified by Choo and his co-workers in 1989 when a positive stranded RNA virus was isolated from the serum of an individual with non-A, non-B hepatitis in the USA, after molecular cloning and was designated HCV (2). Hepatitis C is a liver disease caused by HCV and is a major cause of chronic liver diseases including cirrhosis (60-85%), liver cancer (hepatocellular carcinoma) and lymphomas in humans (3,4,5).

HCV is a member of the genus *Hepacivirus* of the family *Flaviviridae*, and is an enveloped, positive-sense, single-stranded RNA virus measuring 55-65nm in size (6,7). HCV can cause both acute and chronic hepatitis. Acute hepatitis C is usually asymptomatic that does not lead to a life-threatening case, and 15-45% of infected persons clear the virus within 6 months without treatment while about 55-85% of persons will develop chronic HCV infection which will put them at a risk of 15%-30% of developing cirrhosis within 20 years (8,9). It is estimated that as at 2015, 170 million people have chronic hepatitis C virus infection globally and those that are chronically infected will develop cirrhosis or liver cancer (3).

The diagnosis of HCV infection is rare during the acute phase because at this stage it is asymptomatic and can remain like that for a period of 6 months before symptoms develop or serious liver damage is observed (10). Hepatitis C diagnosis is based on the detection of both anti-HCV antibodies and HCV RNA in the presence of biological or histological signs of chronic hepatitis (11). A molecular-based testing for HCV RNA is carried out to confirm the presence of active HCV infection when anti-HCV antibodies are detected (12). There are many diagnostic techniques used in the diagnosis and genotyping of HCV which include HCV enzyme immunoassay (ELISA), quantitative HCV-RNA polymerase chain reaction (PCR), recombinant immunoblot assay-3 (RIB A-3) and the most recent, immunochromatographic (ICT)-based rapid tests (13).

There is currently no effective vaccine available for HCV (12), but the disease is treated based on therapy with interferons and four classes of direct acting antivirals (DAAs). The four classes include; protease inhibitors targeting NS3/5 protein, nucleoside polymerase inhibitors (NPIs) targeting NS5B proteins, non-nucleoside polymerase inhibitors (NNPIs) targeting NS5B protein and NS5A inhibitors (14, 15). The ultimate goal of HCV treatment is to achieve significant sustained virologic response (SVR) rate by interfering with HCV replication (16). Therefore, this study was conducted to determine the prevalence of anti-HCV antibodies and HCV RNA among students of the University of Calabar.

Materials and method:

Study setting, design and participants:

This was a cross-sectional descriptive study of 200 randomly selected students conducted between May and November 2023 to determine the prevalence of HCV infections among students of the University of Calabar, Nigeria

Ethical consideration:

Ethical approval was obtained from the Health Research Ethical Committee of the University of Calabar Teaching Hospital. In addition, individual consent was dully obtained before enrollment.

Data and sample collection:

Blood samples were collected from the selected participants for HCV detection by serology and PCR assay. The blood samples were centrifuged at 2000 rpm for 2 minutes and the sera were taken and stored in cryo-

vials in the refrigerator (2-8^{0}C) for further analysis. A part of the sera was transported on frozen ice pack to a DNA laboratory in Bayelsa State, Nigeria, for molecular analysis.

A structured questionnaire was interviewer-administered to obtain socio-demographic data and potential risk factors such as history of blood transfusion, alcohol consumption, smoking habit, sexual life style, and skin piercing/tattoos.

Detection of antibodies to HCV:

Antibodies to HCV (anti-HCV Ig) was detected using rapid immunochromatographic (ICT) diagnostic test kit (CTK Biotech, Inc., USA). This test is a lateral flow ICT for the qualitative detection of antibodies to HCV in the human blood. This detection card applies the principle of indirect gold immunochromatographic (GICA) method. The detection zone of nitrocellulose membrane is coated with mouse anti-human monoclonal antibody, and the glass fibre paper is pre-coated with gold-labeled natural HCV antigen.

The reagent and sample were adjusted to room temperature (28±2^{0}C) before use according to manufacturer's instruction. The detection card was removed and placed on flat and clean table, 5µl of sample was added to the sample well and 2-3 drops (100-150µl) of sample diluents were added, the result was observed within 15-20 minutes. The samples were carried out upward chromatography by capillary effect after being added into the sample well of the detection card. If there is a certain concentration of HCV antibody in sample, the antibody will combine with the gold-labeled natural HCV antigen on the detection line (T-line). Gold-labeled antigen-antibody complex will accumulate in the detection area and indicates a red line. Any shade of red line in the detection area (T-line) as well as control area (C-line) was taken as positive. If little or no HCV antibody exists, red line will not appear in the detection area (T-line) but only in the control area (C-line) and it was taken as negative.

The quality control area (C-line) on the detection card is the standard reference to determine whether the chromatography is normal and the detection system is effective. The red line is expected to appear on the C-line under all conditions, otherwise the result is considered invalid and re-test will be required. The result is invalid after 30 minutes of the test.

HCV RNA extraction:

HCV RNA was extracted from the samples using the ZYMO Quick RNA MiniPrep extraction kit. Two hundred microliters of the blood samples were mixed with equal volume of the RNA shield in a 1.5 ml micro-centrifuge tube, and 400µl of RNA buffer was added, and mixed thoroughly. The mixture was transferred into IC spin column placed in a collection tube and spun at 12000 rpm. The flow through in the collection tube was discarded and the spin column was placed back in the same collection tube and 200µl of pre-wash buffer was added and spun at 12000 rpm, followed by 500µl of wash buffer and spun at 12000 rpm. The spin column IC was transferred to a clean 1.5 ml micro-centrifuge tube, 15 µl of elution buffer was added and spun at 12000 rpm. The eluted RNA was immediately converted to cDNA using Tehe Biolabs Reverse Transcriptase kit following the manufacturer's instructions.

Reverse transcription:

Reverse transcription (first strand cDNA synthesis) of the extracted RNA was performed using One Taq® RT-PCR Kit (New England BioLabs Inc.). The following components, 1µl Template RNA, 2µl of Random Primer Mix (60µM), M-MuLV Reaction Mix (2X), M-MuLV Enzyme Mix (10X) and Nuclease-free water were mixed together to a total volume of 20µl assay, and the Random Primer Mix was used, the mixture was first incubated at 25^{O}C for 5 minutes before the 42^{O}C incubation for 1 hour to synthesize the first strand cDNA.

PCR amplification:

Amplification of the gene was carried out in a 2700ABI thermal cycler using the primer pair HCVF: 5'-HEX-AAGGACCCGGTCGTC CT-3' and HCVR: 5'-CCGGTTCCGCAGACCACT-3' in a final volume of 25µl. The following components were mixed in a PCR tube on ice rack, 12.5µl 2x Master Mix, 0.5µl 0.5µM Forward Primer, 0.5µM Reverse Primer, 5µl of Diluted cDNA and 1.5µl of water. The PCR cycling conditions were as follows; initial denaturation at 95^{O}C for 3mins and 35 cycles of denaturation at 30secs, annealing at 55^{O}C for 30secs, and extension at 72^{O}C for 1min. The products were resolved on 1% agarose gel electrophoresis and visualized on a blue light transilluminator for a 500bp amplicon band using a 100 base pair as molecular ladder.

Statistical analysis:

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20.0 for windows (Inc., Chicago, IL) and presented as percentages and frequencies whereas differences in proportions were compared using Chi-square test. Odds ratio (OR) with 95% confidence interval (CI) was used to measure the predictive risk factors independently associated with HCV infection, and *p* value was considered significant if < 0.05.

Results:

A total of 200 participants, made of

136 (68.0%) males and 64 (32.0%) females, were recruited into the study. The prevalence of HCV infection using rapid ICT and PCR methods is shown in Table 1. The seroprevalence of anti-HCV was 15.0% (n=30), with females (18.8%, 12/64) more infected than their male counterpart (13.2%, 18/136), although the difference was not statistically significant (p=0.079). A total of 19 participants were positive by RT-PCR, giving a prevalence of HCV infection of 9.5% (19/200), with 8.8% in males and 10.9% in females (p=0.036) (Table 1). The age group with the highest rate of HCV infection by both rapid ICT and PCR techniques was 20-29 years, but there was no statistically significant difference in prevalence with respect to age group (p=1.22) (Table 1).

The result presented in Table 2 shows the association between seroprevalence of HCV infections and some selected risk factors among the study participants in the study area. Out 200 participants, 71 students consumed alcohol regularly but 20 (28.2%) had HCV infections while 129 students do not consumed alcohol but 10 (7.7%) had HCV infections. Thirteen students who indulged in cigarette smoking had HCV seroprevalence of 30.8% (n=4), while 186 students who do not indulge in cigarette smoking had HCV seroprevalence of 13.9% (n=26).

Fourteen students who indulged in the act of skin piercing/tattooing had HCV seroprevalence of 71.4% (n=10) while 186 who do not indulge in skin piercing/tattooing had HCV seroprevalence of 10.8% (n=20). A total of 91 students with multiple sex partners had HCV seroprevalence of 24.2% (n=22) while 109 who do not have multiple sex partners had HCV seroprevalence of 7.3% (n=8). Twenty students who had received blood transfusion had HCV seroprevalence rate of 50.0% (n=10) while those who had not received blood transfusion had HCV seroprevalence of 11.1% (n= 20). Based on marital status, 47 married participants had HCV seroprevalence of 8.5% (n=4), 148 single students had HCV seroprevalence of 16.9% (n=25) while the 4 divorced and the 1 widowed student had HCV seroprevalence of 25.0% (n=1) and 0% respectively.

The significant risk factors associated with seroprevalence of HCV infections include alcohol consumption (OR=4.67, 95% CI=2.04 -10.67, p=0.002), skin piercing (OR=32.99, 95% CI=5.95-72.37, p<0.0001), multiple sex partners (OR=4.03, 95% CI=1.7-9.6, p=0.0018), and history of blood transfusion (OR=8.00, 95% CI=2.97-21.58, p<0.001), while smoking (OR=2.57, 95% CI=0.79-9.6, p=0.2131) and marital status (x^2=2.46, p=0.483) were not significantly associated with HCV infection (Table 2).

Table 1: Prevalence of hepatitis C virus infection by rapid ICT and RT-PCR with respect to gender and age groups of participants

Variables	Total no of participants tested	No (%) of positive participants by rapid ICT	No (%) of positive participants by PCR	Statistical analysis
Gender				
Males	136	18 (13.2)	12 (8.8)	x^2= 3.84, df=1, p=0.036
Females	64	12 (18.8)	7 (10.9)	
Total	**200**	**30 (15.0)**	**19 (9.5)**	
Age group (years)				
20 -29	75	13 (17.3)	10 (13.3)	
30 -39	58	10 (17.2)	6 (10.3)	x^2=5.99, df=2, p=1.22
40-49	41	4 (9.8)	2 (4.9)	
50 -59	26	3 (11.5)	1 (3.8)	
Total	**200**	**30 (15.0)**	**19 (9.5)**	

ICT = immunochromatographic test; RT-PCR= Reverse transcriptase-polymerase chain reaction[i]

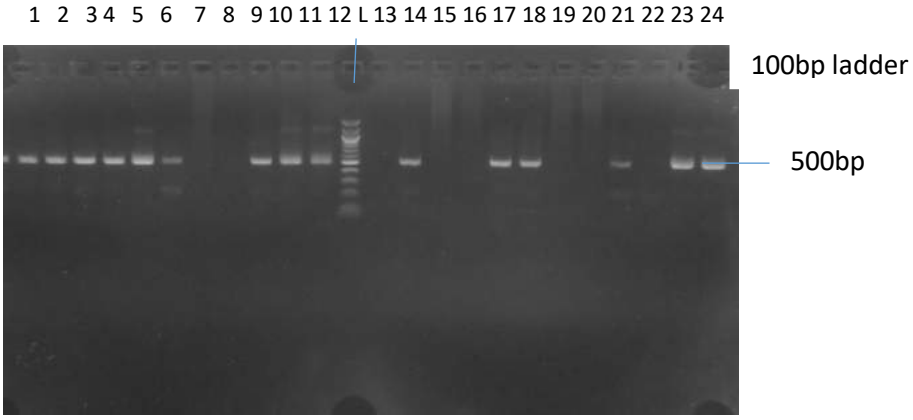
Table 2: Bivariate analysis of selected risk factors for sero-prevalence of hepatitis C virus infections in the study participants

Risk factors		**No of participants**	**No (%) of sero-positive participants**	x^2	**OR (95% CI)**	***p*-value**
Alcohol consumption	Yes	71	20 (28.2)	13.414	4.67 (2.04-10.67)	0.0002*
	No	129	10 (7.8)			
Smoke cigarette	Yes	13	4 (30.8)	1.550	2.75 (0.79-9.60)	0.2131
	No	187	26 (13.9)			
Skin piercing	Yes	14	10 (71.4)	20.75	32.99 (5.95-72.37)	<0.0001*
	No	186	20 (10.8)			
Multiple sex partners	Yes	91	22 (24.2)	9.75	4.03 (1.70-9.60)	0.0018*
	No	109	8 (7.3)			
Have had blood transfusion	Yes	20	10 (50.0)	18.41	8.00 (2.97-21.58)	<0.0001*
	No	180	20 (11.1)			
Marital status						
Married		47	4 (8.5)	2.46	NA	0.483
Single		148	25 (16.9)			
Divorced		4	1 (25.0)			
Widowed		1	0 (0.0)			

x^2 = Chi square; OR = Odd ratio; CI = Confidence interval; NA = Not applicable; * = statistically significant at $p<0.05$

Fig 1 shows agarose gel electrophoresis of the C100 protein gene of HCV, the amplicons obtained from the amplification of HCV RNA of the samples studied. The 5' UTR of the HCV genome region with 100bp was amplified using PCR method. A total of 19 samples were PCR positive.

Table 3 shows the diagnostic performance of rapid ICT for HCV detection in comparison to PCR assay. The sensitivity of rapid ICT was 100.0%, specificity 93.0%, positive predictive value 63.0%, negative predictive 100% and false discovery rate of 36.0%.



Lanes 1-7, 10-12, 14, 17, 18, 21, 23 and 4 represent the C100protein gene bands while lane L represents the 100bp molecular ladder.

Fig 1: Agarose gel electrophoresis of the amplified C100 protein gene of HCV

Table 3: Diagnostic performance of rapid ICT for HCV detection in comparison with PCR assay

Rapid ICT	**PCR**		**Total**
	Positive	Negative	
Positive	19 (TP)	11 (FP)	**30**
Negative	0 (FN)	170 (TN)	**170**
Total	**19**	**181**	**200**

TP = True positive = number positive according to both PCR and rapid ICT
FN = False negative = number positive for PCR and negative by rapid ICT
FP = False positive = number positive for rapid ICT and negative for PCR
TN = True negative = number negative according to both PCR and rapid ICT
Sensitivity of HCV rapid ICT = TP/(TP + FN) = 19/ (19 + 0) = 19/19 = 100%
Specificity of HCV rapid ICT = TN/(FP + TN) = 170/(11 + 170) = 170/181 = 93.9%
Positive predictive valve (PPV) of HCV rapid ICT = TP/(TP + FP) = 19/(19 + 11) = 19/30 = 63%
Negative predictive value (NPV) of HCV rapid ICT = TN/(TN + FN) = 170 /(170 + 0) = 100%
False Discovery rate (FDR) = FP/(FP + TP) = 11/(11 + 19) = 11/30 = 36%

Discussion:

Hepatitis C virus infects 170 million persons worldwide and is a public health problem considering that HCV is principally transmitted by exposure to infected blood (3). Multi-transfused patients constitute one of the most important risk groups in developing countries. The prevalence and molecular detection of HCV infections was performed on students of the University of Calabar after administration of questionnaires and preformed consents.

The overall prevalence of HCV in this study is 9.5% (19/200) which is lower than the prevalence reported by Okafor et al., (17) in the study done on HCV infection and its associated factors among prisoners in a Nigerian prison, where 29.6% (42/142) inmates were seropositive for HCV. However, it is higher than the prevalence of 1.4% (7/500) reported by Ogefere et al., (18) in the study of potential risk factors and seroprevalence of HCV infection among students of a tertiary institution in southern Nigeria and also in the study by Nwokedi et al., (19) on HCV infection among teaching hospital patients in Kano, Nigeria.

Gender stratification in our study showed that 13.2% of males and 18.8% of females were seropositive for HCV. This finding disagrees with that of Umumaranungu et al., (20) where males (31.0%) were more infected than females (15.4%), but agrees with that of Nwokedi et al., (19) which reported higher HCV infection in females (7.4%) than males (5.6%). The prevalence of HCV infection by age of participants showed that the age group 20-29 years had higher prevalence (though not statistically significant) of 17.3% for RDTs and 13.3% for PCR methods. The age bracket (20-29 years) is the most sexually active age as HCV is sexually transmitted. This study found a statistically significant association between HCV infection and multiple sex partners. This finding agrees with that of Ogefere et al., (18), where age groups of 21-24 and 17-20 years had the highest HCV infection rates respectively but disagrees with the finding of Nwokedi et al., (19), which reported higher HCV infection (10.1%) in the age group 31-40years.

The prevalence of HCV infection by PCR method in this study is 9.5% (19/200), which is in agreement with the study of Karoney and Siika (21), who reported prevalence of 9.6% (31/152) for HCV RNA using PCR method in patients attending Rwanda military hospital, but disagrees with Iduh et al., (22) who reported higher prevalence (20.5%, 9/44) of HCV RNA using PCR method in Niger State, Nigeria and Sheyin et al., (23), who reported lower prevalence (4.5%) of HCV RNA using PCR method amongst pregnant women in Kaduna State, Nigeria.

The prevalence of HCV RNA by age of participants showed that the age group 20-29 years had higher prevalence of 13.3% (10/75) and age group of 50-59 years had lower prevalence of 3.8% (1/26). These findings differ from the studies of Sheyin et al., (23) who reported higher prevalence in age group 31-40 years. The prevalence of HCV RNA by gender of participants showed that females (10.9%, 7/64) had higher prevalence compared to males (8.8%, 12/136). This study disagrees with the work of Karoney and Siika (21) which reported high prevalence of 9.8% (3/31) in males and low prevalence of 9.4% (18/31) in females.

This current study showed that not all seropositive HCV samples can be positive for HCV RNA (PCR), which is in agreement with the reports of Karoney and Siika (21) and Mora et al., (25) but disagrees with that of Pawlotsky (24). Only 19 of 30 HCV seropositive participants in our study were HCV RNA (PCR) positive. This disparity could be attributed to factors such as low HCV nucleic acid concentration in clinical samples, infection clearance, improper storage of test kits and cross-reactivity (25,26). HCV RNA (PCR) positive samples may imply that the individual has an active or ongoing infection and there is active viremia. The detection of HCV RNA by PCR always gives direct detection of the presence of the virus and also detection during seronegative window period immediately after infection. Using PCR to detect HCV RNA is more reliable and sensitive than rapid ICT in persons with impaired immune system (26).

Among the potential risk factors studied, skin piercing/tattooing, history of blood transfusion, multiple sexual partners, and alcohol consumption were significantly prevalent among students who tested positive to HCV antibodies. This implies that there is a significant contribution to the disease burden by these unwholesome practices. These findings corroborated previous studies, Ejiofor et al., (27) reported that skin piercing/tattooing is significantly associated with HCV infection among children in Enugu. Similarly, multiple sex partners, alcohol abuse and history of blood transfusion are critical in HCV transmission and could be classified as deferral criteria for blood transfusion (28,29).

Conclusion:

The prevalence of HCV infections among University of Calabar Students was 15.0% for HCV antibody and 9.5% for HCV RNA PCR. The results of this study showed that female students had significantly higher prevalence of HCV infection than male students. Skin piercing/tattoos, history of blood transfusion, multiple sexual partners, and alcohol consumption

are the most prevalent associated risk factors. There is need for awareness campaign on the possible transmission of HCV infection among the human populace. Also, behavioral change among students on sex, alcohol use and safe tattooing is encouraged.

Contributions of authors:

VNO conceptualized and designed the study; collected, analyzed, and interpreted the data. MM wrote the first draft of the manuscript, critically reviewed the manuscript and gave final approval for submission of manuscript. SSA critically reviewed the manuscript, and gave final approval for submission of manuscript. EET handle the statistical analysis, and gave final approval for submission of manuscript. EEB critically reviewed the manuscript, and gave final approval for submission of manuscript. All authors have read and approved the manuscript.

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No conflict of interest is declared

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Original Article **Open Access**

Molecular detection of antimicrobial resistance genes in multidrug-resistant Gram-negative bacteria isolated from clinical samples in two hospitals in Niger

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Abstract:

Background: According to the World Health Organization (WHO), bacterial resistance to antibiotics is a global public health challenge, which is also developing in Niger. The aim of this study was to determine the prevalence of antibiotic resistance genes in Gram-negative bacilli isolated from clinical samples in the biological laboratories of two selected health facilities in Niger.
Methodology: Clinical bacterial isolates were randomly collected from two biological laboratories of Zinder National Hospital and Niamey General Reference Hospital. These were multi-resistant Gram-negative bacteria that have been routinely isolated from pathological samples of patients. Molecular detection of resistance genes was carried out by polymerase chain reaction (PCR) amplification using specific primers. These include plasmid-mediated AmpC beta lactamase genes (*bla*$_{CITM}$, *bla*$_{DHAM}$, *bla*$_{FOXM}$), 'Cefotaxime-Munich' type beta lactamase genes (*bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$), KPC-type beta lactamase gene (*bla*$_{KPC}$), Oxa-type beta lactamase gene (*bla*$_{OXA-48}$), SHV-type beta lactamase gene (*bla*$_{SHV}$), TEM-type beta lactamase gene (*bla*$_{TEM}$), quinolone resistance genes (*qnrA*, *qnrB*, *qnrS*), and sulfonamide resistance genes (*sul1*, *sul2*, *sul3*).
Results: A total of 24 strains of multidrug-resistant Gram-negative bacteria isolated from different clinical samples were analysed. The distribution of the resistance genes detected is as follows; AmpC *bla*$_{CITM}$ (n=6; 25.0%), AmpC *bla*$_{DHAM}$ (n=4; 17.0%), AmpC *bla*$_{FOXM}$ (n=0), *bla*$_{CTX-M-1}$ (n=11; 46.0%), *bla*$_{CTX-M-2}$ (n=0), *bla*$_{CTX-M-9}$ (n=0), *bla*$_{KPC}$ (n=0), *bla*$_{OXA-48}$ (n=2; 8..0%), *bla*$_{SHV}$ (n=5; 21.0%), *bla*$_{TEM}$ (n=0), *qnrA* (n=0), *qnrB* (n=5; 21.0%), *qnrS* (n=17; 71.0%), *sul1* (n=22; 92.0%), *sul2* (n=12; 50.0%), and *sul3* (n=0). All isolates tested had at least two resistance genes.
Conclusion: The results of this study provide a better understanding of the resistance situation of clinical isolates in Niger. Therefore, it is more than necessary to intensify the detection on a larger number of samples and on a national scale. This will make it possible to assess the true extent of the phenomenon and consequently guide control strategies through a national multisectoral plan.

Keywords: Gram-negative bacilli, Resistance genes, PCR, Hospitals, Niger.

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Détection moléculaire de gènes de résistance aux antimicrobiens chez des bactéries Gram-négatives multirésistantes isolées à partir d'échantillons cliniques dans deux hôpitaux du Niger

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Résumé:

Contexte: Selon l'Organisation Mondiale de la Santé (OMS), la résistance bactérienne aux antibiotiques constitue un défi mondial de santé publique, qui se développe également au Niger. Le but de cette étude était de déterminer la prévalence des gènes de résistance aux antibiotiques chez les bacilles Gram négatif isolés à partir d'échantillons cliniques dans les laboratoires de biologie de deux formations sanitaires sélectionnées au Niger.
Méthodologie: Des isolats bactériens cliniques ont été collectés de manière aléatoire dans deux laboratoires de biologie de l'Hôpital National de Zinder et de l'Hôpital Général de Référence de Niamey. Il s'agissait de bactéries Gram-négatives multirésistantes qui ont été systématiquement isolées à partir d'échantillons pathologiques de patients. La détection moléculaire des gènes de résistance a été réalisée par amplification par réaction en chaîne par polymérase (PCR) à l'aide d'amorces spécifiques. Il s'agit notamment des gènes de bêta-lactamase AmpC à médiation plasmidique (*bla*$_{CITM}$, *bla*$_{DHAM}$, *bla*$_{FOXM}$), des gènes de bêta-lactamase de type «Céfotaxime-Munich» (*bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$), du gène de bêta-lactamase de type KPC (*bla*$_{KPC}$), du gène de bêta-lactamase de type Oxa (*bla*$_{OXA-48}$), le gène bêta-lactamase de type SHV (*bla*$_{SHV}$), le gène bêta-lactamase de type TEM (*bla*$_{TEM}$), les gènes de résistance aux quinolones (*qnrA, qnrB, qnrS*) et les gènes de résistance aux sulfamides (*sul1, sul2, sul3*).
Résultats: Au total, 24 souches de bactéries Gram-négatives multirésistantes isolées de différents échantillons cliniques ont été analysées. La répartition des gènes de résistance détectés est la suivante; AmpC *bla*$_{CITM}$ (n=6; 25,0%), AmpC *bla*$_{DHAM}$ (n=4; 17,0%), AmpC *bla*$_{FOXM}$ (n=0), *bla*$_{CTX-M-1}$ (n=11; 46,0%), *bla*$_{CTX-M-2}$ (n=0), *bla*$_{CTX-M-9}$ (n=0), *bla*$_{KPC}$ (n=0), *bla*$_{OXA-48}$ (n=2; 8,0%), *bla*$_{SHV}$ (n=5; 21,0%), *bla*$_{TEM}$ (n=0), *qnrA* (n=0), *qnrB* (n=5; 21,0%), *qnrS* (n=17; 71,0%), *sul1* (n=22; 92,0%), *sul2* (n=12; 50,0%) et *sul3* (n=0). Tous les isolats testés possédaient au moins deux gènes de résistance.
Conclusion: Les résultats de cette étude permettent de mieux comprendre la situation de résistance des isolats cliniques au Niger. Il est donc plus que nécessaire d'intensifier la détection sur un plus grand nombre d'échantillons et à l'échelle nationale. Cela permettra d'évaluer l'ampleur réelle du phénomène et par conséquent d'orienter les stratégies de lutte à travers un plan national multisectoriel.

Mots clés: Bacilles Gram négatif, Gènes de résistance, PCR, Hôpitaux, Niger.

Introduction:

Antimicrobial resistance (AMR) is a global and sub-regional problem. Today, the evolution and spread of bacterial resistance is evident from the results observed in the field (1,2). As in other West African countries, AMR is growing exponentially in Niger, and threatens patient care, economic growth, public health, agriculture and, most importantly, economic security (3).

One of the main limitations in assessing the extent of the phenomenon is the lack of data from field studies. Indeed, it is from these data that strategies to combat bacterial resistance to antibiotics should be derived, in particular data on the epidemiology of multi-resistant (MDR) bacterial strains. In this context, we set out to determine the prevalence of antibiotic resistance genes in pathogenic and MDR Gram-negative bacteria commonly isolated in healthcare facilities in Niger.

Materials and method:

Study sites:

The study was conducted at the Niamey General Reference Hospital, the Zinder National Hospital and the Centre de Recherche Sanitaire et Médicale (CERMES), Niamey.

Study type and period:

This was a laboratory-based study to detect the genes mediating resistance in MDR Gram-negative bacteria isolated from clinical samples of infected patients in two hospitals in Niger from June 2021 to December 2022. Characterization of antibiotic resistance genes was performed at CERMES, Niamey, Niger in October 2023.

Ethical approval:

The study was approved by the ethics committees of both hospitals for the use and analysis of data from medical laboratories. Anonymity and confidentiality of the data were guaranteed.

Bacterial isolates collection:

Clinical bacterial isolates were collected randomly from two biological laboratories in National Hospital in Zinder and General Reference Hospital in Niamey. The isolates were MDR Gram-negative bacteria isolated from pathological samples of infected patients for diagnostic purposes.

DNA extraction:

Genomic DNA was extracted from the various isolates using the "heat shock" method. Four to five young colonies obtained by subculture were resuspended in 200µl sterile

distilled water and placed at -20°C for at least 30 minutes. The suspension was then heated to boiling (100°C) for 10 minutes and centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant obtained was used as DNA template.

PCR amplification:

Simplex PCR assay was used. A negative control consisting of extraction water and a positive control were used for all gene amplification reactions. The 22.5µl reaction mix consisted of 12.5µl Quanta® Master, 1.5µl forward (sense) and reverse (antisense) primers (Table 1) and 7µl water. A volume of 2.5µl of DNA extract was added to the reaction mix to give a final volume of 25µl in each of the appropriate wells and a thermal cycle in a thermocycler of 95°C for 5 minutes (initial denaturation) followed by 35 cycles of 94°C for 30 sec (denaturation), a variable hybridization temperature depending on the primer for 30 sec and 72°C for 60 seconds for primer extension (Table 1).

Detection of the PCR reaction:

The PCR products were migrated on a 2% agarose gel prepared in TAE (Tris-EDTA) buffer and visualized using a Trans illuminator to show the expected sizes using a molecular weight scale (DNA molecular weight 100µg, Promega).

Table 1: Detail of the primers used in the study

Gene	Primer sequence	Hybridization temperature	Amplicon size	Reference
bla$_{TEM}$	F - ATA-AAA-TTC-TTG-AAG-ACG-AAA R - GAC-AGT-TAC-CAA-TGC-TTA-ATC-A	40.8°C	861 pb	AB282997
bla$_{SHV}$	F - CGC-CGG-GTT-ATT-CTT-ATT-TGT-CGC R - TCT-TTC-CGA-TGC-CGC-CGC-CAG-TCA	54.85°C	927 pb	X98098
bla$_{KPC}$	F - CGT-TCT-TGT-CTC-TCA-TGG-CC R - CCT-CGC-TGT-GCT-TGT-CAT-CC	63°C	798 pb	Poirel et al., (4)
bla$_{OXA-48}$	F - TTG-GTG-GCA-TCG-ATT-ATC-GG R - GAG-CAC-TTC-TTT-TGT-GAT-GGC	53.65°C	281 pb	Poirel et al., (4)
bla$_{CTX-M-1}$	F - GGT-TAA-AAA-ATC-ACT-GCG-TC R - TTG-GTG-ACG-ATT-TTA-GCC-GC	58°C	944 pb	X92506
bla$_{CTX-M-2}$	F - ATG-ATG-ACT-CAG-AGC-ATT-CG R - TGG-GTT-ACG-ATT-TTC-GCC-GC	60°C	351 pb	X92507
bla$_{CTX-M-9}$	F - ATG-GTG-ACA-AAG-AGA-GTG-CA R - CCC-TTC-GGC-GAT-GAT-TCT-C	59°C	876 pb	AF174129
AmpC *bla*$_{FOXM}$	F - AAC-ATG-GGG-TAT-CAG-GGA-GAT-G R - CAA-AGC-GCG-TAA-CCG-GAT-TGG	50.45°C	190 pb	X77455
AmpC *bla*$_{CITM}$	F - TGG-CCA-GAA-CTG-ACA-GGC-AAA R - TTT-CTC-CTG-AAC-GTG-GCT-GGC	50.2°C	465 pb	X78117
AmpC *bla*$_{DHAM}$	F - AAC-TTT-CAC-AGG-TGT-GCT-GGG-T R - CCG-TAC-GCA-TAC-TGG-CTT-TGC	50.45°C	405 pb	Y16410 AF189721
qnrA	F - TTC-TCA-CGC-CAG-GAT-TTG-AG R - TGC-CAG-GCA-CAG-ATC-TTG-AC	61°C	516 pb	AY070235
qnrB	F - TGG-CGA-AAA-AAA-TTR-ACA-GAA R - GAG-CAA-CGA-YGC-CTG-GTA-G	52°C	526 pb	DQ351241
qnrS	F - GAC-GTG-CTA-ACT-TGC-GTG-AT R - AAC-ACC-TCG-ACT-TAA-GTC-TGA	52.65°C	417 pb	DQ485529
sul1	F - CGG-CGT-GGG-CTA-CCT-GAA-CG R - GCC-GAT-CGC-GTG-AAG-TTC-CG	67°C	433 pb	AY655484.1
sul2	F - GCG-CTC-CAA-GGC-AGA-TGG-CAT-T R - GCG-TTT-GAT-ACC-GGC-ACC-CGT	53.3°C	293 pb	AY360321.1
sul3	F - GGA-AGA-AAT-CAA-AAG-ACT-CAA R - CCT-AAA-AAG-AAG-CCC-ATA-CC	49.7°C	569 pb	AJ459418.2

Data analysis:

Data were collected using a standard biological information form. Analysis was performed using Excel 2013 software and results were presented as tables and figures.

Results:

Of the 24 MDR Gram-negative bacterial isolates collected from pathological specimens, *Escherichia coli* was the most frequent accounting for 58.3% (n=14). All strains were extended-spectrum beta-lactamase (ESBL) phenotype except one, which was MDR *Pseudomonas aeruginosa* (MDRPA) (Table 2).

Prevalence of resistance genes:

The frequency of detection of resistance genes in the bacterial isolates is shown in Fig 1 with; (i) Plasmid-mediated AmpC beta lactamases: AmpC bla_{CITM} (n=6, 25.0%), AmpC bla_{DHAM} (n=4, 17.0%), and AmpC bla_{FOXM} (n=0); (ii) 'Cefotaxime-Munich' type beta lactamases: $bla_{CTX\text{-}M\text{-}1}$ (n=11, 46.0%), $bla_{CTX\text{-}M\text{-}2}$ (n=0), and $bla_{CTX\text{-}M\text{-}9}$ (n=0); (iii) KPC-type beta lactamases: bla_{KPC} (n=0); (iv) Oxa-type beta lactamases: $bla_{OXA\text{-}48}$ (n=2, 8.0%); (v) SHV-type beta lactamases: bla_{SHV} (n=5, 21.0%); (vi) TEM-type beta-lactamases: bla_{TEM} (n=0); (vii) Quinolone resistance genes: *qnrA* (n=0), *qnrB* (n=5, 21.0%), and *qnrS* (n=17, 71.0%); and (viii) Sulfonamide resistance genes: *sul1* (n=22, 92.0%), *sul2* (n=12, 50.0%), and *sul3* (n=0).

Fig 2 shows the gel electrophoresis pictures of the 16 amplified genes across the 24 bacterial isolates.

Table 2: Source and antibiotic phenotype profiles of bacterial isolates in the study

Serial No	Isolate No	Sample source	Isolate genus/species	Antibiotic phenotype
1	2245HGR	Urine	*Escherichia coli*	ESBL
2	2543HGR	Urine	*Klebsiella oxytoca*	ESBL
3	2543HGR	Urine	*Escherichia coli*	ESBL
4	2548HGR	Urine	*Escherichia coli*	ESBL
5	2559HGR	Urine	*Escherichia coli*	ESBL
6	1800HGR	Urine	*Escherichia coli*	ESBL
7	2258HGR	Urine	*Pseudomonas aeruginosa*	ESBL
8	1644HNZ	Urine	*Escherichia coli*	ESBL
9	1595HNZ	Urine	*Escherichia coli*	ESBL
10	1597HNZ	Urine	*Serratia odorifera*	ESBL
11	1567HNZ	Urine	*Escherichia coli*	ESBL
12	1215HNZ	Urine	*Pseudomonas aeruginosa*	ESBL
13	1599HNZ	Urine	*Escherichia coli*	ESBL
14	1791HNZ	Urine	*Klebsiella pneumoniae*	ESBL
15	1539HNZ	Urine	*Escherichia coli*	ESBL
16	1878HNZ	Urine	*Klebsiella oxytoca*	ESBL
17	1662HNZ	Urine	*Serratia odorifera*	ESBL
18	1601HNZ	Urine	*Pseudomonas aeruginosa*	MDRPA
19	1657HNZ	Urine	*Klebsiella oxytoca*	ESBL
20	118HGR	Pus	*Escherichia coli*	ESBL
21	148HGR	Pus	*Escherichia coli*	ESBL
22	175HGR	Urine	*Escherichia coli*	ESBL
23	166HGR	Pus	*Escherichia coli*	ESBL
24	1791HGR	Urine	*Klebsiella pneumoniae*	ESBL

ESBL: Extended spectrum b-lactamase; MDRPA: Multi-drug resistant *Pseudomonas aeruginosa*

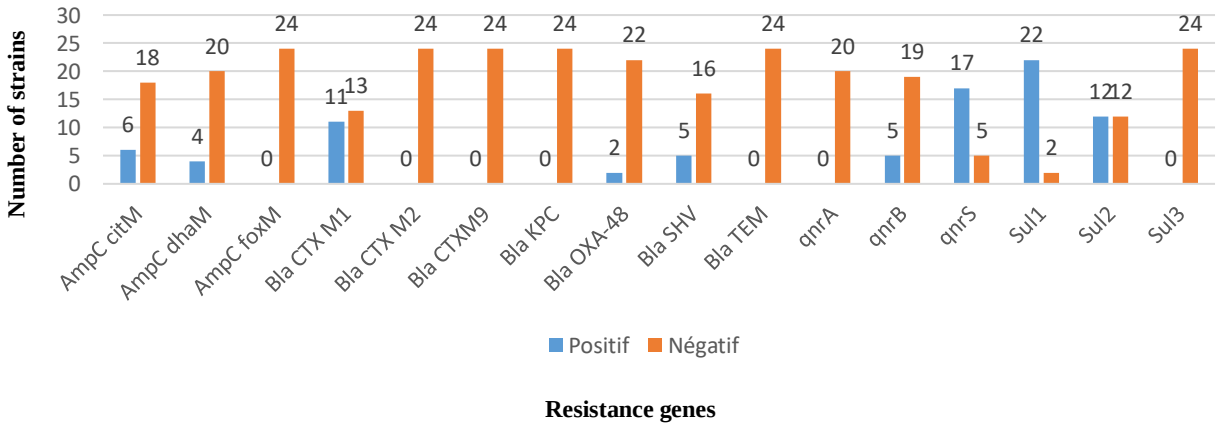
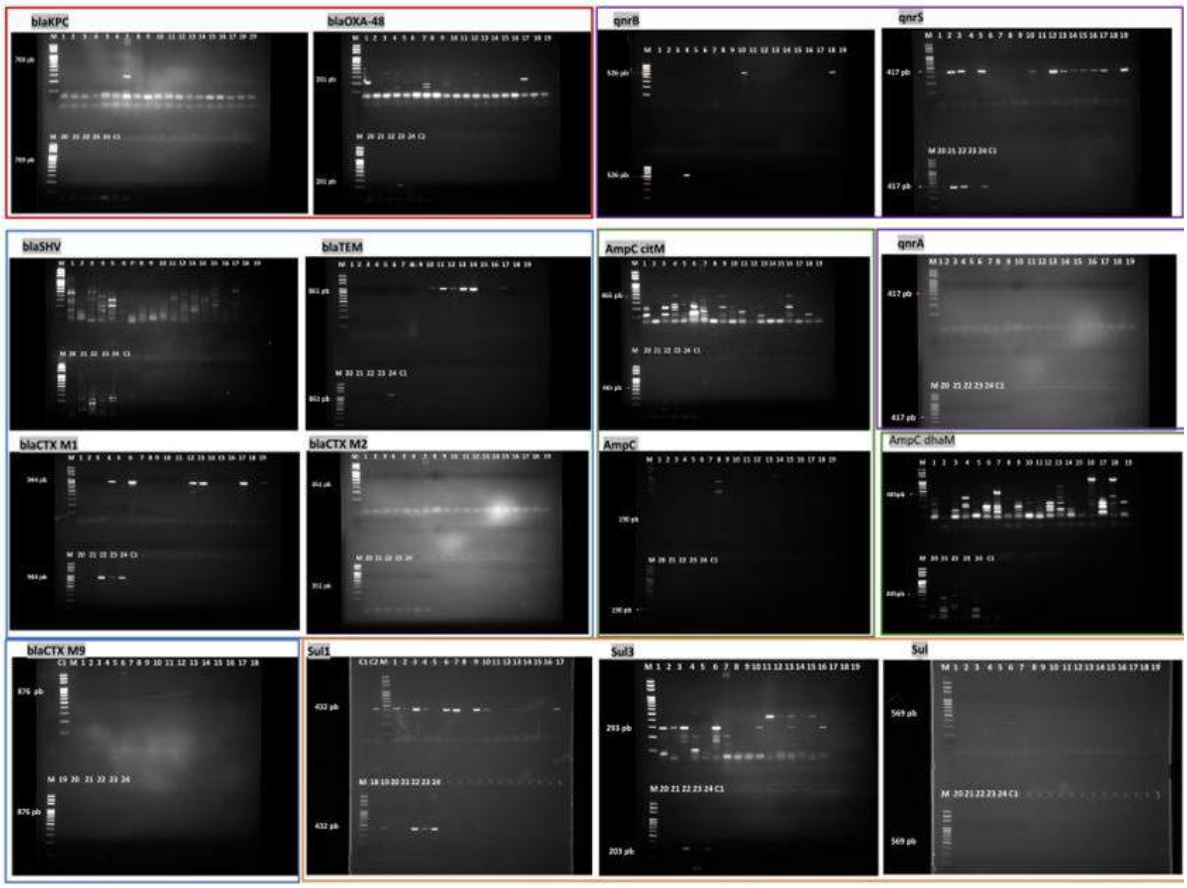


Fig 1: Prevalence distribution of the 16 resistance genes in the bacterial isolates



C1: Negative control; C2: Positive control; M: Size marker; Bacterial isolates: 1, 2, 3, 4, 5, 6; 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24.

Fig 2: Gel electrophoresis pictures of the 16 amplified genes across the bacterial isolates

Discussion:

The threat to public health posed by AMR remains a major and growing challenge, especially in Gram-negative bacteria (5). The aim of our study was to identify the genetic determinants of resistance to different families of antibiotics in pathogenic and MRD Gram-negative bacteria in the biology laboratory of the National Hospital of Zinder and the General Reference Hospital of Niamey in Niger. Of the total of 24 MDR GNB isolates from different clinical samples, all (100%) the isolates carried at least two resistance genes, confirming them to be multidrug resistant.

The AmpC bla_{CITM} gene represented 25.0% and AmpC bla_{DHAM} 17.0% of our isolates. A similar study conducted in Nigeria reported that 4.5% (6/134) of the strains studied carried plasmid-mediated AmpC variants; bla_{ACT-1}, bla_{DHA-1} and bla_{CMY-2} in 1, 4 and 1 isolates respectively (6). It should be noted that AmpC (ampicillin class C beta-lactamase) genes, in particular *MOXM, CITM, DHAM, EBCM, FOXM* and *ACCM*, are responsible for the development of broad-spectrum resistance to most β-lactams except cefepime and carbapenems (7).

CTX-M-type β-lactamases are among the most common and widespread ESBLs, mainly found in Enterobacteriaceae (8). Although discovered later than TEM- and SHV-type ESBLs, it is now clear that CTX-M-type β-lactamases play an important role as emerging resistance determinants in Enterobacteriaceae (8,9). In our study, the gene encoding CTX-M-1-type β-lactamases ($bla_{CTX-M-1}$) was found in almost half of the isolates (46.0%, n=11). The study conducted in Nigeria by Ogbolu et al., (6) reported comparably lower rate of 18.7% (25 out of 134 isolates). In addition, the study by Bonnet (10) reported a worldwide distribution of these enzymes. In a study conducted in central Iran, Abbassi et al., (11) also reported that carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains contained $bla_{CTX-M-1}$ (100.0%) and $bla_{CTX-M-15}$ (96%) genes. Similar results to ours were also reported by Omneya and Mona (9) in Egypt, where the ESBL resistance genes $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ were detected in 60.0% and 24.0% isolates, respectively.

