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Original Article **Open Access**

Discovery of potential antiviral compounds against Dengue virus using virtual screening and physicochemical profiling from zinc database

[1]Ramdeen, S. J., [1]Usoro, N., [1]Giordano, K., [1]Garza, P. E., [2]Quadri, P., [3]Esan, T. E., and [*1]Olanrewaju, A. A.

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Abstract:

Background: Dengue, a mosquito-borne virus infection, reportedly affected over 7.6 million people in early 2024, subsequently leading to approximately 3000 deaths. Dengue is primarily spread by *Aedes* (*A. aegypti and A. albopictus*) mosquitoes. There is currently no clinically approved preventive vaccine against Dengue virus (DENV), and according to the Center for Disease Control (CDC), the only acceptable vaccine for DENV is Dengvaxia, which has efficacy and safety issues. This makes it unsuitable for use against DENV. Drug repurpose is an alternative approach for the discovery of novel therapeutics.
Methodology: In this study, we screened over one million compounds in the zinc database against the DENV NS2B/NS3 protease (PDB ID: 2FOM). Screening yielded about 122 compounds with potential activity against DENV. Among the 122 compounds, the top four were selected based on their docking scores and commercial availability. The cytotoxicity study showed that a concentration of up to 100 µM was non-toxic. Based on the cytotoxicity results, we selected non-toxic concentrations (500nM, 1µM, 10µM, and 20µM) to test against DENV2 infection. The results were analyzed using RT-qPCR and plaque assays.
Results: Compound treatment before DENV2 infection leads to a significant reduction in viral replication, which confirms the screening result. This gives a promising platform against DENV2 therapy.
Conclusion: This shows a significant step towards the discovery of an antiviral drug against the dengue virus.

Key words: Dengue virus; Antiviral compound; Repurposing; Molecular docking; Therapeutics

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Découverte de composés antiviraux potentiels contre le virus de la dengue par criblage virtuel et profilage physico-chimique à partir d'une base de données sur le zinc

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Résumé:

Contexte: La dengue, une infection virale transmise par les moustiques, aurait touché plus de 7,6 millions de personnes début 2024, entraînant environ 3000 décès. La dengue est principalement transmise par les moustiques *Aedes* (*Aedes aegypti* et *Aedes albopictus*). Il n'existe actuellement aucun vaccin préventif contre le virus de la dengue (DENV) approuvé cliniquement. Selon les Centres pour le contrôle et la prévention des maladies (CDC), le seul vaccin acceptable contre le DENV est le Dengvaxia, qui présente toutefois des problèmes d'efficacité et d'innocuité. De ce fait, il est inadapté à la lutte contre le DENV. Le repositionnement de médicaments constitue une approche alternative pour la découverte de nouvelles thérapies.
Méthodologie: Dans cette étude, nous avons criblé plus d'un million de composés de la base de données sur le

zinc contre la protéase NS2B/NS3 du DENV (identifiant PDB: 2FOM). Ce criblage a permis d'identifier environ 122 composés présentant une activité potentielle contre le DENV. Parmi ces 122 composés, les quatre meilleurs ont été sélectionnés en fonction de leurs scores d'amarrage moléculaire et de leur disponibilité commerciale. L'étude de cytotoxicité a montré qu'une concentration allant jusqu'à 100 µM était non toxique. D'après les résultats de cytotoxicité, nous avons sélectionné des concentrations non toxiques (500nM, 1µM, 10µM et 20µM) pour tester leur efficacité contre l'infection par le DENV2. Les résultats ont été analysés par RT-qPCR et par test de plages de lyse.

Résultats: Le traitement par le composé avant l'infection par le DENV2 induit une réduction significative de la réplication virale, confirmant ainsi les résultats du criblage. Ceci constitue une plateforme prometteuse pour le traitement du DENV2.

Conclusion: Ces résultats représentent une avancée significative vers la découverte d'un médicament antiviral contre le virus de la dengue.

Mots-clés: Virus de la dengue; Composé antiviral; Repositionnement; Modélisation moléculaire; Thérapeutique

Introduction:

Dengue virus (DENV) infection is regarded as one of the most common arthropod-borne diseases in the tropical and subtropical regions. Cases of DENV infection reported by the World Health Organization (WHO) are about 5.2 million in 2019 (1,2). The WHO has reported dengue to be one of the most rapidly spreading mosquito-borne viral infections worldwide. It is estimated that approximately 60% of the world's population is at risk of infection by the year 2080 (2). Dengue viruses spread to people through the bite of infected *Aedes* mosquitoes (*Aedes aegypti* or *Aedes albopictus*) (3).

There are four DENV serotypes (DENV-1, DENV-2, DENV-3, and DENV-4), with DENV 2 and 3 being the most virulent forms (4). All four serotypes can cause hemorrhage (5). DENV can cause illnesses ranging from asymptomatic to dengue hemorrhagic fever (DHF), dengue fever (DF), and dengue shock syndrome (DSS). Clinical manifestations of DENV infection include fever, rash, hemorrhagic symptoms, nausea, vomiting, and ocular pain (6).

Dengue virus is a member of the genus Flavivirus of the family Flaviviridae (7). DENV has a small icosahedral envelope (8, 9) with an 11 kb positive-sense single-stranded RNA (10). This encloses the three structural proteins, including the capsid (C), envelope (E), and membrane (M), as well as seven non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (11,12). The mature DENV particles had a diameter of approximately 50 nm (7). The surface is composed of a lipid bilayer that incorporates two transmembrane viral proteins to form a glycoprotein shell (13). The core contains a nucleocapsid formed by the viral RNA genome complex with the capsid protein. The glycoprotein shell contains 180 copies of the envelope (E) and membrane proteins (M or prM). DENV infection depends largely on the conformational changes in the M and E proteins brought about by the pH of the environment (14).

The crystal structure of the dengue virus NS2B-NS3 protease revealed a pocket that can potentially be occupied by various compounds. This protease is one of the major virulent factors of the virus. It is important in viral integration with the host genome. This opens a novel way to identify antiviral agents that are active against the early stages of dengue virus infection (15). Currently, there are no FDA-approved therapeutic drugs against dengue virus infection, and a potent vaccine platform is still in its infancy. Significant work on vaccine development has yielded a single licensed vaccine with strict restrictions on use (11).

The major aim of this pilot study was to verify the safety profile of selected compounds using a cytotoxicity assay and test their antiviral activity against DENV. For this experiment, compound 1 was selected for antiviral assay against DENV2 based on availability and highest docking score compared to other compounds.

Materials and method:

In-silico analysis:

The crystal structure of the DENV protein (PDB ID: 2FOM) was retrieved from the Protein Data Bank. The Protein Preparation Wizard in Maestro (Schrödinger) was used to clean and optimize protein structure. The following steps were carried out; (i) removal of water molecules and other heteroatoms irrelevant to binding, (ii) assignment of bond orders, and (iii) addition of missing hydrogens. Optimization of protonation states for amino acids, especially those involved in catalysis or those close to the ligand-binding site, was performed using PROPKA at pH 7.0. The protein structure was refined by energy minimization using the OPLS4 force field. Additionally, a grid box of 20 Å was generated around the active site to define the docking space and ensure accurate positioning of the ligands during docking simulations.

