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July 2025; Volume 26: Number 3: Pages 200-306

TABLE OF CONTENTS

EDITORIAL:

REVIEW ARTICLES:

ORIGINAL ARTICLES:

ISSN 1595-689X

SHORT COMMUNICATION:

Afr. J. Clin. Exper. Microbiol. 2025; 26 (3): i - iii https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jul 2025; Vol.26 No.3
AJCEM/2402. https://www.ajol.info/index.php/ajcem

Editorial **Open Access**

Ending nuclear weapons, before they end us

This May, the World Health Assembly (WHA) will vote on re-establishing a mandate for the World Health Organization (WHO) to address the health consequences of nuclear weapons and war (1). Health professionals and their associations should urge their governments to support such a mandate and support the new UN comprehensive study on the effects of nuclear war. The first atomic bomb exploded in the New Mexico desert 80 years ago, in July 1945. Three weeks later, two relatively small (by today's standards), tactical-size nuclear weapons unleashed a cataclysm of radioactive incineration on Hiroshima and Nagasaki. By the end of 1945, about 213,000 people were dead (2). Tens of thousands more have died from late effects of the bombings.

Last December, Nihon Hidankyo, a movement that brings together atomic bomb survivors, was awarded the Nobel Peace Prize for its "efforts to achieve a world free of nuclear weapons and for demonstrating through witness testimony that nuclear weapons must never be used again" (3). For the Norwegian Nobel Committee, the award validated the most fundamental human right: the right to live. The Committee warned that the menace of nuclear weapons is now more urgent than ever before. In the words of Committee Chair Jørgen Watne Frydnes, "it is naive to believe our civilisation can survive a world order in which global security depends on nuclear weapons. The world is not meant to be a prison in which we await collective annihilation." (4) He noted that our survival depended on keeping intact the "nuclear taboo" (which stigmatises the use of nuclear weapons as morally unacceptable) (5).

The nuclear taboo gains strength from recognition of compelling evidence of the catastrophic humanitarian consequences of nuclear war, its severe global climatic and famine consequences, and the impossibility of any effective humanitarian response. This evidence contributed significantly to ending the Cold War nuclear arms race (6,7). While the numbers of nuclear weapons are down to 12,331 now, from their 1986 peak of 70,300 (8), this is still equivalent to 146,605 Hiroshima bombs (9), and does not mean humanity is any safer (10). Even a fraction of the current arsenal could decimate the biosphere in a severe mass extinction event. The global climate disruption caused by the smoke pouring from cities ignited by just 2% of the current arsenal could result in over two billion people starving (11).

A worldwide nuclear arms race is underway. Deployed nuclear weapons are increasing again, and China, India, North Korea, Pakistan, Russia and UK are all enlarging their arsenals. An estimated 2,100 nuclear warheads in France, Russia, UK, US and, for the first time, also in China, are on high alert, ready for launch within minutes (8). With disarmament in reverse, extensive nuclear modernisations underway, multiple arms control treaties abrogated without replacement, no disarmament negotiations in evidence, nuclear-armed Russia and Israel engaged in active wars involving repeated nuclear threats, Russia and the US deploying nuclear weapons to additional states, and widespread use of cyberwarfare, the risk of nuclear war is widely assessed to be greater than ever. This year the Doomsday Clock was moved the closest to midnight since the Clock's founding in 1947 (10).

Led by Ireland and New Zealand, in late 2024, the United Nations General Assembly (UNGA) voted overwhelmingly to establish a 21-member independent scientific panel to undertake a new comprehensive study on the effects of nuclear war (12), with its final report due in 2027. Noting that "removing the threat of a nuclear war is the most acute and urgent task of the present day", the panel has been tasked with examining the physical effects and societal consequences of a nuclear war on a local, regional and planetary scale. It will examine the climatic, environmental and radiological effects of nuclear war, and their impact on public health, global socioeconomic systems, agriculture and ecosystems.

The resolution calls upon UN agencies, including WHO, to support the panel's work, including by "contributing expertise, commissioned studies, data and papers". All UN Member States are encouraged to provide relevant information, scientific data and analyses; facilitate and host panel meetings, including regional meetings; and make budgetary or in-kind contributions. Such an authoritative international assessment of evidence on the most acute existential threat to humankind and planetary health is long overdue. The last such

report dates from 1989. It is shameful that France, UK and Russia opposed this resolution (13).

In 1983 and 1987 (14), the WHO convened an international committee of scientists and health experts to study the health effects of nuclear war. Its landmark, authoritative reports were influential and an excellent example of WHO fulfilling its constitutional mandate "to act as the directing and coordinating authority on international health work". In 1993, WHO produced an additional shorter report on the health and environmental effects of nuclear weapons, which included discussion of the production chain of nuclear weapons, including processing, testing and disposal (15). However, despite WHA having mandated WHO to report periodically on relevant developments, no further work was undertaken and in 2020 WHO's mandate on nuclear weapons and health lapsed.

The Marshall Islands, Samoa and Vanuatu, supported by seven co-sponsoring States and International Physicians for the Prevention of Nuclear War (IPPNW), are working to renew WHO's mandate. They are seeking wide support for a resolution on the health effects of nuclear weapons/war at this year's WHA in Geneva on 19-27 May (1). WHO would then re-establish a programme of work on this most critical threat to health, and be able to lead strongly in providing the best health evidence to the UN panel.

Health professionals are well aware how crucial accurate and up-to-date evidence is to making good decisions. We and our organisations should support such a renewed mandate by urging our national WHA delegates to vote in support and commit the modest funds needed to re-establish WHO's work programme, especially now, as the organisation faces severe financial strain with the US decision to withdraw its membership.

Our joint editorial in 2023 (16) on reducing the risks of nuclear war and the role of health professionals, published in over 150 health journals worldwide, urged three immediate steps by nuclear-armed states and their allies: adopt a "no first use" policy, take their nuclear weapons off hair-trigger alert, and pledge unequivocally that they will not use nuclear weapons in any current conflicts they are involved in. We also urged nuclear-armed states to work for a definitive end to the nuclear threat by urgently starting negotiations for a verifiable, timebound agreement to eliminate their nuclear arsenals, and called on all nations to join the Treaty on the Prohibition of Nuclear Weapons (17).

It is an alarming failure of leadership that no progress has been made on these needed measures, nor on many other feasible steps away from the brink, acting on the obligation of all states to achieve nuclear disarmament. Nine States jeopardise all humanity and the biosphere by claiming an exclusive right to wield the most destructive and inhumane weapons ever created. The world desperately needs the leaders of these states to freeze their arsenals, end the modernisation and development of new, more dangerous nuclear weapons, and ensure that new technology such as artificial intelligence can never trigger the launch of nuclear weapons.

The UN scientific panel and a renewed mandate for WHO's work in this area can provide vital authoritative and up-to-date evidence for health and public education, evidence-based advocacy and policies, and the mobilised public concern needed to trigger decisive political leadership. This is a core health imperative for all of us.

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Review Article **Open Access**

Recent update on the mechanism and epidemiology of metallo-β-lactamases in nosocomial infections: A mini review

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Abstract:

Metallo-β-lactamases are different types of biological enzymes that breakdown a broad spectrum of beta-lactam antibiotics such as carbapenems. Antibiotic resistance exhibited by many Gram-negative bacteria is due to acquisition of genes encoding these enzymes, which underscores their importance in antibiotic resistance and has led to an increase in nosocomial infections caused by multi-drug-resistant pathogens. This review analyzes recent updates on the mechanism of antibiotic resistance and epidemiology of metallo-β- lactamases in nosocomial infections. Carbapenem antibiotics are regarded as effective and reserve antibiotics for the treatment of multidrug-resistant bacteria infections. Currently, there is paucity of inhibitors that prevent the activities of metallo beta-lactamase clinically. Carbapenem resistance in bacteria is a global health issue, associated with increased morbidity and mortality in the healthcare setting. There is a need for continuous research on metallo-β-lactamases inhibitors, rational use of carbapenems and appropriate infection control measures in order to curb the increase in infections. This mini-review is aimed to provide a broader scope on the current trends of antimicrobial resistance in metallo-β-lactamase-associated nosocomial infections. This will lead to a better understanding of the public health implication of the unabated spread of these infections, the need for judicious utilization of the limited therapeutic options, resulting to efficient antimicrobial stewardship.

Keywords: Metallo-β-lactamases, β-lactam, Carbapenem, Nosocomial, Multidrug resistant

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Mise à jour récente sur le mécanisme et l'épidémiologie des métallo-β-lactamases dans les infections nosocomiales: une mini-synthèse

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Résumé:

Les métallo-β-lactamases sont différents types d'enzymes biologiques qui dégradent un large spectre d'antibiotiques bêta-lactamines tels que les carbapénèmes. La résistance aux antibiotiques de nombreuses bactéries à Gram négatif est due à l'acquisition de gènes codant ces enzymes, ce qui souligne leur importance dans la résistance aux antibiotiques et a entraîné une augmentation des infections nosocomiales causées par des agents pathogènes multirésistants. Cette revue analyse les dernières avancées sur le mécanisme de résistance aux antibiotiques et l'épidémiologie des métallo-β-lactamases dans les infections nosocomiales. Les antibiotiques carbapénèmes sont considérés comme efficaces et réservés au traitement des infections bactériennes multirésistantes. Actuellement, il existe une pénurie d'inhibiteurs inhibant cliniquement l'activité des métallo-β-lactamases. La résistance bactérienne aux carbapénèmes est un problème de santé mondial, associé à une augmentation de la morbidité et de la mortalité dans le milieu de soins. Il est nécessaire de poursuivre les recherches sur les inhibiteurs des métallo-β-lactamases, d'utiliser rationnellement les carbapénèmes et de mettre en place des mesures appropriées de contrôle des infections afin de freiner l'augmentation des infections. Cette mini-revue vise à élargir le champ d'analyse des tendances actuelles en

matière de résistance aux antimicrobiens dans les infections nosocomiales associées aux métallo-β-lactamases. Elle permettra de mieux comprendre les implications pour la santé publique de la propagation incontrôlée de ces infections, la nécessité d'une utilisation judicieuse des options thérapeutiques limitées et une gestion efficace des antimicrobiens.

Mots-clés: Métallo-β-lactamases, β-lactamines, carbapénèmes, nosocomiales, multirésistance

Introduction:

Nosocomial infections are infections acquired in the hospital and are now referred to as healthcare-associated infections (HAIs). At the time of patient admission, these infections are absent and occur when caring for patients. They may also occur after patient discharge from the hospital and are the main sources of infections among healthcare workers. Nosocomial infections are a threat to the safety of patients and have major socioeconomic implications (1). These infections have a prevalent rate of up to 40% in developing nations compared to 5% in developed countries (2). Healthcare-associated infections result in increased hospital stay, death, disability, and emergence of antimicrobial resistance (3).

Antibiotic resistance develops from acquisition of resistance gene or by plasmid transfer between same or different species of bacteria. A bacterium carrying many genes that express antibiotic resistance is referred to as multi-drug resistant (MDR) or informally 'superbug'. The World Health Organization (WHO) states that 15% and 7% of patients in developing and developed countries respectively will get at least one HAI during their stay in the hospital, and an average of 10% of these patients will die from their HAIs (4). In the USA, the Centre for Disease Control (CDC) reported that 1 in 31 patients in the hospital have at least one nosocomial infection (5). Antibiotics misuse has serious effect on public health, causing emergence of 'super bugs' that results in life-threatening MDR infections.

Antibiotic resistance and nosocomial infections have been known globally for several years, and nosocomial infections such as urinary tract infection, pneumonia and bacteraemia are mostly associated with *Escherichia coli*, *Staphylococcus* and *Pseudomonas* spp (6). There is a global rise in infections caused by MDR bacteria and a looming threat of untreatable infections since the inception of the 21st century (7). The WHO reported that approximately 1.27 million global deaths are due to bacterial antimicrobial resistance (AMR) and AMR contributed to 4.95 million deaths (8). The members of the family Enterobacteriaceae producing extended spectrum beta-lactamases (ESBL) are known as very important nosocomial pathogens in the world today and their mechanism of resistance is also globally spread (9,10).

The prevalence of the pathogens causing HAIs and their methods of antibiotic resistance should determine the therapeutic pattern in different hospitals. Antibiotic resistance is mostly exhibited in bacteria that have high virulence, depends on the history of antibiotic use and could be innate or acquired (11). In this review, the global rise of carbapenem resistance in bacteria due to metallo-beta-lactamases (MBLs) and their mechanism of resistance are discussed. The types of MBLs, the classes of antibiotic they act upon, epidemiology of these enzymes in HAIs and possible therapeutic options to curb increase in nosocomial infections are also reviewed.

β-lactam antibiotics:

Antimicrobial drugs such as β-lactam antibiotics are mostly used in hospitals and their increased incidence is a great concern in clinical setting. They have a wide range of activities against different forms of bacteria. They have unique qualities making them, the most broadly used antibiotics in the world (9). They act by preventing transpeptidase enzymes and these enzymes are mainly responsible in synthesis of the bacterial cell wall peptidoglycan layer (12). The action of β-lactam antibiotics is exerted by blocking the synthesis of the peptidoglycan layer with resultant death of the bacteria (13).

Classes of β-lactam antibiotics:

Different types of β-lactams exist, and their structure determines the class they belong. The clinically important β-lactams are penicillins, carbapenems, cephalosporins and monobactams. The β-lactam ring of penicillin and cephalosporin is joined to five- and six-member rings, respectively which has carboxyl group at the C3 and C4 positions. These antibiotics have a broad-spectrum of actions against many types of bacteria (17). Unlike the penicillins and cephalosporins, the monobactams are not made up of a fusing ring but have a sulfunic acid group linked to its β-lactam at similar sites of carboxylate group as in penicillins and cephalosporins (18), and they show activities against Gram-negative pathogens that are aerobic.

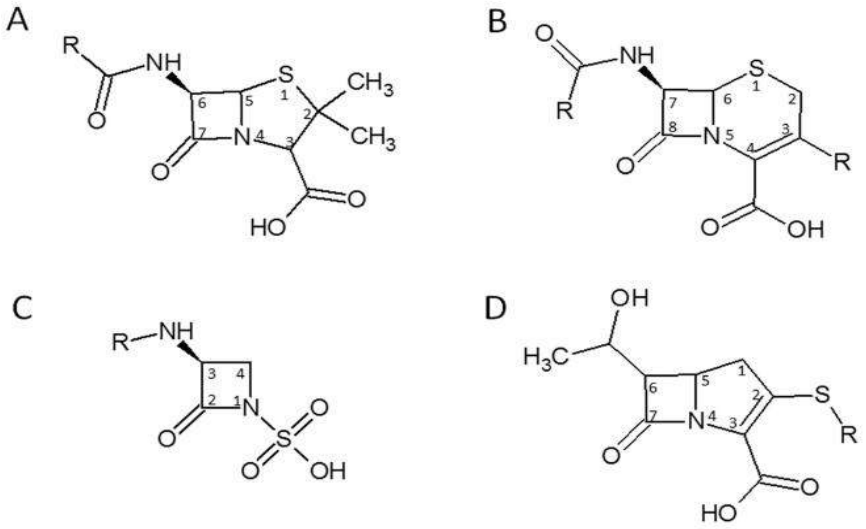

Fig 1: Classes of β-lactam antibiotics (19)

A - Basic penicillin structure; R - groups distinguish different types of penicillins; B - Core structure of cephalosporin; C-Basic structure of Monobactam; D - Carbapenem core structure; Atoms were numbered for reference in the discussion text

The carbapenems are very useful for the treatment of infections caused by bacteria that are MDR and they have a wide range of activities against these bacteria (15). The carbapenems are resistant to hydrolytic activities of β-lactamases and are an inhibitor of most β-lactamases. However, in the last ten years, few classes of β-lactamases are able to hydrolytically breakdown carbapenems and these are major sources of resistance (16). Fig 1 shows the classes of β-lactam antibiotics with the atoms numbered for reference (19).

Resistance to β-lactam antibiotics:

In other to withstand the antimicrobial effects of these antibiotics, bacteria developed some defensive strategies to combat the activities of antibiotics (14). The unguarded use of beta-lactam antibiotics has resulted to increased bacterial resistance thereby threatening antibiotic therapy (15). Although beta-lactam antibiotics have low toxicity and are frequently used antibiotic drugs, there are increasing resistance to these drugs (16). The subclass of the beta-lactamases determines the different enzyme processes which differ based on the number of zinc molecules bound to the enzyme active site.

The spread of genes that encode β-lactamases in Gram-negative bacteria made them a major key for resistance. One of the resistance strategies of Gram-negative bacteria against beta-lactam antibiotics is the production of metallo-β-lactamases (MBLs). Currently, MBLs cannot be prevented by any clin ically available inhibitors (14).

Mechanisms of β-lactam resistance:

The commonest method of antibiotic resistance in Gram-negative bacteria is the hydrolysis of beta-lactam ring by beta-lactamases. Other mechanisms include changes in porin channels, activities of efflux pumps leading to reduced permeability of the cell wall, by the actions of altered transpeptidases and β-lactamase inactivation of the antibiotics (15). In *Streptococcus pneumoniae*, enzymes bind loosely to β-lactam due to changes that occur in the sequences of target transpeptidases through mutation or recombination and these results to resistance. The transpeptidase in *Staphylococcus aureus*, aids in resistance by slowly binding to beta-lactam antibiotics (20).

i. β-lactamases:

Beta-lactamase breaks down the amide bond in beta-lactam ring and this results to products that are not effective, which is the basis of resistance in bacteria. The genes that express β-lactamases are mostly on the plasmids or other mobile genetic elements such as transposons and integrons of the bacteria. Basically, there are four structural classes of beta-lactamases; A, B, C and D (21). Classes A, C and D are serine-based enzymes and they activate the breakdown of β-lactam through a serine-bound acyl intermediate (22). Class B enzymes need zinc for its activities, although catalysis is not through covalent intermediate but by breakdown of hydroxide ion which the zinc in the active site stabilizes (23). Class A carbapenemases and class B MBLs are able to break down carbape-

nems and these are major sources of resistance (23).

ii. Class B Metallo beta-lactamases:

Metallo β-lactamases of class B β-lactamases have wide range of substrates and catalyzes the breakdown of most β-lactam antibiotics excluding monobactams. Their activities are not prevented by clavulanate, sulbactam, or tazobactam which are inhibitors to serine-based beta-lactamases (24). Unlike the serine-based enzymes, MBLs are destroyed by metal chelator like ethylene diamine tetra-acetic acid (EDTA) (24). Metallo-β-lactamases have been in existence for more than forty years and are encoded in the chromosome and were also found in organisms that are not pathogenic, and therefore did not pose challenge for antibiotic therapy (25). But in the 1990s there was wide dissemination of Imipenemase (IMP) and Verona integron-encoded metallo-β-lactamase (VIM) among Gram-negative bacteria such as *Enterobacteriaceae, Pseudomonas aeruginosa and Acinetobacter baumannii.* Table 1 shows the characteristics of class B metallo-β-lactamases.

Integron structures associated with transposons carry genes for VIM- and IMP-type enzymes within them and these genes can be inserted on the chromosome or in plasmids of the bacteria (23). The resultant effect is the basis for the dissemination of MBL genes and their existence in MDR bacteria. In 2008, the newly described New Delhi metallo-β-lactamase-1 (NDM-1) was initially discovered in *Klebsiella pneumoniae* and *Escherichia coli,* found in a patient travelling from India to Sweden (26). Since then, they have spread globally due to rapid exchange of genes among species. The rapid spread of NDM MBLs is because of the bacterial promiscuity exhibited by its genetic element (23). All MBLs hydrolyse carbapenems but not all serine based β-lactamases are carbapenemases (14) (Table 1).

Epidemiology of metallo-β-lactamases in nosocomial infections:

Carbapenemase genes were first identified in the chromosomes of Gram-positive bacilli but by 1980s, MBLs were found in non-lactose-fermenting Gram-negative bacteria (23). By 1990s, they were discovered in the plamids of many species of bacteria isolated from clinical settings (25). Metallo-β-lactamases have been reported as being ubiquitous in nosocomial settings (14). The most affected patients are those in units such as intensive care (ICUs), organ transplant receivers, burns and neonates.

There are reports from a retrospective study carried out in Serbia, that not less than one hospital-acquired infection affects about one-third of patients admitted in intensive care units (28). The WHO classified important pathogens causing MDR hospital-acquired infections based on their global risk into critical, high and medium priority (29). The first two groups are very important pathogens known as 'ESKAPE' and these include *Enterococcus faecium*, *Staphylococcus aureus, K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp. The WHO 2024 Bacterial Priority Pathogens List (WHO BPPL) is an updated version of the 2017 edition which addresses the evolving challenges of antibiotic resistance, and notable bacteria on this list are carbapenemase-producing Gram-negative bacteria (30). These pathogens cause infection with high mortality rate of patients because of their plasmid-borne MBLs (31,32).

Table 1: Characteristics of class B Metallo-β-Lactamases (23)

Ambler structural Class	Functional Class[a]	Active Site[b]	Inhibitors	Notable Gene	Mobile genetic Elements	Multilocus STs[c]	Retained β-lactam Susceptibility
B	3	Zinc	EDTA	VIM	IncN, Incl1, multiple types; class1 integrons	ST147, ST11, others	Monobactam spared
B	3	Zinc	EDTA	IMP	IncL/M, IncA/C, multiple types; class 1 integrons		Monobactams spared
B	3	Zinc	EDTA	NDM	IncA/C, multiple; ISAba125	ST101, ST11, several others	Monobactam spared

EDTA - Ethylene diamine tetra-acetic acid; IMP - Imipenem metallo-beta-lactamase; Inc - Plasmid incompatibility type; NDM - New Delhi metallo-β-lactamases; ST - Sequence type; VIM - Verona integron-encoded metallo-β-lactamase; [a]Bush-Jacob-Medeiros classification scheme [27]; [b]Hydrolytic method; [c] Only *Klebsiella pneumoniae* and *Escherichia coli*-associated STs are listed

Members of the family Enterobacteriaceae carrying MBL genes are a major world threat in healthcare environment (15). Integron-encoded IMP-1 MBL was first reported in *Serratia marcescens* in 7 Japanese hospitals in Okazaki, Japan during a plasmid-mediated outbreak. Presently, there are at least 52 variants of IMP genes in many bacteria species distributed globally, and they are endemic in Taiwan and Japan (33). The bla_{IMP} gene has both broad and narrow host-range plasmids aiding their increased dissemination and a surveillance study conducted in Australia shows further spread of these genes (34).

The VIM MBLs were found in *P. aeruginosa* in 1996 and 1997 in Verona, Italy (VIM-1) and Marseilles France (VIM-2) respectively (23). VIM-2 is the commonest type globally and has at least 46 bla_{VIM} variants which are distributed worldwide with Greece as the epicentre (26,35,36). In 2008, the bla_{NDM-1} MBL gene was discovered in ST14 *K. pneumoniae* from a Swedish patient in a hospital in New Delhi, India. There are about 24 variants of NDM MBL genes distributed worldwide with India, Middle East and Balkans as the epicentre (37,38). In a study on the outbreak of NDM-1 in South Africa, researchers found that New Dehli MBL is a major cause of hospital mortality (39). In Tuscany,Italy, a rapid spread of NDM-producing *K. pneumoniae* from 2018-2019 was reported (40). Some risk factors for nosocomial infections are existence of co-morbid disease, piperacillin or tazobactam exposure, and the use of mechanical ventillators. Metallo-β-lactamases (MBLs) have been observed to persist in environments like burn unit, thus there is a significant relationship between spread of MBLs in the burns unit environment and patient colonization subsequently (41).

In the last 20 years, carbapenems were regarded as 'reserve' antibiotics used basically against bacteria carrying extended spectrum β-lactamase or Gram-negative bacteria that produces AmpC beta-lactamases. Lately, serine carpenemases and MBLs are more frequent and widespread in many parts of the world (14). In the United States, physicians in tertiary healthcare centres, acute care hospitals for long term diseases and nursing homes are concerned because Gram-negative bacteria that produces carbapenemases such as *K. pneumoniae* caused significant rise in death rates in patients with these bacterial infections (42). Studies in the US and Brazil shows that MBLs produced by *P. aeruginosa* resulted in higher mortality rates and an increase in severity of infections (18, 43). Another study in a tertiary hospital in Abuja, Nigeria, concluded that the increase in the occurrence of MBL-producing strains of *P. aeruginosa* is a major threat to therapeutics, and infection prevention and control. Therefore, rational use of carbapenems, regular surveillance and proper infection prevention and control measures should be put in place to curb MBLs dissemination in hospitals (44).

Metallo beta-lactamase inhibitors:

Metallo-beta-lactamases have catalytic sites, mechanism and structures different from serine beta-lactamases because both are not evolutionarily related (45). There are limited options for inhibiting the activities of pathogens that produce MBLs. One of such options is aztreonam-ceftazidime-avibactam combination, which is effective, although the optimization of pharmacokinetics of the combination has not been concluded (46). Recently, the Food and Drug Administration (FDA) approved the clinical use of β-lactam/β-lactamase inhibitor for the treatment of diseases caused by MDR ESKAPE pathogens (47). The FDA approved β-lactam/β-lactamase inhibitor combinations are ceftazidime-avibactam, imipenem-relebactam, the siderophore cephalosporin (cefiderocol), meropenem-vaborbactam and ceftolozane-tazobactam (47). Non-ribosomal peptide antibiotic such as colistin (produced by *Paenibacillus polymyxa)* is active against pathogens that are carbapenem resistant (48). Although there are six approved serine β-lactamase inhibitors, none inhibits the activities of MBLs because their catalytic sites are different (14).

Tebipenem, the foremost oral carbapenem, is undergoing clinical trials (phase 3). If approved, this could be combined with other antibacterial drugs for treatment of nosocomial MBL bacterial pathogens (46). Novel boronate compounds which are effective against MBL are taniborbactam, which is a MBL inhibitor in phase 3 clinical trial (NCT03840 138), QPX7728 in phase 1 clinical trial (NCT0 4380207) and xeruborbactam in phase 1 clinical trial (49,50). Other MBL inhibitors are metal chelators such as aspergllomarasmine A (14). Bicyclic boronates have been developed as inhibitors to serine beta-lactamases and MBLs although the genetic diversity of MBL is a major challenge in developing MBL inhibitors (49).

Conclusion:

There are basically two groups of beta-lactamases in bacteria, which are the cause of resistance to carbapenems, exhibited by many Gram-negative bacteria in clinical settings. The first is the serine carbapenemases (use serine as the active site amino acid) and the second is the MBLs (those that use a Zn^{2+} ion). In recent years, MBLs have become one of the most dreaded resistance systems due to their ability to break down

almost all beta-lactams such as carbapenems and the genes expressing these enzymes are mostly on bacteria plasmids.

Therapeutic choices for nosocomial infections caused by Gram-negative bacteria is largely limited due to the dissemination of MBLs in these pathogens. Healthcare institutions with high occurrence of MBLs should review their empirical treatments. Local, national and international monitoring system, should be developed and organised to monitor threats of MBLs infections. Research studies aimed at identyfying, observing, controlling and reviewing the threats posed by these infections should also be encouraged.

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Contributions of authors:

IFE conceptualized and wrote the manuscript, MOA edited the manuscript, MAA managed the literature searches, and OOO reviewed the final draft of the manuscript. All authors read and approved the final manuscript.

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Review Article **Open Access**

A mini-review of the global implications of the emerging SARS-CoV-2 XEC variant: Epidemiology, mutations, and mitigation strategies

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Abstract:

The newly emerged SARS- CoV-2 XEC subvariant (XEC) is a global public health concern. Since its first detection in May 2024, it has spread rapidly to involve more than 45 countries globally. The World Health Organization and the Africa Centers for Disease Control and Prevention designated XEC a variant under monitoring (VUM), highlighting its global concern. XEC sub-variant is a descendant of the omicron lineage and a recombinant product of K.S.1.1 and KP3.3. Increased infectivity, enhanced immune evasion, and immune resistance, have been linked to specific spike protein mutations, S: F59S and S: T22N, alongside Apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3)-driven mutations that facilitate immune escape and viral survival. Like its predecessor, the novel SARS-CoV-2 XEC variant exhibits flu-like symptoms. However, there is no evidence that it causes more severe illness than previously circulating Omicron variants. Nevertheless, the high reproduction number (Re) of the XEC variant suggests a higher chance of out competing the other major lineages. Wastewater surveillance has shown that XEC variant circulation is approximately 2 to 19 times higher than the number of cases that have been identified. This suggests that underreporting, decreased testing and declining genomic surveillance in many countries may be masking the true epidemiological burden of XEC. Mitigation strategies must prioritize mass vaccination campaigns, incorporating updated 2024-2025 vaccines and infection prevention and control measures. Sadly, vaccine hesitancy poses a significant challenge to the achievement of herd immunity. To address this, targeted public enlightenment campaigns are essential. Additionally, proactive and collaborative global efforts are necessary to curb the spread of emerging variants like XEC. Strengthened IPC practices, and sustained genomic surveillance will be critical in averting future waves and mitigating public health impacts. This review serves to provide insight into the XEC variant and call for action to prevent another looming pandemic.

Keywords: SARS-CoV-2; XEC; subvariant; mitigation; variant of concern

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Mini-analyse des implications mondiales du variant émergent XEC du SARS-CoV-2: Epidémiologie, mutations et stratégies d'atténuation

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Résumé :

Le nouveau sous-variant XEC du SARS-CoV-2 (XEC) constitue un problème de santé publique mondial. Depuis sa première détection en mai 2024, il s'est rapidement propagé, touchant plus de 45 pays. L'Organisation mondiale de la Santé et le Centre africain pour le contrôle et la prévention des maladies (CDC) ont désigné le XEC comme variant sous surveillance (VUM), soulignant ainsi son importance mondiale. Le sous-variant XEC est un descendant de la lignée omicron et un produit recombinant de K.S.1.1 et KP3.3. Une infectiosité accrue, une évasion immunitaire accrue et une résistance immunitaire accrue ont été associées à des mutations spécifiques

de la protéine Spike, S : F59S et S : T22N, ainsi qu'à des mutations induites par l'enzyme d'édition de l'ARNm de l'apolipoprotéine B, le polypeptide catalytique 3 (APOBEC3), qui facilitent l'évasion immunitaire et la survie virale. Comme son prédécesseur, le nouveau variant XEC du SARS-CoV-2 présente des symptômes pseudo-grippaux. Cependant, rien ne prouve qu'il provoque une forme plus grave que les variants Omicron précédemment en circulation. Néanmoins, le taux de reproduction élevé (Re) du variant XEC suggère une probabilité plus élevée de supplanter les autres lignées principales. La surveillance des eaux usées a montré que la circulation du variant XEC est environ 2 à 19 fois supérieure au nombre de cas identifiés. Cela suggère que la sous-déclaration, la diminution des tests et la baisse de la surveillance génomique dans de nombreux pays pourraient masquer le véritable fardeau épidémiologique du XEC. Les stratégies d'atténuation doivent privilégier les campagnes de vaccination de masse, intégrant les vaccins 2024-2025 mis à jour et les mesures de prévention et de contrôle des infections. Malheureusement, l'hésitation vaccinale constitue un obstacle majeur à l'obtention de l'immunité collective. Pour y remédier, des campagnes ciblées de sensibilisation du public sont essentielles. De plus, des efforts proactifs et collaboratifs à l'échelle mondiale sont nécessaires pour freiner la propagation des variants émergents comme le XEC. Le renforcement des pratiques de prévention et de contrôle des infections et une surveillance génomique soutenue seront essentiels pour éviter de futures vagues et atténuer les impacts sur la santé publique. Cette analyse vise à mieux comprendre le variant XEC et à appeler à l'action pour prévenir une nouvelle pandémie imminente.

Mots-clés: SARS-CoV-2; XEC; sous-variant; atténuation; variant préoccupant

Introduction:

Severe acute respiratory syndrome coronavirus-2 (SARS- CoV-2) XEC sub variant (XEC) is a global public health concern and currently the only SARS-COV-2 variant under monitoring (VUM) exhibiting increasing prevalence worldwide. The recent upsurge in COVID-19 cases linked to the newly emerged SARS-CoV-2 XEC variant has caused a global stir. XEC has spread to all three World Health Organization (WHO) regions and has been confirmed in 45 countries across different continents including Europe, Asia, North America and Botswana in Africa (1,2). No cases have been recorded in Nigeria, the most populous country in sub-Saharan Africa.

The WHO and the Africa Center for Disease Control and Prevention designated XEC a variant under monitoring (VUM) highlighting it as a global issue (2). XEC is a recombinant mutant of SARS CoV-2, the cause of the pandemic coronavirus disease 2019 (COVID-19) that shut down the world. SARS CoV-2 is an enveloped, positive-sense, single stranded ribonucleic acid (RNA) virus that belongs to the Coronaviridae family. The genome of SARSCoV-2 is approximately 30kb in length (3).

Immunological adaptations and epidemiological outcomes:

The major variants of SARS-CoV-2 include Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) (4). Among these, the Omicron variant is the predominant variant of concern in circulation and was named variant of concern (VOC) by the WHO based on the detrimental change it caused in COVID-19 epidemiology. The Omicron variant was first identified in South Africa and reported to the WHO by the Genomics Surveillance on November 14, 2021. This newly discovered variant which contained a constellation of mutations quickly spread to other parts of the world. Omicron gradually and continuously undergoes antigenic shifts and recombinant mutations resulting in circulating sub-variants (5). The main sub-variants of omicron currently circulating around the world include BA.2.86, JN.1, KP.2, KP.3 and XEC (2).

The XEC sub-variant, first detected in Italy in May 2024 (2) is a descendant of the Omicron lineage and a recombinant product of JN.1 lineage of KS.1.1 (JN.13.1.1.1) and KP. 3.3 (JN.1.11.1.3.3). XEC acquired two spike (S) protein substitutions, S: T22N and S: F59S through recombination when compared with the parent KP.3 lineage (6). In addition, the relative effective reproduction number (Re) of the XEC sub-variant is 1.13-fold higher than that of KP.3.1.1. This implies that it has a higher chance of out competing the other major lineage including KP.3.1.1. The resulting epidemiological outcome of these mutations include increased host viral infectivity attributed to the S: F59S and enhanced immune resistance and evasion attributed to the presence of S: T22N and S: F59S (1.5-fold and 1.6-fold) when compared with the previously circulating variants. The S: T22N mutation does not affect viral infectivity. Moreover, XEC and KP.3.1.1 have been found to have significantly higher infectivity than KP.3 (7).

Additionally, the accumulation of Apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3)-type mutations confer an epidemiological advantage to the emergent XEC subvariant (8,9). These mutations represent a host defense mechanism, targeting the viral genome during replication when single strands are exposed. This process leads to nucleotide substitutions and changes that enhance viral survival and immune evasion. The APOBEC3A (A3A) cytidine deaminase plays a critical role in the induction of cytosine to uracil substitution in the SARS-CoV-2 genome (8).

The SARS-CoV-2 infected cells exogenously express A3A without the expression of other APOBEC proteins-induced uracil-uracil to uracil-cytosine mutations in the viral RNA (v

RNA). The A3A mRNA expression is drastically increased by increased release of interferons and tumor necrosis factor-α (TNF-α) by the virally infected epithelial cells of the respiratory system, a site for efficient replication of the SAR-CoV-2. This represents a primary host derived mutation involved in editing the viral RNA genome (8,9).

The XEC variant like its predecessor exhibits a wide range of flu-like symptoms ranging from mild to severe illness including but not limited to fever (38^0C or higher), chills, cough, shortness of breath, sore throat, headache, congestion, loss of taste such as high body temperature, body aches, and tiredness (2,10). However, experimental data from researchers across the globe have shown that there is no evidence that it causes more severe illness than previously circulating Omicron variants (2). Notwithstanding, the WHO has highlighted the worrying finding that XEC and KP.3.1.1 (both VUMs) show increasing prevalence globally, despite declining levels of other VUMs (2).

From 16 September to 13 October 2024 (during the 4 weeks period), 90 countries reported COVID-19 cases, and 30 countries reported COVID-19 related-deaths attributed to XEC. Within this period, available data showed 24,000 new cases and 830 new ICU admissions. The quoted figures may just be the tip of the iceberg, and not reflective of the actual number of cases or deaths. This underestimation may be attributable to under reportage, missed diagnosis, decreased testing and sequencing as well as the fact that many countries are no longer reporting or have changed the frequency of reporting of COVID-19 (11).

Furthermore, estimates from wastewater surveillance on the circulation of SARS-CoV-2 revealed that XEC variant circulation is approximately 2 to 19 times higher than the number of cases identified and reported in 30 countries across the WHO five regions as featured on the WHO COVID-19 dashboard (11). Hence, if effective mitigation measures are not put in place, another COVID-19 pandemic may be inevitable.

Mitigation strategies:

Vaccines have demonstrated enormous protective effects in human and animal health (12,13). Hence, effective mass vaccination incorporating all the circulating SAR-CoV-2 sub variants including the newly emergent XEC will ensure the development of herd immunity. The updated 2024-2025 COVID-19 vaccines incorporate the currently circulating strains, and it is highly recommended for persons aged 65 years and above, are at high risk for severe COVID-19, or have never received a COVID-19 vaccine.

The 2024-2025 vaccine protects humans from severe illness, hospitalization, and death. However, as vaccine immunity wanes over time, it is important to ensure compliance with the 2024–2025 COVID-19 vaccine schedule. Sadly, vaccine hesitancy remains a problem which has persisted since the era of the COVID-19 pandemic. Vaccine acceptance rates have ranged from 14-68% (14-17) and researchers have attributed this to bias, propaganda, ignorance, myths, fear of adverse effects and conspiracy theories (15). This development undermines the achievement of the objectives and expectations towards vaccine protection against the circulating sub variants.

It is important that vaccination programs be implemented in conjunction with an effective infection prevention and control (IPC) measures (18) as outlined by the apex health governing stake holders during the COVID-19 pandemic which is now lax in many communities and countries across the globe. These IPC measures include but not limited to physical distancing at 1 meter between person, avoidance of the 3Cs (crowds, close contact and closed spaces), regular wearing of properly fitted face mask preferably covering the nose, mouth and chin when physical distancing is unavoidable and in poorly ventilated settings, adequate hand hygiene with alcohol-based hand rub or soap and water on a regular basis, proper cough etiquette into a bent elbow or use of tissue when coughing and/or sneezing (18).

Other measures include self-isolation following suggestive symptoms or following positive test for COVID-19 until recovery, regular cleaning and disinfection of surfaces including frequently touched sur- faces, such as phones, door handles, faucets and office tabletops (18). Regular public health education and health information dissemination in local languages (12). Reliable sources such as the WHO, local and national health authorities, should provide regular updates on emerging trends on the new variants under monitoring.

Conclusion:

The newly emerged mutant SARS CoV 2-XEC subvariant currently circulating across the globe has resulted in an increased number of cases of COVID-19 with large scale illnesses and fatalities. The resulting rise in viral infectivity and immune evasion have been attributed to the S: F59S and S: T22N substitutions at the spike protein rendering the previous vaccine less effective.

The updated 2024-2025 COVID-19 vaccines confer some protection, although vaccine effectiveness against variants has decreased due to immune escape mutations. Booster and seasonal COVID-19 vaccines are reco-

mmended as strategies for mitigation. Furthermore, public health education should be prioritized to enhance vaccine acceptance and updates should be provides on the newly emergent circulating XEC. These measures should be combined with the institution of effective IPC strategies to further mitigate these ongoing challenges and prevent another looming pandemic.

Contributions of authors:

SOE conceptualized the review. SCE, INN, UCO, INO and SOE wrote the original draft of the manuscript. All authors reviewed, read and approved the final version of the manuscript submitted for publication.

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Original Article **Open Access**

Cytomegalovirus co-infection with human immunodeficiency virus among children in State Hospital, Ibadan, Nigeria

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Abstract:

Background: Cytomegalovirus (CMV) co-infection with human immunodeficiency virus (HIV) poses significant global challenges with possible complications of disease progression in children and adolescents, especially in resource-limited settings. This study investigates the prevalence of CMV among children living with HIV as well as their viral load dynamics and immunological status correlates.
Methodology: A total of 169 HIV infected participants, including 37 children (2 months to 10 years) and 132 adolescents (11–19 years) on antiretroviral therapy (ARTs), were enrolled from the State Hospital HIV Clinic, Ibadan, Nigeria. The CMV-DNA cycle threshold (Ct) and HIV viral loads were determined using real-time polymerase chain reaction (rt PCR) assay while $CD4^+$ cell counts were assessed with CyFlow method. Socio-demographic and clinical data of participants were analyzed using SPSS (version 25.0) and statistical comparisons and correlations between variables were evaluated at significant level of $p<0.05$.
Results: The mean age of all HIV-infected participants was 11.89±3.16 years, and were predominantly males (65.1%, n=110). The overall prevalence of CMV was 13.0%, with higher rates among males (20.9%) compared to females (5.4%) (OR=3.897, 95% CI=1.103-13.776, $p=0.03$). The prevalence of low and high CMV DNA Ct were 8.9% and 4.1% respectively. Most of the participants had low HIV loads (<19 copies/μL) (n=102, 60.4%) and high $CD4^+$ counts (>500 cells/μL) (n=134, 79.3%). However, a subset of the participants (n=12, 7.1%) had high CMV DNA levels despite their low HIV loads. A negative correlation between CMV DNA Ct values and age ($r<0$) suggested increased CMV loads in older participants.
Conclusion: Cytomegalovirus co-infecting with HIV is present at a prevalence of 13.0% in this study, and significantly higher in males and older age groups, depicting a significant concern among male children and adolescents living with HIV. These findings highlight the need for routine CMV screening among children and adolescents living with HIV, age- and gender-sensitive interventions, as well as enhanced ART adherence, to mitigate the risks associated with co-infection in this vulnerable population.

Keywords: CMV; HIV; co-infection; real time PCR; cycle threshold; $CD4^+$ T cell

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Co-infection par le cytomégalovirus et le virus de l'immunodéficience humaine (VIH) chez des enfants à l'hôpital public d'Ibadan, au Nigéria

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Résumé:

Contexte: La co-infection par le cytomégalovirus (CMV) et le virus de l'immunodéficience humaine (VIH) pose des défis mondiaux importants, avec un risque potentiel complications de la progression de la maladie chez les enfants et les adolescents, en particulier dans les milieux à ressources limitées. Cette étude examine la prévalence du CMV chez les enfants vivant avec le VIH ainsi que la dynamique de leur charge virale et les corrélats de leur statut immunologique.
Méthodologie: Un total de 169 participants infectés par le VIH, dont 37 enfants (2 mois à 10 ans) et 132 adolescents (11-19 ans) sous traitement antirétroviral (TAR), ont été recrutés à la clinique VIH de l'hôpital d'État d'Ibadan, au Nigéria. Le seuil du cycle CMV-ADN (Ct) et les charges virales VIH ont été déterminés à l'aide d'un test de réaction en chaîne par polymérase en temps réel (rt PCR) tandis que le nombre de cellules $CD4^+$ a été évalué avec la méthode CyFlow. Les données sociodémographiques et cliniques des participants ont été analysées à l'aide de SPSS (version 25.0) et les comparaisons statistiques et les corrélations entre les variables ont été évaluées à un niveau significatif de $p<0,05$.
Résultats: L'âge moyen de tous les participants infectés par le VIH était de 11,89±3,16 ans et il s'agissait principalement d'hommes (65,1%, n=110). La prévalence globale du CMV était de 13,0%, avec des taux plus élevés chez les hommes (20,9 %) que chez les femmes (5,4%) (OR=3,897, IC à 95%=1,103-13,776, $p=0,03$). La prévalence d'un Ct d'ADN CMV faible et élevé était respectivement de 8,9% et 4,1%. La plupart des participants avaient une faible charge de VIH (< 19 copies/µL) (n=102, 60,4%) et un taux élevé de $CD4^+$ (>500 cellules/µL) (n=134, 79,3%). Cependant, un sous-ensemble de participants (n=12, 7,1%) avait des taux élevés d'ADN CMV malgré leur faible charge de VIH. Une corrélation négative entre les valeurs de Ct de l'ADN du CMV et l'âge ($r<0$) suggère une augmentation des charges de CMV chez les participants plus âgés.
Conclusion: La prévalence de la co-infection par le cytomégalovirus et le VIH est de 13,0% dans cette étude, et significativement plus élevée chez les hommes et les groupes d'âge plus avancés, ce qui témoigne d'une préoccupation importante chez les enfants et les adolescents de sexe masculin vivant avec le VIH. Ces résultats soulignent la nécessité d'un dépistage systématique du CMV chez les enfants et les adolescents vivant avec le VIH, d'interventions tenant compte de l'âge et du sexe, ainsi que d'une meilleure observance du traitement antirétroviral, afin de réduire les risques associés à la co-infection dans cette population vulnérable.

Mots-clés: CMV; VIH; co-infection; PCR en temps réel; seuil de cycle; lymphocytes T $CD4^+$

Introduction:

Viral co-infections with human immunodeficiency virus (HIV) are common findings in infected individuals especially the viruses with similar route of infection, and they are implicated as a contributory factor to the progression of disease development (1). Some of the co-infections can be silent without notable impact on HIV disease progression while HIV co-infection with certain viral infections can affect the natural history of HIV infection leading to severe complications. Among the infectious agents that can disrupt the natural history and course of HIV infection towards severe diseased state when co-infecting the same host are hepatitis C virus (HCV), hepatitis B virus (HBV), human T-cell lymphotrophic virus-1 (HTLV-1), human herpes virus-8 (HHV-8), human papilloma virus (HPV), cytomegalovirus (CMV) and others (1,2).

HIV remains a significant public health concern, particularly in sub-Saharan Africa, accounting for two-thirds of the overall world HIV infections, where the burden of the disease is disproportionately high among children (3). In Nigeria, pediatric HIV presents unique challenges, especially when complicated by co-infections (4). One such co-infection is cytomegalovirus (CMV), a common herpes virus (human herpes virus-5) that poses serious risks to immunocompromised individuals, including children living with HIV (5).