In our study, the bla_{SHV} was seen in 5 out of the 24 (21.0%) isolates tested, mainly in *E. coli*, *S. odorifera*, *Pseudomonas* spp and *K. pneumoniae*. A relatively low prevalence

(6.7%, 30/128) was reported by Socohou et al., in Benin (12). However, Codjoe et al., (13) reported that all carbapenemase-producing strains (100.0%) carried the *bla*$_{TEM}$ and *bla*$_{SHV}$ ESBL genes in their study conducted in Ghana. The *bla*$_{OXA-48}$ gene was found in our study with a frequency of 8.0% (2/24). These results are comparable to those reported by Elbadawi et al., (14) in Sudan and Dikoumba et al., (15) in Gabon with frequencies of 2.4% (5/121) and 40%, respectively. In 2014, Dortet et al., (6) reported that OXA-48-like enzymes accounted for 86.0% of a panel of more than 1,000 French carbapenemase-producing Enterobacteriaceae (EPC) strains, well ahead of the New Delhi metallo-beta-lactamases (NDM) of 8%.

In South Africa, a review of the literature on antibiotic resistance reported that the NDM-1 and OXA-48 genotypes were the most common in Gram-negative bacteria involved in clinical infections (7). This *bla*$_{OXA-48}$ gene, which belongs to class D of the Ambler class has hydrolytic activity towards penicillins and carbapenems, including imipenem, ertapenem and meropenem, but little or no activity towards third-generation cephalosporin (8). Given this diversity of carbapenemase genes, it is now important to know the specific gene produced by an EPC isolate in order to guide therapeutic decisions and avoid therapeutic impasse. Fortunately, cefiderocol is effective against all carbapenemases, making it the last therapeutic option in the treatment of difficult-to-treat GNB infections. Cefiderocol has demonstrated clinically relevant success rates and reduced renal risk (19).

In our study, plasmid-mediated quinolone resistance gene, *qnrS*, was seen in 71.0% of the clinical isolates (17/24) and *qnrB* in 21.0% (5/24) but no *qnrA* gene was observed. Presumably, the overuse of quinolones in our context has contributed to this high prevalence rate compared with similar studies conducted in hospitals in the subregion such as Côte d'Ivoire (20), Cameroon (21), Nigeria (6), and Togo (22). Our results on the prevalence of quinolone resistance genes thus confirm previous work carried out in Niger. Indeed, Illa et al., (23) had reported prevalence rates of 18.75% for the *qnrB* gene and 6.25% for *qnrS* gene in a study of antibiotic-resistant *Salmonella* and *Shigella*. Moumouni et al., (24) also reported rates of 9.5% (4/78) for *qnrA* gene, 26.2% (11/78) for *qnrB* and 64.3% (27/78) for *qnrS* in 2016.

Type 1 and 2 sulfa resistance genes (*sul1* and *sul2*) were observed in almost all the isolates, with *sul1* in 92.0% (22/24) and *sul2* in 50.0% (12/24) but no *sul3* was seen. These results are similar to those reported by Frank et al., (25) in Central Africa with *sul1* (67/78), sul2 (72/78) and both genes (62/78) and that reported by Dahmen et al., (26) in Tunisia with *sul1* (22/80), *sul2* (5/80) and both genes in 49/80.

Conclusion:

Our study, although preliminary, shows the importance of research into resistance genes for the various antibiotics we use routinely. Other rarely used antibiotics have been the subject of resistance when they should not have been. However, it is important to ensure the rational and appropriate use of medicines in general and antibiotics in particular. Antibiotic resistance is a real and growing problem that will reach dramatic proportions if we are not careful. In Africa, this problem is particularly acute, given the ubiquitous availability of low-quality and low-cost antibiotics. Unfortunately, the failure of healthcare workers to follow prescribing guidelines also contributes to the emergence of resistance.

In Niger, more widespread detection is needed to create a global map of antibiotic resistance. The environment and animals will not be left out of the "One Health" strategy.

Contributions of authors:

All authors contributed equally to the study design, methodology, manuscript preparation and approval of the original version submitted for publication.

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Original Article **Open Access**

Urinary tract infections in pregnancy caused by carbapenem-resistant *Enterobacteriaceae* in University of Calabar Teaching Hospital, Nigeria: An emerging therapeutic threat

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Abstract:

Background: Severe infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE) have mortality rate exceeding 50%. On the strength of this, this study sought to determine the prevalence of urinary tract infection (UTI) in pregnancy caused by CRE and associated risk factors in University of Calabar Teaching Hospital (UCTH), Nigeria, with the aim of making recommendations that can stem the tide of UTI caused by this bacterial strain in the hospital.
Methodology: This was a descriptive cross-sectional study of 349 consecutively selected pregnant women attending antenatal clinic of UCTH, Calabar, Nigeria, between September 2020 and August 2021. Demographic/ clinical data and risk factors were collected with semi-structured interviewer-administered questionnaire. Voided mid-stream urine (MSU) sample was collected from each participant and transported to the medical microbiology laboratory of the hospital for microbiological analysis using conventional culture and biochemical identification methods. Antimicrobial susceptibility test (AST) on each isolate was performed by the disk diffusion technique against selected antibiotics. Phenotypic carbapenemase production from presumptive carbapenem resistant isolates following AST was confirmed by the modified Hodge test (MHT). Data analysis was done on SPSS version 19.0. Association of risk factors with prevalence of UTI caused by CRE was determined using Chi square or Fisher Exact test, with $p<0.05$ considered statistically significant.
Results: The prevalence of UTI among the pregnant women was 10.0% (35/349), with prevalence of 6.6% for *Escherichia coli* (23/349) and 3.5% (12/349) for *Klebsiella pneumoniae*. Antibiotic susceptibility test result showed that piperacillin-tazobactam was the most active antibiotic *in vitro*, with 82.9% isolates sensitive to it while sensitivity to imipenem (60.0%) and meropenem (40.0%) was low. A total 17 (48.6%) of the 35 isolates were resistant to carbapenems in the AST and 12 (34.3%) were carbapenemase-producing strains on MHT while 5 (14.3%) were non-carbapenemase-mediated resistance (NCMR). None of the demographic characteristics or risk factors analysed was significantly associated with UTI caused by CRE in the pregnant women ($p>0.05$).
Conclusion: To stem the rising trend of UTIs in pregnancy caused by carbapenem resistant uropathogens, pregnant women receiving antenatal care in UCTH, Calabar should be routinely screened for UTI and offered appropriate treatment if indicated based on microbiological test results

Keywords: UTI; Pregnancy, *Enterobacteriaceae*, carbapenem-resistant, emerging threat

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Infections des voies urinaires pendant la grossesse causée par des *Entérobactéries* résistantes aux carbapénèmes à l'hôpital universitaire de Calabar, Nigéria: une menace thérapeutique émergente

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Résumé:

Contexte: Les infections graves causées par des entérobactéries résistantes aux carbapénèmes (CRE) ont un taux de mortalité supérieur à 50 %. Forte de ces éléments, cette étude a cherché à déterminer la prévalence des infections des voies urinaires (IVU) pendant la grossesse causée par la CRE et les facteurs de risque associés à l'hôpital universitaire de Calabar (UCTH), au Nigeria, dans le but de formuler des recommandations qui peuvent endiguer la marée d'infections urinaires causées par cette souche bactérienne à l'hôpital.
Méthodologie: Il s'agit d'une étude transversale descriptive portant sur 349 femmes enceintes sélectionnées consécutivement fréquentant la clinique prénatale de l'UCTH, Calabar, Nigeria, entre septembre 2020 et août 2021. Les données démographiques/cliniques et les facteurs de risque ont été collectés à l'aide d'un questionnaire semi-structuré administré par un intervieweur. Un échantillon d'urine mi-jet (MSU) a été collecté auprès de chaque participant et transporté au laboratoire de microbiologie médicale de l'hôpital pour analyse microbiologique à l'aide de méthodes de culture et d'identification biochimiques conventionnelles. Le test de sensibilité aux antimicrobiens (AST) sur chaque isolat a été réalisé par la technique de diffusion sur disque contre des antibiotiques sélectionnés. La production phénotypique de carbapénémase à partir d'isolats présumés résistants aux carbapénèmes après AST a été confirmée par le test de Hodge modifié (MHT). L'analyse des données a été effectuée sur SPSS version 19.0. L'association des facteurs de risque avec la prévalence des infections urinaires causées par la CRE a été déterminée à l'aide du test du Chi carré ou de Fisher Exact, avec p <0,05 considéré comme statistiquement significatif.
Résultats: La prévalence des infections urinaires chez les femmes enceintes était de 10,0% (35/349), avec une prévalence de 6,6% pour *Escherichia coli* (23/349) et de 3,5% (12/349) pour Klebsiella pneumoniae. Les résultats du test de sensibilité aux antibiotiques ont montré que l'association pipéracilline-tazobactam était l'antibiotique le plus actif in vitro, avec 82,9% des isolats qui y étaient sensibles, tandis que la sensibilité à l'imipénème (60,0%) et au méropénème (40,0%) était faible. Au total, 17 (48,6%) des 35 isolats étaient résistants aux carbapénèmes dans l'AST et 12 (34,3%) étaient des souches productrices de carbapénémase sur MHT, tandis que 5 (14,3%) étaient des souches non médiées par la carbapénémase (NCMR). Aucune des caractéristiques démographiques ou des facteurs de risque analysés n'était associée de manière significative aux infections urinaires causées par la CRE chez les femmes enceintes (p>0,05).
Conclusion: Pour endiguer la tendance croissante des infections urinaires pendant la grossesse causée par des uropathogènes résistants aux carbapénèmes, les femmes enceintes recevant des soins prénatals à l'UCTH de Calabar devraient être systématiquement dépistées pour les infections urinaires et se voir proposer un traitement approprié si cela est indiqué sur la base des résultats des tests microbiologiques.

Mots-clés: IVU; Grossesse; *Enterobacteriaceae*; résistantes aux carbapénèmes; menace émergente

Introduction:

The rapid spread of multidrug-resistant bacteria has become a public health concern, particularly in countries where the spread of carbapenem-resistant microorganisms is endemic (1). Severe infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE) have mortality rate exceeding 50% (2-4). As part of their virulent factors, microorganisms causing urinary tract infections (UTIs) produce enzymes that hydrolyze therapeutic agents rendering them ineffective for treatment of conditions related to them. Carbapenemases production is one of such mechanisms of antibiotic resistance amongst species of *Enterobacteriaceae* and Gram-negative non-fermenters such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (5), which are common isolates of UTIs.

Urinary tract infections in pregnancy are common worldwide and if untreated can lead to poor perinatal and maternal outcomes (6). A combined effects of hormonal and physiological changes occurring during pregnancy predispose pregnant women to UTIs. Carbapenemases constitute a group of β-lactam hydrolyzing enzymes that break down the carbapenem class of antibiotics. This class of antibiotics has broad spectrum activities and is reserved for the treatment of serious infections arising from multidrug resistant organisms (7). The carbapenem class of antibiotics is readily the drugs of choice for the treatment of infections caused by Gram-negative bacteria producing extended spectrum β-lactamases (ESBLs), a group of enzymes that hydrolyzes third generation cephalosporins and aztreonams but inhibited by β-lactamases inhibitor antibiotics such as clavulanic acid (8).

There is association between ESBL-production and resistance to other classes of antibiotics (9). This turn of events further causes serious therapeutic difficulties in the management of infections caused by ESBL- producing organisms which often culminates in poor treatment outcomes. Persistence of this trend of carbapenem resistance will further compound the problem associated with antibiotic management of serious infections caused by ESBL-producing Gram-negative bacteria. This study is intended to determine the prevalence

of UTI caused by CRE and other Gram-negative bacteria, and associated risk factors, with the aim of promoting and implementing prevention and control measures in the wards and antenatal clinic (ANC) of the hospital.

Materials and method:

Study design, setting and period:

This was a descriptive cross-sectional study carried out among pregnant women attending the ANC of the University of Calabar Teaching Hospital (UCTH), Calabar, Cross River State, Nigeria between 22nd September, 2020 and 14th August, 2021. Cross River State is located in the south-south geopolitical zone of Nigeria and embedded in the Niger Delta region. In 2015, it has a total projected of 3,756,403 and a population density of 170 person/Km^2. It has component units of 18 local government areas. The main occupation of the people includes farming. fishing and petty trading except for those in the few urban towns who are either civil servants or artisans.

The UCTH is a 260-bed tertiary facility located in Calabar the seat of Government of Cross River State, Nigeria. The hospital is patronized by inhabitants of the State and adjoining States and countries. It has several departments and units including the department of Obstetrics and Gynaecology in charge care of women care and child-bearing related conditions.

Ethical approval:

Ethical approval was given by the University of Calabar Teaching Hospital Health Research Ethical Committee. Informed consent was also obtained from each participant before the study was conducted, with assurance of safety, confidentiality of data, freedom to opt out of the study without any consequence, and assurance of no cost on the part of the participants.

Study participants and sample size:

The participants included in this study were consenting pregnant women attending the antenatal clinic and those with obstetric referrals to the hospital. The sample size was determined using the sample size formula for proportion (10), $n = Z^2pq/d^2$, where n is the calculated sample size, Z is 1.96 (95% confidence interval), p is 7.7% (0.077), obtained from the prevalence of UTI in UCTH, Calabar in a previous study (11), q is 1-p, and d is the acceptable margin of error for proportion (which was taken as 0.03 or 3%). This gave the calculated sample size of 303.36, which was adjusted for 15% non-response/attrition, to give the desired sample size of 349.

Method of sampling and data collection:

A total of 349 participants who met the inclusion criteria were consecutively recruited from the ANC over the period of study. All eligible participants for the study were enlisted on their first antenatal visit only. Semi-structured interviewer-administered questionnaires were used to collect socio-demographic data from the participants. The questionnaire consisted of 5 sections which, collected information on participant's demographics, social lifestyle, obstetric, medical and drug history, respectively.

Sample collection:

Each participant was given a sterile appropriately labeled universal container and instructed to collect a clean-catch mid-stream urine specimen after adequate instruction on how to do this. The urine specimens were transported to the medical microbiology laboratory of the hospital for analysis

Urine microscopy:

The urine samples were first examined macroscopically for colour and consistency. A drop of well-mixed uncentrifuged urine sample was then made on a clean grease-free glass slide and covered with cover slip to make a thin film. The thin film of urine was then examined, using light microscope at x10 and x40 for pus cells, epithelial cells, bacteria and were reported on count per high power field (hpf).

Culture isolation and bacterial identification:

Each urine sample was well-mixed by gently tilting the sample bottle after which a loopful was inoculated on Cysteine Lactose Electrolytes Deficient (CLED) media plate using a standard wire-loop (0.005mm diameter) and incubated at 37^OC for ≥ 16 hours in ambient air to yield discrete colonies.

Significant bacteriuria was determined from each culture plate when there are $\geq 10^5$ CFU/ml of urine (≥500 colonies on a culture plate). Discrete colonies from plates with significant bacteriuria were examined for colony morphology and Gram-staining reaction, and bacteria isolates were identified to species level using conventional biochemical test scheme (12).

Antibiotic susceptibility test:

Antibiotic susceptibility testing of each isolated bacterial species was done using the Kirby Bauer disk diffusion technique according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (13), against 7 selected antibiotics (Oxoid, UK); amoxicillin-clavulanic (20+10µg), piperacillin-tazobactam (110µg), ceftriaxone (30µg), ciprofloxacin (30µg), imipenem (10µg) and meropenem (10µg).

Inoculum suspension of bacterial isolates and the control strain (*E. coli* ATCC 25922) were separately made in sterile normal

saline, and the turbidity adjusted to match that of 0.5 McFarland standard. The inoculum suspensions were inoculated on separate Mueller Hinton (MH) agar plates to make thin homogeneous lawns using sterile swab sticks. Antibiotic sensitivity discs were then placed on the inoculated MH agar plate and incubated at 35^{O}C for 18 hours in ambient air. The diameter of zone of inhibition around each antibiotic disc was measured using a metre ruler and interpreted as sensitive, intermediate sensitive or resistant based on stipulated breakpoints according to the CLSI criteria (13).

Phenotypic screening for carbapenemase production:

Enterobacteriaceae species (or miscellaneous Gram-negative bacterial solates) with diameter of zones of inhibition ≤ 21 mm to at least one carbapenem (imipenem or meropenem) in the AST were considered to be resistant to carbapenem and presumptive carbapenemase-producing isolates, which were confirmed by the modified Hodge test (13).

Phenotypic confirmation of carbapenemases using modified Hodges test (MHT):

An inoculum suspension of *Escherichia coli* (ATCC 25922) was prepared and the turbidity adjusted to match that of 0.5 McFarland standard. The suspension was inoculated on MH agar plate to make a thin uniform lawn. A meropenem disk (10µg) was placed in the centre of the lawn. Suspensions of the test bacterial isolates and the control strains (MHT positive *Klebsiella pneumoniae* ATCC1705 and MHT negative *Klebsiella pneumoniae* ATCC1706) were separately prepared and their turbidity adjusted to match 0.5McFarland standard and each was then used to make a straight-line streak from the edge of the meropenem disk at the centre outwards to the edge of the MH agar plate. The plates were incubated for ≥ 16 hours at 35^{O}C in ambient air.

Carbapenemase-producing (MHT positive) isolate displayed a 'clover-leaf' like indentation of the zone of inhibition due to the growth of *E. coli* (ATCC 25922) along the line of streak of the isolate as a result of inactivation of meropenem by the isolate. MHT negative isolate did not show these indentations.

Statistical analysis of data:

The data were analysed using SPSS version 19.0 (SPSS, Chicago, Illinois, USA). Chi-square test was used to assess differences between proportions, and *p* value <0.05 was considered to be the statistical significance.

Results:

Sociodemographic characteristics of the study participants:

A total of 349 participants were included in the study and their socio-demographic characteristics are detailed in Table 1. Majority of the study participants (170, 48.7%) were in the age group 30-34 years, closely followed by those in age group 24-29 years (103, 29.5%). The mean age of the participants is 29.5 ± 6.3 years.

Most participants (145, 41.5%) were business women while 2 (0.6%) were farmers. Majority, (260, 75.5%) had tertiary education with only 8 (2.3%) having primary education. Over half of the participants (182, 52.1%) were indigenes of Cross River State while 165 (48.4%) were non-indigenes of the State. Majority of the participants (208, 59.6%) were low-income earners, with monthly income of 20,000-50,000 Nigeria Naira, and only 18 (5.2%) earned 100,000.00 Naira or more.

Obstetrics and medical history of the study participants:

Table 2 shows that majority of the participants (189, 54.2%) were primigravida and 6 (1.7%) were multiparous (Para^{4}). Most of the participants (190, 54.4%) were in the 25-40 weeks gestation, 113 (32.4%) were in 13-24 weeks gestation and only 1 (0.3%) was post-date/post-term. Majority of participants (296, 84.8%) admitted to having obstetric care and hospital delivery in their previous confinements whereas 37 (10.6%) were cared for and delivered by traditional birth attendants.

A total of 30 (8.6%) participants had history of contraceptive use, 11 (3.2%) used oral contraceptives and 19 (5.4%) used injectable contraceptives, while 319 (91.4%) have no history of contraceptive use. Fifteen (4.3%) participants had positive history of painful micturition with vaginal discharge during the last menstrual period (LMP), 5 (1.7%) had painful micturition without discharge during LMP, 41 (11.7%) had painful micturition in the index pregnancy at the time of sample collection, while there was no history of painful micturition in 288 (82.5%) in the index pregnancy before or during sample collection.

Most participants (223, 92.6%) had no sugar in urine while 26 (7.4%) had glycosuria. A total of 66 participants (19.1%) had fever in present pregnancy.

Table 1: Frequency distribution of socio-demographic characteristics of the student participants

Socio-demographic variables	Number (%)
Age group (years)	
<16	1 (0.3)
16-19	1 (0.3)
20-23	23 (6.6)
24-29	103 (29.5)
30-34	170 (48.7)
35-39	42 (12.0)
>40	9 (2.6)
Mean age (years)	**29.5 ± 6.3**
Occupation	97 (27.8)
Civil Servant	145 (41.5)
Business	2 (0.6)
Farming	71 (20.3)
Others	34 (9.7)
Unemployed (No Reliable Income)	
Religion	
Christian	344 (98.6)
Muslim	5 (1.4)
Others	0
State of origin	
Cross Rivers	182 (52.1)
Other States	165 (48.4)
Non-Nigerian	2 (0.6)
Monthly Income Level (₦)	
<20000	55(15.7)
20-50000	208(59.6)
60000-100,000	68(19.5)
>100,000	18(5.2)
Educational Level	
No Formal	0
Primary	8 (2.3)
Secondary	81 (23.2)
Tertiary	260 (74.5)

There was no history of smoking in majority of participants (348, 99.7%) but 1 (0.3%) participant admitted to occasional smoking. No history of alcohol consumption in 278 (79.7%), 69 (19.7%) participants consumed alcohol occasionally while 2 (0.6%) were habitual alcohol consumers.

Most of the participants (315, 90.3%) had not used antibiotics in the index pregnancy but 34 (9.7%) had used antibiotics, with 21 (6.0%) being inappropriate use (no medical prescription) while 13 (3.7%) were appropriately prescribed by medical doctor. There was no use of steroidal drug in 319 (91.4%) while 30 (8.6%) admitted to using steroidal contraceptives prior to index pregnancy.

Prevalence of UTI and uropathogenic isolates from the study participants:

Out of the 349 participants included in the study, urine samples of 314 (89.9%) yielded no growth of bacterial uropathogens while 35 (10.0%) yielded significant growth of Gram-negative uropathogenic bacilli, thus giving a UTI prevalence of 10.0% among the participants. The bacterial uropathogens isolated were *Escherichia coli* in 23 participants (6.6%) and *Klebsiella pneumoniae* in 12 participants (3.5%).

Antimicrobial susceptibility of uropathogenic isolates:

The result of the AST shows that piperacillin-tazobactam was most active *in vitro* on the isolates, with 29 of 35 (82.9%) isolates sensitive to it, followed by ciprofloxacin (26, 74.3%), ceftazidime (25, 71.4%), ceftriaxone (25, 71.4%), imipenem (21, 60.0%), amoxicillin-clavulanate (18, 51.4%) and meropenem (14, 40.0%) (Table 3).

A total of 17 (48.6%) urinary isolates were resistant to carbapenems (resistance to imipenem and/or meropenem), 12 (34.3%) of which were carbapenemase-producing on modified Hodge test, while 5 (14.3%) were non-carbapenemase-mediated antibiotic resistance (NCMR) (Fig 1).

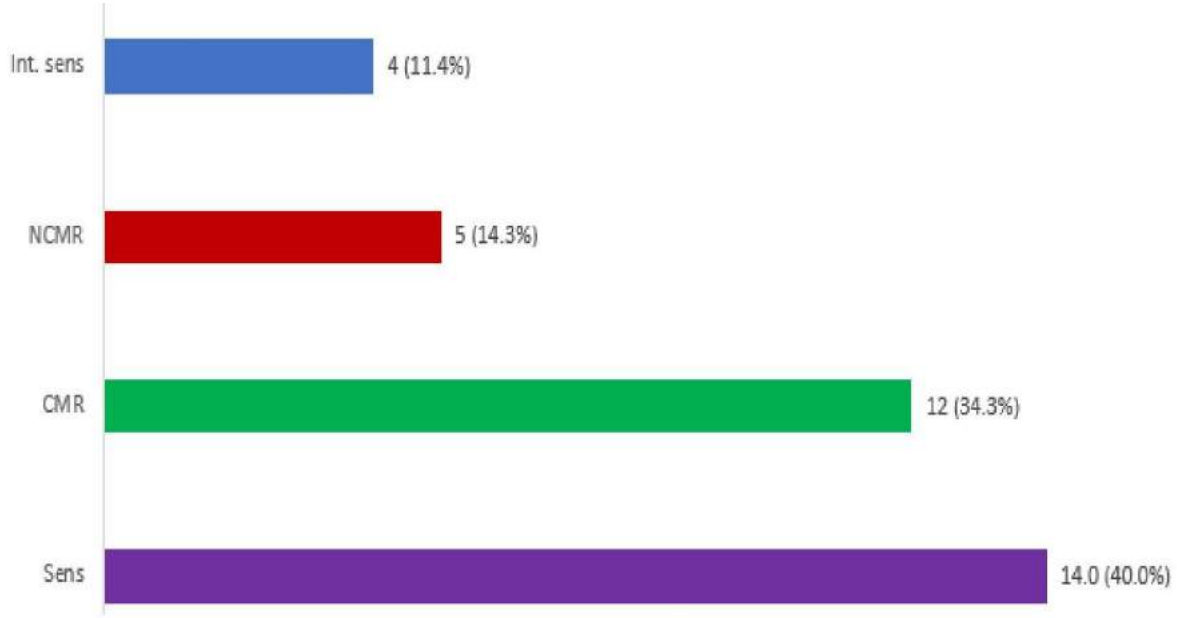
Table 2: Obstetrics and medical history of the student participants

Obstetrics and medical variables	**Number (%)**
Parity	
Primigravida	189 (54.2)
2	96 (27.5)
3	40 (11.5)
4	19 (5.4)
> 4	5 (1.4)
Gestation age of present pregnancy (weeks)	
< 2	1 (0.3)
2-12	44 (12.6)
13-24	113 (32.4)
25-40	190 (54.4)
≥ 40	1 (0.3)
Place of previous deliveries	
Hospital	296 (84.8)
Traditional Birth Attendants	37 (10.6)
Home	15 (4.3)
Never had previous delivery	1 (0.3)
Previous use of contraceptives	
No	319 (91.4)
Yes (Oral)	11 (3.2)
Yes (Injectable)	19 (5.4)
History of painful micturition with/without discharge	
None	288 (82.5)
Yes (≤5 days with discharge during LMP)	15 (4.4)
Yes (≤5 days without discharge during LMP)	5 (1.4)
Presently manifesting ± discharge	41 (11.7)
History of sugar in urine	
Yes	26 (7.4)
No	323 (92.6)
History of fever in current pregnancy	
Yes	66 (18.9)
No	283 (81.1)
Smoking status	
No	348 (99.7)
Yes (Occasionally)	1 (0.3)
Alcohol use	
No	278 (79.7)
Yes (Occasionally)	69 (19.7)
Yes (1 Unit daily)	1 (0.3)
Yes (>1 Unit daily)	1 (0.3)
History of antibiotic use	
No	315 (90.3)
Yes (≤ 4 weeks ago unprescribed)	15 (4.3)
Yes (≤ 4 weeks ago prescribed but abused)	6 (1.7)
Yes (Presently and prescribed)	13 (3.7)
Presently on steroidal drug	
No	319 (91.4)
Yes	30 (8.6)

Table 3: Antibiotic susceptibility of uropathogenic isolates causing urinary tract infections among the student participants

Degree of susceptibility	Antibiotics (n, /%)						
	IMP	CRO	AUG	CIP	TZP	CAZ	MERO
Resistant	11 (31.4)	7 (20)	9 (25.7)	5 (14.3)	5 (14.3)	8 (22.9)	17 (48.6)
Intermediate sensitive	3 (8.6)	3 (8.6)	8 (22.9)	4 (11.4)	1 (2.9)	2 (5.9)	4 (11.4)
Sensitive	21 (60.0)	25 (71.4)	18 (51.4)	26 (74.3)	29 (82.9)	25 (71.4)	14 (40.0)

IMP= Imipenem, CRO= Ceftriaxone, AUG= Amoxicillin/Clavulanate, CIP= Ciprofloxacin, TZP= Piperacillin/Tazobactam, CAZ= Ceftazidime, MERO= Meropenem.



Sens=Sensitive; Int sens = intermediate sensitivity; NCMR = non-carbapenemase resistance; CMS=carbapenemase producing

Fig 1: Susceptibility of urinary bacterial isolates to carbapenems

Risk factors associated with UTI caused by CRE amongst the study participants:

Regarding risk of UTI caused by CRE in Table 4, none of the factor analysed was a significant risk factor for UTI in pregnancy caused by CRE

Discussion:

Physiological and hormonal changes that occur in the course of pregnancy, the short female urethra with proximity to the anal verge and urinary tract instrumentation are among the major factors predisposing pregnant women to acquiring UTI (13). The prevalence of UTI among pregnant women in the study was 10.0%, which is similar to 15.8% reported in Kano (14) and 13.5% reported in Saudi Arabia (13). However, this prevalence is far lower than the prevalence of 56.7% reported in Ile-Ife (15), 31.0% in Ogun State (16), and 61.0% in Akure, Nigeria (17). These variations may be due to prevalent factors bordering on environmental, economic and sociocultural differences between study locations.

Piperacillin-tazobactam had the highest *in vitro* activity (82.9% sensitivity) against the CRE isolates, which agrees with the report of a study in Lagos (9). However, sensitivity of the CRE isolates to meropenem (48.6%) and imipenem (31.4%) was low, which is at variance with the study in Lagos (9), where *in vitro* sensitivity of 99.2% and 93.4% were reported for imipenem and meropenem respectively against the tested organisms. The reason for this variation may be due to differences in 'selection pressure' for antibiotics at the different locations of study.

The prevalence of phenotypic carbapenem resistance in this study among the *Enterobacteriaceae* isolates was 46.8% (17/35) with 34.3% (12/35) being carbapenemase-mediated resistance and 14.3% (5/35) non-carbapenemase-mediated resistance. Carbapenemases production remains a major factor in the spread of carbapenem resistance. Rising antimicrobial resistance to carbapenems creates difficulties in the management of severe infections caused by Gram-negative bacteria as carbapenems remain the drug of choice for the management of such conditions.

In our study, none of the variables tested was identified as significant risk factor associated with UTI in pregnancy caused by CRE, which disagrees with the study by Belete et al., (13) which reported socioeconomic factors, level of education and sexual activities as major reasons for acquiring multi-resistant Gram-negative organisms.

Table 4: Analysis of risk factors associated with urinary tract infection caused by carbapenemase-resistant *Enterobacteriaceae* among study participants

Risk factors	CRE (%) (n=17)	Non-CRE (%) (n=18)	x^2	OR (95% CI)	*p* value
Parity					
Primigravida	8 (47.1)	10 (55.6)	7.018	NA	0.0713
2	4 (23.5)	7 (38.9)			
3	5 (29.4)	0			
4	0	1 (5.6)			
Gestation of index pregnancy (weeks)					
2-12	3 (17.6)	4 (22.2)	1.258	NA	0.7391
13-24	3 (17.6)	4 (22.2)			
25-40	10 (58.9)	10 (55.6)			
Postdate	1 (5.9)	0			
Place of previous delivery					
Hospital	10 (58.9)	10 (55.6)	0.06239	NA	0.9693
Traditional Birth Attendant	5 (29.5)	6 (33.3)			
Home	2 (11.8)	2 (11.1)			
History of contraceptive use					
No	9 (52.9)	10 (55.6)	0.024	0.90	0.8767
Yes	8 (47.1)	8 (44.4)		(0.238-3.41)	
Painful micturition ± discharge					
No	13 (76.5)	14 (77.8)	1.152	NA	0.562
Yes (≥ 2wks post LMP)	1 (5.9) .0	0			
Yes (currently)	3 (17.6)	4 (22.2)			
History of sugar in urine					
No	14 (82.4)	16 (88.9)	0.0048	0.583	0.6581
Yes	3 (17.6)	2 (11.1)		(0.085-4.011)	
History of fever in pregnancy					
No	14 (82.4)	13 (72.2)	0.0965	1.795	0.6906
Yes	3 (17.6)	5 (27.8)		(0.36-9.06)	
Alcohol consumption					
No	17 (100.0)	14 (77.8)	2.352	10.862	0.1039
Yes	0	4 (22.2)		(0.539-219.1)	
History of antibiotic use					
No	16 (94.1)	18 (100.0)	0.0008	0.297	0.4857
Yes (presently)	1 (5.9)	0		(0.011-7.82)	
Present steroidal drug use					
No	15 (88.2)	17 (94.4)	0.00268	0.4412	0.6026
Yes	2 (11.8)	1 (5.6)		(0.036-5.37)	

x^2=Chi square; OR=Odd ratio; CI=Confidence interval; NA=Not applicable; LMP=last menstrual period

Conclusion:

The prevalence of UTI in this study was 10.0% (35/349), with prevalence of 6.6% for *E. coli* and 3.5% for *K. pneumoniae.* Piperacillin-tazobactam was most active antibiotic *in vitro* while sensitivity to imipenem and meropenem was low, with 48.6% of the isolates resistant to the carbapenems, mainly through carbapenemase production (34.3%). This situation will create obvious difficulties in antibiotic management of severe infections caused by Gram-negative bacteria particularly ESBL-producing strains since carbapenems have been the antibiotic class of choice for treatment of severe infections caused by these organisms.

To stem this rising trend of UTIs in pregnancy caused by CRE, pregnant women receiving antenatal care in UCTH, Calabar should be screened for UTI and offered appropriate treatment if required based on microbiological analysis of their urine samples.

Contributions of authors:

The study was conceptualized by OGI and IUC. All authors were involved in the literature searches. IAA analysed the data, while OGI, USN and EUE wrote the manuscript. EUE, AAI and IUC edited the final copy of the manuscript which was read and approved by all the authors.

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Original Article **Open Access**

Detection of microbial pathogens colonizing foot ulcers of diabetic patients in Enugu, Nigeria

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Abstract:

Background: Diabetic foot ulcer (DFU) is a major complication of diabetes mellitus (DM) which is associated with high morbidity and mortality. There is high rate of bacteria colonization especially in those with tendencies for poor wound dressing. This is accompanied by high rate of inappropriate antibiotic usage. The aim of this study is to characterize microbial pathogens colonizing foot ulcers of diabetic patients in Enugu, Nigeria, and to determine the antibiotic susceptibility of these isolates.
Methodology: This was a descriptive cross-sectional study of consecutively enrolled diabetic patients with foot ulcers in two tertiary healthcare facilities in Enugu, Nigeria, between May 2021 and February 2022. A structured questionnaire was used to obtain socio-demographic and clinical data of the patients. Pus samples and/or tissues were collected from the ulcer lesion of each patient for aerobic and anaerobic microbial cultures and biochemical identification using standard conventional techniques.
Results: A total of 310 diabetic patients with foot ulcers were recruited into the study, with 62.3% (193/310) males and 37.7% (117/310) females, and mean age of 56.0±13.9 years. Bacteria and yeast were isolated from samples of 280 (90.3%) patients while samples of 30 (9.7%) patients had no microbial growth. Males had higher frequency of microbial isolates (90.7%, 175/193) than females (89.7%, 105/117), while the age group ≤ 40 years had higher frequency of microbial isolates (100%, 43/43) compared to other age groups, although the differences are not statistically significant ($p>0.05$). The distribution of the isolates showed that 15.7% (44/280) were monomicrobial while 84.3% (236/280) were polymicrobial. The highest single isolate was *Bacteroides fragilis* with 5.0% (14/280), followed by *Staphylococcus aureus* with 3.2% (9/280). *Bacteroides fragilis* and *S. aureus* occurred as the highest combined bacteria isolates with 5.7% (16/280). Most of the patients were colonized by combination of bacterial isolates. The susceptibility indicates that most of the anaerobic bacteria were sensitive to metronidazole while *S. aureus* isolates were resistant to ofloxacin at a rate of 65.0%.
Conclusion: The findings in this study showed that there is high bacteria and fungi colonization of foot ulcers of diabetic patients in Enugu, Nigeria. Routine care of wounds especially frequent changes of dressing materials and the use of potent antiseptics, are recommended.

Keywords: Diabetic foot ulcer; chronic wounds; polymicrobial; antimicrobial resistance

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Détection d'agents pathogènes microbiens colonisant les ulcères du pied de patients diabétiques à Enugu, Nigeria

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Résumé:

Contexte: L'ulcère du pied diabétique (UPD) est une complication majeure du diabète sucré (DM) associée à une morbidité et une mortalité élevées. Il existe un taux élevé de colonisation bactérienne, en particulier chez les

personnes ayant tendance à mal panser les plaies. Cela s'accompagne d'un taux élevé d'utilisation inappropriée d'antibiotiques. Le but de cette étude est de caractériser les agents pathogènes microbiens colonisant les ulcères du pied des patients diabétiques à Enugu, au Nigeria, et de déterminer la sensibilité aux antibiotiques de ces isolats.

Méthodologie: Il s'agissait d'une étude transversale descriptive portant sur des patients diabétiques recrutés consécutivement et souffrant d'ulcères du pied dans deux établissements de soins de santé tertiaires à Enugu, au Nigeria, entre mai 2021 et février 2022. Un questionnaire structuré a été utilisé pour obtenir des données sociodémographiques et cliniques du les patients. Des échantillons de pus et/ou des tissus ont été prélevés sur la lésion ulcéreuse de chaque patient pour des cultures microbiennes aérobies et anaérobies et une identification biochimique à l'aide de techniques conventionnelles standard.

Résultats: Au total, 310 patients diabétiques souffrant d'ulcères du pied ont été recrutés dans l'étude, avec 62,3% (193/310) d'hommes et 37,7% (117/310) de femmes, et un âge moyen de 56,0±13,9 ans. Des bactéries et des levures ont été isolées à partir d'échantillons de 280 (90,3%) patients, tandis que des échantillons de 30 (9,7%) patients ne présentaient aucune croissance microbienne. Les hommes présentaient une fréquence plus élevée d'isolats microbiens (90,7%, 175/193) que les femmes (89,7%, 105/117), tandis que le groupe d'âge ≤ 40 ans présentait une fréquence plus élevée d'isolats microbiens (100.0%, 43/43) par rapport aux autres groupes d'âge, bien que les différences ne soient pas statistiquement significatives (p>0,05). La répartition des isolats a montré que 15,7% (44/280) étaient monomicrobiens tandis que 84,3% (236/280) étaient polymicrobiens. L'isolat le plus élevé était *Bacteroides fragilis* avec 5,0% (14/280), suivi de *Staphylococcus aureus* avec 3,2% (9/280). *Bacteroides fragilis* et *S. aureus* étaient les isolats bactériens combinés les plus élevés avec 5,7% (16/280). La plupart des patients étaient colonisés par une combinaison d'isolats bactériens. La sensibilité indique que la plupart des bactéries anaérobies étaient sensibles au métronidazole tandis que les isolats de S. aureus étaient résistants à l'ofloxacine à un taux de 65,0%.

Conclusion: Les résultats de cette étude ont montré qu'il existe une forte colonisation bactérienne et fongique des ulcères du pied des patients diabétiques à Enugu, au Nigeria. Des soins de routine des plaies, des changements particulièrement fréquents des matériaux de pansement et l'utilisation d'antiseptiques puissants sont recommandés.

Mots clés: Ulcère du pied diabétique; plaies chroniques; polymicrobien; résistance aux antimicrobiens

Introduction:

Diabetes mellitus is a debilitating ailment that affect every race throughout the world. The International Diabetes Federation (IDF) estimated that about 537 million people are living with diabetes worldwide with a projected rise to 783 million people in 2045 (1). Over 6.7 million deaths from diabetes were reported in low-and-middle-income-countries in 2021. Africa has 24 million adults living with diabetes and also the highest level of undiagnosed diabetic cases, reaching 53.6% (2). In sub-Saharan Africa, Nigeria is reported to have the highest burden of diabetes with parallel increase in the prevalence of diabetes-related complications and death (3).

Diabetic foot ulcer (DFU) is one of the most frequent complications in diabetic patients with a prevalence rate of 6.4% worldwide (4), 7.2% in Africa and rate ranging from 11 to 32% in Nigeria among hospitalized patients (5). In Nigeria, DFU accounts for a quarter of diabetic-related prolonged hospital admission with amputation and mortality rates of 35.4% and 20.5% respectively (3). DFU is a breech in the skin epithelium of the host distally from the ankle, with a multi-factorial aetiology and associated with neuropathy, different grades of ischaemia and infection (6).

Established risk factors that precipitate the development of ulcer includes increased body weight, peripheral vascular disease, retinopathy, hypertension, poor glycaemic control, high foot plantar pressure, duration of diabetes, age, race, ethnicity, socio-economic status, presence of callus, smoking, and trauma (7-10). It has been estimated that 40-80% infection rate in DFU has resulted in amputation of lower limb extremities in majority of cases (11). A Nigerian study reported 22.3%-29.3% of non-traumatic amputation due to DFU (12).

Aside hyperglycaemic emergencies, DFU is the commonest cause of diabetes related death in Nigeria (13). Several factors pathogenetically work together to create the onset of foot ulcerations in diabetic patients. DFU are chronic wounds that are frequently colonized by wide range of pathogenic bacteria, which are predominantly polymicrobial with multiple bacteria as the most prevalence organisms. There is sparse knowledge about the ecology of such chronic infections but biofilm formation seems to play a major role (14). The interaction and synergism of the polymicrobial community leads to production of extracellular matrix of hydrated polymeric substances. This becomes irreversibly attached to the biological surface of the ulcer, making them recalcitrant to the action of most antibiotics and also resistant to the immune system (15). A wide range of bacterial pathogens have been identified with diverse antibiotic susceptibility patterns patterns in different geographical regions (16,17).

Chronic infections of diabetic foot make treatment more complex and difficult, increases cost of management, prolong hospital stay as well as increase morbidity and mortality (18). Amputation of the lower limb extremity on its own is associated with significant disabilities including loss of productivity, psychological burdens and reduced quality of life (19).

Report in southwest Nigeria has shown more mean annual expenditure on diabetics with complications such as foot ulcer than diabetics without complications (20). This has been attributed to higher rates of hospital admission, emergency department visits and home health care utilization. There is an under estimation to the true economic burden of DFU as regards to loss of productivity and decreased employment associated with DFU (21). The goal of this study is to determine microbial pathogens colonizing foot ulcer of diabetic patients and their susceptibility to commonly used antimicrobial agents in Enugu, southeast Nigeria.

Materials and method:

Study setting and design:

This was a cross sectional descriptive multicenter study conducted in Enugu State, southeast Nigeria, involving two tertiary healthcare facilities within the State; University of Nigeria Teaching Hospital (UNTH), Ituku Ozalla and Enugu State University Teaching Hospital (ESUTH), Park-lane Enugu.

Ethical issues:

The study protocol was reviewed and approved by Health Research Ethics Committee of the Teaching Hospitals; UNTH Ituku-Ozalla with reference number UNTH/HREC/2021/04/116 and ESUTH Parklane with refere nce number ESUTHP/CMAC/RA/034/vol-2/106. Participation in the study was voluntary and each patient gave their consent before administration of any questionnaire and before wound assessment.

Study participants and data collection:

A total of 310 diabetic patients (hospitalized and outpatient) with clinically infected lower extremity (below the ankle) ulcer were consecutively recruited into the study between May 2021 and February 2022. Information on demographic factors and social life style were obtained from each participant using a pretested structured questionnaire in a face-to-face interview.

Clinical parameters, which included type of lesion, duration of ulcer, location of foot ulcer, duration of hospital stay, were also collected. These were further linked to their microbiological samples through numerical codes for easy identification. Foot ulcers were clinically assessed and diagnosis of infection was determined by the presence of at least two of these indicators; local swelling or indurations, tenderness or pain, purulent discharge, erythema, and heat/warmth.