High-Throughput Virtual Screening (HTVS):

The prepared zinc compounds underwent an initial docking step using the High-Throughput Virtual Screening (HTVS) mode within Schrödinger's Glide module. This screening step allows rapid evaluation of large libra-

ries by focusing on the best-fitting compounds for the protein-binding site. Over 100,000 compounds with the best docking scores were selected for further analysis. This stage prioritizes speed and efficiency and provides rough estimates of the binding affinity.

Standard Precision (SP) Docking:

The compounds selected from the HTVS step were re-docked using the Standard Precision (SP) docking mode in Glide. SP docking involves more thorough exploration of the binding site and ligand conformations, yielding more accurate docking scores. This step reduced the number of compounds from 100,000 to 250 based on the binding affinity, protein-ligand interactions, and key docking parameters. Additionally, a visual inspection of the binding modes was performed to ensure that only the most promising ligands progressed to the next stage.

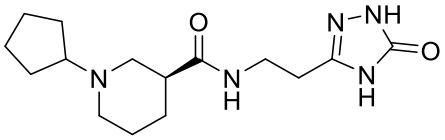
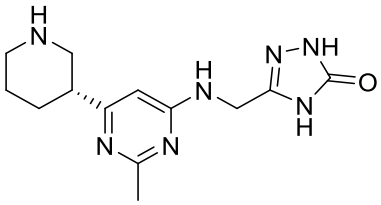
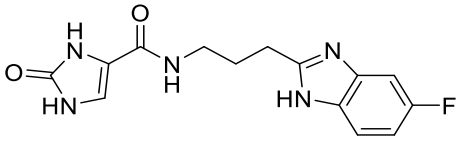
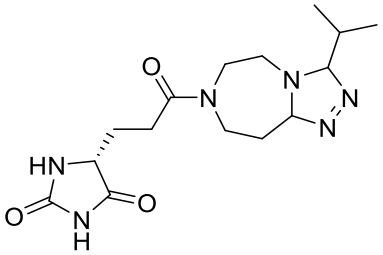
Extra Precision Docking (XP):

The 250 compounds from the SP step were subjected to Extra Precision (XP) docking. XP docking employs a more refined scoring function, penalizing unfavorable interactions, and rewarding favorable interactions, resulting in a more reliable ranking of ligands. After rigorous evaluation, 125 compounds were selected for the final stage. The criteria included docking scores, presence of key hydrogen bonds or hydrophobic interactions, and favorable energy conformations.

Virtual Inspection and Physicochemical Profiling:

The top 125 compounds from the XP docking step underwent virtual inspection to assess their drug-likeness and potential absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties. This involved the evaluation of physicochemical properties, such as molecular weight, logP, hydrogen bond donors and acceptors, and polar surface area. These factors were crucial for narrowing down the selection of 100 final compounds for biological testing. Compounds exhibiting optimal physicochemical profiles as well as those fitting the binding site were prioritized (Table 1).

Table 1: The top four compounds identified by virtual screening and physicochemical profiling and selected for biological testing

Compound ID	Compound Formula	Docking Score	Structure
ZINC000095356735	$C_{15}H_{25}N_5O_2$	-9.695	
ZINC000067908645	$C_{13}H_{19}N_7O$	-8.613	
ZINC000218827339	$C_{14}H_{14}FN_5O_2$	-8.583	
ZINC000091593654	$C_{15}H_{22}N_6O_3$	-8.113	

Final Compound Selection for Biological Testing:

The top four compounds identified by virtual screening and physicochemical profiling were selected for subsequent biological testing (Table 1). These compounds represent those

with the highest binding affinity for the DENV protein and the best overall ADMET profiles. The selected compounds were expected to undergo experimental validation to confirm their inhibitory activity against DENV1-4, focusing on their interactions with the 2FOM protein structure.

Cell Culture Reagents and Virus Strains:

Vero (African green monkey kidney) cells obtained from ATCC (CRL-1587) were maintained in Dulbecco's modified minimum essential medium (DMEM) (Fisher Scientific, 11-995-073) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) from Neuromics (FBS002), 1% L-glutamine (Fisher Scientific, 25-030-081), and 1% penicillin/streptomycin (Fisher Scientific, 15-140-122). *Aedes albopictus* clone C6/36, also from ATCC (CRL-1660), was grown in EMEM (ATCC, 30-2003) supplemented with 10% FBS. All cell lines were maintained at 37°C in 5% CO_2. Dengue virus type 2 (DENV2) was purchased from ATCC (VR-1584).

Cell Viability Assay:

About 1.25 x 10^5 Vero cells were seeded overnight in 1mL of DMEM per well of a 12 well plate. The Vero cells were treated with varying concentrations (100 µM, 50 µM, 20 µM, and 10 µM) of the compounds to be tested and allowed to incubate at 37°C in 5% CO_2. At 24, 48, and 72 h post-treatment, the supernatant was removed, and each well was washed once with sterile phosphate-buffered saline (PBS). The cells were trypsinized, transferred to a 1.5 mL microcentrifuge tube, and spun down to remove trypsin.

The cell pellet was resuspended in 500 µL of complete DMEM. For this experiment, 10 mM of the compound and DMEM was used as positive and negative control respectively. Each aliquot was mixed with stain-acridine orange/propidium iodide (AO/PI) at 1:9 ratio, and sample fluorescence was quantified using a Luna-FL cell counter (Logos Biosystems). Assays were performed in technical duplicates and biological triplicates (16).

Plaque Assay:

Vero cells were placed in a 12-well culture plate at a density of 1.5 × 10^5 cells per well and incubated for about 24 h to reach 90%-100% confluency. Culture supernatant from treatments containing DENV type 2 strain was serially diluted ten-fold in complete DMEM from 10^{-1} to 10^{-4} and 200 µL of diluted inoculum was used for Vero cells infection. After the cells were infected for approximately 2 h, the virus inoculum was removed, and 1 ml overlay of a 1:1 mixture of 0.6% agarose in deionized H_2O (diH_2O) and 2x unstained EMEM supplemented with 5% FBS, 1% Sodium Pyruvate, 2% penicillin/streptomycin, 1% nonessential amino acids and 1% L-glutamine was added to the inoculum in the wells.

The overlay was left at room temperature to solidify and was incubated for 7 days at 37°C in 5% CO_2. After 7 days, the cells were fixed with 10% formalin for 20 min at room temperature. The formalin and the agarose plugs were removed and subsequently, the cells were stained with the mixture of 1% crystal violet and 20% methanol in diH_2O solution for 30–60 s at room temperature (17).