Cytomegalovirus is a highly prevalent viral pathogen belonging to the beta herpesvirinae subfamily, known for its ability to establish lifelong latency in the host. CMV infection is widespread globally, but its impact is most profound in regions with lower socioeconomic status (6). While CMV is often asymptomatic in healthy individuals, it poses significant risks to immunocompromised poplations, notably in those living with HIV, and it can lead to severe complications such as retinitis, esophagitis, encephalitis, hepatitis and pneumonia amongst others (7). CMV is primarily transmitted through bodily fluids such as saliva, urine, breast milk, and blood, with infants often acquiring the virus through breastfeeding, delivery via the birth canal, or in-utero. The virus can also spread among young children through contact with contaminated saliva, in settings where shared toys and objects are common (8).

The use of combination antiretroviral therapy (cART) has drastically reduced the incidence of CMV-related complications in HIV positive individuals, especially those with severe immunosuppression. Before the widespread availability of ART, approximately 40% of severely immunocompromised HIV-positive individuals experienced symptoms of CMV during their lifetime (9). Nevertheless, the effectiveness of ART has not eliminated the need for awareness and early screening for CMV, as this continues to pose a serious risk, more so in regions where access to healthcare remains limited.

Despite the known detrimental effects of CMV co-infection in HIV-positive individuals, there is a paucity of data on its prevalence, impact, and the demographic factors

influencing its occurrence in pediatric populations and effective management strategies in Sub-Saharan Africa (10).

This study is designed to determine the prevalence of CMV among children living with HIV in Ibadan, Nigeria, and to evaluate the impact of antiretroviral therapy (ART) on their immune function through viral load and $CD4^+$ T cells treatment indices. The findings from this study aimed to contribute to the understanding of how CMV affects HIV-positive children, clinical practice and highlight the importance of regular screening of CMV among vulnerable populations.

Materials and method:

Study setting, design and participants:

This was a hospital based cross-sectional study, which recruited a total of 169 participants comprising 37 children (2 months to 10 years) and 132 adolescents (11-19 years) from September 2023 to August 2024. The participants were made up of 110 males and 59 females, who were confirmed to be HIV positive by serology and PCR, already on antiretroviral therapy (ART) and accessing care at the out-patient HIV clinic of the State Hospital, Ibadan, Nigeria.

Laboratory analyses were performed at the Biorepository and Clinical Virology Laboratory (BCVL) and the Haematology laboratory of the Department of Biomedical Laboratory Science (BMLS), College of Medicine, University of Ibadan.

Ethical consideration:

Ethical approval was obtained from Oyo State Ethics Research Committee, Ministry of Health, Ibadan, Nigeria (NHREC/OYO SHRIEC/10/11/22) and informed consents were obtained from the parents/caregivers of the children while those above 12 years were given child assents form before being enrolled into the study.

Inclusion and exclusion criteria:

The inclusion criteria were confirmed positive HIV status, ART initiation and accessing care at the State Hospital, while the exclusion criteria were non-consenting individuals, children without HIV infection and children experiencing a current ongoing illness.

Data and sample collection, handling and processing:

The socio-demographic data and clinical information of the participants were collected using interviewer administered questionnaires and abstraction of data from patients' case notes. Venous blood samples (5ml) were aseptically collected by a professional Phlebotomist into two sterile EDTA sample bottles from the participants and transported to the Clinical Virology Laboratory where aliquot of blood samples from the first bottle was used for viral load analysis and $CD4^+$ T cell count. Blood samples in the second bottle was centrifuged, and the plasma separated and stored at -20°C for CMV DNA molecular analysis.

$CD4^+$ T cell count:

The $CD4^+$ T cell count was measured using automated Sysmex Partec Cyflow device with $CD4^+$ Monoclonal easy count kit (Sysmex-Partec Germany).

Molecular analysis of samples for CMV and HIV detection:

Nucleic acid extraction:

DNA and RNA were extracted from 200 µL of each plasma sample obtained from the participants whole blood using the Qiagen RNA/DNA Extraction Kit, which has a specific polymer group that absorbs nucleic acid from the samples.

Quantitative detection of CMV DNA by real time fluorescence PCR:

The presence of CMV DNA and HIV 1 RNA were detected in the extract using commercially available kit (Guangdong Huayin Medicine Science Co., Ltd.). Amplification of the extracted nucleic acid was performed on a multiple fluorescent real-time PCR technology to quantitatively detect HIV-1 and HCMV DNA levels in samples using specific CMV primers and probes, with an internal control to monitor PCR inhibitor levels and prevent false negative PCR.

The PCR reaction tube, CMV PCR Master Mix, which contains the CMV primers and probes (forward primer: 5'-GAGCCTTTGGAG GA-3' and reverse primer: 5'-GGCTGAGTTCT TGG-3'), along with reaction buffers, Mg^{2+}, dNTPs, Taq DNA Polymerase, was mixed with the extracted DNA samples, CMV positive control (recombinant plasmid containing target gene), negative control (physiological saline) and redissolved diluent (purified water). The reaction tube was placed in the automatic fluorescent PCR instrument and PCR performed in accordance with the operating instructions of the instrument. After the reaction was completed, the results were automatically saved. The start, end and threshold values of the baseline were adjusted according to the analysed image.

The cycle threshold (Ct) value was used as proxy for HCMV detection (qualitative rt PCR), with Ct>0.00 as positive (≥200 IU/ml), Ct=0.00 as negative (<200 IU/ml), Ct< 30 ($1.00x10^3$-$1.00x10^8$ IU/ml) as high positive and Ct>30 (≥200 IU/ml-$1.00x10^3$IU/ml) as low positive.

Data analysis:

Data were analyzed using Statistical Package for Social Sciences (SPSS version 25.0). Descriptive statistic was used to summarize the qualitative (categorical) data. The quantitative data were presented as mean± standard deviation ($\bar{x}$±SD), median (interquartile range) and rates.

The prevalence of CMV co-infection was determined, and the mean HIV loads was compared between CMV-positive and CMV-negative participants. Correlation analysis was performed to explore the relationship between ART duration, HIV load, and CMV positivity. A *p*-value of <0.05 was considered significant.

Results:

Socio-demographic characteristics of the participants:

The participants had a mean age of 11.89±3.16 years with age range of 2 months-19 years. The age group 11-15 years has the highest frequency, followed closely by age group 6-10 years while those in age group 6-19 years were the lowest in frequency. The male participants were predominant and the participants had a mean weight of 32.48kg (Table 1).

Table 1: Demographic data and anthropometric measurements of the study participants

Variable	Frequency (%)
Age group	
2 months – 5 years	12 (7.1)
6-10 years	25 (14.8)
11-15 years	127 (75.1)
16-19 years	5 (2.0)
Mean age ± SD (years)	11.89±3.16
Mean weight ± SD (kg)	32.48±10.59
Gender	
Male	110 (65.1)
Female	59 (34.9)

HIV load, CD4$^+$ cell count and HCMV cycle threshold values of the participants:

Table 2 displayed the mean HIV load and CD4$^+$ T-cells count as well as HCMV cycle threshold (Ct) of all the participants. The mean Ct of HCMV-DNA for all the positive participants is 2.52±0.65, while the mean Ct of HCMV for participants with Ct<30 (high) is 10.97±8.85 (range 1.26-29.49) and for participants with Ct>30 (low) is 37.47±11.08 (range 31.96-39.59) (p<0.05). The mean HIV-1 RNA viral load for all the study participants is 21250.80±11271.32 while the mean CD4$^+$ T cells is 893.84±520.37.

Table 2: Mean of CD4$^+$ T- cells count, HIV-1 RNA load and HCMV DNA Cycle threshold values among the study participants

Variable	Mean± SD
CD4$^+$ T cell count (cells/µL)	893.84±520.37
HIV load (copies/mL)	21250.80±11271.32
HCMV DNA Ct value (Total)	2.52±0.65
HCMV DNA Ct value <30 (1.26-29.49)	10.97±8.85*
HCMV DNA Ct value >30 (31.96-39.59)	37.47±11.08*

CD4- Cluster of Differentiation 4; HCMV DNA Ct- Cytomegalovirus deoxyribonucleic acid Cycle threshold. *p-value significant at ≤0.05

Analysis of prevalence of HCMV with characteristics of the study participants:

Table 3 shows HCMV prevalence of 13.0% (22/169) in the study participants with high and low HCMV prevalence of 8.9% and 4.1%, respectively. Most of the study participants had low HIV-1 RNA viral load (<19 copies/mL) (60.4%, 102/169) and high CD4$^+$ T cell count (>500 cells/µL) (79.3%, 134/169).

Table 3: Distribution and prevalence of HCMV, HIV-1 RNA viral load and CD4$^+$ T cell count values in the participants

Parameters	Frequency (%)
HCMV DNA	
Positive (Ct<30 - high)	15 (8.9)
Positive (Ct >30 - low)	7 (4.1)
Positive Ct > 0.00 -Total)	22 (13.0)
Negative Ct = 0.00	147 (87.0)
HIV-1 RNA Viral Load (copies/mL)	
≤19	102 (60.4)
20-200	19 (11.2)
>200	48 (28.4)
CD4$^+$ T cell count (cells/µL)	
<200	10 (5.9)
200-500	25 (14.8)
>500	134 (79.3)

CD4-Cluster of Differentiation 4; HCMV DNA- Cytomegalovirus deoxyribonucleic acid; Ct - Cycle threshold

The prevalence of HCMV in the HIV infected children by gender is 5.4% (3/56) and 20.9% (19/91) in the female and male participants respectively (OR=3.897, 95% CI =1.103-13.776, p=0.03) (Fig 2). Fig 2a compared the distribution of participants levels of HIV-1 RNA viral load with HCMV DNA Ct values, which shows that those with zero HCMV DNA Ct values were the majority with low (<19 copies/mL), intermediate (20-200 copies/mL) and high (>200 copies/mL) HIV-1 RNA viral load (p<0.05). However, there were notable participants (n=12, 7.1%) with both low HCMV DNA Ct value (high HCMV level) and low HIV load.

Fig 2b compared the distribution of the HIV-1 RNA and HCMV DNA Ct values where majority of those with zero HCMV DNA

had high CD4⁺ T cell count (>500 cells/µL), others had intermediate (200 - 500 cells/µL) and low CD4⁺ T cell count (< 200 cells/µL), while all participants with positive HCMV DNA at low or high Ct values had a high CD4⁺ T cell count.

Fig 3 shows the correlation of HCMV DNA Ct values with the age of the participants, with a moderate negative correlation ($r<0$) which inferred that as the age increases, the HCMV Ct value decreases thereby implying increased HCMV load in older participants.

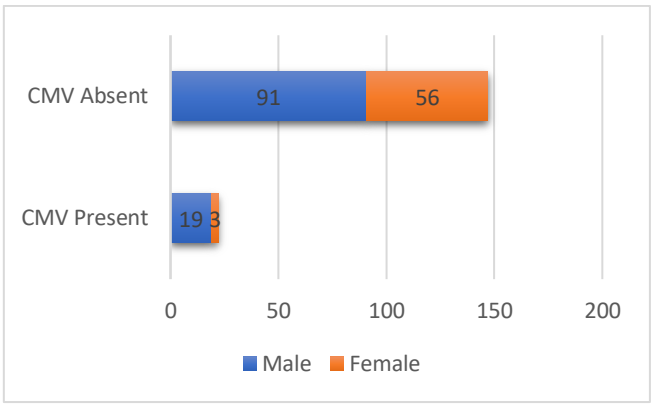
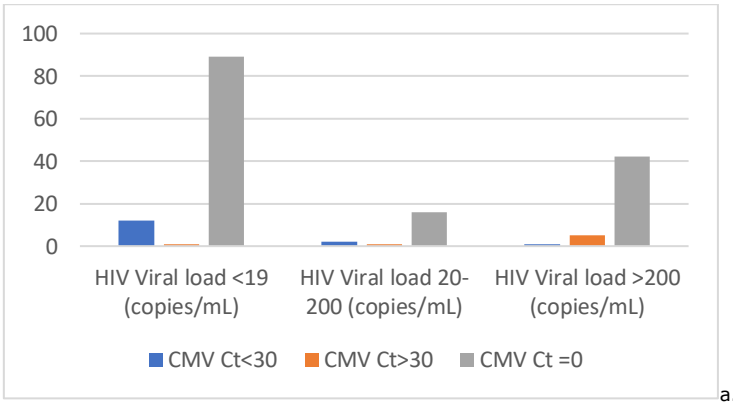
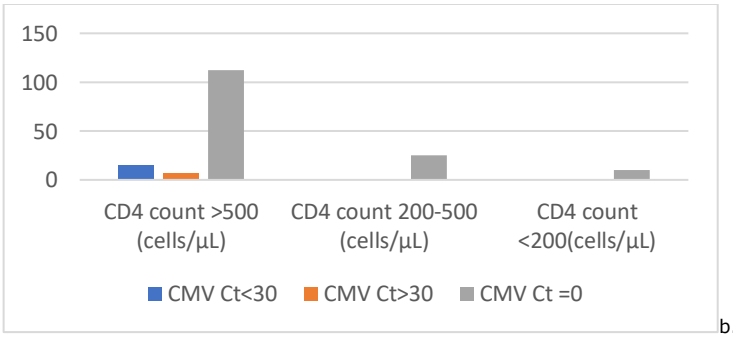


Fig 1: Prevalence of CMV by gender among the study participants



a.



b.

Fig 2: Comparison of distribution of HCMV DNA Ct value with **a:** HIV-1 RNA viral load **b:** CD4 T-cells count.

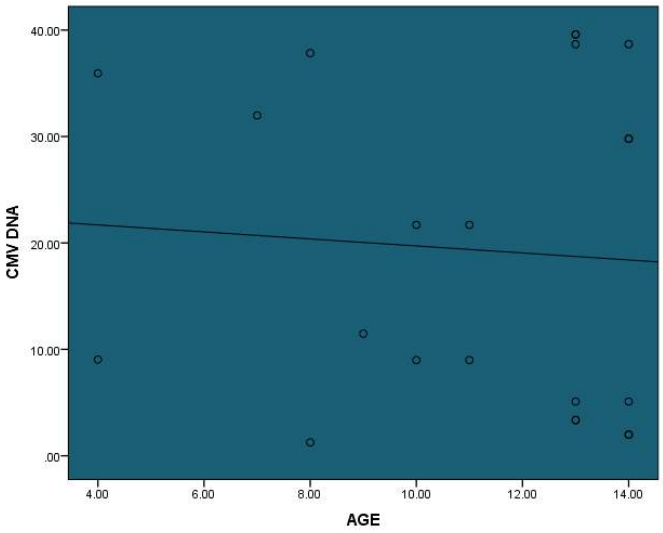


Fig 3: Correlation of HCMV DNA Ct values with the age of the study participants

Discussion:

The co-infection of CMV can be a major complication in children with HIV and could be associated with increased risk of AIDS-defining criteria leading to higher mortality and morbidity. In this study, the socio-demographic results of the mean age and broad age range of the participants displayed the predominance of older children and adolescents in HIV clinics thereby reflecting the success of earlier pediatric HIV interventions that enable longer survival into adolescence. This aligned with previous studies on paediatric and adolescent HIV population described by Mwaanza et al., (11) and by WHO (12), where children aged 10-15 years have been estimated to be the highest population with HIV by the end of the year 2023. The lower representation in the age-group 16–19 years may be indicative of attrition due to delayed diagnosis, ineffective treatment or treatment interruptions which are concerns previously noted in studies examining survival trends among HIV-positive adolescents (13,14).

Also, this study revealed that the male outnumbered female participants, which may be as a result of regional differences in health-seeking behaviors or sampling variations. This is in contrast with some other reported observation where females showed dominance among adult and pediatric HIV cohorts (15). In addition, the mean weight of 32.48 kg reflected the nutritional and health status of the cohort depicting an average low weight of the participants which could be attributable to growth delays and nutritional deficiencies in HIV-positive children despite being on ART. Similar findings were reported in some Nigeria-based studies where HIV-positive children and adolescents in the country demonstrated weight ranges indicative of chronic disease impact, despite ART (16,17).

In this study, the prevalence of HCMV is 13.0% in the children and adolescents' participants who were living with HIV. This aligned with findings from similar studies conducted among HIV-positive pediatric populations ranging from 10% to 33%, reflecting the endemic nature of HCMV in regions with high HIV burdens (2,8,18). The mean CMV Ct value of 2.52±0.65 further highlighted the sensitive detection method resulting in display of active replication of CMV represented by the PCR Ct values within the population, which is suggestive of the need for routine CMV screening among HIV-positive individuals especially the children and adolescents to prevent complication of disease progression.

Furthermore, the distinction between the high (8.9%) and low (4.1%) HCMV DNA PCR Ct level is suggestive of the heterogeneity in viral replication among those with detectable HCMV DNA. The participants with lower HCMV Ct levels indicated a corresponding high CMV DNA viraemia in the system, who may therefore be at increased risk for CMV-associated complications, such as immune activation and end-organ disease, particularly if their immune system is not fully restored by ART. However, those with high PCR Ct level, indicating HCMV DNA load were low, are the majority in the participants, hence it poses a reasonable explanation for most of the participants having a good status of HIV-1 RNA viral load and $CD4^+$ T cell count.

The high number of participants with low HIV viral load and high $CD4^+$ T cell levels explained success of the various ARTs in achieving viral suppression and immune recons-

titution among the participants in the Ibadan, Nigeria. The group represented individuals whose HIV infection were being effectively managed, thereby reducing their susceptibility to opportunistic infections such as CMV. Similar pattern was reported in the study by Albasanz-Puig et al., (19) where the prevalence of CMV-specific immune response was high and endured over time despite the presence of CMV viraemia. However, participants with contrasting level of higher HIV loads and lower $CD4^+$ T cell count reflected a population at greater risk of co-infection complications which cut across individuals with ART non-adherence, treatment failure, or advanced diseases, therefore stressing the need for targeted interventions to improve outcomes.

In furtherance to the prevalence of CMV in the HIV infected pediatric participants, there was an observed significant disparity in CMV prevalence between genders, with 5.4% in females and 20.9% in males (p=0.03). This highlighted an important variation which could be majorly traced to the higher male frequency gender distribution in the entire population, as well as reports of higher male susceptibility to CMV reactivation or acquisition due to biological, behavioral, or environmental factors. These were considered based on similar reports from other studies among paediatric populations with and without HIV infection (8,20-23).

Additionally, most participants without detectable CMV DNA Ct had low (<19 copies/mL), intermediate (20–200 copies/mL), and high (>200 copies/mL) HIV loads, an observation that stresses the effectiveness of ART in suppressing HIV replication and reducing the risk of CMV reactivation (19,24-26). However, the presence of a notable subset (n=12 participants) with both low CMV DNA Ct values (indicating high CMV levels) and low HIV loads suggests a unique interaction between these infections which likely represent cases where CMV reactivation occurred despite effective HIV suppression. Participants with no CMV DNA predominantly had high $CD4^+$ T cell counts (>500 cells/μL), which is consistent with effective immune reconstitution and efficacy of their ARTs (19). Conversely, those with intermediate (200–500 cells/μL) or low (<200 cells/μL) $CD4^+$ T cell counts may be at greater risk for CMV reactivation, where immune suppression correlated strongly with CMV replication (7,27).

Interestingly, all participants with detectable CMV DNA, whether at low or high Ct values, had high $CD4^+$ counts (>500 cells/μL) and this could reflect individuals in a phase of immune recovery, where ART has restored sufficient $CD4^+$ levels, however, CMV reactivation persists temporarily. This finding aligned with studies that highlighted the dynamic nature of immune recovery in HIV, where opportunistic infections such as CMV may still posed a threat even after initial immune reconstitution (28-30).

Finally, the moderately negative correlation (r<0) between CMV DNA Ct values and the age of participants suggests that CMV load increases as participants grew older, which highlights a possible cumulative effect of CMV exposure or reactivation over time. This aligned with findings from studies such as Slyker et al., (31) and Redpath et al., (32) where it was demonstrated that older children and adolescents with HIV often have a longer duration of immune dysregulation, increasing their susceptibility to CMV reactivation or higher viral loads. Also, studies in sub-Saharan Africa have noted that adolescents living with HIV often experienced higher rates of co-infections, reinforcing the need for age-specific approaches in addressing therapeutic options (8).

Conclusion:

Our study reported the prevalence and dynamics of CMV co-infection in children and adolescents living with HIV in Ibadan, Nigeria, emphasizing the complex interplay between demographic factors, viral loads, immune recovery by $CD4^+$ T cell count assessment. A CMV prevalence of 13.0% was observed with variable levels of replication and a significantly higher prevalence rates in males compared to females.

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Contributions of authors:

OEB and EOC conceived and designed the study; EOC, MMB, MEO and OLS collected and analyzed the samples; OEB wrote the initial draft, OEB, OLS, MMB, and MEO reviewed the final draft, while OEB, ECO and OLS supervised the entire research.

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Conflict of interest:

Authors declare no conflict of interest

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Original Article **Open Access**

Molecular characterization of *Cryptosporidium* spp from stool samples of people living with human immunodeficiency virus in Maiduguri, Nigeria

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Abstract:

Background: *Cryptosporidium* is an important cause of diarrhea in immuno-compromised individuals including children. Recent advances in molecular techniques have led to the discovery of different species that are more commonly associated with diarrhea. This study aimed at isolating and characterizing the C*ryptosporidium* spp among HIV patients with diarrhea in Maiduguri, Nigeria.
Methodology: Stool samples were collected from 269 adult HIV patients with diarrhea at the antiretroviral clinic of University of Maiduguri Teaching Hospital. The stool samples were first screened for *Cryptosporidium* using nested PCR after DNA extraction with QIAamp DNA stool mini-extraction kit following the manufacturer's protocol. The identified *Cryptospodium* parasites were further characterized into different species using resyriction fragment polymorphism (RFLP) analysis that targets the amplified small subunit (SSU) ribosomal DNA and glycoprotein 60 (gp60) genes.
Results: From the 269 HIV patients studied, 52 (19.6%) were positive for *Cryptosporidium* by the nested PCR assay. RFLP analysis characterized the *Cryptosporidium* species into *C. hominis* (40.4%, n=20) and *C. parvum* (59.6%, n=32).
Conclusion: Majority of the *Cryptosporidium* species in this study were *C. parvum*, supporting the notion that zoonotic transmission is the main route of cryptosporidiosis transmission. Nonetheless, the observation of *C. hominis* in some patients suggests that anthroponotic transmission is also an important contributor to *Cryptosporidium* pathogenesis in Maiduguri.

Keywords: *Cryptosporidium*; HIV; immuno-compromised; diarrhea; PCR-RFLP; gp60; SSU

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Caractérisation moléculaire de *Cryptosporidium* spp à partir d'échantillons de selles de personnes atteintes du virus de l'immunodéficience humaine à Maiduguri, au Nigéria

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Résumé:

Contexte: *Cryptosporidium* est une cause importante de diarrhée chez les personnes immunodéprimées, y compris les enfants. Les progrès récents des techniques moléculaires ont permis la découverte de différentes espèces plus fréquemment associées à la diarrhée. Cette étude visait à isoler et à caractériser les *Cryptosporidium* spp chez des patients atteints du VIH et souffrant de diarrhée à Maiduguri, au Nigéria.
Méthodologie: Des échantillons de selles ont été prélevés auprès de 269 patients adultes atteints du VIH et souffrant de diarrhée à la clinique antirétrovirale de l'Hôpital universitaire de Maiduguri. Les échantillons de selles ont d'abord été analysés pour *Cryptosporidium* par PCR nichée après extraction d'ADN à l'aide du kit de mini-extraction d'ADN de selles QIAamp, conformément au protocole du fabricant. Les parasites *Cryptosporidium* identifiés ont été caractérisés en différentes espèces à l'aide d'une analyse du polymorphisme des fragments de réplication (RFLP) ciblant les gènes de l'ADN ribosomique de la petite sous-unité amplifiée (SSU) et de la glycoprotéine 60 (gp60).
Résultats: Parmi les 269 patients VIH étudiés, 52 (19,6 %) étaient positifs pour *Cryptosporidium* par PCR nichée. L'analyse RFLP a caractérisé les espèces de *Cryptosporidium* en *C. hominis* (40,4%, n=20) et *C. parvum* (59,6%, n=32).
Conclusion: La majorité des espèces de *Cryptosporidium* dans cette étude étaient *C. parvum*, ce qui étaye l'idée que la transmission zoonotique est la principale voie de transmission de la cryptosporidiose. Néanmoins, l'observation de C. hominis chez certains patients suggère que la transmission anthroponotique est également un contributeur important à la pathogenèse de *Cryptosporidium* à Maiduguri.

Mots-clés: *Cryptosporidium*; VIH; immunodéprimé; diarrhée; PCR-RFLP; gp60; SSU

Introduction:

Cryptosporidium species are Apicomplexan protozoan that is recognized as one of the most important diarrheal pathogens affecting people worldwide, particularly in Africa (1). *Cryptosporidium* is second only to rotavirus in causing diarrhea and death in children in developing countries, responsible for 2.9 million cases annually in children aged <24 months in sub-Saharan Africa (2). People living with HIV/AIDS suffer from numerous and frequent opportunistic infections, many of which pose a serious threat to their health and are life-threatening. Cryptosporidiosis is one of the most common causes of gastroenteritis in immunocompromised people (3). In people with weakened immune systems (HIV-infected people, cancer patients, and organ recipients), cryptosporidiosis can be severe, long-lasting, and sometimes fatal.

Of the 40 currently recognized *Cryptosporidium* species, *Cryptosporidium hominis* and *Cryptosporidium parvum* are responsible for most human infections. However, other species have also been found in immunocompromised patients, including *C. meleagridis, C. canis,* and *C. felis* (1). Cryptosporidiosis remains a common cause of chronic diarrhea in AIDS patients in developing countries, with up to 74% of AIDS patients with diarrhea harboring this parasite in their stool (4).

The main challenge in the proper identification of *Cryptosporidium* spp is to distinguish oocysts from other small particles in fecal and environmental specimens such as yeasts, molds, algae, and plant debris (5). Of the nearly 20 *Cryptosporidium* species and genotypes reported in humans*, C. hominis* and *C. parvum* are responsible for most infections (3). Therefore characterization of *Cryptosporidium* at the species level will be useful in identifying infection and contamination sources (6).

Detection of cryptosporidiosis using PCR-based methods is more sensitive than conventional microscopical and serological methods for detecting oocysts in faeces. Molecular methods can also identify the species/ genotypes and subtypes of *Cryptosporidium,* important for epidemiology of cryptosporidiosis and predicting transmission routes (6). Since the description of the first PCR-based tool for the differentiation of *C. parvum* and *C. hominis*, molecular techniques in the diagnosis of cryptosporidiosis became popular, especially due to their genotyping capabilities (6). This study was conducted to determine the prevalence and identify the different *Cryptosporidium* species among people living with HIV in Maiduguri, Borno State, Northeastern Nigeria using the nested-PCR technique and restricted fragment length polymorphism (RFLP) analysis.

Materials and method:

Study area:

The study was carried out at the University of Maiduguri Teaching Hospital, Borno State, Nigeria. Borno State, also called Yerwa by its locals, is one of the northeast states with Maiduguri as its largest city and

the capital. Maiduguri was founded in 1907 as a military outpost by the British and is home to the Kanem-Bornu Empire. The residents are mostly Muslims, including Kanuri, Hausa, Shuwa, Bura, Marghi, and Fulani ethnic groups. There is also a considerable Christian population.

Borno State has been affected by the Boko Haram insurgency, and almost all the local government areas have relocated to the metropolitan areas, of which Maiduguri is one. The internally displaced individuals are accommodated in camps with inadequate social amenities. The University of Maiduguri Teaching Hospital is a tertiary health center located in Maiduguri with a bed capacity of 650 and 21 departments.

Study design, participants and ethical approval:

This descriptive cross-sectional study was carried out from March 2020 to April 2021 at the University of Maiduguri Teaching Hospital, a tertiary referral health institute, and a center for HIV /AIDS management under the PEPFAR. A total of 269 consenting adults living with HIV who had complained of diarrhea were consecutively enrolled into the study.

A pre-designed structural questionnaire was used to collect socio-demographic characteristics of the patients. Informed consent was obtained from all study participants. The study was approved by the ethical committee of the University of Maiduguri Teaching Hospital, Borno State, Nigeria.

Inclusion and exclusion criteria:

All consenting adult HIV-infected patients presenting with diarrhea attending the ARV clinic in UMTH or were admitted at the time of the study were recruited while all HIV-infected patients who have diarrhea and are on any of the prophylactic drugs against parasitic infection were excluded from the study.

Study instrument:

A structured questionnaire was used to collect information from all the respondents. The tool extracted information about demography, socio-economic characteristics of the respondents, history of risk factors, history of medication, clinical history, and antiretroviral therapy. The data collection tool was adapted from a similar previous study (7).

The questionnaire was pretested at a secondary health facility in the same geographical region as the study area. Before the commencement of data collection, we ensured that the questionnaire issues were identified and corrected.

Specimen collection:

Stool samples from recruited HIV-infected patients with diarrhea were collected by the patients in clinics, wards, and some at home in a wide-mouth screw cap labeled container and transported to the Medical Microbiology laboratory of UMTH Maiduguri for analysis. Each sample was allocated a laboratory number. In case of delay in sample processing, it was stored at 2-8°C for 24 hours.

Macroscopic examination:

The samples were examined macroscopically to detect the presence of any color, consistency (whether formed, semi-formed, soft, or watery), and presence of blood, mucus, and adult worms. The sample was then divided into two portions, one for molecular studies and the other portion for microscopy.

DNA extraction:

DNA extraction was done on the aliquots of the stool sample after defrosting itusing commercially available QIaAmp stool Minikit (Qiagen, Germany) as per the manufacturer's instructions, with some modifications as described by Okojokwu et al., (8). Fecal suspensions were prepared by adding saline (0.8% w/v) in a ratio of 1:1 (v/v). Next, fecal material (0.1ml) was added to 1.4 ml of alkali wash solution (0.5M NaOH and 0.05 M sodium citrate) in a 1.5ml Eppendorf tube and mixed for 30 seconds at room temperature by repeated inversion.

The mixture was subsequently centrifuged at 13,000g for 5min, and the cell pellet containing any cryptosporidial DNA was re-suspended in 0.5ml of 0.5M Tris-HCl (pH 8.0) and centrifuged at 13,000g for 5min. This latter step was repeated, and the resulting pellet was re-suspended in Tris-EDTA (0.1 ml, ten mM Tris-HCl, pH 8.0, containing one mM EDTA) and the DNA extracted from the oocysts by 10-14 cycles of freeze-thawing (in ice for 1 min; in a water bath at 100°C for 1 min). The resulting extract was centrifuged at 13,000g for 15min, and the supernatant containing cryptosporidia DNA was transferred to a clean tube and stored frozen before PCR. This procedure was performed at the University of Maiduguri Biotechnology Center.

Nested PCR amplification:

A nested PCR targeting the small subunit of the 18S rRNA gene was performed at University of Maiduguri Biotechnology center with the use of initial primers Cr18PA: (5′-TTCTAGAGCTAATACATGCG-3′) and Cr18PB: (5′-CCCATTTCCTTCGAAACAGGA-3′), which amlified a 1.3 kb fragment of 18S-rRNA gene. The inner primer Cr18NA: 5′-GGAAGGGTTGT ATTTATTAGATAAAG-3′ and Cr18NB: 5′-CTCA

TAAGGTGCTGAAGGAGTA-3' then amplified a 826-864bp fragment of initial amplified sequence.

The reaction mixture of the PCR consists of 3µL, 1xPCR buffer (50mM KCl, 20 mM Tris-HCL, 2.5 mM $Mgcl_2$, pH 8.4) and 1µL of 0.1µg/ml BSA, 2µL of the initial primers, and 1µL of 0.3mM concentration of each of dNTPs, 0.5µL of recombinant Taq polymerase and 2 µL of the purified DNA and 2µL of 1.5 mM $Mgcl_2$. The reaction mixture was prepared as described above for the second round of amplification, except for the primers and DNA template. Again, 1 µL of the first PCR product as a source of DNA and the inner primers was used.

Primary amplification was carried out in 35 cycles, each consisting of denaturation at 95ºC for 30 seconds, annealing at 45ºC for 30 seconds, and extension at 68ºC for 30 seconds, with an initial denaturation at 95ºC for 3 min and a final extension at 68ºC for 5 min. The secondary round of amplification consisted also of 35 cycles with identical temperatures and times,and annealing temperature of 48 ºC. All PCRs were run in a Thechne PCR thermocycler.

***Cryptosporidium* speciation by restriction fragment length polymorphism analysis:**

RFLP analysis of the secondary PCR products using restriction enzymes *SspI* (New England Biolab, Ipswich, USA) and *VspI* (Promega, Madison, USA) was used to characterize *Cryptosporidium* species. Six microliters (6µL) of the PCR product were digested with 6 units of each enzyme in the same reactions at 37ºC for 4 hours. Positive controls, including PCR mixture reaction with the DNA extract of known *Cryptosporidium* positive stool samples were used in both PCR assays while PCR mixture reaction without DNA was used as negative control in both PCR.

Loading and electrophoresis of the sample:

Five microliters (5µL) of amplified undigested and digested PCR products were mixed with 2.0µL of loading buffer. The mixture was slowly loaded into the well using disposable micropipette tips. A 1500 bp molecular weight marker was loaded in one well to determine the size of the amplified PCR products and 1000 bp for RFLP analysis. Electrophoresis was carried out at 100 volts for 35 minutes. The intensity and size of all amplicons were assessed by electrophoresis in 1.5% ethidium bromide-stained agarose gels using Tris Borate EDTA (65 mMTris-HCl, 27 mM boric acid, one mM Ethylene diamide tetra acetate, pH 9; Bio-Rad, USA) as the buffer and 1.5kbp molecular marker (Fermentas).

The amplified products of the study samples were visualized in a trans-illuminator, and the gel was photographed by a digital camera and transferred data to a computer for further documentation. Negative control was used for each amplification.

Data analysis:

The information obtained from the patient questionnaire and other relevant data from this study was entered into a computer program. Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 23.0. The data were presented in tables/figures as frequencies and proportions for univariate analysis, while for bivariate analysis Chi-square test was used to test for association between independent variable of socio-demographic/risk factors and dependent variables.

Results:

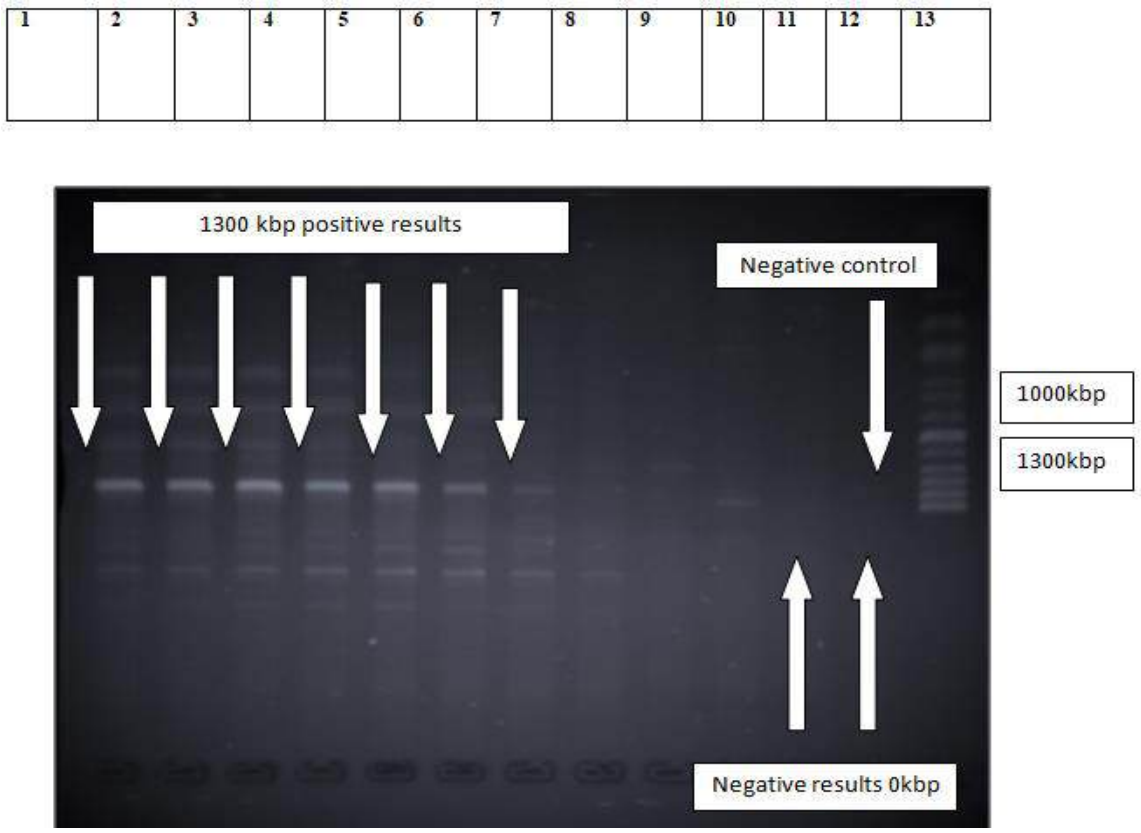
Two hundred and sixty-nine HIV patients were recruited and their samples analyzed. Majority of the patients were females (n=189, 70.3%) while 80 (29.7%) were males (Table 1). Stool samples of 52 of the 269 HIV patients were amplified by PCR, giving the prevalence of cryptospiridiosis of 19.3% in the study. The PCR SSU rRNA genes of *Cryptosporidium* produced bands with a molecular size of 1,300 bp (Fig 1). The secondary PCR products of the 18S rRNA gene yielded bands that coincided with the expected 830 bp for *Cryptosporidium* species (Fig 2).

The prevalence of *Cryptosporidium* infection was higher in males (n=20, 25.0%) compared to females (n=32, 16.9%) but the prevalence difference was not significantly different (p=0.1729) (Table 1). The prevalence was highest in the age group 18-27 years (n=7, 23.5%), while the lowest prevalence (n=5, 11.6%) was in the age group 48-57 years, but there was nosignificant difference in prevalence with respect to age group (p=0.4905) (Table 1). Patients with no or primary education had the highest prevalence (n=19, 24.1%), while patients with tertiary education had the lowest prevalence (n=7, 16.3%) but the prevalence difference was not significantly different with respect to education (p=0.4404) (Table 1). Based on occupational status, the prevalence was highest among the patients who are farmers (n=5, 31.3%) but there was no significant difference in prevalence with respect to occupation (p=0.5440) (Table 1).

Representative electrophoresis profiles of the restriction enzymes digests of the PCR amplified products are presented in Fig 3 and Fig 4. The bands were of molecular sizes 450 bp, 260 bp, 111 bp, and 108 bp and were indicative of *C. hominis* and *C. parvum* (Fig 3). Of the 52 positive samples, 40.4% were *C. hominis,*and 59.6% were *C. parvum* (Fig 5).

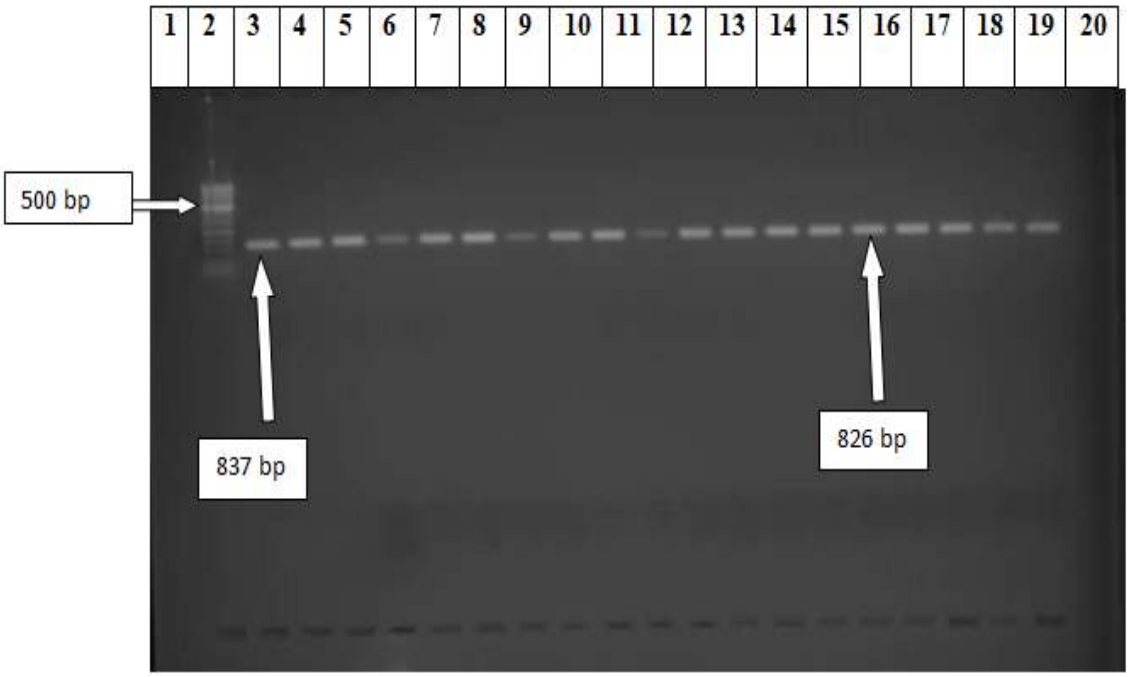
Table 1: Socio-demographic characteristics and prevalence of cryptosporidiosis in the study participants

Variable	Frequency (%) (n=269)	Prevalence by PCR (%) (n=52)	x^2	*p* value
Age group (years)				
18-27	28 (10.4)	7 (25.0)	3.417	0.4905
28-37	85 (31.6)	20 (23.5)		
38-47	100 (37.2)	8 (18.0)		
48-57	43 (15.9)	5 (11.6)		
≥ 58	13 (4.8)	2 (15.4)		
Sex				
Male	80 (29.7)	20 (25.0)	1.858	0.1729
Female	189 (70.3)	32 (16.9)		
Level of education				
None/Primary	79 (29.4)	19 (24.1)	1.640	0.4404
Secondary	147 (54.6)	26 (17.7)		
Tertiary	43 (16.0)	7 (16.3)		
Occupation				
Business	82 (30.5)	12 (14.6)	0.3083	0.5440
Civil service	68 (25.3)	13 (19.1)		
Farming	16 (5.9)	5 (31.3)		
Housewife	91 (33.8)	20 (22.0)		
Student	12 (4.7)	2 (16.7)		



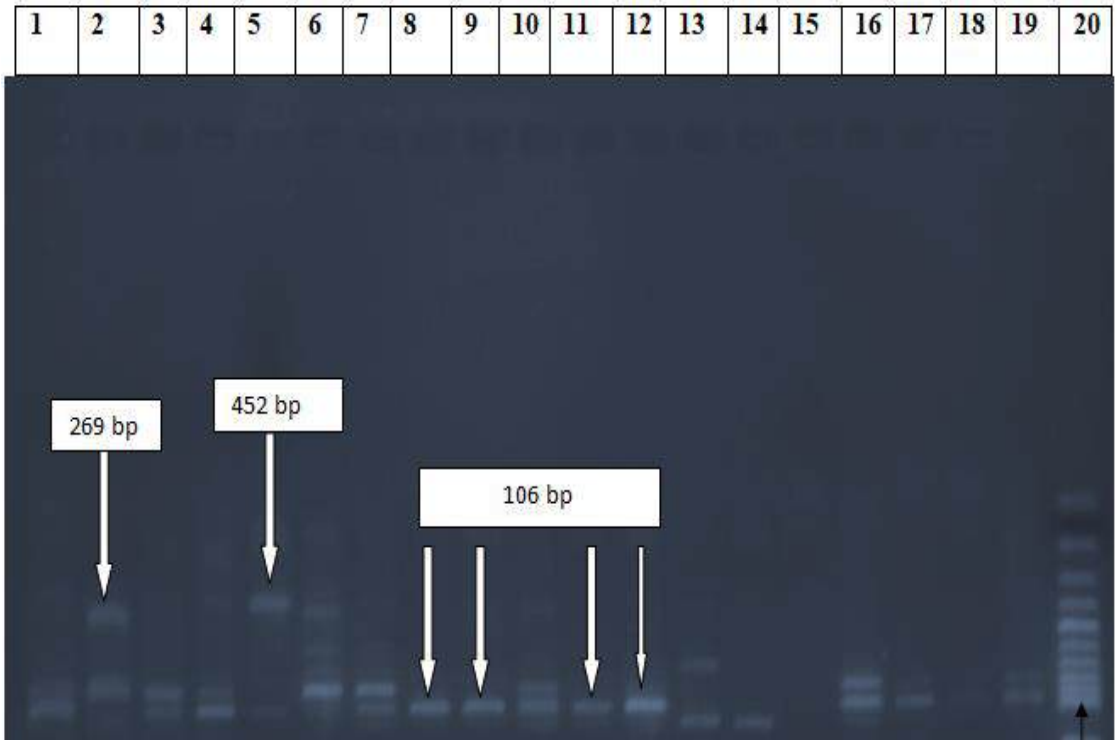
Lane 13 - 1.5 kbp molecular maker; Lane 12 - Negative control for *Cryptosporidium* spp; Lane 1-7 - Clinical samples positive for *Cryptosporidium* spp; Lane 8-11 - Clinical samples negative for *Cryptosporidium* spp

Fig 1: Gel electrophoresis picture of PCR amplification products of SSU rRNA gene



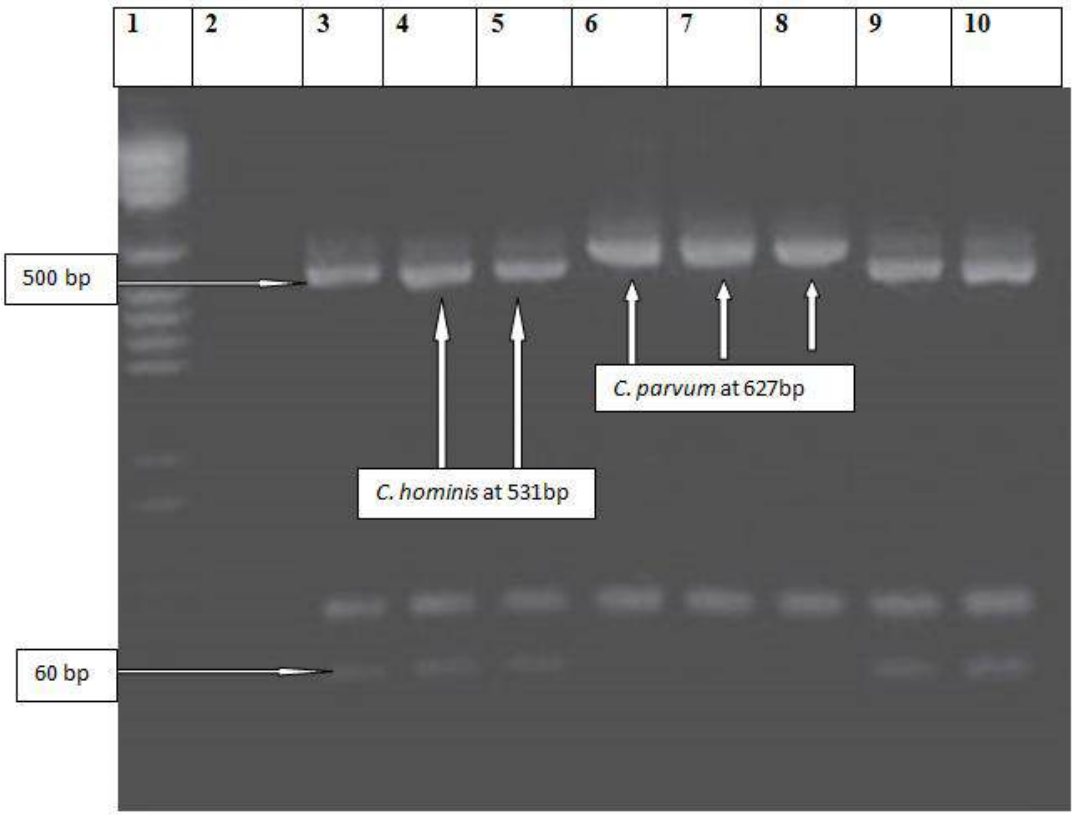
Lane 1 - molecular marker; Lane 2-20 - Positive samples between 826 – 837 bp.