Microbiological sample collection:

Foot ulcers were cleaned vigorously with sterile saline solution and extensively debrided of superficial exudates to reduce the chances of isolating colonizing flora. For superficial ulcers, two sets of sterile swabs were used to collect wound swab from the base of ulcer. By rotating a sterile swab over a 1 cm^2 area of the wound bed for 5 seconds, samples were collected from each patient. The two swab specimens were immediately transported to the laboratory for microbial analysis.

Microbiological culture procedure:

One of the swab specimens was used for Gram staining reaction to identify Gram-positive and Gram-negative bacteria present in the sample (22). The second swab specimen was inoculated onto Blood, MacConkey, and Mannitol salt agar plates as well as into thioglycolate broth medium, and incubated at 37^{o}C for 24 hours. Subcultures from the thioglycolate broth was done Blood agar plate and incubated anaerobically using Gas pack (AnaeroPack® - anaerobic gas generating system). All bacteriological cultures and biochemical identification tests for the isolates were carried out by conventional methods as described in Cowan and Steel's Manual for Identification of Medical Bacteria (23).

Antibiotic susceptibility testing:

Antimicrobial susceptibility testing (AST) was carried out on each isolate by the modified Kirby-Bauer disc diffusion method (24). Briefly, sterile Mueller-Hinton (MH) agar plate was swabbed with standardized inoculum suspension of each isolated bacteria isolates. Antibiotic discs were placed on inoculated MH plate using a sterile forcep and plates were incubated at 37^{o}C for 24 hours. The diameter of zone of inhibition for each isolate was measured with a calibrated ruler and interpreted as sensitive or resistance in line with the CLSI guideline (24). For streptococci, 5% horse blood was added to MH agar for the AST.

The antibiotic discs used in the AST included metronidazole (5µg), cefoxitin (30µg), ampicillin/sulbactam (10/10µg), imipenem (10 µg), ciprofloxacin (10µg), ampicillin (30µg), levofloxacin (10µg), norfloxacin (10µg), ofloxacin (10 µg), erythromycin (10 µg), cefixime (5 µg), ceftriaxone (30µg), penicillin (10µg), ampicillin/cloxacillin (30µg), clindamycin (2µg), chloramphenicol (30µg), and clarithromycin (10µg).

Statistical analysis:

Data were summarized by descriptive statistics and analysed using omnibus model of Chi-square test and logistic regression. The strength of association between bacterial infected or colonized ulcer and selected factors was estimated by calculating the Odds ratios (OR) with 95% confidence intervals (95% CI) and probability value less than 0.05 was considered statistical significance.

Results:

Socio-demographic and clinical characteristics of diabetic patients:

A total of 310 diabetes patients with foot ulcer were enrolled into the study, made of 193 (62.3%) males and 117 (37.7%) females, with age range of 21-92 and mean age of 56.02±13.9 years. The age group 50-59 years made up 30.0% (93/310). Participants with secondary and primary school education constituted 49.4% and 22.3% respectively. Traders were the most represented occupational group with 42.6% (132/310). Participants residing in semi urban and rural areas constituted 42.3% and 40.6% respectively. Majority of the diabetic patients (40.7%, 126/310) had the foot ulcer for > 3 years. About 69.0% (214/310) of the participants had indulged in self-medications especially with antibiotics. Most ulcer positions were located at the dorsal portion of the feet (45.5%, 141/310), followed by plantar area (20.6%, 64/310) (Table 1).

Prevalence of bacterial colonization of ulcer:

Of the 310 diabetic patients with foot ulcers whose samples were analysed, 280 (90.3%) had bacterial isolates while 30 (9.7%) showed no bacterial growth. The distribution of the bacterial isolates showed that 15.7% (44/280) had single isolate while 51.1% (143/280), 28.2% (79/280) and 5.0% (14/280) had two, three and four bacterial isolates respecttively.

The predominant monomicrobial isolate was *Bacteroides fragilis* (5.0%, 14/280) followed by *Staphylococcus aureus* (3.2%, 9/280) (Table 2a). In the group of diabetic patients with two isolates, *S. aureus* and *B. fragilis* constituted 5.7% (16/280) while *Enterococcus faecalis* and *Propionibacterium* spp represented 3.6% (10/280). Some other combined isolates included 3.2% (9/280) *S. aureus* and *Propionibacterium* spp. The frequency of *S. aureus*/*Escherichia coli, B. fragilis*/coagulase negative staphylococcus (CoNS), and *B. fragilis*/*Proteus mirabilis* were 2.9% (8/280) each (Table 2b). The highest frequency for cultures with three isolates were *S. aureus*/*B. fragilis*/*P. mirabilis*; *E. coli*/*P. mirabilis*/*Peptostreptococcus* spp; *E. coli*/*Peptostreptococcus* spp/CoNS; *S. aureus*/*B. fragilis*/*P. mirabilis* and *S. aureus*/*E. coli*/*Propionibacterium* spp, with 1.4% (4/280) each (Table 2c). Diabetic patient ulcers colonized with four bacterial isolates included *E. coli*/*Pseudomonas aeruginosa*/*Peptostreptococcus* spp/CoNS with 0.8% (3/280).

Analysis of socio-demographic and clinical characteristics of participants with respect to microbial isolates:

Table 3 shows bivariate analysis of the sociodemographic and clinical characteristics of the study participants with respect to the distribution of the microbial isolates. Male participants had higher frequency of microbial isolates of 90.7% (175/193) compared to females with 89.7% (105/117) but this difference was not statistically significant (p=0.788). Drivers (7/7), students (12/12) and clergy (2/2) had the highest frequency of bacteria isolates with 100.0% each. Traders as well as unemployed also had high frequency of bacterial isolates with 89.4% and 87.9% respectively. A total of 116 (92.1%) bacterial isolates were recovered from participants who used antibiotics without medical prescription compared to 86 (89.6%) isolates from those who did not (p=0.80). In all, none of the characteristics analysed was significantly associated with bacterial colonization of ulcers in the participants as rates were high across board.

Antibiotic susceptibility of bacterial isolates in diabetic ulcer patients:

The antibiotic susceptibility of the isolated bacteria indicated that Gram-positive bacteria have the highest sensitivity rate to chloramphenicol followed by fluoroquinolones (levofloxacin and norfloxacin) respectively. Ampicillin/sulbactam, ciprofloxacin, as well as amoxicillin/clavulanate had the highest inhibitory activity against Gram-negative aerobes. Ciprofloxacin had moderate inhibitory activity against both Gram-negative and Gram-positive aerobic bacteria but with low activity against *Klebsiella pneumoniae*. Aerobic bacteria were moderately susceptible to gentamicin, ceftriaxone, clindamycin and ofloxacin.

Anaerobic isolates were less resistant to metronidazole, penicillin and ampicillin/sulbactam. The bacteria most resistant to the antimicrobials tested are *Clostridium perfringes* while *Fusobacterium* spp were the most susceptible to the antimicrobials tested with exception of imipenem.

Table 1: Socio-demographic and clinical characteristics of diabetic patients with foot ulcers in Enugu, Nigeria

Variables	Categories	Frequency (n)	Percentage (%)
Gender			
	Male	193	62.3
	Female	117	37.7
Age group (years)			
	≤ 40	45	14.5
	41- 49	44	14.2
	50 – 59	93	30.0
	60 – 69	89	28.7
	> 70	34	12.6
Educational status			
	None	33	10.6
	Primary	69	22.3
	Secondary	153	49.4
	Tertiary	55	17.7
Occupation			
	Unemployed	83	26.8
	Civil Servant	48	15.5
	Trader	132	42.6
	Driver	7	2.3
	Farmer	26	8.4
	Student	12	3.9
	Clergy	2	0.6
Marital status			
	Single	20	6.5
	Married	232	74.8
	Divorced	8	2.6
	Separated	8	2.6
	Widowed	42	13.5
Residency			
	Urban	53	17.1
	Semi-Urban	131	42.3
	Rural	126	40.6
Type of house			
	Duplex/Bungalow	70	22.6
	Flat	121	39.0
	One Room Apartment	119	38.4
Duration of ulcer (years)			
	< 1	106	34.2
	1 – 2	46	14.8
	2 – 3	32	10.3
	>3	126	40.7
Position of ulcer			
	Planter	64	20.6
	Dorsal Portion	141	45.5
	Toes (left Foot)	31	10.0
	Toes (right Foot)	46	14.8
	Ankle	28	9.0
Treatment assessment			
	Hospitalized	179	57.7
	Out-Patient	131	42.3
Antibiotic use without prescription			
	Yes	214	69.0
	No	96	31.0

Table 2: Microbial isolates colonizing foot ulcers of diabetic patients in Enugu, Nigeria

2 (a): Single microbial isolate (monomicrobial)

Isolate	Frequency	Percentage
Staphylococcus aureus	9	3.2
Bacteroides fragilis	14	5.0
Escherichia coli	3	1.1
Pseudomonas aeruginosa	4	1.4
Proteus mirabilis	1	0.4
Candida albicans	6	2.1
Coagulase-negative staphylococcus	3	1.1
Streptococcus pyogenes	2	0.7
Peptostreptococcus spp	2	0.7
Total	**44**	**15.7**

2 (b): Two microbial isolates

Isolates	Frequency	Percentage
Staphylococcus aureus + *Bacteroides fragilis*	16	5.7
Staphylococcus aureus + *Enterococcus faecalis*	1	0.4
Staphylococcus aureus + *Escherichia coli*	1	0.4
Staphylococcus aureus + *Peptostreptococcus* spp	8	2.9
Staphylococcus aureus + *Fusobacterium* spp	3	1.1
Staphylococcus aureus+ *Propionibacterium* spp	9	3.2
Staphylococcus aureus + *Clostridium perfringens*	1	0.4
Escherichia coli + *Proteus mirabilis*	1	0.4
Escherichia coli + *Peptostreptococcus* spp	2	0.7
Escherichia coli + Coagulase-negative staphylococcus	5	1.9
Escherichia Coli + *Propionibacterium* spp	4	1.4
Escherichia coli + *Clostridium perfringens*	2	0.7
Bacteroides fragilis + Coagulase-negative staphylococcus	8	2.9
Bacteroides fragilis + *Enterococcus faecalis*	4	1.4
Bacteroides fragilis + *Streptococcus pyogenes*	1	0.4
Bacteroides Fragilis + *Candida albicans*	2	0.7
Bacteroides fragilis + *Escherichia coli*	6	2.1
Bacteroides fragilis + *Pseudomonas aeruginosa*	2	0.7
Bacteroides fragilis + *Proteus mirabilis*	8	2.9
Bacteroides fragilis + *Peptostreptococcus* spp	6	2.1
Pseudomonas aeruginosa + *Clostridium perfringens*	1	0.4
Pseudomonas aeruginosa + *Peptostreptococcus* spp	5	1.9
Pseudomonas aeruginosa + *Klebsiella pneumoniae*	3	1.1
Pseudomonas aeruginosa + *Propionibacterium* spp	1	0.4
Pseudomonas aeruginosa + *Proteus mirabilis*	1	0.4
Proteus mirabilis + *Candida albicans*	1	0.4
Proteus mirabilis + *Peptostreptococcus* spp	1	0.4
Proteus mirabilis + Coagulase-negative staphylococcus	1	0.4
Proteus mirabilis + *Propionibacterium* spp	2	0.7
Coagulase-negative staphylococcus + *Propionibacterium* spp	4	1.4
Coagulase-negative staphylococcus + *Clostridium perfringens*	1	0.4
Coagulase-negative staphylococcus + *Klebsiella pneumoniae*	1	0.4
Coagulase-negative staphylococcus +*Fusobacterium* spp	2	0.7
Peptostreptococcus spp + Coagulase-negative staphylococcus	2	0.7
Peptostreptococcus spp + *Propionibacterium* spp	2	0.7
Peptostreptococcus spp + *Candida albicans*	2	0.7
Enterococcus faecalis + *Clostridium perfringens*	1	0.4
Enterococcus faecalis + *Fusobacterium* spp	3	1.1
Enterococcus faecalis + *Propionibacterium* spp	10	3.6
Enterococcus faecalis + *Peptostreptococcus* spp	5	1.9
Candida albicans + *Clostridium perfringens*	1	0.4
Candida albicans + *Fusobacterium* spp	1	0.4
Candida albicans + *Klebsiella pneumoniae*	1	0.4
Streptococcus pyogenes + *Fusobacterium* spp	1	0.4
Total	**143**	**51.1**

2(c): Three microbial isolates

Isolates	Frequency	Percentage
Staphylococcus aureus+ Bacteroides fragilis+ Escherichia coli	3	1.1
Staphylococcus aureus+ Enterococcus faecalis+ Escherichia coli	3	1.1
Bacteroides fragilis+ Enterococcus faecalis + Pseudomonas aeruginosa	1	0.4
Staphylococcus aureus + Bacteroides fragilis + Proteus mirabilis	4	1.4
Bacteroides fragilis + Enterococcus faecalis + Proteus mirabilis	1	0.4
Bacteroides fragilis+ Escherichia coli+ Proteus mirabilis	1	0.4
Staphylococcus aureus+ Enterococcus faecalis + Peptostreptococcus spp	2	0.7
Bacteroides fragilis, Enterococcus faecalis, Peptostreptococcus spp	1	0.4
Bacteroides fragilis+ Escherichia coli + Peptostreptococcus spp	2	0.7
Bacteroides fragilis +Pseudomonas aeruginosa + Peptostreptococcus spp	2	0.7
Bacteroides fragilis + Proteus mirabilis + Peptostreptococcus spp	1	0.4
Escherichia coli + Proteus mirabilis + Peptostreptococcus spp	4	1.4
Pseudomonas aeruginosa + Proteus mirabilis + Peptostreptococcus spp	1	0.4
Staphylococcus aureus + Bacteroides fragilis + Escherichia coli	1	0.4
Escherichia coli + Peptostreptococcus spp + *Candida albicans*	1	0.4
Escherichia coli+ Peptostreptococcus spp + Coagulase-negative staphylococcus	4	1.4
Proteus mirabilis+ Peptostreptococcus spp+ Coagulase-negative staphylococcus	2	0.7
Staphylococcus aureus+ Bacteroides fragilis+ Klebsiella pneumoniae	2	0.7
Staphylococcus aureus+ Escherichia coli +Klebsiella pneumoniae	1	0.4
Bacteroides fragilis + Escherichia coli + Klebsiella pneumoniae	1	0.4
Enterococcus faecalis + Peptostreptococcus spp + *Klebsiella pneumoniae*	1	0.4
Staphylococcus aureus+ Bacteroides fragilis + Fusobacterium spp	1	0.4
Staphylococcus aureus +Escherichia coli + Fusobacterium spp	1	0.4
Escherichia coli, Pseudomonas aeruginosa, Fusobacterium spp	1	0.4
Staphylococcus aureus+ Proteus mirabilis+ Fusobacterium spp	1	0.4
Escherichia coli+ Proteus mirabilis+ Fusobacterium spp	2	0.7
Proteus mirabilis + Candida albicans + Fusobacterium spp	1	0.4
Proteus mirabilis+ Streptococcus pyogenes + Fusobacterium spp	2	0.7
Enterococcus faecalis + Proteus mirabilis + Propionibacterium spp	2	0.7
Staphylococcus aureus+ Bacteroides fragilis+ Propionibacterium spp	4	1.4
Enterococcus faecalis+ Peptostreptococcus spp + *Propionibacterium* spp	1	0.4
Escherichia coli+ Peptostreptococcus spp + *Propionibacterium* spp	1	0.4
Staphylococcus aureus+ Escherichia coli+ Propionibacterium spp	4	1.4
Bacteroides fragilis+ Peptostreptococcus spp +Propionibacterium spp	3	1.1
Staphylococcus aureus +Enterococcus faecalis + Propionibacterium spp	1	0.4
Enterococcus faecalis + Coagulase-negative staphylococcus + *Propionibacterium* spp	2	0.7
Enterococcus faecalis+ Klebsiella pneumoniae + Propionibacterium spp	1	0.4
Pseudomonas aeruginosa+ Klebsiella pneumoniae + Propionibacterium spp	1	0.4
Escherichia coli + Peptostreptococcus spp + *Clostridium perfringens*	1	0.4
Escherichia coli+ Proteus mirabilis + Clostridium perfringens	1	0.4
Staphylococcus aureus + Escherichia coli + Clostridium perfringens	1	0.4
Escherichia coli+ Pseudomonas aeruginosa + Clostridium perfringens	1	0.4
Escherichia coli + Proteus mirabilis + Clostridium perfringens	2	0.7
Enterococcus faecalis + Proteus mirabilis + Clostridium perfringens	1	0.4
Enterococcus faecalis + Coagulase-negative staphylococcus + *Clostridium perfringens*	3	1.1
Proteus mirabilis + Streptococcus pyogenes + Enterococcus faecalis	1	0.4
Total	**79**	**28.2**

2(d): Four microbial isolates

Isolates	Frequency	Percentage
Staphylococcus aureus+ Bacteroides fragilis + Escherichia coli + Peptostreptococcus spp	2	0.7
Staphylococcus aureus + Escherichia coli + Pseudomonas aeruginosa + Peptostreptococcus spp	1	0.4
Staphylococcus aureus+ Bacteroides fragilis+ Enterococcus faecalis + Coagulase-negative staphylococcus	1	0.4
Staphylococcus aureus + Bacteroides fragilis + Escherichia coli + Coagulase-negative staphylococcus	2	0.7
Bacteroides fragilis + Pseudomonas aeruginosa + Proteus mirabilis + Coagulase-negative staphylococcus	1	0.4
Escherichia coli + Pseudomonas aeruginosa + Peptostreptococcus spp + Coagulase-negative staphylococcus	3	0.7
Bacteroides fragilis + Pseudomonas aeruginosa + Coagulase-negative staphylococcus + *Streptococcus pyogenes*	1	0.4
Enterococcus faecalis + klebsiella pneumoniae + Streptococcus pyogenes + Fusobacterium spp	1	0.4
Bacteroides fragilis+ Enterococcus faecalis + Peptostreptococcus spp + *Propionibacterium* spp	1	0.4
Enterococcus faecalis + Proteus mirabilis + klebsiella pneumoniae + Clostridium perfringens	1	0.4
Total	**14**	**5.0**

Table 3: Bivariate analysis of socio-demographic and clinical characteristics of diabetic patients with foot ulcers with respect to prevalence of colonization by microbial isolates

Characteristics	**Categories**	**Microbial isolates**		x^2	**OR (95% CI)**	***p*-value**
		Yes (%)	**No (%)**			
Gender	Male	175 (90.7)	18 (9.3)	.072	1.11 (.52-2.39)	.788
	Female	105 (89.7)	12 (10.3)			
Age group (years)	≤ 40	43 (100.0)	0	8.135	NA	.078
	41- 49	42 (91.3)	4 (8.7)			
	50 – 59	82 (88.2)	11 (11.8)			
	60 – 69	80 (89.9)	9 (10.1)			
	> 70	33 (84.6)	6 (15.4)			
Educational Status	None	28 (84.8)	5 (15.2)	2.273	NA	.518
	Primary	62 (89.9)	7 (10.1)			
	Secondary	138 (90.2)	15 (9.8)			
	Tertiary	52 (94.5)	3 (5.5)			
Occupation	Unemployed	73 (87.9)	10 (12.1)	2.585	NA	.831
	Civil Servant	43 (89.6)	5 (10.4)			
	Trader	118 (89.4)	14 (10.6)			
	Driver	7 (100.0)	0			
	Farmer	25 (96.2)	1 (3.8)			
	Student	12 (100.0)	0			
	Clergy	2 (100.0)	0			
Residency Type	Urban	49 (92.5)	4 (7.5)	1.676	NA	.433
	Semi-Urban	115 (87.8)	16 (12.2)			
	Rural	116 (92.1)	10 (7.9)			
Marital Status	Single	20 (100.0)	0	2.794	NA	.545
	Married	208 (89.7)	24 (10.3)			
	Divorced	7 (87.5)	1 (12.5)			
	Separated	7 (87.5)	1 (12.5)			
	Widowed	38 (90.5)	4 (9.5)			
House Type	Duplex/Bungalow	66 (94.3)	4 (5.7)	1.776	NA	.411
	Flat	107 (88.4)	14 (11.6)			
	One-room apartment	107 (89.9)	12 (10.1)			
Duration of ulcer (years)	< 1	99 (93.4)	7 (6.6)	7.826	NA	.043
	1 – 2	36 (78.3)	10 (11.6)			
	2 – 3	29 (90.6)	3 (9.4)			
	>3	116 (92.1)	10 (7.9)			
Position of ulcer	Planter	58 (90.6)	6 (9.4)	.342	NA	.995
	Dorsal Portion	127 (90.1)	14 (9.9)			
	Toes (left Foot)	28 (87.5)	3 (12.7)			
	Toes (right Foot)	41 (89.1)	5 (10.9)			
	Ankle	26 (92.9)	2 (7.1)			
Treatment assessment	Hospitalized	165 (92.2)	14 (7.8)	1.772	1.664 (.78-3.54)	.183
	Out-Patient	115 (87.8)	16 (12.2)			
Use antibiotics without prescription	Yes	194 (90.7)	20 (9.3)	.0641	1.109 (.49-2.47)	.800
	No	86 (89.6)	10 (10.4)			

NA=Not applicable; OR=Odd ratio; CI=Confidence interval

Table 4 (a): Antibiotic susceptibility of isolated anaerobic bacteria

Antibiotics	***Bacteroides fragilis*** **(n =103)**		***Peptostreptococcus*** **spp (n = 69)**		***Propionibacterium* spp (n= 53)**		***Fusobacterium* spp (n =20)**		***Clostridium perfringes*** **(n= 19)**	
	R	**S**	**R**	**S)**	**R**	**S)**	**R**	**S**	**R**	**S**
Penicillin	101 (98.0)	2 (2.0)	3 (4.3)	66 (95.6)	6 (11.3)	47 (88.7)	6 (30.0)	14 (70.0)	0	19 (100)
Metronidazole	3 (2.9)	100 (97.1)	10 (14.5)	59 (85.5)	0	53 (100)	0	20 (100)	0	19 (100)
Clindamycin	41 (39.8)	63 (60.2)	0	69 (100)	6 (11.3)	47 (88.7)	0	20 (100)	18 (94.7)	1 (5.3)
Cefoxitin	43 (41.7)	60 (58.3)	10 (14.5)	66 (85.5)	15 (28.3)	38 (71.7)	0	20 (100)	10 (52.6)	9 (47.4)
Ampicillin/ Sulbactam	4 (3.9)	99 (96.1)	19 (27.5)	50 (72.5)	15 (28.3)	38 (71.7)	6 (30.0)	14 (70.0)	10 (52.6)	9 (47.4)
Imipenem	11 (5.3)	92 (94.7)	20 (28.9)	49 (71.1)	18 (44.0)	35 (66.0)	10 (50)	10 (50.0)	6 (31.6)	13 (68.4)
Ceftriaxone	23 (22.3)	80 (77.7)	35 (50.7)	34 (49.3)	30 (56.6)	23 (43.4)	6 (30.0)	14 (70.0)	9 (47.4)	10 (52.6)
Ampicillin	103 (100)	0	35 (50.7)	34 (49.3)	6 (11.3)	47 (88.7)	0	20 (100)	14 (73.7)	5 (15.3)

Table 4(b): Antibiotic susceptibility of isolated aerobic Gram-positive bacteria

Antibiotics	***Staphylococcus aureus*** **(n = 83)**		**CoNS (n = 46)**		***Streptococcus pyogenes*** **(n = 9)**		***Enterococcus faecalis*** **(n=48)**	
	R	**S**	**R**	**S**	**R**	**S**	**R**	**S**
Erythromycin	41 (49.4)	42 (50.6)	34 (74.0)	12 (26.0)	4 (44.4)	5 (55.6)	48 (100.0)	0
Ceftriaxone	40 (48.2)	43 (51.8)	46 (100)	0	4 (44.4)	5 (55.6)	22 (45.8)	26 (54.2)
Ampicillin/Cloxacillin	40 (48.2)	43 (51.8)	46 (100)	0	5 (55.6)	4 (44.4)	47 (97.9)	1 (2.1)
Cefixime	41 (49.4)	42 (50.6)	46 (100)	0	9 (88.9)	1 (11.1)	46 (95.8)	2 (4.1)
Levofloxacin	0	83 (100)	5 (10.9)	41 (89.1)	2 (22.2)	7 (77.8)	19 (39.6)	29 (60.4)
Norfloxacin	0	83 (100)	5 (10.9)	41 (89.1)	3 (33.3)	6 (66.7)	19 (39.6)	29 (60.4)
Gentamicin	42 (50.6)	41 (49.4)	24 (52.2)	22 (47.8)	5 (55.6)	4 (44.4)	24 (50.0)	24 (50.0)
Ofloxacin	54 (65.1)	29 (34.9)	10 (21.7)	36 (78.3)	5 (55.6)	4 (44.6)	31 (65.0)	17 (35.0)
Clindamycin	40 (48.2)	3 (51.8)	25 (54.3)	21 (45.7)	3 (33.3)	6 (66.7)	36 (75.0)	12 (25.0)
Ciprofloxacin	15 (18.1)	68 (81.9)	30 (65.2)	16 (34.8)	1 (11.1)	9 (88.9)	22 (45.8)	26 (54.2)
Chloramphenicol	19 (22.9)	64 (77.1)	4 (8.6)	42 (91.4)	1 (11.1)	8 (88.9)	1 (2.1)	47 (97.9)

CONS=Coagulase negative staphylococcus; R=Resistance; S=Sensitive

Table 4 (c): Antibiotic susceptibility of isolated aerobic Gram-negative bacteria

Antibiotics	***Escherichia coli*** **(n = 67)**		***Proteus mirabilis*** **(n = 45)**		***Pseudomonas aeruginosa*** **(n = 30)**		***Klebsiella pneumoniae*** **(n = 14)**	
	R	**S**	**R**	**S**	**R**	**S**	**R**	**S**
ceftriaxone	33 (49.2)	34 (50.8)	14 (31.1)	31 (68.9)	5 (16.7)	25 (83.3)	6 (42.9)	8 (57.1)
Ciprofloxacin	1 (1.5)	66 (98.5)	16 (35.6)	29 (64.4)	0	30 (100)	8 (57.1)	6 (42.9)
Gentamycin	29 (43.3)	38 (56.7)	23 (51.1)	22 (48.9)	22 (73.4)	8 (26.6)	12 (85.7)	2 (14.3)
Ofloxacin	31 (46.3)	36 (53.7)	33 (73.3)	12 (26.7)	22 (73.4)	8 (26.6)	14 (100)	0
Clarithromycin	9 (13.4)	58 (86.6)	23 (51.1)	22 (48.9)	18 (60.0)	12 (40.0)	11 (78.6)	3 (21.4)
Ampicillin	17 (25.4)	50 (74.6)	8 (20.0)	37 (80.0)	0	30 (100)	0	14 (100)
Chloramphenicol	45 (67.2)	22 (32.8)	24 (53.3)	21 (46.7)	10 (33.3)	20 (66.7)	10 (71.4)	4 (28.6)
Amoxicillin/Clavulanate	1 (1.5)	66 (98.5)	11 (24.4)	34 (75.6)	0	30 (100)	0	14 (100)

Discussion:

Diabetic foot ulceration still remains the most severe complication affecting diabetic patients globally. The prevalence rate of 90.3% microbial colonization of diabetic foot ulcer in our study is very high. Similar studies in Nigeria (25-27) reported lower rate of bacterial colonization, with amputation rate and mortality as the end results in most cases. This can be attributed to the high cost of burden in treatment, lack or inadequate knowledge about DFU and diabetes, unqualified medical personnel, lack of structural management and poor drug supply chain.

The DFU participants in this study were predominantly male with 62.3% against 37.7% females. This is in line with the findings of other studies in Ethiopia (28), India (29) and Nigeria (30,31). This can be due to differences in lifestyle and professional activities, job exposing men to more risks and trauma. A study in Kuwait by Alhubali et al., (32) reported DFU prevalence twice in men than women with more likelihood in younger men who presents with deeper and complex DFU. This can be explained further by tendency of women to take more responsibility in medical care and hygiene. Therefore, gender can be said to be a risk factor for DFU (33).

Similar to gender, participants in our study were predominant within the age group 50-69 years with mean age of 56.02±13.99 years. This is in agreement with the study carried out in Ile Ife Nigeria, with mean age of 54.7±12.8 years and highest prevalence of 48.0% for 50-69 years (30). Another study by Ugwu et al., (3) reported highest prevalence between 45-64 years (mean age of 55.9±12.5 years). This finding highlights the socio-economic burden of diabetes on the predominant active working class of the country.

DFU is usually colonized by pathogenic bacteria, predisposing patients to infection of the lower extremity. In our study, we observed that majority of ulcer were located on the dorsal portion (45.5%) and plantar regions (20.6%). This was similar to the study carried out in Brazil (34) which reported plantar and dorsal regions of the feet as the most common regions for DFU. This can be attributed to dryness of the dorsal part of the feet (from peripheral neuropathy), habits of not wearing protective footwears or ill-fitting foot wear and pressure on the plantar surfaces which are weight bearing area of the body.

High frequency of microbial isolates was seen among traders with 89.4% (118/132) rate. This may be due to constant contact with environmental air pollutions with accompanying bacteria being carried along with the wind, since their trading is majorly on open market stalls. Participants who reside in rural area also presented with high prevalence of microbial isolates with 92.1% (116/126) rate. This is similar to the study by Abuhay et al., (35), who reported higher rate of DFU and microbial isolates among those living in rural areas. Inadequate knowledge of the disease in the rural areas can be a contribution to this high rate amongst the lower class. This can equally be viewed through the lens of the educational status which shows 90.2% and 89.8% rates among those who had secondary and primary education. This is however contrary to the study of Unegbu et al, (31), who reported that farmers and rural communities were the most prevalent with DFU and microbial isolates.

In this study, prevalence of microbial colonization of 92.2% (165/179) was seen in participants who were hospitalized and 87.8% (115/131) of those attending outpatient clinics. Although high numbers of participants claimed to be compliant with hospital attendance for treatment, microbial organisms were isolated from their ulcers in higher frequency. This may indicate non-compliance to drug therapy, poor diabetic care (diet and foot) and inconsistent hospital visits. Odusan et al., (27) reported 30.5% of DFU participants in a Lagos State study who had prior knowledge of the disease but were not compliant to visit and drug treatment, hence had recurrent infections and persistent ulcer occurrence. On the other hand, indiscriminate use of antibiotics was reported in 209 participants accounting for 67.4%, and 90.4% of isolated organisms were reported for the 209 participants who used antibiotics without medical prescription. Apparently, this misuse of antibiotics can result in emergence of resistant organisms leading to treatment failure in most cases. A study reported 11.8% multi-drug resistant *Staphylococcus* or MRSA isolated from patients with DFU who had used antibiotics for more than 20 years (31).

Our study showed the predominance of aerobic bacteria with 54.9% over anaerobic bacteria with 42.4% and fungi with 2.7%. Higher prevalence of aerobes has been reported with 88.0% aerobes and 12.0% anaerobes in one study (36), and 86.3% aerobes and 13.3% anaerobes in another study (37). *Bacteroides fragilis* was the predominant anaerobe isolated with 17% rate and also the most common bacteria isolated in monomicrobial culture. This has also been reported by some studies as the most common anaerobe with 8.0% prevalence rate (36). Otta et al., (33) reported similarity to this but with reference to isolation of *B. fragilis* from grade 4-5 ulcer cases, followed by *Peptostreptococcus* spp, which agrees with our report of 11.0% prevalence rate for *Peptostreptococcus* spp. Higher prevalence of these anaerobes is an indication of chronic infections and infections beyond the superficial skin layer (11).

Anaerobic bacteria showed less resistance to metronidazole, clindamycin, penicillin and ampicillin/sulbactam in our study. Higher resistance was seen to ceftriaxone and ampicillin than other antibiotics. A Nigerian study by Anyim et al., (17) and many others have reported similar susceptibility pattern with little difference over time. This shows that our susceptibility results are still in range of previously researched studies and can be trusted to guide empirical treatment of serious bacterial infections in patients before microbiological results are available.

Gram-positive aerobes were the largest isolated group of microorganisms in this study with 30.0% as against 25.0% for Gram-negative aerobes (ratio of 1.2). *Staphylococcus aureus* was the predominant aerobic bacteria (13.3%), with *Enterococcus faecalis* as the second largest (7.7%). Anyim et al., (17), reported similar predominance of *S. aureus* with *Streptococcus pyogenes* as the second. Other studies in Ethiopia (28) also reported similar observation. On the contrary, Gol et al., (36) reported predominance of Gram-negative bacteria with 54% prevalence rate. The differences in these rates may be as a result of geographical variation, types and severity of infection, changes in causative organisms over time, inadequate sample collection, poor handling techniques and poor preservation methods for anaerobes.

High prevalence rate of Gram-negative aerobes was reported (25.0%) with *Escherichia coli* and *Proteus mirabilis* as the predominant isolates with 10.7% and 7.2% respectively. Amaefule et al., (38) reported predominance of Gram-negative isolates similar to our study, with *Proteus* spp (18.0%) and *E. coli* (16.0%) being the most common Gram-negative isolates in their study. This high prevalence of Gram-negative bacteria can be attributed to severity of ulcers grade 4 and 5, which are known to be colonized predominantly by Gram negative organisms (38).

Both Gram-positive and Gram-negative aerobes were shown in this study to exhibit resistance to gentamicin, ceftriaxone and cefixime, making the drug of choice for empirical therapy to be levofloxacin, norfloxacin, and ciprofloxacin. This similarity can be seen in other studies (17) but contrary to report that countered the use of ciprofloxacin for empirical treatment (36). Indiscriminate use of antibiotics, over-the-counter drugs and inconsistency during treatment with drugs are factors that predict the emergence of resistant bacteria. Gram-negative bacteria can also be treated with amoxicillin/clavulanate as these isolates showed high sensitivity to this beta-lactam and beta-lactam inhibitor combination.

Conclusion:

The findings in this study shows that there is increased rate of DFU within our communities with wide range of different microorganisms present. High rate of polymicrobial community in DFU has been demonstrated as a major contributing factor to increased duration of ulcer, mortality and amputation rate. Detailed knowledge of antimicrobial susceptibility for isolated bacteria within our community was a major finding in this study. This will help create a detailed work sheet for empirical treatment on any patient with DFU emergency at first clinical visit.

Contribution of authors:

UOB conceptualized the study and designed the laboratory methods along with UTKC. UTKC, UOB and ACL were involved in material preparation, data collection and analysis. UOB and ACS provided funding and prepared initial manuscript draft. All authors read and approved the final manuscript.

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Original Article **Open Access**

Phylogenetic diversity and susceptibility of *Candida* species from women using contraceptive devices in northcentral Nigeria

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Abstract:

Background: The use of contraceptive devices predisposes women to vulvovaginal candidiasis (VVC) globally. Despite the high incidence of VVC and antifungal resistance to azoles, the genetic diversity and resistance pattern among contraceptive users in Nigeria is poorly investigated. This study therefore sought to characterize and determine the phylogenetic breadth of *Candida* species as well as their resistance to antifungal agents.
Methodology: This study recruited 1,600 women using contraceptive devices who visited selected gynaecology and obstetrics clinics in northcentral Nigeria. *Candida* species were isolated and characterized using conventional methods and sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). Bayesian phylogenetic analysis was used to characterize the diversity of *Candida* species and primer-specific PCR was used to detect the presence of resistant genes. Agar well diffusion technique was used for the determination of antifungal susceptibility profiles. Data analysis was done by Kruskal-Wallis Chi-square test on R Console software version 3.2.2, followed by post-hoc Wilcoxon rank sum test with Bonferroni correction for multiple pairwise comparisons of means where there was a significant difference between the antifungal agents. The level of significance was set at $p < 0.05$.
Results: A total of 710 (44.3%) out of the 1,600 women using contraceptive devices had VVC with five species of *Candida* identified in them. Although *Candida albicans* was the predominant (43.2%, n=307) species, other non-albicans *Candida* species include *Candida* (*Nakaseomyces*) *glabrata* (19.0%, n=135), *Candida tropicalis* (15.8%, n=112), *Candida parapsilosis* (8.9%, n=63), and *Candida akabanensis* (13.1%, n=93) which were phenotypically identified as *Candida* (*Nakaseomyces*) *glabrata*. All the *Candida* species showed varying degrees of susceptibilities to voriconazole, fluconazole and nystatin. However, resistance of *C. albicans* to fluconazole was 29.0%, *C. tropicalis* to nystatin (46.0%) and to voriconazole (14.0%), while *C. akabanensis* was 100.0% resistant to voriconazole and fluconazole. Kruskal-Wallis Chi-square test showed nystatin as the most effective antifungal agent against the *Candida* species (χ^2=786.03, df=2, p<0.001). Also, resistant gene *Erg11* was identified in all the *Candida* species that were phenotypically resistant to the antifungal agents tested.
Conclusion: Women using contraceptive devices in northcentral Nigeria harbor phylogenetically diverse *Candida* species including *C. akabanensis*, an uncommon cause of VVC. Of these *Candida* species, *C. albicans, C. tropicalis* and *C. akabanensis* were notable for multidrug drug resistance as well as harboring *Erg11* resistance gene.

Keywords: *Candida*, Mycobiome, Contraceptives, Resistance

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Diversité phylogénétique et sensibilité des espèces de *Candida* chez les femmes utilisant des dispositifs contraceptifs dans le centre-nord du Nigeria

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Résumé:

Contexte: L'utilisation de dispositifs contraceptifs prédispose les femmes à la candidose vulvo-vaginale (CVV) à l'échelle mondiale. Malgré l'incidence élevée de la CVV et de la résistance antifongique aux azoles, la diversité génétique et les modèles de résistance parmi les utilisatrices de contraceptifs au Nigéria sont peu étudiés. Cette étude a donc cherché à caractériser et déterminer l'étendue phylogénétique des espèces de *Candida* ainsi que leur résistance aux agents antifongiques.
Méthodologie: Cette étude a recruté 1600 femmes utilisant des dispositifs contraceptifs qui ont visité des cliniques de gynécologie et d'obstétrique sélectionnées dans le centre-nord du Nigeria. Les espèces de *Candida* ont été isolées et caractérisées à l'aide de méthodes conventionnelles et du séquençage de la région de l'espaceur transcrit interne (ITS) de l'ADN ribosomal (ADNr). L'analyse phylogénétique bayésienne a été utilisée pour caractériser la diversité des espèces de *Candida* et la PCR spécifique aux amorces a été utilisée pour détecter la présence de gènes résistants. La technique de diffusion dans des puits de gélose a été utilisée pour la détermination des profils de sensibilité aux antifongiques. L'analyse des données a été effectuée par le test du chi carré de Kruskal-Wallis sur la version 3.2.2 du logiciel R Console, suivi d'un test de somme de rangs de Wilcoxon post-hoc avec correction de Bonferroni pour de multiples comparaisons par paires de moyennes où il y avait une différence significative entre les agents antifongiques. Le niveau de signification a été fixé à $p<0,05$.
Résultats: Au total, 710 (44,3%) des 1600 femmes utilisant des dispositifs contraceptifs présentaient une VVC contenant cinq espèces de *Candida*. Bien que *Candida albicans* soit l'espèce prédominante (43,2%, n=307), d'autres espèces de *Candida* non albicans comprennent *Candida* (*Nakaseomyces*) *glabrata* (19,0%, n=135), *Candida tropicalis* (15,8%, n=112), *Candida parapsilosis* (8,9%, n=63) et *Candida akabanensis* (13,1%, n=93), phénotypiquement identifiés comme étant *Candida* (*Nakaseomyces*) *glabrata*. Toutes les espèces de *Candida* présentaient divers degrés de sensibilité au voriconazole, au fluconazole et à la nystatine. Cependant, la résistance de *C. albicans* au fluconazole était de 29,0%, celle de *C. tropicalis* à la nystatine (46,0%) et au voriconazole (14,0%), tandis que celle de *C. akabanensis* était de 100,0% résistante au voriconazole et au fluconazole. Le test du Chi carré de Kruskal-Wallis a montré que la nystatine était l'agent antifongique le plus efficace contre l'espèce *Candida* (x^2=786,03, df=2, $p<0,001$). En outre, le gène résistant *Erg11* a été identifié chez toutes les espèces de *Candida* qui étaient phénotypiquement résistantes aux agents antifongiques testés.
Conclusion: Les femmes utilisant des dispositifs contraceptifs dans le centre-nord du Nigéria abritent des espèces de *Candida* phylogénétiquement diverses, notamment *C. akabanensis*, une cause rare de CVV. Parmi ces espèces de *Candida*, *C. albicans*, *C. tropicalis* et *C. akabanensis* se distinguaient par leur multirésistance aux médicaments et par l'hébergement du gène de résistance *Erg11*.

Mots clés: *Candida*, Mycobiome, Contraceptifs, Résistance

Introduction:

The vaginal microecosystem consists of a complex and dynamic microbiome that coexists in a symbiotic relationship with the host (1) and the diversity of the vaginal microbiome plays a vital role in vaginal health. The vaginal normal flora of women within the reproductive age harbors 10^{10}–10^{11} bacteria predominantly *Lactobacillus* species (2,3). Despite the vast number of bacteria in the vaginal ecosystem, fungi contribute to the promotion of vaginal health. Using the sequences of the internal transcribed spacer 1 (ITS1) of vaginal samples from healthy women, Drell et al., (4) identified two fungal phyla as the major constituents of the vaginal mycobiome; Ascomycota (58.0%), predominated by the *Candida* genera, and Basidiomycota (3.0%). Similarly, *Candida* species colonization constitute 21.0-65.0% of the vaginal mycobiome of healthy women (4,5,6). However, these *Candida* species can transit from colonization and cause symptomatic infections including vulvovaginal candidiasis.

Vulvovaginal candidiasis (VVC) is caused by the overgrowth of *Candida* species in the vagina which is characterized by vulva irritation and may also present with white 'cheese-like' vaginal discharge (7). Globally, *Candida albicans* accounts for between 85-90.0% cases of VVC (8-11). However, recent studies have implicated non-*albicans Candida* (NAC) species including *Candida tropicalis, Candida* (*Nakaseomyces*) *glabrata, Candida krusei/ Pichia kudriavzevii* and *Candida parapsilosis* as emerging aetiological agents of VVC (12, 13).

VVC is a global health risk that contributes to significant morbidity and economic burden in women. It has been estimated that 70.0% of all women will have at least one episode of VVC, and 372 million women are affected by recurrent vulvovaginal candidiasis (RVVC) during their reproductive years (14, 15). Aging, pregnancy, use of contraceptives, diaphragms, vaginal douching, prolonged chemotherapy or antibiotic use, metabolic diseases especially diabetes mellitus and immunosuppression predispose women to VVC (16). The association between VVC and the use of injectables, intrauterine devices (IUD) and oral contraceptive pills has been documented (8,17,18).

In recent times, non-pathogenic species and emerging fungal agents are agents of

human disease. These emerging species express important virulence factors and possess antimicrobial resistant genes. The emerging fungal pathogen, *Candida auris*, known for invasive candidiasis was reported in a case of vulvovaginitis (19). There are various oral and topical treatments available for the treatment of VVC (20,21). However, local and global antifungal susceptibility surveillance has revealed decreased susceptibility of some *Candida* isolates to some antifungal in recent times (22,23,24).