RNA Isolation and Quantitative RT-PCR (RT-qPCR) Analysis of Viral Genome:

Total RNA was isolated from different samples using Direct-zol RNA Miniprep from Zymo research (R2052). The following reagents were obtained from BEI Resources, NIAID, NIH: Dengue Virus type 2 primers (NR-12079). Purification was performed following the manufacturer's protocol. For RT-qPCR, the Thermo Fisher RNA Ultra Sense One-Step Quantitative RT-PCR System (117 32927) was used for actin detection (Thermo Fisher; 401846), while the Power SYBR™ Green RNA-to-CT™ 1-Step Kit (Thermo Fisher- 4389986) was used to detect the DENV 2 region of the E gene (BEI resources; NR-12079). Primer pairs used for PCR were as follows; A) E gene: DENV2-F Polymerase Forward (5'–TCCATACACGCCAAACATGAA–3'), and DENV2-R Polymerase Reverse (5'–GGGATTTCCTCCCATGATTCC–3'); B) Actin: Forward (5'-CACCATTGGCAATGAGCGGTTC-3') and Reverse (5'-AGGTCTTTGCGGATGTCCACGT-3').

The PCR cycling conditions for the Power SYBR™ Green RNA-to-CT™ 1-Step Kit and the RNA Ultra Sense One-Step Quantitative RT-PCR System were performed according to the manufacturer's protocol. The RNA count of the samples was determined relative to the reference gene actin or based on the cycle threshold (Ct) value relative to the standard curve. All assays were performed in triplicate and biological triplicate.

Viral Replication Assay:

Approximately 5 × 10^5 Vero cells were seeded in a 12-well culture plate and incubated overnight at 37°C, 5% CO_2 to allow the cells to attach. The cells were then treated with different concentrations of compounds (500nM, 1 µM, 10 µM, and 20 µM) for 4 h. The compound was aspirated from the wells and washed three times using 1x PBS before subsequent infection with DENV2 at a multiplicity of infection (MOI) of 0.1 for 2h at 37°C, 5% CO_2. The inoculum was then removed, and the cells were washed twice with PBS and incubated in culture medium plus the compound at the respective concentrations for 48 h. Plaque assays and RT-qPCR were performed to determine viral load and viral genetic expression.

Assays were performed in technical duplicates and biological triplicates.

Statistical Analysis:

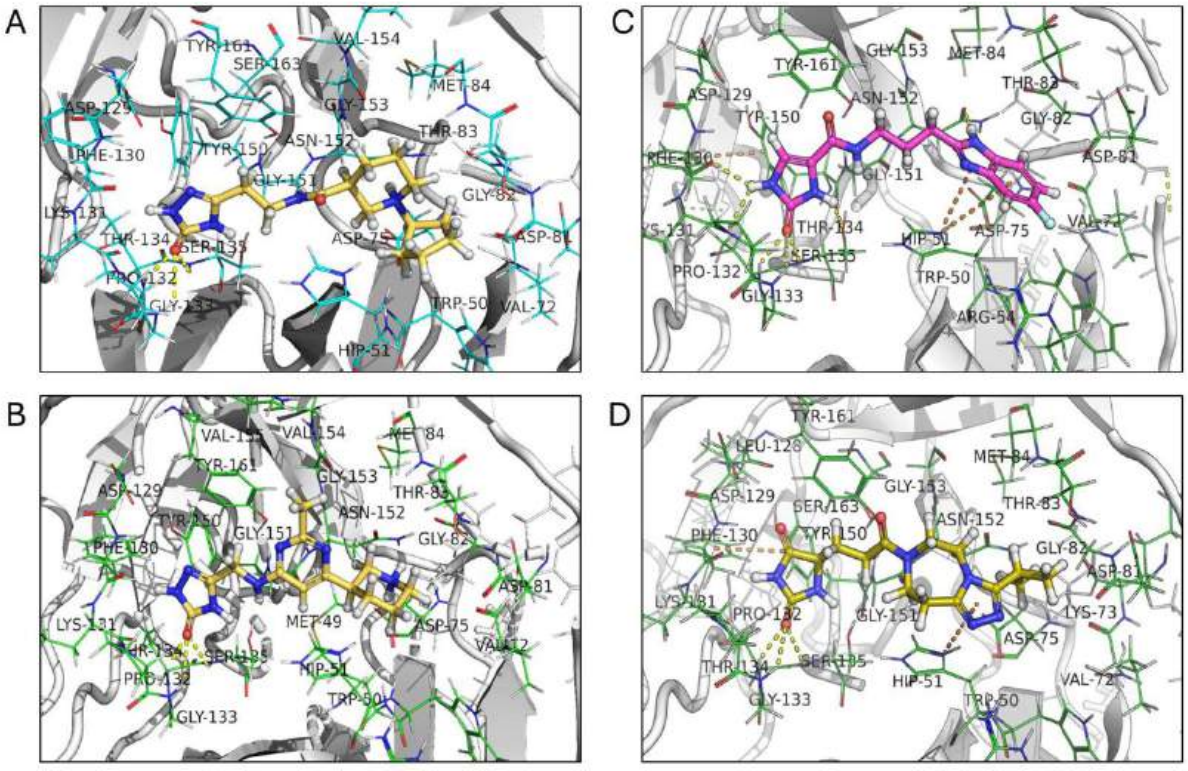
Statistical analysis was performed using GraphPad Prism, unless otherwise noted. For the parametric data, the significance was determined using the Student's *t*-test or one-way ANOVA and $p<0.05$.

Results:

Molecular docking of DENV protease:

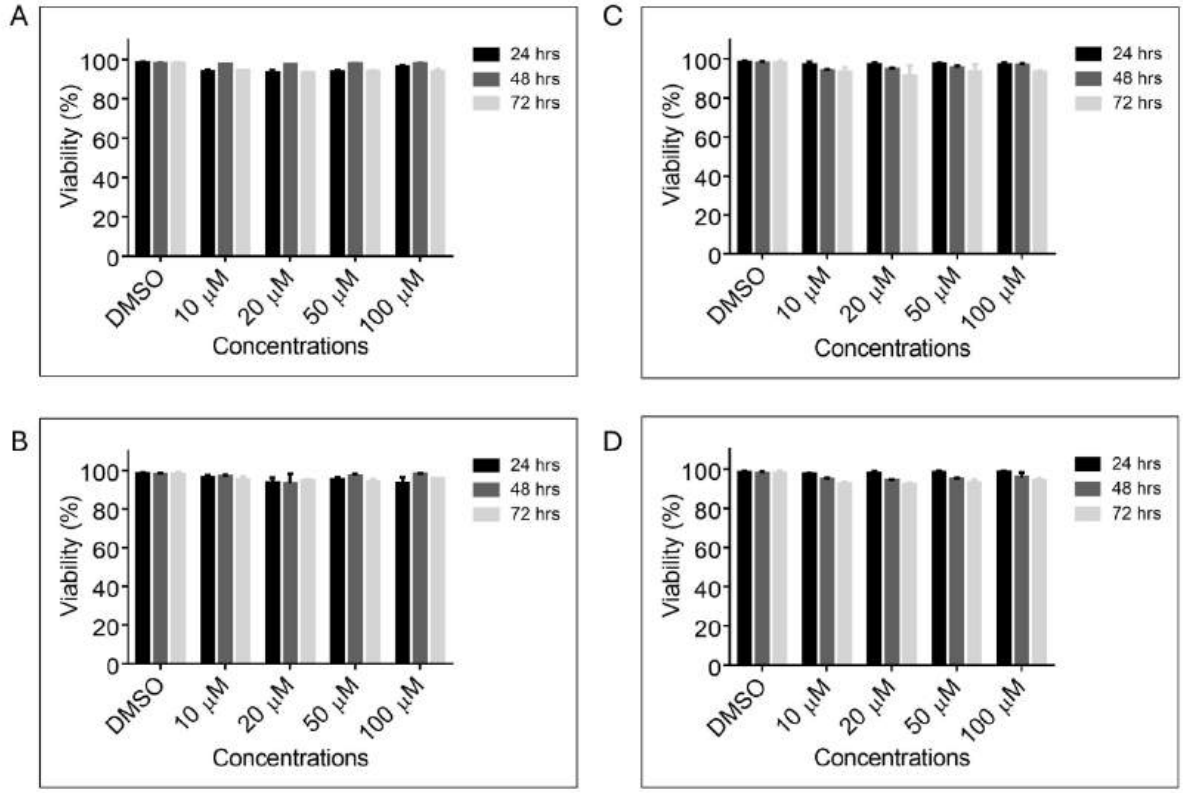
A dataset of over 1 million compounds was sourced from the 3D tranches dataset of the Zinc 21 database for virtual screening of the DENV protein (PDB ID: 2FOM). The compounds were first filtered based on their drug-likeness using Lipinski's Rule of Five to ensure their commercial availability. This process applies strict criteria such as molecular weight (250–500 Da), LogP (-1 to 4), and charge (-2 to +2) to focus on compounds with potential for drug development. Once the initial screening was complete, the selected compounds were downloaded as a PowerShell file, renamed as name_of_file.ps1, and converted into SDF (Structure Data File) format using PowerShell. This conversion enabled the compounds to be uploaded to Maestro, a docking program from Schrödinger LLC.

After importing the compounds into Maestro, further refinement was conducted using QikProp analysis to evaluate their ADME properties. To further prioritize the compounds, a STAR analysis was performed, ranking the compounds on a scale from 0 to 5 stars, with 0 stars as the cutoff for removal. Using these rigorous filtering steps and defined cutoffs for key ADME properties, the initial library of over a million compounds was narrowed down to four candidate compounds with strong drug-like characteristics, ready for further evaluation and testing in subsequent stages of the drug discovery process (Fig 1).



(A) ZINC95356735 (C, yellow; H, white; N, blue; O, red), docked into DENV-2 (PDB: 2FOM), using the induced fit docking program (Schrödinger, LLC). Broken lines show interaction type; yellow for H-bonding and green for all π interaction**. (B)** ZINC000067908645 (C, yellow; H, white; N, blue; O, red), docked into DENV-2 (PDB: 2FOM), using the induced fit docking program (Schrödinger, LLC). Broken lines show interaction type; yellow for H-bonding and green for brown for all π- interaction. (**C)** ZINC000218827339 (C, pink; H, white; N, blue; O, red; F, Cyan), docked into DENV-2 (PDB: 2FOM), using the induced fit docking program (Schrödinger, LLC). Broken lines show interaction type; yellow for H-bonding and green for brown for all π- interaction. (**D)** ZINC000091593654 (C, gold; H, white; N, blue; O, red), docked into DENV-2 (PDB: 2FOM), using the induced fit docking program (Schrödinger, LLC). Broken lines show interaction type; yellow for H-bonding and brown for all π- interaction. Docking pictures was generated with PyMOL molecular graphics system, version 2.0 Schrödinger, LLC.

Fig 1: The predicted binding interaction pose of zinc compounds

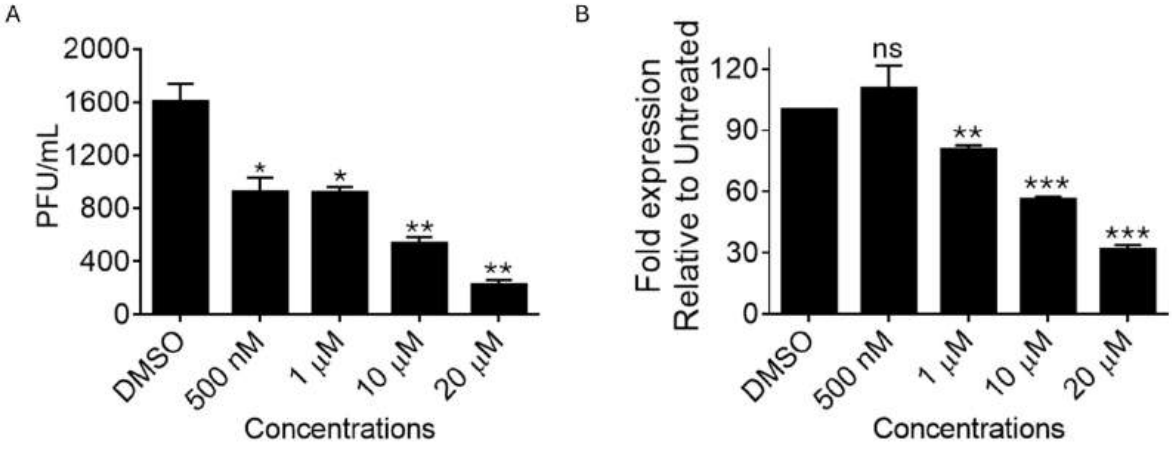


Naïve Vero cells were treated with increasing concentrations (10, 20, 50, and 100 μM) of compounds 1–4 (in Fig 1A - Fig 1D respectively), and cell viability was measured using acridine orange/propidium iodide (AO/PI) staining for up to 72 h post-treatment. DMSO was used as a control. The mean values ± SEM from three biological replicates are shown.

Fig 2: Cytotoxic effect of zinc compounds against Vero cells.

Compound show no cytotoxicity on Vero cell in the cell viability assay:

First, we assessed the cytotoxicity of the top four compounds against Vero cells. Cells were either treated with compounds or DMSO control for up to 72 h, after which viability was examined using acridine orange/ propidium iodide (AO/PI). Compound concentrations of up to 100 μM showed no significant cytotoxic effects in Vero cells after 72 h of incubation (Fig 2A–2D).