Fig 2: Gel electrophoresis of *Cryptosporidium* 18 NA amplified products with the inner primer Cry18NA.



Lane 20 - Molecular marker at 1000 bp; Lanes 2 and 5 - *Cryptosporidium hominis* at 269 bp and 452 bp respectively; Lane 6 - *Cryptosporidium hominis* positive control; Lane 1 - *Cryptosporidium hominis* negative control; Lanes 3,7,8,9,10,11,12,16,17 - *Cryptosporidium parvum* positive (106 bp); Lane 19 - *Cryptosporidium parvum* positive control; Lane 18 - *Cryptosporidium parvum* negative control

Fig 3 PCR-RFLP analysis using *Sspl* and *Vspl* restriction enzymes for differentiating *Cryptosporidium* species.



Lane 1; MW, Lane 2; negative control, Lane 3; positive control for *C. hominis*(531 bp),Lane 6; positive control for *C. parvum* (627 bp), Lanes 4,5,9,10; *C. hominis* positive isolates, Lanes 7 & 8; *C. parvum* positive isolates.

Fig 4: Species identification of *Cryptosporidium* by RFLP analyses of 18S rRNA gene with *Vspl* restriction enzyme

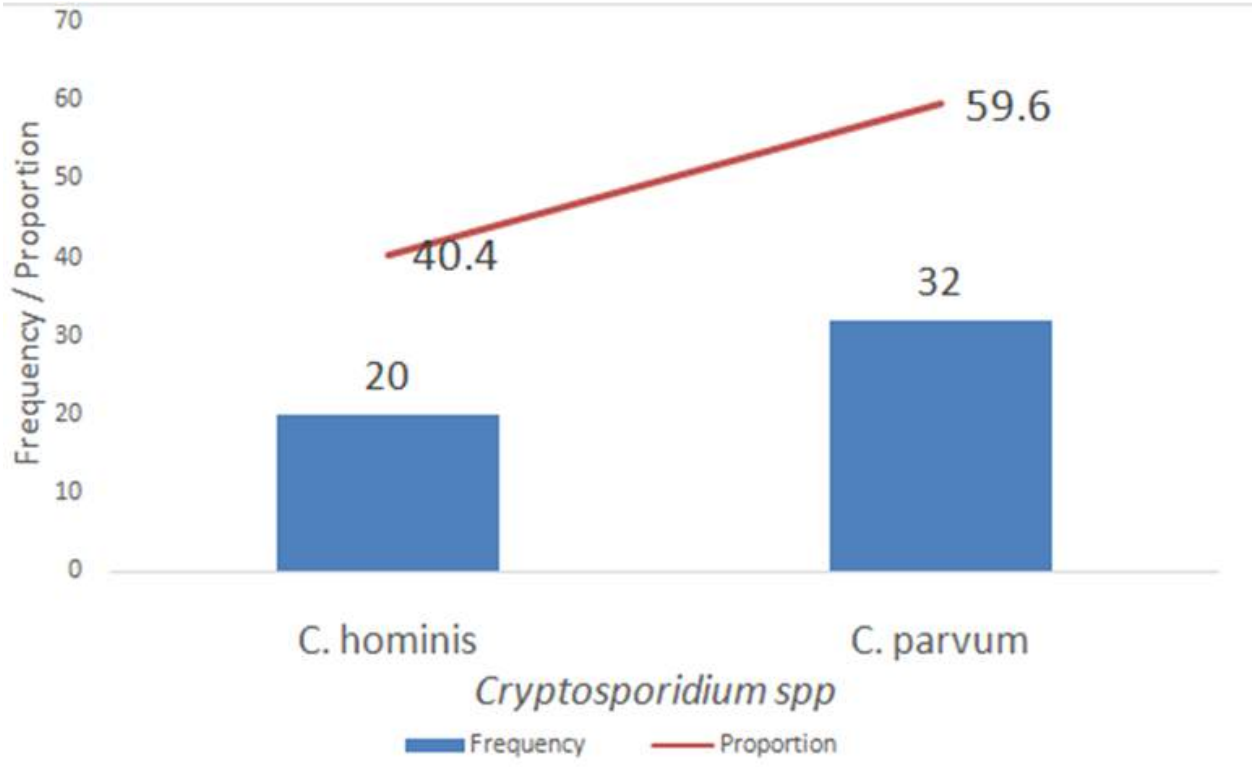


Fig 5 : Frequency of *Cryptosporidium* species identified among HIV-infected patients with diarrhea in Maiduguri

Discussion:

This study reported a prevalence of 19.6% for cryptosporidiosis amongst HIV-positive patients in Maiduguri. This prevalence is similar to earlier reported findings among HIV-infected patients in Michika, northeast Nigeria (9), andin a study done in Jos North central Nigeria, where the prevalence of cryptosporidiosis was 27% in HIV-infected patients (10). This study reported a higher prevalence compared to a study by Olopade et al., (11). This disparity could be a result of the nature of the stool sample used, as it has been shown that diarrhoeic samples yield more *Cryptosporidium* oocyst than non-diarrhoeic stool (12). Many of the participants in our study were residents of internally displaced persons (IDP) camps with poor water and sanitation hygiene, poor access to affordable and quality education, and inadequate healthcare services, among others. This setting promotes the feaco-oral transmission of infection, and may partly explain the high prevalence of cryptosporidium infection observed in the study. Immuno-suppressed individuals show a two-fold increase in prevalence rate when compared with immune-competent individuals (13).

The molecular assays revealed that *C. parvum* is the most common species found in HIV-infected patients in Maiduguri. This may be due to the fact that participants have a history of contact with animals and reside in overcrowded areas. This will explain both zoonotic and anthropogenic transmission. *Cryptosporidium parvum* was slightly more prevalent than *C. hominis* as the leading cause of human cryptosporidiosis in this study. Another reason could be the increased likelihood of animal-human contact among residents of the study area. Both domesticated animals are allowed to roam freely on the streets and animal dung is used as fertilizer during irrigation farming in the study area. This finding is in keeping with what was found in other parts of the world (14). Our study is in concordance with the results by Xin Yang et al., (15), and also with studies from other parts of Nigeria (7,16). The prevalence and distribution of this coccidian disease tend to vary from one locality to another and from one country to another based on the contamination rate of food, water, and contact with animals.

Furthermore, variations in sample size and sampling techniques play a pivotal role in the differences in results (7). Differences in the distribution of *Cryptosporidium* species are considered an indication of differences in the source of infection. The predominance of *C. parvum* in a population could result from both zoonotic and anthroponotic transmission, while *C. hominis* is considered to be a result of anthroponotic routes (17). Our study is limited by the fact that only two species of *Cryptosporidium* could be identified in the study because the restriction enzymes used could only detect *C. parvum* and *C. hominis.*

Conclusion:

Our study found a prevalence of 19.6% of cryptosporidiosis among HIV patients in Maiduguri with a predominance of *C. parvum.* There is need for health education of people at risk of cryptosporidiosis to interrupt transmission of this opportunistic parasite. Potable drinking water should be provided to the populace with particular emphasis on people with compromised immune status. Routine use of PCR for identification of *Cryptosporidium* in stool samples of HIV patients should be adopted.

Contributions of authors:

MUK, YMY, GBG and KBA contributed to writing the concept notes and formulating the study design, JA and ASB, MY, AUM and ZBS worked on data collection, data analysis and interpretation, laboratory analysis and interpretation. BBG, ABS, MD, ZG and all the authors contributed significantly during the manuscript drafting, revision and accepts responsibility for the integrity of the data and accuracy of analyses.

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No funding was received for the study

Conflict of interest:

No conflict of interest is declared.

Availability of data and material:

The datasets are available on reasonable request from the corresponding author

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Original Article **Open Access**

Prevalence of enteric parasitic infection among people living with HIV in parts of southeastern Nigeria

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Abstract:

Background: Intestinal parasites in HIV/AIDS patients increase the risk of gastroenteritis, adding to the complexity of the disease. The interactions of intestinal parasites are one of the factors leading to HIV replication and progression of AIDS. Chronic immunosuppression caused by HIV infection makes people vulnerable to parasitic infestations, and this is associated with a $CD4^+$ cell count of less than 200/mm^3. This study is aimed at determining the prevalence of enteric parasitic infection among people living with HIV in southeast Nigeria.
Methodology: This was a cross-sectional study of 535 patients recruited from Government owned tertiary health institutions with HIV referral and center for HIV/AIDS management under the Presidents Emergency Plan for Aids Relief (PEPFAR) in the 5 states of southeast Nigeria (Abia, Anambra, Ebonyi, Enugu and Imo) from September 2018 to December 2023. The institutions included FMC-Umuahia, NAUTH-Nnewi, FMC-Abakaliki UNTH-Enugu and FMC-Owerri. Stool and blood samples collected from the participants were examined using standard methods for parasitological and haematological assessment according to the guidelines of the World Health Organization. Clinical and laboratory data were analysed using the statistical software INSTAT and SPSS version 22.0, with statistical significance set at $p<0.05$.
Results: The prevalence of intestinal parasites among the 535 patients was 43.2% (n=231). *Ascaris lumbricoides* followed by hookworm emerged as the most prevalent parasites identified with 33.3% (77/231) and 29.9% (69/231) respectively while *Schistosoma japonicum, Trichuris trichiura, and Enterobius. vermicularis* had very low prevalence of 0.9% (2/231) each. Imo State had the highest prevalence of intestinal parasitic infection (48.0%, 48/100), followed by Anambra State (46.0%, 46/100), Abia State (44.4%, 60/135), Ebonyi State (42.0%, 42/100) and Enugu State (35.0, 35/100) but the prevalence was not significantly different between the States ($p=0.3870$). Patients in the age groups 21-30, 31-40, 41-50 and 51-60 years had statistically significant higher prevalence and egg burden ($p=0.000$). The $CD4^+$ T cell count of the participants showed a statistically significant decrease ($p<0.05$) as the egg burden increases.
Conclusion: In view of the high prevalence and egg burden of intestinal parasites among HIV infected patients in this study, serious efforts must be made by appropriate government authorities to institute a regular deworming process for the populace, to reduce the egg burden of intestinal parasitosis in southeast Nigeria.

Keywords: Enteric Parasite; HIV/AIDS; $CD4^+$ T cell: egg burden; southeast Nigeria

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Prévalence des infections parasitaires entériques chez les personnes vivant avec le VIH dans certaines régions du sud-est du Nigéria

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Résumé:

Contexte: Les parasites intestinaux chez les patients atteints du VIH/sida augmentent le risque de gastro-entérite, ce qui complexifie la maladie. Les interactions entre parasites intestinaux sont l'un des facteurs favorisant la réplication du VIH et la progression du sida. L'immunosuppression chronique causée par l'infection par le VIH rend les personnes vulnérables aux infestations parasitaires, ce qui est associé à un taux de $CD4^+$ inférieur à 200/mm^3. Cette étude vise à déterminer la prévalence des infections parasitaires entériques chez les personnes vivant avec le VIH dans le sud-est du Nigéria.
Méthodologie: Il s'agissait d'une étude transversale portant sur 535 patients recrutés dans des établissements de santé tertiaires publics avec référence VIH et centre de gestion du VIH/SIDA dans le cadre du Plan d'urgence du Président pour la lutte contre le sida (PEPFAR) dans les 5 États du sud-est du Nigéria (Abia, Anambra, Ebonyi, Enugu et Imo) de septembre 2018 à décembre 2023. Les institutions comprenaient FMC-Umuahia, NAUTH-Nnewi, FMC-Abakaliki UNTH-Enugu et FMC-Owerri. Les échantillons de selles et de sang prélevés sur les participants ont été examinés à l'aide de méthodes standard d'évaluation parasitologique et hématologique conformément aux directives de l'Organisation mondiale de la santé. Les données cliniques et de laboratoire ont été analysées à l'aide du logiciel statistique INSTAT et SPSS version 22.0, avec une signification statistique fixée à $p<0,05$.
Résultats: La prévalence des parasites intestinaux parmi les 535 patients était de 43,2% (n=231). Français *Ascaris lumbricoides* suivi de l'ankylostome sont apparus comme les parasites les plus répandus identifiés avec respectivement 33,3% (77/231) et 29,9% (69/231) tandis que *Schistosoma japonicum, Trichuris trichiura* et *Enterobius vermicularis* avaient une très faible prévalence de 0,9% (2/231) chacun. L'État d'Imo avait la plus forte prévalence d'infection parasitaire intestinale (48,0%, 48/100), suivi de l'État d'Anambra (46,0%, 46/100), de l'État d'Abia (44,4%, 60/135), de l'État d'Ebonyi (42,0%, 42/100) et de l'État d'Enugu (35,0%, 35/100) mais la prévalence n'était pas significativement différente entre les États (p=0,3870). Les patients des tranches d'âge 21-30, 31-40, 41-50 et 51-60 ans présentaient une prévalence et une charge en œufs significativement plus élevées (p=0,000). Le taux de lymphocytes T $CD4^+$ des participants diminuait significativement ($p<0,05$) à mesure que la charge en œufs augmentait.
Conclusion: Compte tenu de la prévalence et de la charge en œufs élevées des parasites intestinaux chez les patients infectés par le VIH participant à cette étude, les autorités gouvernementales compétentes doivent déployer des efforts importants pour instaurer un déparasitage régulier auprès de la population afin de réduire la charge en œufs des parasitoses intestinales dans le sud-est du Nigéria.

Mots-clés: Parasite entérique; VIH/SIDA; cellules T $CD4^+$: charge en œufs; sud-est du Nigéria

Introduction:

Enteric parasitic infections are significant health concern among people living with the human immunodeficiency virus (PLWHIV) and accounts for up to 80% of HIV-related death (1). HIV exposes a person to so many opportunistic infections by side-stepping and suppression of the immune system leading to high morbidity and mortality. Enteric parasitic infections in HIV infected individual weakens the immune system faster and hasten the progression of HIV to AIDS (2). Helminthes and intestinal protozoa are the mostly implicated enteric parasite globally and have a high negative effect among HIV/AIDS patients. Co-infections of HIV/AIDS and enteric parasite is a crucial health issue in Africa where HIV infection is endemic (2). These enteric parasitic infections cause lingering diarrhea, weight loss, malnutrition making it a health threat in people living with HIV/AIDS (1).

The immunopathologic activities of enteric parasite involve the activation of T helper cells. Research has shown that HIV/AIDS infected individuals are the most vulnerable to enteric parasite infestation and a greater percentage die as a result of HIV related issues than from HIV infection alone. A decrease in the number of $CD4^+$ T helper cells below 200/mm^3 exposes the patients to opportunistic infections (3).

Enteric parasites have been reported to be the cause of diarrhea that cut across all age groups with severe impact among people with weaken immune system or life treating diseases as well as in children (1). These enteric parasites such as *Cryptosporidium parvum, Isospora belli, Microsporidia* spp, *Strongyloides stercoralis* and *Trichuris trichiura* causes severe diarrhea in immunocompromised individuals which can lead to dehydration, loss of electrolytes, malnutrition and in some cases, death (1). The mode of transmission of these enteric parasites is through fecal-oral ingestion of contaminated water or food, penetration of unbroken skin and person to person transmission (4).

There is paucity of information on the dynamics of parasitic infection in HIV/AIDS patients in southeastern part of Nigeria. The objective of this study is to determine the prevalence of enteric parasitic infection among people living with HIV/AIDS in southeastern Nigeria.

Materials and method:

Study design and setting:

A descriptive cross-sectional study was carried out to determine the prevalence of enteric parasitic infections among people living with HIV/AIDS attending the HIV clinics of selected tertiary hospitals with referral status and center for HIV/AIDS in southeast Nigeria. The hospitals are University of Nige-

ria Teaching Hospital (UNTH)-Enugu, Federal Medical Center (FMC)-Abakaliki, Nnamdi Azikiwe University Teaching Hospital (NAUTH)-Nnewi, Federal Medical Center (FMC)-Umuahia and Federal Medical Center (FMC)-Owerri.

Ethical approval:

Ethical clearance was obtained from the Ethics Committee of University of Nigeria Teaching Hospital, Ituku-Ozalla, Enugu State, Nigeria

Sample size calculation:

A single population-proportion was used to determine the sample size with the formula; $n = Z^2 P (1-P)/d^2$, where n = sample size, p = prevalence of intestinal parasitic infection of 40% (0.4) from a previous study determined by Abdulkadir et al., (5), d = margin of error, and Z = standard deviation at 95% confidence interval corresponding to 1.96. To account for attrition arising from the likelihood of non-compliance, 10% of the calculated sample size was adjusted to give a total of 535 patients for the study.

Inclusion/exclusion criteria and method of sampling:

The inclusion criteria for the study were patients confirmed with HIV attending the HIV clinic at the time of the study, who were willing to participate by signing the informed consent form, and who have not taken anti-parasitic drug in the one month preceding the study.

Participants were recruited using systematic sampling technique, taking the ART clinic registration book as a sampling frame. Participants who had taken any anti-parasitic drugs within the prior 4weeks were excluded.

Data collection:

A pre-designed structured questionnaire was used to collect socio-demographic characteristics of the participants in a face-to-face interview. Information such as age, sex, occupation, hand washing hygiene practices, marital status educational status etc were collected from each participant

Sample collection and processing:

Freshly voided faecal sample was collected by each participant into a stool specimen container and 5 ml of venous blood into EDTA bottle. The bottles were properly labeled with patient identification number and transported to the laboratory for processing.

Laboratory analysis of specimen:

Macroscopic examination of the stool sample was done to observe adult worms of enteric parasites, and to note the consistency and colour of the stool samples. Microscopic examination of the stool samples for parasite ova, larva, cysts or trophozoites was done using wet mount, concentration methods (formolether concentration and brine floatation method), Gram-chromotrope staining technique for *Microsporidia* and modified Ziehl Neelsen staining technique as described by Abayneh et al., (3).

The Stoll's egg count was carried out on all the faecal samples that were positive for parasitic ova in wet preparation in order to determine the egg count burden of the parasite per gram faeces of the participants as described by Jawetz et al (6).

Flow cytometer (Partec Immunocytometry system, Singapore) was used to analyze the $CD4^+$ T lymphocyte estimation as described by Flynn and Gorry (7), and haemoglobin estimation using autoanalyzer (Zybio Full Automation) according to manufacturer's instruction.

Data analysis:

Data obtained were analysed with the Chi-square (x^2) test and Odds ratio (OR) for qualitative variables and ANOVA and Student's *'t'* test for quantitative variables using the statistical software INSTAT and SPSS version 22.0. Statistical significance was set at 95% level of significance.

Results:

A total of 535 patients (167 males, 368 females) were recruited for sample collection, 231 (43.2%) were infected with one or two enteric parasites, giving an overall prevalence of intestinal parasitosis of 43.2% in the study population (Table 1).

Table 1: Prevalence of Intestinal parasitic infection in the study population

Characteristics	No of participants recruited	No of participants positive for parasite (prevalence in %)	Chi square (X^2)	Odds ratio (95% CI)	p-value
Gender					
Male	167	71 (42.5)	0.01305	0.962	0.8511
Female	368	160 (43.5)		(0.6643-1.391)	
Total	535	231 (43.2)			
Age group (years)					
<10	9	2 (22.2)	4.306	NA	0.8285
11 – 20	24	11 (45.8)			
21 – 30	49	23 (46.9)			
31 - 40	172	72 (41.9)			
41 – 50	172	77 (44.8)			
51 – 60	76	29 (38.2)			
61 - 70	26	14 (53.8)			
71 – 80	5	2 (40.0)			
81 – 90	2	1 (50.0)			
Total	535	231 (43.2)			
State					
Abia	135	60 (44.4)	4.143	NA	0.3870
Anambra	100	46 (46.0)			
Ebonyi	100	42 (42.0)			
Enugu	100	35 (35.0)			
Imo	100	48 (48.0)			
Total	535	231 (43.2)			

NA = Not applicable; CI = Confidence interval

Table 2: Frequency distribution of intestinal parasites in people living with HIV/AIDS in southeast Nigeria.

Parasites	Frequency	Percentage (%)
Ascaris lumbricoides	77	33.3
Hookworm	69	29.9
Giardia lamblia	24	10.4
Entamoeba histolytica	19	8.2
Strongyloides stercoralis	17	7.4
Cryptosporidium parvum	8	3.5
Microsporidia spp	6	2.6
Isospora belli	5	2.2
Schistosoma japonicum	2	0.9
Trichuris trichiura	2	0.9
Enterobius vermicularis	2	0.9
Total	**231**	**100.0**

Ascaris lumbricoides was the most frequently identified parasite in 77 (33.3%) of the 231 positive patients, followed closely by hookworm from 69 (29.9%), *G. lamblia* from 24 (10.4%) and *E. histolytica* from 19 (8.2%) patients. Other parasites included *S. stercoralis* from 17 (7.4%), *C. parvum* from 8 (3.5%), *Microsporidia* spp from 6 (2.6%), *I. belli* from 5 (2.2%) and *Schistosoma japonicum*, *T. trichiura*, and *Enterobius vermicularis* from 2 (0.9%) patients each (Table 2).

Table 3 provides the frequency of patients in each State who were positive for specific parasites, offering a comparative view of parasitic infection rates across the regions. Hookworm and *A. lumbricoides* were the most frequently detected parasites among the participants across the States. For example, the frequency of hookworm infection was relatively similar among the States, ranging from 23.8% in Ebonyi to 33.3% in Abia. *Ascaris lumbricoides* also shows high frequency, especially in Imo (39.6%) and Ebonyi (35.6%).

Other parasites such as *E. histolytica, S. stercoralis*, and *G. lamblia* were moderate in frequency, with *G. lamblia* showing the highest frequency in Enugu at 17.1%. *Microsporidia* spp and *S. japonicum,* showed low frequency across the States, and sometimes entirely absent, indicating that they are of less concern in the population. The frequency of other parasites such as *I. belli, T. trichiura,* and *E. vermicularis* was very low but consistent in a few States.

Table 3: Frequency distribution of intestinal parasites detected from study participants across the States

Parasites	**Abia (%)**	**Anambra (%)**	**Ebonyi (%)**	**Enugu (%)**	**Imo (%)**
Hookworm	20 (33.3)	13 (28.3)	10 (23.8)	11 (31.4)	15 (31.3)
Ascaris lumbricoides	19 (31.7)	14 (30.4)	15 (35.6)	10 (28.6)	19 (39.6)
Entamoeba histolytica	5 (8.3)	4 (8.7)	4 (9.5)	3 (8.6)	3 (6.3)
Strongyloides stercoralis	5 (8.3)	4 (8.7)	4 (9.5)	1 (2.9)	3 (6.3)
Giardia lamblia	4 (6.7)	4 (8.7)	5 (11.9)	6 (17.1)	5 (10.4)
Microsporidia spp	2 (3.3)	2 (4.3)	1 (2.4)	1 (2.9)	0
Cryptosporidium parvum	3 (5.0)	2 (4.7)	1 (2.4)	1 (2.9)	1 (2.1)
Schistosoma japonicum	0	0	0	1 (2.9)	1 (2.1)
Isospora belli	1 (1.7)	1 (2.2)	1 (2.4)	1 (2.9)	1 (2.1)
Trichuris trichiura	1 (1.7)	1 (2.2)	0	0	0
Enterobius vermicularis	0	1 (2.2)	1 (2.4)	0	0
Total	**60 (100.0)**	**46 (100.0)**	**42 (100.0)**	**35 (100.0)**	**48 (100.0)**

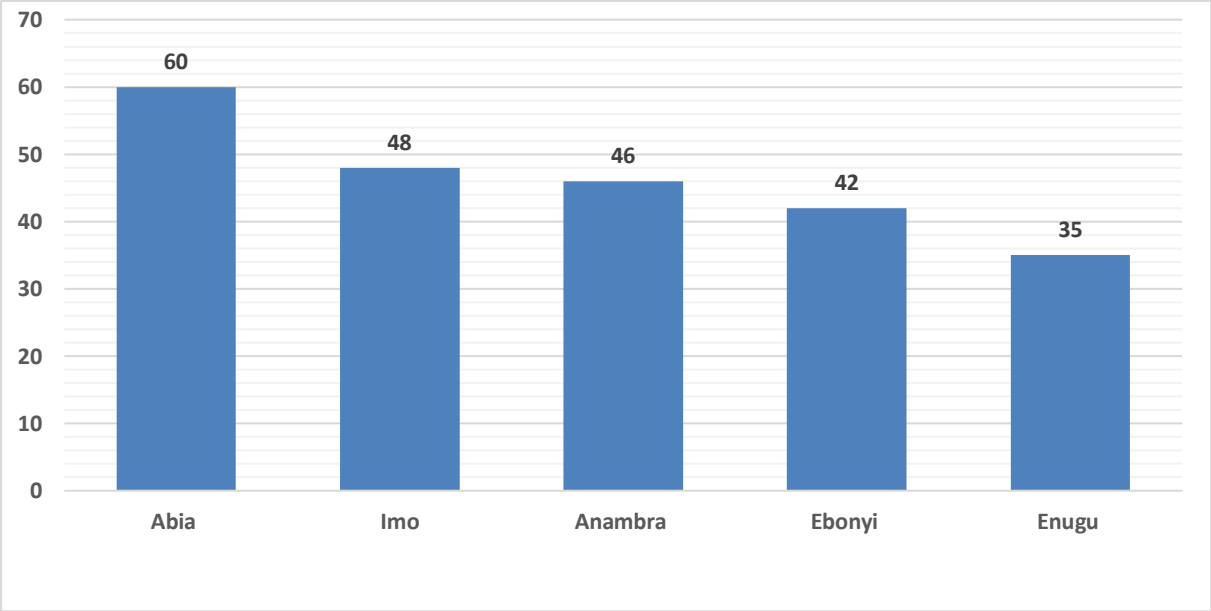

Fig 1: Frequency distribution of intestinal parasites from the study participants with respect to States

The number of participants with parasitic infection from each State (ranging from 35 in Enugu to 60 in Abia) show no statistically significant difference in the distribution of parasites across the states (p= 0.3870). This uniformity suggests that common environmental or socio-economic factors across these states might influence the overall distribution of parasitic infections among HIV/AIDS patients (Fig 1).

The frequency distribution of various parasites from HIV/AIDS patients according to sex is shown in Table 4. The total number of participants with intestinal parasitic infection in the study is 231, giving an overall prevalence of 43.2%, with a slightly higher prevalence in the females (43.5%, 160/368) than in the males 42.5%, 71/167) but no significant difference (p=0.8511). The most prevalent parasites among the patients are *A. lumbricoides* (77, 33.3%) and hookworm (69, 29.9%), with both being more frequent in females (36.9% and 31.3%) than in males (25.4% and 26.8%).

Other parasites such as *E. histolytica*, *S. stercoralis*, and *G. lamblia* show varied distributions but are less common. There is no statistically significant difference in the distribution of parasites between male and female patients (p=0.34). As shown in Fig 2, females are more frequently infected than the males across all the 5 States.

Table 4: Frequency distribution of intestinal parasites in people living with HIV/AIDS according to gender

Parasites	**Male (%)**	**Female (%)**	**Total (%)**	**X^2**	***p*-value**
Hookworm	19 (26.8)	50 (31.3)	69 (29.9)	0.622	0.430
Ascaris lumbricoides	18 (25.4)	59 (36.9)	77 (33.3)	5.110	0.024
Entamoeba histolytica	8 (11.3)	11 (6.9)	19 (8.2)	0.511	0.475
Strongyloides stercoralis	5 (7.0)	12 (7.5)	17 (7.4)	0.000	0.998
Giardia lamblia	10 (14.1)	14 (8.8)	24 (10.4)	3.373	0.338
Microsporidia spp	1 (1.4)	5 (3.1)	6 (2.6)	0.598	0.439
Cryptosporidium parvum	3 (4.2)	5 (3.1)	8 (3.5)	0.149	0.699
Schistosoma japonicum	1 (1.4)	1 (0.6)	2 (0.9)	0.330	0.566
Isospora belli	4 (5.6)	1 (0.6)	5 (2.2)	5.595	0.018
Trichuris trichiura	1 (1.4)	1 (0.6)	2 (0.9)	0.330	0.566
Enterobius vermicularis	1 (1.4)	1 (0.6)	2 (0.9)	0.330	0.566
Total	**71 (30.7)**	**160 (69.3)**	**231 (100.0)**		

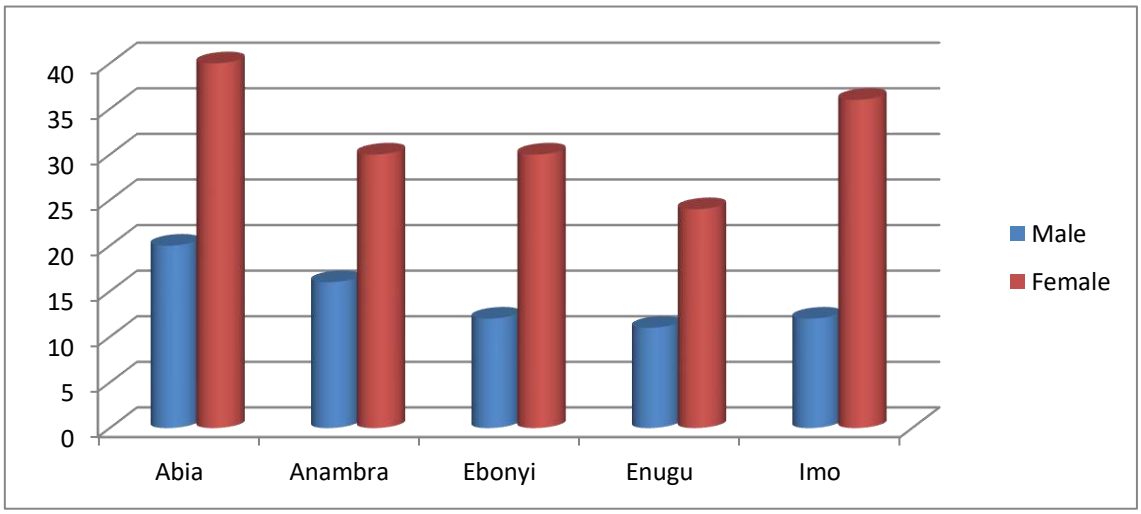

Fig 2: Frequency distribution of intestinal parasites in people living with HIV/AIDS according to States and gender

The distribution of parasitic egg burdens in relation to the $CD4^+$ T cell count of the HIV/AIDS patients is presented in Fig 3. Patients with lower $CD4^+$ T cell counts (100-500 cells/mm^3) had higher percentages of egg burdens, suggesting that a weaker immune system is associated with a higher load of parasitic eggs. For instance, patients with $CD4^+$ T cell count in the range of 801-900/mm^3 have 66.7% egg burden, which significantly increases to 100.0% with $CD4^+$ T cell count in the range of 1001-1100/mm^3, indicating severe parasitic infections as the immune system further weakens. Conversely, as $CD4^+$ T cell counts increase, the percentage of parasitic egg burden generally decreases, indicating better immune responses capable of controlling parasitic infections.

The highest $CD4^+$ T cell counts range of 1101-1500/mm^3 show minimal to no egg burden in the stool of the participants. The ANOVA test showed statistical significance (p=0.0005) between egg burden and $CD4^+$ T cell count.

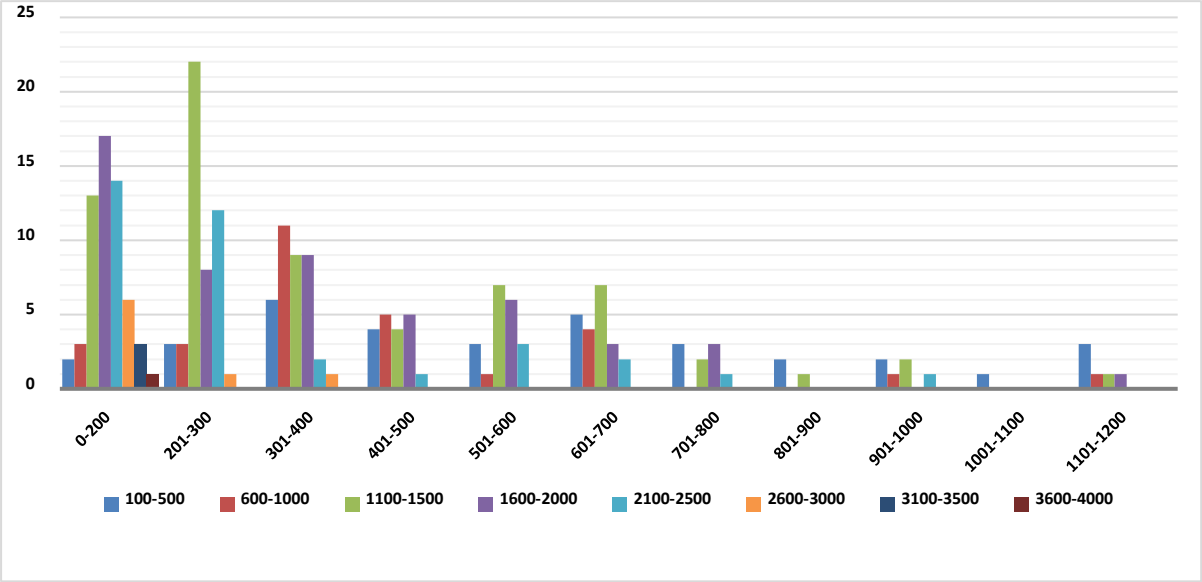

Fig 3: Distribution of egg burden with respect to $CD4^+$ T cell count

The distribution of parasitic egg burdens among HIV/AIDS patients with respect to levels of hemoglobin (Hb) is shown in Fig 4, with a critical measure of blood health, across several defined ranges of 3 - >20.9 g/dl. The figure categorized patients into Hb level groups and related these with the intensity of their parasitic egg burdens, expressed in percentages within specific egg burden ranges (100-4000). A notable trend observed is the concentration of higher egg burdens within intermediate Hb levels, particularly in the 9-11.9% and 12-14.9% Hb ranges, where parasitic egg burdens are most prevalent. For instance, patients with Hb levels between 9-11.9% and 12-14.9% display significant percentages of egg burdens.

As Hb levels increase, (15-17.9% and 18-20.9%), there is a notable decrease in the egg burden percentages, except for a 100% occurrence in the highest Hb level (18-20.9%) within the 600-1000 egg burden range. Conversely, very low and very high Hb levels show fewer incidences of egg burdens. The ANOVA test showed statistically significant association (p=0.0001) between egg burden across different Hb levels, suggesting a complex interaction between parasitic infections and patient health as reflected in Hb levels.

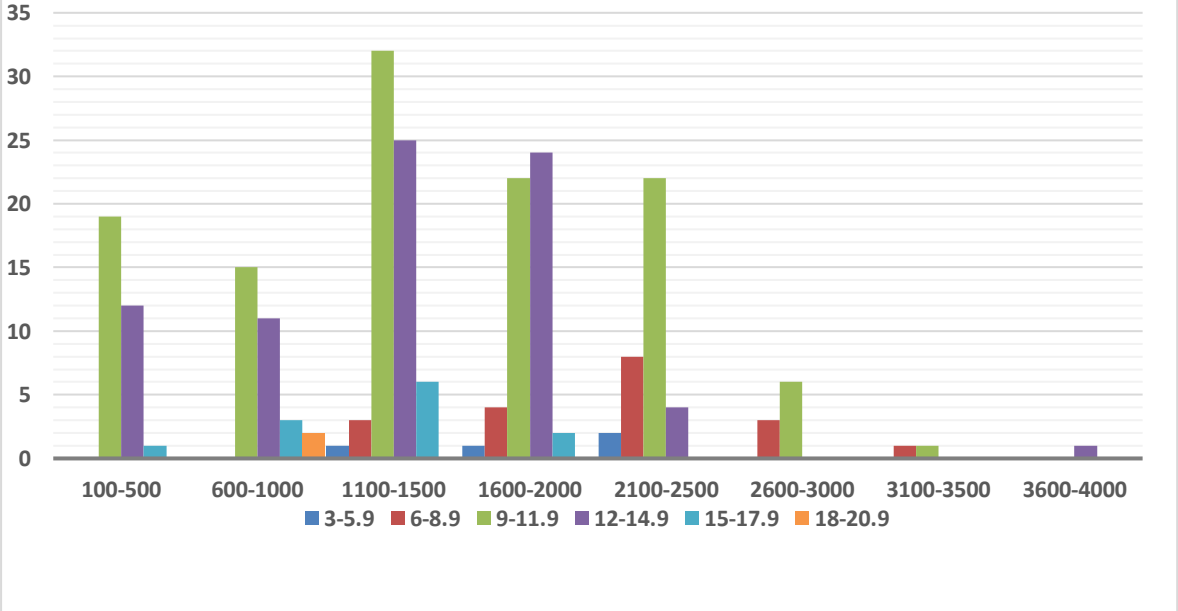

Fig 4: Distribution of egg burdens with respect to haemoglobin level

The distribution of parasitic egg burdens among various age groups, highlighting how these burdens vary across different stages of life is shown in Table 5. The highest concentrations of egg burdens are found in the middle age brackets, particularly from 21-60 years. For instance, individuals aged 21-30, 31-40, 41-50 and 51-60 years show significantly higher percentages of egg burdens (30.4%, 24.0%, 27.3% and 44.4% respectively in the 1100-1500/gram of faeces).

The table also reveals that the youngest (0-10 years) and oldest (81-90 years) age groups have lower frequencies of parasitic eggs. There was a sharp decrease in egg burdens for those above 60 years, except for a few cases in the 51-60 years age brackets. ANOVA test indicates that there is a statistically significant difference in the parasitic egg burdens across the age groups (p= 0.000).

Fig 5 provides an analysis of parasitic egg burdens across different ranges with respect to gender. The distribution indicates that females generally have higher frequency of egg burden across almost all ranges compared to males. For instance, in the 100-500 range, females show a higher frequency of 16.3% compared to 8.5% for males. This trend continues across other burden ranges, with female consistently showing higher frequency, particularly noticeable in the 1100-1500 range where females have 26.3% frequency compared to 35.2% for males.

Although males show a substantial presence in the mid to high burden ranges (1100-2000), their frequencies taper off sharply above the 2100-2500 range, indicating fewer high burden cases among males as compared to females. The Students' 't' test showed no significant difference (p=0.09) in the egg burdens between gender.

Table 5: Distribution of egg burden according to age group

Egg burden (g)/ Age group (years)	**100-500**	**600-1000**	**1100-1500**	**1600-2000**	**2100-2500**	**2600-3000**	**3100-3500**	**3600-400**
<10 (n=2)	1 (50.0)	1 (50.0)	0	0	0	0	0	0
11-20 (n=10)	1 (10.0)	0	2 (20.0)	3 (30.0)	3 (30.0)	1 (10.0)	0	0
21-30 (n=23)	3 (13.0)	2 (8.7)	7 (30.4)	6 (26.1)	4 (17.4)	1 (4.3)	0	0
31-40 (n=75)	14 (18.7)	8 (10.7)	18 (24.0)	18 (24.0)	13 (17.3)	3 (4.0)	1 (1.3)	0
41-50 (n=77)	7 (9.1)	15 (19.5)	21 (27.3)	18 (23.4)	12 (15.6)	3 (3.9)	1 (1.3)	0
51-60 (n=27)	3 (11.1)	4 (14.8)	12 (44.4)	4 (14.8)	2 (7.4)	1 (3.7)	0	1 (3.7)
61-70 (n=14)	3 (21.4)	0	6 (42.9)	4 (28.6)	1 (7.1)	0	0	0
71-80 (n=2)	0	1 (50,0)	0	0	1 (50.0)	0	0	0
81-90 (n=1)	0	0	1 (100.0)	0	0	0	0	0
Total (n=231)	**32 (13.9)**	**31 (13.4)**	**67 (29.0)**	**53 (22.9)**	**36 (15.9)**	**9 (3.9)**	**2 (0.9)**	**1 (0.4)**

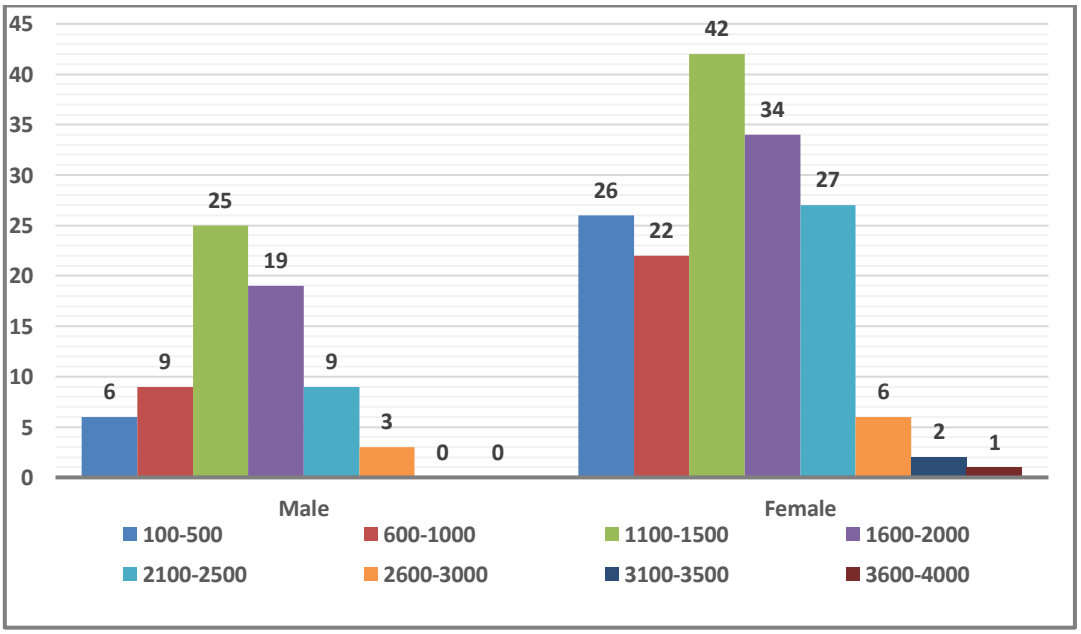

Fig 5: Distribution of egg burdens with respect to gender

Discussion:

Enteric parasitic infections are among the leading causes that intensify the condition of HIV/AIDS patients and a major cause of morbidity and mortality (8). HIV/AIDS significantly weaken the body immune system, rendering it susceptible to various opportunistic parasitic infections (9). The prevalence of enteric parasite in this study is 43.2%. This rate is similar to the study of Obature et al., (10) with 42.6%, but higher than the rate of 35.1% reported by Abanye et al., (3) in southwest Nigeria and 11.4% in a study from northern Nigeria (11). The prevalence reported is lower than those reported from other parts of the world such as 68.2% in Gabon by Legongo et al., (12), 57.5% in Cameroun (13) and 50.9% in Kenya (14), although higher than 21.1% reported by Feleke et al., (2). The variation in prevalence rates could be attributed to different sociocultural differences, parasite distribution, period of study, method of diagnosis used hygiene practices of the participants, awareness on the knowledge of prevention and control of enteric parasite in each locality.

Imo State had the highest prevalence of intestinal parasitic infection (48.0%, 48/100), followed by Anambra State (46.0%, 46/100), Abia State (44.4%, 60/135), Ebonyi State (42.0%, 42/100) and Enugu State (35.0, 35/100) but the prevalence was not significantly different between the States (p= 0.3870). This indicates that the prevalence of these parasites is relatively uniform across the regions studied. This uniformity suggests that common environmental or socio-economic factors across these States.

Ascaris lumbricoides was the most prevalent parasite identified from stool samples of people living with HIV in this study with 33.3%, followed by hookworm (29.9%), *G. lamblia* (10.4%) and *E. histolytica* (8.2%). This agrees with the work of Dibua et al., (15) in Nsukka, Nigeria, which reported *A. lumbricoides* prevalence of 28.0% and hook worm of 20.0%. Akinbo et al., (16) in Benin City, Nigeria also reported *A. lumbricoides* as the most predominant parasite amongst HIV patients and controls but contradicted the study by Akinbo and Omoregie (15) which reported a low prevalence of 5.3% for intestinal parasitic infection in HIV patients in Benin City.