In Nigeria, the increasing number of women using contraceptive devices can be linked to the increased advocacy on the use of devices to promote health and reduce infant and maternal mortality. However, the risk of vaginal microbiome modulation using these devices, pathogen's carriage, dissemination, biofilm formation, complex community interaction, changes in hormonal level are often neglected. We hypothesized that the use of contraceptive devices modifies the vaginal mycobiome leading to phylogenetic diverse species responsible for VVC among contraceptive users. In addition, the use of advance molecular technologies for the identification of *Candida* species is rarely carried out in most clinical settings in Nigeria because the procedure is relatively expensive, hence, patients are treated empirically on the basis of traditional diagnostic results thereby promoting drug resistance. Furthermore, there is paucity of epidemiological data on the phylogenetic diversity and susceptibility patterns of *Candida* species involved in VVC among contraceptive users in Nigeria, hence, this study was designed to bridge the gap by providing relevant information on VVC and contraceptive usage.

Materials and Method:

Study setting:

This research was carried out in three States of the northcentral Nigeria (Nasarawa, Niger, Benue) and the Federal Capital Territory (FCT) Abuja, which were selected by simple random sampling technique.

Ethical approval:

Ethical approval was obtained from the Health Research Ethics Committees of the hospitals with reference numbers (MOH/STA/204/Vol.1/96; STA/495/Vol/136; FHREC/2017/01/109/11-12-17; NHREC18/06/ 2017). The samples were obtained with the informed consent of the women.

Study design, participants & sampling method:

This was a descriptive cross-sectional study of 1600 randomly selected women on contraceptive devices conducted from January 2018 to May 2019. The sample size was determined by the Cochran formula (25) using a previous prevalence of 0.155% to determine the sample size per hospital in each State and the FCT. A total of 710 consenting women with contraceptive devices with vaginal *Candida* isolates but asymptomatic for VVC, across eight secondary health facilities in the study area were enrolled. Only women using contraceptives were included while those who were not contraceptive users and those pregnant were excluded.

Data and sample collection:

A structured questionnaire with both open and closed ended questions was used to collect risk factors and obtain biodemographic data from the study participants. With the assistance of a gynecologist, two cotton-tipped sterile swabs sticks were used to collect high vaginal swab (HVS) samples from each woman who met the inclusion criteria. The HVS was collected by inserting a sterile vaginal speculum into the vagina; a sterile cotton wool swab was inserted into the posterior vaginal fornix and rotated gently as previously described (26). The swab stick was withdrawn and replaced in its case and labeled appropriately with the participant's information.

Isolation and phenotypic identification of *Candida* species:

One swab was used subjected to 10% KOH direct smear examination while the other swab was cultured on Sabouraud Dextrose Agar (SDA, HiMedia, India) plates, supplemented with 50 mg/L chloramphenicol, and incubated for 48-72 hours at 35°C. Phenotypic identification of the isolated strains was carried out on the basis of microscopic and cultural features. All *Candida* species were differentiated on the CHROMagar *Candida* medium, (Difco™, CHROMagar™) after incubation for 48h at 37°C. In addition to Gram staining procedure, germ tube test was performed by inoculating the *Candida* isolate into 0.5 mL human serum, incubating at 37°C for 3 hours, and observing for sprouting yeast cells under the microscope.

Antifungal susceptibility test:

Antifungal susceptibility was performed by the modified disc diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI) guideline (27), which required the addition of 2% glucose and 0.5 µg/mL methylene blue dye into Mueller-Hinton agar (Oxoid, UK). A suspension was prepared by adding five distinct *Candida* colonies into a 5ml test tube containing 0.85% sterile saline solution and incubated overnight. The suspension was then compared to 0.5 McFarland standards. Cotton swab moistened with the fungal suspension was streaked on modi fied Mueller-Hinton media. Commercially avai-

lable antifungal disks for nystatin (100 units), voriconazole (10µg) and fluconazole (10µg) (Oxoid, UK) were aseptically dispensed onto the surface of the inoculated agar plates and placed in an incubator at 37^0 C for 24 hours.

The diameters of zone of inhibition were measured in millimetres and reported as susceptible (S), susceptible dose dependent (SSD) or resistant (R) in accordance with CLSI M44A document guideline (27). Quality control tests were performed daily to check for the precision and accuracy of the results of disk diffusion testing.

Genomic DNA extraction:

The genomic DNA was extracted from the *Candida* isolates using a ZymoResearch (ZR) fungal/bacterial DNA mini prep extraction kit (Cat.D6005; South Africa) according to the manufacturer's instructions. Briefly, into each ZR BashingBead Lysis tubes, colonies from the pure culture of the isolates were added into 200 µL of isotonic buffer and 750µL of lysis buffer was added to the tube secured in the 2mL tube holder assembly of the Disruptor Genie™ and centrifuged at 10,000x*g* for 1 min.

Four hundred microlitres (400µL) of supernatant was transferred to a Zymo-Spin IV spin filter in a collection tube and centrifuged at 7000x*g* for 60sec. One thousand two hundred microlitre (1200µL) of fungal DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600µL, 800µL was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000x*g* for 60sec, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred microlitres (200µL) of the DNA pre-wash buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000x*g* for 60sec followed by the addition of 500 µL of fungal DNA and wash buffer. This was centrifuged at 10,000x*g* for 60sec. The Zymo-spin IIC column was transferred to a clean 1.5µL centrifuge tube, 100µL DNA elution buffer was added to the column matrix and centrifuged at 10,000x*g* for 30sec to elute the DNA. The pure DNA was then stored at -20^OC for further analysis.

The quantity and quality of extracted DNA was estimated using a NanoDrop™1000 spectrophotometer (Thermo Fisher Scientific, USA) at 260 nm. The purified DNA was maintained at -20 °C until used in the PCR assay.

PCR amplification of ITS region:

The ITS region of the isolates was amplified using the specific primer pair of ITS1 forward (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 reverse (5'-TCCTCCGCTTAT TGATA TGC-3') primers (28). The PCR conditions were as follows; initial denaturation at 95ºC for 5 min; final denaturation at 95ºC for 30 sec; annealing at 53ºC for 30 sec; initial extension at 72ºC for 30sec for 35 cycles and final extension at 72ºC for 5min in a Thermocycler (Applied Biosystems, UK). The integrity of the amplified product was evaluated by electrophoresis in a 1% (w/v) agarose gel at 120V for 15 min in 1 X Tris-borate-EDTA (TBE) buffer, stained with 2µL ethidium bromide and visualized on a blue light transilluminator.

Sequencing of amplified products and bioinformatic analysis:

Sequencing of the purified and amplified products was performed as previously described (29), using standard methods. Sequencing was done in Pretoria, South Africa, with BigDye Terminator kit on 3510 ABI sequencer (Applied Biosystems, UK) using standard protocols and previously designed primers. The sequencing was performed at a final volume of 10µL, the elements included 0.25 µL BigDye® terminator v1.1/v3.1, 2.25µL of 5xBigDye sequencing buffer, 10µL primers, PCR primers and 2-10ng PCR template per 100bp.

The bioinformatics algorithm Trace edit was used to edit the obtained sequences and similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLASTn) for species identification. The sequences obtained were aligned using Multiple Alignment Fast Fourier Transform (MAFFT).

Identification of ergosterol resistant (*Erg11*) gene:

Detection of *Erg11* resistant gene was done on representative isolates from each *Candida* species. Amplification of the gene was performed using the Erg F: 5'–GTTGA AACTGTCATTGAT-3' and Erg R: 5'-TCAGAACA CTGAACTGAAA-3' primers on ABI 9700 Thermal Cycler (Applied Biosystems, UK). The PCR conditions were strictly followed, and the resulting products were resolved on 1% agarose gel at 120V for 25min and visually observed on UV transilluminator with expected *Erg11* amplicon size of 500bp (30).

Bioinformatic and statistical analyses:

Bayesian phylogenetic analysis was used, and the evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (31,32). Confidence limits for phylogenetic trees were estimated by bootstrap consensus tree inferred from 500 replicates (33). This was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the method of Jukes and Cantor (34).

Other data obtained were analyzed

using R Console software (version 3.2.2). Shapiro-wilk normality test was carried out and the data was observed not to be normally distributed. Hence, Kruskal-Wallis Chi-square test was used to compare the mean response of the fungi species in relation to antifungal agents. Kruskal-Wallis Chi-square test was followed by a post-hoc Wilcoxon rank sum test with Bonferroni correction was used for multiple pairwise comparisons of means where there was a significant difference between the treatments. The level of significance was set at $p<0.05$.

Results:

A total of 710 (44.3%) women using contraceptive devices had VVC and five species of *Candida* were phenotypically identified; *C. albicans, C. glabrata (Nakaseomyces glabrata*), *C. tropicalis, C. parapsilosis* and *C. krusei* (*Pichia kudriavzevii*). *Candida albicans* (43.2%, n=307) was the most predominant species isolated, other non-albicans *Candida* species included *C. glabrata* (*N. glabrata*) (19.0%, n=135), *C. tropicalis* (15.8%, n=112), *C. parapsilosis* (8.9%, n=63) and *C. krusei (P. kudriavzevii*) (13.1%, n=93).

Candida albicans was the most predominant species (49.4%) identified among women within the age group 20-24 years. The highest distribution of *C. tropicalis* was seen in older women aged 45-50 years. A high frequency of *C. parapsilosis* (16.0%) was observed within the age group 30-35 years, no *C. parapsilosis* was isolated amongst women within age group 15-19 and 45-50 years. *Candida* (*Nakaseomyces) glabrata* was observed to have a high frequency distribution (32.0%) among women within the age group 30-34 years. *Candida krusei* (*P. kudriavzevii*) had a high frequency (22.0%) among women within the age group 15-19 years (Table 1).

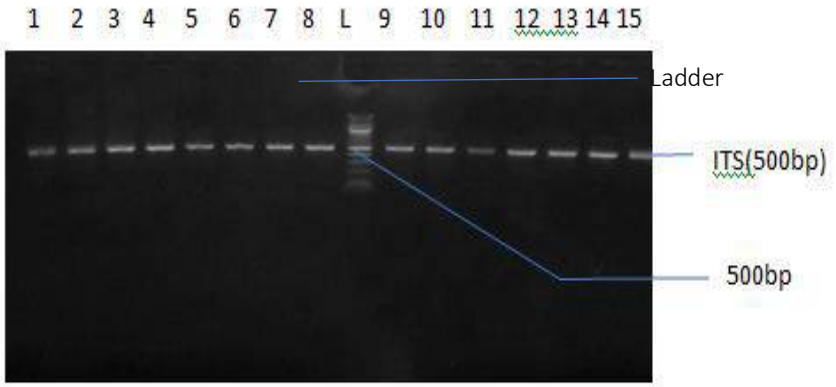
The most predominant *Candida* species solated from women who use contraceptive pills was *C. albicans. Candida tropicalis* and *C. glabrata* (*N. glabrata*) (25.0%) were the most frequent isolates recovered from the study participants that used injectable contraceptives. *Candida parapsilosis* (18.0%) and *C. glabrata* (*N. glabrata)* (25.0%) were mostly isolated from IUCD and implant users. The diversity of *Candida* species may be attributed to the use of contraceptive devices which modified the vaginal mycobiome. *Candida albicans* was the most recovered isolate from participants with informal secondary and tertiary education. The highest frequency of *C. tropicalis* was seen amongst women with primary education. The distribution of *Candida* species among the married women was higher than the single ladies (Table 1).

The amplification of the ITS DNA region revealed that all the isolates have a fragment of 500bp which indicated they were all *Candida* species (Fig 1). The obtained ITS sequence from the isolate produced an exact match during the MegaBlast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. *Candida* isolates designated 1,3,5,6,8,9,11,12,13,14, 15,17,23,25 and 27 were identified as *C. albicans;* isolates 24,2,22,4,19 and 20 were *C. glabrata* (*N. glabrata)*; isolates 16,21 and 26 were *C. tropicalis*; and isolate 18 was identified as *C. parapsilosis.* The ITS sequence identified isolates 7 and 10 as *Candida akabanensis* but were phenotypically identified as *C. krusei* (*P. kudriavzevii*).

The susceptibility pattern of *Candida* species against the 3 antifungal drugs tested is shown in Table 2. *Candida albicans* was 100.0% susceptible to nystatin, 92.1% susceptible to voriconazole and 71.1% susceptible to fluconazole (28.9% resistant to fluconazole). All isolates of *C. glabrata* (*N. glabrata)* were 100.0% susceptible to nystatin, voriconazole and fluconazole. *Candida tropicalis* was susceptible to nystatin (53.5%), voriconazole (85.7%) and fluconazole (100.0%) although some isolates of *C. tropicalis* were 46.4% and 14.3% resistant to nystatin and voriconazole. *Candida parapsilosis* was 100.0% susceptible to nystatin and fluconazole while 82.5% were susceptible to voriconazole. *Candida akabanensis* was 100.0% susceptible to nystatin but 100.0% resistant to voriconazole and fluconazole.

Table 1: Frequency distribution of *Candida* species with respect to demographic characteristics of the women with contraceptive devices in northcentral Nigeria

Parameters	**Frequency of *Candida* isolation (%)**					
	Total	***Candida albicans***	***Candida tropicalis***	***Candida parapsilosis***	***Candida krusei* (*Pichia kudriavzevii*)**	***Candida* (*Nakaseomyces*) *glabrata***
Age group in years						
15-19	63	29 (46.0)	11 (17.5)	0	14 (22.2)	9 (14.3)
20-24	81	40 (49.4)	20 (24.7)	6 (7.4)	10 (12.3)	5 (6.2)
25-29	208	93 (44.7)	38 (18.3)	18 (8.7)	21 (10.1)	38 (18.3)
30-34	187	72 (38.5)	21 (11.2)	14 (7.5)	19 (10.2)	61 (32.6)
35-39	123	51 (41.5)	10 (8.1)	20 (16.3)	21 (17.1)	21 (17.1)
40-44	42	20 (47.6)	9 (21.4)	5 (11.9)	8 (19.0)	0
45-50	6	2 (33.3)	3 (50.0)	0	0	1 (16.7)
Contraceptive types						
IUCD	157	70 (44.6)	17 (10.8)	29 (18.5)	21 (13.4)	10 (6.4)
Injectables	252	76 (30.1)	64 (25.4)	33 (13.1)	15 (6.0)	64 (25.4)
Implants	96	45 (46.9)	11 (11.5)	0	24 (25.0)	16 (16.7)
Pills	205	116 (56.6)	10 (4.9)	1 (0.5)	33 (16.1)	45 (21.9)
Educational status						
Informal	69	30 (43.5)	21 (30.4)	0	0	18 (26.1)
Primary	195	89 (45.6)	38 (19.5)	11 (5.6)	15 (7.7)	42 (21.5)
Secondary	178	82 (46.1)	23 (12.9)	13 (7.3)	39 (21.9)	21 (11.8)
Tertiary	268	106 (39.6)	30 (11.2)	39 (14.5)	39 (14.5)	54 (20.2)
Marital status						
Single	64	14 (21.9)	11 (17.2)	11 (17.2)	12 (18.8)	16 (25.0)
Married	646	293 (45.4)	101 (15.6)	52 (8.0)	81 (12.5)	119 (18.4)
Total number of participants with VVC	**710**	**307 (43.2)**	**112 (15.8)**	**63 (8.9)**	**93 (13.1)**	**135 (19.0)**



Lanes 1-15: ITS bands at 500bp; lane L: 500bp molecular ladder

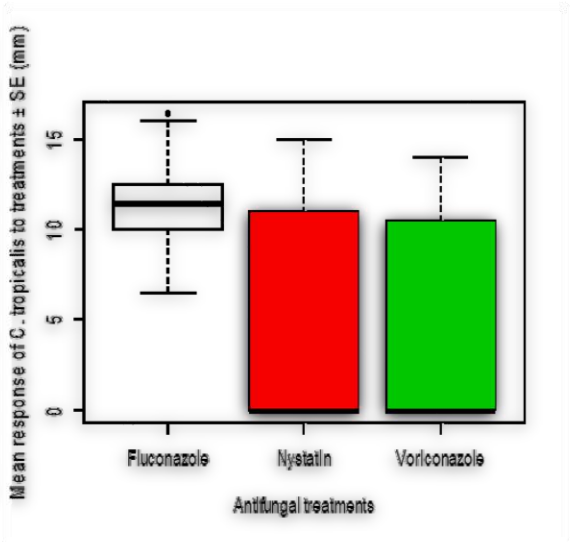
Fig 1. Agarose gel electrophoresis picture of amplified ITS bands of representative *Candida* isolates

Table 2: *In vitro* antifungal susceptibility and mean inhibitory response of the isolates

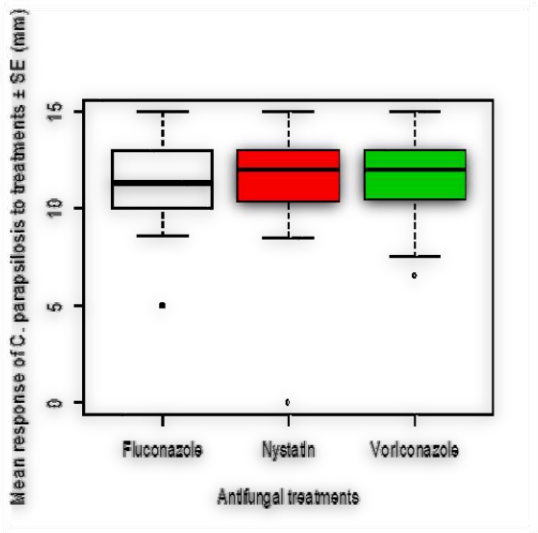
***Candida* species**	**Antifungal agents**	**No (%) of susceptible isolates**	**No (%) of intermediate isolates**	**No (%) of resistant isolates**	**Kruskal-Wallis (x^2)**
Candida albicans (n=307)	Nystatin	307 (100.0)	0	0	181.71
	Voriconazole	283 (92.1)	24 (7.8)	0	
	Fluconazole	218 (71.1)	0	89 (28.9)	
Candida (*Nakaseomyces*) *glabrata* (n=135)	Nystatin	135	0	0	9.091
	Voriconazole	135	0	0	
	Fluconazole	135	0	0	
Candida tropicalis (n=112)	Nystatin	60 (53.5)	0	52 (46.4)	229.52
	Voriconazole	96 (85.7)	0	16 (14.3)	
	Fluconazole	112 (100.0)	0	0	
Candida parapsilosis (n=63)	Nystatin	63 (100.0)	0	0	9.091
	Voriconazole	52 (82.5)	11 (17.5)	0	
	Fluconazole	63 (100.0)	0	0	
Candida akabanensis (n=93)	Nystatin	93 (100.0)	0	0	786.03
	Voriconazole	0	0	93 (100.0)	
	Fluconazole	0	0	93 (100.0)	

Fig 3 shows the mean diameters of zones of inhibition. Nystatin was the most effective antifungal agent against *C. albicans* while fluconazole was the least effective. Consequently, the mean inhibitory response of *C. albicans* to antifungal agents showed a very high significant difference (Kruskal-Wallis χ^2= 181.71, df=2, p<0.0001). The mean zones of inhibition revealed that nystatin was the most effective antifungal agent against *N. glabrata* (*C. glabrata)* followed by fluconazole and voriconazole respectively (Kruskal-Wallis χ^2=9.09 df=2, p=0.01061). The mean zone of inhibition showed that fluconazole was the most effective antifungal agent against *C. tropicalis* while voriconazole was the least effective (Kruskal-Wallis χ^2=229.52, df=2, p<0.0001).

The mean zones of inhibition revealed that nystatin was the most effective antifungal agent against *C. parapsilosis,* thus, the mean inhibitory response of *C. parapsilosis* to antifungal agents showed a significant difference (Kruskal-Wallis χ^2=6.9564, df=2, p=0.0307). The mean zones of inhibition revealed that nystatin was the most effective antifungal agent against *C. akabanensis* whereas voriconazole and fluconazole were not effective. Therefore, there was a very high significant difference (Kruskal-Wallis χ^2=792.14, df=2, p<0.001) on the mean inhibitory response of *C. akabanensis* to antifungal agents.



(a)



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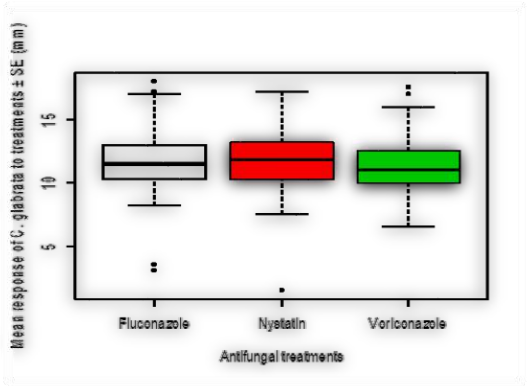

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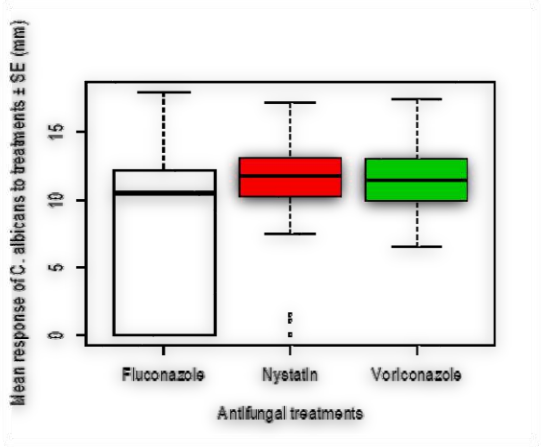
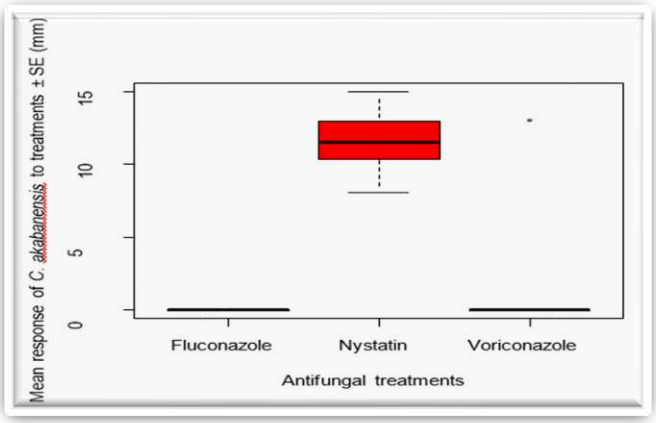


(d)



(e)

Fig 3: Mean inhibitory responses to antifungal agents of; (a) *Candida tropicalis*; (b) *Candida parapsilosis*; (c) *Nakaseomyces* (*Candida*) *glabrata*; (d) *Candida albicans*; (e) *Candida akabanensis*

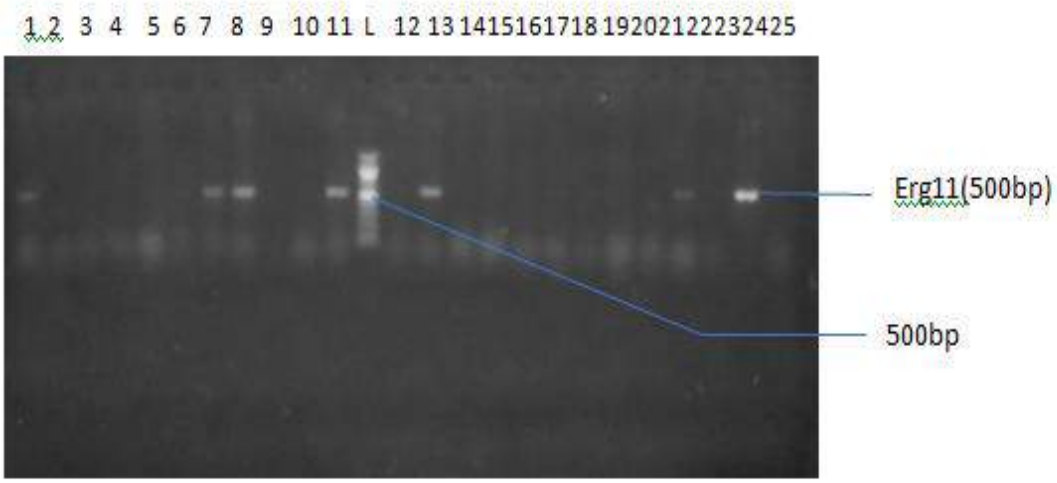


Fig 4: Agarose gel electrophoresis picture showing the bands of the amplified *Erg11* genes

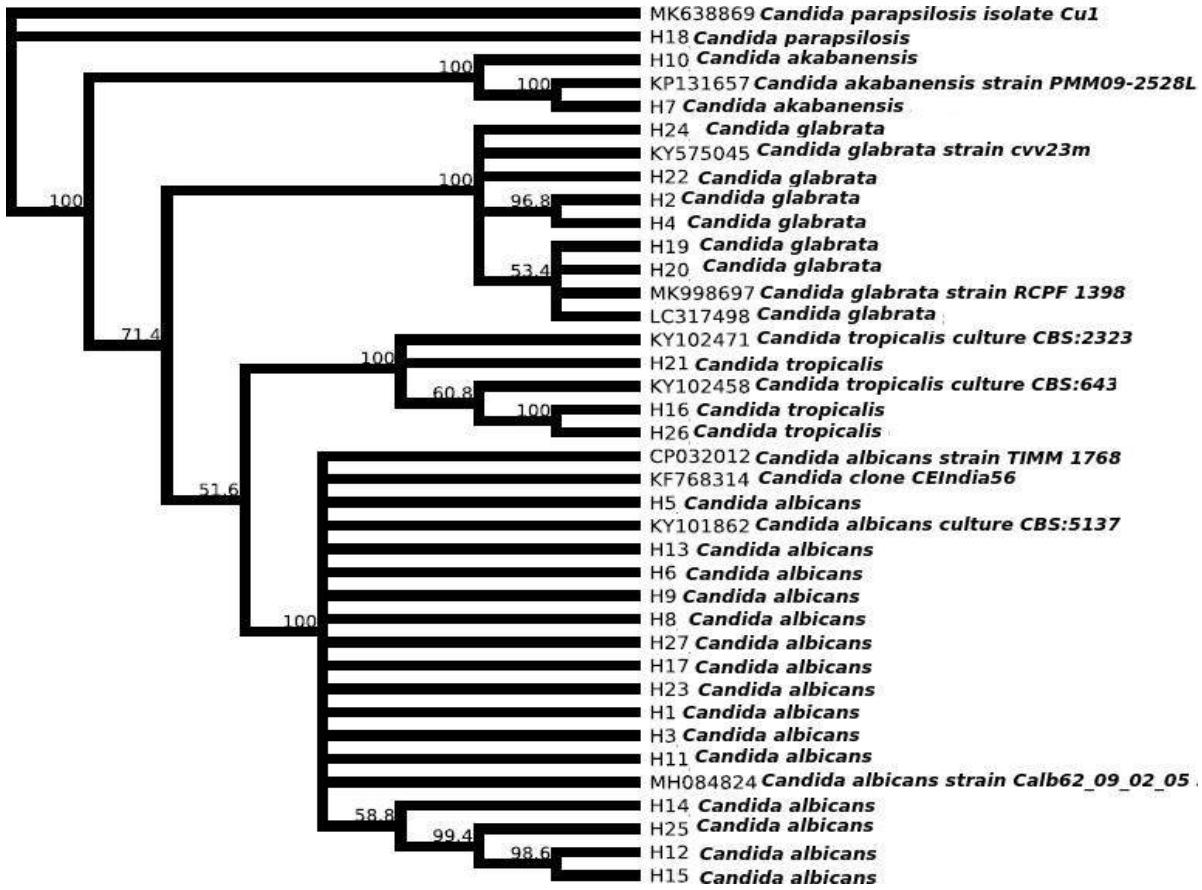


Fig 5: Phylogenetic analysis showing the evolutionary distance between the *Candida* isolates

The resistant gene, *Erg11,* was identified in all the *Candida* species resistant to the antifungal agents tested (Fig 4). *Candida albicans, C. glabrata (N. glabrata) and C. akabanensis* depicted on lanes isolates 1,7,8,11,13, 22 and 24 carried the *Erg11* gene. Lane L represents the 500bp molecular ladder. Fig 5 shows the evolutionary distances of the *Candida* isolates expressed on a phylogenetic tree. The *Candida* isolates from this study are coded as H1 to H25 while the others are reference strains obtained from the GenBank.

Discussion:

Our study observed the diversity of *Candida* species among the study participants regardless of the contraceptive type used, age marital or educational status (demographic information). Contraceptive users are more prone to VVC. Younger women had a high distribution of *Candida* species which suggest the role of estrogen in contraceptives while menopausal (45-50 years) women had the least distribution of *Candida* species. Similarly, our results revealed a high frequency of diverse *Candida* species among users of oral contraceptive pills which has been reported to increase the glycogen content of the vagina thereby increasing the accessibility of sugars that enhance *Candida* replication (35,36). We hypothesized that the use of contraceptive devices modifies the vaginal mycobiome by decreasing bacterial diversity leading to phylogenetic diverse *Candida* species responsible for VVC.

Candida albicans was the most prominent species isolated from the women diagnosed with VVC followed by non-albicans isolates, *C. glabrata* (*N. glabrata*) (19.0%) and *C. tropicalis* (16.0%). Our study results are consistent with those of Amouri et al., (37) and Yassin et al., (38) who reported that *C. albicans* represented the predominant strain responsible for VVC. This frequency of *C. albicans* may be attributed to the role of estrogen which has been reported to decrease the ability of vaginal epithelial cells to inhibit the growth of *C. albicans* or its ability to exhibit unique virulence factors which elicits robust immunopathogenicity (39-43).

Of the non-albicans *Candida* (NAC) species, *C. glabrata* (*N. glabrata*) had the highest distribution which may be due to the genomic plasticity reported in *C. glabrata* (44,45). The loss and gain of relevant genes may be crucial for its adaptation in the vaginal microbiota. The high occurrence of *C. tropicalis* may

be due to its proficient biofilm production which promotes antifungal resistance and promotes the acquisition of genetic modification in the vaginal ecosystem (46,47). Furthermore, the transition from commensalism to pathogenicity of *Candida* species may result to dysbiosis of the microbiome. Similarly, fungal-bacterial interaction may result in the modulation of the vaginal ecosystem (48).

Phenotypically, 5 *Candida* species (*C. albicans*, *C. glabrata (N. glabrata)*, *C. tropicalis*, *C. parapsilosis* and *P. kudriavzevii*/*C. krusei*) were identified from contraceptive users. DNA sequencing confirmed the phenotypic classification of 4 *Candida* isolates using CHROMagar used in our study. However, the results of the DNA sequencing revealed that the previous classification of *P. kudriavzevii* (*C. krusei*) was misrepresented as the linear order of nucleotide bases in the DNA of the isolates and MegaBlast search revealed 100% similar sequence with *C. akabanensis* which buttress the relevance of molecular diagnosis. Historically, *C. albicans*, *C. glabrata* (*N. glabrata*), *C. tropicalis* and *C. parapsilosis* have been frequently identified in women with VVC (15,49). Interestingly, *C. akabanensis*, a non-pathogenic species with no history of VVC was identified. In our opinion, the use of contraceptive devices modified the vaginal mycobiome leading to this phylogenetic diverse species responsible for VVC among the study participants.

All the *Candida* species showed varied susceptibility patterns to the three antifungal drugs and this variation may be an indication that these drugs are still potent for the treatment of VVC. However, *C. albicans* recorded 28.9% resistance to fluconazole. This may be due to the role of hormonal contraceptives which have been reported to modulate *Candida* vaginal isolates biofilm formation and decrease their susceptibility to azoles. Our observation is similar to Ruchi et al., (50) who reported a higher resistance of 40.6% to fluconazole by *C. albicans*. The robust formation of biofilm by *C. tropicalis* may be responsible for increased resistance to nystatin and voriconazole.

Ergosterol (Erg) is a vital constituent of fungal cell membranes, consequently inhibition of Erg11 protein (cytochrome P450 lanosterol 14-α-demethylase) reduces cellular ergosterol and results in the accumulation of toxic methylated sterol intermediates in the cell membrane, thereby halting cell growth (51). *Candida albicans* expressed the resistant *Erg11* gene which is consistent with the findings of Caban et al., (52). The *Erg11* resistant gene was not expressed in *C. tropicalis* yet 46.4% and 14.3% resistance to nystatin and voriconazole were phenotypically observed. This may be due to the expression of other resistant genes which were not evaluated in this study. Similarly, *C. glabrata* (*N. glabrata*) demonstrated 100.0% susceptibility to the azoles phenotypically, nevertheless, *C. glabrata* (*N. glabrata*) isolates carried the *Erg11* gene and this phenomenon may be due to mutations or increased expression of *Erg11*. The study of Yang et al., (53) reported the carriage of *Erg11* resistant gene in *C. glabrata* (*N. glabrata*).

Candida akabanensis was 100% resistant to fluconazole and voriconazole. Although *C. akabanensis* is reported to be non-pathogenic, this *Candida* specie may employ the mechanism of horizontal gene transfer through natural genetic transformation with transfer of vital genes such as antifungal resistance or virulence genes, thereby constituting a public health concern. The phylogenetic analysis confirmed the evolutionary relationships among the *Candida* isolates examined.

Conclusion:

Women using contraceptive devices in Central Nigeria harbors phylogenetically diverse *Candida* species including *C. akabanensis* an uncommon cause of VVC. Of these *Candida* species, *C. albicans*, *C. tropicalis* and *C. akabanensis* were noted for multidrug drug resistance as well as harboring *Erg11* resistant gene. The susceptibility and resistance patterns of the *Candida* species to azole antifungal drugs observed in this study can guide the appropriate treatment protocol to be initiated.

Contributions of authors:

ALY prepared the manuscript, AC and NF reviewed the manuscript, ALY and AB carried out data collection and analysis. OA and RCR performed data analysis. All authors reviewed the results and approved the final version of the manuscript.

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Authors declare no conflict of interest.

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Hydroxychloroquine and zinc ameliorate interleukin-6 associated hepato-renal toxicity induced by *Aspergillus fumigatus* in experimental rat models

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Abstract:

Background: In Nigeria, immunocompromised persons, particularly those living with HIV, are at an increased risk of developing invasive pulmonary aspergillosis caused by *Aspergillus fumigatus*. Interestingly, this condition produces symptoms that can be easily mistaken for those of COVID-19. This misdiagnosis results in their treatment with zinc and hydroxychloroquine (HCQ). To better understand the pathophysiology of aspergillosis and determine the therapeutic and toxic effects of zinc and HCQ, this study examined liver and renal functions in experimental rat models.
Methodology: Twenty-eight Albino rats, randomised into 7 groups (n=4 each) designated A to G, were used for this study. Group A rats received standardized rat chow and distilled water only. Group B rats received moderate dose of HCQ only. Group C to G rats received immunosuppressive agents (an alkylating agent: cyclophosphamide and a steroid: hydrocortisone) to simulate an immunocompromised state before being infected with *A. fumigatus* suspension (AFS). Group C rats received AFS without treatment. Group D rats simultaneously received AFS and low dose of HCQ. Group E rats simultaneously received AFS and moderate dose of HCQ. Group F rats simultaneously received AFS and high dose of HCQ, and Group G rats simultaneously received AFS and moderate dose of HCQ and zinc. Serum levels of interleukin (IL)-6 and IL-10, liver enzymes, and renal parameters were measured using standard methods. The weights of the lungs, liver, and kidneys of each rat were measured after being sacrificed. One-way analysis of variance (ANOVA) was used to compare the means (±SD) of the biochemical variables and relative weight of the organs, while Post Hoc test was used for group comparison. Pearson's correlation was used to determine relationship between parameters, with significant levels established at $p<0.05$.
Results: Higher levels of serum alanine transaminase, creatinine, and urea and lower relative lung weight were observed in group C rats (infected but untreated) compared to rats in other groups ($p<0.001$). Higher IL-6 levels and IL-6/IL-10 ratio were also observed in group C rats compared to rats in other groups ($p>0.05$).
Conclusion: This study revealed that HCQ and zinc ameliorate oxidative stress and hepato-renal damage induced by *A. fumigatus* in Albino rats.

Keywords: Cytokine; Oxidative stress; Aspergillosis; Interleukin-6; Interleukin-10

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L'hydroxychloroquine et le zinc améliorent la toxicité hépato-rénale associée à l'interleukine-6 induite par *Aspergillus fumigatus* dans des modèles expérimentaux de rats

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Résumé:

Contexte: Au Nigeria, les personnes immunodéprimées, en particulier celles vivant avec le VIH, courent un risque accru de développer une aspergillose pulmonaire invasive causée par *Aspergillus fumigatus*. Il est intéressant de noter que cette maladie produit des symptômes qui peuvent facilement être confondus avec ceux du COVID-19. Cette erreur de diagnostic entraîne leur traitement au zinc et à l'hydroxychloroquine (HCQ). Pour mieux comprendre la physiopathologie de l'aspergillose et déterminer les effets thérapeutiques et toxiques du zinc et de l'HCQ, cette étude a examiné les fonctions hépatiques et rénales dans des modèles expérimentaux

de rats.
Méthodologie: Vingt-huit rats albinos, randomisés en 7 groupes (n=4 chacun) désignés de A à G, ont été utilisés pour cette étude. Les rats du groupe A ont reçu uniquement de la nourriture pour rats standardisée et de l'eau distillée. Les rats du groupe B ont reçu uniquement une dose modérée de HCQ. Les rats des groupes C à G ont reçu des agents immunosuppresseurs (un agent alkylant: cyclophosphamide et un stéroïde: hydrocortisone) pour simuler un état d'immunodépression avant d'être infectés par une suspension d'*A. fumigatus* (AFS). Les rats du groupe C ont reçu de l'AFS sans traitement. Les rats du groupe D ont reçu simultanément de l'AFS et une faible dose de HCQ. Les rats du groupe E ont reçu simultanément de l'AFS et une dose modérée de HCQ. Les rats du groupe F ont reçu simultanément de l'AFS et une dose élevée de HCQ, et les rats du groupe G ont reçu simultanément de l'AFS et une dose modérée de HCQ et de zinc. Les taux sériques d'interleukine (IL)-6 et d'IL-10, les enzymes hépatiques et les paramètres rénaux ont été mesurés à l'aide de méthodes standard. Le poids des poumons, du foie et des reins de chaque rat a été mesuré après sacrifice. Une analyse de variance unidirectionnelle (ANOVA) a été utilisée pour comparer les moyennes (±SD) des variables biochimiques et du poids relatif des organes, tandis que le test Post Hoc a été utilisé pour la comparaison de groupes. La corrélation de Pearson a été utilisée pour déterminer la relation entre les paramètres, avec des niveaux significatifs établis à $p<0,05$.
Résultats: Des taux sériques plus élevés d'alanine transaminase, de créatinine et d'urée et un poids relatif inférieur des poumons ont été observés chez les rats du groupe C (infectés mais non traités) par rapport aux rats des autres groupes ($p<0,001$). Des taux d'IL-6 et un rapport IL-6/IL-10 plus élevés ont également été observés chez les rats du groupe C par rapport aux rats des autres groupes ($p>0,05$).
Conclusion: Cette étude a révélé que l'HCQ et le zinc atténuent le stress oxydatif et les lésions hépato-rénales induites par *A. fumigatus* chez les rats albinos.

Mots clés: Cytokine; Stress oxydatif; Aspergillose; Interleukine-6; Interleukine-10

Introduction:

Aspergillus is a filamentous mold that may cause a wide spectrum of infections including acute life-threatening infections, chronic pulmonary infections, hypersensitivity and allergic diseases depending on the host immune status or pulmonary structure (1). Aspergillosis is caused by the species of mold called *Aspergillus fumigatus*. An invasive variant of aspergillosis is a devastating illness, with mortality rates in some patient groups reaching as high as 90% (2). Over the previous two decades, the incidence of invasive aspergillosis has increased tenfold (3). Aspergillosis is seen in immunocompromised patients and can also be symptomatic in certain cases. A prevalence of 3.1% was reported among individuals living with HIV in Nigeria (4).

Studies have shown that hydroxychloroquine (HCQ) modulates the immune system by interfering with lysosomal acidification, inhibition of antigen presentation, and down-regulation of cytokine production and secretion by monocytes and T-cells (5). Hydroxychloroquine inhibits cytokine production and modulation of certain co-stimulatory molecules (6) while zinc is an essential vital exogenous mineral that aids DNA synthesis, enzymatic reactions, immune functions, protein synthesis, wound healing, growth, and development (7).

Inflammatory cytokines such as interleukin (IL)-6 and IL-10 are important biomarkers for distinguishing infections caused by various pathogens (8). IL-6 and interferon gamma (IFN-γ) were reported to be predominantly elevated in invasive pulmonary aspergillosis and *Pneumocystis pneumonia* (8). IL-10 increases the host susceptibility to lethal fungal infection, possibly because IL-10 is associated with T_{h2} response, down-regulation of T_{h1} response, and macrophage activation (9).

With the rising frequency of pulmonary aspergillosis diagnosis and chronic obstructive pulmonary disease in Nigeria, it has become critical to assess the efficacy of HCQ and zinc in alleviating respiratory distress associated with pulmonary aspergillosis. For the first time, this study investigated the curative and toxic effects of different doses of HCQ and zinc on experimentally induced pulmonary aspergillosis and respiratory distress in Albino rats following exposure to *A. fumigatus*.

Materials and method:

Study site and ethical considerations:

This experimental study was carried out at the vivarium in the Department of Physiology, Nnamdi Azikiwe University, Nnewi Campus, Nigeria. Ethical approval for the research was obtained from the College of Health Sciences Ethics Committee at the Nnamdi Azikiwe University (NAU/FHST/2021/MLS68 and NAU/FHST/2021/MLS105). The rats were carefully handled in line with the 2011 ethical standards and protocols for the care and use of laboratory animals of the National Institute of Health.

Collection of *Aspergillus* isolate for induction of aspergillosis:

Aspergillus fumigatus isolate used in the study was obtained from a patient sample in Onitsha and this was sub-cultured on Sabouraud dextrose agar (SDA) at 37°C for 2 days. The culture growth was identified microscopically following the Lactophenol cotton blue (LCB) staining technique as described by Moore and Jaciow (10). The conidia were extracted by washing the plates with sterile

0.2% Tween 20 and Normal Saline, followed by centrifugation and filtration of the suspension.

Median effective dose (ED50) of the drugs used in the study:

The oral LD_{50} of HCQ in rats is reported to be 1240 mg/kg while the therapeutic dose is 10 mg/kg (11). The oral LD_{50} of zinc salts in rats is 237–623 mg/kg (12) while the LD_{50} of hydrocortisone acetate is 150 mg/kg. The intraperitoneal LD_{50} of cyclophosphamide mixed with halothane is 152 mg/kg (13). Both cyclophosphamides mixed with halothane and hydrocortisone were used as immunosuppressive agents.

Study design and animal handling:

The test system for this study was Albino rats (n=28) weighing 80±20g (Table 1). The rats were allowed to acclimatize in the vivarium for two weeks before the start of the experiment and their daily consumption of food was noted. They were then randomized into seven groups (A-G), with each group containing four rats.

On day 1, rats in groups C to G were given moderate dose of steroid-sparing agent (cyclophosphamide 75 mg/kg, ~0.3 ml intraperitoneally) and steroid (hydrocortisone acetate 80 mg/kg, ~0.32 ml intraperitoneally) to simulate immunocompromised state, replicating real-life situation as aspergillosis, which majorly affects immunosuppressed individuals, thus facilitating the development of the disease. The steroids were administered with syringes while HCQ and zinc were administered orally with an oral cannula.