(A) Naïve Vero cells were treated with either compound 1 (500 nM, 1 μM, 10 μM and 20 μM) or DMSO control. After four hours, the cells were infected with DENV2 for 48 hours. Subsequently, supernatant samples were obtained and analyzed using plaque assay for viral load determination. (**B)** Naïve Vero cells were treated with either compound 1 (500 nM, 1 μM, 10 μM and 20 μM) or DMSO control. After four hours, the cells were infected with DENV2 for 48 hours. Subsequently, cell samples were obtained and analyzed using RT-qPCR to determine DENV2 genetic expression relative to DMSO control. For each treatment condition and time point assayed, mean values ± SEM from three biological replicates is shown. ns (no significance), *P ≤ 0.03; **P ≤ 0.01; ***P ≤ 0.005.

Fig 3: Pre-treatment of Naïve Vero Cells with Compound 1 Significantly Reduces Viral Replication in the Cells.

Pre-treatment of Vero cells with compound 1 reduces viral replication and viral release post-infection:

Compound 1 was analyzed for its potential role in reducing viral replication or release using Monkey Vero cells, which is an established cell infection model for DENV 2. We first treated the cells with different concentrations of the compounds (500 nM, 1 µM, 10 µM, and 20 µM) for 4 h, followed by infection with DENV 2. At 72 h post-infection, the supernatant samples were collected to determine viral release. The results shown in Fig 3A demonstrate that pretreatment with compounds at different concentrations resulted in a significant decrease in DENV release. An approximately two-fold decrease was observed at 500 nM, while an up to eight-fold decrease in viral release was observed at 20 µM (Fig 3A).

We also treated the cells with TRIzol to determine the intracellular viral load using RT-qPCR. Our results showed that while the 500 nM concentration did not show a statistically significant difference compared to the control, other higher concentrations showed a significant reduction in the intracellular viral load compared to the DMSO control (Fig 3B). These results collectively show that Compound 1 has antiviral activity against DENV 2.

Discussion:

Repurposing drugs is currently a major practice used to identify novel drug candidates (18,19,20,21). This strategy is based solely on knowledge of pharmacology and the clinical effects of the compound based on its original use (1,4,22). For this study, a dataset of over 1 million compounds was sourced from the 3D tranches dataset of the Zinc 21 database for virtual screening of the DENV protein (PDB ID: 2FOM). The compounds were first filtered based on their drug-likeness using Lipinski's Rule of Five to ensure their commercial availability. This process applies strict criteria such as molecular weight (250–500 Da), LogP (-1 to 4), and charge (-2 to +2) to focus on compounds with potential for drug development.

The compounds were streamlined down to 100 compounds based on criteria such as docking score. The top four compounds were selected based on their availability and docking scores for testing against DENV2. Our experiments using the Vero cell line as a model to test different compounds against DENV2 demonstrated that compound 1(2-(tert-butyl amino)-4-[[(1R,3S)-3-hydroxycyclohexyl]amino]pyrimidine-5-carboxamide) robustly decreased the viral load along the concentration gradient (Fig 3), suggesting that the compound interferes with the viral replication process. We do not know yet if the interference is due to modulation of host response to Dengue infection or the direct inhibition of dengue protease activity. Understanding this will explain the antiviral activity observed.

Compound 1 is a c-Jun N-Terminal Kinase 1 (JNK1) inhibitor. The compound has been utilized to demonstrate efficacy against pulmonary fibrosis in animal models via oral administration (23,24). Therefore, it is currently being evaluated in phase II clinical trials for fibrosis (25). Although the mode of action of 2-(tert-butylamino)-4-[[(1R,3S)-3-hydroxycyclohexyl]amino]pyrimidine-5-carboxamide is yet to be determined, as this is the first report of this compound to be used as a potential antiviral candidate against DENV2, we believe that the compound does not exert its major function to prevent viral entry. To this effect, further studies need to be carried out to determine the mechanistic framework of 2-(tert-butylamino)-4-[[(1R,3S)-3-hydroxycyclohexyl]amino]pyrimidine-5-carboxamide. In addition to this, the remaining compounds should be tested for their antiviral properties against dengue virus infection.

The development of genetic alterations to control mosquito populations is an ongoing endeavor to control dengue infection. While this has very high potential, environmental concerns are a major factor affecting this approach (21,26). This with other concerns made anti-dengue drug development a top priority for the control of dengue infection. The challenges that occur during therapeutic drug development for the dengue virus include the existence of four DENV (1-4) serotypes, a lack of appropriate animal models, and the need for large trials because of different populations and clinical outcomes (13). Therefore, it is important to identify potential candidates to mitigate these challenges.

Conclusion:

In this pilot study, we tested the antiviral activity of the top compound (2-(tert-butylamino)-4-[[(1R,3S)-3-hydroxycyclohexyl]amino]pyrimidine-5-carboxamide) from an in-silico study against type 2 dengue virus NS2B/NS3 protease (PDB ID: 2FOM) and observed significant antiviral activity against DENV2. This shows a significant step towards the discovery of an antiviral drug against the dengue virus. It is important to note that this is a preliminary pilot study, and more work needs to be done to know the mechanism of action of this compound. Also, dengue virus serotypes share conserved regions, and future work should check if this compound is able to produce antiviral activity against other serotypes.

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Contributions of authors:

All authors contributed significantly to the manuscript. AAO and TEE designed the experiment. AAO, SJR, NU, KG, PEG and OQ were involved in the analysis. AAO and TEE drafted and reviewed the write-up. All authors critically reviewed the final manuscript.

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Conflict of interest:

Authors declare no conflict of interest.

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Original Article **Open Access**

Exploring lytic *Salmonella* phages as potential alternatives to antibiotics: Isolation, characterization, and stability assessment of *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe

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Abstract:

Background: Typhoid fever, caused by antibiotic-resistant *Salmonella enterica* serovar Typhi (*Salmonella* Typhi), is a significant public health issue, especially in Nigeria, necessitating exploration of alternative treatment strategies such as the use of bacteriophages. Bacteriophages are viruses that specifically infect and replicate within bacteria and play important roles in medicine. The objective of this study is to isolate and characterize two lytic phages against *S*. Typhi, assess their stability under various environmental conditions and determine their potentials as biocontrol agents and alternatives to antibiotics for treatment of *S.* Typhi infections.
Methodology: *Salmonella* Typhi strains previously isolated from stool samples of patients in Jos, Nigeria, were used. Phages were isolated from hospital and poultry wastewater samples using the double-layer agar method. Phage purification and stock preparation were performed using the double-layer agar overlay technique. Stability was assessed by incubating phages at different temperatures, pH levels, and NaCl concentrations. The DNA of the phages were extracted using the DNeasy Blood and Tissue Kit (Qiagen) and the whole genome was then sequenced using the Illumina NextSeq 500 platform. Bioinformatic analysis was conducted using various computational tools.
Results: Two phages, *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe, were isolated and characterized. The phages were stable at 4°C-37°C which serves as control and maintained a reasonable titre at 45 and 55° C. Phages were also stable at 0.5% NaCl and pH 8 for at least one hour. *Jerseyvirus* Ijeoma, with a genome size of 41,747 bp and 56 coding sequences (CDS), showed 81% similarity to *Jerseyvirus* SS3e. *Apdecimavirus* Ayanbimpe, with a genome size of 39,601 bp and 48 CDS, exhibited 83% similarity to *Apdecimavirus* AP10.
Conclusion: Both phages demonstrated good stability under various abiotic factors, maintaining reasonable titres in alkaline and high-salt conditions and at elevated temperatures. *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe show potential as biocontrol agents and alternatives to antibiotics for treating *S*. Typhi infections. Their stability and tolerance to diverse conditions make them suitable candidates for phage therapy.