The frequency of identifications of *S. stercoralis* (7.4%), *C. parvum* (3.5%), *Microsporidia* spp (2.6%), *I. belli* (2.2%), *S. japonicum* (0.9%), *T. trichiura* (0.9%) and *E. vermicularis* (0.9%) were very low, which contrasts the study by Faleke et al., (2) who reported *E. histolytica* and *E. vermicularis* as the most frequent enteric parasites and the study by Miressa et al., (17) which reported *G. lamblia* as the most frequent enteric parasite. The discrepancy could be as a result of the time of study and different parasite burden in each locality.

In this study, the prevalence of intestinal parasitic infection of 43.5% (160/368) in the females was slightly higher than 42.5% (71/167) in the males, but this was not statistically significant (p=0.8511). This agrees with the studies by Udeh et al., (18) and Jegede et al., (11) in Nigeria, who reported higher prevalence in females than males but disagrees with the study of Hotez (19) in the United States of America, where overall prevalence and intensity were higher in males than females, attributed to males being more exposed to environment conducive to the parasite vectors in agricultural settings. The slightly higher prevalence in females in our study may be due to exposure frequency because females are more engaged in agricultural activities, household activities and rural dwelling in our setting.

The effect of the parasite egg burden across different $CD4^+$ T cell and haemoglobin (Hb) ranges were demonstrated. The result indicated that there is a statistically significant relationship between $CD4^+$ T cell counts (200 cell/cm^3) and anaemia (p<0.05). The egg burden is high when $CD4^+$ T cell count and haemoglobin ranges decreases, and low as the $CD4^+$ T cell count increases. Thus, these parameters decrease in proportion to the intensity of the parasite infection. It was observed from the study, that in chronic moderate or heavy infection, there were remarkable loss of blood and depletion of the immune system resulting in marked reduction in $CD4^+$ T cell count and haemoglobin level, which leads to fatigue, loss of appetite, weight loss, gastrointestinal disorder, cough, pica, facial and peripheral edema, anaemia and other pathological features. It was also observed from the study that the higher the intensity and duration of egg burden, the worse the symptoms and pathological features observed in infected patients. Our findings agree with the studies of Abarver et al., (12), Akinbo et al., (17) and Amoo et al., (1), who reported that there are correlations between parasite intensity and reduction in haemoglobin level and $CD4^+$ T cell count.

The frequency of enteric parasites was higher in patients whose $CD4^+$ T cell counts were lower than 200 cells/µl. This observation is in line with similar studies conducted elsewhere (1,17,20). Poor immune status predisposes patients to opportunistic pathogen (3). Decreased $CD4^+$ T cell count weakens the immune system making it very difficult for patients to overcome infections they would ordinarily have overcome thus are

difficult to eliminate once established (21). Wakokoo et al., (22) reported that the baseline for $CD4^+$ T cell is 500/µl and is linked to significant suppression of viral load.

The findings of our study on egg burden with respect to the age of the participants indicated that participants in the age brackets 21-30 (30.4%), 31-40 (32.5%), 41-50 (33.3%) and 51-60 (44.4%) years had statistically significant higher egg burden than other age groups ($p<0.05$). This suggests the influence of factors such as immune system variability, exposure risks, and social or environmental conditions unique to this specific age group. It could also be due to that fact that participants in these age groups were mainly youths who may be active and adventurous in life and therefore involved in activities that encourages transmission of infections. Also, there is the possibility that participants may come from poor rural home and filthy environment characterized by open defecation, bushy surroundings and habit of walking barefooted, which increase contact with contaminated soil that predisposes to parasitic infection. This is in line with the reports of previous studies (23,24).

Conclusion:

In this study, a high prevalence of enteric parasitic infection of 43.2% is reported and low $CD4^+$ T cell count was associated with high parasite egg burden. Therefore, regular screening of persons living with HIV/AIDS for enteric parasite and appropriate treatment for those positive should be encouraged to help reduce the burden and associated morbidity and mortality.

Contributions of authors:

ACC designed the study, wrote the protocol and contributed to the literature search, performed statistical analysis of data, wrote the initial manuscript draft and the final manuscript; ACC and ONF performed the laboratory works and contributed to the discussion; ONF supervised the study and proof read the manuscript. Both authors approved the final manuscript submitted for publication.

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Conflict of interest:

No conflict of interest is declared.

Declaration of authors:

Authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Original Article **Open Access**

Assessment of markers of platelet immune activation in malaria, tuberculosis and human immunodeficiency virus infection

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Abstract:

Background: Malaria, tuberculosis (TB) and human immunodeficiency virus (HIV) infection remain issues of public health concern in Nigeria. This study assessed parameters of platelet immune function with a view to understanding their role in the immune response against malaria, TB and HIV infection at the University of Calabar Teaching Hospital and Dr Lawrence Henshaw Memorial Hospital, Calabar, Nigeria.
Methodology: Following ethical approval and informed consent, 180 participants (males and females) aged 15-45 years were enrolled, comprising 90 malaria patients, 20 TB patients, 25 HIV patients, and 45 apparently healthy participants as control. Platelet indices were determined by haemocytometry. Platelet factor 4, P-selectin and cluster of differentiation 40 ligand (CD40L) were measured by enzyme linked immunosorbent assay (ELISA). One-way analysis of variance was used to analyse data, with statistical significance set at $p<0.05$.
Results: The platelet count of the TB patients was significantly higher ($p=0.017$) than that of malaria and HIV patients, and the control. The mean platelet volume was significantly increased ($p=0.001$) for HIV patients compared to malaria and TB patients, and the control. Similarly, platelet distribution width was significantly higher for HIV patients ($p=0.020$) compared to the other groups. Platelet factor 4 and CD40L levels were higher for HIV patients, followed by malaria and TB patients, compared to the control ($p=0.001$). P-selectin values were also significantly higher for malaria, TB and HIV patients compared to the control participants ($p=0.001$).
Conclusion: This study shows significant association of immune effectors such as platelet factor 4, CD40L and P-selectin released by platelets, with malaria, TB and HIV infections. Targeting these immune effectors may open new pathways for therapeutic interventions against these diseases.

Keywords: Platelets, immune activation, malaria, tuberculosis, human immunodeficiency virus.

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Évaluation des marqueurs de l'activation immunitaire plaquettaire dans le paludisme, la tuberculose et l'infection par le virus de l'immunodéficience humaine (VIH)

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Résumé:

Contexte: Le paludisme, la tuberculose (TB) et l'infection par le virus de l'immunodéficience humaine (VIH) demeurent des problèmes de santé publique préoccupants au Nigéria. Cette étude a évalué les paramètres de la fonction immunitaire plaquettaire afin de comprendre leur rôle dans la réponse immunitaire contre le paludisme, la tuberculose et l'infection par le VIH à l'hôpital universitaire de Calabar et à l'hôpital Dr Lawrence Henshaw Memorial, à Calabar, au Nigéria.
Méthodologie: Après approbation éthique et consentement éclairé, 180 participants (hommes et femmes) âgés de 15 à 45 ans ont été recrutés, comprenant 90 patients atteints de paludisme, 20 patients atteints de tuberculose, 25 patients atteints du VIH et 45 participants apparemment en bonne santé comme témoins. Les

indices plaquettaires ont été déterminés par hémocytométrie. Le facteur plaquettaire 4, la P-sélectine et le ligand du cluster de différenciation 40 (CD40L) ont été mesurés par dosage immuno-enzymatique (ELISA). Une analyse de variance à un facteur a été utilisée pour analyser les données, avec une signification statistique fixée à $p<0,05$.
Résultats: La numération plaquettaire des patients atteints de tuberculose était significativement plus élevée ($p=0,017$) que celle des patients atteints de paludisme et de VIH et du témoin. Le volume plaquettaire moyen était significativement augmenté ($p=0,001$) pour les patients atteints du VIH par rapport aux patients atteints de paludisme et de tuberculose et au témoin. De même, la largeur de distribution plaquettaire était significativement plus élevée pour les patients atteints du VIH ($p=0,020$) par rapport aux autres groupes. Les taux de facteur plaquettaire 4 et de CD40L étaient plus élevés chez les patients atteints du VIH, suivis des patients atteints de paludisme et de tuberculose, par rapport au groupe témoin ($p=0,001$). Les valeurs de P-sélectine étaient également significativement plus élevées chez les patients atteints de paludisme, de tuberculose et du VIH par rapport aux participants témoins ($p=0,001$).
Conclusion: Cette étude montre une association significative entre les effecteurs immunitaires, tels que le facteur plaquettaire 4, le CD40L et la P-sélectine libérés par les plaquettes, et les infections par le paludisme, la tuberculose et le VIH. Cibler ces effecteurs immunitaires pourrait ouvrir de nouvelles voies thérapeutiques contre ces maladies.

Mots-clés: Plaquettes, activation immunitaire, paludisme, tuberculose, virus de l'immunodéficience humaine.

Introduction:

Platelets (thrombocytes) are formed elements of blood alongside red and white blood cells. Their indispensable role in the haemostatic function of blood is well established; however, the role of platelets in immunity has been the subject of literature, opening up new insights into the contribution platelets make in immune response to infectious diseases and inflammatory states. Platelets are known to cover a broad range of functions. Besides their role in haemostasis, they have immunological functions and thus participate in the interaction between pathogens and host defense.

The role of platelets in immunity have been extended to include but not limited to intervention against infectious pathogens, recruitment of other immune cells, enhancement of effector cell functions, modulation of antigen presentation and amplification of the adaptive immune responses to infection (1). Platelets are able to sense invading pathogens and infection-induced inflammation; hence, they initiate an intense crosstalk with other arms of the innate and adaptive immunity, including neutrophils, monocytes/macrophages, dendritic cells, B cells and T cells. On stimulation by bacteria or thrombin, platelets release the content of their alpha-granules, which include bioactive peptides, such as chemokines and lymphokines (2).

Malaria, tuberculosis (TB) and human immunodeficiency virus (HIV) infection are major infectious diseases that have persisted as issues of public health concern globally and in Nigeria. One of the United Nations Sustainable Development Goals (SDGs) aims to "*Ensure healthy lives and promote well-being for all at all ages*". The World Health Organization (WHO) in line with the SDGs had set targets to reduce the burden and subsequently eradicate these infectious diseases between 2020 and 2030 (3,4,5). Assessing the immune function of platelets may improve our understanding of the immune response and the interaction between immunologic and inflammatory processes that greatly influences the pathogenesis of malaria, TB and HIV infection.

The objective of this study is to provide information on the immune function of platelets by determining parameters of platelet function and markers of inflammatory and immune response in the trio of malaria, TB and HIV infections. The findings of this study may reveal specific pathways and immunological markers that could be useful targets of preventive and therapeutic interventions. Overall, this could improve the care and management of malaria, TB and HIV patients and ultimately reduce the burden of these diseases in Nigeria.

Materials and method:

Study setting and ethical approval:

The study sites are University of Calabar Teaching Hospital and Dr Lawrence Henshaw Memorial Hospital, Calabar. These two centers are tertiary institutions designated for the treatment of HIV, Malaria and TB in Cross-River state, Nigeria. Ethical approval for this study was obtained from the Health Research Ethics Committee of University of Calabar Teaching Hospital (UCTH/HREC/33/Vol.III/036) and informed consent was obtained from all the participants.

Study design and participants enrolment:

Based on a case-control study design requiring no follow-up, a total of 180 study participants (males and females) within the age range of 15-45 years were enrolled. This comprised of 90 malaria, 20 TB and 25 HIV-infected participants including newly diagnosed patients and those on treatment, attending clinics in the two hospitals. Patients with co-morbidities and those who did not give consent were excluded from the study.

Forty-five apparently healthy participants were recruited as controls from among the staff and students of both hospitals. They were individuals who had been screened negative for latent TB infection (LTBI) using the

Mantoux test in the preceding six months. The control participants were also screened negative for malaria and HIV antibodies using RDT test and Determine HIV strip respectively. The diagnostic criteria for recruitment of control participants, malaria, TB and HIV patients are presented in Fig. 1.

Data and sample collection and laboratory analysis:

The socio-demographic data of all recruited participants were collected using a designed data collection form. Five milliliters (5ml) of venous blood were collected from each participant with minimum stasis; 2ml was added to ethylene diamine tetra-acetic acid (EDTA) to a final concentration of 2 mg/ml while 3ml was dispensed into a plain container. The samples were transported in cold chain to Haematology laboratory of the University of Calabar Teaching Hospital for analysis.

The sample in the plain container was allowed to clot and then centrifuged to obtain serum. The serum was dispensed into a separate container and stored at -20^{o}C until used for ELISA tests. Platelet counts, mean platelet volume, platelet distribution width and plateletcrit were determined for all participant samples by haemocytometry using an autoanalyzer. Platelet factor 4, P-selectin and CD40L were measured by ELISA technique for the 25 control participants, 20 malaria, 20 TB and 25 HIV patients.

Data analysis:

Results were expressed as mean±SD. One-way analysis of variance and post-hoc test on the Statistical Package for the Social Sciences (SPSS) version 22.0, was used to test hypotheses. P value < 0.05 was considered to be statistically significant.

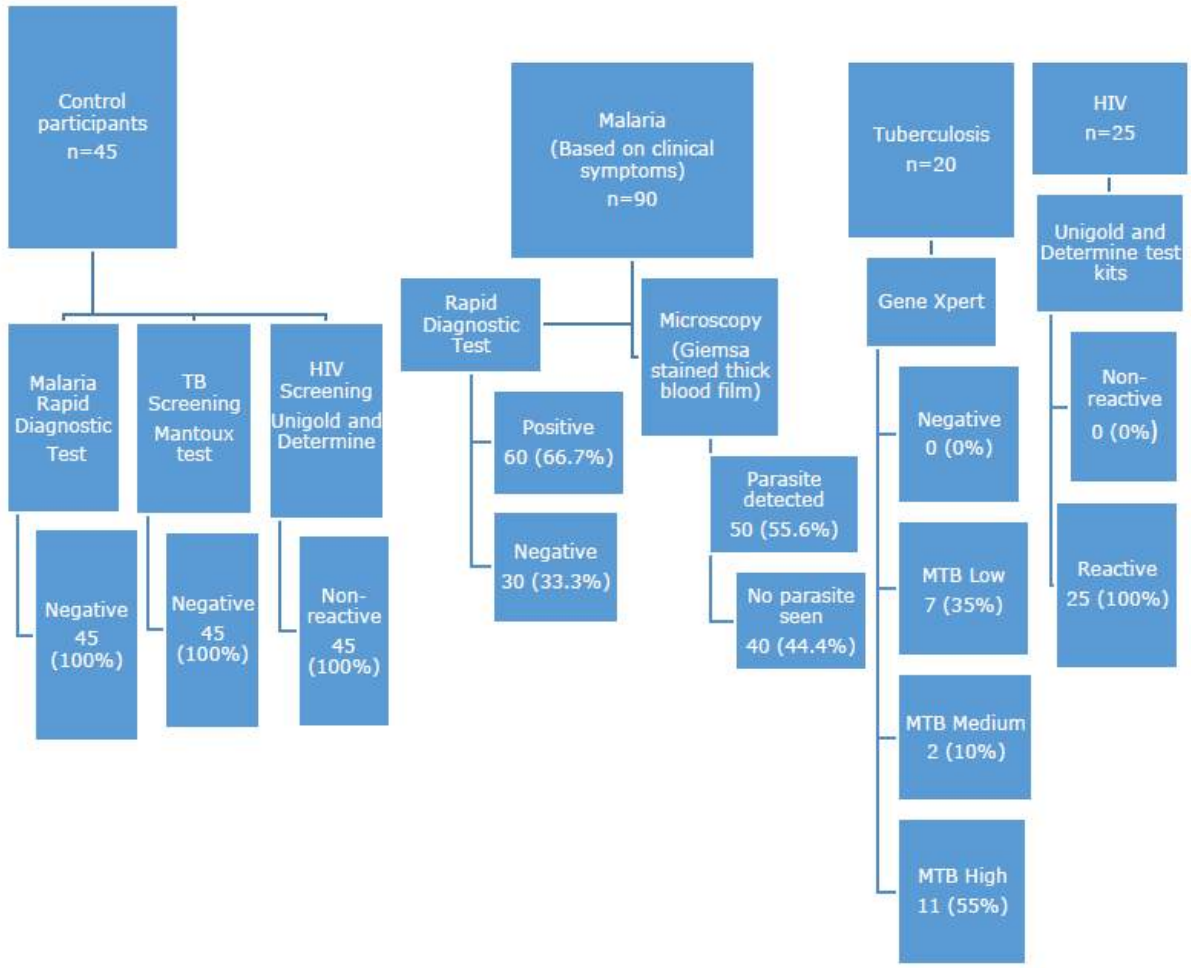

Fig 1: Diagnostic criteria for recruitment of malaria, tuberculosis and HIV patients, and the control participants

Results:

Table 1 shows the demographic data of the study participants. The control was equally distributed, 15 (33%) among three age groups of 15-24, 25-34 and 35-45 years; malaria patients were 15, 38 and 37 for the three age groups respectively. Tuberculosis patients were 4, 5 and 11 while HIV patients were 0, 3 and 22 for the three age groups respectively. The controls comprised 46.7% males and 53.3% females. The gender distribution for malaria was 25.6% males, 74.4% females, for TB 75% males, 25% females and for HIV 48% males and 52% females. Sixty percent of the control were single while 75.5% of malaria patients were married; TB patients were equally distributed (50.0%) each for single and married categories while the HIV patients comprised more of the married (60%).

Majority (75.5%) of the control as well as 50.0% of malaria patients and 40.0% of HIV patients had tertiary level of education while 55% of TB patients were secondary school leavers. The control were mostly students (55.6%), malaria and TB patients were spread across business operators (40% and 25%) and students (31.1% and 25%) while HIV patients comprised mostly of business operators (40%).

As shown in Table 2, the platelet count of TB patients was significantly higher (p= 0.017) when compared to the values for the control, malaria and HIV patients. The mean platelet volume significantly increased (p= 0.001) for HIV patients compared to malaria and TB patients, and the control. Similarly, platelet distribution width was significantly higher for HIV patients (p=0.020) compared to the other groups.

Table 3 shows that platelet factor 4, CD40L and P-selectin levels of malaria, TB and HIV patients were significantly higher (p= 0.001) in comparison with the control values. Post hoc analysis revealed that platelet factor 4 and CD40L were significantly higher for malaria compared to TB patients, as well as for HIV compared to TB patients. Also, platelet factor 4 was observed to be significantly higher for HIV compared to malaria patients.

Table 1: Socio-demographic characteristics of malaria, tuberculosis and HIV patients, and the control participants

Parameters	Control (%) (n=45)	Malaria patients (%) (n=90)	Tuberculosis patients (%) (n=20)	HIV patients (%) (n=25)
Age group (years)				
15-24	15 (33.3)	15 (16.7)	4 (20.0)	0
25-34	15 (33.3)	38 (42.2)	5 (25.0)	3 (12.0)
35-45	15 (33.3)	37 (41.1)	11 (55.0)	22 (88.0)
Gender				
Male	21 (46.7)	23 (25.6)	15 (75.0)	12 (48.0)
Female	24 (53.3)	67 (74.4)	5 (25.0)	13 (52.0)
Marital status				
Single	27 (60.0)	15 (16.7)	10 (50.0)	10 (40.0)
Married	18 (40.0)	68 (75.5)	10 (50.0)	15 (60.0)
Divorced	0	0	0	0
Widowed	0	7 (7.8)	0	0
Education				
Non-formal	0	8 (8.9)	2 (10.0)	0
Primary	3 (6.7)	14 (15.5)	3 (15.0)	8 (32.0)
Secondary	8 (17.8)	23 (25.6)	11 (55.0)	7 (28.0)
Tertiary	34 (75.5)	45 (50.0)	4 (20.0)	10 (40.0)
Occupation				
Civil/Public servants	20 (44.4)	10 (11.1)	3 (15.0)	8 (32.0)
Business/trading	0	36 (40.0)	5 (25.0)	10 (40.0)
Artisans	0	0	4 (20.0)	1 (4.0)
Students	25 (55.6)	28 (31.1)	5 (25.0)	4 (16.0)
Farmers	0	16 (17.8)	3 (15.0)	2 (8.0)

Table 2: Platelet count and indices of malaria, tuberculosis and HIV patients, and the control participants

Parameters	Control (n=45)	Malaria patients (n=90)	Tuberculosis patients (n=20)	HIV patients (n=25)	*p*-value
Platelet count x 10^9/L	179.80±58.37	225.47±98.09	276.63±61.45[a]	221.92±89.51	0.017
Mean platelet volume (fl)	9.18±0.57	8.87±0.57[c]	9.03±0.55	9.76±0.84[b]	0.001
Platelet distribution width	14.96±0.41	15.05±0.51	15.14±0.49	15.33±0.34[b]	0.020
Plateletcrit	0.95±0.43	0.20±0.10	0.27±0.12	0.23±0.10	0.664

Post Hoc: [a] = difference between control vs TB; [b] = difference between control vs HIV; [c] = difference between malaria vs HIV

Table 3: Platelet factor 4, CD40L and P-selectin levels of malaria, tuberculosis and HIV patients, and the control participants

Parameters	Control (n=25)	Malaria patients (n=20)	Tuberculosis patients (n=20)	HIV patients (n=25)	*p*-value
Platelet factor 4 (pg/ml)	21.83±0.95	53.61±2.85 [a]	40.92±4.34 [a, b]	67.08±3.76 [a, c, d]	0.001
CD40L (ng/ml)	3.33±1.21	6.79±1.00 [a]	5.11±1.29 [a, b]	6.80±1.13 [a, c]	0.001
P-selectin (pg/ml)	26.18±5.24	126.63±19.25 [a]	143.88±20.30 [a]	130.76±20.16 [a]	0.001

Post Hoc: [a]= Significantly different from control; [b]= difference between malaria and tuberculosis; [c]= difference between tuberculosis and HIV; [d]= difference between malaria and HIV; CD40L = Cluster of differentiation 40 ligand

Discussion:

Markers of platelet immune activation were assessed for malaria, TB and HIV patients in this study. About 33% of the malaria patients who were recruited based on clinical symptoms as determined by a physician were found to be negative for malaria using the rapid diagnostic test kit. Furthermore, the parasite was not detected microscopically for 44.4% of these malaria patients. A possible reason for this finding may be the practice of self-medication that is rampant in Nigeria (6,7). Several of these patients use over-the-counter antimalarial drugs and only present themselves at the clinic when symptoms do not improve. This underscores the need for proper laboratory testing especially in an endemic region where many patients are treated for malaria based on a combination of clinical symptoms that may or may not be caused by malaria parasite. The World Health Organization had directed that treatment for malaria should only be initiated after proper testing (8) but this is yet to be a reality in practice.

Most of the patients who presented with malaria, TB and HIV (83.3%, 80% and 100%) were between 25-45 years age group with lower percentage observed for patients <25 years of age. This reflects the enormous impact of these infectious diseases on the economically productive age group and is a cause for serious concern. Gender differences were observed among the patient population with more females presenting with malaria and more males presenting with TB. Females have been reported to contribute more to the burden of malaria with higher risk in child-bearing age as well as more frequent visits to health facilities as possible reasons (9). This is further confirmed as the findings of this study shows that more married persons were affected by malaria, with women being the majority.

One study suggested that women were more exposed to mosquito bites due to traditional roles which requires them to be outside during dangerous hours and the fact that self-medication was practiced more by men when compared to women (10). Again, it has been reported previously that TB affects more males than females due to socio-demographic factors and lifestyle that pre-disposes the male to infection with an airborne disease such as TB (11). Low socio-economic status has been implicated as a favorable factor for the acquisition of TB (12,13), and this is supported by the finding that 80% of the TB patients in this study had secondary school level of education and below.

Platelet count was observed to be significantly higher for TB patients in comparison to the control whereas the mean platelet volume which reflects the size and volume occupied by platelets, was found to be lower in malaria and higher in HIV infection with the platelet distribution width being higher in HIV infection. Increase in platelet count and mean platelet volume as observed is suggestive of platelet activation as a reaction to the presence of infection. Again, platelet factor 4, CD40L

and P-selectin levels were observed to be significantly higher in malaria compared to the apparently healthy controls in this study. Platelet activation and release of chemokines such as platelet factor 4 has been reported previously in malaria patients of Caucasian origin. The present study highlights the role platelets play in immunity against malaria in Africans, particularly for our local population in Nigeria.

Platelets contribute to the host defense against malaria through direct killing of the *Plasmodium* species (14-18). When platelets bind to malaria infected red cells, they become activated and release platelet factor 4 (CXCL4), a chemokine present in the alpha granules of platelets. Platelet factor 4 inhibits the growth of the malaria parasite resulting in parasite death; this is beneficial in malaria pathogenesis and may be useful in the formulation of antimalarial drugs targeted at enhancing the release and function of platelet factor 4. The CD40L is a transmembrane protein expressed on platelets, where it contributes to platelet activation and release of inflammatory mediators. Another transmembrane protein, P-selectin, is stored in the alpha granules of platelets and released upon platelet activation in inflammatory states (19). Increases in these proteins in malaria, TB and HIV infections as observed in this study, signifies platelet activation that suggests that platelets play a role in the immune response against these infections.

In our study, platelet factor 4, CD40L and P-selectin were observed to be significantly higher in TB patients, suggesting that platelet activation is part of the immune response against TB. This has been previously reported (20). Platelets engulf the bacteria and release antimicrobial peptides, which interact with internalized *M. tuberculosis*. In fact, *M. tuberculosis* modulates the production of cytokines by platelets. Furthermore, it has been shown that platelets are recruited into the granuloma formed in the late stages of TB. They internalize the *Mycobacterium* and produce host defense peptides (HDPs) such as RNase 7 and platelet factor 4 suggesting the participation of platelets in the immune response against TB through HDPs and production of cytokines (21).

The participation of platelets in infection and chronic inflammation such as TB is linked to their granules and organelles that contain chemokines, cytokines, enzymes and growth factors which function as immune effectors that are often released in response to damaged epithelium and interaction with leucocytes (22,23). Although these pro-inflammatory properties of platelets are beneficial, they could also lead to pathology in chronic infections such as TB. Hence, platelets appear to support the host response when the infection is extra-cellular, while in intracellular infections, they may contribute to immune evasion by TB pathogen, thereby making platelets a contributor to immunopathology (24). These two platelet effects may be explored as possible targets for anti-TB therapy.

Platelet factor 4, CD40L and P-selectin are released following activation of platelets and their levels were significantly increased in the HIV patients in our study. Platelets have been reported to harbor or hide HIV in infected person prior to the initiation of anti-retroviral therapy, thus infection of $CD4^+$ T cells via HIV-platelet complex is enhanced by platelet activation and subsequent platelet–T-cell complex formation (25,26). It has been suggested that platelets suppress HIV-1 spread in co-cultured T cells in a concentration-dependent manner and platelets containing granules inhibited HIV-1 spread in T cells more efficiently than degranulated platelets, indicating that granule content might exert antiviral activity. Indeed, supernatant from activated and degranulated platelets suppressed HIV-1 infection. The chemokine, CXCL4 (platelet factor 4) which is a major component of platelet granules, was found to block HIV-1 entry, and neutralization of CXCL4 in platelet supernatants largely abrogated their anti-HIV-1 activity. The inhibitory activity of platelet-derived CXCL4 suggests the role of platelets in the defense against infection by HIV-1 and potentially other pathogens (27). Patients with high levels of biomarkers of coagulation/inflammation such as CXCL4 are at an increased risk of developing non-AIDS defining serious illnesses such as cardiovascular diseases (28). This increased risk has serious implications for the HIV patients in our study.

The trio of malaria, TB and HIV infection induce a state of inflammation in the body. Infection-induced inflammation involves recruitment and activation of platelets, as observed in our study. Excessive activation of platelets may result in thrombosis, which could be localized or widespread, with possible consequences of organ dysfunctions (29,30). The most severe result of platelet activation as part of the immune response is the condition of disseminated intravascular coagulation, characterized by intense microvascular thrombosis and consumption of platelets (31,32). Thus, the participation of platelets in the immune response against malaria, TB and HIV, may be both beneficial and harmful.

Conclusion:

This study shows significant association of immune effectors such as platelet factor 4, CD40L and P-selectin released by platelets with malaria, TB and HIV infections. Targeting the functions of these immune effectors may open new pathways for therapeutic inter-

ventions against these diseases by the development of drugs that will enhance platelet immune response while addressing the detrimental effects of excessive platelet activation.

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Contributions of authors:

PA designed the study and wrote the first draft of the manuscript; EA performed the statistical analysis; JE managed the literature searches; All authors were involved in the laboratory analysis and the approval of the final manuscript.

Conflict of interest:

No conflict of interest is declared.

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Original Article **Open Access**

Diagnostic utility of p16 and Ki-67 immunohistochemical markers in cervical intraepithelial neoplasia: Association with HPV status and histological grade

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Abstract:

Background: Cervical intraepithelial neoplasia (CIN) is a precursor to cervical cancer, often associated with high-risk human papillomavirus (HPV) infection. Accurate diagnosis and grading of CIN are critical for effective management. Immunohistochemical markers like p16 and Ki-67 can enhance diagnostic precision by identifying oncogenic HPV-induced changes and cellular proliferation. This study evaluates the diagnostic utility of p16 and Ki-67 expression in CIN lesions and association with HPV status and histological grade.
Methodology: This retrospective study analyzed archived cervical biopsy samples diagnosed with CIN over a 5-year period in a tertiary care center in Sagamu, Ogun State, Nigeria. Histological grading of CIN was confirmed, and immunohistochemical staining for p16 and Ki-67 was performed. HPV status was determined using polymerase chain reaction (PCR) on the biopsy sample. Marker expression patterns were compared across CIN grades and with HPV status. Statistical association of p16 and Ki-67 expression with CIN grades and HPV was done using Chi-square or Fisher Exact test, with $p<0.05$ considered as statistical significance.
Results: Of the 72 samples analyzed, p16 expression showed a significant association with higher CIN grades and HPV positivity ($p<0.001$), with sensitivity and specificity values of 92.0% and 88.0%, respectively. Ki-67 expression was also associated with increasing CIN grades and HPV status ($p=0.001$), and demonstrated a sensitivity of 85.0% and specificity of 82.0% in distinguishing CIN lesions.
Conclusion: p16 and Ki-67 immunohistochemical markers are valuable adjuncts in the diagnosis and grading of CIN, particularly when correlated with HPV status. Their combined use can enhance diagnostic accuracy, supporting their incorporation into routine pathology workflows for CIN evaluation.

Keywords: Biomarkers; cervical biopsy; CIN; HPV; Ki67; p16

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Utilité diagnostique des marqueurs immunohistochimiques p16 et Ki-67 dans les néoplasies intraépithéliales cervicales: Association avec le statut HPV et le grade histologique

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Résumé:

Contexte: La néoplasie intraépithéliale cervicale (CIN) est un précurseur du cancer du col de l'utérus, souvent associée à une infection par le virus du papillome humain (VPH) à haut risque. Un diagnostic et une classification précis de la CIN sont essentiels à une prise en charge efficace. Les marqueurs immuno-histochimiques tels que p16 et Ki-67 peuvent améliorer la précision du diagnostic en identifiant les modifications oncogènes induites par le VPH et la prolifération cellulaire. Cette étude évalue l'utilité diagnostique de l'expression de p16 et Ki-67 dans les lésions de CIN et leur association avec le statut VPH et le

grade histologique.
Méthodologie: Cette étude rétrospective a analysé des échantillons de biopsies cervicales archivés chez lesquels une CIN a été diagnostiquée sur une période de 5 ans dans un centre de soins tertiaires de Sagamu, dans l'État d'Ogun, au Nigéria. La classification histologique de la CIN a été confirmée et une coloration immunohistochimique pour p16 et Ki-67 a été réalisée. Le statut HPV a été déterminé par PCR (amplification en chaîne par polymérase) sur l'échantillon de biopsie. Les profils d'expression des marqueurs ont été comparés entre les grades de CIN et le statut HPV. L'association statistique de l'expression de p16 et Ki-67 avec les grades de CIN et le HPV a été réalisée par test du Chi carré ou test exact de Fisher, avec une valeur de $p<0,05$ considérée comme statistiquement significative.
Résultats: Sur les 72 échantillons analysés, l'expression de p16 a montré une association significative avec des grades de CIN plus élevés et une positivité HPV ($p<0,001$), avec des valeurs de sensibilité et de spécificité de 92,0% et 88,0%, respectivement. L'expression de Ki-67 a également été associée à des grades de CIN plus élevés et au statut HPV ($p=0,001$), et a démontré une sensibilité de 85,0% et une spécificité de 82,0% pour distinguer les lésions de CIN.
Conclusion: les marqueurs immunohistochimiques p16 et Ki-67 sont des compléments précieux pour le diagnostic et la classification des CIN, en particulier lorsqu'ils sont corrélés au statut HPV. Leur utilisation combinée peut améliorer la précision du diagnostic, favorisant ainsi leur intégration dans les procédures de routine de pathologie pour l'évaluation des CIN.

Mots-clés: Biomarqueurs; biopsie cervicale; CIN; HPV; Ki67; p16

Introduction:

Cervical cancer remains a significant public health challenge (1), particularly in low-and-middle-income countries where it is a leading cause of cancer-related morbidity and mortality among women (2). Persistent infection with high-risk human papillomavirus (HPV) is recognized as the primary etiological factor in the development of cervical intraepithelial neoplasia (CIN) and its progression to invasive carcinoma (3). Early detection and appropriate management of CIN are pivotal in preventing cervical cancer, underscoring the importance of accurate diagnostic techniques (4).

Histopathological examination of cervical biopsies remains the gold standard for diagnosing CIN (4), however, the subjective interpretation of histology can lead to observers' variability, particularly in distinguishing between low-grade (CIN I) and high-grade (CIN II/III) lesions (5). The advent of immunohistochemical markers, such as p16 and Ki-67, offers a promising adjunct to traditional histopathology by providing objective and reproducible insights into the biological behavior of these lesions (6).

As a surrogate marker for oncogenic HPV activity, p16 reflects the overexpression of the CDKN2A gene, which occurs due to HPV-mediated inactivation of tumor suppressor proteins. Ki-67, a nuclear protein expressed during active phases of the cell cycle, indicates cellular proliferation and abnormal growth patterns (6). Individually, these markers have demonstrated utility in distinguishing CIN from benign and reactive lesions. Combined, they provide complementary information, enhancing diagnostic precision (7). Despite the availability of these markers, their integration into routine practice varies, particularly in resource-limited settings (6). Furthermore, the relationship between p16 and Ki-67 expression, HPV status, and CIN grade requires further exploration to optimize diagnostic algorithms (7).

In recent years, the combination of p16 and Ki-67 has gained attention as a reliable method for improving the accuracy of CIN diagnosis and grading (8). However, the diagnostic performance of these markers in different clinical settings and populations remains under-explored (9). This study aims to address this gap by evaluating the role of p16 and Ki-67 in diagnosing CIN and exploring their correlation with HPV status and histological grading. By providing a clearer understanding of these markers' diagnostic utility, this research seeks to contribute to the refinement of screening and diagnostic protocols, particularly in settings with limited access to advanced molecular testing.

Materials and method:

Study setting and design:

A retrospective study was conducted in the Department of Morbid Anatomy and Histopathology at Olabisi Onabanjo University Teaching Hospital, Sagamu, southwest Nigeria, covering the period from December 2019 to November 2024. The study was approved by the ethical review board of the hospital, and patient confidentiality was maintained throughout the study.

Study population:

The study included archived formalin-fixed, paraffin-embedded (FFPE) cervical biopsy specimens from women diagnosed with cervical intraepithelial neoplasia (CIN) during the study period. Cases without adequate histological samples or complete clinical data were excluded from the study to ensure accuracy.

Histopathological grading:

Cervical biopsy specimens were examined and reviewed microscopically. They were graded as CIN lesions according to the

Bethesda system (10). CIN lesions were classified as CIN I (mild dysplasia), CIN II (moderate dysplasia), or CIN III (severe dysplasia/carcinoma in situ).

Immunohistochemical staining:

Immunohistochemical staining for the p16 and Ki-67 was performed on all eligible specimens. Briefly, 4-µm thick sections were cut from FFPE blocks and mounted on slides. For p16, the slides were incubated with a monoclonal antibody (clone E6H4, Dako, Denmark) at a dilution of 1:100. For Ki-67, the slides were incubated with a monoclonal antibody (clone MIB-1, Dako, Denmark) at a dilution of 1:200. Following incubation, the slides were processed with appropriate secondary antibodies, and the results were visualized using 3,3'-diaminobenzidine (DAB) as the chromogen. The slides were counterstained with hematoxylin.

Scoring and interpretation of immunohistochemical staining:

The expression of p16 and Ki-67 was assessed by the percentage of positive cells and the intensity of staining. p16 expression was considered positive when more than 70% of the cells showed strong diffuse staining, consistent with the overexpression typical of HPV-induced lesions. Ki-67 expression was scored based on the percentage of positive cells in the epithelial layer, with a cutoff of >10% staining indicating high proliferative activity. Both markers were classified as positive or negative based on these criteria.

HPV testing:

HPV testing was performed on cervical biopsy specimens using polymerase chain reaction (PCR) to detect the presence of high-risk HPV types (16, 18, and other high-risk strains). HPV DNA was extracted from FFPE tissue sections using the Qiagen DNA extraction kit (Qiagen, Germany) following the manufacturer's instructions.

Amplification by PCR was performed using type-specific primers targeting the L1 region of high-risk HPV genotypes, including HPV types 16, 18, 31, 33, and 45. Each 25 µL PCR reaction mixture contained 10–100 ng of extracted DNA, 0.2 µM of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, and 1 unit of Taq DNA polymerase. Thermal cycling conditions included an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 7 minutes.

PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV illumination. A sample was considered HPV-positive if at least one high-risk HPV genotype was detected.

Data analysis:

Descriptive statistics were used to summarize patient demographics and clinicopathological features. The association between p16 and Ki-67 expression and CIN grade was analyzed using the Chi-square test or Fisher's exact test, as appropriate. The sensitivity and specificity for each marker and their combined use in diagnosing CIN were calculated. A *p*-value of <0.05 was considered statistically significant.

Results:

Clinicopathological characteristics of the patients with cervical biopsy samples:

As depicted in Table 1, archived cervical biopsy samples of a total of 72 patients were involved in the study. The mean age (standard deviation) of the patients is 31.61 ±8.75 years, with predominant age group between 21-40 years (n=58 cases, 80.6%).

All samples were positive for HPV except 5 (6.9%), with low-risk (LR)-HPV (n= 37, 51.4 %) and high-risk (HR)-HPV cases (n=30, 41.7 %). CIN I was the diagnosis in 37 cases (51.4 %), while CIN II and CIN II were seen in 19 cases (26.4 %) and 16 cases (22.2 %) respectively.

Table 1: Clinicopathological characteristics of women whose cervical samples were used in the study

Parameter	Frequency (%)
Age group (years)	
1-20	5 (6.9)
21-40	58 (80.6)
41-60	9 (12.5)
Mean age (± SD)	31.60 ± 8.75
HPV status	
HPV negative	5 (6.9)
LR HPV	37 (51.4)
HR HPV	30 (41.7)
CIN grade	
CIN I	37 (51.4)
CIN II	19 (26.4)
CIN III	16 (22.2)

CIN=cervical intraepithelial neoplasia; HPV=Human papilloma virus, LR=Low-risk; HR-High-risk; SD=Standard deviation

Association between p16 expression and CIN grades:

A significant association was found between expression of p16 with increased severity of the cervical lesion ($p<0.001$) as showed in Table 2. Only cases of CIN I were negative for p16 (5.4%, 2/37), with 27 of the 37 (73.0%) CIN I cases showing weak staining (+). In contrast, the majority of CIN II cases showed intermediate staining (++) intensity (9/19, 47.4%) while most of CIN III cases showed strong staining (+++) intensity

for p16 (13/16, 81.3%). There was no strong staining for p16 amongst the CIN I cases.

Association between Ki67 expression and CIN grades:

As illustrated in Table 3, a significant association was found between Ki-67 expression and increase in the severity of the cervical lesion (p=0.001). Most cases of CIN I showed weak p16 stain (24/37, 64.9%). In contrast, most of CIN II showed intermediate staining (9/19, 47.4%) and strong staining (8/19, 42.1%), while the majority of CIN III cases showed strong staining (11/16, 68.8%). CIN III did not show weak staining at all, while only 2 cases (2/19, 10.5%) of CIN II showed weak staining.

Association between p16 and Ki67 staining patterns:

Cervical samples with intermediate or strong intensity for p16 did not show negativity or weak staining for Ki-67 as illustrated in Table 4. Cases negative for p16 did not show intermediate or strong staining for Ki-67, however, 1 of the 2 (50.0%) p16 negative staining showed a weak staining pattern for Ki-67. Cases with weak staining for p16 did not show strong staining for Ki-67, however, they showed mostly weak staining (23/30, 76.7%) and to a lesser extent, intermediate staining (7/30, 23.3%), and this finding was significantly associated (p<0.001)

Association between HPV status and CIN grades:

As shown in Table 5, 5 patients with a history of CIN I were negative for HPV while majority were positive for LR-HPV (30/37, 81.1%). Patients with history of CIN II and CIN III mostly showed positivity for HR-HPV (13/19, 68.4%) and (15/16, 93.8%), and this was significantly associated (p=0.001).

Association between HPV status and p16 and Ki67 staining score:

As observed in Table 6, a significant association was found between HPV genotype and the expression of either p16 or Ki-67 (p<0.001 for each). HPV negative cases did not show intermediate or strong staining scores for either p16 or Ki67 but showed weak staining for p16 (3/5, 60.0%) and Ki-67 (4/5, 80%). Most of HR-HPV positive cases showed strong staining score (+++) for both p16 (19/30, 63.3%) and Ki67 (17/30, 56.7%) with no negative staining score for both. LR-HPV positive cases mostly showed weak staining pattern for p16 (23/37, 62.2%) or Ki-67 (18/37, 48.6%) or showed intermediate staining pattern for p16 (13/37, 35.1%) and Ki-67 (17/37, 45.9%).

Table 2: Association of p16 expression with cervical intraepithelial neoplasia grade

CIN grades	**No of sample with p16 expression level (%)**					***p* value**
	-	**+**	**++**	**+++**	**Total**	
CIN I	2 (5.4)	27 (73.0)	8 (21.6)	0	**37 (51.4)**	<0.001*
CIN II	0	3 (15.8)	9 (47.4)	7 (36.8)	**19 (26.4)**	
CIN III	0	0	3 (18.8)	13 (81.3)	**16 (22.2)**	
Total	**2 (2.8)**	**30 (41.7)**	**20 (27.8)**	**20 (27.8)**	**72 (100.0)**	

* = Statistically significant; + = weak staining; ++ = moderate staining; +++ = strong staining; - = no staining

Table 3: Association of Ki-67 expression and cervical intraepithelial neoplasia grade

CIN grades	**No of samples with Ki-67 expression level (%)**					***p* value**
	-	**+**	**++**	**+++**	**Total**	
CIN I	1 (2.7)	24 (64.9)	12 (32.4)	0	**37 (51.4)**	<0.001*
CIN II	0	2 (10.5)	9 (47.4)	8 (42.1)	**19 (26.4)**	
CIN III	0	0	5 (31.3)	11 (68.8)	**16 (22.2)**	
Total	**1 (1.4)**	**26 (36.1)**	**26 (36.1)**	**19 (26.4)**	**72 (100)**	

* = Statistically significant; + = weak staining; ++ = moderate staining; +++ = strong staining; - = No staining

Table 4: Association between p16 and Ki-67 expression levels

No of samples with p16 expression level (%)	**No of samples with Ki-67 expression level (%)**					***p* value**
	-	**+**	**++**	**+++**	**Total**	
-	1 (50.0)	1 (50.0)	0	0	**2**	<0.001*
+	0	23 (76.7)	7 (23.3)	0	**30**	
++	0	2 (10.0)	17 (85.0)	1 (5.0)	**20**	
+++	0	0	2 (10.0)	18 (90.0)	**20**	
Total	**1**	**26**	**26**	**19**	**72**	

* = Statistically significant; + = weak staining; ++ = moderate staining; +++ = strong staining; - = No staining

Table 5: Association of human papillomavirus status with cervical intraepithelial neoplasia grade

CIN grades	**HPV status (%)**				*p* value
	HPV –ve	**LR-HPV**	**HR-HPV**	**Total**	
CIN I	5 (13.5)	30 (81.1)	2 (5.4)	**37 (51.4)**	<0.001*
CIN II	0	6 (31.6)	13 (68.4)	**19 (26.4)**	
CIN III	0	1 (6.2)	15 (93.8)	**16 (22.2)**	
Total	**5 (6.9)**	**37 (51.4)**	**30 (41.7)**	**72 (100.0)**	

* = Statistically significant; CIN=cervical intraepithelial neoplasia; HPV=Human papilloma virus, LR=Low-risk; HR-High-risk

Table 6: Association of HPV status with p16 and Ki-67 intensity scores

HPV	**No of samples with p16 expression level (%)**				**No of samples with Ki-67 expression level (%)**			
	-	**+**	**++**	**+++**	**-**	**+**	**++**	**+++**
HPV–ve (n=5)	2 (40.0)	3 (60.0)	0	0	1 (20.0)	4 (80.0)	0	0
LR-HPV (n=37)	0	23 (62.2)	13 (35.1)	1 (2.7)	0	18 (48.6)	17 (45.9)	2 (5.4)
HR-HPV (n=30)	0	4 (13.3)	7 (23.3)	19 (63.3)	0	4 (13.3)	9 (30.0)	17 (56.7)
p value	<0.001*				<0.001*			

* = Statistically significant; + = weak staining; ++ = moderate staining; +++ = strong staining; - = No staining; CIN=cervical intraepithelial neoplasia; HPV=Human papilloma virus, LR=Low-risk; HR-High-risk

Discussion:

This study aimed to evaluate the diagnostic utility of p16 and Ki-67 immunohistochemical markers in the grading of CIN in relation to HPV genotype. The results demonstrated significant associations between p16 and Ki-67 expression and CIN grades, with strong associations between HR HPV infection and higher CIN grades. These findings are consistent with the growing body of evidence that highlights the role of immunohistochemical markers in the early diagnosis and grading of cervical lesions (11,12).