On day 6, the rats were anaesthetized with 0.5 ml ketamine, administered slowly over 60 seconds. Still on day 6, aspergillosis was sufficiently induced with *Aspergillus* suspension in drops into the right nasal cavity using a tuberculin syringe, with the rat nostrils pointed facing upwards. The suspension of *A. fumigatus* conidia was dropped at a slow rate (150 µl) into the right nasal cavity with the tuberculin string gently inserted 1.5 cm deep into the nasal cavity.

On day 7, a second batch of immunosuppressants was administered (cyclophosphamide intraperitoneally at a dose of 60 mg /kg) to ensure immunosuppression. On days 8, 9, and 10, rats were also exposed to *A. fumigatus* again via inhalation of the conidia spore. Starting from day 13 to day 15, the respective rats (groups D-G) were given their corresponding doses of HCQ and zinc. On day 16, the rats were made to fast and then sacrificed on day 17.

Sample collection and laboratory analysis:

On day 17, the rats were anaesthetized by placing them in an air-tight jar containing cotton wool soaked in chloroform. Following loss of reflexes, response to stimulus, and reduction in the animal respiratory rate, blood samples were collected by ocular puncture. The blood samples were collected into EDTA (Alpha Surgicare) and plain bottles, labelled and left undisturbed for 15 minutes. The whole blood samples were centrifuged for 10 minutes at 3000 rpm to separate the serum.

The lungs, kidneys, and liver of the rats were subsequently harvested, processed, and stained using the Haematoxylin and Eosin (H & E) staining technique as described by Feldman and Wolfe (14).

Table 1: Description and regimen for rats in the control and experimental groups

Groups of rats		Description	Treatment
Control	Group A	Neutral control	The animals in this group received the standardized rat feed and distilled water for 28 days
	Group B	Negative control	The animals received the standardized rat feed and distilled water, alongside moderate dose of HCQ (40 mg/kg ~0.17 ml)
	Group C	Positive control	Infected with *A. fumigatus* (conc. = 5 × 10^7 CFU/ml) without any treatment
Experimental	Group D	Treatment	Infected with *A. fumigatus* and treated with a low dose of the HCQ orally (20 mg/kg ~0.085 ml)
	Group E	Treatment	Infected with *A. fumigatus* and treated with a moderate dose of HCQ orally
	Group F	Treatment	Infected with *A. fumigatus* and treated with a high dose of HCQ orally (80 mg/kg ~0.34 ml)
	Group G	Treatment	Infected with *A. fumigatus* and treated with a moderate dose of HCQ and Zinc (15 mg/kg ~0.063 ml)

All animals received standard feed and distilled water *ad libitum*

Determination of serum levels of interleukins 6 and 10:

Before the biochemical assays were carried out, all reagents, serum, calibrators, and controls were brought to room temperature (27°C). The serum levels of IL-6 and IL-10 were determined using ELISA Microwell Test Kit at 450 nm absorbance in a microplate reader. The IL-6 ELISA kit Fine (Test ER0042) possessed a reactivity range of 62.5 - 4000 pg/ml and a sensitivity of 37.5 pg/ml, while the IL-10 ELISA test kit Fine (Test ER0033) possessed a reactivity of 31.25 - 2000 pg/ml and a sensitivity of 18.75 pg/ml.

Statistical analysis:

The Statistical Package for the Social Sciences (SPSS) version 25.0 and GraphPad Prism (version 6.0) were used to analyze the data. One-way analysis of variance (ANOVA) was used to compare the means (±SD) of the variables from the biochemical assay as well as relative organ weights (organ weight over total body weight multiplied by 100).

The Post Hoc test was used for group comparison. To determine the relationships between parameters, Pearson's correlation was used. Data were considered significant at $p \leq 0.05$, ≤ 0.01, and ≤ 0.001. Relative organ weight was calculated as the weight of the organ (lungs, liver, and kidney) divided by total body weight multiplied by 100.

Results:

Following intraperitoneal injection of cyclophosphamide and hydrocortisone acetate, experimental rats in groups C, D, E, F, and G had decreased appetite; a reduction in consumption of daily ration compared to the amount consumed during the acclimatization period. Four days post-induction of immunosuppression, the rats exhibited lethargy and decrease in their reflexes. The rats recovered from the lethargy and decreased reflexes 9 days post-immunosuppression.

On day 7, a day after the first fungal inoculation, sneezing and nose scratching were observed in the *Aspergillus*-infected groups and these increased with subsequent fungal inoculations.

Interleukin 6 and 10 levels and ratio:

The pro-inflammatory cytokine (IL-6) value was higher for rats in group C (infected but untreated) compared with rats in other groups (p=1.34>0.05), but this value only reached significant level (p<0.05) when compared with rats in treatment group D (p=0.018), group E (p=0.046), and group F (p=0.049), but not with rats in treatment group G (p=0.704) (Table 2). Hydroxychloroquine treatment caused a decrease in IL-6 for rats in group D (infected but treated with low dose HCQ) compared with rats in other treatment groups (p=1.34>0.05) but this decrease was significant when compared with rats in only treatment group G (p=0.041), while at moderate (group E) and high (group F) dosages of HCQ (towards the LD_{50}), the decrease in IL-6 was minimal (not statistically significant), although this minimal decrease could be attributed to oxidative stress produced by moderate/high dose of HCQ.

A higher IL-10 value was observed for rats in group G compared with rats in the other groups, but this did not reach a significant level (F=0.969, p=0.470). There was a direct correlation between IL-6 and IL-10 (r= 0.649, p<0.001). Since group C had a higher IL6/IL-10 ratio compared with other groups (Fig 1), it could be argued that group C rats had the worst outcome (p> 0.05).

Dysregulation of liver enzymes:

There was a direct relationship between AST and ALT (r=0.565, p=0.002), AST and ALP (r=0.391, p=0.044), and ALT and ALP (r=0.588, p=0.001). A higher AST was observed in group B, treated with HCQ alone, compared with the neutral control (group A-uninfected and untreated) (p=0.029<0.05) (Table 2). This suggests that HCQ can induce hepatoxicity in animals that have no underlying disease. However, treatment with HCQ among rats with aspergillosis (groups D, E and F) resulted in reduced AST levels compared with the infected and untreated group (group C) although the AST levels were not significantly different between the groups (p>0.05).

In group B, a higher level of ALT was also observed compared with other groups (p<0.05) except group C, which had the highest ALT value. The highest levels of both ALT (p=0.000) and ALP (p=0.077) were observed in group C rats (infected but untreated), compared to the control and treatment groups. Furthermore, infected rats treated with moderate dose of HCQ and zinc had significantly lower levels of ALP compared to other groups (p<0.05), except for group A, which had comparative ALP levels (p=0.498> 0.05). This also suggests that the addition of zinc to the treatment regime mitigated the hepatic damage induced by aspergillosis and moderate doses of HCQ.

Table 2: Mean comparison of interleukins and liver enzyme levels across experimental groups

Groups	IL-6 (pg/ml)	IL-10 (pg/ml)	AST (U/L)	ALT (U/L)	ALP (U/L)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Group A	4.41 ± 0.78	112.92 ± 24.04	10.50 ± 1.29	9.50 ± 2.38	54.25 ±19.52
Group B	4.58 ± 0.30	109.34 ± 14.82	13.75 ± 1.26	12.50 ± 0.58	71.75 ±19.52
Group C	5.11 ± 0.36	115.37 ± 12.30	12.50 ± 4.51	15.75 ± 2.50	104.50 ± 33.73
Group D	3.61 ± 1.18	101.23 ± 24.39	9.75 ± 2.06	8.75 ± 0.96	81.50 ±21.30
Group E	3.87 ± 0.56	102.24 ± 10.94	10.25 ± 0.96	10.00 ± 2.31	65.50 ±9.88
Group F	3.89 ± 0.51	116.3 ± 3.63	9.75 ± 2.06	9.75 ± 1.71	80.00 ±23.51
Group G	4.88 ± 1.40	127.92 ± 27.26	11.00 ± 1.15	9.50 ± 1.29	64.75 ±15.73
F-value	1.870	0.969	1.864	7.422	2.258
P-value	0.134	0.470	0.135	0.000*	0.077
A vs B	0.777	0.788	0.050[a]	0.029[a]	0.264
A vs C	0.248	0.854	0.215	0.000[c]	0.003[b]
A vs G	0.432	0.267	0.753	1.000	0.498
A vs D	0.182	0.384	0.637	0.565	0.088
A vs E	0.359	0.426	0.875	0.701	0.469
A vs F	0.376	0.799	0.637	0.848	0.106
B vs C	0.378	0.652	0.434	0.019[a]	0.043[a]
B vs D	0.110	0.544	0.018[a]	0.008[b]	0.529
B vs E	0.234	0.595	0.036[a]	0.065	0.686
B vs F	0.247	0.602	0.018[a]	0.044[a]	0.594
B vs G	0.612	0.172	0.094	0.029[a]	0.651
C vs D	0.018[a]	0.294	0.094	0.094	0.146
C vs E	0.046[a]	0.330	0.166	0.166	0.018[a]
C vs F	0.049[a]	0.944	0.094	0.094	0.123
C vs G	0.704	0.350	0.349	0.349	0.016[a]
D vs G	0.041[a]	0.055	0.434	0.434	0.284
D vs E	0.663	0.939	0.753	0.291	0.306
D vs F	0.639	0.264	1.000	0.393	0.923
E vs F	0.973	0.297	0.753	0.710	0.352
E vs G	0.096	0.064	0.637	0.836	0.961
F vs G	0.103	0.387	0.434	0.868	0.328

Statistical analysis: ANOVA and Post hoc Test. *Significance is set at $p \leq 0.05$ = [a], ≤ 0.01= [b], ≤ 0.001= [c] Number of animals per group = 4.

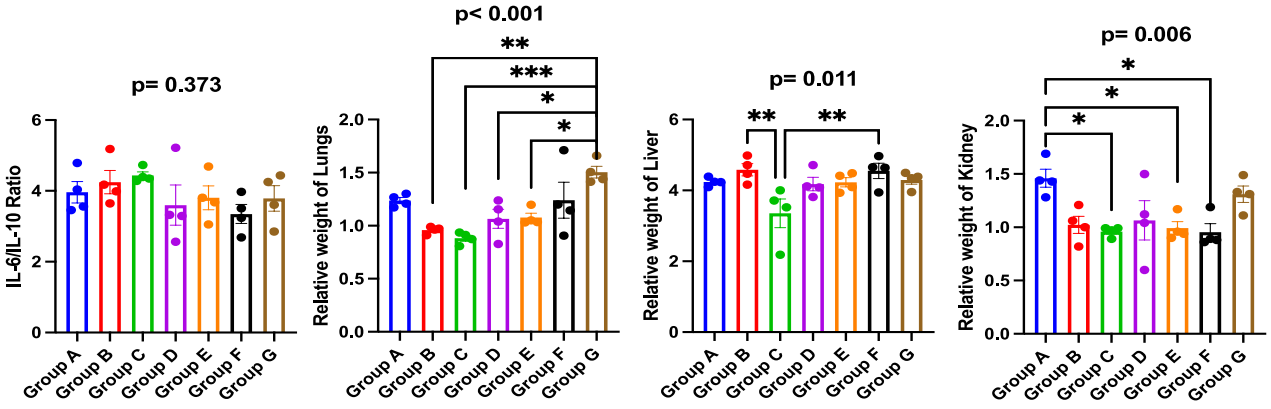

Fig 1: Relative organ weight and Interleukin ratio across experimental groups

In Fig I, rats in group C (infected but untreated) had a higher IL-6/IL-10 ratio (but this is not statistically significant at p=0.373) and lower relative weight of the lungs, liver, and kidneys (consistent with atrophy) compared with the control and treatment groups.

The IL-6/IL-10 ratio and relative organ weight of Group G rats (infected but treated with moderate dose of HCQ and zinc) were like those of Group A rats (uninfected and untreated), which suggests that addition of zinc to moderate dose of HCQ improved treatment outcomes in infected rats.

Table 3: Mean comparison of renal function parameters across the experiment groups

Groups	Electrolytes (mEq/L)				Creatinine (mg/dl)	Urea (mg/dl)
	Potassium	Sodium	Chloride	Bicarbonate		
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Group A	4.83 ± 0.25	141.75 ± 3.30	100.50 ± 4.12	24.25 ± 1.26	9.25 ± 1.26	42.00 ± 8.76
Group B	4.40 ± 0.59	142.00 ± 1.41	103.25 ± 0.96	23.25 ± 1.71	9.75 ± 0.50	44.75 ± 4.79
Group C	5.18 ± 0.83	141.50 ± 2.38	101.25 ± 1.50	25.00 ± 0.82	14.75± 2.22	132.25 ± 34.33
Group D	4.28 ± 0.34	145.00 ± 1.41	104.00 ± 1.41	25.00 ± 0.96	8.50 ± 1.29	49.00 ± 2.83
Group E	4.63 ± 0.43	142.50 ± 2.65	102.75 ± 2.50	23.75 ± 1.71	9.50 ± 0.58	44.25 ± 6.50
Group F	4.80 ± 0.64	143.25 ± 3.10	103.75 ± 2.06	24.50 ± 1.29	8.75 ± 0.96	45.50 ± 9.25
Group G	4.95 ± 0.39	142.50 ± 1.73	102.50 ± 1.29	24.75 ± 0.96	8.75 ± 1.50	49.25 ± 10.01
F-value	1.388	0.985	1.354	1.201	11.252	19.873
p-value	0.265	0.460	0.278	0.344	0.000*	0.000*
A vs C	0.362	0.884	0.637	0.419	0.000[c]	0.000[c]
A vs D	0.158	0.069	0.036[a]	0.284	0.425	0.508
A vs F	0.948	0.386	0.050[a]	0.786	0.594	0.740
B vs C	0.052	0.771	0.215	0.068	0.000[c]	0.000[c]
B vs D	0.742	0.091	0.637	0.039[a]	0.190	0.687
C vs D	0.026[a]	0.051	0.094	0.786	0.000[c]	0.000[c]
C vs E	0.158	0.561	0.349	0.184	0.000[c]	0.000[c]
C vs F	0.329	0.313	0.125	0.588	0.000[c]	0.000[c]
C vs G	0.555	0.561	0.434	0.786	0.000[c]	0.000[c]

Statistical analysis: ANOVA and Post hoc Test. *Significance is set at $p \leq 0.05$ = a, ≤ 0.01= b, ≤ 0.001= c Number of animals per group = 4.

Alterations in renal function of rats:

Higher levels of serum creatinine and urea were observed in group C rats (infected but untreated) compared to rats in the treatment groups ($p<0.05$) (Table 3), which suggests that aspergillosis induces renal damage as well. The result in Table 3 suggests that a low dose of HCQ reduced the elevated level of potassium induced by aspergillosis in the Group D rats ($p<0.05$). A dose-dependent increase in potassium levels was also observed among rats treated with low, moderate, and high doses of HCQ ($p>0.05$).

Rats that received moderate dose of HCQ had better renal function compared to rats that received low or high doses of HCQ. Results indicate that adding zinc to moderate dose of HCQ did not improve renal function compared to treatment with HCQ alone. Pearson's correlation revealed a direct relationship between potassium and urea (r=0.481, $p=0.011$), sodium and chloride (r=0.864, $p< 0.001$), and urea and creatinine (r=0.700, $p< 0.001$).

Discussion:

This study assessed the immunological and biochemical effects of HCQ and zinc therapy in experimentally induced invasive pulmonary aspergillosis. Inflammatory cytokines, especially IL-6, are important biomarkers for distinguishing trauma or inflammation associated with infections caused by pathogens such as *Aspergillus* (8,15,16). This study revealed a lower IL-6 level and improved glomerular filtration rate in *A. fumigatus*-infected groups (D, E, and F) treated with HCQ or combined therapy of HCQ and zinc, compared to *A. fumigatus*-infected but untreated group (group C). This finding suggests that HCQ possess immunosuppressive effects and could be used in the management of many inflammatory diseases.

Studies have shown that, to restore homeostasis, the release of pro-inflammatory cytokines (primarily IL-6) results in subsequent release of anti-inflammatory cytokines such as IL-10 (15,17). However, the compensatory increase in IL-10 levels is often ineffective in counteracting increasing levels of IL-6 (18). This could be the explanation for the sustained elevation of IL-6 levels despite high IL-10 levels in Group C rats in our study. Interestingly, Feng et al., (19) reported a consistent rise in serum IL-6, IL-10 and white blood cells in mild sepsis, and severe sepsis to septic shock (19). This systemic inflammatory response syndrome depicts the host acute phase immune reaction to infec-

tion and trauma, which could lead to multiple organ dysfunctions due to cytokine storm and neutrophil activation (20).

Trauma-related elevation of pro-inflammatory cytokines have been associated with respiratory distress syndrome and organ failure (21). Immune cells and cytokines play crucial roles in the pathogenesis of disease and treatment outcomes. In recent years, the ratio of pro-inflammatory (IL-6) to anti-inflammatory cytokine (IL-10) i. e. IL-6/IL-10 ratio has been used as a reliable marker of inflammation (21,22). The IL-6/IL-10 ratio was higher in groups B and C rats causing them to have low organ weight and body mass in relationship to the other groups. An increase in IL-6/IL-10 ratio has been shown to be directly associated with a poor outcome (21). Our study revealed that the IL-6/IL-10 ratio decreased significantly in group F rats, which correlated with a decrease in oxidative stress and organ damage. This is evidenced by a reduction in IL-6 level and increase in IL-10 level, especially in treatment Groups D and E. This elevation in IL-10 serves to suppress immune responses, warding off autoimmunity, and decreasing oxidative stress levels (23).

Upon the administration of zinc as an immune booster (Group G rats), there was an increase in the level of IL-6, irrespective of the synergized effects of the HCQ (which acted as an immunosuppressant in aspergillosis-induced groups). Zinc induces monocytes to produce interleukin-1 (IL-1), IL-6 and tumour necrosis factor-α (24). Similarly, decreased production of T_{h1} cytokines and interferon-α (IFN-α) by leukocytes in healthy elderly persons is correlated with low zinc serum levels. It is important to note that in response to zinc supplementation, plasma cytokines exhibit a dose-dependent response (25). According to the findings of this study, the use of HCQ in the absence of aspergillosis (group B negative control) resulted in an augmentation of organ weight and elevated liver enzymes. The shift in liver function could potentially be attributed to heightened levels of IL-6.

Research indicates that IL-6 may substantially impact B cell hyperactivity and directly impact tissue harm (26). However, other research showed that HCQ was able to ameliorate aspergillosis-induced hepatoxicity through concomitant reduction of IL-6 levels and decreased polymorphonuclear infiltrates (27). Although zinc supplements have not been shown to result in higher expression of IL-10 (28), the results from our study revealed higher expression of IL-10 when HCQ and zinc were simultaneously administered to the experimental rats. Despite the fact that HCQ has shown promise in treating respiratory diseases, there are concerns about its potential side effects such as renal toxicity and heart failure, particularly when taken at high doses, as noted by Alanagreh et al., (29). Nevertheless, the finding of our study suggests that low dose of HCQ or combination of moderate dose of HCQ and zinc may improve renal function when dealing with aspergillosis. This finding is reinforced by a previous study that highlighted the potential beneficial effects of HCQ on renal dysfunction, as outlined by Bourke et al., (30). Taken together, it could be argued that other immunological factors may be associated with the drop in IL-6 in the HCQ treatment groups. This warrants future studies.

Conclusion:

Our research revealed that both HCQ and zinc have the potential to alleviate cytokine levels and relative organ weight. This indicates that HCQ might be able to lower the risk of autoimmunity and systemic inflammatory response syndrome associated with systemic fungi infection such as invasive aspergillosis that can be life-threatening.

Contributions of authors:

JOO conceptualized the research and was involved in data analysis and original manuscript draft preparation; ATB conceptualized the research and was involved in literature searches, visualization and data curation; OGO and POA were involved in literature searches, visualization and data curation; POA and NEE cultured and isolated *A. fumigatus* for inoculation in the rat models. All authors read and approved the final version of the manuscript.

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Original Article **Open Access**

Molecular detection of hepatitis E virus among swine and poultry birds in Lagos, Nigeria

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Abstract:

Background: Hepatitis E virus (HEV), the only hepatitis virus that replicates in humans and a wide range of animal hosts, is a significant public health enteric virus with a growing trend of infection globally. The public and environmental implications associated with HEV as a zoonotic transmitted virus remain to be fully elucidated. Thus, with the limited information on HEV in other species other than humans in Nigeria, this study aimed to detect by molecular methods HEV among some livestock in Lagos, Nigeria.
Methodology: A cross-sectional study of 172 (42.0%) poultry birds aged between 5 and 18 months, and 238 (58.0%) swine aged between 2 and 18 months purposively selected from Ojo, Ikorodu and Agege Local Government Areas (LGAs) of Lagos State, Nigeria between November 2017 and July 2019 was conducted. A total of 410 non-repetitive stool samples collected were analysed by molecular technique for the detection of HEV RNA. Descriptive statistics were computed for all relevant data. The association between gender and age with HEV RNA positivity was tested using Chi-square. All significant associations were recorded at $p \leq 0.05$.
Results: On the overall, 15 (3.7%) of the 410 stool samples were positive for HEV RNA with 5 (2.9%) and 10 (4.2%) of the 172 and 238 poultry birds and swine respectively. More female livestock (6.0%) had detectable HEV RNA than their male counterparts (1.0%) and the infection clustered majorly among age groups 1-6 months, and 7-12 months with a detection rate of 9.3%, 3.2% and 5.6%, 3.2% for both the swine and poultry birds respectively. Approximately 11.1% of the swine and 5.0% of the poultry birds' samples from Ikorodu LGA were positive for HEV RNA. Only 3.0% of the swine samples from Ojo LGA had detected HEV RNA. No sample from Agege LGA had detectable HEV RNA.
Conclusion: The detection of HEV in both the swine and poultry birds in Lagos, Nigeria further confirms the endemicity of HEV and a cause for public health concern regarding the epidemiology of HEV in Nigeria. There is an urgent need for active and continuous surveillance to further detect and subtype the circulating HEV among livestock to prevent the advent of virulent strains that may be transmitted to handlers and the community at large.

Keywords: Hepatitis E Virus, Swine, Poultry birds, Zoonotic Transmission, Polymerase Chain Reaction.

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Détection moléculaire du virus de l'hépatite E chez les porcs et les volailles à Lagos, Nigeria

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Contexte: Le virus de l'hépatite E (VHE), le seul virus de l'hépatite qui se réplique chez l'homme et chez un large éventail d'hôtes animaux, est un virus entérique important pour la santé publique avec une tendance croissante d'infection à l'échelle mondiale. Les implications publiques et environnementales associées au VHE en tant que virus transmis par des zoonoses restent à élucider pleinement. Ainsi, compte tenu des informations limitées sur le VHE chez d'autres espèces autres que les humains au Nigeria, cette étude visait à détecter par des méthodes moléculaires le VHE chez certains animaux d'élevage à Lagos, au Nigeria.
Méthodologie: Une étude transversale portant sur 172 (42,0%) volailles âgées de 5 à 18 mois et 238 (58,0%) porcs âgés de 2 à 18 mois, sélectionnés à dessein dans les zones de gouvernement local (LGA) d'Ojo, Ikorodu et Agege de L'État de Lagos, au Nigéria, a été mené entre novembre 2017 et juillet 2019. Au total, 410 échantillons de selles non répétitifs collectés ont été analysés par technique moléculaire pour la détection de l'ARN du VHE. Des statistiques descriptives ont été calculées pour toutes les données pertinentes. L'association entre le sexe et l'âge avec la positivité de l'ARN du VHE a été testée à l'aide du chi carré. Toutes les associations significatives ont été enregistrées à $p \leq 0,05$.
Résultats: Au total, 15 (3,7%) des 410 échantillons de selles étaient positifs pour l'ARN du VHE, dont 5 (2,9%) et 10 (4,2%) des 172 et 238 volailles et porcs respectivement. Un plus grand nombre de femelles (6,0%) présentaient un ARN du VHE détectable que leurs homologues mâles (1,0 %) et l'infection se concentrait principalement dans les tranches d'âge de 1 à 6 mois et de 7 à 12 mois avec un taux de détection de 9,3%, 3,2% et 5,6%, 3,2% respectivement pour les porcs et les volailles. Environ 11,1% des échantillons de porcs et 5,0% des échantillons de volailles de la LGA d'Ikorodu étaient positifs pour l'ARN du VHE. Seulement 3,0% des échantillons porcins d'Ojo LGA avaient détecté l'ARN du VHE. Aucun échantillon d'Agege LGA ne contenait d'ARN du VHE détectable.
Conclusion: La détection du VHE chez les porcs et les volailles à Lagos, au Nigeria, confirme en outre l'endémicité du VHE et une source de préoccupation pour la santé publique concernant l'épidémiologie du VHE au Nigeria. Il existe un besoin urgent d'une surveillance active et continue pour détecter et sous-typer le VHE en circulation parmi le bétail afin de prévenir l'avènement de souches virulentes susceptibles d'être transmises aux manipulateurs et à la communauté dans son ensemble.

Mots clés: Virus de l'hépatite E; porcs; volailles; transmission zoonotique; réaction en chaîne par polymérase

Introduction:

Globally, Hepatitis E virus (HEV) has received recognition as a significant public health enteric ribonucleic acid (RNA) virus that infects and replicates in humans and a wide range of animal hosts such as wild and domestic swine, deer, chicken, mongoose, rat, ferret, fish, and rabbits, with an ever-expanding host range (1-5). The peculiarity of HEV is that among all known major hepatitis viruses (A, B, C, E and G), HEV is the only one with animal reservoirs and is zoonotic (2,6-9).

Hepatitis E virus belongs to a group of non-segmented, non-enveloped and single-stranded RNA family Hepeviridae, which is divided into two genera, Orthohepevirus and Piscihepevirus (5,9,10-12). The genus, Orthohepevirus, where HEV is domiciled has four designated species known as Orthohepevirus A to D (9,12,13). Orthohepevirus A contains eight genotypes, HEV-1 to HEV-8 (9,13).

HEV-1 and HEV-2 are only implicated in human infections and are responsible for large hepatitis E outbreaks described in developing regions like Africa and Asia (5,9,14,15). HEV-3 and HEV-4 have been isolated in humans and other animals and are the main cause of sporadic infection among humans in develped countries. These two genotypes are considered zoonotic, and pigs and other animal species are reservoirs of these viruses which are transmissible to humans (5,9,12). HEV-5 and HEV-6 have been identified in Japanese wild boars; HEV-7 has been described recently in an immunocompromised transplant patient and dromedary camels, while HEV-8 was detected recently in Bactrian camels in China (5, 9,12). During an outbreak of HEV in 2017 in Nigeria, analysis of HEV genotypes responsible for the outbreak revealed the predominance of HEV genotypes 1 (HEV-1) and 2 (HEV-2), the generation of the second full-length HEV-1e genome available till date in the country (16, 17).

It has been estimated that about 20 million cases of HEV infections resulting in 3.4 million clinical cases, 30,000 stillbirths and

about 70,000 deaths, occur worldwide each year (5,18). In developing countries, HEV is generally considered a waterborne disease, transmitted by poor sanitation and faecal contamination of water supplies, and has also been associated with high morbidity and mortality in pregnant women (15,16,19,20). In the developed world, it is primarily a zoonotic disease and transmission is by consumption of undercooked infected meat, especially pork (5,9,11,14,15,19).

Hepatitis E virus is still regarded as an emerging pathogen in developing countries including sub-Saharan Africa, where information regarding the actual burden of the disease in humans and most particularly animals, is currently lacking due to inattention to the disease and limited public health responses (20). The public and environmental health concerns and risks associated with HEV infection, with more emphasis on zoonotic transmission, remain to be fully elucidated in this part of the world. However, documented evidence of HEV infections/outbreaks particularly in humans with few studies in animals have been described in Kenya, Sudan, Uganda, the Democratic Republic of Congo (DRC), Cameroon and the Central African Republic (14,15,21,22).

In Nigeria, varying prevalences of HEV infections have also been documented in different States; Jos-42.7% (23); Oyo-1.7% (24); Ekiti-13.4% (25); Osun-43% (26) and Lagos-17.8% (27). These infections have mostly been linked to human populations (9,17,28,29). Just a few studies have documented HEV in animals in Nigeria (17). Osanyinlusi et al., (30) in 2020 reported a high prevalence of HEV RNA detected in *Rattus norvegicus*, a rodent predominantly present within human dwelling, makes rodents an obvious target for further investigations for their roles in HEV epidemiology in Nigeria.

Based on the dearth of information on HEV in species other than humans in Nigeria, this study aimed to detect, using molecular methods, HEV among animals in Lagos State, the most populous and economic nerve center of Nigeria.

Materials and method:

Study area:

This study was conducted in three (Ojo, Ikorodu and Agege) selected local government areas (LGAs) of Lagos State, Nigeria, based on the locations having farms with mixed husbandry practice. Lagos is the commercial nerve centre of Africa and the most populous black city with over 21 million people (31). Lagos State is in South-Western region of Nigeria, with a total land area of 356,861 hectares (h), including 75,755 h of wetlands (31).

Lagos State is divided into five divisions consisting of Ikeja, Badagry, Ikorodu, Lagos Island and Epe (IBILE), with different farms spreading across the divisions where animals such as swine, chicken, cattle, and the likes are reared and sold in mixed husbandry practice. It shares its northern and eastern boundaries with Ogun State, its western boundary with the Republic of Benin, and its southern boundary with the Atlantic Ocean. It consists of 20 LGAs, with 37 local council development areas. It has a very diverse population due to heavy migration from other parts of Nigeria and surrounding countries.

Study design:

This was a cross-sectional study on molecular detection of the HEV among poultry birds, and swine in selected LGAs of Lagos State, Nigeria, between 2017 and 2019.

Ethical approval:

Approval for the study was obtained from the Health Research Ethics Committee (HREC) of the College of Medicine, University of Lagos with approval identification code CM/HREC/2/17/103, and the Lagos State Ministry of Agriculture.

Sample collection, transportation, & storage:

A total of 410 stool samples from active and inactive animals of both sexes and all ages, consisted of 172 chicken and 238 swine were collected using random sampling technique during the study period. The distribution of the samples within the LGAs sampled were; 164 from 101 chicken and 63 from swine in Ikorodu LGA; 171 from 71 chicken and 100 from swine in Ojo LGA; and 75 from 15 chicken and 60 from swine in Agege LGA.

Freshly voided droppings were collected using sterile universal sample containers with scoops and transported immediately in cold chain using triple-level packaging to the Centre for Human and Zoonotic Virology (CHA ZVY), College of Medicine of the University of Lagos, Idi-Araba, Lagos. All the samples were stored at -80ºC until required for RNA extraction, amplification, and detection by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

Viral RNA extraction, RT-PCR and nested PCR:

RNA extraction of HEV was done in a class II biosafety cabinet using the QIAamp Viral RNA Mini Spin kit (Qiagen, Germany), according to the manufacturer's instructions. In the first round, the RT-PCR was performed with the extracted RNA using the One-Step RT-PCR Master Mix Reagent (Jena Bioscience, Germany) in a total volume of 25µl comprising of 1µl each of the primers; HEV-cs 5'-TCG CGC ATC ACM TTY TTC CAR AA (23)-3'and HEV-cs 5-GCC ATG TTC CAG ACD GTR TTC CA (23)-3', 12.5µl one strength (1x) PCR Buffer, 1µl 1x enzyme mix, 5µl of extracted RNA and 4.5µl

molecular grade water. The RT-PCR cycling condition used was 30 minutes at 50^0C for reverse transcription reaction, followed by 5 minutes at 95^0C for reverse transcriptase inactivation/denaturation of the cDNA, followed by 40 cycles amplification of 15 seconds at 95^0C for denaturation and 15 seconds at 55^0C for annealing, with an extension of 20 seconds at 68^0C and a final extension at 3 minutes at 68^0C.

The first round of RT-PCR products was then prepared for nested PCR using 2µl of RT-PCR product, 5µl PCR master-mix buffer, 1µl forward and reverse primers (the nested primer HEV-csn neu 5-TGT GCT CTG TTT GGC CCN TGG TTY MG (26)-3'and HEV-casn 5-CCA GGC TCA CCR GAR TGY TTC CA (26) 3', and 16µl molecular grade water to make a final volume of 25µl. The nested PCR was carried out using the following cycling conditions; 5 minutes at 95^0C for initial denaturation, followed by 35 cycles amplification of 30 seconds at 95^0C for denaturation, and 30 seconds at 55^0C for annealing with an extension of 30 sec at 72^0C, and a final extension of 5 minutes at 72^0C.

The nested PCR amplicons were examined by electrophoresis on a 2.5% agarose gel, stained with 1.5µl 1x SYBR safe (Invitrogen, Carlsbad, California, United States) at 120 volts for 30 minutes, and the images of the amplicon band size of 345 base pair (bp) were visualized under ultraviolet (UV) illumination and documented with BioDoc Analyze 2.0imager (Biometra, Goettingen, Germany).

Data analysis:

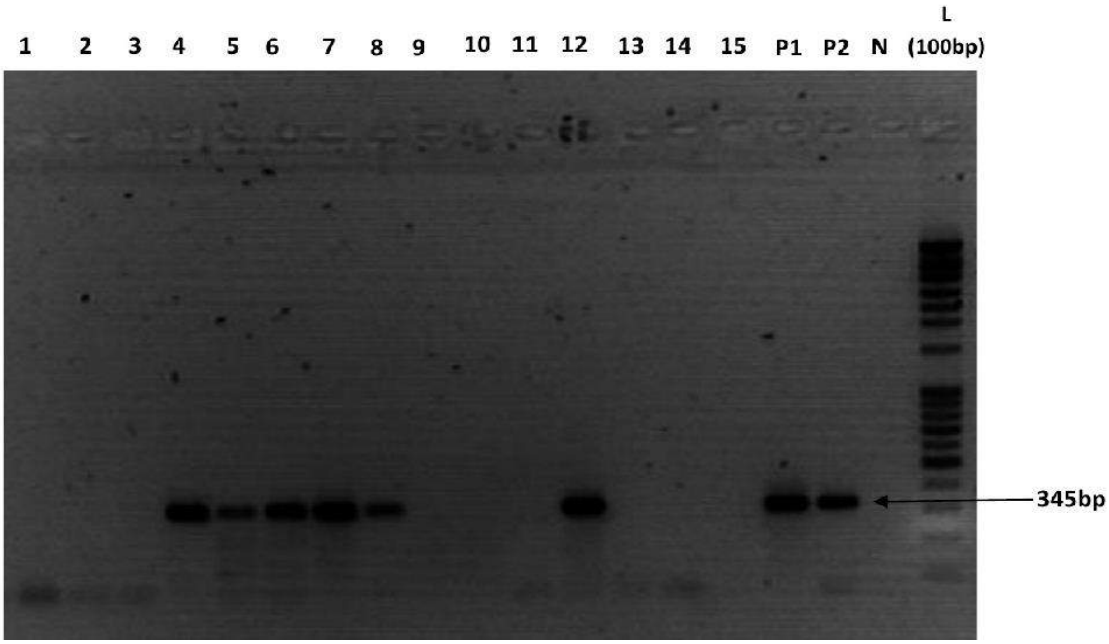
All qualitative/quantitative data were entered in the computer and analysed using SPSS version 26.0 for Windows (IBM Corp, 2021). Descriptive statistics were computed for all relevant data. The association between gender and age with HEV RNA positivity was tested using Chi-square. All significant associations were recorded at $p \leq 0.05$.

Results:

The analysis of all the 410 stool samples from 172 (42%) poultry birds aged between 5 and 18 months and 238 (58%) swine aged between 2 and 18 months, respectively, by the RT-PCR and the nested PCR revealed positive amplicons with band size of 345 bp for HEV RNA target and corresponding with the positive control band as shown in the image of the agarose gel electrophoresis (Fig 1.0). On the overall, 15 (3.7%) of these stool samples were positive for HEV RNA. Based on the different livestock sampled, 5 (2.9%) and 10 (4.2 %) of the 172 and 238 poultry birds and swine respectively, were positive for HEV RNA with an infection ratio of 1:2 between the poultry birds and swine (Table 1).

The detected HEV RNA were more prevalent in the female livestock (6.0%) than the male counterparts (1.0%) (Tab 1). The detected HEV RNA were mainly clustered among age groups 1-6 months, and 7-12 months for both animal groups, with a detection rate of 9.3%, 3.2% and 5.6%, 3.2% for both the swine and poultry bird samples respectively (Table 1). However, there was no statistically significant difference observed in the detection rate of HEV RNA across the gender (p=0.27) and age groups (p=0.20 and p=0.28) sampled for both livestock samples (Table 1). The HEV RNA was not detected in any sample from age group 13-18 months for both the swine and poultry bird samples (Table 1).

In addition, considering the distribution of HEV among the livestock sampled across the three LGAs, most of the positive swine (11.1%) and all positive poultry birds (5.0%) were from samples collected within Ikorodu LGA. However, only 3.0% of the positive swine samples originated from Ojo LGA. No HEV RNA was detected from both the poultry birds and swine stool samples collected from Agege LGA. The study showed statistically significant association between HEV RNA detection and LGAs from which samples originated (x^2=10.835, p=0.0044)) with significantly higher HEV detection rate in Ikorodu (7.3%, 12/164) compared to Ojo (1.8%, 3/171) and Agege (0%) (Table 1).



Lanes 1 – 15 represent the samples (Lanes 4, 5, 6, 7, 8 & 12 positive for HEV RNA), P1 & P2 (positive controls), N (negative control) and L (100bp ladder)

Fig 1: Agarose gel electrophoresis image showing the PCR amplicon band size (~345 bp) for targeted HEV RNA

Table 1: Characteristics and molecular parameters of livestock from three Local Government Areas (LGAs) in Lagos, Nigeria

Parameters	Total samples (%)	No positive (%)	*p* value
Livestock			
Swine	238 (58.0)	10 (4.2)	0.35
Poultry Birds	172 (42.0)	5 (2.9)	
Gender			
Male	194 (47.3)	2 (1.0)	0.27
Female	216 (52.7)	13 (6.0)	
Age group (months)			
Swine age group (months)			
1-6	54 (22.7)	5 (9.3)	0.20
7-12	157 (66.0)	5 (3.2)	
13-18	27 (11.3)	0	
Poultry birds age group (months)			
1-6	36 (21.0)	2 (5.6)	0.28
7-12	95 (55.0)	3 (3.2)	
13-18	41 (24.0)	0	
Locations			
Ikorodu LGA			
Swine	63	7 (11.1)	0.0044*
Poultry birds	101	5 (5.0)	
Ojo LGA			
Swine	100	3 (3.0)	
Poultry birds	71	0	
Agege LGA			
Swine	60	0	
Poultry birds	15	0	

* = statistically significant

Discussion:

Hepatitis E virus (HEV) is generally considered a waterborne disease in developing countries and a zoonosis in developed countries. However, the actual burden and transmission dynamics of this emerging endemic disease found across Africa are yet to be fully elucidated. In Sub-Saharan African countries including Nigeria, the level of awareness reg-

arding the zoonotic transmission of HEV is still very low as the sources of infection require further investigations. The detection of HEV RNA in livestock within farms in this study suggests there is a salient circulation of HEV among livestock in our environment and these animals could serve as main reservoirs of HEV infection and an important driver of the animal-to-human and human-to-animal transmission chains of HEV.

Domestic pigs and wild boar have been confirmed by serologic and molecular techniques to be major reservoirs of HEV and potential zoonotic transmission sources (5,32-37). The interactions of the handlers of these HEV-infected animals remain a risk within our environment. The transmission chain of the virus could be enhanced when infected and may serve as source of infection to other members of the community where they live, thereby spreading the virus to humans and possibly animals in reverse zoonosis. Thus, active surveillance of HEV in both animals and humans directly in contact with these animals will help to elucidate the actual burden and transmission patterns of the virus within our environment.

The detection of HEV in swine and poultry birds as documented in this study is also of significant public health importance, particularly to the communities harboring farms. The waste and effluents from the farms could be another source of transmission for HEV within the communities if not properly decontaminated. HEV, based on its properties, has been documented to be highly stable and resistant to environmental stress such as heat, extreme pH and desiccation (38,39). This permits the virus to be maintained within an environment for a long time and as such could result in contamination of food and surfaces. Therefore, strict application of hygienic measures during food production is crucial to prevent HEV's persistence on surfaces and subsequent cross-contamination. Owing to the high population density of the LGAs sampled in this study and associated sanitary challenges, water contamination by HEV and foodborne transmission cannot be ignored as the possible means of HEV transmission these areas and in Nigeria.

The prevalence of 4.2% among swine as compared to the 2.9% in poultry birds reported in this study is in line with the study of Adelabu *et al*. in 2016 [40] that reported an HEV prevalence rate of 4.4% among swine in South Africa. However, this prevalence rate is much lower than the prevalence rate of 76.7% of HEV detected by RT-PCR among swine in Plateau State, Nigeria (19). The disparities in this prevalence could be attributed to the fact that the stool samples used in this study were obtained from swine reared in confinement for commercial purposes and as such some levels of improved hygienic condition and animal health care service might have been put in place to avoid economic loss. In most situations in Nigeria, domestic animals like swine are managed in such a way that they are either housed or allowed to roam freely to scavenge for food and water. They visit waste heaps and stagnant or flowing water bodies thereby contaminating such areas with feces and urine. This system predisposes swine to diverse infections including HEV and thus facilitates subsequent transmission to humans, especially in environments where there are close association of swine and people (19,28). This detection in poultry birds could also result in commercial chicken farmers incurring losses in their businesses (41).

Considering gender, higher detection rate was recorded among the female compared to the male livestock, particularly in the swine population, although slightly more females were sampled in this study. This might not be unconnected with the rearing of offspring nature of the female swine. However, there was no statistically significant association between gender and HEV RNA detection among the livestock recruited in this study. Furthermore, the detection rate of HEV RNA in stool samples of both swine and poultry birds aged 1-6 months agreed with the findings of Martelli et al., (42), Owolodun et al., (19) and Abrantes et al., (43). However, both swine and poultry birds aged 7-12 months had a higher rate of HEV RNA in our study, suggesting that HEV could be circulating in all age groups and that swine that are close to slaughtering age can still be HEV-infected (44-49). It is important to note that in Nigeria, swine are usually slaughtered at 9 months of age, which raises concern about the potential zoonotic transmission of this virus by consumption and handling of raw or undercooked food products from pork meat which has been documented in the literature (5,50-52). However, there was no statistically significant association between age group and HEV RNA positivity among both livestock in this study.

There was a statistically significant difference (p=0.0044) in the association between the detection of HEV RNA among swine and poultry birds and the LGAs from which the samples originated. This finding is also of a significant public health concern in our environment, as the high population density and the high movement of people due to commercial and other purposes out of these LGAs could facilitate an enhanced salient transmission of this agent in Lagos State, Nigeria. There is a need for the implementation of comprehensive public health measures in educating farmers and other related workers such as abattoir workers, on infection prevention and

control by the public health policy makers, since there is no specific treatment for HEV infection (9,53-55). Although, the only currently available HEV vaccine was licensed in the year 2012 in China, a recommendation for its routine use has not been issued by the World Health Organization (WHO) (9). Therefore, the search for an effective vaccine against HEV for both humans and animals continue and requires urgent concerted efforts globally to minimize the risk of HEV transmission in endemic regions of the world (9).

Conclusion:

The detection of HEV in both the swine and poultry birds in Lagos, Nigeria further confirms the endemicity of HEV in the country and a cause for public health concern regarding the huge human population of the state and the country in general. HEV deserves increased monitoring and surveillance in humans and livestock within our locality for early detection and to forestall possible outbreaks. Also, further research needs to focus on the investigation of transmission chains from animal reservoirs to humans, including the food-borne route.