Keywords: Lytic Phages, Phage Stability, Typhoid fever, Antibiotic resistance, Phage Genome, *Salmonella* Typhi

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Exploration des phages lytiques de *Salmonella* comme alternatives potentielles aux antibiotiques: Isolement, caractérisation et évaluation de la stabilité de *Jerseyvirus* Ijeoma et d'*Apdecimavirus* Ayanbimpe

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Résumé:

Contexte: La fièvre typhoïde, causée par *Salmonella enterica* sérovar Typhi (*Salmonella* Typhi), une bactérie résistante aux antibiotiques, représente un problème de santé publique majeur, notamment au Nigéria, ce qui rend nécessaire l'exploration de stratégies thérapeutiques alternatives telles que l'utilisation de bactériophages. Les bactériophages sont des virus qui infectent et se répliquent spécifiquement au sein des bactéries et jouent un rôle important en médecine. L'objectif de cette étude est d'isoler et de caractériser deux phages lytiques contre *S.* Typhi, d'évaluer leur stabilité dans différentes conditions environnementales et de déterminer leur potentiel en tant qu'agents de biocontrôle et alternatives aux antibiotiques pour le traitement des infections à *S.* Typhi.
Méthodologie: Des souches de *Salmonella* Typhi précédemment isolées d'échantillons de selles de patients de Jos, au Nigéria, ont été utilisées. Les phages ont été isolés à partir d'échantillons d'eaux usées hospitalières et d'eaux usées d'élevages avicoles par la méthode de la double couche d'agar. La purification des phages et la préparation des stocks ont été réalisées par la technique de la double couche d'agar. La stabilité a été évaluée en incubant les phages à différentes températures, différents pH et différentes concentrations de NaCl. L'ADN des phages a été extrait à l'aide du kit DNeasy Blood and Tissue (Qiagen), puis le génome entier a été séquencé sur la plateforme Illumina NextSeq 500. Une analyse bioinformatique a été réalisée à l'aide de divers outils de calcul.
Résultats: Deux phages, *Jerseyvirus* Ijeoma et *Apdecimavirus* Ayanbimpe, ont été isolés et caractérisés. Les phages étaient stables entre 4°C et 37°C (température servant de contrôle) et ont conservé un titre satisfaisant à 45°C et 55°C. Ils étaient également stables à 0,5% de NaCl et à pH 8 pendant au moins une heure. *Jerseyvirus* Ijeoma, dont le génome mesure 41747 pb et comporte 56 séquences codantes (CDS), présente une similarité de 81% avec *Jerseyvirus* SS3e. L'*Apdecimavirus* Ayanbimpe, dont le génome mesure 39601 pb et comporte 48 CDS, présente une similarité de 83% avec l'*Apdecimavirus* AP10.
Conclusion: Les deux phages ont démontré une bonne stabilité face à divers facteurs abiotiques, conservant des titres satisfaisants en milieu alcalin, en forte salinité et à températures élevées. Le *Jerseyvirus* Ijeoma et l'*Apdecimavirus* Ayanbimpe présentent un potentiel en tant qu'agents de biocontrôle et alternatives aux antibiotiques pour le traitement des infections à *S.* Typhi. Leur stabilité et leur tolérance à diverses conditions en font des candidats prometteurs pour la phagothérapie.

Mots-clés: Phages lytiques, Stabilité des phages, Fièvre typhoïde, Résistance aux antibiotiques, Génome phagique, *Salmonella* Typhi

Introduction:

Typhoid fever is an infectious disease caused by the Gram-negative bacterium, *Salmonella enterica* subspecies *enterica* serovar Typhi (*S*. Typhi). Typically, this infection is contracted through the consumption of contaminated food or water sources (1). Typhoid fever remains a major concern in Nigeria due to urbanization, insufficient water, regional migration, poor waste management, strained health care, and antibiotic overuse, fostering antibiotic-resistant *S.* Typhi (2). Annually, there are approximately 11 million reported cases of typhoid fever globally, leading to over 100,000 fatalities (3). The greatest burden of this disease is observed in low- and middle-income countries (LMICs), where access to clean water and proper sanitation facilities is often limited (4). The effectiveness of treating typhoid fever with antibiotics is progressively diminishing due to the emergence of drug-resistant strains (5). There is, therefore, need to explore alternatives to antibiotics in the control and management of typhoid fevers.

With rise in multi-drug-resistant *S*. Typhi, bacteriophages (phages), with their potent bactericidal activity, host specificity, self-limiting nature, and genetic manipulability, are emerging as promising treatments of bacterial infections in humans and poultry, offering potential alternatives to antibiotics (6). Phages have been used to eliminate *Salmonella* in *Galleria* model (7). A study has shown that *Salmonella* phage reduced the burden of salmonellosis in a mammalian host by inhibiting *S.* Typhi invasion into the liver and spleen tissue (8). In this study, two lytic phages against *S*. Typhi were isolated from hospital and wastewater, their stability in temperature, pH, NaCl and genomic characteristics were reported.

Materials and method:

Bacterial strain isolation and characterization:

In this study, *Salmonella* Typhi strains SO1 previously isolated from Jos by Olorundare et al., (9), and which genome sequence was determined and deposited into the GenBank (Bio Sample/Accession number SAMN168652 06) were used. Approval for this previous study was obtained from the State Emergency Management Agency (SEMA) Plateau State, Nigeria. Informed consent forms and questionnaire were administered to the individuals recruited from the study population (internally displaced camp). All methods were carried out in compliance with the applicable guidelines and regulations.

Bacteriophage isolation protocol:

Sewage samples are always ideal for isolation of phages for enteric bacteria since they contain diverse enteric bacterial hosts

(10). In this study environmental samples were used as source of isolation of phages in the hospital and poultry since isolates are commonly found in this environment and phages are specific and are found where the organisms are present.