In the present study, a significant association (p<0.001) was detected between p16 expression and the severity of cervical lesion; the strongest staining (+++) was most frequently found in CIN III (65.0%), intermediate staining (++) was mostly detected in CIN II (45.0%), while no cases of CIN I showed p16 strong staining score. This result agrees with previous studies which reported that diffuse intense p16 expression was found in 88-100% of invasive carcinoma, 40- 100% of CIN II/III, and 0-15% in non-dysplasia (13,14,15). One study which reported a higher rate of p16 expression in CINs and invasive cancer also found a higher rate of expression in non-dysplasia (32%) than other studies (16), which may be attributed to the criteria used in that study or to HPV infection genotypes.

This study also reported a significant association between ki-67 expression and severity of the cervical lesion (p=0.001). CIN II and CIN III showed strong positive staining for Ki-67 (42.1% and 68.8% respectively) while cases of CIN I show 0%. These results agree with what was reported earlier that Ki-

67 expression was seen in 90-100% of invasive carcinoma, 20-70% in CIN II/III, 70-90% in CIN I, and 0-20% in non-dysplasia (13,14,15). Immunopositivity for Ki-67 linearly increases as the CIN grade is higher (17). A study by Kruse et al., (18) also found Ki-67 to be useful in distinguishing different grades of CIN lesions, and a potential method of quality control and possible indicator of progression in low-grade lesions.

A significant positive association was found between combined p16 and Ki67 expression in this study (p<0.000). The expression levels of p16 and Ki67 gradually increase with the development of CIN lesions and cervical cancer as previously reported (17). Moreover, p16/Ki67 dual staining has been used for cervical cancer screening (19). The current study revealed that most patients with CIN I are either negative for HPV or positive for LR-HPV (13.5% and 81.1% respectively), while patients with CIN II and CIN III are mostly positive for HR-HPV (68.4%, and 93.8% respectively) and this association was statistically significant (p=0.001). These results agree with what was reported by Nam et al., (20) who showed that in low-grade CIN, HPV test was negative in 54.5% of patients. HPV positivity rates tend increase directly with the cervical lesion severity (17).

Regarding the association of p16 and Ki-67 with HPV status, our study showed that HPV negative cases did not have intermediate or strong staining score for either p16 or Ki-67 while most of the HR-HPV positive cases had strong positive staining for both p16 and Ki-67and this was statistically significant (p <0.001 for each). This agrees with a study by Bosch et al., (21), who found that the p16 over-expression is rare in patients infected with a low-risk HPV because E7 protein of low-risk HPV has a lower affinity for pRb than that of high-risk HPV. Klaes et al., (22) found that over-expression of p16 has a close association with high-risk HPV infection and correlates with the degree of cervical neoplasia. Keating et al., (23) also showed a strong relationship between Ki-67, cyclin E, and p16 in the recognition of HPV-associated precursors as well as in distinguishing normal squamous mucosa from precancerous lesions and, found that with the increase of p16 and Ki-67 expression levels, the number of HR-HPV copies increase.

Several studies have highlighted the diagnostic value of p16 and Ki-67 in distinguishing high-grade CIN (CIN II/III) from low-grade lesions (CIN I). In our study, p16 demonstrated high sensitivity and specificity for high-grade lesions, consistent with its established role as a surrogate marker for transforming high-risk HPV infection. Ki-67 also showed increased expression with rising CIN grade, supporting its utility as a proliferation marker. Previous studies have reported sensitivities ranging from 86% to 100% and specificities between 70% and 95% for p16 in detecting CIN II/III lesions (8,17,20). Ki-67, while slightly less specific when used alone, provides added diagnostic value when co-expressed with p16, especially in ambiguous or borderline cases. The combined use of p16 and Ki-67 has been shown to enhance diagnostic accuracy and interobserver agreement, offering a reliable adjunct to routine histology. These findings support the incorporation of both markers into routine practice for more accurate grading of CIN.

Conclusion:

This study highlights the diagnostic value of p16 and Ki-67 immunohistochemical markers in the assessment of CIN, particularly in distinguishing high-grade lesions (CIN II and CIN III) from low-grade lesions (CIN I). The significant association between these biomarkers and CIN grade, coupled with the strong association of HR HPV with high-grade lesions, highlights the utility of a multi-marker approach in the diagnostic and prognostic evaluation of cervical dysplasia.

Our results also suggest that both p16 and Ki-67 are highly accurate in identifying high-grade CIN lesions, with p16 demonstrating slightly higher diagnostic accuracy. The combined use of p16 and Ki-67 may improve the overall diagnostic performance in distinguishing between low- and high-grade lesions. Future studies should aim to further validate these findings in larger cohorts and explore the potential role of p16 and Ki-67 in monitoring disease progression and predicting the risk of cervical cancer.

Contribution of authors:

DTO conceived and designed the study, acquired, analyzed and interpreted the data. HAS acquired and interpreted data. OS and SK interpreted the data. All the authors drafted, revised, read and approved the manuscript.

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No conflict of interest is declared

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Original Article **Open Access**

Phenotypic and molecular detection of methicillin-resistance and virulence factors in coagulase-negative staphylococci isolated from patients with urinary tract infections in southeastern Nigeria

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Abstract:

Background: The emergence of antibiotic resistance among coagulase-negative staphylococci (CoNS) is an important factor in nosocomial infections outcomes in the healthcare system. This study aimed to phenotypically detect methicillin resistance and genotypically detect *mec*A gene and other virulence markers in coagulase-negative staphylococci isolated from patients with suspected urinary tract infection (UTI) in Alex Ekwueme Federal University Teaching Hospital (AE-FUTHA), Abakaliki, Ebonyi State, Nigeria.
Methodology: A total of 704 patients (adults and children) with clinical features suggestive of UTI, attending the Alex-Ekwueme Federal University Teaching Hospital, were randomly selected for the study. Urine samples were collected from the patients, cultured in mannitol salt agar (MSA) and CoNS identified using conventional microbiological methods. Genotypic confirmation of CoNS was done for 15 randomly selected isolates using the 16S rRNA gene amplification and Big Dye terminator sequencing. Phenotypic detection of methicillin resistance was done using cefoxitin disc diffusion test and confirmed by PCR amplification of a 533-base pair *mec*A gene fragment. Data were analyzed using SPSS version 27.0, and Chi-square test was used to determine association between qualitative variables obtained in this study.
Results: A total of 50 CoNS were isolated from the samples of 704 patients with suspected UTI, 29 (58.0%) of which were phenotypically methicillin-resistant-CoNS and included all the *mec*A gene positive isolates. Of the 15 confirmed CoNS by 16S rRNA sequencing, 4 were *Staphylococcus condimenti*, 4 were *Staphylococcus gallinarum*, 3 were *Staphylococcus simulans*, 3 were *Staphylococcus haemolyticus* and 1 was *Staphylococcus sciuri.*
Conclusion: The study recorded a high rate of methicillin resistance among CoNS isolates, indicating that there is need for consistent monitoring and evaluation of antibiotic use in hospital settings to curtail the concerning increase in prevalence of *mec*A gene-positive CoNS isolates.

Keywords: methicillin-resistance; coagulase-negative staphylococci; *mec*A; 16S rRNA; sequencing

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Détection phénotypique et moléculaire des facteurs de résistance à la méthicilline et de virulence chez les staphylocoques à coagulase négative isolés de patients atteints d'infections des voies urinaires dans le sud-est du Nigéria

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Résumé:

Contexte: L'émergence d'une résistance aux antibiotiques chez les staphylocoques à coagulase négative (CoNS) est un facteur important dans les résultats des infections nosocomiales dans le système de santé. Cette étude visait à détecter phénotypiquement la résistance à la méthicilline et à détecter génotypiquement le gène mecA et d'autres marqueurs de virulence chez les staphylocoques à coagulase négative isolés de patients suspectés d'infection des voies urinaires (IVU) à l'hôpital universitaire fédéral Alex Ekwueme (AE-FUTHA), Abakaliki, État d'Ebonyi, Nigéria.
Méthodologie: Au total, 704 patients (adultes et enfants) présentant des caractéristiques cliniques suggérant une infection urinaire, fréquentant l'hôpital universitaire fédéral Alex-Ekwueme, ont été sélectionnés au hasard pour l'étude. Des échantillons d'urine ont été prélevés sur les patients, cultivés dans de la gélose au mannitol et au sel (MSA) et les CoNS ont été identifiés à l'aide de méthodes microbiologiques conventionnelles. Français La confirmation génotypique de la CoNS a été effectuée pour 15 isolats sélectionnés au hasard en utilisant l'amplification du gène de l'ARNr 16S et le séquençage du terminateur Big Dye. La détection phénotypique de la résistance à la méthicilline a été effectuée en utilisant le test de diffusion sur disque de céfoxitine et confirmée par amplification PCR d'un fragment du gène mecA de 533 paires de bases. Les données ont été analysées à l'aide de SPSS version 27.0, et le test du Chi carré a été utilisé pour déterminer l'association entre les variables qualitatives obtenues dans cette étude.
Résultats: Un total de 50 CoNS ont été isolés à partir des échantillons de 704 patients suspectés d'infection urinaire, dont 29 (58,0%) étaient des CoNS phénotypiquement résistants à la méthicilline et comprenaient tous les isolats positifs pour le gène mecA. Parmi les 15 CoNS confirmés par séquençage de l'ARNr 16S, 4 étaient des *Staphylococcus condimenti*, 4 des *Staphylococcus gallinarum*, 3 des *Staphylococcus simulans*, 3 des *Staphylococcus haemolyticus* et 1 des *Staphylococcus sciuri*.
Conclusion: L'étude a enregistré un taux élevé de résistance à la méthicilline parmi les isolats de CoNS, ce qui indique qu'il est nécessaire de surveiller et d'évaluer régulièrement l'utilisation des antibiotiques en milieu hospitalier pour limiter l'augmentation inquiétante de la prévalence des isolats de CoNS positifs au gène mecA.

Mots-clés: résistance à la méthicilline; staphylocoques à coagulase négative; *mec*A ; ARNr 16S; séquençage

Introduction:

Nosocomial infections are complicated by the fact that antibiotic resistance (AMR) has developed in coagulase-negative staphylococci (CoNS) population (1). A number of researchers have recently concentrated on the capacity of CoNS to produce different types of extracellular enzymes. Enzymes such as proteases, phospholipases, esterases, lipases, and elastases are part of this group (1,2). There are some enzymes that are toxins such as those in the enterotoxins, haemolysins, exfoliative toxins, and toxic shock syndrome toxin-1 (TSST-1) families (3,4). These enzymes and toxins are virulence factors of staphylococci responsible for invasion and death of nearby tissues (5). In addition, β-lactam antibiotics are ineffective against methicillin-resistant CoNS (MR-CoNS) (6), and over 80% of hospital isolates of MR-CoNS have been shown to be multi-drug resistant (MDR) (7). These pathogens infect hosts and survive by employing a wide variety of strategies.

According to Kumurya (7), the health-care system is facing an increasingly critical problem with AMR in CoNS population. The purpose of this study was to phenotypically identify methicillin-resistant coagulase-negative staphylococci (MR-CoNS) in patients with suspected urinary tract infections (UTIs) at the Alex-Ekwueme Federal University Teaching Hospital, Abakaliki, Nigeria, and to detect *mec*A gene and virulence markers in the isolates.

Materials and method:

Study period, participants and sample collection:

Between April 2018 and March 2019, 704 inpatients and outpatients suspected of urinary tract infection at the Alex Ekwueme Federal University Teaching Hospital (AE-FUT HA), Abakaliki, Ebonyi State, Nigeria, were consecutively selected for the study.

Clean-catch mid-stream urine samples were collected from the patients into sterile

universal containers and transported immediately to the microbiology laboratory for analysis.

Ethical approval:

Approval to conduct the study was obtained from the Ethical and Research Committee of the Alex Ekwueme Federal University Teaching Hospital, Abakaliki, Ebonyi State, with approval number FETHA/REC/VOL 2/2019/260

Culture and isolation of CoNS bacteria:

Standard bacteriological examinations were carried out on the urine samples. Using aseptic technique, each urine sample was cultured on mannitol salt agar (MSA) and aerobically incubated at 37^{o}C for 24 hours. In order to obtain pure colonies and determine the haemolytic pattern of the bacteria, the presumed CoNS colonies were sub-cultured onto fresh MSA and 5% sheep blood agar (3).

Gram staining, mannitol fermentation, catalase, coagulase, deoxyribonuclease, haemolysin, ornithine decarboxylase, pyrrolidonyl arylamidase, and other conventional biochemical tests were then performed on the isolates to phenotypically identify them to species level.

Phenotypic screening for virulence and detection of methicillin resistance:

Using blood agar, Tween 80, and Tween 20 as substrates, the isolates were examined for their ability to produce haemolysin, lipase, and esterase. Phenotypic methicillin resistance was detected on isolated CoNS by the cefoxitin disc diffusion method.

Molecular detection of CoNS isolates and 16S rRNA gene sequencing:

Molecular analysis (16S rRNA amplification sequencing and *mec*A gene amplification) was performed on 15 randomly selected CoNS isolates

DNA extraction:

The DNA miniprep (Zymo, catalogue number: D6005) was used for DNA extraction. A ZR bashing™ lysis tube was used to mix 2ml of bacterial cell broth with 750ml of lysis solution. The mixture was then centrifuged in a microcentrifuge at > 10,000 x g for 1 min. After transferring 400 µL of supernatant to a collection tube with an orange-top Zymo-Spin™ IV spin filter, it was spun at 7,000 x g for 1 minute. Next, 1,200 µL of DNA binding buffer was added to the mixture following filtration. After 800 µL of the mixture was spun in a collection tube at 10,000 x g for one minute, it was moved to a Zymo-Spin™ IIC column. The gathered flow was then discarded, and the last process was repeated.

The Zymo-Spin™ IIC column was mixed with 200 µL of DNA Pre-Wash buffer using a fresh collection tube, centrifuged at 10,000 x g for 1 minute, and the mixture subsequently applied. After a minute of spinning at 10,000 x g, the Zymo-Spin™ IIC column was washed with 500 µL of bacterial/fungal DNA wash buffer. Then, 100 µL of DNA elution buffer was mixed directly with the Zymo-Spin™ IIC column matrix after its transfer to a fresh 1.5 µL microcentrifuge tube. To extract the DNA, the mixture was spun in a centrifuge at 10,000xg for 30 seconds.

PCR amplification of 16S rRNA gene:

The following reagents were used to prepare the 16S rRNA gene PCR sequencing cocktail; 10 µL of 5x Go Taq colorless reaction, 1 µL of 10 mM of dNTPs mix, 3 µL of 25 mM $MgCl_2$, 1 µL of 10 pmol each of the forward primer (27F: AGAGTTTGATCMTGGCTCAG) and reverse primer (1525R: AAGGAGGT GWTCC ARCCGCA), and 0.3 units of Taq DNA polymerase (Promega, USA) mixed with 8 µL of DNA template to achieve 42 µL solution.

The PCR assay was carried out in a GeneAmp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA) with a PCR cycling condition consisting of an initial denaturation at 94°C for 5 min; followed by 30 cycles consisting of 94°C for 30secs, annealing of primer at 56^{o}C for 30secs and 72°C for one and half minutes, and a final termination at 72°C for 10 mins and chilled at 4^{o}C.

PCR amplification of mecA gene:

The *mec*A-specific primer pairs used to amplify a 533-base pair fragment were; forward 5′-AAAATCGATGGTAAAGGTTGGC-3′ and reverse 5′-AGTTCTGGAGTACCGGATTTGC-3′ (8). A volume of 1 µL of the prepared DNA (0.5 µg) was incorporated into a final volume of 25 µL PCR mixture, which consisted of 10 µL of 2× Master Mix (Ampliqon, Denmark), containing 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.15 mmol/L dNTP, and 1.25 IU Taq DNA polymerase (Ampliqon Co., Denmark), along with 0.7 µL of 0.8 µmol/L of each primer and 12.6 µL of sterile distilled water.

The thermal cycling condition for PCR included an initial step at 95 °C for 3 minutes, followed by 33 cycles at 94°C for 1 minute, 53°C for 30 seconds, and 72°C for 1 minute, and a final extension at 72°C for 6 minutes.

Agarose gel electrophoresis of PCR amplicons:

The agarose powder and 100 mL of 1 x TAE were mixed in a microwavable flask. Microwaving the mixture for one to 3 minutes dissolved the agarose completely. Ten µL of EZ vision DNA stain was added once the agarose solution had cooled to around 50^{o}C. After positioning the well comb on a gel tray, the agarose was carefully added. In order to ensure that the freshly poured gel solidified, it was allowed to sit at room temperature for 20-30 minutes or cooled to 4°C for 10-15 minutes.

The loading buffer was applied to all DNA samples and PCR products. The gel elect-

rophoresis box was used to transfer the set agarose gel. The gel box was filled to the top with 1xTAE (or TBE) after a molecular weight ladder was loaded into the first lane. A voltage range of 80 to 150 V was applied to the samples for about one to one and a half hours after they were delicately placed into the additional gel wells. The power was turned off and the electrodes were disconnected from the power source after the gel was carefully removed from the gel box.

The PCR amplicons were observed with the aid of the UV transilluminator. The gel band was compared to that of a hyper ladder1 (molecular weight ladder) to determine the sizes of the PCR amplicons. The amplicon has a size of about 1200 base pairs for 16S rRNA gene and 533 base pairs for *mec*A gene.

Purification of 16S rRNA gene amplicon:

The PCR reagents were removed by ethanol-amplified fragment removal following gel integrity. In a new sterile 1.5 µl tube from Eppendorf, 7.6 µL of Na acetate 3M and 240 µl of 95% ethanol were added to around 40 µL of PCR amplified result. After the mixture was thoroughly mixed by vortexing, it was kept at -20°C for at least 30 minutes to guarantee proper storage. Before being centrifuged for 15 minutes at 7500 g and 4°C, the pellet was rinsed with 150 µL of 70% ethanol and mixed after being inverted onto the trash once.

Centrifugation was then done for 10 minutes at 13,000 g and 4°C to remove the supernatant. After turning the tube upside down and placing it in the trash, the supernatant was collected again. Once it had dried out in the fume hood for 10-15 minutes at room temperature, it was mixed with 20 µL of sterile distilled water and kept at -20^{o}C until sequencing. Once again, the presence of the purified product was verified by electrophoresis of the isolated fragment on 1.5% agarose gel at 110 V for about an hour.

16S rRNA gene sequencing:

Utilizing the Big Dye terminator v3.1 cycle sequencing kit, the instructions of the Applied Biosystems Genetic Analyzer 3130xl sequencer was followed to sequence the amplified fragments. Bio-Edit and MEGA 6 were employed for all phylogenetic analysis. The 16S rRNA gene sequences of all 1,1820 strains currently available in the Type Strain Genomic Server (TYGS) database were BLASTed (9) against each of the sequences obtained. The genomes of CoNS isolates were used to extract them using RNA primer (10). This was utilized as a stand-in for each genome bitscore to identify the most closely related type strains, and then utilized the Genome BLAST Distance Phylogeny method (GBDP), with the 'coverage' algorithm and the d5 distance calculation to determine exact distances (11).

Data analysis:

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 27.0. Association between qualitative variables was done using Chi-square test with $p<0.05$ considered statistically significant.

Results:

Of the total of 704 participants (196 males and 508 females) with suspected UTI whose mid-stream urine samples were cultured, 29 (4.1%) grew CoNS. Tables 1 and 2 show the distribution of CoNS isolates among the patients with respect to sex and age. There was no significant difference in the frequency of CoNS isolation from urine with respect to sex (OR=1.384, p=0.4038) or age group (OR =0.83, p=1.000).

Out of the 65 male patients below 18 years of age in the study, 4 (6.2%) were positive for CoNS isolates while only 1 (1.3%) of the 75 female patients below 18 years of age was positive (OR=4.852, p=0.1830). Six (4.6%) of the 131 adult male patients (18-84 years) were positive for CoNS while 18 (4.2%) of the 433 adult female patients (18-84 years) were positive for CoNS (OR=1.107, p= 0.8071).

Table 1: Frequency of coagulase-negative staphylococci isolation from urine of selected patients with respect to sex

Sex	Number positive for CoNS (%)	Number negative for CoNS (%)	Total	x^2	OR (95% CI)	*p* value
Male	10 (5.1)	186 (94.9)	196	0.5462	1.384 (0.03-3.03)	0.4038
Female	19 (3.7)	489 (96.3)	508			
Total	**29 (4.1)**	**675 (95.9)**	**704**			

CoNS = Coagulase negative staphylococci; OR = Odd ratio; CI – Confidence interval

Table 2: Frequency of coagulase-negative staphylococci isolation from urine of selected patients with respect to age group

Age group (years)	Number positive for CoNS (%)	Number negative for CoNS (%)	Total	x^2	OR (95% CI	*p* value
<18	5 (3.6)	135 (96.4)	140	0.016	0.83 (0.31-2.23)	1.000
18-84	24 (4.3)	540 (95.7)	564			
Total	**29 (4.1)**	**675 (95.9)**	**704**			

CoNS = Coagulase negative staphylococci; OR = Odd ratio; CI – Confidence interval

Table 3: Reactions of different CoNS isolates to haemolysis, lipase production and esterase

Age group (years)	No of isolates	Haemolysis Production		Lipase Production	Esterase Fermentation
		Weak/delayed	Strong		
Children (< 18)	5	1 (20.0)	4 (80.0)	5 (100.0)	5 (100.0)
Adults (18-84)	24	4 (16.7)	20 (83.3)	24 (100.0)	24 (100.0)
Total	**29**	**5 (17.2)**	**24 (82.7)**	**29 (100.0)**	**29 (100.0)**

Table 4: The phylogenetic tree showing the genetic relationship of the 15 CoNS isolates

S/N	% pair wise identity (Similarity index)	CoNS strain
1	90.80	*Staphylococcus condimenti* strain CIP105760
2	91.70	*Staphylococcus condimenti* strain DSM 11674
3	92.90	*Staphylococcus condimenti* strain FAN 4249
4	90.9	*Staphylococcus condimenti* strain CIP105760
5	92.9	*Staphylococcus simulans* strain GZ202
6	94.9	*Staphylococcus simulans* strain GZ202
7	95.00	*Staphylococcus simulans* strain MS8-25
8	90.30	*Staphylococcus gallinarum* strain F4
9	92.10	*Staphylococcus haemolyticus* strain SHKS1
10	89.90	*Staphylococcus haemolyticus* strain M1-1
11	93.70	*Staphylococcus gallinarum* strain VIIII
12	90.7	*Staphylococcus gallinarum* strain OTM101-2
13	84.60	*Staphylococcus haemolyticus* strain M1-1
14	93.4	*Staphylococcus gallinarum* strain ABR
15	93.1	*Staphylococcus. sciuri* strain LZH-22

CoNS = Coagulase negative staphylococci

Table 3 shows the reactions of the different CoNS isolates to haemolysis, lipase production and esterase activity. All 29 CoNS isolates were positive for lipase, esterase and haemolysis, with 5 (17.2%) being weakly haemolytic. Table 4 show the similarity index of the various isolates. Among the 15 CoNS isolates, 4 *S. condimenti* strains, 4 strains of *S. gallinarum*, 3 strains of *S. simulans*, 3 strains of *S. haemolyticus* and 1 strain of *S. sciuri* were observed.

Fig 1 shows the frequency of phenotypic methicillin resistance among the CoNS isolates with respect to sex and age of the patients from whom the isolates were recovered. Of the 29 CoNS isolates, 16 (55.2%) were MR-CoNS, while 13 (44.8%) were methicillin-sensitive-CoNS. The frequencies of MR-CoNS isolation in the male and female children were 1 (3.5%) and 0 (0%) respectively, and 3 (10.3%) and 12 (41.4%) for adult male and female respectively.

Fig 2 confirms that all the isolates are of bacterial origin by amplification of the 16S rRNA gene (1200bp). Fig 3 is the gel image of amplification of *mec*A gene at 150bp. All selected CoNS isolates expressed the *mec*A gene which renders these organisms resistant to β-lactam antibiotic.

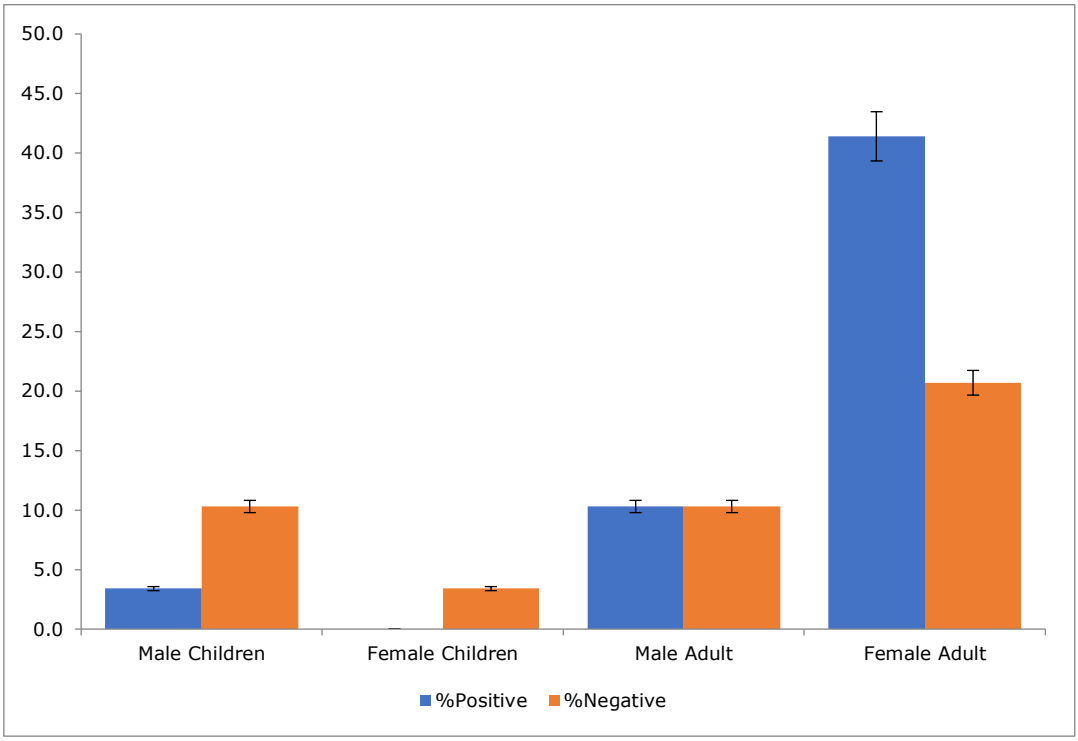

Fig 1: Frequency of methicillin resistance among coagulase negative staphylococci with respect to age and sex

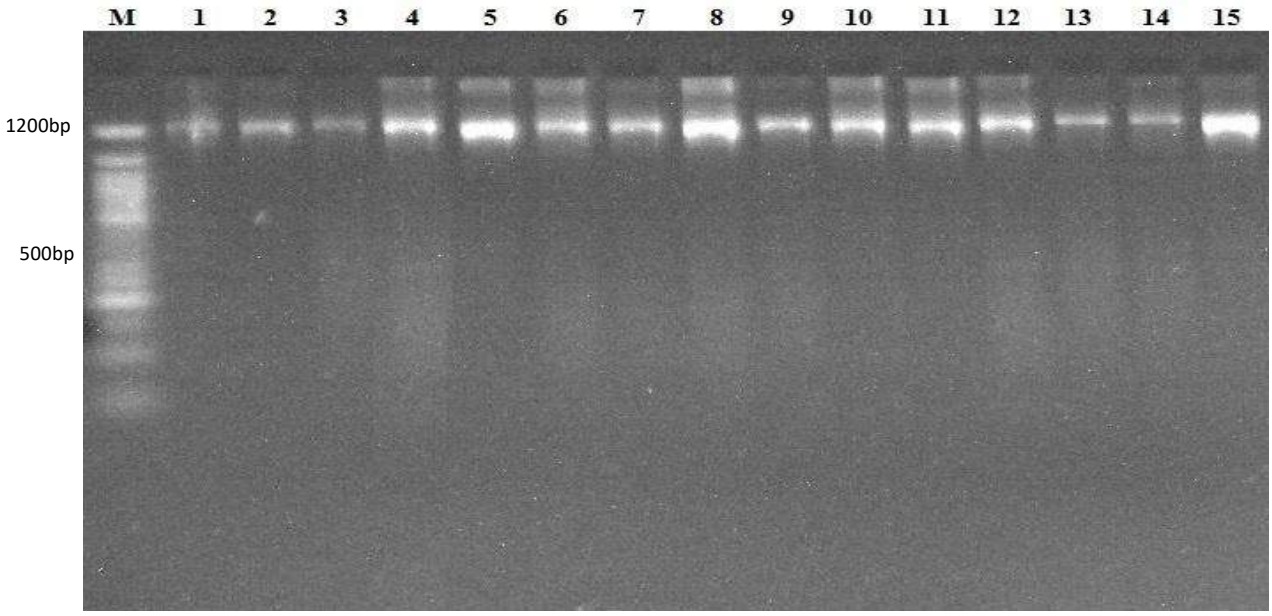

Fig 2: Amplification of bacteria 16S RNA gene region at 1200bp

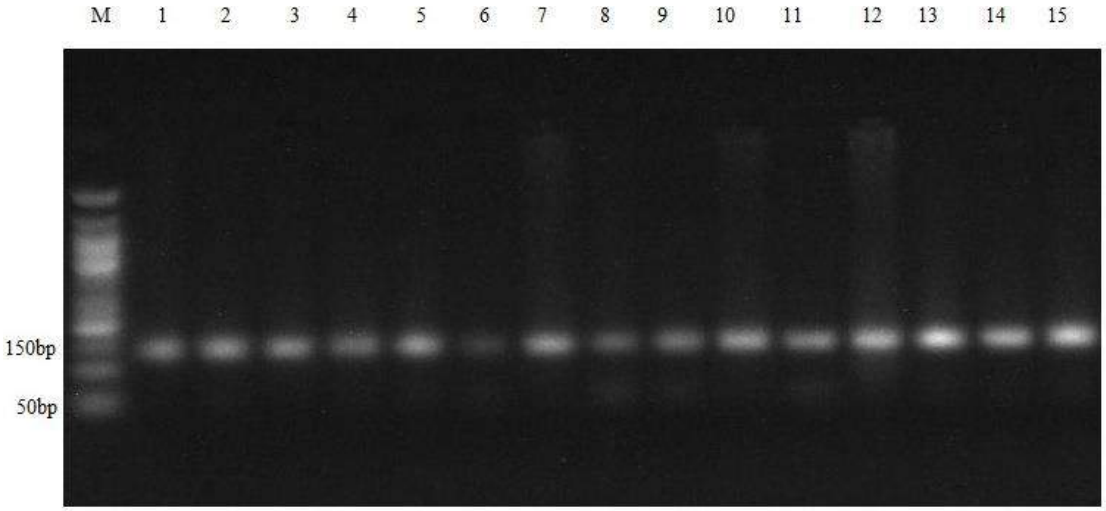

Fig 3: Gel image showing the amplification of *mec*A gene

Discussion:

Over the period of this study (April 2019–March 2019), CoNS were recovered from 29 of the 704 patients with suspected UTIs, recruited into the study. These isolates tested positive hydrolytic enzymes such as lipase, esterase, and haemolysin. While only 5 of the isolates (17.2%) showed mild haemolysis, all of them tested positive for lipase and esterase which can be considered virulence factors of CoNS. Sixteen (55.2%) of the CoNS isolates tested positive for methicillin-resistance as determined by the zone of inhibition to cefoxitin (30µg). This finding is consistent with previous studies that showed large proportion of CoNS isolates tested positive for methicillin resistance (12).

The 16S rRNA sequence analysis on 15 randomly selected isolates confirmed the isolates to be CoNS from the urine samples of the patients. The *mec*A gene was detected in all the 15 isolates, which agrees with a previous study in Japan that reported *mecA*-positive CoNS in 7.9% of the genitourinary tract isolates, although the CoNS isolates in the study were *Staphylococcus saprophyticus* (13). Methicillin resistance in staphylococci is conferred by the production of abnormal penicillin binding protein (PBP2a), that is encoded by *mec*A gene.

At genus-level phylogenetic analysis, 16S rRNA gene sequence analysis has been helpful, but at species-level, its usefulness has been called into question (14). In our study, 14 of the 15 CoNS had 16S rRNA sequence similarity of 90–99%. Four of the CoNS isolates were identified as *S. condimenti*, 4 as *S. gallinarum*, 3 as *S. simulans*, 3 as *S. haemolyticus* and 1 as *S. sciuri*, while only *S. haemolyticus* strain M1-1 had less than 90% (84.6%) sequence similarity. Our finding agrees with what has been previously established on the use of 16S rRNA sequencing for bacterial identification at genus and species levels (15). Using 16S rRNA gene sequencing, the clinical importance of the strain of CoNS species isolated from blood culture could be established, enabling optimal care of patients (16).

Conclusion:

The high carriage of *mec*A gene by the CoNS isolates in this study is a worrying development. This warrants continuous surveillance and review of the antimicrobial use as well as infection control, in order to contain emergence and spread of drug-resistant bacteria in our hospitals.

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Contributions of authors:

IJK was involved in acquisition of data, analysis and interpretation of data, drafting the manuscript and critically revising it for important intellectual content; IOO was involved in analysis and interpretation of data, drafting the manuscript or critically revising it for important intellectual content; CRI, IOO and CMO were involved in acquisition of data, drafting the manuscript and critically revising it for important intellectual content; IOO and SOA were involved in data analysis and inter-

pretation, drafting the manuscript and critically revising it critically for important intellectual content; IOO, UCA, EUU, SIO, OO, and PUM were involved in conception and design of the study, analysis and interpretation. All authors approved the final manuscript submitted for publication.

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Authors declare no conflict of interests

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Original Article **Open Access**

Prevalence and molecular detection of ESBL genes in uropathogens isolated from children and adolescents in a General Hospital in Lagos, Nigeria

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Abstract:

Background: Urinary tract infections (UTIs) are a significant global health concern with rising antimicrobial resistance (AMR) posing a serious challenge to effective treatment. The increasing prevalence of extended-spectrum beta-lactamase (ESBL)-producing uropathogens further complicates management, as these bacteria are resistant to third-generation cephalosporins and other β-lactam antibiotics. The objective of this study is to determine the prevalence and characterize ESBL genes in uropathogens isolated from children with UTIs in Ajeromi General Hospital, Ajegunle, Lagos, Nigeria.
Methodology: A total of 165 children and adolescents presenting with symptoms of UTI were consecutively recruited into the study. Socio-demographic and clinical characteristics were collected from the participants using a structured data collection developed for the study. Voided midstream urine sample was collected from each participant. Standard microbiological methods were used to isolate and identify uropathogens from the sample, followed by antibiotic susceptibility testing (AST) using the Kirby-Bauer disk diffusion method. ESBL production was phenotypically detected by the double-disk synergy test (DDST), and conventional polymerase chain reaction (PCR) assay was performed to detect *bla*$_{TEM}$ and *bla*$_{CTX-M1}$ ESBL genes.
Results: Of the 165 children and adolescents whose urine samples were collected, 49 (26.7%) tested positive for significant bacterial growth, with *Escherichia coli* (53.1%) and *Klebsiella pneumoniae* (16.3%) being the predominant bacterial isolates. The AST revealed high resistance rates to third-generation cephalosporins (with 76.9% to ceftriaxone) and carbapenem (imipenem). While the phenotypic DDST did not confirm ESBL production in any of the isolates, PCR detected ESBL genes in 37 (75.5%) isolates.
Conclusion: Our study highlights the widespread presence of ESBL-producing uropathogens, despite limitations in phenotypic detection methods, underscoring the need for routine molecular screening in AMR surveillance. The findings emphasize the critical role of antibiotic stewardship, infection control measures, empirical treatment guidelines, and promoting targeted molecular diagnostics in preventing the spread of multidrug-resistant (MDR) uropathogens.

Keywords: Urinary tract infections; ESBL; Multidrug-resistant; Molecular screening

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Prévalence et détection moléculaire des gènes BLSE chez des uropathogènes isolés chez des enfants et des adolescents dans un hôpital général de Lagos, au Nigéria

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Résumé:

Contexte: Les infections urinaires (IU) constituent un problème de santé mondial majeur, la résistance croissante aux antimicrobiens (RAM) posant un sérieux défi à l'efficacité de leur traitement. La prévalence croissante des uropathogènes producteurs de bêta-lactamases à spectre étendu (BLSE) complique encore davantage la prise en charge, car ces bactéries sont résistantes aux céphalosporines de troisième génération et à d'autres antibiotiques bêta-lactamines. L'objectif de cette étude est de déterminer la prévalence et de caractériser les gènes BLSE chez les uropathogènes isolés chez des enfants atteints d'IU à l'hôpital général d'Ajeromi, à Ajegunle, à Lagos, au Nigéria.

Méthodologie: Au total, 165 enfants et adolescents présentant des symptômes d'IU ont été recrutés consécutivement pour l'étude. Les caractéristiques sociodémographiques et cliniques des participants ont été recueillies à l'aide d'un recueil de données structuré développé pour l'étude. Un échantillon d'urine mictionnelle a été prélevé chez chaque participant. Français Des méthodes microbiologiques standard ont été utilisées pour isoler et identifier les uropathogènes de l'échantillon, suivies d'un test de sensibilité aux antibiotiques (AST) à l'aide de la méthode de diffusion sur disque de Kirby-Bauer. La production d'BLSE a été détectée phénotypiquement par le test de synergie à double disque (DDST), et un test de réaction en chaîne par polymérase (PCR) conventionnel a été réalisé pour détecter les gènes ESBL *bla*$_{TEM}$ et *bla*$_{CTX-M-1}$.

Résultats: Sur les 165 enfants et adolescents dont les échantillons d'urine ont été collectés, 49 (26,7%) ont été testés positifs pour une croissance bactérienne significative, *Escherichia coli* (53,1%) et *Klebsiella pneumoniae* (16,3%) étant les isolats bactériens prédominants. L'AST a révélé des taux de résistance élevés aux céphalosporines de troisième génération (avec 76,9% à la ceftriaxone) et aux carbapénèmes (imipénème). Bien que le test phénotypique de détection des antigènes de surface (DDST) n'ait confirmé la production de BLSE dans aucun des isolats, la PCR a détecté des gènes de BLSE dans 37 isolats (75,5%).

Conclusion: Notre étude met en évidence la présence généralisée d'uropathogènes producteurs de BLSE, malgré les limites des méthodes de détection phénotypique, soulignant la nécessité d'un dépistage moléculaire systématique dans la surveillance de la RAM. Les résultats soulignent le rôle crucial de la gestion des antibiotiques, des mesures de contrôle des infections, des recommandations thérapeutiques empiriques et de la promotion de diagnostics moléculaires ciblés dans la prévention de la propagation des uropathogènes multirésistants (MR).

Mots-clés: Infections urinaires; BLSE; Multirésistance; Dépistage moléculaire

Introduction:

Urinary tract infections (UTIs) are common medical conditions resulting from the invasion of pathogenic microorganisms into the urinary tract, leading to infections affecting various parts of the urinary system (1,2). The condition is more prevalent in certain populations, such as newborns, young children, sexually active women, and the elderly (3-6). Antimicrobial resistance (AMR) presents an increasing challenge in UTI management, particularly with uropathogenic bacteria exhibiting high resistance rates to multiple antibiotics (7,8). Reports have shown that resistance rates to ciprofloxacin, the most commonly prescribed antibiotic for UTIs, range from 8% to 93% for *Escherichia coli* and from 4% to 79% for *Klebsiella pneumoniae* (7). This increasing prevalence of multi-drug-resistant (MDR) organisms, particularly Gram-negative bacteria, complicates treatment, as they exhibit notable resistance to commonly prescribed antibiotics (9,10).

Studies have shown varying UTI prevalence rates among children, including 26.8% in Uganda (11), 20.65% in Tanzania (12), 11.0% in Nigeria (13), and 27.0% in Ethiopia (9). Also, recent studies have reported notable rise in extended-spectrum β-lactamase (ESBL)-producing *E. coli* in paediatric UTIs. In Duhok City in Iraq, 64.0% of *E. coli* isolates tested positive for ESBL production, with molecular analysis revealing that over 90% harbored *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes (14). A meta-analysis by Azzam et al., (15) found an overall ESBL prevalence rate of 62.0% among pediatric UTI cases, while Kibwana et al., (16) reported 97.0% prevalence of *bla*$_{CTX-M}$ genes in phenotypically detected ESBL-PE isolates in Tanzania.

These findings highlight the importance of thoroughly characterizing ESBL-resistant genes in uropathogens linked to UTIs in children and adolescents to guide treatment approaches and limit the spread of MDR infections. Nevertheless, there exists a scarcity of evidence-based research concerning antibiotic-resistant UTIs in children and adolescents in Nigeria. Consequently, this study determined the prevalence and antimicrobial susceptibility patterns, and detected ESBL genes in bacterial uropathogens involved in UTIs among clinically suspected children and adolescents in a General Hospital in Lagos, Nigeria.

Materials and method:

Clinical setting, study participants and ethical approval:

This was a descriptive cross-sectional

study of 165 consecutively selected children and adolescents with clinical symptoms of UTI attending the Ajeromi General Hospital, Ajegunle, Lagos, Nigeria. The study protocol was approved by the Health Research Ethics Committee of the University of Lagos College of Medicine (Approval Number: CMUL/HREC/01/24/1342). Informed consent was obtained from the parents/guardians/caregivers of the children and informed assent of adolescents.

Data collection:

Demographic and clinical information were obtained from each participant using a structured data collection form developed for the study. Trained healthcare personnel collected the data during routine clinical consultation at the pediatric outpatient department of the hospital. Clinical information collected included presenting symptoms (such as fever, dysuria, urinary frequency, urgency, suprapubic pain), history of previous UTI and patient location (inpatient or outpatient).

Sociodemographic variables collected included age, sex, type of school attended, history of use of school toilets, knowledge of participants toilet use and personal hygiene. These data were obtained directly from the caregivers/parents of the participants through interview.

Urine sample collection:

Voided mid-stream urine sample was collected from each participant by the parent/caregiver into sterile sample bottle, with clear instruction on how urine should be collected. The urine samples were transported immediately to the laboratory for analysis.

Culture isolation of bacteria from urine:

The urine samples were carefully inoculated on Blood and Cystine-Lactose-Electro lyte-Deficient (CLED) agar plates using the streak plate method. The culture plates were incubated at 37°C for 24 hours in aerobic incubator. Culture plates showing significant bacteriuria ($\geq 10^5$ colony-forming units/ml of a single uropathogen on culture plate) were characterized further and isolate identified based on colony morphology, hemolysis patterns on blood agar, lactose fermentation on CLED agar, and conventional biochemical test schemes, including indole, citrate, urease, methyl red (MR), and Voges Proskauer (VP) tests, in accordance with the protocol outlined in Cheesbrough's guidelines for diagnostic microbiology (17). Culture plates with mixed growths or with colony counts of less than 10^5 CFU/ml were excluded from further analysis.

Antibiotic susceptibility test:

The Kirby-Bauer disk diffusion method was used to determine the susceptibility of each isolate to 11 selected antibiotics (18). The antibiotic discs included ofloxacin (OFX, 5µg), cefixime (CFM, 5µg), nalidixic acid (NA, 30µg), ceftriaxone-sulbactam (CRO, 30/15µg), cefuroxime (CXM, 30µg), cefotaxime (CTX, 30µg), imipenem/cilastatin (IMP, 10/10µg), amoxicillin-clavulanic acid (AUG, 20/10µg), levofloxacin (LEV, 5µg), gentamicin (GN, 10 µg) and nitrofurantoin (NIT, 300µg).

The plates were incubated at 37°C in an aerobic environment for 18-24 hours. The zone diameter of inhibition around the disk was measured with a graduated ruler to the nearest millimetre, and interpreted as sensitive, intermediate or resistant according to the CLSI guideline (18).

Phenotypic ESBL screening and confirmation of Gram-negative bacteria:

Gram-negative bacterial isolates resistant to at least one third-generation cephalosporin (cefotaxime, ceftriaxone or cefixime) in the AST were suspected to be presumptive ESBL producers. These isolates were confirmed as ESBL producers using the double disk synergy test (DDST).

Inoculum of the bacterial isolates was standardized to 0.5 McFarland standard, which was then used to inoculate Mueller-Hinton agar plates. Amoxicillin-clavulanate disc was placed in the center of the plate, and cefotaxime and ceftriaxone discs were then placed 15mm away from the amoxicillin-clavulanate disc. After incubation, an ESBL-producing bacteria was confirmed if there was enhancement of inhibition zones of cefotaxime or ceftriaxone around the amoxicillin-clavulanate disc to produce the characteristic 'keyhole' or 'champagne cork' shape.

Genomic DNA extraction:

Fresh bacterial cultures were used for genomic DNA extraction using the boiling method, as described by Egwuatu et al., (19). The resulting supernatant, containing the extracted DNA, was then stored at -20°C for subsequent PCR analysis.

PCR amplification of ESBL genes:

The ESBL genes (bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$) were amplified by using gene-specific primers (Table 1). Each PCR reaction (12.5 µL) included a master mix of One Taq Quick-Load 2x Master Mix, forward and reverse primers, template DNA, and molecular-grade water. The mixture was amplified in a thermocycler with the following reaction cycle parameters; an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing (temperature specified in Table 1) for 1 minute, and extension at 72°C for 1 minute.

Electrophoresis of amplified products:

The amplified PCR products were analyzed using 2% agarose gel electrophoresis, run-

ning at 80V for 90 minutes. The DNA bands were visualized after ethidium bromide staining, and a 100 bp DNA ladder was used to determine the size of the amplified DNA fragments.