Implementation of a notification system for cases of HEV to help estimate the burdens and impact of infection in humans and animals, and an analysis of the genetic variability of HEV circulating in our environment is strongly advocated for in Nigeria. The analysis of the genetic variability of the strains of HEV circulating could not be analysed and reported in this study due to the high ambiguities and background noise of the generated nucleotide sequence data of HEV RNA and therefore remains a limitation.

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Contributions of authors:

SOB, EMJ, and OMR conceptualized the study and were responsible for the experimental and project design, analysis of data and writing the manuscript; MBP, ARA, AMA, and AIA made conceptual contributions, performed experimental analysis, and assisted in preparing the manuscript; SBA and MRM made conceptual contributions and assisted in preparing the manuscript; while OSA was the laboratory director, team lead of the Centre for Human and Zoonotic Virology and was responsible for the experimental and project design, analysis of data and writing of the manuscript. All authors read and approved the manuscript.

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No conflict of interest is declared.

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Original Article **Open Access**

Antimicrobial resistance profiles of bacteria from *Enterobacteriaceae* family of laying chicken in Ibadan, southwestern Nigeria

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Abstract:

Background: Antibiotics are significant for improving the health and productivity of chickens, but overuse and misuse of antibiotics have led to the development of antimicrobial resistance (AMR), which has resulted in ineffective treatment of infectious diseases with associated mortality in chicken and potential spread of AMR pathogens to humans. The objective of the study was to evaluate the AMR profiles of *Enterobacteriaceae* from faecal samples of laying chicken in Ibadan, southwestern Nigeria.
Methodology: This was a cross-sectional study of 200 apparently healthy laying hens from 10 selected local government areas of Ibadan, Nigeria, and from which cloacal samples were collected for isolation of *Enterobacteriaceae*. Samples were first inoculated on tryptone soy broth (TSB) for enrichment and then sub-cultured on MacConkey agar plates. Presumptive *Escherichia coli* isolates were sub-cultured on Eosin Methylene Blue (EMB) agar and greenish metallic sheen colonies on EMB agar were identified as *E. coli* by colony morphology and Gram stain microscopy. Commercial API (Analytical Profile Index) kit was used to confirm the identity of the *Enterobacteriaceae* isolates. Antimicrobial susceptibility testing of the isolates was performed by the disc diffusion technique and result interpreted using the guideline of Clinical and Laboratory Standards Institute. Data were analysed on STATA and $p<0.05$ was considered statistical significance.
Results: The results showed that out of 200 chicken samples, 190 were cultured positive, giving a colonization rate of 95.0%, with 287 *Enterobacteriaceae* isolates. *Escherichia coli* (59.6%), *Enterobacter* spp., (27.9%), and *Klebsiella pneumoniae* (12.5%) were the bacterial isolates identified. For antibiotic susceptibility, *E. coli* had sensitivity rate of 78.2% to ciprofloxacin, 73.4% to ofloxacin, 71.8% to sparfloxacin, and 70.9% to pefloxacin, and resistant rates to cotrimoxazole of 73.4%, streptomycin 65.4%, and other antibiotics 63.7%. *Klebsiella pneumoniae* was sensitive to gentamicin (33.3%), ofloxacin (33.3%), and ciprofloxacin, but resistant to other antibiotics. *Enterobacter* spp. was sensitive to amoxicillin-clavulanic acid (93.8%), pefloxacin, and streptomycin (70.3%), but resistant to ofloxacin (100.0%), cotrimoxazole (84.5%), chloramphenicol (68.8%), gentamicin (64.1%), amoxicillin (60.9%) and ciprofloxacin (60.9%). A total of 29 resistance patterns were observed in 50 resistant *Enterobacteriaceae* isolates with 12 MDR patterns observed in 54.0% (n=27) of the isolates.
Conclusion: This study reports faecal *Enterobacteriaceae* colonization rate of 95% of commercial poultry chicken in Ibadan, southwest Nigeria, belonging to three members of the family *Enterobacteriaceae,* with high MDR patterns. The high AMR rates can lead to ineffective treatment of infectious diseases in chicken, with associated mortality and a potential source for transmission of AMR pathogens to humans.

Keywords: Antimicrobial resistance; *Enterobacteriaceae*; Laying chicken; Ibadan; Nigeria

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Profils de résistance aux antimicrobiens des bactéries de la famille des Enterobacteriaceae des poules pondeuses à Ibadan, dans le sud-ouest du Nigeria

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Résumé:

Contexte: Les antibiotiques sont importants pour améliorer la santé et la productivité des poulets, mais leur utilisation excessive et inappropriée a conduit au développement d'une résistance aux antimicrobiens (RAM), qui a entraîné un traitement inefficace des maladies infectieuses avec une mortalité associée chez les poulets et une propagation potentielle de ces maladies. Agents pathogènes de la RAM pour les humains. L'objectif de l'étude était d'évaluer les profils de RAM des *Entérobactéries* à partir d'échantillons fécaux de poules pondeuses à Ibadan, dans le sud-ouest du Nigeria.
Méthodologie: Il s'agissait d'une étude transversale portant sur 200 poules pondeuses apparemment en bonne santé provenant de 10 zones de gouvernement local sélectionnées d'Ibadan, au Nigeria, et à partir desquelles des échantillons cloacaux ont été collectés pour l'isolement des *Entérobactéries*. Les échantillons ont d'abord été inoculés sur un bouillon tryptone soja (TSB) pour enrichissement, puis repiqués sur des plaques de gélose MacConkey. Des isolats présumés d'*Escherichia coli* ont été sous-cultivés sur une gélose à l'éosine et au bleu de méthylène (EMB) et des colonies à reflets métalliques verdâtres sur une gélose EMB ont été identifiées comme étant *E. coli* par la morphologie des colonies et la microscopie à coloration de Gram. Un kit commercial API (Analytical Profile Index) a été utilisé pour confirmer l'identité des isolats d'entérobactéries. Les tests de sensibilité aux antimicrobiens des isolats ont été réalisés par la technique de diffusion sur disque et les résultats ont été interprétés selon les directives d'Institut des Normes Cliniques et de Laboratoire. Les données ont été analysées sur STATA et $p<0,05$ a été considéré comme statistiquement significatif.
Résultats: Les résultats ont montré que sur 200 échantillons de poulets, 190 étaient cultivés positifs, soit un taux de colonisation de 95,0%, avec 287 isolats d'*Entérobactéries*. *Escherichia coli* (59,6%), *Enterobacter* spp. (27,9%) et *Klebsiella pneumoniae* (12,5%) étaient les isolats bactériens identifiés. Pour la sensibilité aux antibiotiques, *E. coli* avait un taux de sensibilité de 78,2% à la ciprofloxacine, de 73,4% à l'ofloxacine, de 71,8% à la sparfloxacine et de 70,9% à la péfloxacine, et des taux de résistance au cotrimoxazole de 73,4%, à la streptomycine de 65,4% et à d'autres antibiotiques de 63,7%. *Klebsiella pneumoniae* était sensible à la gentamicine (33,3%), à l'ofloxacine (33,3%) et à la ciprofloxacine, mais résistante à d'autres antibiotiques. *Enterobacter* spp était sensible à l'amoxicilline-acide clavulanique (93,8%), à la péfloxacine et à la streptomycine (70,3%), mais résistant à l'ofloxacine (100,0%), au cotrimoxazole (84,5%), au chloramphénicol (68,8%), à la gentamicine (64,1%), à l'amoxicilline (60,9 %) et ciprofloxacine (60,9%). Au total, 29 profils de résistance ont été observés dans 50 isolats d'*Enterobacteriaceae* résistants, avec 12 profils MDR observés dans 54,0% (n=27) des isolats.
Conclusion: Cette étude rapporte un taux de colonisation fécale des Enterobacteriaceae de 95.0% des volailles commerciales d'Ibadan, dans le sud-ouest du Nigeria, appartenant à trois membres de la famille des Enterobacteriaceae, avec des profils MDR élevés. Les taux élevés de RAM peuvent conduire à un traitement inefficace des maladies infectieuses chez le poulet, avec une mortalité associée et une source potentielle de transmission d'agents pathogènes RAM aux humains.

Mots-clés: Résistance aux antimicrobiens; *Entérobactéries*; Poulet pondeur; Ibadan; Nigeria

Introduction:

The *Enterobacteriaceae* is a broad family of Gram-negative bacteria that comprise both free-living and indigenous flora of the lower gastrointestinal tract of various animals and humans (1,2). The family comprises 51 genera and 238 species (3–5). Most of their members live in the intestines of chickens and humans, and have been widely studied due to their obvious impact on human and animal health as well as agricultural practices (5,6). The important members of the *Enterobacteriaceae* family that cause food poisoning, gastroenteritis, enteric fever, and plague include *Salmonella* spp., *Shigella* spp., *Enterobacter* spp., *Citrobacter* spp., *Yersinia* spp., *Klebsiella* spp., and *Escherichia coli* (3) .

Antimicrobials are important in improving chicken health and productivity in poultry, cattle, and aquaculture (7–9). However, excessive use of antimicrobials has environmental and health implications due to the creation of antimicrobial resistance (AMR), leading to ineffective treatment of infectious diseases and death of animals and humans (10,11). In most rural and peri-urban areas of Nigeria and other developing countries, antimicrobials are readily purchased for use in chickens and humans without prescriptions, resulting in misuse, which has negatively affected their efficacy (3,12).

In a global context, antimicrobials such as antibiotics are widely used, at non-therapeutic dosages, as growth promoters, and prophylaxis in chicken feeds, and water, which sometimes last for long period, thereby predisposing to emergence of AMR strains of microorganisms (13). Also, when antibiotics are excreted in chicken wastes, they can contaminate the environment, thereby facilitating the widespread dissemination of AMR traits in the community (7–9,14).

The objective of this study is to det-

ermine the AMR profiles of *Enterobacteriaceae* isolates of faecal samples of laying chicken in Ibadan, southwestern Nigeria, with the aim of bridging significant knowledge gaps regarding the prevalence of this problem for urgent public health responses as well develop recommendations for farmers on the appropriate ways to apply antimicrobial medications to increase poultry productivity, improve food security and prevent emergence of AMR pathogens.

Materials and method:

Study area:

The study was conducted in Ibadan, a city in the southwestern region of Nigeria, at coordinates of latitude 7° 22' 36.2496'' N and longitude 3° 56' 23.2296'' E (Fig 1). Ibadan is located at an elevation of 230 meters above sea level and is divided into several LGAs (15,16).

Study design, participants and sample collection:

This was a descriptive cross-sectional study of 200 apparently healthy chickens randomly selected from 10 poultry farms (20 per farm) in 10 LGAs (1 farm per LGA) in Ibadan, southwestern Nigeria between January 1 and 31, 2023 (Table 1). Chicken that exhibited symptoms and/or signs of sickness were excluded from the study.

Cloacal faecal samples were collected using sterile swab sticks, placed in cold box with ice packs, and transported to the laboratory of Veterinary Microbiology Department of the University of Ibadan for microbiological analysis.

Isolation and identification of *Enterobacteriaceae* isolates:

Each sample was pre-enriched by inoculating cloacae faecal swab into 9ml sterile tryptone soy broth (TSB) in universal bottles, and incubated at 37^0C for 18-24hrs. A loopful of the pre-enrichment culture was inoculated on MacConkey agar and incubated at 37^0C for 18-24hours. Rose pink colonies from MacConkey agar were sub-cultured onto Eosin Methylene Blue agar (EMB) and incubated at 37^0C for 24 hrs.

The rose-pink colonies on MacConkey agar plates (putative *E. coli*) that showed greenish metallic sheen colonies on EMB agar were selected for phenotypic identification, which included observation of colony morphology, Gram stain microscopy reaction, and commercial biochemical identification with Analytical Profile Index (API) to confirm the identity of *E. coli* and other bacteria isolates.

Antibiotic susceptibility testing (AST):

Antimicrobial susceptibility testing of the isolates was performed by the disc diffusion technique recommended by the Clinical and Laboratory Standards Institute (17). Five classes of antibiotics were tested which included fluoroquinolones (ofloxacin, ciprofloxacin, pefloxacin, sparfloxacin); sulfonamide (co-trimoxazole); phenicol (chloramphenicol); penicillins (amoxicillin, amoxicillin-clavulanic acid); and aminoglycoside (gentamicin, streptomycin). The antibiotic discs used were amoxicillin (AM, 30µg); amoxicillin-clavulanic acid (augmentin or AU 10µg); ofloxacin (tarivid or OFX 10µg); sparfloxacin (SP 10µg); ciprofloxacin (CPX 30µg); pefloxacin (PEF 30µg); chloramphenicol (CH 30µg); sulfamethoxazole-trimethoprim (septrin or SXT 30µg); streptomycin (S 30µg) and gentamicin (CN 30 µg).

Inoculum suspension of the overnight colonies of each isolate was prepared and adjusted to match the turbidity of 0.5 McFarland standards. Mueller-Hinton (MH) agar was inoculated with the suspension using a sterile swab. A sterile forcep was used to place the antibiotic discs on the inoculated MH agar plate, and the seeded agar plates were incubated for 24 hours at 37^0C. The measurement of the diameter of inhibition zones around the antibiotic discs was done to the nearest millimeter, and interpreted according to the guidelines of the Clinical and Laboratory Standards Institutes (17). Multidrug resistance (MDR) in any isolate was defined as resistance to antibiotics in 3 or more of the 5 classes of antibiotics tested (18).

Data analysis:

Data were entered into the Microsoft Excel 2010, including the samples checklist, and laboratory results, before sending them into STATA 17.0 version for analysis. All the results are presented in tables and graphs as frequencies, and percentages.

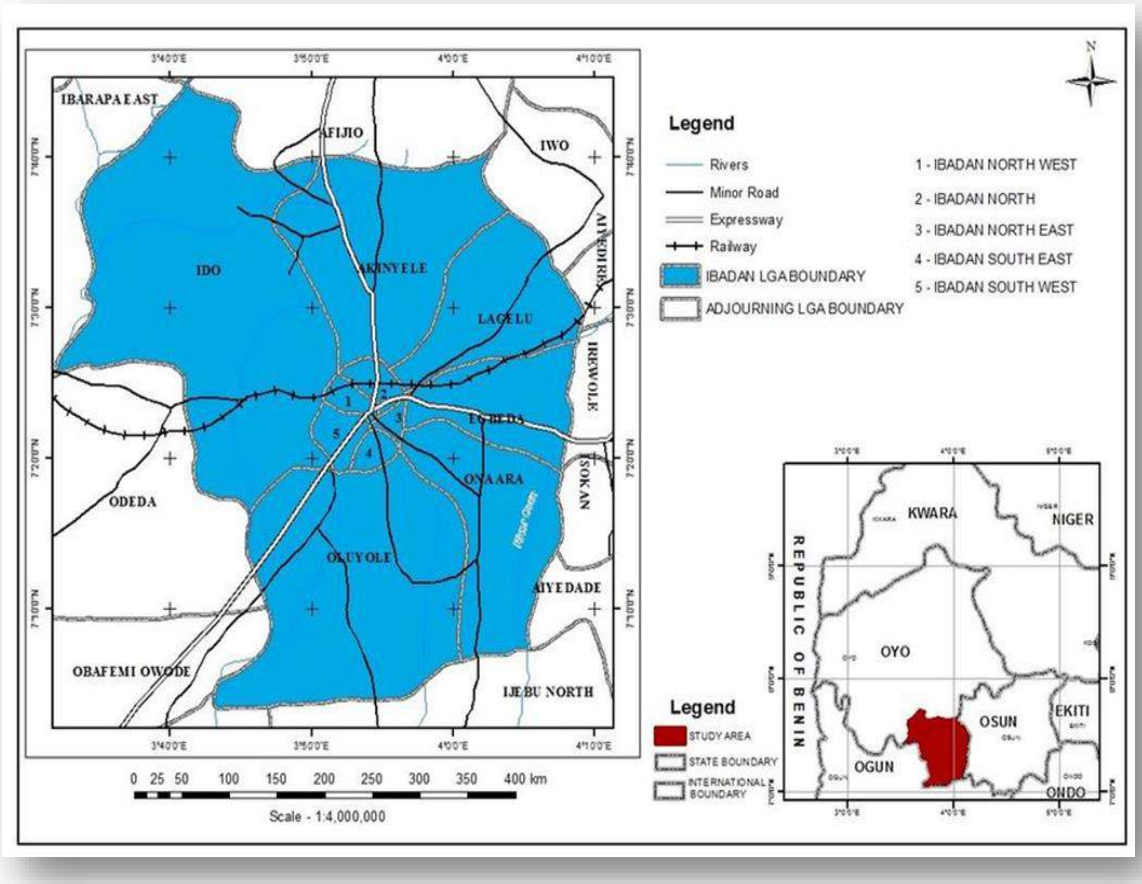

Fig 1: Map of the study area

Table 1: Distribution of laying chickens and sample collection with respect to the study location and local government areas (LGAs) in Ibadan, Nigeria

Local Government Areas	Farms	Poultry type	Samples collected	Number of chicken samples collected
Akinyele	Elyon	Laying Hens	Cloacal fecal	20
Ibadan North	BL Goshe	Laying Hens	Cloacal fecal	20
Lagelu	Olu Oluwa	Laying Hens	Cloacal fecal	20
Ibadan North East	Freedom	Laying Hens	Cloacal fecal	20
Ido	His Grace	Laying Hens	Cloacal fecal	20
Ibadan North West	James's	Laying Hens	Cloacal fecal	20
Ona Ara	Kay's	Laying Hens	Cloacal fecal	20
Egbeda	Ayo's	Laying Hens	Cloacal fecal	20
Ibadan South East	Twins'	Laying Hens	Cloacal fecal	20
Oluyole	Sun Boy's	Laying Hens	Cloacal fecal	20

Results:

Prevalence of faecal colonization of chicken by *Enterobacteriaceae:*

Cloacal faecal samples of 190 chickens were culture positive for *Enterobacteriaceae,* giving a colonization rate of 95.0% while 10 (5.0%) were culture negative. Three species of bacteria belonging to family *Enterobacteriaceae* were isolated, with *E. coli* in 121 chickens (60.5%), *Enterobacter* spp. in 46 chickens (23.0%), and *Klebsiella pneumoniae* in 23 chickens (11.5%) as shown in Fig 2.

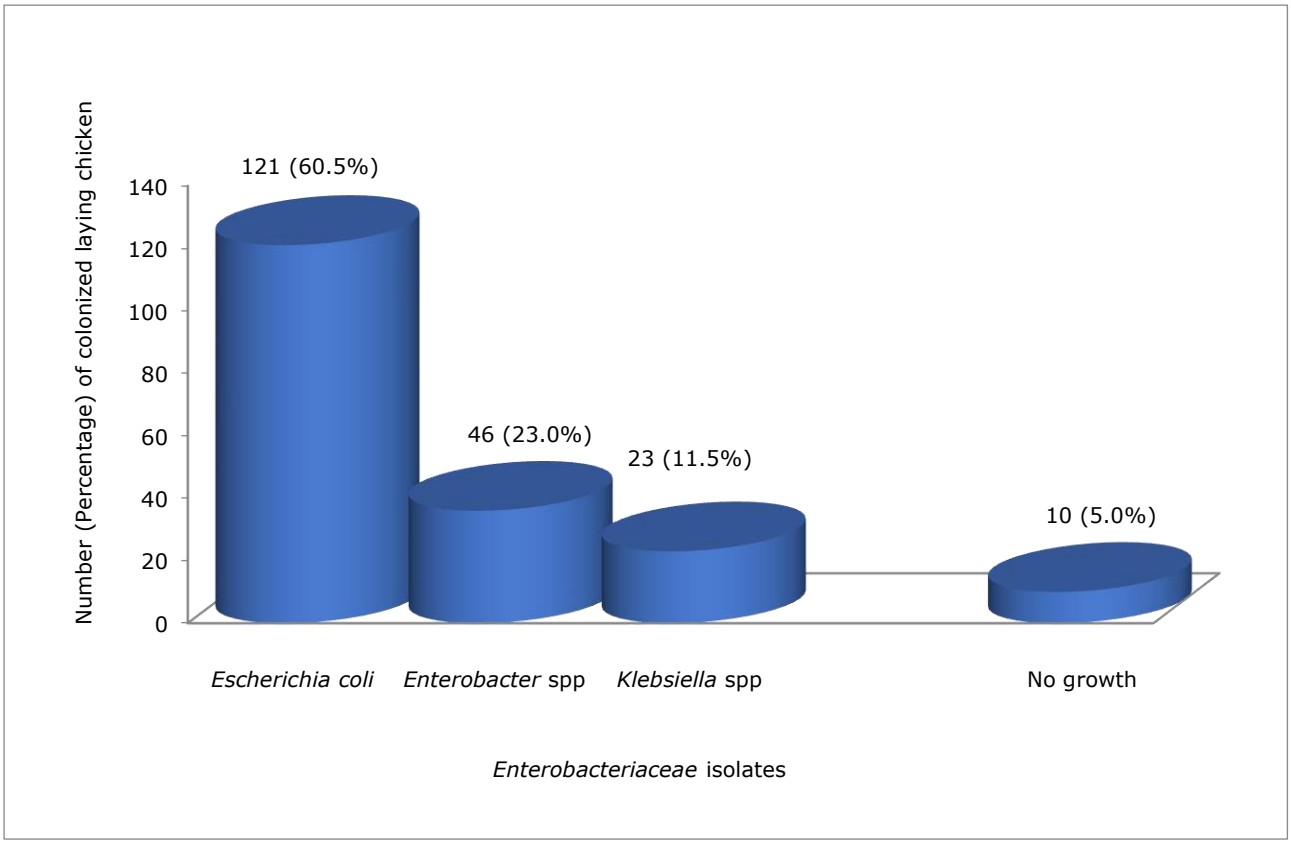

Fig 2: Prevalence of faecal colonization of laying chickens by *Enterobacteriaceae*

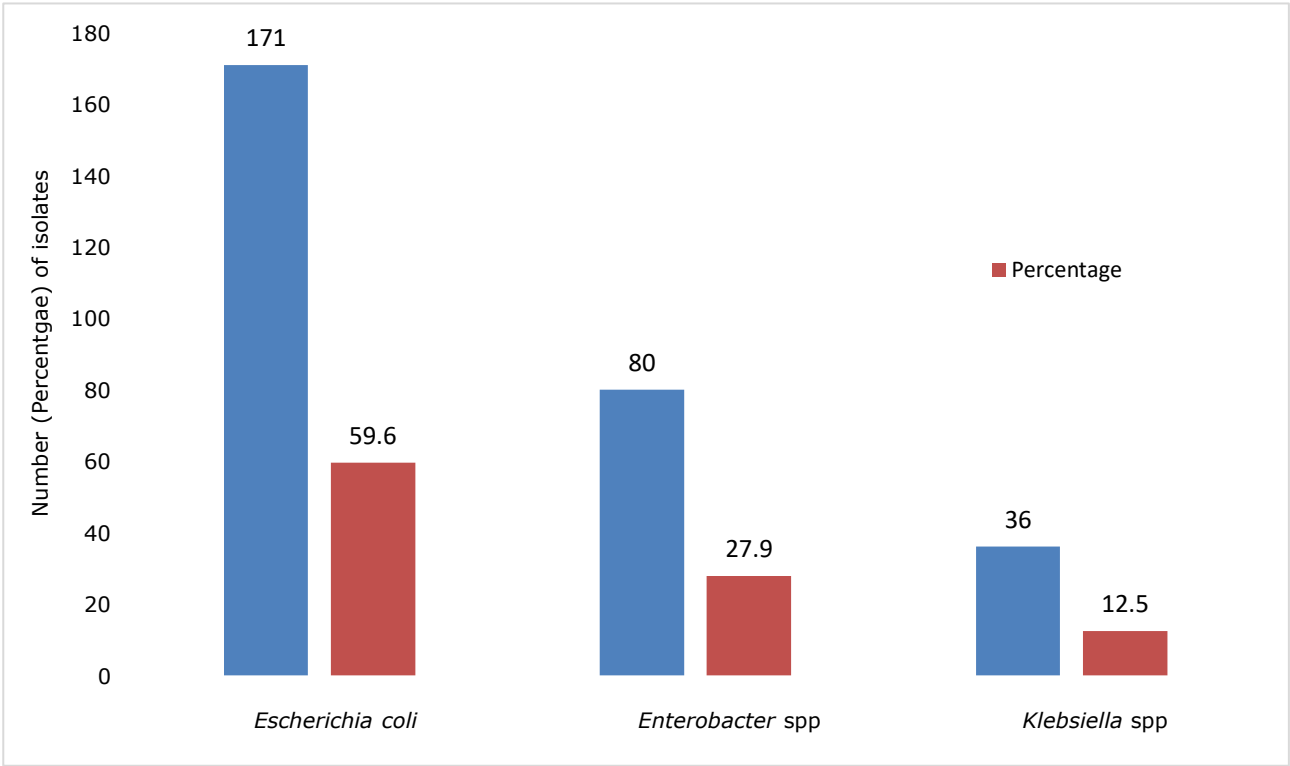

Fig 3: Frequency distribution of bacterial isolates from positive cultures

Frequency distribution of the *Enterobacteriaceae* isolates:

A total of 287 bacterial isolates were recovered from the 190 positive cultures, with the most frequent being *E. coli* (171, 59.6%), *Enterobacter* spp. (80, 27.9%), and *Klebsiella pneumoniae* (36, 12.5%) as shown in Fig 3.

The farms with the highest number of isolates were Elyon and BL Goshe farm with 39 (13.6%) each, followed by Freedom farm with 33 (11.5%), and Sun's Boy farm with 19 (6.6%) ($p>0.05$) as shown in Table 2.

Table 2: Frequency distribution of *Enterobacteriaceae* isolates with respect to farms and local government areas in Ibadan

Sample location/ farm	LGAs	No of chicken sampled	No of LF isolates	No of NLF isolates	Total no of isolates (%)	*p* value
Elyon	Akinyele	20	25	14	39 13.6)	
BL Goshe	Ibadan North	20	19	20	39 (13.6)	
Olu Oluwa	Lagelu	20	21	09	30 (10.5)	
Freedom farm	Ibadan North East	20	17	16	33 (11.5)	
His Grace	Ido	20	11	12	23 (8.0)	
James's farm	Ibadan North West	20	15	07	22 (7.7)	$p>0.05$
Kay's farm	Ona Ara	20	18	3	21 (7.3)	
Ayo's farm	Egbeda	20	24	8	32 (11.5)	
Twins	Ibadan South East	20	22	7	29 (10.1)	
Sun Boy's farm	Oluyole	20	11	8	19 (6.6)	
Total		**200**	**183**	**104**	287 (100.0)	

LF: Lactose fermenter; NLF: Non-lactose fermenter; LGA: Local Government Area

Table 3: Antibiotic susceptibility of *Enterobacteriaceae* isolates from laying chicken in Ibadan, Nigeria

Antibiotic class	*Escherichia coli*		*Klebsiella pneumoniae*		*Enterobacter* spp	
	Sensitive n (%)	Resistant n (%)	Sensitive n (%)	Resistant n (%)	Sensitive n (%)	Resistant n (%)
Penicillin						
AM	171 (100.0)	0	12 (33.3)	24 (66.7)	25 (39.1)	39 (60.9)
AU	128 (75.0)	43 (25.0)	10 (27.8)	26 (72.2)	60 (93.8)	4 (6.2)
Fluoroquinolones						
OFX	118 (69.0)	53 (31.0)	19 (52.8)	17 (47.2)	0	64 (100)
SP	10 (6.0)	161 (94.0)	17 (47.2)	19 (52.8)	30 (46.9)	34 (53.1)
CPX	147 (86.0)	24 (14.0)	25 (69.4)	11 (30.6)	25 (39.1)	39 (60.9)
PEF	125 (73.0)	46 (27.0)	19 (52.8)	17 (47.2)	45 (70.3)	19 (29.7)
Phenicol						
CH	100 (58.0)	71 (42.0)	10 (27.8)	26 (72.2)	20 (31.3)	44 (68.8)
Sulfonamide						
SXT	50 (29.0)	121 (71.0)	8 (22.2)	28 (77.8)	10 (15.6)	54 (84.4)
Aminoglycoside						
S	48 (28.0)	123 (72.0)	12 (33.3)	24 (66.7)	45(70.3)	19 (29.7)
CN	21(12.0)	150 (88.0)	0	36 (100.0)	23 (35.9)	41 (64.1)

AM=amoxicillin; AU=amoxicillin-clavulanic acid (Augmentin); OFX=ofloxacin (Tarivid); SP=Sparfloxacin; CPX=ciprofloxacin; PEF=pefloxacin; CH=chloramphenicol; SXT=sulfamethoxazole-trimethoprim (Septrin); S=streptomycin; CN=gentamicin

Table 4: Antimicrobial resistance patterns of *Enterobacteriaceae* isolates from laying chicken in Ibadan, Nigeria

Antibiotics	Resistance pattern	Number of resistant isolates			
		Escherichia coli	***Enterobacter* spp**	***Klebsiella* spp**	**Total isolates**
OFX	Mono resistance	2	0	0	2
S	Mono resistance	0	2	0	2
CH	Mono resistance	0	0	1	1
SP	Mono resistance	1	0	0	1
CPX	Mono resistance	0	0	1	1
AU	Mono resistance	0	1	1	2
AM	Mono resistance	1	0	0	1
CN	Mono resistance	0	1	1	2
PEF	Mono resistance	0	0	1	1
SXT	Mono resistance	1	0	0	1
OFX-S	Double resistance	0	2	0	2
OFX-CH	Double resistance	0	0	2	2
OFX-CPX	Double resistance	1	0	0	1
OFX-AU	Double resistance	0	1	0	1
OFX-AM	Double resistance	1	0	0	1
OFX-CN	Double resistance	0	1	0	1
OFX-SXT	Double resistance	1	0	0	1
OFX-S-CH	MDR (Triple resistance)	1	1	0	2
OFX-S-AU	MDR (Triple resistance)	0	0	1	1
OFX-S-AM	MDR (Triple resistance)	0	1	0	1
OFX-S-SXT	MDR (Triple resistance)	1	0	0	1
OFX-S, CH-AU	MDR (Quadruple resistance)	1	0	0	1
OFX-S, CH-AM	MDR (Quadruple resistance)	0	0	1	1
OFX-S-CH-SXT	MDR (Quadruple resistance)	0	1	0	1
S-CH-CPX-AU	MDR (Quadruple resistance)	0	1	1	2
S-CH-CPX-AM	MDR (Quadruple resistance)	1	0	0	1
S-CH-CPX-AU-SXT	MDR (Quintuple resistance)	0	1	0	1
OFX-S-CH-AU-SXT	MDR (Quintuple resistance)	2	0	0	2
S-CH-AM-PEF-SXT	MDR (Quintuple resistance)	7	4	2	13
Total		**21**	**17**	**12**	**50**

A total of 29 resistance patterns were observed in 50 resistant *Enterobacteriaceae* isolates, with 10 mono-resistance (14 isolates, 28.0%), 7 double resistance (9 isolates, 18.0%) and 12 multi-drug resistance patterns (27 isolates, 54.0%). AM=amoxicillin; AU=amoxicillin-clavulanic acid (Augmentin); OFX=ofloxacin (Tarivid); SP=Sparfloxacin; CPX=ciprofloxacin; PEF=pefloxacin; CH=chloramphenicol; SXT=sulfamethoxazole-trimethoprim (Septrin); S=streptomycin; CN=gentamicin; MDR= Multidrug resistance

Antibiotic susceptibility test results:

Escherichia coli isolates showed 78.2% sensitivity to ciprofloxacin, 73.4% to ofloxacin, 71.8% to sparfloxacin, and 70.9% to pefloxacin but resistant to sulfamethoxazole-trimethoprim (73.4%), streptomycin (65. 4%), and to other antibiotics (63.7%). About 22% of *Klebsiella pneumoniae* were susceptible to gentamicin, 33.3% to ofloxacin, 33.3% to ciprofloxacin, and 44.5% to other antibiotics. *Enterobacter* spp were resistant to ofloxacin (100%), sulfamethoxazole-trimethoprim (84.5%), chloramphenicol (68.8%), gentamicin (64.1%), ciprofloxacin (60.9%) while being sensitive to amoxicillin-clavulanic acid (93.8%), pefloxacin (70.3%) and streptomycin (70.3%) (Table 3).

A total of 29 resistance patterns were observed in 50 resistant *Enterobacteriaceae* isolates, with 10 mono-resistance (14 isolates, 28.0%), 7 double resistance (9 isolates, 18.0%) and 12 MDR patterns (27 isolates, 54.0%), with MDR patterns varying from resistance to 3 to 5 antibiotic classes (Table 4).

Discussion:

Antimicrobial drugs are commonly used in poultry, livestock, and aquaculture at non-therapeutic dosages as growth promoters and prophylaxis in feeds and water for protracted periods thereby contributing to the development and spread of resistant organisms (8). Additionally, the misuse of antimicrobials and other drugs in household chickens may promote the zoonotic transmission of AMR pathogens, thereby posing a threat to animal and human health (10). The failure to complete the prescribed course of antimicrobials or the preservation of medication for future use in

chickens and humans can lead to inappropriate usage, which could trigger the development of resistance in bacterial strains (19). The over-reliance on antibiotics such as tetracycline as growth promoters in chickens can also lead to the development of resistance, and consuming poultry products can transmit antimicrobial resistance to humans. However, in Nigeria, there has been report of circulation of *Enterobacteriaceae* strains among chickens and animal populations (7).

In this study, the overall prevalence of the chicken colonization by *Enterobacteriaceae* was 95.0% (190/200) with isolation of three members of this family; *E. coli* in 60.5% (121/200), *Enterobacter* spp in 27.9% (46/200) and *Klebsiella pneumoniae* in 11.5% (23/200) from commercial laying chickens in the 10 LGAs studied. Regrettably, poultry farms have contributed to environmental contamination with antibiotic-resistant bacteria, primarily members of the family *Enterobacteriaceae*, which are transmitted to chickens, livestock, and humans through direct contact or through contaminated food products (20,21). Similar studies carried out in Nigeria in 2011 and 2019 showed comparatively similar results (7,22). Furthermore, Ibrahim et al., (23) reported *E. coli* as a common enteric pathogen, specific strains of which can cause human and animal disease.

In the current study, *E. coli* was the most frequently recovered bacteria in 59.6% (171/287). This might be due to poor hygiene practices and sanitation status, as well as lack of biosecurity in the farms, consistent with previous research conducted in Ethiopia (24). Most of the previous studies demonstrated that the frequency of *E. coli* from chicken faeces could vary greatly with the time of sample collection, chicken age, and the diet (21). The high prevalence of bacteria from the *Enterobacteriaceae* family is a significant problem for commercial poultry breeders, as reported by Khouja et al (13).

The *Enterobacteriaceae* isolates in our study were sensitive to ciprofloxacin, ofloxacin, sparfloxacin, and pefloxacin but were largely resistant to cotrimoxazole, streptomycin, and other antibiotics. According to Awogbemi et al., (14), *E. coli* isolated from Portuguese poultry were reported to be highly resistant to tetracycline (70.0%) and ampicillin (63.0%) while low level of resistance was observed with co-trimoxazole (33.0%), gentamicin (17.0%) and co-amoxiclav (17%). Our study agreed with the previous work done by Kaushik et al., (25), where the growing threat of MDR observed more frequently in some Gram-negative bacteria such as *E. coli* continues to cause concern.

Antimicrobial resistant bacteria are detected in poultry wastes, poultry products, and poultry environments. Humans through consumption of contaminated poultry products (7,14), may acquire these resistant bacterial strains. Our study isolated and identified a high number of AMR pathogenic bacteria from cloacae swabs, showing that these bacteria could contaminate poultry meats and other poultry products.

Conclusion:

This present study showed high rates of faecal colonization of commercial poultry chickens in Ibadan, southwest Nigeria with MDR *Enterobacteriaceae* isolates (*E. coli, Enterobacter* spp., and *K. pneumoniae*). The high prevalence of these MDR isolates may be attributed to poor hygiene, improper use of biosecurity measures, poor sanitation, and excessive administration of antibiotics in poultry production. Our study recommends the need to ensure appropriate use of antibiotics in poultry production in order to prevent severe economic implications for poultry production and threats of AMR emergence and spread to the human population.

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Contributions of authors:

OCV was involved in data collection, isolation and write up, while EAA and EBO were involved in research supervision, guidance and correction of the manuscript. All authors approved the manuscript submitted for publication.

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Conflict of interest:

No conflict of interest is declared.

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Original Article **Open Access**

Evaluation of antimicrobial properties of five medicinal plants used against bacterial infections in Jalingo, Nigeria

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Abstract:

Background: The prevalent utilization of medicinal plants in communities underscores their promise as antimicrobial agents amid rising antibiotic resistance. This study assesses five medicinal plants; *Bambusa vulgaris*, *Hibiscus sabdariffa*, *Heteropogon contortus*, *Moringa oleifera*, and *Carica papaya* against clinical isolates of *Salmonella* Typhi and *Shigella dysenteriae*.
Methodology: Five medicinal plants were chosen based on traditional knowledge and ethnobotanical practices. Phytochemical analysis followed standard methods. Plant extracts were prepared using ethanol, ethyl acetate, dichloromethane, and hexane. Various concentrations (R conc., D1 conc., D2 conc, D3 conc, and D4 conc) of the extracts were evaluated using Kirby-Bauer disk diffusion and broth dilution methods to ascertain antimicrobial properties, including minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC).
Results: Phytochemical analysis revealed abundant saponins, cardiac glycosides, terpenoids, steroids, flavonoids, phenolics, and tannins, notably higher with ethanol extraction. *Hibiscus sabdariffa* demonstrated potent activity against *S.* Typhi with inhibition zone diameters of 29.00 mm (R conc), 27.00 mm (D1 conc), 14.00 mm (D2 conc), and 4.00 mm (D3 conc). *Heteropogon contortus* exhibited activity against *S. dysenteriae* with inhibition zone diameter of 25.05 mm (R conc), 15.00 mm (D1 conc), 10.00 mm (D2 conc), and 5.00 mm (D3 onc). The inhibition zone diameters of *B. vulgaris* were 18.50 mm (R conc), 17.00 mm (D1 conc), and 10.00 mm (D2 conc) against *S. dysenteriae*. The MIC and MBC were similar for both organisms, with *H. sabdariffa* (MIC: D3-4.27 mg/mL, MBC: D1-68.25 mg/mL) and *H. contortus* (MIC: D3-4.69 mg/mL, MBC: R-75.00 mg/mL), while *M. oleifera, C. papaya*, and *B. vulgaris* had negligible antimicrobial activity.
Conclusion: *Hibiscus sabdariffa* and *H. contortus* exhibited potent antimicrobial effects against *Salmonella*, with MICs of 4.27 mg/mL and 4.69 mg/mL, and MBCs of 68.25 mg/mL and 75.00 mg/mL respectively. Their consistent low MICs against *Shigella* suggest their potentials for antibiotic production.

Keywords: Antimicrobial agent; Antibiotic resistance; Plant extracts; MIC; MBC

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Évaluation des propriétés antimicrobiennes de cinq plantes médicinales utilisées contre les infections bactériennes à Jalingo, Nigeria

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Résumé:

Contexte: L'utilisation répandue des plantes médicinales dans les communautés souligne leur promesse en tant qu'agents antimicrobiens dans un contexte de résistance croissante aux antibiotiques. Cette étude évalue cinq plantes médicinales; *Bambusa vulgaris, Hibiscus sabdariffa, Heteropogon contortus, Moringa oleifera* et *Carica papaya* contre les isolats cliniques de *Salmonella* Typhi et *Shigella dysenteriae*.
Méthodologie: Cinq plantes médicinales ont été choisies sur la base des connaissances traditionnelles et des pratiques ethnobotaniques. L'analyse phytochimique a suivi les méthodes standard. Des extraits de plantes ont été préparés en utilisant de l'éthanol, de l'acétate d'éthyle, du dichlorométhane et de l'hexane. Diverses concentrations (R conc., D1 conc., D2 conc., D3 conc et D4 conc) des extraits ont été évaluées à l'aide des méthodes de diffusion sur disque Kirby-Bauer et de dilution en bouillon pour vérifier les propriétés antimicrobiennes, y compris les concentrations minimales inhibitrices (CMI) et les concentrations minimales concentrations bactéricides (MBC).
Résultats: L'analyse phytochimique a révélé une abondance de saponines, de glycosides cardiaques, de terpénoïdes, de stéroïdes, de flavonoïdes, de composés phénoliques et de tanins, notamment plus élevés avec l'extraction à l'éthanol. *Hibiscus sabdariffa* a démontré une activité puissante contre *S. Typhi* avec des diamètres de zone d'inhibition de 29,00 mm (conc R), 27,00 mm (conc D1), 14,00 mm (conc D2) et 4,00 mm (conc D3). *Heteropogon contortus* a présenté une activité contre *S. dysenteriae* avec un diamètre de zone d'inhibition de 25,05 mm (R conc), 15,00 mm (D1 conc), 10,00 mm (D2 conc) et 5,00 mm (D3 onc). Les diamètres des zones d'inhibition de *B. vulgaris* étaient de 18,50 mm (conc R), 17,00 mm (conc D1) et 10,00 mm (conc D2) contre *S. dysenteriae*. La CMI et la MBC étaient similaires pour les deux organismes, avec *H. sabdariffa* (CMI: D3-4,27 mg/mL, MBC: D1-68,25 mg/mL) et *H. contortus* (CMI: D3-4,69 mg/mL, MBC: R -75,00 mg/mL), tandis que *M. oleifera, C. papaya* et *B. vulgaris* avaient une activité antimicrobienne négligeable.
Conclusion: *Hibiscus sabdariffa* et *H. contortus* ont présenté de puissants effets antimicrobiens contre *Salmonella*, avec des CMI de 4,27 mg/mL et 4,69 mg/mL et des MBC de 68,25 mg/mL et 75,00 mg/mL respectivement. Leurs CMI constamment faibles contre *Shigella* suggèrent leur potentiel de production d'antibiotiques.

Mots-clés: Agent antimicrobien; Résistance aux antibiotiques; Extraits de plantes; CMI; MBC

Introduction:

Throughout history, certain plants have played significant roles in traditional medicine, serving as remedies for various ailments and contributing to preservation methods. Long before discovering microorganisms, the belief in the healing potential of certain plants was widely accepted. Human utilization of plants for medicinal purposes dates back millennia, reflecting the enduring relationship between humanity and therapeutic properties of plants. The extensive array of plant species employed for healing purposes encompasses a wide spectrum, including analgesics, anticancer, antipyretic and antihypertensive agents (1).

Earth is home to approximately 250, 000 higher plant species, with over 80,000 reported to possess medicinal value and around 5,000 recognized for specific therapeutic benefits. Notably, more than 80% of the global population relies primarily on plants and plant extracts for healthcare (2,3). There is a growing interest in plant-derived drugs and dietary supplements, particularly in the treatment of infectious diseases. Traditional healers, inspired by the historical use of plants, have contributed to the integration of plant-based remedies into Western medicine. The secondary metabolites found in plants, such as tannins, terpenoids, alkaloids, and flavonoids, are well-known for their in vitro antimicrobial properties. Plant-based antimicrobials may offer alternative mechanisms for inhibiting bacteria, potentially contributing to the treatment of resistant microbial pathogens (4).