The isolation of *Salmonella* phages from hospital and poultry samples was carried out using the standard spot and double-layer agar method (11) with slight modifications. Initially, 50 ml each of hospital and poultry sample were collected and centrifuged at 10,000×g for 10 minutes to eliminate debris. For phage isolation, the supernatant was filtered using a 0.45µm syringe filter. 10ml of filtrate was then mixed with 10ml of double-strength brain heart infusion (BHI) broth supplemented with $CaCl_2$ and an overnight 100 µl of logarithmic growth phase of *S.* Typhi culture was added. This mixture was incubated at 37°C for 48 hours with gentle shaking at 50 rpm. After of incubation, the sample was centrifuged, and supernatant was collected and filtered using a 0.45µm syringe to obtain the phage lysate.

To detect the phages, the double-agar method was employed. Specifically, 100µl of logarithmic growth phase *S.* Typhi grown overnight was added to 5 ml of soft-top agar [Brain Heart Infusion Agar (BHIA) Lifesave Biotech /USA, containing 0.4% (w/v) agar] and then poured onto solid bottom agar [BHIA containing 1.5% (w/v) agar]. The 10 µl phage lysate was spotted on the *S.* Typhi double agar plate and incubated at 37°C for 24 hours. Zones of lysis/clearance on bacterial lawns were observed the next day. Finally, lysed zones were scraped out, transferred to 1 ml sodium magnesium (SM) buffer, and incubated at 4°C for 30 minutes to allow phage particle to diffuse and then filtered through 0.45µm filters.

Plaque purification and phage stock preparation:

The double layer agar overlay technique was employed for the subsequent purification of plaques from two *S.* Typhi phages, namely *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe. A single plaque from the phage-agar overlay plate was carefully picked using a standard pipette tip, and the resulting agar plug was expelled into 300 µl of SM buffer (pH 7.0). Afterward, the tubes were incubated at 4°C overnight to allow phage to diffuse. This was followed by centrifugation at 10,000 ×g at 4°C for 10 minutes. The supernatant was then filtered through 0.45µm syringe filters and stored at 4°C until further utilization. The phages were purified three times to obtain single plaques (12).

Phage stability assay:

The stability of *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe was evaluated through incubation at different temperatures, pH values, and NaCl concentrations, following a previously established protocol (13) For the assessment of thermal stability, 10^9 pfu/ml of each phage was subjected to incubation at 4°C, 37°C, 45°C, 50°C, and 55°C for 1 hour. Phages at 4°C and 37°C were considered as control since it's the temperature used in the storage of bacteria stock and incubation respectively.

To evaluate their ability to withstand various pH values, SM Buffer solutions with pH levels of 2, 4, 6, 8, 10 and 12 were prepared, adjusting the pH with either 6 N HCl or 6 M NaOH. The phages were suspended in these buffers to a final concentration of 10^9 PFU. The pH solutions were then incubated at 37°C for 1 hour, and the surviving phages were immediately quantified using the spot assay technique and double layer agar overlay plating method, as described previously. Phage suspended in SM buffer at pH 7 was used as control.

To assess phage stability in salt, phage at 10^9 pfu were suspended at different concentrations of NaCl (0.5% 5%, 10%, and 15%). The solutions were incubated at 37°C for 1 hour, and the titre of the phages were determined by spot assay. Phage in SM buffer was used as control. The results are presented as the average of triplicates.

Sequence and bioinformatic analysis:

DNA extraction was performed during the sequencing process using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Following that, a sequencing library was painstakingly created using the Nextera XT DNA Sample Preparation Kit (Illumina) in accordance with the manufacturer's methodology. The whole genome was then sequenced using the Illumina NextSeq 500 platform at the Antimicrobial Resistance Laboratory within the Microbial Genomics Reference Laboratory, CIDMLS, ICPMR, Westmead Hospital (Australia). The raw data underwent trimming through Fastp (14) and assembly using SPAdes v3.13.0 (15) Evaluation of genome quality was conducted using FastQC (v0.11.7) Assembly statistics were generated through BBMap (v38.88) (16), SAM tools (v0.1.8) (17), and QUAST (v5.2.0) (18).

Subsequently, BACterioPHage Lifestyle Predictor (BACPHLIP v0.9.6) (19) was employed to predict the lifestyle (temperate or virulent) of each phage, leveraging a local dataset comprising 634 phage genomes (20). BACPHLIP identifies conserved domains and utilizes them in predicting lifestyle through a random forest classifier. Genome annotation was carried out utilizing Prokka v1.14.6 (21) with PHROGS (Prokaryotic Virus Remote Homologous Groups database) (22) using a previously established method (23), and the results were

visualized with Proksee Genome relatedness was determined through BLAST, and the genome sequence with the highest similarity percentage was selected for downstream analyses to ascertain if the phages were unique. Phage genomes displaying varying degrees of similarity were analyzed using the VIRIDIC (24) web-based version with default settings. Genome visualization was performed using a linear genome plot (20) accessed via https://cpt.tamu.edu/galaxy page

Results:

Isolation of phages:

Two phages were isolated from hospital and poultry sewage specific to *S.* Typhi isolate in Jos, Plateau State, Nigeria. The plaques morphology appears to be large, clear and round on the lawn of *S.* Typhi (Fig 1B), distinct plaques were selected, purified and stored for use.

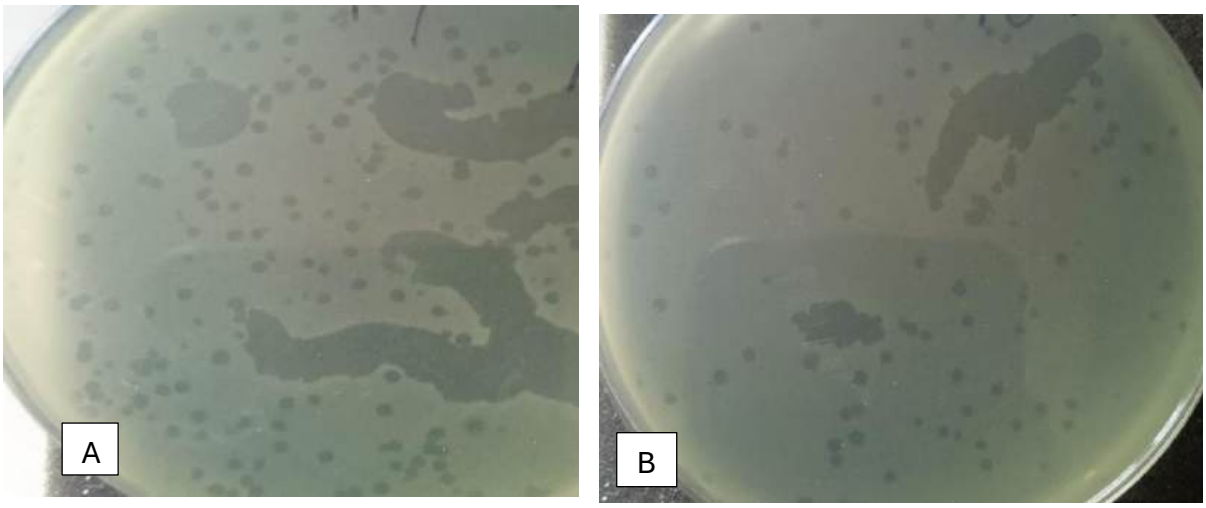

Fig 1: Plaque morphology of *Apdecimavirus* Ayanbimpe on lawn of *Salmonella* Typhi (A); Plaque morphology of *Jerseyvirus* Ijeoma on lawn of *Salmonella* Typhi (B)