Results:

Prevalence of urinary tract infection with respect to age and gender:

A total of 165 children and adolescents with clinical features of UTI were recruited into the study, with 51.5% (n=85) males and 48.5% (n=80) females. The age group 11-18 years constitute the majority of the participants (63.6%, n=105), followed by age group 6-10 years (20. 0%, n=33) and age group 0-5 years (16.4%, n=27) (Table 2).

Urine samples of 49 participants showed significant bacterial (monomicrobial) growth, giving a 29.7% prevalence of UTI in the study (Table 2). The UTI prevalence of 32.5% in the females is higher than 27.1% in the males but this difference is not statistically significant (p= 0.5525). The prevalence of UTI among age group 0-5 years (29,6%), 6-10 years (33.3%) and 11-18 years (28.6%) was not significantly different (p=0.8725) (Table 2).

Bacterial isolates from urine specimens:

Of the 49 clinical isolates identified, 26 (53.1%) are *E. coli,* 8 (16.3%) *K. pneumoniae,* 6 (12.2%) *Proteus mirabilis,* 3 (6.1%) *P. aeruginosa* and 6 (12.2%) *S. aureus* (Table 3).

Table 1: Primers used for PCR assay of ESBL genes in the study

Target gene	Primer sequence (5' - 3')	Annealing temperature (°C)	Product size (bp)	References
$bla_{CTX\text{-}M\text{-}1}$	F-CGTCACGCTGTTGTTAGGAA R-ACGGCTTTCTGCCTTAGGTT	55	780	Zeynudin et al., (20)
bla_{TEM}	F-GTATCCGCTCATGAGACAATAACCCTG R-CCA ATG CTT AAT CAG TGA GGC ACC	62	918	Kaur and Aggarwal., (21)

Table 2: Prevalence of urinary tract infection with respect to gender and age of the study participants

Variable		Urine culture positive (n, %)	Urine culture negative (n, %)	x^2	OR (95% CI)	p value
Sex						
	Male (n=85)	23 (27.1)	62 (72.9)	0.3529	0.7705 (0.3945-1.505)	0.5525
	Female (n=80)	26 (32.5)	54 (67.5)			
	Total (n=165)	**49 (29.7)**	**116 (70.3)**			
Age group (years)						
	0–5 (n=27)	8 (29.6)	19 (70.3)	0.2728	NA	0.8725
	6–10 (n=33)	11 (33.3)	22 (66.7)			
	11–18 (n=105)	30 (28.6)	75 (71.4)			
	Total (n=165)	**49 (29.7)**	**116 (70.3)**			

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

Table 3: Frequency of bacterial isolates recovered from urine samples of the study participants

Bacterial isolates	**Number of isolates**	**Frequency (%)**
Escherichia coli	26	53.1
Klebsiella pneumoniae	8	16.3
Proteus mirabilis	6	12.2
Pseudomonas aeruginosa	3	6.1
Staphylococcus aureus	6	12.2
Total	**49**	**100.0**

Table 4: Association of UTI prevalence with socio-demographic characteristics of the study participants

Variables	**No positive for UTI (%)**	**No negative for UTI (%)**	**x2**	**OR (95% CI)**	***p* value**
Type of school					
Public	39 (36.8)	67 (63.2)	13.988	NA	0.0009*
Private	8 (14.0)	49 (86.0)			
Home-based	2 (100.0)	0			
Use of school toilets					
Yes	49	112	0.5807	3.960	0.3194
No	0	4		(0.2090 - 75.02)	
Knowledge of personal hygiene for toilet use					
Yes	37	83	0.1092	1.226	0.7033
No	12	33		(0.5698 - 2.637)	
History of UTI					
Yes	6	14	0.001	1.017	1.000
No	43	102		(0.3663 - 2.822)	
Location					
Outpatient	49	102		14.01	0.0111
Inpatient	0	14		(0.818 - 239.76)	

UTI = urinary tract infection; OR = Odd ratio; CI = confidence interval; NA = Not applicable

Association of sociodemographic characteristics of the study participants with UTI prevalence:

The prevalence of UTI was significantly higher among children who attends home-based school (100.0%) compared to those who attend public (36.8%) or private school (14.0%) (p=0.0009) (Table 4). However, other factors such as children use of school toilets, knowledge of personal hygiene on the use of the toilets, and history of UTI were not significantly associated with UTI prevalence (p>0.05).

Antibiotic susceptibility results of bacterial isolates:

The antibiotic susceptibility test result for *E. coli* in Table 6 shows that all 26 isolates were 100% resistant to cefuroxime, cefotaxime, ceftriaxone, imipenem, amoxicillin-clavulanate and cefixime. The isolates were sensitive to ofloxacin (100.0%), levofloxacin (80.8%) and gentamicin (100.0%) (Table 5).

Klebsiella pneumoniae isolates was 100.0% sensitive to ofloxacin, levofloxacin and gentamicin but 100.0% resistant to cefotaxime, imipenem, cefuroxime, cefixime, ceftriaxone and amoxicillin-clavulanate. *Proteus mirabilis* isolates were 100.0% sensitive to ofloxacin, levofloxacin and gentamicin but 100.0% resistant to cefixime, cefuroxime, cefotaxime, nitrofurantoin, amoxicillin-clavulanate, ceftriaxone and imipenem (IMP). *Pseudomonas aeruginosa* isolates were 100.0% sensitive to ofloxacin, levofloxacin and gentamicin but 100.0% resistant to ceftriaxone, cefotaxime and cefuroxime and imipenem

Staphylococcus aureus isolates were sensitive to ofloxacin (100.0%), levofloxacin (83.3%), and gentamicin (83.3%) but 100.0% resistant to cefixime, cefuroxime, ceftriaxone, amoxicillin-clavulanate and cefotaxime (Table 6).

Table 5: Antibiotic susceptibility test results of Gram-negative bacterial isolates to selected antibiotics

Antibiotic/Bacteria isolate	*Escherichia coli* (%)		*Klebsiella pneumoniae* (%)		*Proteus mirabilis* (%)		*Pseudomonas aeruginosa* (%)	
	S	R	S	R	S	R	S	R
β-lactam- β-lactamase inhibitor combination								
Amoxicillin-clavulanic acid	0	26 (100.0)	0	8 (100.0)	0	6 (100.0)	0	3 (100.0)
Cephalosporins								
Cefotaxime (CTX)	0	26 (100)	0	8 (100.0)	0	6 (100.0)	0	3 (100.0)
Cefuroxime (CXM)	0	26 (100)	0	8 (100.0)	0	6 (100.0)	0	3 (100.0)
Ceftriaxone (CRO)	0	26 (100)	0	8 (100.0)	0	6 (100.0)	0	3 (100.0)
Cefixime (CFM)	0	26 (100)	0	8 (100.0)	0	6 (100.0)	0	3 (100.0)
Quinolones								
Ofloxacin (OFX)	26 (100.0)	0	8 (100)	0	6 (100.0)	0	3 (100.0)	0
Levofloxacin (LEV)	21 (80.8)	5 (19.2)	8 (100)	0	6 (100.0)	0	3 (100.0)	0
Nalidixic acid (NA)	5 (19.2)	21 (80.8)	1 (12.5)	7 (87.5)	2 (33.3)	4 (66.7)	2 (66.7)	1 (33.3)
Carbapenems								
Imipenem (IMP)	0	26 (100.0)	0	8 (100.0)	0	6 (100.0)	0	3 (100.0)
Nitrofurans								
Nitrofurantoin (NIT)	4 (15.4)	22 (84.6)	0	8 (100.0)	0	6 (100.0)	0	3 (100.0)
Aminoglycosides								
Gentamicin (GEN)	26 (100.0)	0	8 (100.0)	0	6 (100.0)	0	3 (100.0)	0

Table 6: Antibiotic susceptibility test results of *Staphylococcus aureus* isolate to selected antibiotics

Antibiotics/Bacterial isolate	*Staphylococcus aureus* (%)	
	S	R
β-lactam- β-lactamase inhibitor combination		
Amoxicillin-clavulanic acid (AUG)	0	6 (100.0)
Cephalosporins		
Cefotaxime (CTX)	0	6 (100.0)
Cefuroxime (CXM)	0	6 (100.0)
Ceftriaxone (CRO)	0	6 (100.0)
Cefixime (CFM)	0	6 (100.0)
Quinolones		
Ofloxacin (OFX)	6 (100.0)	0
Levofloxacin (LEV)	5 (83.3)	1 (16.7)
Nalidixic acid (NA)	1 (16.7)	5 (83.3)
Carbapenems		
Imipenem (IMP)	0	6 (100.0)
Nitrofurans		
Nitrofurantoin (NIT)	0	6 (100.0)
Aminoglycosides		
Gentamicin (GEN)	5 (83.3)	1 (16.7)

Phenotypic confirmation of ESBL production and ESBL genes:

Although all the 43 Gram-negative and 6 Gram-positive bacterial isolates were resistant to the 3rd generation cephalosporins used in the AST, none of the 43 Gram-negative isolates was positive for the confirmatory ESBL test with the double disc synergy test. However, 37 of the 43 (86.0%) Gram-negative isolates carried ESBL genes on PCR amplification. The most prevalent ESBL gene carried was the bla_{CTXM-1} harbored by 33 (89.2%) of the 37 ESBL Gram-negative

Discussion:

The global increase in bacterial resistance to extended-spectrum cephalosporins presents a major obstacle to effective antibiotic treatment. This rising resistance reduces the range of available therapeutic options, complicating the management and control of infections. In this study, the prevalence of bacterial UTI among the selected children and adolescents was 29.7%, which agrees with findings of studies conducted in Hawassa, Ethiopia, where a prevalence of 27.5% was reported (9), Uganda with 26.8% (11), and Tanzania with 20.65% (12).

However, the prevalence in our study is higher than those from other studies with same population range conducted in Bahirdar, Ethiopia with 16.7% (10), Abakaliki, Nigeria with 3% (22), Enugu, Nigeria with 11% (14), and Dar es Salaam, Tanzania with 16.7% (23). This difference in the prevalence may be due to variations in nutritional status of the population studied, immunity, and geographical location of the study (24).

Table 7: Frequency of ESBL genes carriage among the Gram-negative bacterial isolates

ESBL gene	*Escherichia coli*	*Klebsiella pneumonia*	*Proteus mirabilis*	*Pseudomonas aeruginosa*
bla_{CTXM-1} only	16	0	5	3
bla_{TEM} only	2	2	0	0
bla_{TEM} + bla_{CTXM-1}	2	6	1	0
Total (%)	**20 (46.5)**	**8 (18.6)**	**6 (13.9)**	**3 (6.9)**

Although the age group 11-18 years constituted the majority of the children and adolescents (63.6%) with clinical symptoms of UTI recruited in this study, the prevalence of UTI by culture was highest among the age group 6-10 years (33.3%), although the UTI prevalence difference was not statistically significant with respect to age group of the participants (p=0.8725). However, the recruitment of high number of children with symptoms of UTI in this age group may not be unrelated to increased sexual activity being reported within this age demographic. A study in Benin, Nigeria reported that approximately 31.6% of the study respondents had had sexual intercourse before the age of 15 years (25), a predisposing factor to UTI. Another study in Nigeria reported mean age of onset of sexual activity of 11.68±1.98 years and prevalence of premature sexual activity of approximately 11.0% (26). Additionally, community-based studies in Ghana, Malawi, Uganda and Burkina Faso reported that 45.0% of young men and nearly 60.0% of young women in sub-Saharan Africa had had sexual intercourse before the age of 18 years (27).

In this study, the prevalence of UTI was higher in the females (32.5%) than males (27.1%) although this difference was not statistically significant (p=0.5525). However, it has long been established in literature that females are at higher risk of UTI than males due to the short urethra in females, and the close proximity of vaginal and anal canal with the urinary system, which facilitate entry of vaginal and rectal flora into the urinary tract.

Escherichia coli is the most predominant uropathogen in our study, responsible for over half of the UTI followed by *K. pneumoniae* (16.3%), which is consistent with trends reported globally, where *E. coli* is recognized as the leading cause of UTIs in paediatric populations, accounting for up to 90% of cases (28-30). The predominance of these two bacterial uropathogens also agrees with findings of other studies in Africa such as Ethiopia and Tanzania, which also reported a high prevalence of *E. coli* and *Klebsiella* in paediatric UTIs (9,12). The prevalence rate of 53.1% for *E. coli* UTI in our study is comparable with the rate of 48.6% reported in Hawassa, Ethiopia (9), 63.6% in Bahir Dar, Ethiopia (10), 71.7% in Iran (31), 31.8% in Enugu, Nigeria (13), 35.7% in Dar es Salaam, Tanzania (23), and 41.2% in Mwanza, Tanzania (12). However, our finding contrasts the study conducted in Abakaliki, Nigeria, where the most common bacterial uropathogen was *Klebsiella* spp with 24.5% (22), and in Uganda where *Proteus* spp was the predominant uropathogen with 39.5% (11). The possible explanation for these differing rates may be due to variations in sample size of the studies, specimen collection technique, host immunity system, and nutritional state (32,33).

Being a common gut commensal, *E. coli* is the leading cause of community-acquired UTI and its increased prevalence among pediatric UTI cases may be partly attributed to inadequate personal hygiene practices, which can facilitate faecal contamination of the periurethral area. However, this is considered alongside other contributory factors such as age, sex, and individual host susceptibility. Additionally, *E. coli* has a special structure that encourages urinary tract colonization and evades the immune system, as well as being able to move from the perineum area that is contaminated with faecal matter, invading the host tissues within the urinary tract (34). *Staphylococcus aureus* was isolated from 12.2% of the children which is unexpected because this pathogen has been implicated in predominantly nosocomial infection. However, recent epidemiological trends have shown increasing prevalence of community-acquired *S. aureus* infections including UTIs among individuals without prior hospitalization or known risk factors (35).

The prevalence of UTI was significantly higher among children who attended home-based school (100.0%) compared to those who attended public (36.8%) or private school (14.0%) (p=0.0009). This observation may however need to be cautiously interpreted in view of the few numbers of children attending home-based school in the study, which could affect the validity of the statistical analysis. Although regular urination and prompt bladder emptying are essential components of good toilet hygiene that help

prevent UTIs (36), our study found no significant association of children use of school toilets or knowledge of personal hygiene on the use of toilets and UTI prevalence. The prevalence of UTI among children from outpatient department was 100% compared to 0% among those from inpatient wards (p= 0.0111). This appears to suggest that community-associated paediatric UTI is significantly higher than nosocomial paediatric UTI, however, the prevalence of 100% reported is far higher than the global prevalence, that ranges between 13 and 33% (37). Our finding, which must be interpreted with caution, may be due to the small number of paediatric inpatients with symptoms of UTI recruited into the study.

Antibiotic resistance in microbial population have become one of the most pressing concerns in healthcare worldwide. The emergence and spread of antibiotic-resistant bacteria significantly complicate the treatment of infections and increase mortality, as infections become harder to treat with conventional antimicrobial therapies (38,39). In this study, we assessed the antibiotic resistance of the bacterial uropathogens to eleven selected antibiotics because of previous reports from the Global Antimicrobial Resistance and Use Surveillance System (GLASS), which indicate rising resistance levels among uropathogens, particularly to first-line antibiotics (7). Resistance rates were high to the antimicrobial agents tested, especially the 3rd generation cephalosporins, amoxicillin-clavulanate and carbapenem, to which all the bacterial isolates were 100.0% resistant. The observed high resistance rates may be attributed to the widespread misuse and over-use of antibiotics in our community which stemmed from lack of robust antibiotic stewardship programs in the region (40,41)

Although all the 43 Gram-negative uropathogens isolated in our study were resistant to 3rd generation cephalosporins in the susceptibility testing, making them to be presumptive ESBL producers, none of them were confirmed to produce ESBL by the double disc synergy test. However, molecular analysis by PCR showed that 37 (86.0%) of the 43 isolates harbored ESBL ($bla_{\text{CTX-M-1}}$ and bla_{TEM}) genes, which are well-documented genetic basis of resistance to 3rd generation cephalosporins such as cefotaxime, ceftriaxone and ceftazidime. This molecular finding aligns with the antibiotic susceptibility results, where 100.0% resistance rate was recorded against the cephalosporins, suggesting potential ESBL production as the major mechanism of resistance in the Gram-negative uropathogens. The multi-drug resistance patterns observed however suggest that some of the Gram-negative uropathogen harboring $bla_{\text{CTX-M-1}}$ and bla_{TEM} genes may in addition contain other resistance determinants, contributing to reduced efficacy of β-lactam/β-lactamase inhibitor combinations, and resistance to fluoroquinolones and aminoglycosides, which could be linked to co-selection pressure from the ESBL plasmids that often carry multiple resistance genes. This is clinically significant as fluoroquinolones and aminoglycosides are often used as alternative therapies for complicated UTIs.

Previous studies have reported rising trend of ESBL-producing pathogens not only in the hospital but also in community settings (42,43). The high carriage rate of $bla_{\text{CTX-M-1}}$ gene in particular aligns with the global reports that identify CTX-M as the most prevalent ESBL type in both community-acquired and healthcare-associated infections (44). The carriage rate of $bla_{\text{CTX-M-1}}$ was highest among *E. coli* isolates (n=18) in our study, which is consistent with previous findings that *E. coli* is a major producer of CTXM-1 enzyme, particularly in healthcare-associated infections. This was followed by *K. pneumoniae* (n=6), which is expected, as this organism is also known to harbour ESBL genes, including CTX-M-1. All the *P. aeruginosa* and *P. mirabilis* isolates in our study also harbored the CTX-M-1 genes. The highest carriage rate of bla_{TEM} gene was among *K. pneumoniae* (n=8), followed by among *E. coli* (n=4). This suggests that these organisms are capable of producing TEM-type beta-lactamases also, which are often encoded on plasmids and can be transferred between different species. *Proteus mirabilis* isolates have a much lower carriage rate of ESBL genes (n=1), indicating that this species may harbour TEM gene but in a smaller proportion of isolates compared to *E. coli* and *K. pneumoniae*.

Our study showed disparity in the phenotypic confirmatory ESBL detection test (double disc synergy test-DDST) and genotypic (PCR) method used. This emphasizes the necessity of more sensitive and precise techniques to detect these resistant species. The observed discrepancy could be caused by a combination of environmental factors, differences in ESBL expression levels, reduced sensitivity of phenotypic tests, and technical issues when performing the phenotypic confirmatory tests. This may compromise patient outcomes by resulting in incorrect diagnoses and ineffective therapy. To guarantee precise identification of ESBL-positive isolates and direct suitable antibiotic therapy, it is essential to integrate cutting-edge molecular approaches into routine susceptibility testing (45, 46).

There are a number of reasons why the DDST approach for ESBL detection may produce false negative results. Without necessarily engaging ESBL genes, resistance to all

β-lactam antibiotics can be conferred by the co-production of enzymes that hydrolyze carbapenem and aztreonam (42,47). Furthermore, phenotypic detection techniques may not be adequate due to the growing complexity of bacterial resistance mechanisms, which include the development of various β-lactamase types and the combination of ESBLs with AmpC β-lactamases. These drawbacks highlight the necessity of more advanced molecular methods to precisely identify bacteria that produce ESBLs and direct the proper course of antibiotic treatment.

Plasmid-mediated AmpC-type β-lactamases have been discovered in *K. pneumo niae* isolates in a number of studies. It has been discovered that some of these species contain both ESBLs and AmpC-type β-lactamases (42,48). The presence of both enzyme types in the same strain raises the minimum inhibitory concentration (MIC) for cephalosporins, but it can also lead to false-negative ESBL detection tests. It is possible that AmpC type β-lactamases obstruct the synergistic impact of clavulanate and cephalosporins against ESBLs because they are resistant to clavulanate inhibition. It is not known how much of one β-lactamase is needed in relation to the other to mask its presence.

Conclusion:

Our study results have significant clinical implications. The high resistance of *E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa,* and *S. aureus* to antibiotics commonly used for treatment of UTI and other infections underscores the necessity of susceptibility testing to guide tailored treatment. The global rise of resistance to third-generation cephalosporins and fluoroquinolones in Gram-negative bacteria raises concerns about their long-term effectiveness. ESBL-producing *E. coli*, along with carbapenem-resistant *P. aeruginosa* and *K. pneumoniae,* pose major treatment difficulties, demanding the development of new antibiotics or alternative therapies.

These findings highlight the critical need to combat antibiotic resistance through enhanced diagnostics, personalized treatment plans, and robust antibiotic stewardship programs. Ongoing monitoring of resistance trends and the discovery of new antimicrobial agents are essential to combat resistant pathogens and preserve antibiotic efficacy for future use.

Contributions of authors:

ENF was involved is study conceptualization, project administration, investigations, methodology, resources and writing of the original draft; UMC was involved in supervision, validation of data, review and editing of the manuscript; AKC was involved in supervision, methodology, visualization, investigation, review and editing of manuscript; CCR was involved in supervision, investigation, cytology validation and review of manuscript; CHN was involved in the design, validation of research data and review of the manuscript; and OLC and MUC were involved in molecular analysis, methodology and data validation. All authors approved the manuscript for submission.

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Original Article **Open Access**

Molecular detection of carbapenemase-producing *Klebsiella* spp from clinical samples in Ibadan, Nigeria

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Abstract:

Background: *Klebsiella* species are significant pathogens responsible for various healthcare-associated infections. Despite the existence of several mechanisms of resistance against high priority antibiotics such as carbapenems, the production of carbapenemases remains the most important mechanism of resistance among *Klebsiella* spp. The aim of this study was to determine the prevalence of carbapenemase-production among *Klebsiella* spp isolated from clinical specimens at the University College Hospital (UCH) Ibadan, Nigeria.
Methodology: A total of 120 clinical bacterial isolates were collected from the routine laboratories in the Department of Medical Microbiology and Parasitology, UCH, Ibadan between January and August 2023. These isolates were re-identified using conventional microbiological methods, including Gram staining, and biochemical identification tests. Eighty viable isolates were screened for carbapenem resistance using imipenem, following CLSI guidelines. Carbapenemase production was confirmed by the modified carbapenem inactivation (mCIM) and EDTA carbapenem inactivation (eCIM) methods. Conventional multiplex polymerase chain reaction (PCR) was used to amplify and detect carbapenemase resistance genes (bla_{NDM-1}, bla_{VIM}, bla_{KPC}, bla_{OXA-48}, and bla_{IMP}) in the isolates, with the amplified products resolved by agarose gel electrophoresis. Data were entered and analyzed using Statistical Package for the Social Sciences (SPSS) version 26.0.
Results: Ninety-five of the 120 isolates were biochemically identified as *Klebsiella* spp while only 80 remained viable. Twenty-three (28.8%) biochemically confirmed isolates were resistant to imipenem (carbapenem resistant *Klebsiella*), and 20 (25.0%) were confirmed as *Klebsiella* spp by PCR, of which 11 (55.0%) were resistant to all tested antibiotics. Carbapenemase production was detected in 5 (6.3%) isolates using mCIM and eCIM. PCR identified bla_{NDM-1} gene in 3 (3.8%) of the isolates, while bla_{VIM}, bla_{KPC}, bla_{OXA-48}, and bla_{IMP} genes were not detected in any isolate.
Conclusion: The detection of bla_{NDM-1} and high resistance levels among isolates is of great concern. The emergence of carbapenem-resistant *Klebsiella*, especially with bla_{NDM-1} gene, threatens the effectiveness of 'last-resort' antibiotics. There is a need for antimicrobial resistance surveillance and antimicrobial stewardship to stem the tide of antimicrobial resistance in healthcare settings.

Keywords: *Klebsiella*, carbapenemase, antimicrobial resistance, Ibadan, Nigeria.

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Détection moléculaire de *Klebsiella* spp productrices de carbapénémases dans des échantillons cliniques à Ibadan, au Nigéria

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Résumé:

Contexte: Les espèces de *Klebsiella* sont des agents pathogènes importants responsables de diverses infections associées aux soins. Malgré l'existence de plusieurs mécanismes de résistance aux antibiotiques prioritaires tels que les carbapénèmes, la production de carbapénémases demeure le principal mécanisme de résistance chez les espèces de *Klebsiella*. L'objectif de cette étude était de déterminer la prévalence de la production de carbapénémases chez les espèces de *Klebsiella* isolées à partir d'échantillons cliniques à Hôpital Universitaire (UCH) d'Ibadan, au Nigéria.
Méthodologie: Au total, 120 isolats bactériens cliniques ont été collectés dans les laboratoires de routine du Département de microbiologie médicale et de parasitologie de l'UCH d'Ibadan entre janvier et août 2023. Ces isolats ont été réidentifiés à l'aide de méthodes microbiologiques conventionnelles, notamment la coloration de Gram et des tests d'identification biochimique. Quatre-vingts isolats viables ont été testés pour leur résistance aux carbapénèmes à l'imipénème, conformément aux directives du CLSI. La production de carbapénémases a été confirmée par les méthodes d'inactivation modifiée des carbapénèmes (mCIM) et d'inactivation des carbapénèmes par EDTA (eCIM). Français La réaction en chaîne par polymérase multiplex (PCR) conventionnelle a été utilisée pour amplifier et détecter les gènes de résistance aux carbapénémases (*bla*$_{NDM-1}$, *bla*$_{VIM}$, *bla*$_{KPC}$, *bla*$_{OXA-48}$ et *bla*$_{IMP}$) dans les isolats, les produits amplifiés étant résolus par électrophorèse sur gel d'agarose. Les données ont été saisies et analysées à l'aide de la version 26.0 de Statistical Package for the Social Sciences (SPSS).
Résultats: Quatre-vingt-quinze des 120 isolats ont été identifiés biochimiquement comme Klebsiella spp tandis que seulement 80 sont restés viables. Vingt-trois (28,8%) isolats confirmés biochimiquement étaient résistants à l'imipénème (*Klebsiella* résistant aux carbapénèmes) et 20 (25,0%) ont été confirmés comme *Klebsiella* spp par PCR, dont 11 (55,0%) étaient résistants à tous les antibiotiques testés. La production de carbapénémase a été détectée dans 5 isolats (6,3%) à l'aide des techniques mCIM et eCIM. La PCR a identifié le gène *bla*$_{NDM-1}$ dans 3 isolats (3,8%), tandis que les gènes *bla*$_{VIM}$, *bla*$_{KPC}$, *bla*$_{OXA-48}$ et *bla*$_{IMP}$ n'ont été détectés dans aucun isolat.
Conclusion: La détection de blaNDM-1 et de niveaux élevés de résistance parmi les isolats est très préoccupante. L'émergence de *Klebsiella* résistantes aux carbapénèmes, en particulier celles porteuses du gène *bla*$_{NDM-1}$, menace l'efficacité des antibiotiques de «dernier recours». Il est nécessaire de surveiller la résistance aux antimicrobiens et d'en assurer la gestion afin d'enrayer la vague de résistance aux antimicrobiens dans les établissements de santé.

Mots-clés: *Klebsiella*, carbapénémase, résistance aux antimicrobiens, Ibadan, Nigéria.

Introduction:

Klebsiella species are significant pathogens responsible for various healthcare-associated infections (1,2). Their ability to form biofilms facilitates persistence and rapid spread in hospital environments, posing a serious threat to public health due to frequent resistance to critically important antibiotics, especially carbapenems (3-5). The rising prevalence of multidrug-resistant (MDR) bacteria (resistance to at least one member in three or more classes of antibiotics), including carbapenem-resistant *Klebsiella* strains, is associated with increased morbidity, mortality (approaching 60%), and healthcare costs (6-9).

In *Klebsiella* spp, carbapenem resistance, primarily due to carbapenemase production, is the most significant β-lactam resistance mechanism. *Klebsiella pneumoniae* harbors several types of carbapenemases, including KPCs (Class A), NDM (Class B), and OXA-type (Class D) (10-12). Non-carbapenemase mechanisms such as loss of outer membrane proteins and efflux pump production, also contribute to carbapenem resistance (13). Despite these mechanisms, carbapenemase production remains the most important resistance mechanism.

The modified carbapenem inactivation method helps distinguish between these detection mechanisms but other standardized phenotypic tests for carbapenemase detection include multi-disk mechanism testing and combined disk synergy test (14). Molecular detection methods such as PCR are favoured due to the low turnaround time, high accuracy, and specificity. The spread of carbapenemase genes among Enterobacteriaceae, coupled with inadequate surveillance and infection control measures, exacerbates the problem. Detection and characterization of carbapenemase-producing *Klebsiella* strains are essential for effective management and infection control.

In Nigeria, lack of adequate surveillance and monitoring of carbapenem-resistant Enterobacteriaceae increases the risk of further dissemination of antibiotic-resistant genes (15-18). Continuous detection and characterization of carbapenemases are crucial for understanding their epidemiology and preventing their spread. Therefore, this study aimed to detect carbapenemase-producing strains of *Klebsiella* spp isolated from clinical samples in University College Hospital, Ibadan, southwest Nigeria.

Materials and method:

Study site:

This laboratory-based study was conducted in the Department of Medical Microbiology and Parasitology of the University College Hospital (UCH) in Ibadan, Nigeria between January and August 2023.

Clinical bacterial isolates:

Presumptively identified *Klebsiella* spp isolates were collected from the Department of Medical Microbiology and Parasitology laboratory. These isolates were recovered from sam-

ples collected from both in-patients and out-patients across 31 different wards and various clinics in UCH and a few other hospitals in Ibadan (Oluyoro Catholic Hospital and Adeoyo Maternity Teaching Hospital, Yemetu).

Ethical approval:

Approval was obtained from the Ethics and Research committee of University College Hospital and University of Ibadan, Nigeria (UI/EC/22/0403).

Bacterial isolates and preliminary analyses:

Presumptively identified non-duplicate *Klebsiella* isolates from clinical samples on the routine bench were re-characterized using standard microbiological techniques (19). Isolates were subcultured onto MacConkey agar (Oxoid, UK) and incubated under aerobic conditions at 37ºC for 24 hours. Re-characteri zation was carried out by Gram stain reaction, urease test, citrate utilization, indole production, and motility assessment, using standard methods (19).

Antibiotic susceptibility testing (AST):

Antibiotic susceptibility testing (AST) for each isolate was done using the modified Kirby-Bauer disk diffusion test on Mueller Hinton agar (Oxoid, UK) according to the Clinical and Laboratory Standards Institute guidelines (20). The antimicrobial discs used included imipenem (IPM, 10µg), ceftazidime (CAZ, 30 µg), levofloxacin (LEV, 5µg), piperacillin (PRL, 30µg), gentamicin (GM, 120µg), cefoxitin (FOX, 30µg), amoxicillin-clavulanate (AUG, 30 µg) and ciprofloxacin (CIP, 5µg). *Escherichia coli* (ATCC 25922) was used as quality control strain to validate media and disc potency.

Phenotypic detection of carbapenemases:

Imipenem-resistant isolates were tested for carbapenemase production using the Modified Carbapenem Inactivation method (m CIM) and EDTA Inactivation Method (eCIM). Isolates were subcultured from Nutrient agar slants onto MacConkey agar and incubated overnight. Overnight cultures of both test isolates and quality control strains were inoculated into 2mL of Typtic Soy Broth (TSB) for mCIM and 2mL of TSB containing 20µL of 0.5M EDTA for eCIM. After homogenization, a meropenem disk was added to each tube, followed by a 4-hour incubation at 32±2˚C. Discs were then placed on *E. coli* ATCC 25922-inoculated Mueller Hinton agar (MHA) plates and incubated at 37˚C for 24 hours.

A zone of inhibition with a diameter of 6-15 mm indicated a positive mCIM. An increase of ≥5mm in the zone size for eCIM compared to mCIM suggested metallo-carbapenemase production. Control strains used were *Klebsiella pneumoniae* ATCC® BAA-1705™ (KPC positive), *Klebsiella pneumoniae* ATCC® BAA-1706™ (carbapenemase negative), and *Klebsiella pneumoniae* ATCC® BAA-2146™ (NDM positive).

Molecular detection of *Klebsiella* isolates:

All molecular analyses were carried out at the molecular unit of the Biorepository and Clinical Virology Laboratory (BCVL), College of Medicine, University of Ibadan.

DNA extraction:

Freshly cultured carbapenem resistant *Klebsiella* isolates were sub-cultured into Tryptic Soy broth (TSB) and stored at -20^{0}C for molecular studies. Stored isolates were recovered by centrifugation. The supernatant was discarded, and the pellet composed of the bacterial cells were subjected to DNA extraction process as described by Trindade et al., (21). The broth culture was centrifuged (10,000rpm for 5 minutes) to collect the cell pellet. Five hundred microliters of Tris-EDTA buffer were added, followed by 15µL of Sodium Dodecyl Sulphate (SDS) and 3µL of proteinase K. The mixture was vortexed thoroughly to facilitate cell lysis and incubated at 37˚C for 1 hour.

After incubation, 100µL of 5M NaCl and 80µL of 10% CTAB solution in 0.7M NaCl were added and vortexed, followed by a 10 minutes' incubation at 65˚C on the heating block. Phenol-Chloroform-Isoamylalcohol (PCI, 25:24:1) was thereafter added to separate DNA from proteins and other cellular debris. After centrifugation (10,000rpm for 10 mins), the DNA-containing supernatant was precipitated with 400µL of isopropanol (8˚C for 1 hr) and further purified with 500µL 70% ethanol. The DNA pellets were re-suspended with 50µL of nuclease-free PCR graded water and stored at -80˚C for further analyses.

Confirmatory identification of Klebsiella isolates by PCR:

The identity of carbapenem-resistant *Klebsiella* isolates (n=23) was confirmed by conventional PCR assay and products were resolved using agarose gel electrophoresis. The set of primers and reaction protocols applied are highlighted in Table 1.

Table 1: Primer sequences for targeted Klebsiella species, with corresponding amplicon sizes and PCR cycling parameters

Target strain	Primer sequence (5' → 3')	Amplicon size (bp)	PCR cycling condition
***Klebsiella* genus**	F:GAGCAAGCGGACCTCATAAA R:CGGTTACCTTGTTACGACTTCA	241	Initial denaturation of 5 min at 94^{0}C followed by 35 cycles, denaturation at 95^{0}C for 30 sec, annealing at 49^{0}C for 40 sec, extension at 72^{0}C for 40 sec, and final extension at 72^{0}C for 10 min
Klebsiella pneumoniae	F: GGAGGTATCAGAAGTGCGAATG R: CACACCTCAGCCTTGATTATCC	300	Initial denaturation of 5 min at 94^{0}C followed by 35 cycles, denaturation at 95^{0}C for 30 sec, annealing at 50^{0}C for 40 sec, extension at 72^{0}C for 40 sec, and final extension at 72^{0}C for 10 min

Table 2: Primer sequences for detection of selected carbapenem resistance genes

Target gene	Primer sequence (5' → 3')	Amplicon size (bp)	PCR cycling condition
bla$_{NDM-1}$	F: GGTTTGGCGATCTGGTTTTC R: CGGAATGGCTCATCACGATC	624	Initial denaturation of 5 min at 94^{0}C followed by 30 cycles, denaturation at 95^{0}C for 30 sec, annealing at 50^{0}C for 40 sec, extension at 72^{0}C for 40 sec, and final extension at 72^{0}C for 5 min
bla$_{OXA-48-like}$	F: TTCTGTTGTTTGGGTTTCGC R: ACGCAGGAATTGAATTTGTTC	190	Initial denaturation of 5 min at 94^{0}C followed by 30 cycles, denaturation at 95^{0}C for 30 sec, annealing at 47^{0}C for 40 sec, extension at 72^{0}C for 40 sec, and final extension at 72^{0}C for 5 min
bla$_{KPC}$	F: CATTCAAGGGCTTTCTTGCTGC R: ACGACGGCATAGTCATTTGC	498	Initial denaturation of 5 min at 94^{0}C followed by 30 cycles, denaturation at 95^{0}C for 30 sec, annealing at 47^{0}C for 40 sec, extension at 72^{0}C for 40 sec, and final extension at 72^{0}C for 5 min
bla$_{VIM}$	F: GGTGTTTGGTCGCATATCGCAA R: ATTCAGCCAGATCGGCATCGGC	502	Initial denaturation of 5 min at 94^{0}C followed by 30 cycles, denaturation at 95^{0}C for 30 sec, annealing at 50^{0}C for 40 sec, extension at 72^{0}C for 40 sec, and final extension at 72^{0}C for 5 min
bla$_{IMP}$	F: TCGTTTGAAGAAGTTAACG R: ATGTAAGTTTCAAGAGTGATGC	568	Initial denaturation of 5 min at 94^{0}C followed by 30 cycles, denaturation at 95^{0}C for 30 sec, annealing at 47^{0}C for 40 sec, extension at 72^{0}C for 40 sec, and final extension at 72^{0}C for 5 min

Detection of carbapenemase genes by multiplex PCR:

Klebsiella spp isolates that exhibited phenotypic resistance to imipenem (n=23) were further screened for relevant carbapenemase-coding genes using conventional multiplex PCR assay and PCR products were viewed using Agarose Gel Electrophoresis (AGE). The set of primers and reaction protocols applied are highlighted in Table 2.

Statistical analysis:

Data were entered into Microsoft Excel and analyzed using Statistical Package for the Social Sciences (SPSS) version 26.0. Descriptive statistics such as frequencies and percentages were used to summarize the distribution of carbapenemase-producing isolates and their antimicrobial susceptibility patterns.

Results:

Frequency distribution of *Klebsiella* isolates across clinical samples and hospital wards:

Of the 120 presumptive *Klebsiella* spp tested, 95 (80.0%) were biochemically confirmed as *Klebsiella* spp while only 80 remained viable after subsequent subculturing. The pathogen was predominantly recovered from urine specimen (n=30, 37.5%).

Majority of the confirmed isolates were recovered from the medical (n=28, 35.0%) and surgical (n=28, 35.0%) wards in UCH while 17 (21.3%) were from outside facilities (Oluyoro Catholic hospital and Adeoyo Maternity Teaching hospital Ibadan) (Table 3 and 4).

Table 3: Distribution of *Klebsiella* spp across clinical samples

Clinical sample	Frequency (%)			
	Klebsiella* spp	**Culture positive *Klebsiella* spp**	**CRKP**	**CpCRKP**
Urine	42 (35.0)	30 (37.5)	12 (40.0)	5 (16.7)
Wound **s**wab	22 (18.3)	14 (17.5)	2 (14.3)	0
Eye **s**wab	3 (2.5)	2 (2.5)	0	0
Pus	4 (3.3)	1 (1.3)	1 (100.0)	0
Wound **a**spirate	6 (5.0)	2 (2.5)	0	0
Sputum	15 (12.5)	12 (15.0)	3 (25.0)	0
High **v**aginal **s**wab	6 (5.0)	5 (6.3)	1 (20.0)	0
Blood **c**ulture	6 (5.0)	6 (7.5)	2 (33.3)	0
Tracheal **a**spirate	1 (0.8)	1 (1.3)	1 (100.0)	0
Wound **b**iopsy	5 (4.2)	1 (1.3)	0	0
Throat **s**wab	3 (2.5)	1 (1.3)	0	0
Stool	3 (2.5)	3 (3.8)	1 (33.3)	0
Ear **s**wab	4 (3.3)	2 (2.5)	0	0
Total	**120 (100.0)**	**80 (100.0)**	**23 (28.8)**	**5 (6.3)**

*Presumptive isolates; CRKP - Carbapenem-resistant *Klebsiella* spp; CpCRKP-Carbapenemase-producing Carbapenem-resistant *Klebsiella* spp

Table 4: Distribution of *Klebsiella* isolates across different wards in UCH Ibadan and outside facility

Ward	Frequency (%)			
	Klebsiella* spp.	**Culture positive *Klebsiella* spp**	**CRKP**	**CpCRKP**
Medical wards	38 (31.7)	28 (35.0)	5 (17.9)	2 (7.1)
Surgical wards	50 (41.7)	28 (35.0)	6 (21.4)	2 (7.1)
Emergency	10 (8.3)	7 (8.8)	1 (14.3)	0
Outside UCH	22 (18.3)	17 (21.3)	11 (64.7)	1 (5.9)
Total	**120 (100.0)**	**80 (100.0)**	**23 (28.8)**	**5 (6.3)**

*Presumptive isolates; CRKP - Carbapenem-resistant *Klebsiella* spp; CpCRKP-Carbapenemase-producing Carbapenem-resistant *Klebsiella* spp

Antibiotic susceptibility test results:

Of 80 viable isolates following sub-culturing, 40 (50.0%) were resistant to ceftazidime, 62 (77.5%) to cefoxitin, 36 (45.0%) to levofloxacin, 49 (61.3%) to ciprofloxacin, 30 (37.5%) to gentamicin, and 23 (28.8%) to imipenem (Fig 1).

All 80 isolates were resistant to both piperacillin and amoxicillin-clavulanate while 50 (62.5%) expressed multidrug resistance phenotype, with 19 different antimicrobial resistant patterns identified, CAZ/FOX/LEV/CIP/PRL/AUG/GM/IPM being the most predominant (n=11, 22.0%) resistance pattern (Table 5).

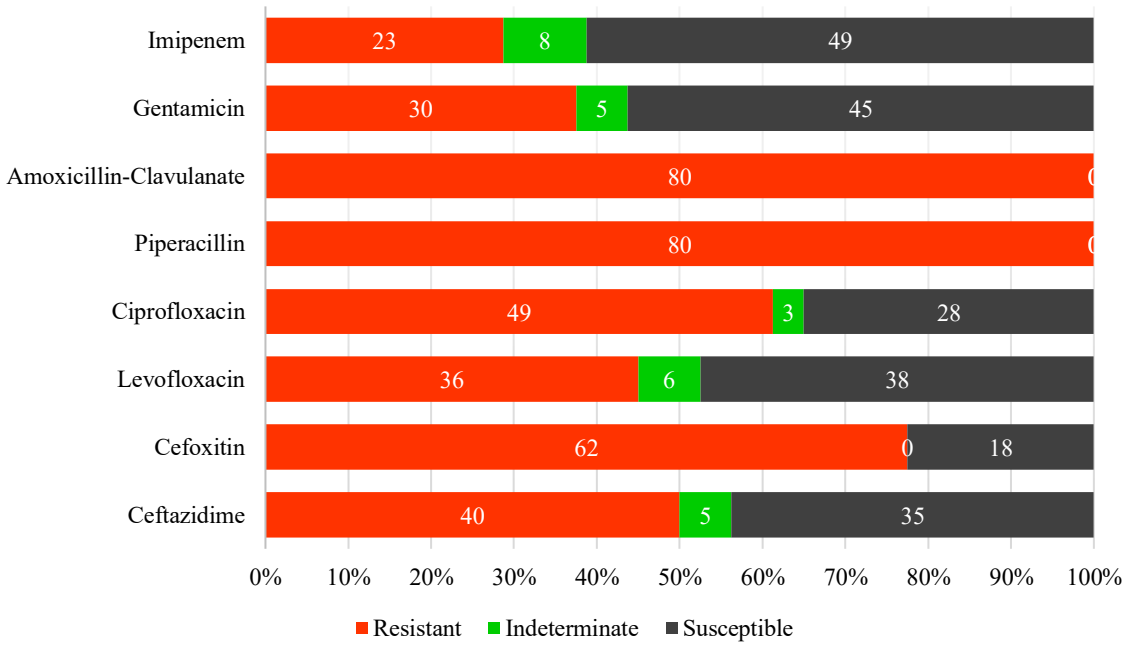

Fig 1: Antibiotic sensitivity pattern of *Klebsiella* isolates

Table 5: Multi-drug-resistant patterns of *Klebsiella* isolates

S/N	MDR patterns	No of isolates (%)	MDR pattern
1	CAZ/FOX/LEV/CIP/PRL/AUG/GM/IPM	11 (22.0)	MDR
2	CAZ/FOX/LEV/CIP/PRL/AUG/GM	6 (12.0)	MDR
3	CAZ/FOX/LEV/CIP/PRL/AUG/IPM	3 (6.0)	MDR
4	CAZ/FOX/CIP/PRL/AUG/GM/IPM	3 (6.0)	MDR
5	CAZ/FOX/CIP/PRL/AUG/IPM	3 (6.0)	MDR
6	CAZ/FOX/LEV/CIP/PRL/AUG	5 (10.0)	MDR
7	CAZ/LEV/CIP/PRL/AUG/GM	1 (2.0)	MDR
8	CAZ/FOX/PRL/AUG/GM/IPM	1 (2.0)	MDR
9	CAZ/FOX/PRL/AUG/GM	1 (2.0)	MDR
10	CAZ/CIP/PRL/AUG/GM	1 (2.0)	MDR
11	CAZ/PRL/AUG/GM	1 (2.0)	MDR
12	CAZ/CIP/PRL/AUG	1 (2.0)	MDR
13	FOX/LEV/CIP/PRL/AUG/GM	2 (4.0)	MDR
14	FOX/CIP/PRL/AUG/GM/IPM	1 (2.0)	MDR
15	FOX/CIP/PRL/AUG	2 (4.0)	MDR
16	FOX/LEV/CIP/PRL/AUG	5 (10.0)	MDR
17	LEV/CIP/PRL/AUG	1 (2.0)	MDR
18	LEV/CIP/PRL/AUG/GM	1 (2.0)	MDR
19	LEV/PRL/AUG/GM	1 (2.0)	MDR
	Total	**50 (100.0)**	**MDR**

Ceftazidime –CAZ; Cefoxitin –FOX; Levofloxacin –LEV; Ciprofloxacin –CIP; Piperacillin –PRL; Amoxicillin-clavulanate -AUG; GM –Gentamicin; IPM- Imipenem.