In response to the escalating issue of antibiotic resistance, numerous studies have explored plants as natural antimicrobials (5). The rise in resistance to conventional antimicrobial agents, particularly antibiotics, has led to alarming mortality rates, with an estimated 100,000 deaths annually and a projected global increase to 10 million by 2050, with Africa accounting for 40% of these deaths (6, 7,8).

In response, the World Health Organization (WHO) published a list of antibiotic-resistant bacteria of public health concerns, signaling the urgent need for new measures (9). Investigating the phytochemical constituents and antimicrobial properties of herbal plants holds promise for developing new drugs to combat antibiotic resistance, with the added benefit of lower toxicity compared to chemically synthesized antimicrobials.

This study aims to assess the antimicrobial properties of five medicinal plants; *Bambusa vulgaris* (Bamboo), *Heteropogon contortus* (Spear grass), *Hibiscus sabdariffa*, *Moringa oleifera*, and *Carica papaya*, traditionally used for medicinal purposes in treating infections caused by *Salmonella* Typhi and *Shigella dysenteriae*.

Materials and method:

Plant collection and identification:

Plants were gathered from Gateri village of Kurmi Local Government Area (LGA) and Kona area of Jalingo LGA Taraba State, Nigeria, in August 2022. The Department of Botany, Taraba State University, Jalingo, identified the plants. The selection was based on

traditional and ethnobotanical usage indicating potential antimicrobial properties.

Plant preparation:

The plant parts to be used were air dried under shade, cut into pieces, and stored at 40C. 300g of the plant part(s) were extracted with solvents of 80% 900ml each of ethanol, ethyl acetate, dichloromethane, and hexane in a shaking incubator at 28^0C for 12 hours. The residues were re-extracted three times. The extracts were pooled and filtered, and the solvent-combined specimen was evaporated to dryness using a vacuum rotary evaporator and weighed to determine the yield of a soluble constituent, labeled and stored in a desiccator, subject to further analysis.

To vary the concentration, extract(s) were dissolved in phosphate-buffered saline (PBS) at a concentration of 100mg/ml for each extract. Five-fold serial dilution were carried out in 10ml sterile test tubes containing PBS. This was used in the preparation of the antibiotic plates. PBS has no antimicrobial properties.

Isolation and identification of test clinical bacteria isolates:

Salmonella Typhi and *S. dysenteriae* used were isolated from patients attending the Specialist Hospital, Jalingo Taraba State. Stool samples were collected into a sterile container and processed immediately. The stool samples were cultured on *Salmonella-Shigella* (SS) agar and incubated at 37°C (98.6°F) for 24 hours. *Salmonella* colonies on SS agar appeared colorless with a black center due to hydrogen sulfide production, while *Shigella* colonies appeared colorless because of their inability to produce hydrogen peroxide.

To confirm the identity of the isolates, biochemical tests such as Triple Sugar Iron (TSI) agar test was used to differentiate based on sugar fermentation and gas production, Urea agar test was used to test for urease production, and motility test with *Salmonella* motile, while *Shigella* is non-motile.

Determination of antimicrobial concentrations of the extracts by serial dilution:

The concentrations (mg/mL) of extracts from *H. sabdariffa*, *M. oleifera*, *C. papaya*, *B. vulgaris*, and *H. contortus* are presented following various dilution levels as shown in Table 1. For *H. sabdariffa*, the undiluted (raw) concentration (R conc) was 273 mg/mL, decreasing to 0.027 mg/mL at the fifth dilution (D5 conc). The concentration of the positive control (ceftriaxone) was a constant of 0.030 mg/mL, while the concentration of the negative control (Dilution Solvent: ethanol) remained 0.00 mg/mL. Other plant extracts had similar concentration trends at different levels of dilution, giving varying concentrations for each extract.

The initial concentrations before dilution (R conc.) for *H. sabdariffa*, *M. oleifera*, *C. papaya, B. vulgaris*, and *H. contortus* were 273 mg/mL, 82 mg/mL, 111 mg/mL, 266 mg/mL, and 75 mg/mL, respectively. After 5-fold serial dilutions (D1 to D5 conc), concentrations varied as shown. Ceftriaxone, the positive control, maintained a constant concentration of 0.030 mg/mL. *Hibiscus sabdariffa* had the highest concentrations, followed by *B. vulgaris, C. papaya, M. oleifera*, and *H. contortus*.

Table 1: Concentrations of the medicinal plant extracts from serial dilution and the controls

	Concentrations in milligrams per milliliter (mg/mL)							
Plants	**R conc**	**D1 conc**	**D2 conc**	**D3 conc**	**D4 conc**	**D5 conc**	**Cef (PC)**	**DS (NC)**
Hibiscus sabdariffa	273	68.25	17.06	4.27	1.066	0.027	0.030	0.00
Moringa olifera	82	20.5	5.13	1.28	0.32	0.08	0.030	0.00
Carica papaya	111	27.75	6.93	1.73	0.44	0.11	0.030	0.00
Bambusa vulgaris	266	66.5	16.63	4.156	1.04	0.26	0.030	0.00
Heteropogon contortus	75	18.75	4.69	1.17	0.30	0.075	0.030	0.00

R con= Undiluted (Raw) concentration, D1 conc = First dilution concentration, D2 conc = Second dilution concentration, D3 conc = Third dilution concentration, D4 conc = Fourth dilution concentration, D5 conc = Fifth dilution concentration, Cef = Ceftriaxone, PC = Positive Control, DS = Dilution Solvent (ethanol), NC = Negative Control

Analysis of phytochemical constituents of the medicinal plants:

The secondary metabolites were identified using previously described standard methods. Cardiac glycosides were identified by the Kellar-Kiliani test, which involves dissol ving 50mg of methanolic extract in 2ml chloroform, and adding H_2SO_4 to produce brown ring at the interface. Flavonoid was detected by the Shinoda test which involves adding to 2-3ml of ethanolic extract, Mg ribbon and HCl to give a pink-red or red solution or by the NaOH test in which extract is treated with dilute NaOH, and then dilute HCl to give a yellow solution that turns colorless. Phenol was identified by the phenol test in which to a spot extract on filter paper is added phosphomolybdic acid reagent, which is then exposed to ammonia vapors to give blue spot coloration.

Phlobatannin was identified by boiling 2ml of the extract with 2ml of 1% HCl leading to the formation of red precipitates. Saponin was identified by the Frothing or Foam test in which 0.5ml filtrate was added to 5ml distilled water and shaken to give persistent frothing. Steroid was detected by the Liebermann-Burchardt test which involves mixing 1ml of the extract with chloroform, acetic anhydride, and sulfuric acid to give dark green coloration, which can be confirmed by mixing 1ml extract with acetic anhydride and sulfuric acid to produce blue or green color change.

Tannin was detected by the Braemer's test which involved mixing ferric chloride with extract to produce dark blue or greenish-grey solution. Terpenoid was also detected by the Liebermann-Burchardt test that involved mixing extract with chloroform, acetic anhydride, and sulfuric acid to produce pink or red coloration or by the Salkowski test in which extract is combined with chloroform and sulfuric acid to produce reddish-brown color interface.

Test of antimicrobial properties by the disk diffusion method:

The Kirby-Bauer disk diffusion method was used to assess the antimicrobial properties of the test extracts. First, the test organisms were inoculated on nutrient agar plates and impregnated sterile filter paper disks with plant extract solution at different concentrations (D1, D2, D3, D4, D4, and R conc.) were placed on inoculated agar using sterile forceps, evenly distributed across plates. The plates were incubated at 37°C for 24 hours, followed by measurement of diameters of zones of inhibition using a ruler and determination of antimicrobial activity. The control disks were prepared with PBS (negative control) and commercially prepared ceftriaxone (Hardy diagnostics, Santa Maria, USA) as positive control. Inhibition zone diameter of 24–30 mm indicated sensitivity, 13–23 mm intermediate and ≤12 mm resistance (10).

Determination of minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of the extracts:

The MIC of the extracts against the test bacterial isolates (*S.* Typhi and *S. dysenteriae*) was determined by diluting an overnight culture growth of a pure culture of the test organisms in nutrient broth to a concentration of 1×10^5 CFU/ml. A 5-fold serial dilution of the antimicrobial test extract was performed in a series of test tubes to establish varying concentrations using the ethanolic extract. One milliliter of each test extract dilution was inoculated with an equal volume of the standardized microorganism. Positive control (ceftriaxone) and negative control (PBS) tubes were included for each test microorganism. The tubes were incubated at 37°C for 24 hrs. The MIC was determined as the lowest concentration of the extract in the tube where no growth was visually observed.

For the MBC determination, dilutions representing the MIC and concentrations above were plated on nutrient agar, incubated overnight, and colonies were enumerated to determine CFU/ml. The MBC is the lowest concentration showing no viable bacterial growth.

Results:

Phytochemical constituents of extracts:

Table 2 indicates the results for the different components (saponins, cardiac glycosides, terpenoids, steroids, flavonoids, phenolics, and tannins) with the four solvents used for the plant extracts (ethanol, ethyl acetate, dichloromethane, hexane). Saponins were extracted by ethanol, ethyl acetate, and hexane. Cardiac glycosides by ethanol, ethyl acetate and hexane. Terpenoids by ethyl acetate and hexane with highest concentration with hexane. Steroids by ethyl acetate, dichloromethane, and hexane with highest concentration in hexane. Flavonoids by ethanol, ethyl acetate, and hexane. Phenolics by ethyl acetate, dichloromethane, and hexane with highest concentration in dichloromethane. Tannins by ethanol, ethyl acetate, and hexane, with the highest concentration in ethanol.

Ethanol had higher yield of the phytochemical components compared to the other solvents. Additionally, ethanol and ethyl acetate also showed the presence of more phytochemical components than other solvents.

Table 2: Phytochemical components of the plant extracts by the four extraction solvents

Phytochemical components	**Ethanol**					**Ethyl Acetate**					**Dichloromethane**					**Hexane**				
	Hs	Mo	Cp	Bv	Hc	Hs	Mo	Cp	Bv	Hc	Hs	Mo	Cp	Bv	Hc	Hs	Mo	Cp	Bv	Hc
Saponins	+	+	-	+	+	+	-	+	-	-	-	+	-	+	+	+	-	+	+	+
Cardiac glycosides	+	+	-	+	+	-	+	+	+	-	+	+	-	-	-	+	+	-	-	+
Terpenoids	-	+	+	-	+	-	+	-	-	+	+	-	-	-	+	+	+	+	-	-
Steroids	-	+	+	-	+	+	-	-	+	+	+	+	+	-	-	+	+	+	+	+
Flavonoids	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	-	+	+	-	+
Phenolics	-	-	+	-	+	+	+	+	-	+	-	+	+	+	-	+	-	-	-	+
Tannins	+	+	+	+	+	-	+	+	-	+	+	+	+	-	-	+	-	-	+	-

+ = Positive, - = Negative, Hs = *Hibiscus sabdariffa, Mo = Moringa oleifera, Cp = Carica papaya, Bv = Bambusa vulgaris, Hc = Heteropogon contortus*

Table 3: Diameter of zone of inhibition of bacterial isolates at various concentrations of the extracts and the controls

Extracts/concentrations	R conc	D1 conc	D2 conc	D3 conc	D4 conc	D5 conc	Cef (PC)	PBS (NC)
	Diameter of zone of inhibition (mm) for *Salmonella* Typhi							
Hibiscus sabdariffa	29.00	27.00	14.00	4.00	0.00	0.00	14.05	0.00
Moringa oleifera	1.00	0.50	0.00	0.00	0.00	0.00	13.80	0.00
Carica papaya	1.00	0.00	0.00	0.00	0.00	0.00	14.50	0.00
Bambusa vulgaris	5.00	5.00	3.00	0.00	0.00	0.00	15.00	0.00
Heteropogon contortus	10.00	4.00	2.00	1.00	0.50	0.00	14.00	0.00
	Diameter of zone of inhibition (mm) for *Shigella dysenteriae*							
Hibiscus sabdariffa	22.05	16.00	5.50	0.50	0.00	0.00	15.00	0.00
Moringa oleifera	1.00	0.00	0.00	0.00	0.00	0.00	14.00	0.00
Carica papaya	1.00	0.7	0.00	0.00	0.00	0.00	14.00	0.00
Bambusa vulgaris	18.50	17.00	10.00	5.00	0.00	0.00	13.50	0.00
Heteropogon contortus	25.05	15.00	10.00	5.00	0.00	0.00	14.00	0.00

R conc=Raw concentration, D1 conc = First dilution concentration, D2 conc = Second dilution concentration, D3 conc = Third dilution concentration, D4 conc = Fourth dilution concentration, D5 conc = Fifth dilution concentration, ZI = Zone of inhibition, Cef = Ceftriaxone, PC = Positive Control, DS = Dilution Solvent (ethanol), NC = Negative Control

Antimicrobial properties of the extracts

Table 3 displays zone of inhibition (ZI) values for *S.* Typhi and *S. dysenteriae* in response to varying concentrations of the plant extracts. Undiluted (273 mg/mL) and D1 concentration (68.2 mg/mL) of *H. sabdariffa* extracts had antibacterial effects on *S.* Typhi and *S. dysenteriae* above the diameter of zone of inhibition breakpoint of ≤ 12 mm, comparable to 0.030 mg/mL of ceftriaxone control. Also, undiluted (266 mg/mL) and D1 concentration (66.5 mg/mL) of *B. vulgaris* extracts had antibacterial effects on *S. dysenteriae* above the diameter of zone of inhibition breakpoint of ≤ 12 mm, comparable to 0.030 mg/mL ceftriaxone control. Similarly, undiluted (75 mg/mL) and D1 concentration (18.75 mg/mL) of *H. contortus* extracts had antibacterial effects on *S. dysenteriae* above the diameter of zone of inhibition breakpoint of ≤ 12 mm, comparable to 0.030 mg/mL ceftriaxone control.

In *S.* Typhi, the zone of inhibition (ZI) of *H. sabdariffa* decreases from 29.00 mm at undiluted concentration to 0.00 mm at the highest dilution (D5). Positive control (ceftriaxone) had a ZI of 14.05 mm, while negative control (NC) was 0.00 mm. Similar concentration-dependent decreases were observed for other plants, indicating reduced inhibitory effects with dilution. For *Shigella dysenteriae*, a similar concentration-dependent decrease in ZI values was noted. For instance, in *H. sabdariffa*, the ZI decreased from 22.05 mm at undiluted concentration to 0.00 mm at D5. The positive control maintained a constant 15.00 mm ZI, while the negative control remained at 0.00 mm.

Table 4: Minimum inhibitory and minimum bactericidal concentration of extracts against the test bacterial isolates

Plant extracts	MIC (mg/mL)	MBC (mg/mL)
	***Salmonella* Typhi**	
Hibiscus sabdariffa	D3 (4.27)	D1 (68.25)
Moringa oleifera	R (82.00)	-
Carica papaya	D2 (6.93)	-
Bambusa vulgaris	D2 (16.63)	-
Heteropogon contortus	D3 (4.69)	R (75.00)
	Shigella dysenteriae	
Hibiscus sabdariffa	D3 (4.27)	D1 (68.25)
Moringa oleifera	R (82.00)	-
Carica papaya	D2 (6.93)	-
Bambusa vulgaris	D2 (16.63)	D1 (66.5)
Heteropogon contortus	D2 (4.69)	D1 (18.75)

MIC= Minimum inhibitory Concentration, MBC= Minimum Bactericidal Concentration, R con= Raw concentration, D1 conc = First dilution concentration, D2 conc = Second dilution concentration, D3 conc = Third dilution concentration

As the test extracts were diluted, the zone of inhibition against both bacteria decreased, suggesting diminishing antimicrobial effects. This dose-dependent response indicates antimicrobial activity with variations among plants and dilutions. The positive control consistently showed approximately 14 mm ZI for both bacteria, highlighting antibacterial efficacy of ceftriaxone, while the negative control consistently exhibited no ZI, indicating the solvent's lack of antibacterial properties.

Minimum inhibitory and bactericidal concentrations of the extracts:

Table 4 represents the MIC and MBC values of the plant extracts for *S.* Typhi and *S. dysenteriae.* For *S.* Typhi, the MIC of *H. sabdariffa* was at D3 conc (4.27 mg/mL) and MBC at D1 conc (68.25 mg/mL). The MIC of *M. oleifera* was at R conc (82.00 mg/mL), with no MBC reached. The MIC of *C. papaya* was at D2 conc (6.93 mg/mL), and *B. vulgaris* was at D2 conc (16.63 mg/mL), both without MBC. The MIC of *H. contortus* was at D3 conc (4.69 mg/mL) and MBC at R conc (75 mg/mL).

For *S. dysenteriae*, the MIC of *H. sabdariffa* was at D3 conc (4.27 mg/mL) and MBC at D1 conc (68.25 mg/mL). The MIC of *M. oleifera* was at R conc (82.00 mg/mL), with no MBC reached. The MIC of *C. papaya* was at D2 conc (6.93 mg/mL), and *B. vulgaris* at D2 conc (16.63 mg/mL) and MBC at D1 conc (66.5 mg/mL). The MIC of *H. contortus* was at D2 conc (4.69 mg/mL) and MBC at D1 conc (18.75 mg/mL).

Discussion:

This study was a thorough investigation into the phytochemical composition, concentrations, and antimicrobial efficacy of extracts from *H. sabdariffa, M. oleifera, C. papaya, B. vulgaris,* and *H. contortus*. Employing various solvents, including ethanol, ethyl acetate, dichloromethane, and hexane, the research explored the diverse phytochemical components using 5-fold serial dilutions, gauging antimicrobial effects against *S.* Typhi and *S. dysenteriae*. Phytochemical analysis revealed the presence of saponins, cardiac glycosides, terpenoids, steroids, flavonoids, phenolics, and tannins, with ethanol consistently exhibiting higher concentrations. The solvent ethanol generally has higher concentrations of these phytochemicals than other solvents. These findings align with prior research (11).

Antimicrobial Activities were measured by the zone of inhibition (ZI) against *S.* Typhi and *S. dysenteriae*, the activity decreases with increasing dilutions, indicating a dose-dependent response and antimicrobial activity. Ceftriaxone served as a positive control, consistently demonstrating effectiveness. Dose-dependent responses and antimicrobial activities from plant extracts were also corroborated by existing studies (12-15).

Specifically, Perera et al., (11) highlighted the antibacterial potential of *H. contortus* extracts against *Escherichia coli* and *Staphylococcus aureus*. Eremwanarue and Shittu (16) demonstrated antibacterial activity of *M. oleifera* against multidrug-resistant bacteria. Abdallah (17) reported the efficacy of *H. sabdariffa* calyces against various bacteria, surpassing penicillin. Emad (18) reported significant antibacterial properties of *H. sabdariffa* against *A. baumannii* strains. Bokaeian et al., (19) explored inhibitory effects of *M. oleifera* against *E. coli* and *S. aureus*, exhibiting dose-

dependent responses. Timothy et al., (20) reported antibacterial activity of *H. sabdariffa* against *S. aureus* and *E. coli*. Guteirrez et al., (21) studied the effective inhibition of *H. sabdariffa* calyx extracts against multidrug-resistant *Salmonella* strains.

The MIC and MBC values determined revealed similar MIC values for both bacterial isolates with varying MBC values. *Salmonella Typhi* exhibited greater resistance than *S. dysenteriae*. Individual plants exhibited unique MIC and MBC profiles, highlighting plant-specific antibacterial properties. For instance, *H. sabdariffa* at D3 conc demonstrated an MIC of 4.27 mg/mL against both bacterial strains, while *B. vulgaris* at D2 conc exhibited an MBC of 16.63 mg/mL against *S.* Typhi and 66.5 mg/mL against *S. dysenteriae*.

Conclusion:

This study provides valuable insights into the phytochemical composition and antimicrobial properties of plant extracts, emphasizing the importance of plant specificity in antibacterial effects. The results have implications for potential therapeutic applications and warrant further investigation into the active compounds responsible for the observed effects. The variations in phytochemical content and antimicrobial efficacy among different solvents and plant species underscore the importance of careful selection in extraction processes for medicinal purposes.

Our findings support the idea that plant extracts could serve as potential sources of antimicrobial agents. However, further research, including toxicity studies and clinical trials, is essential to validate their safety and efficacy for practical applications in medicine. Further studies are also required to determine the bioactive component and antimicrobial mechanisms of actions of the extracts for proper documentation. The study contributes valuable insights into the potential use of these plants in developing natural antimicrobial agents for combating bacterial infections.

Contributions of authors:

DZ designed the study, coordinated the research, monitored the benchwork and wrote the manuscript. JB performed the phytochemical analysis, while HN, EO, AG and HC carried out the bench work. All authors read and approved the manuscript for submission.

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Conflicts of interest:

No conflict of interest is declared.

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Original Article **Open Access**

Isolation and biocontrol of bacteriophages from wastewater in the city of Lomé, Togo: potential application as a novel source for antimicrobial therapy

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Abstract:

Background: Bacteriophages offer one of the most promising solutions to the challenges of antimicrobial resistance in bacteria. The aim of this study is to investigate bacteriophages as a source of new antimicrobial therapy.
Methodology: Waste water samples were randomly collected from 8 different locations in the city of Lomé for bacteriophage isolation. The phages were isolated using multi-resistant clinical isolates (*Escherichia coli* 1642 and *Staphylococcus aureus* 0868) as hosts by means of a spot test. The host range of the phages was determined also by a spot test using 8 other clinical bacterial isolates including two reference strains (*E. coli* ATCC 25922 and *S. aureus* ATCC 29213). The virulence of the phages and their effects on bacterial growth were assessed by *in vitro* experiments using *E. coli* 1642 BBec phage suspension.
Results: Isolation of phages by the spot test was positive only with the host *E. coli* 1642. A reduced host range was observed with the other bacteria. The BBec phage suspension showed a titer of 1.6×10^7 PFU/ml. Virulence studies revealed a latency time of less than 10 minutes, a degree of absorption of 87% and a burst size of 63 PFU/cell. The effect of BBec phage suspension on *E. coli* 1642 showed an almost total reduction in the population of *E. coli* 1642 after 4 hours.
Conclusion: This study provided scientific data showing the antibacterial effect of a phage suspension (BBec) on a multi-resistant clinical isolate of *E. coli* 1642. This phage could therefore be explored as a candidate for the development of new antibacterial therapies.

Keywords: multidrug resistance, bacteriophage, antibacterial effect, wastewater, Togo

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Isolement et biocontrôle des bactériophages à partir des eaux usées de la ville de Lomé, Togo: potentielle application en tant que source de nouvelles thérapies antimicrobiennes

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Résumé:

Contexte: Face aux problèmes de multirésistance des bactéries aux agents antimicrobiens, les bactériophages représentent l'une des solutions les plus prometteuses. L'objectif de ce travail est d'étudier les bactériophages en

tant que source de nouvelles thérapies antimicrobiennes.
Méthodologie: Des échantillons d'eaux usées ont été collectés de manière aléatoire dans 8 endroits différents de la ville de Lomé pour l'isolement des bactériophages. Les phages ont été isolés en utilisant comme hôtes, des isolats cliniques (*Escherichia coli* 1642 et *Staphylococcus aureus* 0868) multirésistants par le biais d'un test ponctuel. La gamme d'hôte des phages a également été déterminée par un test ponctuel utilisant 8 autres isolats dont deux souches de référence (*E. coli* ATCC 25922 et *S. aureus* 29213). L'évaluation de la virulence des phages et leurs effets sur la croissance des bactéries ont été réalisés à travers des expérimentations *in vitro avec* une suspension de phages d'*E. coli* désignée BBec.
Résultats: L'isolement des phages par le test ponctuel s'est révélé positif seulement avec l'hôte *E. coli* 1642. Une gamme d'hôte réduite a été observée avec les autres bactéries. La suspension de phage BBec a présenté un titre de 1,6 x 10^7 UFP/ml. L'étude de sa virulence a révélé un temps de latence inférieur à 10 minutes, un degré d'absorption de 87% et une taille de rafale de 63 UFP/Cellule. L'effet de la suspension phagique BBec sur l'isolat *E. coli* 1642 a montré une réduction quasi totale de la population de l'isolat *E. coli* 1642 au bout de 4 h.
Conclusion: Cette étude a permis de fournir des données scientifiques qui montrent l'effet antibactérien d'une suspension de phage (BBec) sur un isolat clinique multirésistant *E. coli* 1642. Ce phage pourrait donc être exploré comme candidat au développement de nouvelles thérapies antibactériennes.

Mots clés: multirésistance aux médicaments, bactériophages, effet antibactérien, eaux usées, Togo.

Introduction:

Antibiotics have been important therapeutic discovery for human health. Their use has long helped to reduce mortality and morbidity worldwide. However, the increased use and misuse of these antimicrobial agents have resulted in the emergence of resistance in microbial populations counteracting the beneficial effects of antibiotics (1). Thanks to their genetic flexibility and plasticity, pathogenic bacteria are capable of setting up specific resistance mechanisms against a particular antibiotic. In fact, some strains are able to establish multiple resistance to several antibiotics at the same time, giving rise to what is known as multidrug resistant (MDR) bacteria (2).

According to the World Health Organization (WHO), MDR bacteria are one of the most serious threats to global health, food security and development. It is now reaching dangerously high levels in all regions of the world. New resistance mechanisms are emerging and spreading worldwide, compromising our ability to treat common infectious diseases. The MDR bacteria cause serious infections that are difficult or impossible to treat, due to the loss of effectiveness of antibiotics.

The UK Government's Review on Antimicrobial Resistance published in 2014 estimated that up to 10 million people could die each year from AMR infections by 2050 (3). However, in 2019 antibiotic-resistant bacteria directly caused the death of more than 1.2 million patients (4). The situation is alarming in countries with limited resources where infectious diseases, poverty and malnutrition are endemic (5). In sub-Saharan West Africa, 27.3 deaths per 100,000 population (all ages) were attributable to antibiotic-resistant germs (4). The six main pathogens responsible for these resistance-associated deaths are *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* (4,6).

Faced with this growing problem, the search for a new antimicrobial strategy has become one of the highest priorities of modern medicine and biotechnology. Bacteriophages (environmental viruses that naturally infect bacteria) represent one of the most promising solutions (7). In fact, the idea of using bacteriophages to treat bacterial infections (phage therapy) is not new. At the beginning of the 20th century, phage therapy enjoyed successes that were to be the starting point of a true "globalisation" (8). However, with the discovery of penicillin in 1928 and the second World War, which generated an immense need for anti-infective treatments, phagotherapy was gradually abandoned in favour of antibiotic therapy (9). Today, with the emergence of bacteria that are multi-resistant to antibiotics, there is renewed interest in the study of bacteriophages (10).

Recent studies showed that bacteriophages are currently used in the food industry to limit the development of pathogens during processing and/or on surfaces in contact with food. In agriculture, they are used to combat plant pathogenic bacteria (11) and also in human medicine, to treat patients suffering from infection by multi-resistant bacteria (12). This study focuses mainly on the isolation of bacteriophages from the environment and the potential application in the biocontrol of MDR bacteria.

Materials and method:

Study setting:

This study was carried out in Lomé, Maritime region of Togo, which included Bè Lagoon (1.24113176; 6.14959641), West Lake (1.21255321; 6.13774805), Forever Reservoir (1.22217439; 6.16634648), Tokoin Séminaire (1.21201485; 6.15123999), Tokoin Dogbeavou (1.21300797; 6.15174332), Bè Beach (1.2492 3218; 6.1308177), Oando Beach (1.22930563; 6.12336644) and Palm Beach (1.22196697; 6.12 061692).

The Laboratory of Biomedical, Food and

Environmental Health Sciences, High School of Biological and Food Technics at the University of Lomé provided the setting for sample handling.

Bacteria strains:

The bacterial strains used in this study were supplied by the Medical Bacteriology Department of the National Institute of Hygiene of Lomé, Togo. A total of 10 strains were used which include 8 clinical isolates from various samples (urine and pus) and 2 reference strains (*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213).

Sample collection:

Waste water samples were randomly collected on the 15th July 2022 from a total of 8 locations; 5 samples of urban wastewater from Bè Beach (BB), Oando Beach (OB), Palm Beach (PB), Tokoin Séminaire (TS), Tokoin Dogbeavou (TD), and 3 other samples from Bè Lagoon (BL), West Lake (WL) and Forever Water Reservoir (FR). Each sample was collected in a sterile 50 ml bottle. For each sample, the bottle was first rinsed with wastewater before being filled to 30 ml. After collection, the samples were transported to the laboratory as quickly as possible in a pot of ice for bacteriophage isolation (13).

Bacteriophage isolation:

The phage enrichment method was used for the isolation of bacteriophages against the host bacterial strains (*E. coli* 1642 and *S. aureus* 0868) (14). Briefly, under aseptic conditions, 5 ml of overnight-grown bacterial cultures of each host were mixed with 5 ml of wastewater samples from each site. Each mixture was then incubated for 24 h at 37°C in a shaking incubator (70 rpm) with 10 ml of Luria-Bertani (LB) broth in sterile 50 ml bottles. Each mixture was centrifuged at 6,000g for 15 mins. The supernatant was collected in a syringe and filtered directly into 1.5 ml Eppendorf tubes using a 0.20 µm syringe filter. The tubes were stored at 4°C.

The filtrate (phage suspension) was tested for phage activity using the spot test. Briefly, a 5 ml suspension of overnight grown bacterial cultures (each host bacterium) was inoculated by flooding onto LB agar and incubated for 15 mins. Subsequently, 10 µl of each filtrate was deposited on the lawn of the corresponding host bacterium and allowed to dry. For the control, 10 µl of sterile water was also deposited. Each LB plate was then incubated for 24 hours and the appearance of clear spots (plaques) indicated the presence of bacteriophages in the filtrate. This test was performed on the other clinical bacterial isolates as well as on the reference strains of *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 to measure the host range of the phage suspensions. The diameters of the lysis plaques were measured and the data were graphically synthesised to evaluate the lytic capacity of the phage suspensions according to location.

Bacteriophage titration:

The suspension was serially diluted (10^0 to 10^{-8}) with magnesium sulphate buffer (25 ml Tris-HCl, 1 g $MgSO_4$, 2.9 g NaCl, 500 ml H_2O) (14). Prior to this, a 5 ml suspension of overnight grown bacterial cultures of each bacterium was inoculated onto the LB agar by flooding. After 15-20 min of incubation, 10 µl of each dilution was applied to the surface of the agar and allowed to dry. The plates were then incubated at 37°C for 24 h and the titre was obtained by counting the lysis spots observed with the dilution causing the most lysis on the agar using the formula (15); T(pfu)n = (N x F)/V(ml), where T is the titre, 'pfu' is phage format unit, N is the number of phages counted, F is the dilution factor, and V is the volume of phage suspension deposited.

One-step phage multiplication kinetics:

Phage cycle characterisation was performed using BBec suspension and *E. coli* 1642 host. Briefly, 100 µl of bacterial culture (1.5 x 10^8 CFU/ml, *E. coli* 1642) was infected with 100 µl of *E. coli* 1642 BBec suspension in 10 ml of LB broth contained in a sterile 50 ml bottle and incubated at 37°C for 10 mins. The mixture was centrifuged at 12,000 rpm for 5 mins and the supernatant was collected for titration and estimation of the percentage of phages absorbed on the cell surface in 10 mins (degree of absorption) according to the formula (16); DA=[pfu(0)-pfu(10)]/pfu(0) x 100, where DA is the degree of absorption and 'pfu' is the phage format unit.

The pellet containing the infected cells is resuspended in 10 ml of LB broth and incubated at 37°C. An aliquot of 100 µL is taken every 10 mins for 50 mins to titrate the phages. The data obtained, representing the evolution of the phage titre of the culture as a function of time, were synthesised in the form of a graph. The latency period was determined from the graph and the number of phages released per cell was calculated by dividing the maximum viral titre by the initial titre (t=0 min).

Evaluation of the effects of phage on bacterial growth:

A mixture of 100 µl of an 18 h suspension culture of *E. coli* 1642 was added to 10 ml of LB broth and incubated for 10 mins before the addition of 100 µl of the BBec suspension (17). This preparation was carried out in 6 sterile 50 ml bottles labelled (B+P, 0 h); (B+P, 2 h); (B+P, 4 h); (B+P, 6h.); (B+P, 24 h) and (B+P, 48 h) respectively. In parallel, positive controls were prepared under the same condi-

tions by adding 100 µl of overnight-grown bacterial cultures of *E. coli* 1642 to 10 ml of LB in a sterile 50 ml bottle; this preparation was also prepared in six sterile 50 ml bottles labelled (B, 0 h); (B, 2 h); (B+P, 4 h); (B, 6 h); (B, 24h) and (B, 48h).

The optical density (OD) of the flasks (B + P, 0 h) and (B, 0 h) was measured directly at 600 nm. The other flasks were placed in a shaking incubator. After two (2) hours of incubation, the flasks (B+P, 2 h) and (B, 2 h) were removed from the incubation and their ODs were measured at 600 nm. This procedure was repeated with the flasks ((B+P, 4 h), (B, 4 h)); ((B+P, 6 h), (B, 6 h); ((B+P, 24 h), (B, 24 h)) and ((B+P, 48 h), (B, 48 h)) respectively after 4, 6, 24 and 48 h of incubation. The experiment was repeated three times and the data obtained are summarised in the form of graphs where each point corresponds to the average of the OD_{600} nm values of the three measurements of each group of hours.

Statistical analysis of data:

Data were entered in Microsoft Excel 2019 MSO (Version 2209 Build 16.0.15629. 20152) before being exported to GraphPad Prism8 software for statistical analyses. Means were compared using the two-way ANOVA. P-value of less than 0.05 was considered statistically significant.

Results:

Isolated phages:

In all, 16 phage suspensions (8 from *E. coli* 1642 and 8 from *S. aureus*) were obtained from the water samples and recorded as shown in Table 1. The presence of bacteriophages was detected by the spot test. Lysis plaques ranging in diameter from 9 to 11 mm were observed in the spot test results of the 8 filtrates obtained from *E. coli* 1642 phage suspensions (Fig 1A). The other 8 filtrates obtained from *S. aureus* 0868 phage suspensions did not show lysed plaques (Fig 1C).

The host range results for *E. coli* 1642 and *S. aureus* 0868 phage suspensions are shown in Table 2. No interaction (lysis zone) was observed between the suspensions obtained from *E. coli* 1642 and the other bacteria, whereas for the suspensions obtained from *S. aureus* 0868, an interaction was observed only with the isolate *S. aureus* 0931 (Fig 1B). This indicates that these suspensions have a very restricted host range.

Table 1: *Escherichia coli* 1642 and *Staphylococcus aureus* 0868 phage suspensions from different samples

Hosts	**Phage suspension**							
Escherichia coli 1642	BBec	OBec	PBec	TSec	TDec	BLec	WLec	FRec
Staphylococcus aureus 0868	BBsa	OBsa	PBsa	TSsa	TDsa	BLsa	WLsa	FRsa

BB = Bè Beach, OB = Oando Beach, PB = Palm Beach, TS = Tokoin Séminaire, TD = Tokoin Dogbeavou, BL = Bè Lagoon, WL = West Lake, FR = Forever water reservoir, ec = *E. coli* 1642 phage suspensions, sa = *S. aureus* 0868 phage suspensions

Table 2: Bacterial host range of bacteriophage suspensions from wastewater samples in the city of Lome, Togo

Bacteria strains	**Ec**	**Sa**
Escherichia coli 1628	N	N
Escherichia coli 1610	N	N
Escherichia coli 1555	N	N
Escherichia coli ATCC 25922	N	N
Staphylococcus aureus 0931	N	P
Staphylococcus aureus 4031	N	N
Staphylococcus aureus RM	N	N
Staphylococcus aureus ATCC 29213	N	N

Ec = *E. coli* 1642 phage suspensions, Sa = *S. aureus* 0868 phage suspensions, N = indicates that there is no phage-bacteria interaction, P = indicates that there is a phage-bacteria interaction.

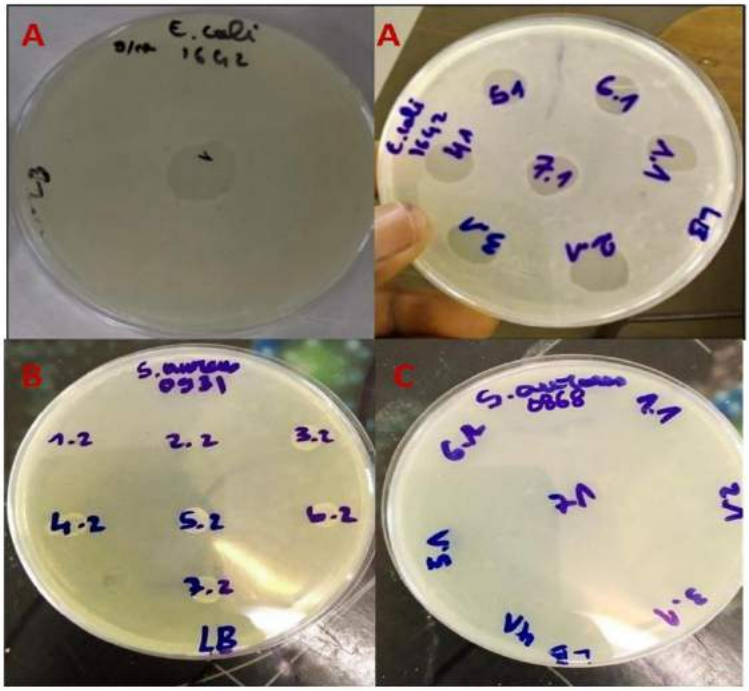

Fig 1: Spot test

A: Spot test using *E. coli* 1642, B: Spot test using *S. aureus* 0931, C: Spot test using *S. aureus* 0868

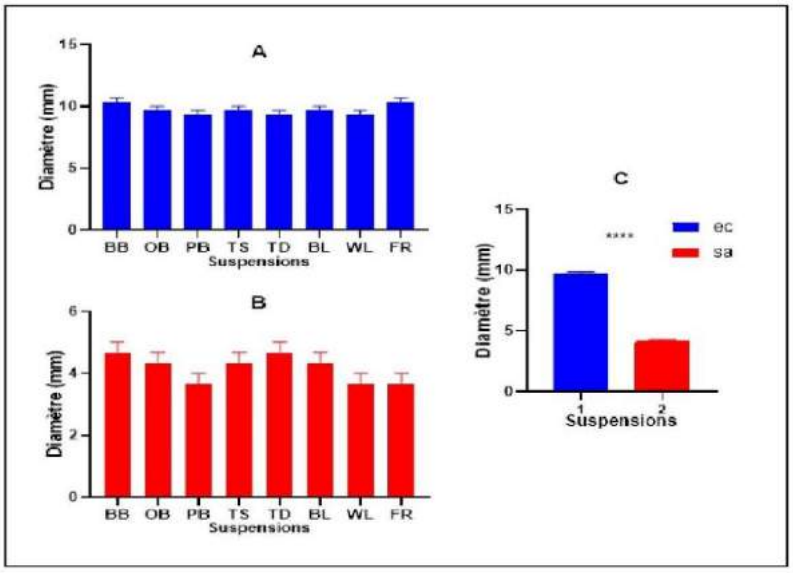

Figure 2: Lytic capacity of phage suspension

BB = Bè Beach, OB = Oando Beach, PB = Palm Beach, TS = Tokoin Séminaire, TD = Tokoin Dogbeavou, BL = Bè Lagoon, WL = West Lake, FR = Forever water reservoir, ec = *E. coli* 1642 phage suspensions, sa = *S. aureus* 0931 phage suspensions

Fig 2 shows in A the diameters of lysis plaques caused by *E. coli* 1642 phage suspensions (ec) from the different samples, in B the diameters of lysis plaques caused by *S. aureus* 0931 phage suspensions (sa), and in C, the set of lysis plaque diameters caused by the two phage suspensions. The differences in the mean diameter of the lysis plaques caused by the suspensions in A are not statistically significant (p=0.42). However, in C, mean differences between "ec" and "sa" are statistically significant (p<0.0001).

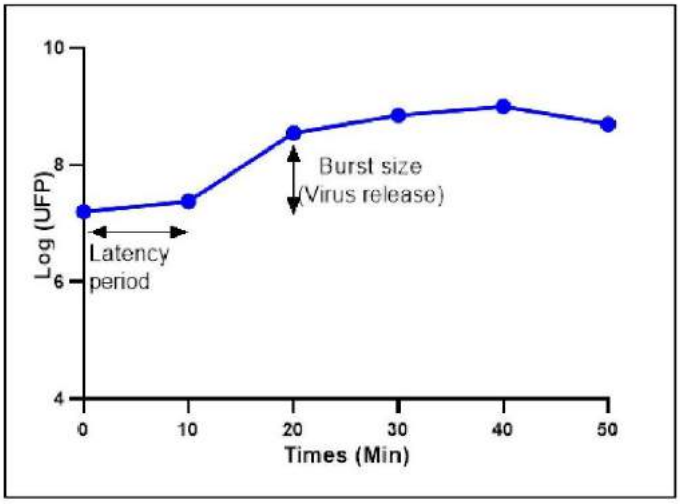

Fig 3: Kinetics of the multiplication of the *E. coli* 1642 BBec phage suspension

Table 3: Virulence data of *Escherichia coli* 1642 BBec phage suspension

Settings	Results
Degree of absorption (%)	87
Burst size (virus release) (UFP/Cell)	63
Latency (mn)	< 10

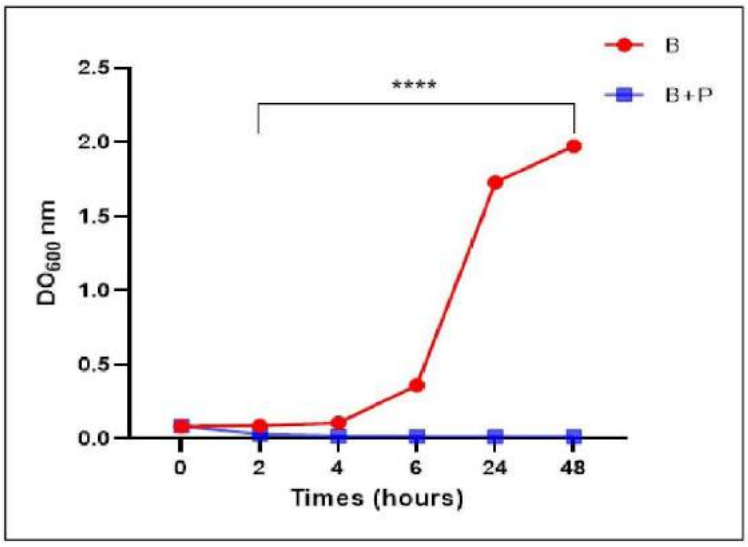

Fig 4: Evolution of the *E. coli* 1642 population

B+P: suspension of BBec mixed with a suspension of *E. coli* 1642 in LB broth, B: Suspension of *E. coli* 1642 alone in LB broth (control positive)

The titer of the BBec suspension obtained after counting and calculation is 1.6×10^7 PFU/ml. The kinetics of the one-step multiplication of the BBec phage are shown in Fig 3 and the virulence parameters are shown in Table 3. The effect of BBec suspension on the growth of *E. coli* 1642 is shown in Fig 4. The evolution of the optical density is used to indicate the population of *E. coli* 1642. The optical density (OD) of the control, absence of BBec suspension (B), increases until t = 48 hours, while that of the test, presence of BBec

suspension (B+P), gradually decreases until t = 4 hours, when it is close to zero. Comparing (B) and (B+P), there is a significant increase in the OD of (B) compared to OD (B+P) over time. From t = 2 hours to t = 48 hours, a statistically significant difference (p<0.0001) was observed between the OD values of the (B) and (B+P) groups.