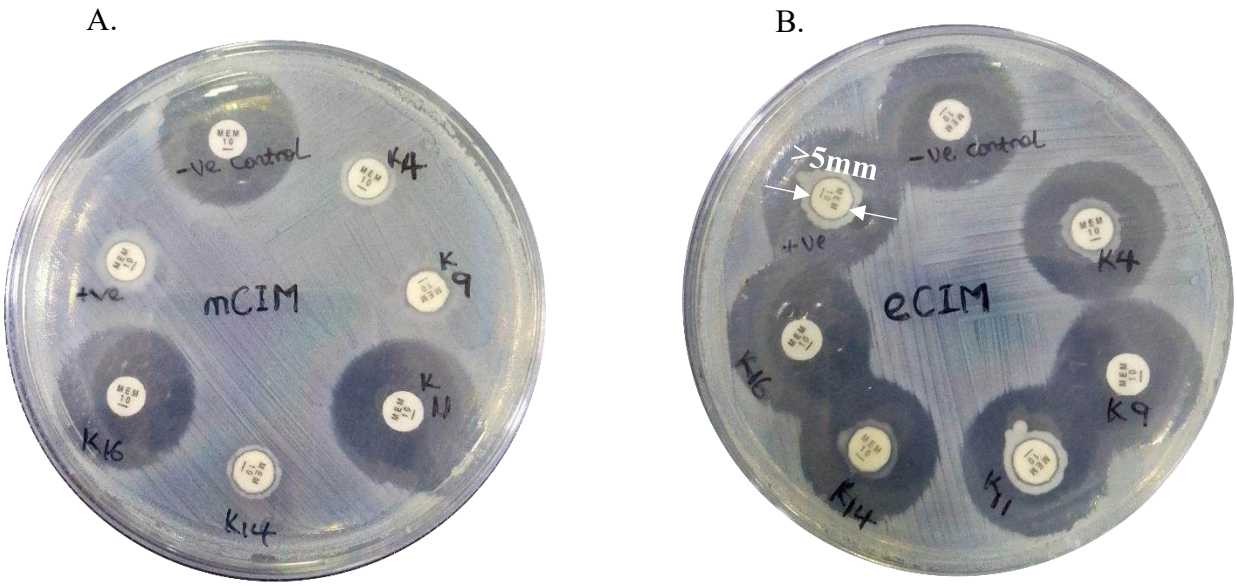

Fig 2: Phenotypic Test result for carbapenemase production. A. Modified Carbapenem Inactivation Method results: K11 and K16 -Negative isolates, K4, K9, & K14 -Positive isolates. B. EDTA- Carbapenem Inactivation Method results: K4, K9, & K14 -Positive isolates.

Phenotypic detection of carbapenemase production:

In this study, 5 (6.3%) of the 80 isolates were phenotypically identified as carbapenemase producers, representing 25.0% of the carbapenem-resistant strains confirmed by both mCIM and eCIM methods. Fig 2 below shows the result of mCIM and eCIM simultaneously carried out on carbapenem-resistant isolates.

Molecular confirmation of carbapenem-resistant *Klebsiella* spp:

Twenty-three (28.8%) biochemically confirmed *Klebsiella* spp isolates were resistant to imipenem, 20 (25.0%) were confirmed as *Klebsiellae* using PCR (Fig 3) and 17 (21.3%) were identified as *Klebsiella pneumoniae* with the aid of specific primers (Fig 4).

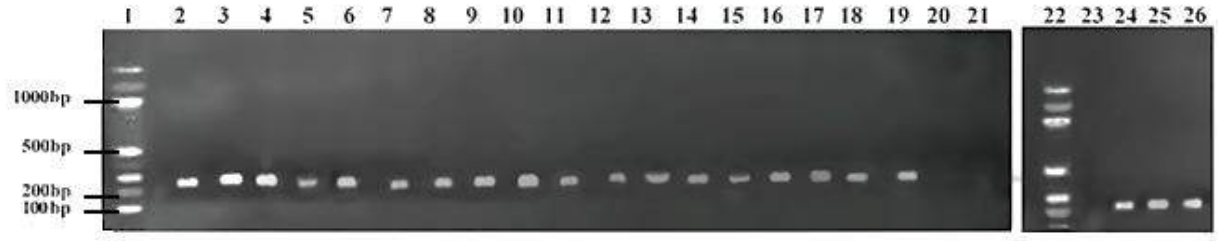

Fig 3: PCR products of the amplification of *Klebsiella* spp.-specific gene. Lane 1 & 22: 100.0 bp molecular weight marker; Lane 2: positive control; Lane 3-19, & 24-26: positive isolates; Lanes 20, 21 & 23: negative isolates.

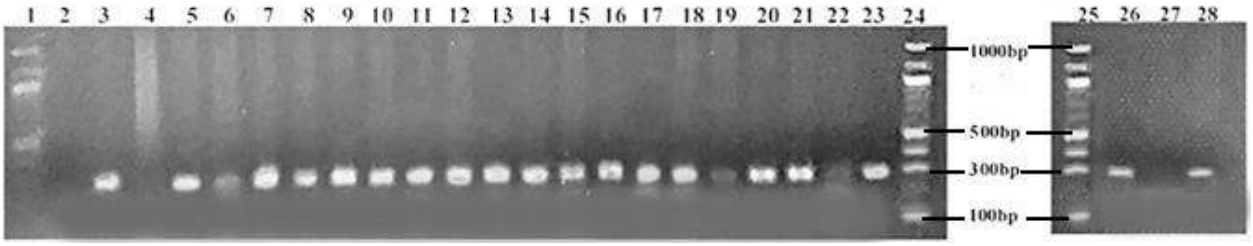

Fig 4: PCR products of the amplification of *Klebsiella pneumoniae*-specific gene. Lane 1, 24 & 25: 100.0 bp molecular weight marker; Lane 2: negative control; Lane 3: positive control; Lanes 4, 22 & 27: negative isolates; Lanes 5-19, 26 & 28: positive isolates. Lanes 20/21 (repeated), 22 & 23: *Klebsiella* spp. negative isolates.

Molecular detection of the relevant carbapenem resistance genes:

The PCR amplification products for the $bla_{\text{NDM-1}}$ gene multiplexed with bla_{VIM}, bla_{KPC} with $bla_{\text{OXA-48}}$, and bla_{IMP} genes are shown in Figs 5 and 6. Of the 5 isolates positive for phenotypic carbapenemase detection, 3 harbored the $bla_{\text{NDM-1}}$ gene, while none expressed other resistance genes.

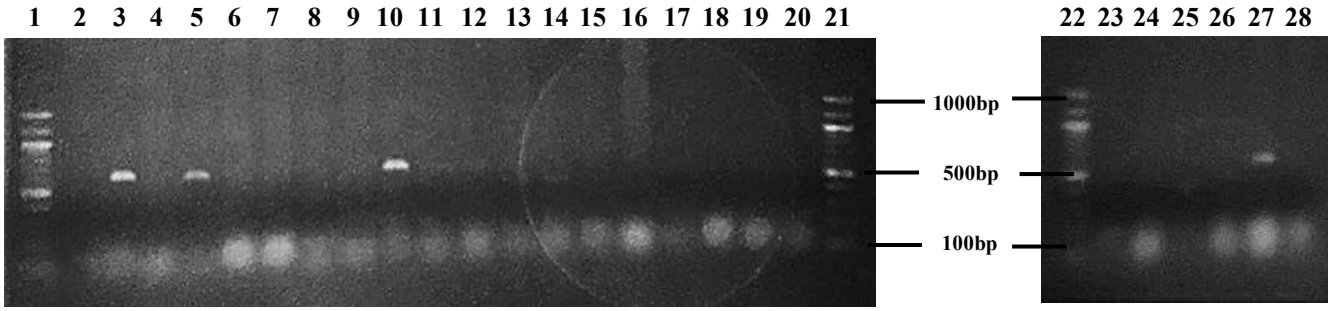

Fig 5: PCR products of the amplification of $bla_{\text{NDM-1}}$ multiplexed with bla_{VIM}, and bla_{KPC} genes. Lane 1, 21 & 22: 100.0 bp molecular weight marker; Lane 2: negative control; Lane 3: NDM positive control; Lanes 4, 6-9, 11-20, 23-26, & 28: negative isolates; Lanes 5, 10 & 27: positive isolates.

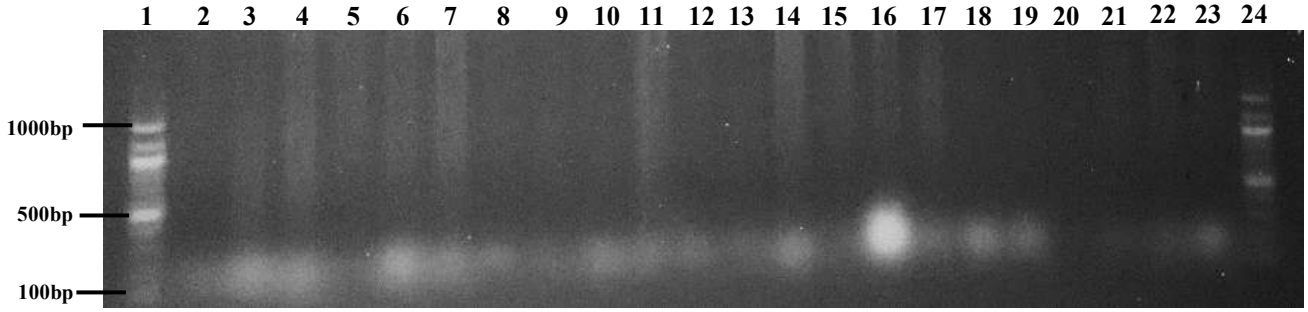

Fig 6: PCR products of the amplification of $bla_{\text{OXA-48}}$ and bla_{IMP} genes. Lane 1, & 24: 100.0 bp molecular weight marker; Lane 2: negative control; Lanes 3-23: negative isolates.

Discussion:

The emergence of antibiotic resistance in Gram-negative bacteria is a significant threat to public health (22). *Klebsiella* spp is associated with infections affecting the lungs, urinary tract, gastrointestinal tract, bloodstream, wound or surgical site and other body sites (23). These bacteria have demonstrated notable resistance to several antibiotics, including carbapenems, the 'last-resort' drug for multidrug-resistant infections. The spread of carbapenemases has worsened this problem, with continuous dissemination across clinical settings, communities and the environment (24).

This study investigated the carbapenemase-producing *Klebsiella* isolates in clinical samples, providing a comprehensive analysis of AMR patterns, MDR, and the mechanisms underlying carbapenem resistance. From this study, *Klebsiella* spp were mostly isolated from urine samples (37.5%), wound swabs (17.5%) and sputum (15.0%). Previous studies show similar distributions with 35.4% in urine, 26.2% in wound swabs, and 13.4% in sputum as reported by Mukail et al., (25), and 45.8% in urine, 29.2% in sputum, and 12.5% in wound swab as reported by Jeremiah et al., (26). The lower rates in our study may be due to differences in sample size. The predominance of isolates from urine samples highlights the urinary tract as a significant reservoir of the pathogen (27,28). Wound swabs and sputum samples contributed to the spectrum, aligning with findings that emphasize the organisms' role in wound and respiratory infections (29).

Klebsiella infections were widespread in different clinical wards, with the highest frequency in both surgical (35.0%) and medical (35.0%) wards. Surgical site infections (SSIs) are common healthcare-associated infections, often caused by bacteria from patients' normal flora introduced during surgery (30-32). Immunocompromised individuals are particularly at risk of developing SSIs (33). Other studies carried out in Russia and Romania have reported *Klebsiella* spp to be the predominant cause of infections in surgical patients (34, 35). In the Romania study, *Klebsiella* infections in hospital wards showed a prevalence range of 5.25% to 19.49% in surgical wards, and 5.15% to 17.36% in medical wards. In our study, similarly high *Klebsiella* prevalence was observed in the medical wards (35.0%), reflecting *Klebsiella* versatile clinical presentations from pneumonia to sepsis. Interestingly, the high frequency of isolation of *Klebsiella* spp from out-patient samples in other hospitals (21.3%) indicates a potential community source.

In this study, antibiotic resistance among *Klebsiella* isolates is quite alarming, with 100.0% resistance to piperacillin and amoxicillin/clavulanate. Similar observations have been reported in other study (36), suggesting a consistent trend of high-level resistance of *Klebsiella* spp to piperacillin; although a lower resistance rate of 54.2% against amoxicillin-clavulanate was observed. A similar observation of 50.0% resistance rate was reported by Mukail and colleagues in Kaduna, northern Nigeria (25). The isolates also exhibited considerable resistance to cefoxitin (77.5%), comparable to an Ethiopian study where 79.3% resistance was reported (37). Resistance to gentamicin, ceftazidime, levofloxacin, and ciprofloxacin was also high, reflecting limited treatment options for infected patients.

The observation of carbapenem resistance among *Klebsiella* isolates in this study highlights a concerning trend with significant clinical implications. Twenty (25.0%) of the 80 PCR-confirmed *Klebsiella* isolates were resistant to imipenem. Other studies have reported lower resistance rates of 4.2%, 10.9% and 21.9% respectively (25,36,38). The *invitro* efficacy of imipenem in our study is however lower compared to previous studies in Nigeria and India (39,40). Differences in antibiotic use and healthcare practices may likely be responsible for these variations.

Reports of carbapenemase production in *Klebsiella* spp which hydrolyze carbapenems and render them ineffective, have increased globally. Our study found a 6.3% prevalence of carbapenemase production, similar to the study of Jeremiah et al., (26) who reported a 6.2% phenotypic expression of carbapenemase in *Klebsiella pneumoniae*. Marked variation in prevalence has also been observed, such as the Kaduna study with prevalence as high as 24.0% (25). Alternative resistance mechanisms, such as efflux pumps or cell wall modifications, may also be involved (26,41)

Molecular detection of carbapenem resistance genes helps to better understand the genetic basis underlying antibiotic resistance. In this study, NDM-1 gene was found in 60.0% (3 of 5) of isolates that produced carbapenemase. The gene coding for NDM-1 is usually found on distinct large plasmids, facilitating its high frequency transfer to susceptible bacteria. These plasmids are implicated in conferring resistance to nearly all antibiotics (42). This can explain why all the isolates harboring the NDM-1 gene in this study were resistant to all antibiotics used. The observed prevalence of NDM-1 (3.8%) in this study is comparable to the findings by Mushi *et al*. (43) and Okoche et al., (44) who reported 3.1% and 2.6% prevalence rates respectively.

The non-detection of other genes tested in this study, however positive Phenotypically, could be explained by the potential occurrence of false negative PCR results associated with mutations that could affect the anne-

aling of primers (45). The non-detection of other resistance genes may also suggest a specific regional prevalence or a unique genetic profile of the studied *Klebsiella* population. This observation is comparable to the findings in a recent study conducted by Onyeji *et al*. (46), who reported the non-detection of carbapenemase genes in 46.0% of the isolates resistant to carbapenems. Likewise, in a study carried out in Iran by Moradi et al., (47), a discrepant result was observed with 15.7% of isolates phenotypically testing positive by modified Hodge Test, while PCR was negative.

Our study is limited by a number of factors. The exact prevalence of carbapenem resistance across individual species of *Klebsiella* was not estimated. Also, other mechanisms responsible for resistance in PCR negative isolates were not investigated. The omission of these investigations was primarily due to limitations in funding, laboratory resources, and time. This could have provided insights on uncommon or emerging carbapenemases and non-carbapenemase-controlled mechanism of resistance in *Klebsiella* spp.

Conclusion:

The detection of bla_{NDM-1} and high antimicrobial resistance rates among clinical *Klebsiella* isolates is alarming in this study. The emergence of carbapenem-resistant *Klebsiella*, especially with the bla_{NDM-1} gene, threatens the effectiveness of last-resort antibiotics. The recovery of both multidrug-resistant and carbapenem-resistant strains from various hospital wards and the community calls for a need to ensure implementation of infection prevention and control policy in the hospital. Additionally, it also justifies the need for antimicrobial stewardship.

Contributions of authors:

SAA was involved in the study conceptualization, design, methodological activities, and initial draft production; KAO was involved in the study conceptualization, design, initial draft production and supervision; AI was involved in data collection and methodological activities; AVO designed all primers used; MOB and OAB were involved in the critical review of the initial draft; All authors were involved in findings interpretation, critical review and approval of the final draft.

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Conflict of interest

Authors declare no conflict of interest.

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Original Article **Open Access**

Antimicrobial susceptibility and plasmid profiles of *Salmonella* Typhi in Sagamu, southwest Nigeria

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Abstract:

Background: *Salmonella enterica* serovar Typhi is the causative agent of typhoid fever, a major public health concern in Nigeria and other low-resource settings. The emergence of antimicrobial resistance (AMR), often plasmid-mediated, poses a serious challenge to treatment. Local surveillance data on resistance and plasmid carriage are critical for guiding effective interventions. This study investigated the antimicrobial susceptibility and plasmid profiles of clinical *Salmonella* Typhi isolates from stool samples in Sagamu, southwest Nigeria.
Methodology: A cross-sectional study of 100 patients with clinical features of typhoid fever and reactive for *S.* Typhi antibody by the Widal agglutination test, was conducted across health facilities in Sagamu, including Olabisi Onabanjo University Teaching Hospital (OOUTH), private hospitals, and selected diagnostic laboratories. Stool samples were collected from each participant and cultured on selenite F broth enrichment, followed by subculture on MacConkey and *Salmonella-Shigella* agar plates. *Salmonella* Typhi was identified by colony morphology, Gram stain reaction, biochemical test scheme of triple sugar iron, citrate, and urease tests, and confirmed by serotyping with agglutination of sample using specific antisera. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method. Plasmid DNA was extracted by the alkaline lysis method, and electrophoresis was used for plasmid size determination. Mating experiments with *Escherichia coli* K-12 were conducted to assess plasmid transferability. Statistical analysis of data was done using SPSS (version 25), with significance set at $p<0.05$.
Results: Sixty-six isolates were confirmed as *S.* Typhi. Of these, 28.8% (n=19) were multidrug-resistant (MDR), although all isolates were susceptible to ciprofloxacin and ofloxacin. Plasmids with sizes ranging from 4.6 to 5.7kb were detected in 16 of the 19 MDR isolates, 9 (56.3%) of which successfully transferred their plasmids to *E. coli* K-12. A significant association was found between plasmid carriage and MDR (OR=447.86, $p<0.0001$).
Conclusion: The findings of this study suggest that plasmid-mediated resistance plays a major role in the emergence of MDR *S.* Typhi strains in the region, posing a challenge to the effective treatment of typhoid fever. Continuous surveillance and prudent use of antibiotics are necessary to combat the rising threat of AMR in *S.* Typhi population.

Keywords: Antimicrobial resistance; plasmid; mating; transfer; *Salmonella* Typhi

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Sensibilité aux antimicrobiens et profils plasmidiques de *Salmonella* Typhi à Sagamu, sud-ouest du Nigéria

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Résumé:

Contexte: *Salmonella enterica* sérotype Typhi est l'agent responsable de la fièvre typhoïde, un problème de santé publique majeur au Nigéria et dans d'autres pays à faibles ressources. L'émergence de la résistance aux antimicrobiens (RAM), souvent d'origine plasmidique, pose un sérieux défi thérapeutique. Français Les données de surveillance locales sur la résistance et le portage plasmidique sont essentielles pour guider des

interventions efficaces. Cette étude a examiné la sensibilité aux antimicrobiens et les profils plasmidiques d'isolats cliniques de *Salmonella* Typhi à partir d'échantillons de selles à Sagamu, dans le sud-ouest du Nigéria.
Méthodologie: Une étude transversale de 100 patients présentant des caractéristiques cliniques de fièvre typhoïde et réactifs aux anticorps *S.* Typhi par le test d'agglutination de Widal, a été menée dans des établissements de santé à Sagamu, y compris l'hôpital universitaire Olabisi Onabanjo (OOUTH), des hôpitaux privés et des laboratoires de diagnostic sélectionnés. Des échantillons de selles ont été prélevés chez chaque participant et cultivés sur un bouillon d'enrichissement au sélénite F, suivis d'une sous-culture sur des plaques de gélose MacConkey et *Salmonella-Shigella*. *Salmonella* Typhi a été identifiée par morphologie des colonies, réaction de coloration de Gram, schéma de test biochimique des tests de fer triple sucre, citrate et uréase, et confirmée par sérotypage avec agglutination de l'échantillon à l'aide d'antisérums spécifiques. Les tests de sensibilité aux antimicrobiens ont été réalisés à l'aide de la méthode de diffusion sur disque de Kirby-Bauer. Français L'ADN plasmidique a été extrait par la méthode de lyse alcaline et l'électrophorèse a été utilisée pour déterminer la taille du plasmide. Des expériences d'accouplement avec *Escherichia coli* K-12 ont été menées pour évaluer la transférabilité du plasmide. L'analyse statistique des données a été effectuée à l'aide de SPSS (version 25), avec une signification fixée à $p<0,05$.
Résultats: Soixante-six isolats ont été confirmés comme étant *S.* Typhi. Parmi ceux-ci, 28,8% (n=19) étaient multirésistants aux antibiotiques (MDR), bien que tous les isolats soient sensibles à la ciprofloxacine et à l'ofloxacine. Des plasmides d'une taille comprise entre 4,6 et 5,7kb ont été détectés dans 16 des 19 isolats MDR, dont 9 (56,3%) ont transféré avec succès leurs plasmides à *E. coli* K-12. Une association significative a été trouvée entre le portage du plasmide et la MDR (OR=447,86, $p<0,0001$).
Conclusion: Les résultats de cette étude suggèrent que la résistance induite par les plasmides joue un rôle majeur dans l'émergence de souches multirésistantes de *S.* Typhi dans la région, ce qui complique le traitement efficace de la fièvre typhoïde. Une surveillance continue et une utilisation prudente des antibiotiques sont nécessaires pour lutter contre la menace croissante de la RAM dans la population de *S*. Typhi.

Mots-clés: Résistance aux antimicrobiens; plasmide; croisement; transfert; *Salmonella* Typhi

Introduction:

Typhoid fever, caused by the bacterium *Salmonella* Typhi, is a significant public health concern in developing countries, including Nigeria (1). The disease is predominantly transmitted through contaminated food and water, and its prevalence is heightened by poor sanitation and inadequate access to clean water (2). Despite advancements in public health, typhoid fever continues to pose a substantial burden, particularly in regions with limited healthcare resources (1).

The clinical management of typhoid fever has been increasingly complicated by the emergence of multidrug-resistant (MDR) *S.* Typhi strains (3). Resistance to first-line antibiotics such as ampicillin, chloramphenicol, and cotrimoxazole has been widely reported, prompting reliance on fluoroquinolones and third-generation cephalosporins (4). However, the growing resistance to these agents indicates the need for continuous surveillance and a deeper understanding of the underlying mechanisms of resistance.

Plasmids are extrachromosomal DNA elements that play important role in the acquisition and dissemination of antimicrobial resistance (AMR) genes among bacterial populations (5). The presence of transmissible resistance plasmids (R-plasmids) in *S.* Typhi has been identified as a major driver of MDR, posing a significant threat to effective treatment strategies (6).

This study aims to explore the antimicrobial susceptibility patterns and plasmid profiles of *S.* Typhi isolates obtained from various health establishments in Sagamu, southwest Nigeria. By highlighting the resistance patterns and the role of plasmids, the find - ings of this study will contribute to the growing body of knowledge on typhoid fever and its management in endemic regions.

Materials and method:

Study design and sample collection:

This was a descriptive cross-sectional study of 100 patients with clinical features of typhoid fever and reactive for *S.* Typhi antibody by the Widal agglutination test conducted in Sagamu, southwest Nigeria between August and November 2022. Stool samples were collected from each participant recruited from Olabisi Onabanjo University Teaching Hospital (OOUTH), selected private diagnostic laboratories (Synlab, Afriglobal and Dew laboratories) and selected private hospitals (Owokoniran Medical Hospital, Ayotola Specialist Hospital and Rene Medical Center). Informed consent was obtained from each participant prior to sample collection.

Culture isolation and identification of *Salmonella* Typhi:

The stool samples were processed using standard microbiological techniques with initial enrichment in Selenite F, followed by culture on selective media (MacConkey and *Salmonella-Shigella* agar plates), and incubating at 37°C for 24 hours. Presumptive colonies of *Salmonella* were identified by colonial morphology, Gram stain reaction, and biochemical test scheme of triple sugar iron (TSI) agar**,** citrate utilization, and urease tests. Serological confirmation of *S.* Typhi was performed using specific antisera, where agglutination of the sample with the antisera confirmed the presence of *S.* Typhi.

Antimicrobial susceptibility testing:

Antimicrobial susceptibility testing of the serologically confirmed *S.* Typhi isolates (n=66) was done by the Kirby-Bauer disk diffusion method against 9 selected antibiotics in line with the guidelines of the Clinical and Laboratory Standards Institute (7). The antibiotics tested included ampicillin (10µg), chloramphenicol (30µg), gentamicin (10µg), tetracycline (30µg), cotrimoxazole (25µg), nalidixic acid (30µg), ciprofloxacin (5µg), ofloxacin (5µg) and ceftriaxone (30µg). Zones of inhibition were measured in mm and interpreted as sensitive or resistant in line with the CLSI guidelines (7).

Plasmid DNA extraction and profiling:

Plasmid DNA extraction was carried out using the alkaline lysis method. Briefly, overnight cultures of *S.* Typhi isolates grown in Luria-Bertani (LB) broth were harvested by centrifugation at 10,000 rpm for 10 minutes. The bacterial pellet was re-suspended in 200 µL of a solution containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA (Solution I).

The cells were lysed with 200 µL of a lysis buffer containing 0.2 M NaOH and 1% SDS (Solution II), mixed gently, and incubated on ice for 5 minutes. To neutralize the solution, 150 µL of a cold potassium acetate solution (Solution III: 3 M potassium acetate, pH 5.5) was added, followed by gentle mixing and incubation on ice for 10 minutes. The mixture was centrifuged at 13,000 rpm for 10 minutes to pellet cellular debris and chromosomal DNA.

The supernatant containing plasmid DNA was transferred to a fresh tube, and plasmids were precipitated by adding 0.6 vol of isopropanol. After centrifugation at 13,000 rpm for 10 mins, the DNA pellet was washed ed with 70% ethanol, air-dried, and re-suspended in 30µL nuclease-free water.

For profiling, the extracted plasmid DNA was resolved on a 0.8% agarose gel in 1X TAE buffer, stained with ethidium bromide (0.5 µg/mL), and visualized under UV light. A 1 kb DNA ladder and a lambda DNA/HindIII marker were used to estimate the sizes of the plasmids.

Plasmid transfer by mating:

Plasmid transfer experiment was conducted by mating *S.* Typhi isolates with *Escherichia coli* K-12 as the recipient strain. The potential donor strain, carrying the plasmid, was grown overnight in Luria-Bertani (LB) broth, while *E. coli* K-12 was prepared by streaking onto LB agar plates and incubating overnight.

Prior to mating, the *E. coli* K-12 recipient strain was confirmed to be susceptible to ampicillin and chloramphenicol by performing antimicrobial susceptibility testing. The donor and recipient strains were mixed in a 1:1 ratio and spotted onto LB agar plates. After overnight incubation at 37°C, the bacterial cells were scraped from the plate, suspended in saline, and plated onto MacConkey agar containing ampicillin and chloramphenicol to select for transconjugants.

Antimicrobial susceptibility testing of the transconjugants was performed using the disk diffusion method. Successful plasmid transfer was confirmed if the *E. coli* K-12 transconjugants were resistant to ampicillin and chloramphenicol used in the selection media.

Data analysis:

Data obtained from antimicrobial susceptibility testing and plasmid profiling were analyzed using descriptive statistics to summarize the resistance patterns and plasmid profiles of S. Typhi isolates. The distribution of antimicrobial resistance among the isolates was expressed as percentages, and the presence of multidrug-resistant (MDR) strains was noted.

Association between plasmid carriage and multidrug resistance was assessed using Chi-square test to determine statistical significance and p value was set at <0.05 for all analyses. Statistical analyses were conducted using SPSS version 25.0 (IBM Corp, USA).

Results:

A total of 66 confirmed *S.* Typhi were isolated from the stool samples of 100 participants by biochemical and serological identification tests (Table 1).

Table 1: Distribution of *Salmonella* Typhi isolated from the study sites

Institution	No of isolates	No identified as *S.* Typhi (%)
OOUTH, Sagamu	50	31 (62.0)
Private laboratories	40	29 (72.5)
Private hospitals	10	6 (60.0)

OOUTH: Olabisi Onabanjo University Teaching Hospital

All 66 isolates were susceptible to ofloxacin, ciprofloxacin and ceftriaxone, while 65 (98.5%) and 62 (93.9%) were susceptible to nalidixic acid and gentamicin respectively (Table 2). The susceptibility pattern to the commonly used first line antibiotics was variable; cotrimoxazole (54.5%), chloramphenicol (45.5%), ampicillin (34.8%) and tetracycline (27.2%). Only 17 (25.8%) isolates were fully susceptible to all the 9 antibiotics tested while 19 (28.8%) isolates were MDR (resistance to three or more antibiotic classes), and 49 (72.2%) were resistant to at

least one antibiotic.

Of the 19 MDR strains, 8 (42.1%) were resistant to chloramphenicol, ampicillin and tetracycline; 6 (31.6%) to ampicillin, chloramphenicol and cotrimoxazole, and 5 (26.3%) to chloramphenicol, ampicillin, tetracycline and cotrimoxazole (Table 2).

Table 2: Antimicrobial drug resistance patterns of 66 strains of *Salmonella* Typhi

Antibiotic resistant pattern	**Number of resistant *Salmonella* Typhi isolates (%)**
Single drug resistance (n=66)	
Ampicillin	43 (65.2)
Tetracycline	48 (72.7)
Cotrimoxazole	30 (45.5)
Chloramphenicol	36 (54.5)
Gentamicin	4 (6.0)
Nalidixic acid	1 (1.5)
Ciprofloxacin	0
Ofloxacin	0
Ceftriaxone	0
Multidrug resistance (MDR) pattern (n=19)	
Ampicillin, Chloramphenicol, Tetracycline	8 (42.1)
Ampicillin, Chloramphenicol, Cotrimoxazole	6 (31.6)
Ampicillin, Chloramphenicol, Cotrimoxazole	5 (26.3)

The electrophoretic profiles of the isolated plasmid DNA of the 66 *S.* Typhi strains is shown in Table 3. Of the 19 MDR isolates, 16 (84.2%) contained plasmids, with sizes of 4.6 kb (n=5 isolates), 4.9 kb (n=3 isolates), 5.1 kb (n=7 isolates), and 5.7 kb (n=1 isolate). Also, 9 (56.3%) of these successfully transferred their plasmids to *E. coli* K-12. In contrast, none of the MDR-negative isolates contained plasmids, as 47 isolates, including the 17 isolates that were sensitive to all 9 antibiotics, were plasmid-negative

Statistical analysis of the data showed a significant association between plasmid carriage and MDR (x^2=54.24, OR=447.86, 95% CI = 21.935-9144.2, p<0.00001), indicating that plasmid-borne resistance may play a significant role in the dissemination of MDR among *S.* Typhi isolates in the study setting.

Discussion:

This study assessed the antimicrobial susceptibility and plasmid profiles of *S.* Typhi isolates from human stool samples in Sagamu, southwest Nigeria. Our findings highlight a high prevalence of AMR, with a significant association between MDR and plasmid carriage among *S.* Typhi isolates. This research provides valuable insights into the evolving challenges in managing typhoid fever in the region and underscores the importance of continuous surveillance to monitor and combat the spread of resistant strains.

We observed high levels of resistance to commonly used antibiotics, particularly ampicillin, tetracycline, and chloramphenicol. This resistance pattern is consistent with previous reports from Nigeria and other sub-Saharan African countries, where *S.* Typhi has shown increasing resistance to first-line antibiotics. A study from Lagos, Nigeria by Akinyemi et al., (8) reported similar resistance rates to ampicillin and tetracycline respectively among *S.* Typhi isolates. Additionally, a study done in Ghana (9) found that antimicrobial resistance in *S.* Typhi isolates was rising, corroborating our findings. These results are concerning, as they suggest a shift towards the use of second-line drugs, which could have implications for treatment efficacy and healthcare costs in resource-limited settings.

Interestingly, our study found that all *S.* Typhi isolates were susceptible to ciprofloxacin and ofloxacin. This finding aligns with other studies that have reported the retention of susceptibility to fluoroquinolones despite the development of resistance to older antibiotics (8,10). However, it is worth noting that emergence of fluoroquinolone resistance in *S.* Typhi has been documented in other parts of the world. For instance, a study in India reported the emergence of ciprofloxacin-resistant *S.* Typhi, which highlights the

Table 3: Molecular size characteristics of *Salmonella* Typhi plasmid DNA

Plasmid presence/sizes	**Number of MDR isolates (%)**	**Number of non-MDR and sensitive isolates (%)**	**Total isolates (%)**
4.6kb	5	0	5
4.9kb	3	0	3
5.1kb	7	0	7
5.7kb	1	0	1
Plasmid negative	3	47	50
Total	**19 (28.8%)**	**47 (71.2%)**	**66 (100%)**

MDR – Multi-drug resistance; x^2=54.24, OR=447.86, 95% CI=21.935 - 9144.2, p<0.0001

potential for fluoroquinolone resistance to emerge, particularly as its use increase in the treatment of typhoid fever (11). Continuous monitoring of fluoroquinolone resistance is essential to prevent the emergence of resistance in the region.

Our findings also showed a concerning 28.8% prevalence of MDR *S.* Typhi strains. This rate is consistent with those reported in studies from other parts of Nigeria, where MDR *S.* Typhi has become a significant clinical challenge (3,12). MDR *S.* Typhi strains (resistant to three or more classes of antibiotics) pose a major challenge to the treatment regimens of typhoid fever and have been associated with increased morbidity and mortality, especially in regions with limited access to alternative antibiotics (3).

The molecular analysis revealed a strong association between plasmid carriage and MDR. In total, 16 out of 19 MDR isolates carried plasmids, with sizes ranging from 4.6 to 5.7 kilobases. This is in line with previous studies that have identified plasmids as key vectors for the transmission of resistance genes in *Salmonella* species. For example, a study by Owoseni and Onilude (13) demonstrated that MDR *S.* Typhi strains in West Africa were often associated with the presence of plasmids carrying resistance genes. The finding that no MDR-negative isolates carried plasmid elements supports the hypothesis that plasmid-mediated resistance plays a crucial role in the spread of MDR *S.* Typhi.

Plasmid transfer experiments using *E. coli* K-12 as the recipient strain confirmed the transferability of resistance genes. This result aligns with previous research, which showed that plasmids carrying AMR genes can be easily transferred between *Salmonella* strains and other enteric bacteria (14). The ability of *S.* Typhi to transfer resistance plasmids to other bacteria increases the potential for resistance to spread beyond a single pathogen species, exacerbating the overall problem of AMR in the region.

Statistical analysis confirmed a significant association between plasmid presence and MDR in *S.* Typhi isolates (OR=447.86, p <0.0001. This statistical significance supports the conclusion that plasmid-borne resistance is a critical factor in the development and spread of MDR strains in the study area. Previous studies have also found strong associations between plasmid presence and resistance profiles in *S.* Typhi, reinforcing the importance of plasmids in the dissemination of antimicrobial resistance (13,15).

Conclusion:

This study highlights the growing concern of antimicrobial resistance in *S.* Typhi isolates in Sagamu, southwest Nigeria, and emphasizes the role of plasmids in mediating multidrug resistance. A significant proportion of *S.* Typhi isolates were resistant to common antibiotics, with a notable prevalence of MDR strains. Plasmid profiling revealed that plasmid carriage is associated with the resistance phenotypes, particularly in the MDR isolates.

Furthermore, the transfer of resistant plasmids between *S.* Typhi and other enteric bacteria further complicates the management of typhoid fever in the region. These findings underscore the urgent need for enhanced surveillance, effective infection control strategies, and the prudent use of antibiotics to mitigate the spread of resistant *S.* Typhi strains. Additionally, the study advocates for the continuous monitoring of AMR patterns and the development of alternative therapeutic approaches to ensure the effective treatment of typhoid fever.

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Conflicts of interest:

No conflict of interest is declared.

Contribution of authors:

DTO conceived and designed the study, acquired, analyzed and interpreted the data. AOO acquired and interpreted data. Both authors drafted, revised, read and approved the manuscript.

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Original Article **Open Access**

Prevalence of fungal isolates in selected poultry farms and outcomes of ocular fungal infections in experimental murine model

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Abstract:

Background: Poultry farm workers, often exposed to fungal-laden environments, are at heightened risk of ocular mycoses, which is a potentially blinding infection that warrants urgent attention and investigation. This study investigates the fungal load and pathogenicity of fungal isolates from ten selected poultry farms in Anambra State, southeast Nigeria, and their potential to cause ocular mycoses in a murine model, simulating occupational exposure.
Methodology: A total of 300 environmental samples (droppings, feed, and litter) from 10 randomly selected poultry farms in 6 local government areas of Anambra State, southeast Nigeria, were examined. Fungal loads were determined, and isolates were characterized macroscopically, microscopically using needle mount and slide culture technique, and genetically using DNA sequencing and Basic Local Alignment Search Tool (BLAST) analysis. Pathogenicity testing of the characterized isolates was conducted in a murine model, wherein 30 albino mice, randomly divided into 6 groups of 5 mice each (with one group as uninfected control), were topically inoculated with 1ml of fungal inoculum (approximately $1x10^6$ spores/ml) directly into the eyes, and monitored twice daily for clinical signs and histopathological changes in the eyes for a period of 10 days. Data analysis was done using ANOVA in SPSS software version 21.0.
Results: The mean fungal loads in the environmental samples significantly differs ($p<0.05$) between dry and rainy seasons. The mean fungal loads (spore forming unit/g) during the rainy season ranged from $1.5x10^4$ - $2.0x10^5$ sfu/g in droppings, $1.1x10^4$ – 'too numerous to count' (TNTC) in litter samples, and $9.5x10^3$ - $2.10x10^5$ sfu/g in feed samples. The mean fungal loads during the dry season in droppings, litter, and feed samples ranged from $1.2x10^4$ - TNTC, $2.2x10^4$ -$9.5x10^4$, and $6.0x10^3$ -$1.7x10^5$ sfu/g, respectively. Fifteen genera of fungi were identified, with yeast being the most prevalent during the dry season and *Aspergillus* species during the rainy season. Pathogenicity testing showed significant differences ($p<0.05$) between the different fungi-induced ocular lesions in the experimental mice, with *Aspergillus tubingensis* and *Curvularia verruculosa* causing the most frequent and severe histopathological changes that included mild to severe erythema, blood congestion, distorted cornea, iris, and ciliary body, and sloughing off of the sclera.
Conclusion: The prevalence of fungi isolates is high in the farms. Due to the pathological changes produced in the eyes of infected experimental mice, ocular exposure to fungi may pose significant health risks to poultry farm workers in Anambra State. This highlights the need for personal protective equipment, improved hygiene practices, proper ventilation, and sanitation in these farms to prevent ocular exposure and potential risks to poultry farm workers.

Keywords: Ocular mycoses, poultry farming, fungal infections, murine model, pathogenicity.

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Prévalence des isolats fongiques dans des élevages avicoles sélectionnés et évolution des infections fongiques oculaires dans un modèle murin expérimental

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Résumé:

Contexte: Les travailleurs des élevages avicoles, souvent exposés à des environnements contaminés par des champignons, présentent un risque accru de mycoses oculaires, une infection potentiellement cécitante qui nécessite une attention et des investigations urgentes. Cette étude examine la charge fongique et la pathogénicité d'isolats fongiques provenant de dix élevages avicoles sélectionnés dans l'État d'Anambra, au sud-est du Nigéria, et leur potentiel à provoquer des mycoses oculaires dans un modèle murin simulant une exposition professionnelle.
Méthodologie: Au total, 300 échantillons environnementaux (fientes, aliments et litière) provenant de dix élevages avicoles sélectionnés au hasard dans six collectivités locales de l'État d'Anambra, au sud-est du Nigéria, ont été examinés. Les charges fongiques ont été déterminées et les isolats ont été caractérisés macroscopiquement, microscopiquement par la technique de culture sur lame et par montage à l'aiguille, et génétiquement par séquençage de l'ADN et analyse BLAST ((Outil de recherche d'alignement local de base). Des tests de pathogénicité des isolats caractérisés ont été réalisés sur un modèle murin. Trente souris albinos, réparties aléatoirement en six groupes de cinq souris chacun (dont un groupe témoin non infecté), ont reçu une inoculation topique de 1ml d'inoculum fongique (environ $1x10^6$ spores/ml) directement dans les yeux. Les signes cliniques et les modifications histopathologiques oculaires ont été surveillés deux fois par jour pendant dix jours. L'analyse des données a été réalisée par ANOVA avec le logiciel SPSS version 21.0.
Résultats: Les charges fongiques moyennes dans les échantillons environnementaux diffèrent significativement (p<0,05) entre la saison sèche et la saison des pluies. Français Les charges fongiques moyennes (unité formant des spores/g) pendant la saison des pluies variaient de $1,5x10^4$ -$2,0x10^5$ sfu/g dans les excréments, $1,1x10^4$– « trop nombreux pour être comptés » (TNTC) dans les échantillons de litière, et $9,5x10^3$ - $2,10x10^5$ sfu/g dans les échantillons d'aliments. Les charges fongiques moyennes pendant la saison sèche dans les échantillons d'excréments, de litière et d'aliments variaient respectivement de $1,2x10^4$ - TNTC, $2,2x10^4$ -$9,5x10^4$ et $6,0x10^3$ -$1,7x10^5$ sfu/g. Quinze genres de champignons ont été identifiés, les levures étant les plus répandues pendant la saison sèche et les espèces d'*Aspergillus* pendant la saison des pluies. Les tests de pathogénicité ont montré des différences significatives (p<0,05) entre les différentes lésions oculaires induites par des champignons chez les souris expérimentales. *Aspergillus tubingensis* et *Curvularia verruculosa* étaient responsables des modifications histopathologiques les plus fréquentes et les plus graves, incluant un érythème léger à sévère, une congestion sanguine, une déformation de la cornée, de l'iris et du corps ciliaire, ainsi qu'une desquamation de la sclère.
Conclusion: La prévalence des isolats de champignons est élevée dans les élevages. En raison des modifications pathologiques produites dans les yeux des souris expérimentales infectées, l'exposition oculaire aux champignons peut présenter des risques sanitaires importants pour les travailleurs des élevages avicoles de l'État d'Anambra. Ceci souligne la nécessité de porter des équipements de protection individuelle, d'améliorer les pratiques d'hygiène, d'assurer une ventilation et un assainissement adéquats dans ces élevages afin de prévenir l'exposition oculaire et les risques potentiels pour les travailleurs.

Mots-clés: Mycoses oculaires, élevage avicole, infections fongiques, modèle murin, pathogénicité.

Introduction:

Poultry farming is a crucial sector of agriculture, providing a significant source of protein for millions worldwide. However, this occupation poses substantial health risks to farmers, particularly ocular mycoses caused by filamentous fungi (1). Fungal keratitis, also known as mycotic keratitis, is a severe corneal infection that can lead to blindness and eye loss. Ocular mycoses can cause severe complications, including corneal ulcers, endophthalmitis, and vision loss (2). Globally, over one million people are affected by fungal keratitis annually, with 8-11% of patients losing their eyes (3). The tedious nature of poultry farming increases the risk of ocular mycoses, as farmers often work long hours in contaminated environments and handle infected birds, feed, and bedding materials (4).

Filamentous fungi, such as *Aspergillus*, *Fusarium*, and *Curvularia*, thrive in these environments, contaminating dust, water, feed, litter, and organic matter. *Aspergillus* and *Fusarium* species are the most common causative agents of fungal keratitis, followed by *Curvularia* and other dematiaceous fungi (5). Among *Aspergillus* species, *Aspergillus fumigatus* and *Aspergillus flavus* are frequently isolated from poultry farms and are also implicated in ocular mycoses (6). *Fusarium* species such as *Fusarium oxysporum* and *Fusarium solani*, have also been reported as significant causes of ocular mycoses among poultry farmers. Additionally, rare cases of fungal keratitis caused by *Cunninghamella bertholletiae* and *Aspergillus tubingensis* have been reported, highlighting the diversity of fungal pathogens involved in ocular infections

(6). *Cunninghamella bertholletiae* has been implicated in a rare case of fungal corneal ulcer and is associated with trauma and immunosuppression (7).

Clinical manifestations of ocular mycoses include blurred vision, eye pain, redness, and discharge (8). Diagnosis remains challenging due to similarities with bacterial and viral infections. This study aims to inve- stigate the prevalence of fungal isolates within poultry farms and the clinical outcomes experimental ocular infections caused by some of the filamentous isolates using a mouse model

Materials and method:

Description of study area:

A large-scale study was carried out on poultry droppings, feed and litter samples in 10 selected poultry farms (AK, JO, OS, CY, AV, GT, VO, TA, EM, EG) from six local government areas (Aguata, Awka South, Idemili North, Nnewi North, Onitsha North, Oyi) of Anambra State, southeast Nigeria (Latitude 6° 20'N and Longitude 7° 00'E). The farms were selected using random sampling method.

Ethical approval, sample and data collection:

Ethical approval was obtained from the university ethical committee before conducting the research. Sample collection and filling of questionnaires were voluntarily done on the site of the farm by the workers, with the permission of the management of the farms.

Sample collection and transportation:

A total of 300 samples (100 samples each of poultry droppings, feeds and litters) were aseptically collected from the selected poultry farms, labeled with the appropriate information (name and location of farm, source of sample, collection time and date), placed in sampling packets and transferred to the Microbiology Laboratory of Nnamdi Azikiwe University, Awka, Anambra State, Nigeria, for analysis.

Enumeration and isolation of fungi:

A 10-fold serial dilution of the environmental sample was prepared in sterile test tubes. One milliliter (1ml) of 10^{-4} dilution of the sample was inoculated on Sabouraud Dextrose Agar (SDA) plates, supplemented with 0.05mg/ml of chloramphenicol. After 3 to 6 days incubation at 25°C, the plates were observed for fungal growth (9,10). The heterotrophic fungal count was estimated in spore forming unit/ml (sfu/ml). Pure colonies of each isolate were sub-cultured on SDA slant and stored at 4°C for further analysis.

Identification of fungal isolates:

The fungal isolates were identified by detailed studies of their macroscopic, microscopic and genetic features using DNA sequencing and BLAST analysis (11).

Pathogenicity testing of fungal isolates:

Experimental and control mice:

Thirty 3-month-old adult albino mice, weighing 28-30g, were used for the experimental study. The mice were housed in cages (divided into 6 groups of 5 mice each) in the animal facility of the Zoology Department, University of Nigeria, Nsukka, Nigeria. The mice were provided with commercial feed and sterile water *ad libitum.* One group served as the control group.

Inoculum preparation:

To prepare the fungal inoculum, the isolates were grown on Sabouraud Dextrose Agar at 25°C for 6 days to induce asexual structures. Fungal spores were harvested by suspending the cultures in phosphate-buffered saline (PBS) containing 0.1% Tween-80. The suspension was filtered through two layers of sterile gauze to remove hyphal fragments. Tween-80 was removed by repeated washing with PBS, and the final spore suspension was adjusted to a concentration of approximately 1×10^6 spores/ml using haemocytometer (12).

Animal inoculation:

Each experimental mouse was topically inoculated with 1ml of the fungal inoculum directly into the eyes. The control group was administered an equivalent volume of phosphate-buffered saline. The mice were monitored twice daily for 10 days for clinical signs, including gross morphological changes, pathological symptoms, and mortality rate.

Necropsy and histopathological examination:

Detailed case histories and observed clinical signs were considered before conducting necropsy. Gross lesions in the eyes of the experimental animals were carefully recorded. Tissue samples from the eyes of infected mice were collected postmortem and fixed in 10% neutral buffered formalin to prevent autolysis.

The fixed tissue samples were processed, embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H & E) following standard protocols (13). Histopathological lesions were examined under a light microscope at 100× magnification. Photomicrographs were captured using a Motic camera, and pathological changes were documented.

Statistical analysis:

The results and data obtained were statistically analyzed using One-way Analysis of Variance (ANOVA) with SPSS Software version of 21.0.

Results:

Enumeration of fungal isolates from environmental samples:

The mean fungal loads in the environmental samples for each selected poultry farm is shown in Table 1 for raining season and Table 2 for dry season. The mean fungal loads during the rainy season ranged from $1.5x10^4$ - $2.0x10^5$ sfu/g in droppings, $1.1x10^4$ to 'Too Numerous To Count' (TNTC) in litter samples, and 9.5×10^3 - 2.10×10^5 sfu/g in feed samples. The mean fungal loads during the dry season in droppings, litter, and feed samples ranged from 1.2×10^4 - TNTC, 2.2×10^4 - 9.5×10^4, and $6.0x10^3$ - 1.7×10^5 spore forming unit (sfu)/g, respectively.

Identification of fungal isolates from the environmental samples:

A total of 15 genera of fungal isolates were recovered from droppings, feed and litter samples from the poultry farms in both seasons (Table 3). The morphological and microscopic features of some of the isolates are presented in Table 4 and Plates 1-5.

Table 1: Mean fungal loads (spore forming unit/g) of environmental samples from selected poultry farms during rainy season

Farm code	Town/Local Government Area	Feed	Litter	Droppings	System
AK	Umuoji/ Idemili North	1.0×10^5	TNTC	1.2×10^4	Floor
JO	Nkpor/ Idemili North	1.2×10^5	1.1×10^4	1.7×10^4	Floor
OS	Ogidi/ Idemili North	4.8×10^4	1.2×10^5	2.0×10^5	Floor
CY	Ogidi/ Idemili North	4.6×10^4	6.8×10^4	2.3×10^4	Floor
AV	Nnewi / Nnewi North	3.6×10^4	1.3×10^5	4.0×10^4	Floor
GT	Onitsha/ Onitsha North	2.6×10^4	-	1.9×10^4	Floor
VO	Ogidi/ Idemili North	2.2×10^4	NA	4.5×10^4	Cage
TA	Awka/Awka South	9.5×10^3	NA	1.6×10^4	Cage
EM	Nteje /Oyi	4.7×10^4	NA	2.0×10^4	Cage
EG	Uga/Aguata	2.1 x 105	NA	1.5×10^4	Cage

TNCT = Too numerous to count; NA = Not available

Table 2: Mean fungal load (spore forming unit/g) of environmental samples from selected poultry farms during dry season

Farm code	Town/Local Government Area	Feed	Litter	Droppings	System
AK	Umuoji/ Idemili North	1.7×10^5	9.7×10^4	2.4×10^5	Floor
JO	Nkpor/ Idemili North	1.1×10^4	2.2×10^4	TNTC	Floor
OS	Ogidi/ Idemili North	2.2×10^4	8.9×10^4	1.9×10^5	Floor
CY	Ogidi/ Idemili North	3.5×10^4	8.1×10^4	1.6×10^5	Floor
AV	Nnewi / Nnewi North	1.7×10^5	6.4×10^4	TNTC	Floor
GT	Onitsha/ Onitsha North	8.5×10^3	-	1.2×10^4	Floor
VO	Ogidi/ Idemili North	1.1×10^5	NA	1.2×10^4	Cage
TA	Awka/Awka South	6.0×10^3	NA	1.5×10^4	Cage
EM	Nteje /Oyi	8.1×10^4	NA	1.7×10^4	Cage
EG	Uga/Aguata	7.6×10^4	NA	1.1×10^4	Cage

TNCT = Too numerous to count; NA = Not available

Table 3: Fungal genera isolated from feed, litter and droppings during rain and dry seasons

Fungal genus	**Isolation**	
	Rain season	**Dry season**
Aspergillus spp	+	+
Yeast	+	+
Penicillium spp	+	+
Fusarium spp	+	+
Lichtheimia spp	+	-
Paecelomyces spp	+	-
Acremonium spp	+	-
Chrysonilia spp	+	-
Curvularia spp	-	+
Cunninghamella spp	-	+
Syncephalis spp	-	+
Aureobasidium spp	-	+
Nathrasia spp	-	+
Trichoderma spp	-	+
Scopulariopsis spp	-	+

+ = isolated, - = not isolated

Table 4: Morphological and microscopic features of some of the fungal isolates observed in the poultry farms

Species of fungi	**Source of isolate**	**Morphological features of isolate**			**Microscopic features of isolate**
		Texture of growth	**Colour on surface**	**Colour on reverse**	
Aspergillus tubingensis	Droppings, Feed and Litter	Fluffy and wrinkled with age	Yellow and white, appear black with age	Yellow	Produced sclerotia
Curvularia verruculosa	Feed, Litter	Lanose	Brown	Dark brown	Branched, simple conidiophores, geniculate near the apex and brown in colour.
Cunninghamella bertholletiae	Feed, Litter	Cottony	Initially white, later turns grey	Pale buff	Broad and septate hyphae, long and branched sporangia, ending in swollen vesicles. Vesicles on lateral branches are generally smaller and are covered with spine-like denticles for supporting a single round to oval sporangium.
Aspergillus fumigatus	Droppings	Dense felt of conidiophore	Initially white then blue-green	Cream	Columnar conidial heads, subclavate vesicles
Fusarium oxysporum	Feed	Velvety	White	Hyaline	Short, single conidiophores lateral monophialides in the aerial mycelium, later arranged in densely branched clusters, slightly curved fusiform macroconidia, pointed at the tips and mostly septate

Plate 1: Morphological and microscopic features of *Aspergillus tubingensis* isolated from litter sample (7thday on SDA)

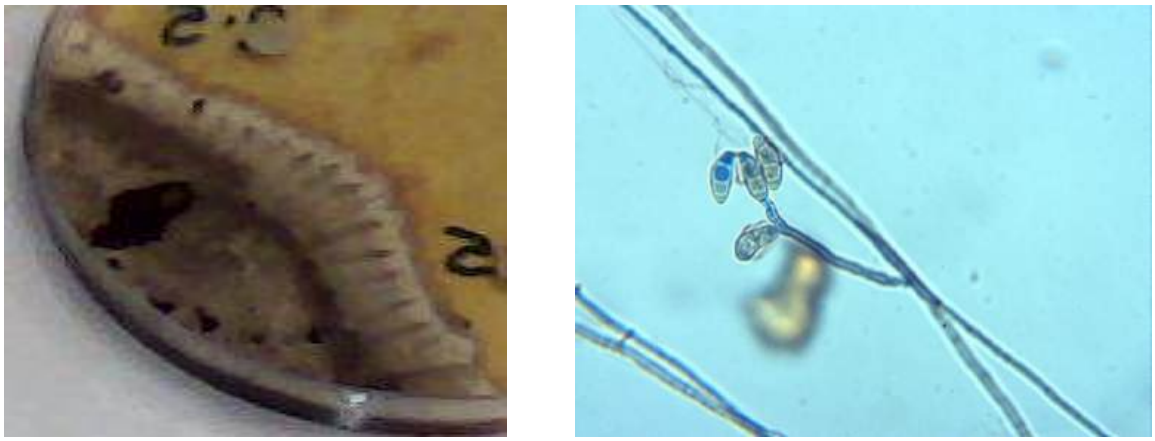

Plate 2: Morphological (2months on SDA) and microscopic (5 days on SDA) features of *Curvularia verruculosa* isolated from birds' droppings

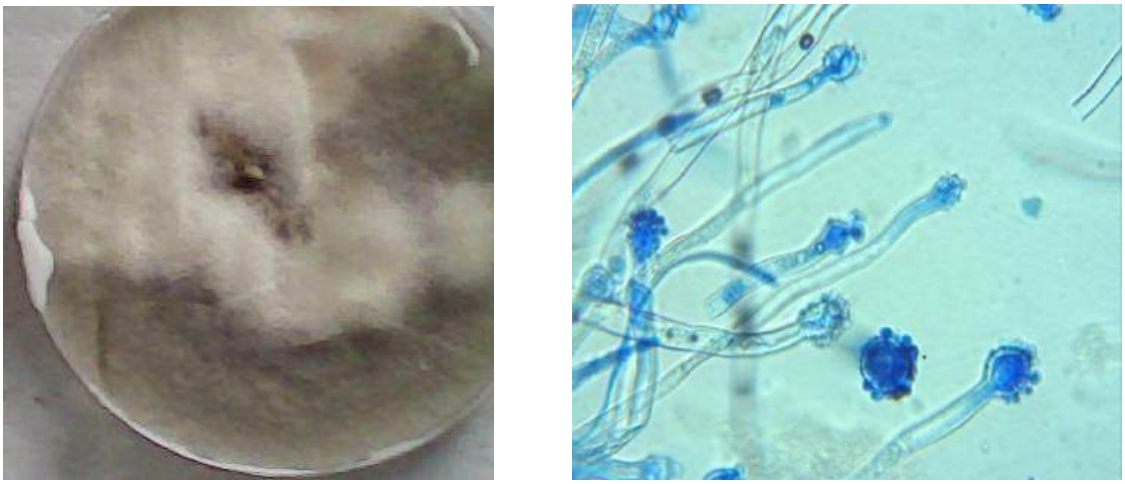

Plate 3: Morphological and microscopic features of *Cunninghamella bertholletiae* isolated from feed (5th day on SDA).

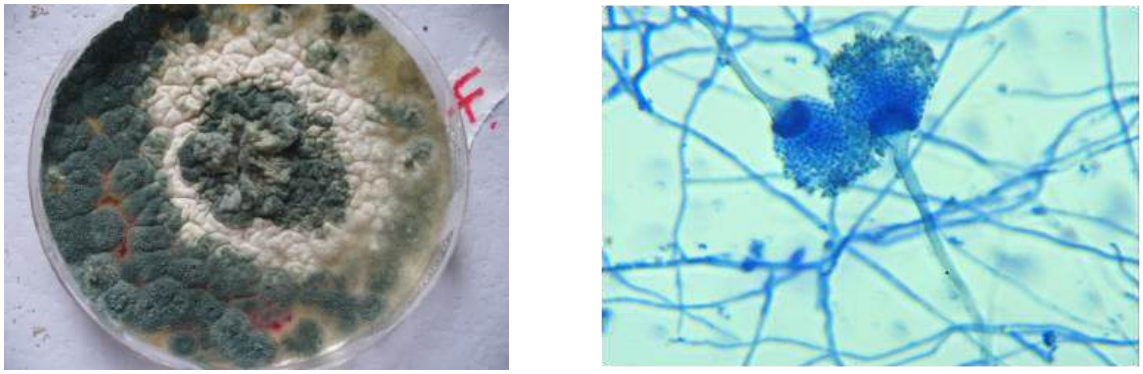

Plate 4: Morphological and microscopic features of *Aspergillus fumigatus* isolated from a bird's droppings (5th day on SDA).

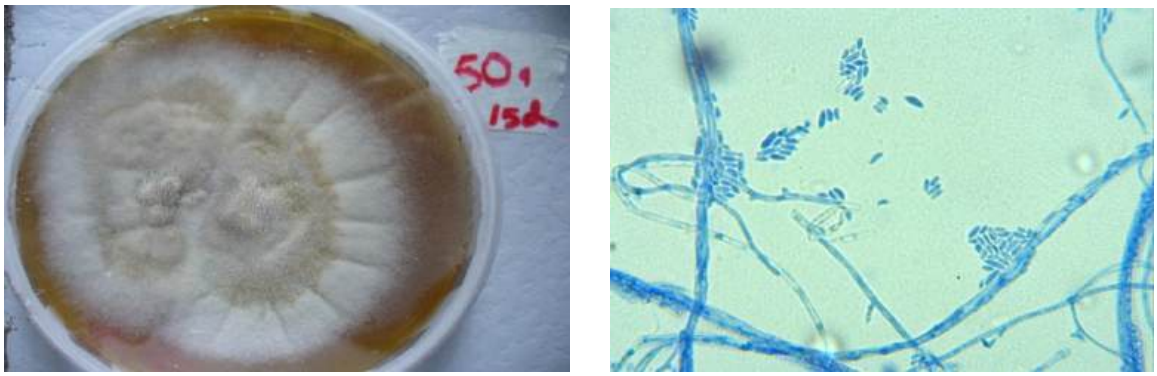

Plate 5: Morphological and microscopic features of *Fusarium oxysporum* isolated from feed (10th day on SDA)

Frequency distribution of fungal isolates during dry and rainy Seasons:

The frequency of fungal isolates in droppings, feed and litter samples from the poultry farms is presented in Fig 1 and Fig 2.

During the dry season, the most prevalent organism is yeast (Fig. 1), while *Aspergillus* species are the most prevalent during the rainy season (Fig 2).

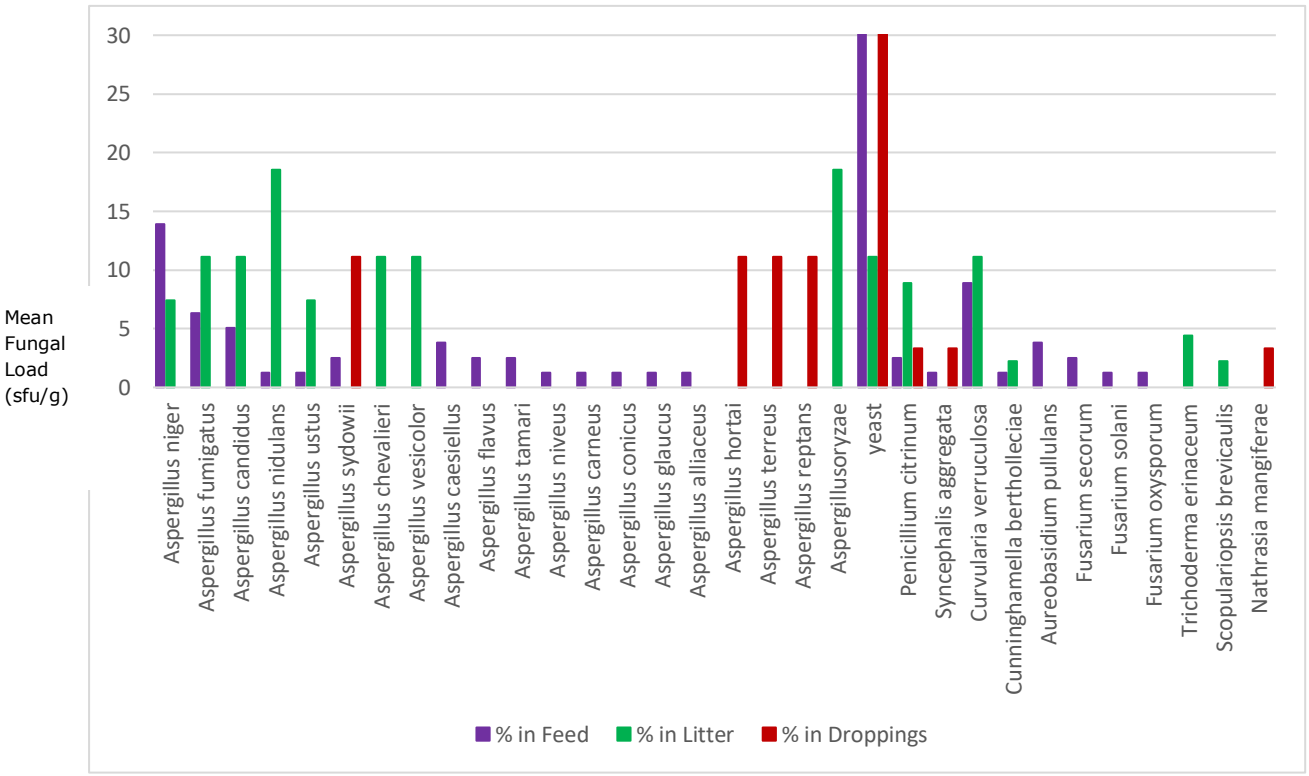

Fig 1. Frequency distribution of fungal isolates in environmental samples during dry season (14)

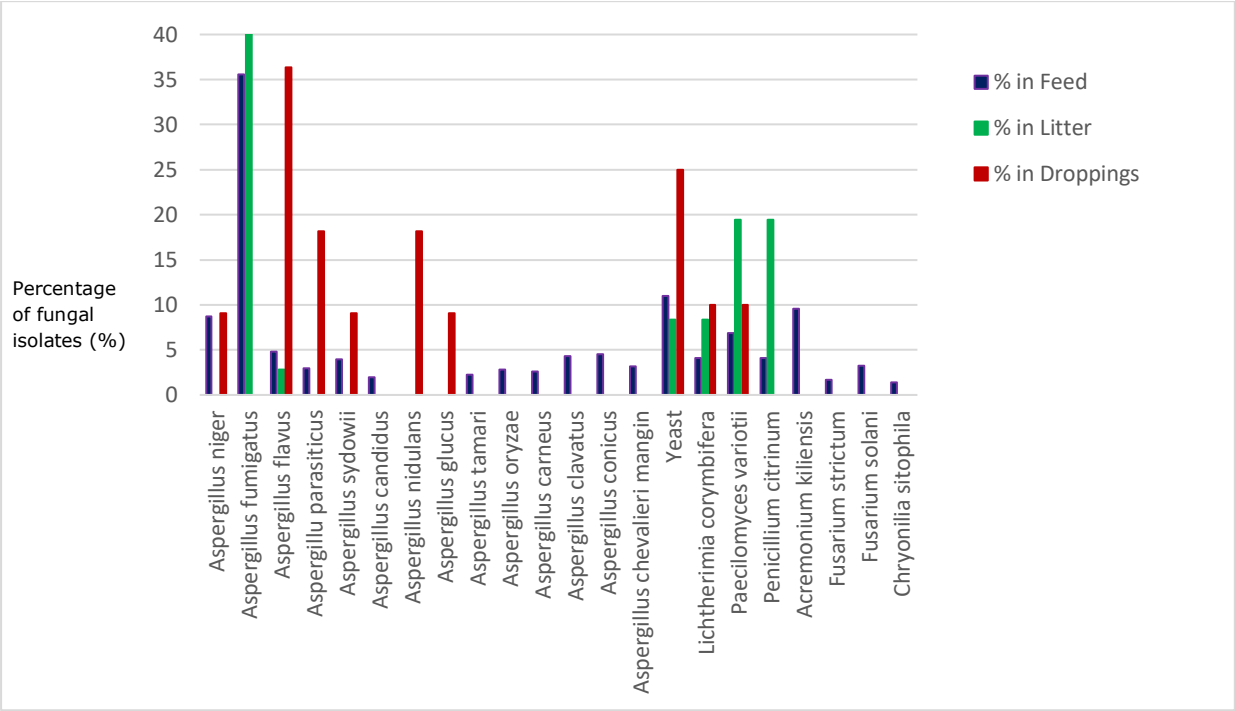

Fig 2: Frequency distribution of fungal isolates in environmental samples during rainy season (14)

Morphological changes in the eyes of fungal-infected mice:

Varying degrees of morphological and histopathological changes were observed in the eyes of mice topically infected with different fungal isolates. Mice infected with *A. tubingensis* showed severe erythema in the ocular region (6A) and histopathological changes including blood congestion, sloughing off of the sclera, distorted cornea, iris, and ciliary body, while the retina and optic nerves remained intact (6B). In contrast, mice infected with *C. bertholletiae* exhibited mild erythema in the cornea (7A) and histopathological changes include blood congestion, loosened strands of retina muscles, and distorted cornea, iris, and ciliary body (7B).

Mice infected with *Fusarium oxysporum* showed mild erythema and swollen cornea (8A), with severe blood congestion observed in histopathological examination (8B). *Curvularia verruculosa* infection resulted in severe erythema in the cornea (9A), damaged cornea and blood congestion (9B). Finally, mice infected with *Aspergillus fumigatus* exhibited slacked lower lid, swollen sclera, mild erythema in the cornea, (10A) and intact iris, with damaged lens, distorted cornea, sclera, retina, and pupil observed in histopathological examination (10B).

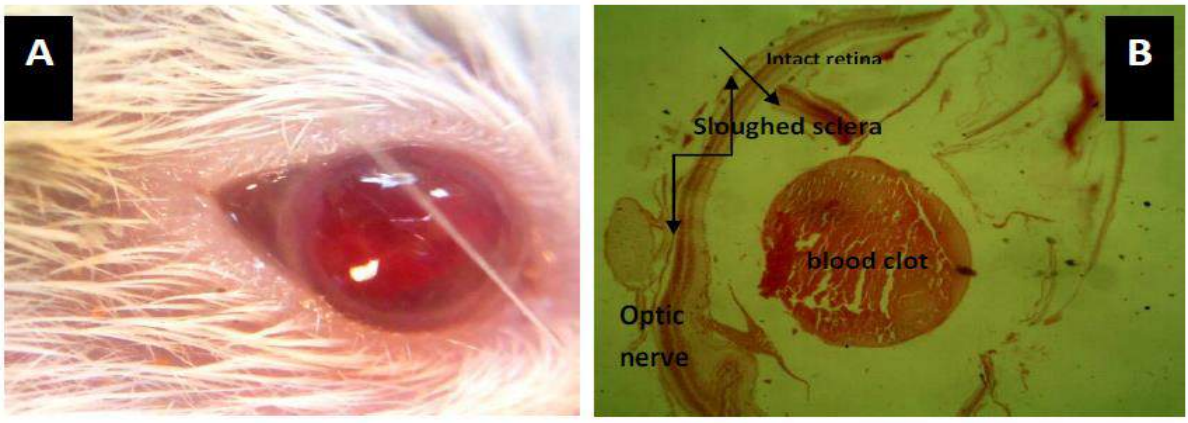

Plate 6: Morphological and histopathological changes in *Aspergillus tubingensis* ocular infection of a mouse
6A: Adult mouse showing severe erythema under steromicroscope and 6B: Photomicrograph of eye of adult mouse showing blood congestion with sloughing off of the sclera, distorted cornea, iris and ciliary body. The retina and optic nerves were intact. (H & E magnification 100x)

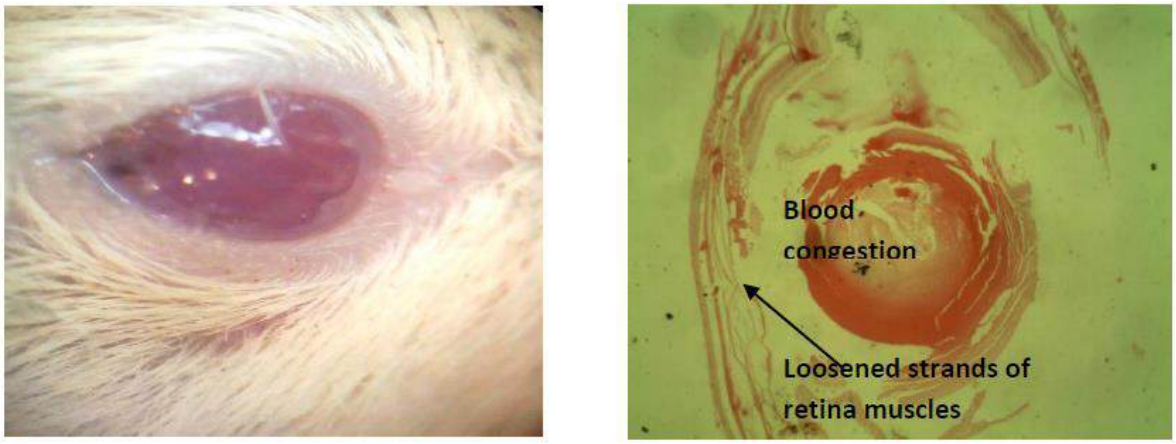

Plate 7: Morphological and histopathological changes in *Cunninghamella bertholletiae* ocular infection of a mouse
7A: Infected eye showing mild erythema in the cornea under steromicroscope. 7B: Photomicrograph of infected eye showing blood congestion and loosened strands of retina muscles. The cornea, iris and ciliary body appeared distorted. (H & E magnification 100x)

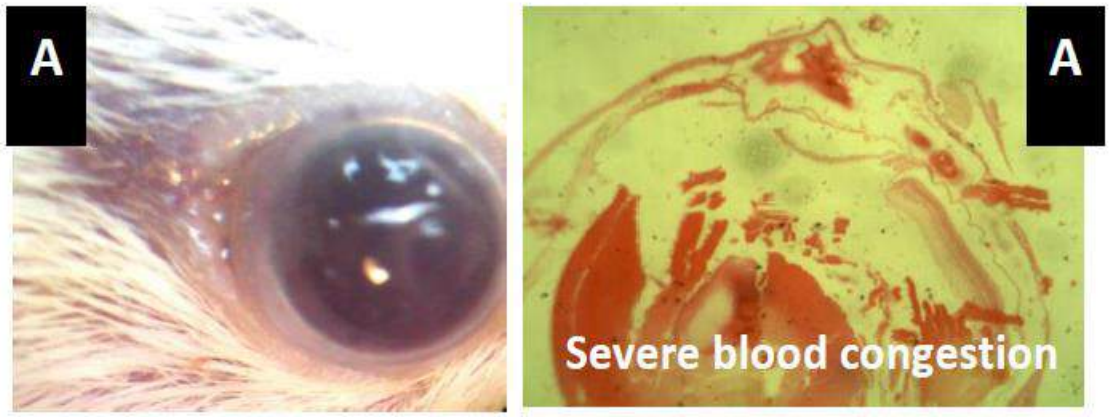

Plate 8: Morphological and histopathological changes in *Fusarium oxysporum* ocular infection of a mouse
8A: Infected mouse eye showing mild erythema and swollen cornea under stereomicroscope. 8B: Photomicrograph of infected mouse eye showing severe blood congestion (H & E magnification 100x)

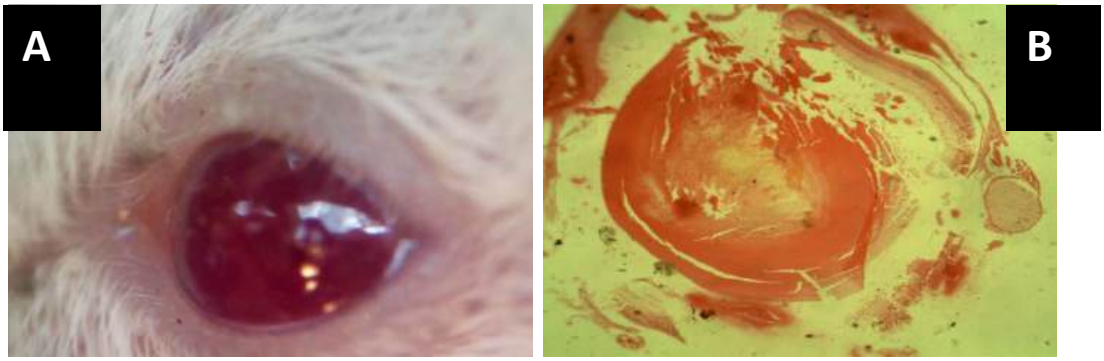

Plate 9: Morphological and histopathological changes in *Curvularia verruculosa* ocular infection of a mouse
9A: Infected mouse showing severe erythema in the cornea under stereomicroscope. 9B: Photomicrograph of infected mouse showing damages and blood congestion in the lens (H & E magnification 100x)

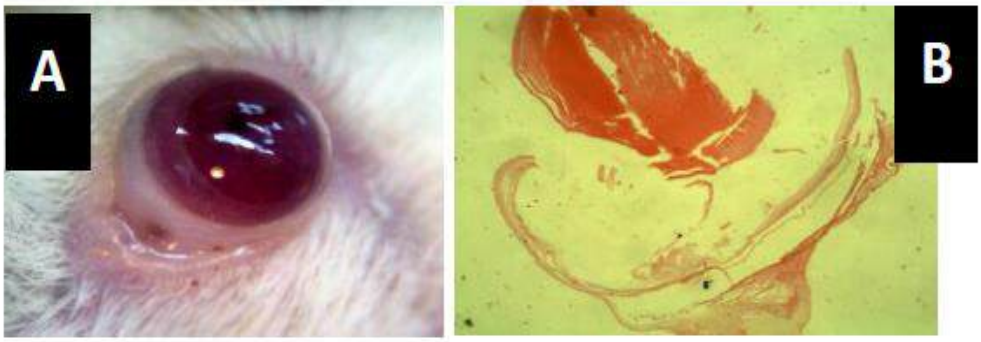

Plate 10: Morphological and histopathological changes in *Aspergillus fumigatus* ocular infection of a mouse
10A: Infected mouse eye under stereomicroscope showing slacked lower lid, swollen sclera, mild erythema in the cornea and intact iris. 10B: Photomicrograph of infected mouse eye showing damaged lens, distorted cornea, sclera, retina and pupil (H & E magnification 100x)

Discussion:

The occurrence and growth of fungi (moulds) in poultry farms is one of the major threats to poultry workers, poultry economy and health (14). In this study, we observed that all the environmental samples were overly contaminated by fungi, although to a variable extent as previously reported by Mba et al., (14). The frequency and distribution of fungal isolates in the environmental samples in this study showed presence of different fungal isolates with *Aspergillus* species having the highest frequency in the litter samples during rainy season and yeast showing the highest frequency in both droppings and feed samples in dry seasons. Contrary to our finding, Cruciani et al., (15) and Ostović et al., (16) reported *Aspergillus* species as the most frequent isolates in litter samples during dry season. However, they also observed *Aspergillus* species as the most frequent in litter samples in the rainy season which is in line with the result obtained in this study.

The unique structure of the eye as well as its exposure directly to the environment renders it vulnerable to a number of uncommon infectious diseases caused by fungi. Once the anatomical barrier is breached, host defenses directed against these microorganisms are often insufficient to prevent loss of vision. Filamentous fungal infections of the eye, known as mycotic keratitis, has been reported in many different parts of the world, particularly tropical area, where it may account for 50% of all ocular mycoses (2) or of all cases of microbial keratitis (4).

Filamentous fungi such as *A. fumigatus, A. flavus, Fusarium* species*, Curvularia, Alternaria* and *Acremonium* as well as yeasts such as *Candida* species have been reported to be implicated in fungal keratitis (17,18). Among these organisms, *Fusarium* species have been reported as the most common cause of keratitis followed by *Aspergillus* species and thirdly, the dematiaceous fungi such as *Curvularia*, with *Fusarium solani* and *Aspergillus* species constituting up to one-third of all cases of traumatic infectious keratitis (19). This agrees with the findings in this study as shown in Plate 6–10, in which topical ocular inoculation of *A. tubingensis* caused severe erythema (Plate 6A). Our findings in mice also agree with that observed by Kredics et al., (6), who reported pain, redness in the eyes, hypopyon and necrosis of the cornea in their patients, but contrary to our result, hypopyon was not observed.

It was also noted that of all the *Aspergillus* species implicated in keratitis, *A. tubingensis* had not been previously reported to cause mycotic keratitis. Studies however, showed that the paucity of information on the pathogenicity of *A. tubingensis* is possibly as a result of mis-leading identification results based on microscopy, morphology and culture, in which isolates of *A. tubingensis* were identified as *A. niger* because of similarities in their morphological and microscopic features (20).

Topical ocular inoculation of *C. bertholletiae* resulted in slight erythema and blurred cornea, with a discharge from the infected eyes. Ribes et al., (21) reported pain, blurring and loss of vision as the manifestations of ocular infection by *C. bertholletiae* which supports our finding however, pain and loss of vision were not observed. Spellberg et al., (8) reported symptoms of ocular infection by *Mucorales* as invasion of blood vessels, which resulted in formation of blood clots and death of surrounding tissues due to loss of blood supply. In our study, infected eye by *C. bertholletiae* showed formation of blood clot. This observation is supported by the study of Spellberg et al., (8) although loss of blood supply was not determined. Other pathological signs such as loosened retina walls, distorted cornea, iris and ciliary body were also reported.

Fusarium species have been reported by Liesegang and Forster (19) and Richard et al., (22) as one of the agents of fungal keratitis and the most common cause of corneal infection in the Southern United States (45-75%). Haynes et al., (1) described *F. oxysporum* as the most common isolate in fungal keratitis. Mikami and Stemmermann (23) in their study observed that keratitis due to *Fusarium* species, especially *F. solani* and *F. oxysporum* resulted in ulceration of the cornea which was progressive and destructive. Ocular infections by *F. oxysporum* in our study resulting in severe erythema, swelling, opacification of the cornea and blood congestion within 7 days (and clearing over 10 days of infection), were not observed by Mikami and Stemmermann (23), but were in line with the study of Kirk et al., (24), who also reported swollen eyes within one-half hour of infection, opacification and presence of edema in the cornea. However, their observation of the whiteness of the lower cornea, superficial ulcer which involved the cornea within 10 days and pus production, presence of pain by the 24th day, perforation of the limbus by the 28th day, with granular mass and mucopurulent discharge along the perforated area, denudation, necrosis, fragmentation and lysis of the cornea were not seen in our study. This may be as a result of non-extension of this study beyond 10days.

Mikami and Stemmermann (23) in their study reported that fungal keratitis by *Curvularia* species is characterized by presence of ulcer which became deep and lead to corneal perforation. Zhong et al., (25) reported superficial, feathery infiltrates of the central cornea, suppurative ulceration of the peripheral cornea and hypopyon in some cases, which resulted in poor vision. These observations by the researchers are at variance with that obtained in this study, in which only destruction of the various parts of the eyes with presence of erythema was observed. Feathery infiltrates, ulceration, perforation of the cornea and hypopyon were not observed.

As observed in our study, the ocular topical inoculation of *A. fumigatus* resulted in slacked lower lid, swollen cornea, mild erythema with intact iris (Plate 10A) while photomicrograph of the same eye showed damaged lens and distortion of the cornea, sclera, retina and pupil (Plate 10B). These findings are similar to the study by Darlene et al., (26) and Walther et al., (27) in which clinical manifestations such as opacification of the cornea, irregularity of the cornea indicating corneal damage were observed. Darlene et al., (26) also reported infiltration of inflammatory cells into the cornea which rapidly increased during the early stage of infection playing dual role of fighting the fungal pathogen and destroying the infected tissue.

Conclusion:

Our study reported a high prevalence of fungi isolates in selected poultry farms in Anambra State, southeast Nigeria. Experimental infections of mice demonstrate morphological and histopathological changes caused by different fungi in the eyes of the mice, providing valuable insights into the pathogenesis of fungal infections of the eyes. Ocular exposure to fungi may pose significant health risks to poultry farm workers.

Contributions of authors:

MAN conceptualize and performed the study, curate the data, and prepared the original manuscript; MAN and EAI reviewed and edited the manuscript; Both authors read and agreed to the published version of the manuscript.

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Conflicts of interest:

No conflict of interest is declared

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Short Communication **Open Access**

Leptospira sp and chronic kidney disease: A preliminary study of the serological status of a population of dialysis patients in Abidjan, Côte d'Ivoire

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Abstract:

Background: Pathogenic strains of *Leptospira* are responsible for leptospirosis, a neglected zoonotic disease, whose pathophysiology involves acute kidney injury. These kidney lesions may progress to chronic forms, suggesting that leptospirosis could be a potential contributor to chronic kidney disease (CKD). This study aimed to determine the serological status related to leptospirosis in dialysis patients in Côte d'Ivoire.
Methodology: This cross-sectional study was conducted from November 15 to December 15, 2024, at the Institut Pasteur de Côte d'Ivoire. It involved serum samples collected from dialysis patients in Abidjan. The presence of anti-*Leptospira* IgM and IgG antibodies was assessed using ELISA techniques, and data were analyzed using the Epi Info software.
Results: A total of 30 patients, predominantly males (sex ratio of 2), were included, with a mean age of 43.7 years. None of the patients tested positive for anti-*Leptospira* IgM or IgG antibodies.
Conclusion: The findings from this preliminary study suggest that leptospirosis likely plays a limited role in the burden of CKD cases in Côte d'Ivoire. However, given the relatively small sample size, these conclusions should be interpreted with caution. Further studies involving a larger population would be necessary to better assess potential links between leptospirosis and chronic kidney disease.

Keywords: *Leptospira*; Chronic kidney disease; Hemodialysis; Serology; Côte d'Ivoire

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Leptospira sp et maladie rénale chronique: étude préliminaire du statut sérologique d'une population de patients dialysés à Abidjan, Côte d'Ivoire

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Résumé:

Contexte: Des souches pathogènes de *Leptospira* sont responsables de la leptospirose, une maladie négligée. Maladie zoonotique, dont la physiopathologie implique une atteinte rénale aiguë. Ces lésions rénales peuvent évoluer vers des formes chroniques, suggérant que la leptospirose pourrait être un facteur contributif potentiel de l'insuffisance rénale chronique (IRC). Cette étude visait à déterminer le statut sérologique lié à la leptospirose chez les patients dialysés en Côte d'Ivoire.
Méthodologie: Cette étude transversale a été menée du 15 novembre au 15 décembre 2024 à l'Institut Pasteur de Côte d'Ivoire. Elle a porté sur des échantillons de sérum prélevés chez des patients dialysés à Abidjan. La présence d'anticorps IgM et IgG anti-*Leptospira* a été évaluée par des techniques ELISA, et les données ont été analysées à l'aide du logiciel Epi Info.
Résultats: Un total de 30 patients, majoritairement des hommes (sex-ratio de 2), ont été inclus, avec un âge

moyen de 43,7 ans. Aucun des patients n'a été testé positif aux anticorps IgM ou IgG anti-*Leptospira*.
Conclusion: Les résultats de cette étude préliminaire suggèrent que la leptospirose joue probablement un rôle limité dans la prévalence des cas d'IRC en Côte d'Ivoire. Cependant, compte tenu de la taille relativement réduite de l'échantillon, ces conclusions doivent être interprétées avec prudence. D'autres études portant sur une population plus large seraient nécessaires pour mieux évaluer les liens potentiels entre la leptospirose et l'insuffisance rénale chronique.

Mots-clés: Leptospirose; Insuffisance rénale chronique; Hémodialyse; Sérologie; Côte d'Ivoire

Introduction:

Pathogenic strains of *Leptospira* are responsible for leptospirosis, a neglected zoonosis that poses a significant public health challenge. Each year, nearly about one million cases are reported globally, with approximately 60,000 deaths (1). Leptospirosis is more prevalent in tropical and subtropical areas where climatic conditions favor the bacterial life cycle (2). In West Africa, the prevalence of leptospirosis varies significantly across countries, reaching up to 33% in some studied populations (3,4). In Côte d'Ivoire, the prevalence is high, estimated at 9.4% by Koffi et al., (5).

While leptospirosis is generally mild, severe forms occur in about 10% of cases, often characterized by multiorgan failure (2,6). Pathophysiological evidence shows that the kidneys are one of the primary target organs, and the bacterial infection could lead to acute kidney injuries (7). These acute injuries, particularly acute interstitial nephritis and acute tubular necrosis, may progress to chronic kidney disease, according to the severity of initial damages (7,8). Consequently, leptospirosis could be a potential contributor to the burden of chronic kidney disease (CKD).

In this context, an essential question arises; what proportion of CKD cases in Côte d'Ivoire could be attributed to leptospirosis? Answering this question could provide critical insights into CKD management, thereby justifying this study, which aims to determine the serological status related to leptospirosis in patients with chronic renal failure.

Materials and method:

Study setting, design, participants and sample collection:

We conducted a cross-sectional study from November 15 to December 15, 2024, at the Institut Pasteur de Côte d'Ivoire. The study involved serum samples collected from dialysis patients at the Cocody site, Abidjan, Côte d'Ivoire, of the Centre National de Prévention et de traitement de l'Insuffisance Rénale (CNPTIR). All consenting patients aged 18 years or older were included. Pregnant women were excluded from the study. Serum samples were collected between July 1 and August 31, 2021, and stored at -20°C at the Institut Pasteur de Côte d'Ivoire until analysis.

Ethical approval:

The study was approved by the Comité national d'Ethique des Sciences de la Vie et de la Santé under the reference number 023-20/MSHP/CNESVS-km.

Serological detection of *Leptospira* in samples:

In the laboratory, anti-*Leptospira* IgM and IgG antibodies were detected using an ELISA technique, with *Leptospira fainei* as the antigen, as previously described by Bourhy et al., (9). The antigen was immobilized on 96-well flat-bottom plates. For IgM detection, we used a goat anti-human IgM conjugated with horseradish peroxidase (Invitrogen®).

For IgG detection, a goat anti-human IgG conjugated with horseradish peroxidase was used (Chemicon®). The presence of anti-*Leptospira* antibodies was revealed by adding the TMB substrate (3,3',5,5'-Tetramethyl benzidine). Negative and positive controls were included to validate the tests.

Data analysis:

Data were analyzed using the CDC's EPI INFO software 7.2. Quantitative variables were expressed as minimum and maximum values, means, and standard deviations. Qualitative variables were presented as absolute numbers and proportions.

Results:

A total of 30 patients were included in this preliminary study. The age of participants ranged from 23 to 76 years, with a mean age of 43.7±17.3 years. There was a predominance of male patients, accounting for 66.7% of the study participants, resulting in a sex ratio of 2. Laboratory analysis revealed no patients with detectable anti-*Leptospira* IgM or IgG antibodies.

Discussion:

The present study aimed to detect anti-*Leptospira* antibodies in dialysis patients with chronic kidney disease (CKD) to explore a potential link between leptospirosis and CKD. Leptospirosis is known to cause acute kidney injuries, which, when severe, can evolve into chronic kidney disease, ultimately requiring dialysis. The study participants were predominantly males, with a relatively young mean age of 43.7 years. These findings are comparable to those of several studies cond-

ucted in Côte d'Ivoire on CKD. Studies by Mondé et al., (10) and Ouattara et al., (11) reported similar mean ages of 39 and 44 years, respectively. Male predominance was also observed in these two studies, although with slightly lower sex ratios of 1.1 and 1.3, respectively.

In the present study, none of the tested samples showed detectable levels of anti-*Leptospira* IgM or IgG antibodies. Based on these findings, leptospirosis does not appear to be a significant contributor to CKD in Côte d'Ivoire. However, these results prompt reflections on both the potential effects of dialysis on antibody levels and the healthcare access of patients undergoing dialysis. Regarding the potential impact of dialysis on antibody levels, it is worth noting that hemodialysis may influence antibody kinetics, either by altering their elimination or by affecting their production. Hemodialysis, which removes metabolic waste and excess electrolytes from the blood, could theoretically lower antibody titers to undetectable levels by clearing them from circulation. This could lead to false-negative results. However, antibodies are large molecules that are not filtered by the membranes used in hemodialysis. Therefore, hemodialysis is unlikely to have affected the presence of anti-*Leptospira* antibodies in the patients included in this study through this mechanism.

Conversely, hemodialysis is known to impair antibody production. Hemodialysis patients often exhibit altered immune responses, making them less responsive to infections and vaccines (12). However, for this hypothesis to be considered, CKD would need to be pre-existing, suggesting that a factor other than leptospirosis initially triggered the renal failure. In severe forms of leptospirosis, multi-organ failure often includes renal involvement (7). Many studies have reported acute kidney injuries associated with severe leptospirosis (13-15). It is therefore essential to investigate whether these acute injuries could progress to chronic kidney disease following a severe episode of leptospirosis.

The study population, composed of dialysis patients, highlights the issue of health care access in Côte d'Ivoire. Despite efforts to improve access to dialysis, it remains limited due to the shortage of dialysis units in public healthcare facilities, the high cost of dialysis in private centers, and the lack of social coverage for most patients (16,17). Consequently, resource-limited populations, who are at higher risk of leptospirosis due to poor hygiene conditions and rodents' proliferation, the primary reservoirs of leptospires worldwide (2), may not have access to dialysis. This situation could underrepresent this segment of the population among dialysis patients, potentially masking a link between CKD and leptospirosis.

Furthermore, the risk of leptospirosis is higher in the western region of the country (5,18), where only one dialysis center exists according to the Ivorian Society of Nephrology (S.I.NEPH, http://www.sinephweb.org/page_indicateur_1.php). This limited access to dialysis services could also contribute to the underrepresentation of a potential link between the two diseases, even though the study population was selected from Abidjan, the economic capital of Côte d'Ivoire. However, the small sample size of this preliminary study warrants caution when interpreting these findings. A larger study, encompassing more regions of the country, would be necessary to better assess the potential association between leptospirosis and CKD in Côte d'Ivoire.

Conclusion:

In conclusion, this study did not detect anti-*Leptospira* IgM or IgG antibodies in the dialysis patients included. These findings suggest that leptospirosis plays a limited role in the burden of CKD in Côte d'Ivoire. However, given the relatively small sample size, these conclusions should be interpreted with caution. A larger study, covering a broader population, would be necessary to more accurately assess the potential link between leptospirosis and chronic kidney disease.

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Contributions of authors:

KKS, BGA and SND designed the study; KKS drafted the protocol and the manuscript; BGA and SND collected the samples; TGG and KKS carried out the laboratory analyses; MS and DM validated the protocol and revised the document. All the authors read and approved the manuscript submitted for publication.

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Conflicts of interest:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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