Discussion:

Several studies have reported isolating bacteriophages from wastewater (18,19). They are usually detected by lytic activity on the host bacteria. In this study, phage isolation from clinical isolates of *E. coli* 1642 and *S. aureus* 0931 with reduced lytic spectrum from wastewater was demonstrated by the spot test and host range. Phage suspensions "ee" and "sa" obtained from different sites all exhibited lytic activity against *E. coli* 1642 and *S. aureus* 0931 respectively. These sampling sites would be contaminated by these pathogens (*E. coli* 1642 and *S. aureus* 0931) because according to Son et al., (20), phages are known to be present wherever hosts are present. This observation may be due either to resistance of these bacteria to bacteriophages, or to the absence of lytic phages specific for these bacteria in the suspension, or even to an insufficient incubation time to allow adsorption of the phages on the cell membrane of these bacteria.

On the basis of the "kill the winner" theory, we may assume that *E. coli* 1642 and *S. aureus* 0931 were isolated from the samples with a low density of lytic phages specific for these bacteria or with a low presence or absence of these bacteria. This theory suggests that phages adapt to preferentially infect the bacterial line with the highest frequency in the population (21). Unfortunately, there are no previously documented reports on the prevalence of these bacteria in these ecosystems. The work of Mirzaei and Nilsson (22) showed that a cocktail of 6 phages isolated from waste water was able to lyse 55 strains of *E. coli*. In addition, obtaining bacteriophages with a broader or different spectrum than that of their host bacterium is challenging because if a bacteriophage targeting proteins are modified, it may lose the ability to recognise its host. As a result, there will be no replication or maintenance of the bacteriophage in its host (23). Evaluating the lytic capacity of phages by measuring the diameter of the lysis plaque showed an independence between lytic capacity and sampling sites. On the other hand, host dependence is very real.

The titer of 1.6 x 10^7 PFU/ml of BBec suspension obtained by counting lysis plaques is within the average range of phages found in liquid media, between 10^7 and 10^8 PFU/ml (8). *S. aureus* phages isolated from urban wastewater and the Ebrié lagoon in Côte d'Ivoire showed titers between 10^5 and 10^8 PFU/ml in the study of Addablah et al., (19). The demonstration of BBec phage replication in the presence of *E. coli* 1642 provided virulence data such as a latency period of less than 10 mins, a degree of uptake of 87% in 10 minutes and an estimated burst size of 63 PFU/cell. *E. coli* phages (SU57) isolated from wastewater by Mirzaei and Nilsson (22) showed a latency of 17 mins, an uptake of 90% in 5 mins and a burst size of 196 PFU/cell. These differences in virulence data probably indicate that the phages isolated in the two studies are genetically different.

Several *in vitro* experimental models have described the effect of phages isolated from wastewater on the evolution of the population of associated pathogens (15,24). In our study, the increase in OD in the absence of BBec suspension (B) reflects the normal growth of the *E. coli* 1642 population in the broth, whereas the decrease in OD in the presence of BBec suspension (B+P) indicates the disruption of the normal growth of *E. coli* 1642. These observations can be explained by the fact that the phages in the BBec suspension infected the *E. coli* 1642 population causing massive lysis, hence the observed OD regression. Previous studies have made similar observations, for example, in the *in vitro* experiments by Wang et al., (17), MDR *E. coli* and methicillin-resistant *S. aureus* phages isolated from wastewater exhibited antibacterial activities against their respective populations.

Conclusion:

In our study, phage from clinical isolates of *E. coli* and *S. aureus* with reduced host range were isolated from random wastewater samples of urban sewage, lakes and lagoons in the city of Lomé, Togo. Demonstrating the virulence of one of the phage suspensions, designated BBec, allowed the determination of the latency of the phage, number of phages released per bacterial cell and degree of phage uptake. In addition, data demonstrating an antibacterial effect of the BBec suspension on the *E. coli* 1642 isolate was obtained by *in vitro* demonstration of the effect of the BBec suspension on the *E. coli* 1642 isolate.

Therefore, we can confirm that BBec suspension phages could be candidates for the development of new antibacterial therapies based on the scientific data obtained in this study. It would be interesting to extend the sampling sites to search for other phages specific to MDR bacterial strains, their characterization, study of the physical and chemical influences on their evolution, and *in vivo* experiments to better understand their application in therapeutics.

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Contributions of authors:

OAK conceived the study, collected the sample, participated in the experiments and analysis of the data, and drafted the manuscript; HY conceived the study, participated in its design, supervised the laboratory experiments, participated in the writing and corresponding author of the manuscript; GHE and PP participated in the experiments and analysis of the data; KK and SK participated in the sample collection, and helped to draft the manuscript; MM and DB were involved in the coordination of the study and corrected the manuscript; TT participated in the design of the study and supervised the work. All authors read and approved the final manuscript.

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Conflict of interest:

No conflict of interest is declared.

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Short Communication **Open Access**

Aerobic vaginitis in women seen at the laboratory of the university hospital of Befelatanana, Antananarivo, Madagascar

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Abstract:

Background: Vaginitis is common in women. The present study aims to identify the factors associated with aerobic vaginitis (AV) in women and evaluate the antibiotic resistance of bacteria responsible for this vaginitis.
Methodology: This was a retrospective cross-sectional study of 840 patients and analysis of the results of their cytobacteriological examinations of cervicovaginal samples from January 01, 2020 to December 31, 2022 at the Centre Hospitalier Universitaire Joseph Raseta Befelatanana (CHUJRB) laboratory, Antananarivo, Madagascar.
Results: Among the 840 patients, 35 had aerobic vaginitis, giving the prevalence of AV of 4.2%. Enterococcal vaginitis was the most common, representing 48.6% (n=17) cases of AV. Regarding associated factors, there was no significant difference in the prevalence of AV between women under age of 40 (4.4%, 29/653) and women over age of 40 years (3.2%, 6/187) (p=0.539); hospitalized (6.6%, 10/152) and non-hospitalized outpatients (3.6%, 25/688) (p=0.115); and pregnant (4.2%, 8/192) and non-pregnant women (4.2%, 27/648) (p=1.000). The antibiotic resistance varies from 0% (vancomycin) to 90.0% (penicillin G) for the Gram-positive bacteria and 0% (imipenem and amikacin) to 100% (cotrimoxazole, ciprofloxacin, cefixime) for Gram-negative bacteria (*Pseudomonas* spp)
Conclusion: Cytobacteriological examination of cervicovaginal specimens in cases of genital disorders is necessary to improve the management of patients with AV in Madagascar. Similarly, empirical treatment should be properly guided and self-medication avoided, in order to limit the emergence of multidrug-resistant bacteria.

Keywords: Antibiotic resistance; bacteria; aerobic vaginitis; women; Madagascar

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Vaginite aérobie chez la femme vue au laboratoire de l'hôpital universitaire de Befelatanana, Antananarivo, Madagascar

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Résumé:

Contexte: La vaginite est fréquente chez les femmes. La présente étude vise à identifier les facteurs associés à la vaginite aérobie (AV) chez la femme et à évaluer la résistance aux antibiotiques des bactéries responsables de cette vaginite.
Méthodologie: Il s'agit d'une étude transversale rétrospective portant sur 840 patientes et analyse des résultats de leurs examens cytobactériologiques de prélèvements cervico-vaginaux du 01 janvier 2020 au 31 décembre 2022 au laboratoire du Centre Hospitalier Universitaire Joseph Raseta Befelatanana (CHUJRB), Antananarivo, Madagascar.
Résultats: Parmi les 840 patientes, 35 avaient une vaginite aérobie, soit une prévalence d'AV de 4,2%. La vaginite à entérocoques était la plus courante, représentant 48,6% (n=17) des cas d'AV. Concernant les

facteurs associés, il n'y avait pas de différence significative dans la prévalence de l'AV entre les femmes de moins de 40 ans (4,4%, 29/653) et les femmes de plus de 40 ans (3,2%, 6/187) (p=0,539); patients hospitalisés (6,6%, 10/152) et non hospitalisés (3,6%, 25/688) (p=0,115); et les femmes enceintes (4,2%, 8/192) et non enceintes (4,2%, 27/648) (p=1.000). La résistance aux antibiotiques varie de 0% (vancomycine) à 90,0% (pénicilline G) pour les bactéries à Gram positif et de 0% (imipénème et amikacine) à 100% (cotrimoxazole, ciprofloxacine, céfixime) pour les bactéries à Gram négatif (*Pseudomonas* spp).
Conclusion: L'examen cytobactériologique des prélèvements cervico-vaginaux en cas de troubles génitaux est nécessaire pour améliorer la prise en charge des patients atteints d'AV à Madagascar. De même, le traitement empirique doit être correctement guidé et l'automédication évitée, afin de limiter l'émergence de bactéries multirésistantes.

Mots-clés: Résistance aux antibiotiques; bactéries; vaginite aérobie; femmes; Madagascar

Introduction:

The female reproductive tract is composed of the vagina, cervix, uterus, fallopian tubes, and ovaries, with the cervix connecting the upper reproductive tract to the vagina (1). The existence and invasion of microbes in the female reproductive tract have long been known to impact genital and reproductive health. Species of *Lactobacillus*, generally the most abundant taxa in the vaginal microbiome (VMB), produce lactic acid and probably bacteriocins that inhibit dysbiosis-associated microbes and work to maintain homeostasis and reduce risks of disease. A *Lactobacillus*-dominated VMB has been the hallmark of female reproductive health (2).

Vaginitis is a term to describe various conditions of infection or inflammation of the vagina. The most common kinds of vaginitis are bacterial vaginosis, vulvovaginal candidiasis or yeast vaginitis, trichomonas vaginitis and aerobic vaginitis (2). Aerobic vaginitis (AV) is an imbalance of the vaginal flora and was first mentioned in 2002 by Donders and others (3). The main characteristic is an abnormal vaginal flora that contains aerobic and intestinal pathogens and different degrees of vaginal inflammation (4). The frequency of AV varies from 12% to 23.7% in symptomatic women who are not pregnant and 4% to 8% during pregnancy (5), and is an increased risk for sexually transmitted diseases (5).

The causative agents of AV include *Enterococcus faecalis*, *Esherichia coli*, group B streptococcus and *Staphylococcus aureus*. The most frequently isolated AV pathogen is *Enterococcus faecalis* in about 31% of cases (6). These AV cause discomfort in women and can have serious repercussions, particularly in pregnant women. Thus, in order to improve the care of women suffering from AV, the present study was carried out with the aim of identify the factors associated with AV in women and evaluate the antibiotic resistance of bacteria responsible for this type of vaginitis.

Materials and method:

Study setting:

This study was carried out at the laboratory of the University Hospital of Befelatanana, Antananarivo, the capital city of Madagascar. This laboratory is versatile and performs haematological, immunological, biochemical and microbiological analyses on clinical samples.

Study design:

This was a descriptive cross-sectional study of 840 patients and analysis of the results of their cytobacteriological examinations of cervicovaginal samples from Jan 01, 2020 to December 31, 2022 at the Centre Hospitalier Universitaire Joseph Raseta Befelatanana (CHUJRB) laboratory.

Study participants:

All women with results of cytobacteriological examinations of cervicovaginal samples during the study period were included in the study. The participants were out-patients or hospitalized patients with various genito-urinary symptoms such as leukorrhea, dyspareunia, lower back pain or patients who came for routine health checks. Women with polymicrobial infections were excluded from the study.

Ethical consideration:

The study was smoothly incorporated into the standard care delivery framework and did not interfere with patient treatment. Given its alignment with routine healthcare procedures and the absence of any negative impact on patient management, no evaluation by the Research Ethics Committee was required in accordance with national regulations of Madagascar. However, patient anonymity and confidentiality were maintained.

Specimen collection and laboratory analysis:

Two sterile swabs were routinely used to collect specimens from the endocervix and ectocervix/vagina of each woman participant.

A disposable speculum was used for the cervicovaginal smear collection except for participants who were virgin, in whom simple only vulvar swabs were collected. The specimens were collected by a doctor, midwife or laboratory technician.

In the laboratory, microbiological culture was done on 2 types of agar plates. The endocervical swab was plated on heated blood agar and incubated at 37°C for 48 hours in an atmosphere rich in CO_2 for isolation of *Neisseria gonorrheae*. The ectocervical swab was plated an ordinary chromogenic agar (Uriselect®) and incubated at 37°C for 24 hours for the detection of vaginal candidiasis and other uropathogens.

Smears of colonies from the culture plates were made on the slide with physiological serum and covered with cover slide for microscopic examination, and also for Gram staining microscopic examination, which allowed for visualization and counting of the vaginal flora and establishment of the Nugent score to classify the vaginal flora of each participant

After 48 hours of incubation, the colonies which appeared on the culture media were identified using identification tests including catalase test (Gilbert®), oxidase test (Oxoid®), API®20 Strep (BioMérieux®) and API® 20 NE (BioMérieux®). Antibiotic susceptibility test was carried out on the isolates by the disc diffusion method on Mueller-Hinton agar and interpreted according to the recommendations of the "Comité de l'Antibiogramme de la Société Française de Microbiologie" (7). The antibiotics (Oxoid®, UK) tested include penicillin G (1U), ampicillin (2µg), cefixime (5µg), cefepime (30µg), cefoxitin (30µg), imipenem (10µg), gentamicin (10µg), tobramycin (10µg), amikacin (30µg), ciprofloxacin (5µg), levofloxacin (5µg), norfloxacin (10µg), cotrimoxazole (1.25/23.75µg), erythromycin (15µg), clindamycin (2µg), tetracycline (30 µg) and vancomycin (30µg).

Data collection and statistical analysis:

Data were collected from microbiological analysis request form, laboratory register notebooks and antibiogram result sheets. Data entry and analysis were done using EPI INFO 3.5.2 software. Association of dependent variables (results of vaginitis) with independent variables (age, hospitalization, pregnancy and antibiogram results) was determined using Pearson Chi square test. The threshold of statistical significance of the results chosen was $p<0.05$.

Results:

Among the 840 patients who participated in the study, 35 had aerobic vaginitis, giving the prevalence of AV of 4.2% (Fig 1). Enterococcal vaginitis was the most common, representing 48.6% of all AV cases (n=17) (Fig 2). Regarding associated factors, there was no significant difference in the prevalence of AV between women under age of 40 (4.4%, 29/653) and women over age of 40 years (3.2%, 6/187) (p=0.539); hospitalized (6.6%, 10/152) and non-hospitalized women (3.6%, 25/688) (p=0.115); and pregnant (4.2%, 8/192) and non-pregnant women (4.2%, 27/648) (p=1.000) (Table 1).

The antibiotic resistance varies from 0% (vancomycin) to 90% (penicillin G) for the Gram-positive bacteria (Fig 3). The 2 isolates of *Pseudomonas* spp., were sensitive to imipenem and amikacin, but resistant to co-trimoxazole, ciprofloxacin and cefixime. One of the 2 *Pseudomonas* isolates was resistant to tobramycin, gentamicin, levofloxacin and cefepime.

Table 1: Factors associated with aerobic vaginitis in women seen at the laboratory of the university hospital of Befelatanana, Antananarivo, Madagascar

Associated factors	Parameters	Aerobic vaginitis (n=35)		Non-aerobic vaginitis & others (n=805)		Total (n=840)	OR (95% CI)	*p* value
		n	%	n	%			
Age (years)	<40	29	4.4	624	95.6	653	1.40 (0.57-3.43)	0.539
	≥40	6	3.2	181	96.8	187		
Status of women	Non-pregnant	27	4.2	621	95.8	648	1.00 (0.45-2.24)	1.000
	Pregnant	8	4.2	184	95.8	192		
Department	Out-patient	25	3.6	663	96.4	688	0.54 (0.25-1.14)	0.115
	In-patient	10	6.6	142	93.4	152		

OR=Odds Ratio; CI=Confidence Interval; n=number

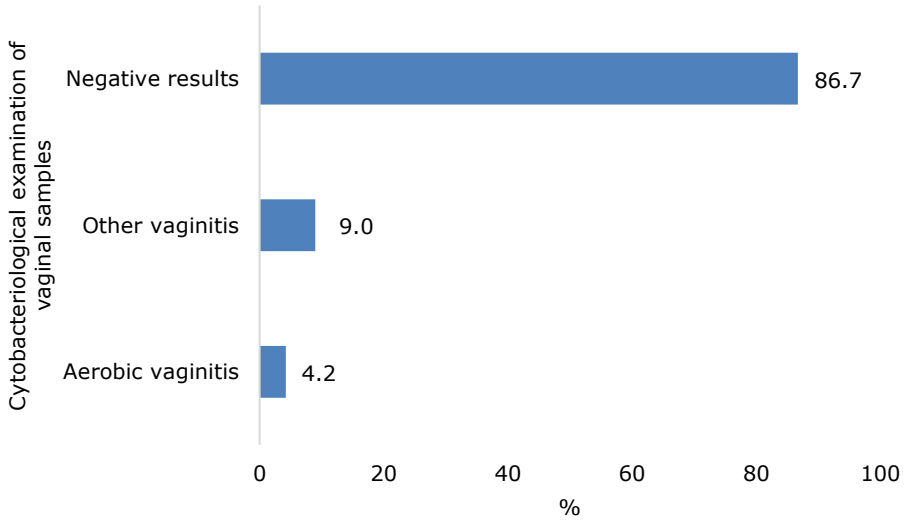

Fig 1: Prevalence of aerobic vaginitis among the study participants

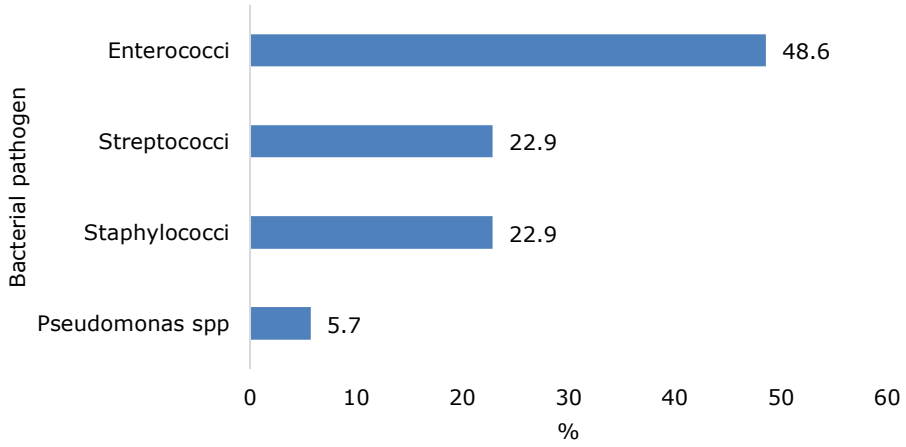

Fig 2: Bacteria responsible for aerobic vaginitis among the study participants

Discussion:

In the present study, the prevalence of AV was 4.2%. Other studies also found low prevalence of <10.0% (8,9). In the literature, bacterial vaginosis (BV) and vaginal candidiasis (VC) are the most predominant among vaginitis. Bacterial vaginosis is considered the most common form of vaginitis and affects approximately 30.0% of women (10). Because most women who have AV exhibit no or minor symptoms, there is a tendency to overlook this condition. Similarly, women who harbor *Candida* organisms in their vaginas have a spectrum of manifestations ranging from asymptomatic colonization to severe acute symptomatic infection. Yeast colonization occurs relatively frequently, with up to 30.0% of healthy asymptomatic women having a positive culture for yeast at any single point of time and up to 70.0% if followed longitudinally over a 1-year period (10). Although rare, AV should not be underestimated because they can cause serious complications if not treated quickly.

In the present study, enterococcal vaginitis was the most common, representing 48.6% of cases AV. Other studies have also reported the similar results (6). The pathogenic effect of *Enterococcus faecalis* shows that it is the cause of spontaneous abortion, premature birth, puerperal sepsis, abscess and infection of the urinary system (11). New reports indicate the presence of HPV 16 gene and genome in *E. faecalis* in biopsied materials from cervical cancer, as well as the ability that HPV 16 genes can be translated and transcribed in these bacteria. The fact that HPV gene can form viral particles in these bacteria leads to certain connection that can be a risk factor in the progression of cervical lesions to cancer (12). *Enterococcus faecalis* as a cause of AV is very often unrecognized or ignored and may be the reason for neglected

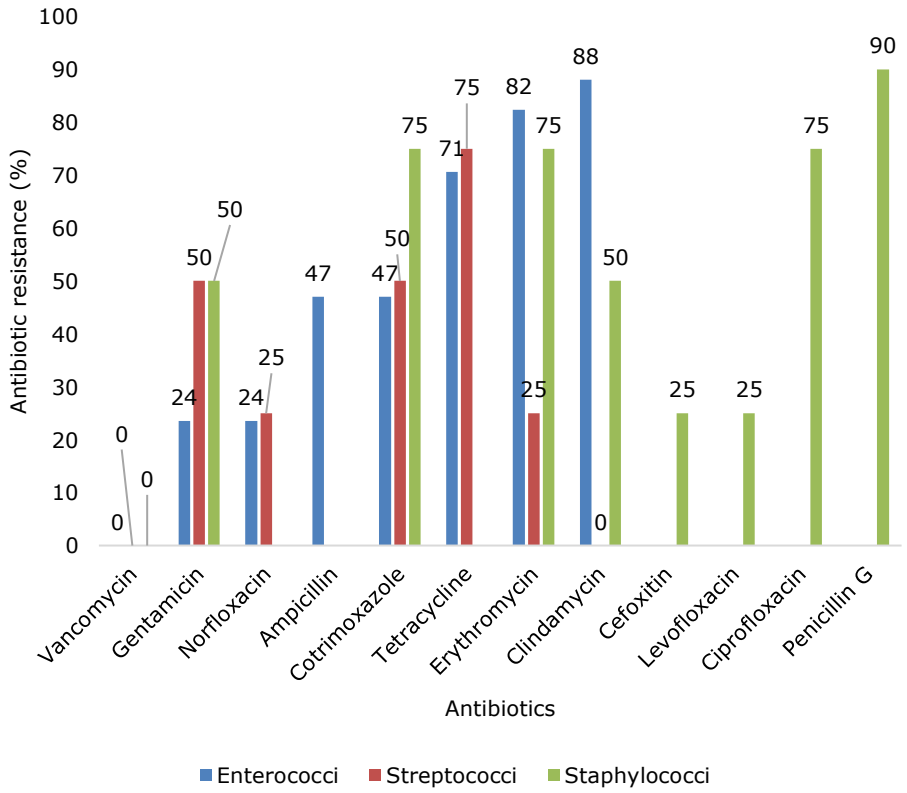

Fig 3: Antibiotic resistance of Gram-positive bacteria responsible for aerobic vaginitis

diagnosis. If this AV is not diagnosed or neglected in HPV-positive women, there is a justified possibility of long-term infection due to the presence of HPV in *E. faecalis*. The presence of HPV in this bacterium can lead to persistent HPV infection and the occurrence of high-grade cervical intraepithelial neoplasia (CIN) as well as progression to cervical cancer (12,13). Thus, in the event of genito-urinary disorders, cytobacteriological examination of the cervicovaginal smear is required in order to quickly diagnose possible vaginitis and treat the patient in time to avoid complications.

With respect to the associated factors analysed, there was no significant association between participants age groups, origin or patient status with prevalence of AV in our study. We can say that aerobic bacteria are present in the community and in hospital environment and can affect all patients regardless of their age. Thus, intimate hygiene is very important and the young individuals should be should be educated about this. However, according to a study carried out in pregnant women, cervical shortening is a risk factor for AV (14). Similarly, the literature confirms that the nosocomial pathogens present in hospitals can contaminate women during invasive healthcare procedures, which may explain the high frequency of AV among hospitalized women (15). The small number of AV cases in our study may be responsible for not obtaining factors significantly associated with AV. A larger sample size or multicenter study will be required to determine the factors significantly associated with AV in women

Regarding antibiotic resistance, Gram positive cocci (GPC) were highly resistant to penicillin and clindamycin and Gram-negative bacilli (GNB) to cotrimoxazole, ciprofloxacin and cefixime. This result is not at all surprising because these antibiotics are easily purchased in pharmacies without the need for a medical prescription in Madagascar. These antibiotics are also sold in small grocery stores, thus, these antibiotics are consumed indiscriminately by the populace because of easy access and poor regulations. Studies in the literature have reported that self-medication by the populace is one of the major factors for the emergence of antibiotic resistance (16). Fortunately, no resistance of GPC to vancomycin was detected in our study. Indeed, vancomycin is a reserved antibiotic for the treatment for multidrug-resistant GPC (17). Furthermore, none of the *Pseudomonas* spp was resistant to amikacin and imipenem. It has been reported in the literature that imipenem represents the 'reserved' drug for treatment of infections caused by multidrug-resistant *Pseudomonas* spp (18). Similarly, amikacin is a broad-spectrum antibiotic that is effective against MDR GNB such as *Pseudomonas* spp and *Acinetobacter* spp (19).

Conclusion:

The present study showed that AV is frequently caused by enterococci and affects young individuals and hospitalized patients in the majority of cases. Enterococci infections in pregnancy may be associated with complications and the pathogen may also promote the development of cervical cancer along with high-risk HPV. Therefore, cytobacteriological examination of cervicovaginal smears must be systematic in cases of genital disorders to improve the management of patients with AV. Similarly, empirical treatments should be well guided and self-medication avoided in order to limit the emergence of MDR bacteria.

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Contributions of authors:

RZD is involved in the study design, data collection, and writing of the manuscript; RII was involved in methodological designs, statistical analysis and presentation of results; RSS was involved in bibliographic search and writing the discussions; RAL was involved in writing the discussions; and RA was involved in correcting and editing the manuscript. All authors approved the final manuscript.

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Conflict of interest:

No conflict of interest is declared

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Short Communication **Open Access**

HIV status of individuals who underwent pre-employment medical screening at a federal tertiary health institution in southeast Nigeria

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Abstract:

Background: The human immunodeficiency virus (HIV) targets the host immune system, particularly the CD4 T cells. The host resistance to opportunistic and non-opportunistic infections such as tuberculosis, fungal infections, severe bacterial infections, and several malignancies is weakened as a result of destruction of these CD4 cells by HIV. The purpose of this study was to determine the prevalence of HIV among individuals who participated in pre-employment medical screening at David Umahi Federal University Teaching Hospital Uburu, Ebonyi State, Nigeria, with the aim of connecting those who are HIV-positive to voluntary counseling and treatment programs.
Methodology: This was a retrospective analysis of the medical records of 537 eligible participants who underwent pre-employment medical screening exercise, and whose blood samples were tested for presence of HIV antibodies at the University Teaching Hospital, using the Determine HIV-1/2 (T1) and Unigold HIV-1/2 (T2), and the tie breaker Statpak HIV-1/2 (T3) tests. The serological results were interpreted according to the national HIV testing algorithm, with test result declared negative for HIV antibodies if T1 was negative or if only T1 was positive but T2 and T3 were both negative.
Results: Of the total record of 756 pre-employment participants for the medical screening exercise, only 537 met the inclusion criteria for the study. The mean age of the 537 participants was 34.2±6.9 and age range of 18-67 years; 325 (61.0%) were females while 212 (39.0%) were males. The seroprevalence of HIV among the study participants was 2.4% (13/537), with 1.4% (3/212) in the males and 3.1% (10/325) in the females (x^2=0.879, OR=0.45; 95% CI=0.12-1.60, p=0.3485). Only participants in the age range 26–35 and 36–45 years were HIV seropositive, with prevalence of 2.9% (9/310) and 2.4% (4/169) respectively but the HIV seroprevalence was not significantly associated with age and gender of the participants (p>0.05).
Conclusion: The study findings provide useful information for the hospital administration of the HIV situation of its planned workforce, which will help with decisions on HIV positive participants to enrol in antiretroviral therapy program.

Keywords: HIV, Prevalence, University Teaching Hospital, Pre-employment, Screening.

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Statut VIH des personnes ayant subi un examen médical préalable à l'emploi dans un établissement fédéral de santé tertiaire du sud-est du Nigeria

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Résumé:

Contexte: Le virus de l'immunodéficience humaine (VIH) cible le système immunitaire de l'hôte, en particulier les lymphocytes T CD4. La résistance de l'hôte aux infections opportunistes et non opportunistes telles que la tuberculose, les infections fongiques, les infections bactériennes graves et plusieurs tumeurs malignes est affaiblie en raison de la destruction de ces cellules CD4 par le VIH. Le but de cette étude était de déterminer la prévalence du VIH chez les personnes ayant participé à un dépistage médical préalable à l'emploi à l'hôpital universitaire fédéral David Umahi d'Uburu, dans l'État d'Ebonyi, au Nigeria, dans le but de connecter les personnes séropositives à des conseils volontaires et des programmes de traitement.
Méthodologie: Il s'agissait d'une analyse rétrospective des dossiers médicaux de 537 participants éligibles qui ont subi un exercice de dépistage médical préalable à l'emploi et dont les échantillons de sang ont été testés pour la présence d'anticorps anti-VIH à l'hôpital universitaire, à l'aide du système de détermination du VIH-1/2 (T1) et Unigold HIV-1/2 (T2), ainsi que les tests de départage Statpak HIV-1/2 (T3). Les résultats sérologiques ont été interprétés selon l'algorithme national de dépistage du VIH, le résultat du test étant déclaré négatif pour les anticorps anti-VIH si T1 était négatif ou si seul T1 était positif mais que T2 et T3 étaient tous deux négatifs.
Résultats: Sur le total de 756 participants préalables à l'emploi pour l'exercice de sélection médicale, seuls 537 répondaient aux critères d'inclusion de l'étude. L'âge moyen des 537 participants était de 34,2±6,9 ans et la tranche d'âge était de 18 à 67 ans; 325 (61,0%) étaient des femmes tandis que 212 (39,0%) étaient des hommes. La séroprévalence du VIH parmi les participants à l'étude était de 2,4% (13/537), dont 1,4% (3/212) chez les hommes et 3,1% (10/325) chez les femmes (x^2=0,879; OR=0,45; 95% IC=0,12-1,60; p=0,3485). Seuls les participants âgés de 26 à 35 ans et de 36 à 45 ans étaient séropositifs au VIH, avec une prévalence de 2,9% (9/310) et 2,4% (4/169) respectivement, mais la séroprévalence du VIH n'était pas significativement associée à l'âge et au sexe des personnes les participants (p>0,05).
Conclusion: Les résultats de l'étude fournissent des informations utiles à l'administration hospitalière sur la situation VIH de sa main-d'œuvre prévue, ce qui aidera à prendre des décisions concernant les participants séropositifs à s'inscrire à un programme de thérapie antirétrovirale.

Mots clés: VIH, Prévalence, Hôpital Universitaire, Pré-emploi, Dépistage

Introduction:

The dreaded acquired immunodeficiency syndrome (AIDS) was first identified as being caused by the human immunodeficiency virus (HIV) on June 5, 1981, when the Center for Disease Control (CDC) reported five cases of *Pneumocystis carinii* pneumonia in active homosexual males from three different hospitals in Los Angeles, California (1). Two strains of HIV virus (HIV-1 and HIV-2) infect humans. While HIV-1 is present in every country, West Africa is mostly the only place where HIV-2 is found. HIV-1 is more easily transmissible than HIV-2 and causes AIDS more quickly after infection (2).

The human immune cells are susceptible to HIV infection, especially the $CD4^+$ T-cell subtype of white blood cells that supports the ability of the body to recognize and eliminate unwanted pathogens such as bacteria, viruses, parasites, and even some cancer cells. HIV infection gradually destroys $CD4^+$ cells, impairing immune function and increasing the risk of infections, serious disease, and death (2). These infections are often referred to as "opportunistic infections" because they are uncommon or moderate in healthy individuals but can take advantage of the opportunity offered by an immune system that has been compromised by HIV and can be fatal.

HIV can also directly harm the tissues, which can result in heart, neurologic and other diseases of the body. The number of $CD4^+$ T-cells in one microliter of blood is measured by the $CD4^+$ cell count, often known as the T-cell count. The $CD4^+$ count is used by HIV-positive individuals and their medical professionals to track the progression of the HIV infection (2). According to the National Library of Medicine (3), AIDS is characterized by an increased susceptibility to opportunistic infections and a $CD4^+$ T-cell count of fewer than 200 cells/μL. A key factor in the pathogenesis of HIV is the gradual depletion of $CD4^+$ T-cells (4).

One of the biggest public health issues in the world today is HIV/AIDS. Only 28.2 million individuals have access to antiretroviral therapy as of June 2021, while in 2020, there were approximately 37.7 million HIV-positive persons living around the world, 680,000 AIDS-related deaths, and 1.5 million new HIV infections (5). In sub-Saharan Africa (SSA), which is home to 71% of all people living with HIV (PLHIV), it is the main cause of illness and mortality (6,7).

The first case of HIV/AIDS on the African continent was documented in Uganda, East Africa in 1982 (8). The first case of HIV/AIDS in Nigeria was identified and reported in

Lagos, Nigeria in 1985 (9). The first two cases reported by the Federal Ministry of Health were a 13-year-old sexually active child and a female commercial sex worker from a neighboring West African country (1,10). Nigeria, the most populous nation in Africa, has the third-highest HIV burden and the highest number of orphans due to AIDS in sub-Saharan Africa (11-14). According to Mitsunaga et al., (15), heterosexual transmission is the main method of transmission for the AIDS epidemic in Nigeria. Nigeria has 1.9 million people living with HIV (PLHIV) between the ages of 15 and 49 years, with prevalence of 1.4%. According to state-level stratification (16), the highest prevalence rates are found in Akwa Ibom (5.6%), Benue (4.9%), Rivers (3.8%), Taraba (2.7%), and Anambra (2.7%) States, and the lowest prevalence rates are found in Jigawa (0.3%) and Katsina (0.3%) States. Other states in the southeast are Ebonyi (0.8%), Abia (2.1%), Enugu (2.1%), and Imo (1.6%).

HIV testing services (HTS) are acknowledged as crucial entry point for attaining epidemic control and quickly accomplishing the aim of HIV care cascade (17). Globally, HIV diagnosis is frequently performed using algorithms based on HIV rapid tests (RTs). In general, RTs have emerged as the preferred method for HIV diagnosis in low-and-middle-income-countries (LMICS) due to the performance characteristics of HIV RT and the added operational benefits, such as ease of use and interpretation, storage at ambient temperature, and no special laboratory equipment requirements (18).

In 2015, Determine HIV-1/2, Unigold HIV-1/2, and Statpak HIV-1/2 (as a tie-breaker test) were chosen as part of a serial testing methodology that was reviewed nationally for usage in Nigeria (19). Clients who test positive for HIV can know their status and start receiving the right care and treatment right away by bringing testing closer to communities, rather than just the laboratories. Three successive reactive tests should be utilized for HIV diagnosis, and they must be evaluated in the context of HIV prevalence because the overall positive predictive value (PPV) in low prevalence settings is anticipated to be low (20,21).

Antiretroviral therapy (ART) has continued to be the only treatment available in the absence of a cure that has the potential to significantly reduce morbidity and mortality associated with HIV/AIDS while enhancing the situation of people living with HIV (22). It has been successful in lowering viral load, boosting immune system performance, and enhancing the quality of life (QOL) of PLHIV (23,24). Due to the convergent AIDS and COVID-19 pandemics, as well as the economic and humanitarian crises, the global HIV response has become increasingly in jeopardy during the past two and half years. Health services have been affected by a number of worldwide instabilities, including COVID-19, and the absence of millions of students has increased their HIV vulnerability (25).

There are some positive indicators, such as notable declines in the number of new HIV infections per year in the Caribbean, Central and Western Africa, and the latter mostly as a result of developments in Nigeria (26). In an effort to support the fight against HIV and AIDS, this study sought to determine the prevalence of HIV infection among people who attended pre-employment medical screening at David Umahi Federal University Teaching Hospital, Uburu, Ohaozara, Local Government Area, Ebonyi State, South East, Nigeria, with the aim of directing HIV-infected persons to appropriate counselling and treatment program.

Materials and method:

Study setting:

The study was carried out at the David Umahi Federal University Teaching Hospital Uburu in Ohaozara Local Government Area (LGA) of Ebonyi State, Nigeria. The LGA has Obiozara Uburu as its administrative center and other towns in the LGA including Ugwulangwu and Okposi. The LGA has a combined area of 312 km^2 (120 sq mi) and 148,626 residents as of the 2006 census. The hospital is situated at these coordinates; Latitude: N 60 1' 53", Longitude: E 70 43' 10", and Altitude: 56.5m.

Ethical consideration:

As at the time of this study, there was no established Research and Ethics Committee in both the hospital and university as the institutions were very new and young, therefore approval for the study was given by the Chairman, Medical Advisory Committee of the hospital who was in charge of Clinical services.

Study design and participants:

This was a retrospective review of prospective staff who presented for pre-employment screening in David Umahi Federal Teaching Hospital, Uburu, Ohaozara, LGA, Ebonyi State, southeast Nigeria from November 2022 to March 2023. Prospective staff who were being considered for full time, part time and locum appointments in the teaching hospital with properly filled laboratory request forms were included while those whose laboratory request forms were not properly filled or lacked information such as age and sex were excluded from the study.

Data collection:

Data were collected from the record book of the Pathology Department of David Umahi Federal University Teaching Hospital, Uburu between November 2022 and March 2023.

HIV testing algorithm:

According to the laboratory record, specimens from participants between the ages of 16 and 67 years were tested for HIV using the Nigeria national HIV RT algorithm, which consists of Determine HIV-1/2 [Test 1 (T1)] (Abbott, California, USA) followed by Unigold HIV-1/2 (T2) (Trinity Biotech Plc., Ireland) if T1 was reactive. If the T1 and T2 results were inconclusive, a tie-breaker test called Statpak HIV-1/2 (T3) (Chembio Diagnostic Systems, Inc., New York, USA) was utilized. The test result was declared negative for HIV antibodies if T1 was non-reactive or if only T1 was reactive but T2 and T3 were both negative (18).

Statistical analysis:

Data were analyzed using Statistical Package for Social Sciences (SPSS) version 25.0. Chi-square test was used to determine the significance of association between HIV status and age group. P value < 0.05 depicts significant association.

Results:

Sociodemographic characteristics of the study participants:

A total of 756 individuals took part in the pre-employment medical examination but only 537 people matched the inclusion criteria. The mean age of the participants is 34.2±6.9 and age range of 18-67 years, with 325 females (60.5 %) and 212 males (39.5%) (Table 1).

The age group 26-35 and 36-45 years constituted the largest proportion of the participants, with 57.7% (310/537) and 31.5% (169/537) respectively, while the age groups 66-75 and 56-65 years constituted the least proportion with 0.2% (1/537) and 1.1% (6/537) respectively.

Seroprevalence of HIV among the study participants:

The overall HIV seroprevalence rate is 2.4% (13/537), with 1.4% (3/212) in the males and 3.1% (10/325) in the females [x^2= 0.879, OR=0.45 (95% CI=0.12-1.60), p= 0.3485] (Table 2). Only participants in the age range 26–35 and 36–45 years were HIV seropositive, with prevalence of 2.9% (9/310) and 2.4% (4/169) respectively but the HIV seroprevalence was not significantly associated with age and gender of the participants (p> 0.05) (Table 2).

Table 1: Gender and age-group distribution of selected staff who participated in the HIV screening exercise

Age group (years)	Gender		Total (%)
	No. of male (%)	No. of female (%)	
16-25	7 (1.3)	23 (4.3)	30 (5.6)
26-35	117 (21.8)	193 (35.9)	310 (57.7)
36-45	72 (13.4)	97 (18.1)	169 (31.5)
46-55	12 (2.2)	9 (1.7)	21 (3.9)
56-65	4 (0.7)	2 (0.4)	6 (1.1)
66-75	0	1 (0.2)	1 (0.2)
Total	212 (39.5)	325 (60.5)	537 (100.0)

Table 2: Association of HIV seropositivity with age group and gender of the study participants

Age group (years)	HIV seropositivity by gender			x^2	OR (95% CI)	*p*-value
	Males (%)	Females (%)	Total (%)			
16-25	0	0	0			
26-35	2 (1.7)	7 (3.6)	9 (2.9)	0.3917	0.46 (0.09-2.26)	0.5314
36-45	1 (1.4)	3 (3.1)	4 (2.4)	0.04364	0.44 (0.05-4.34)	0.8345
46-55	0	0	0			
56-65	0	0	0			
66-75	0	0	0			
Total	**3 (1.4)**	**10 (3.1)**	**13 (2.4)**	**0.8790**	**0.45 (0.12-1.66)**	**0.3485**

x^2=Chi square; OR=Odds ratio; CI=Confidence interval

Discussion:

The goal of this study was to determine the sero-prevalence of HIV among prospective employees of David Umahi Federal University Teaching Hospital (DUFUTH), Uburu, who showed up for pre-employment medical screening in the hospital. The average age of the participants was 34.20±6.9 years. A total of 13 HIV positive cases were detected yielding a seroprevalence of 2.4% (3.1% in females and 1.4% in males). Compared to the newly admitted students at Ebonyi State University in Abakaliki, southeast Nigeria, this prevalence is higher than reported by Nworie et al., (27). This might be because the students were younger, have recently graduated from secondary schools, and have been living under the relatively close supervision of their parents or guardians, who might have shielded them from several risk factors including sexual activity.

The seroprevalence in our study is also higher than the national prevalence of 1.4% in Nigeria and the prevalence of 0.8% in Ebonyi State (28). This discrepancy may be due to the fact that majority of the study population were more youthful and sexually active than the previous study, which included a wider range of populations and age brackets. All the 13 HIV positive cases were detected in the 26-35 and 36-45-years age groups, with prevalence of 2.9% and 2.4% respectively, which are higher (though not statistically significant) in the females (with 3.6% and 3.1%) than in the males (with 1.7% and 1.4%) for the respective age groups. The higher prevalence in the females might be explained by the fact that females within this age groups frequently travel across state lines and briefly stay somewhere other than their homes, which raises the possibility that they can engage in risky sexual behaviors (29).

We noted that the seroprevalence of HIV among females (3.1%) was higher (though not statistically significant) compared to their male counterparts (1.4%). Similar report was made by NAIIS (28) in which a prevalence of 0.9% and 0.7% respectively were reported among female and male adults of age 15-64 years in Ebonyi State, Nigeria. Our finding is also corroborated by previous reports (27,30). The higher prevalence among females has been attributed to a number of factors, such as their vulnerability, which is frequently linked to low socioeconomic status, shyness toward sex education, and abhorrence of extramarital sex, which results in denying young girls access to sexual health and HIV services (27). To stem the tide of HIV spread in Nigeria, it has become imperative to deliberately improve on case finding through sensitization and testing of the general population especially the youths who are at risk of contracting HIV.

Conclusion:

The HIV seroprevalence of 2.4% in our study is higher than the national average rate. In view of this, HIV testing services should be increased in Ohaozara Local Government Area of Ebonyi State and infected persons should be immediately referred for treatment so as to suppress their viral load to levels that cannot be transmitted to others and as well prevent them from progressing to AIDS.

It is recommended that routine HIV screening should be done before employment. Subsequently, staff should be encouraged to check their HIV status and those with positive results should be enrolled into the HIV care. Moreover, HIV awareness and education should be a part of the routine hospital function.

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Contribution of authors:

EFE was involved in the study design, supervision and manuscript editing; AUM was involved in supervision and manuscript editing; EGO was involved in data collection, analysis and manuscript writing; OSE was involved in the study design and implementation; CCG was involved in data collection and analysis; CAN was involved in the study design and manuscript editing; and NMA was involved in the study design and manuscript editing. All authors approved the final manuscript submitted for publication.

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