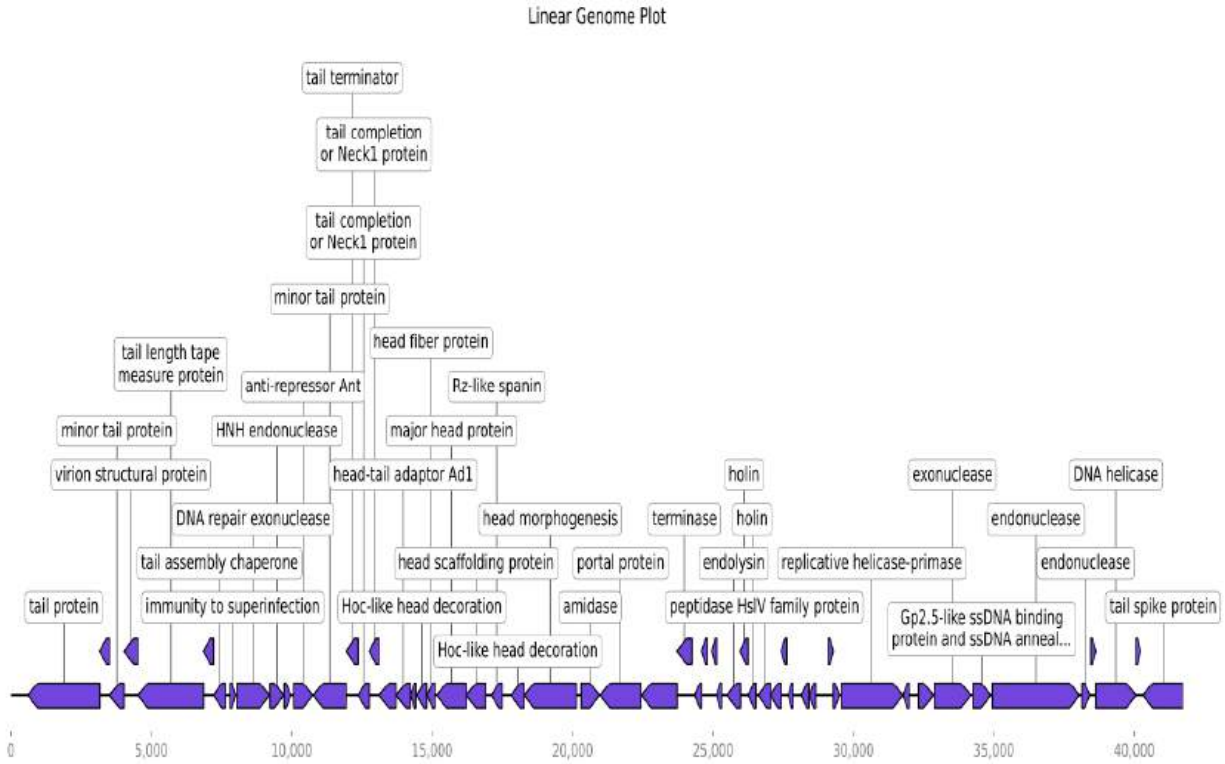

Fig 2: Genome map for *Jerseyvirus* Ijeoma generated using linear genome plot tool

Genomic characterization of phages:

The genome sequence for phage *Jerseyvirus* Ijeoma (Fig 2) isolated from poultry sewage is a double stranded DNA (dsDNA), with a genome size of 41,747 bp, 56 CDS and G+C content 49.99%. The genomic sequence for phage *Apdecimavirus* Ayanbimpe (Fig 3) isolated from hospital sewage is a double stranded DNA (dsDNA) with genomic size of 39,601 bp, 48 CDS and G+C content of 50.72%.

The phylogenic result of the isolated *Salmonella* phages using VIRIDIC showed *Apdecimavirus* Ayanbimpe is 83% similar to *Apdecimavirus* AP10 (KT852574.1) isolated from pig manure in Canada (Fig 4A). *Jerseyvirus* Ijeoma shares 81% similarity to *Jerseyvirus* SS3e isolated from fecal residue in Chile with accession number KR270151 (Fig 4B).

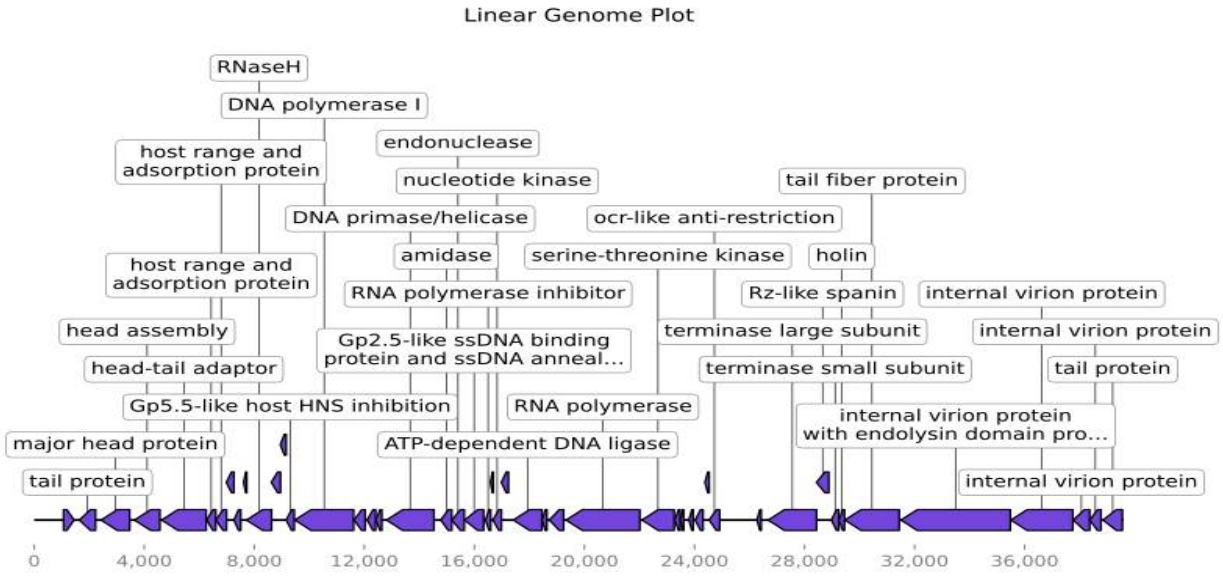

Fig 3: Genome map for *Apdecimavirus* Ayanbimpe generated using linear genome plot tool

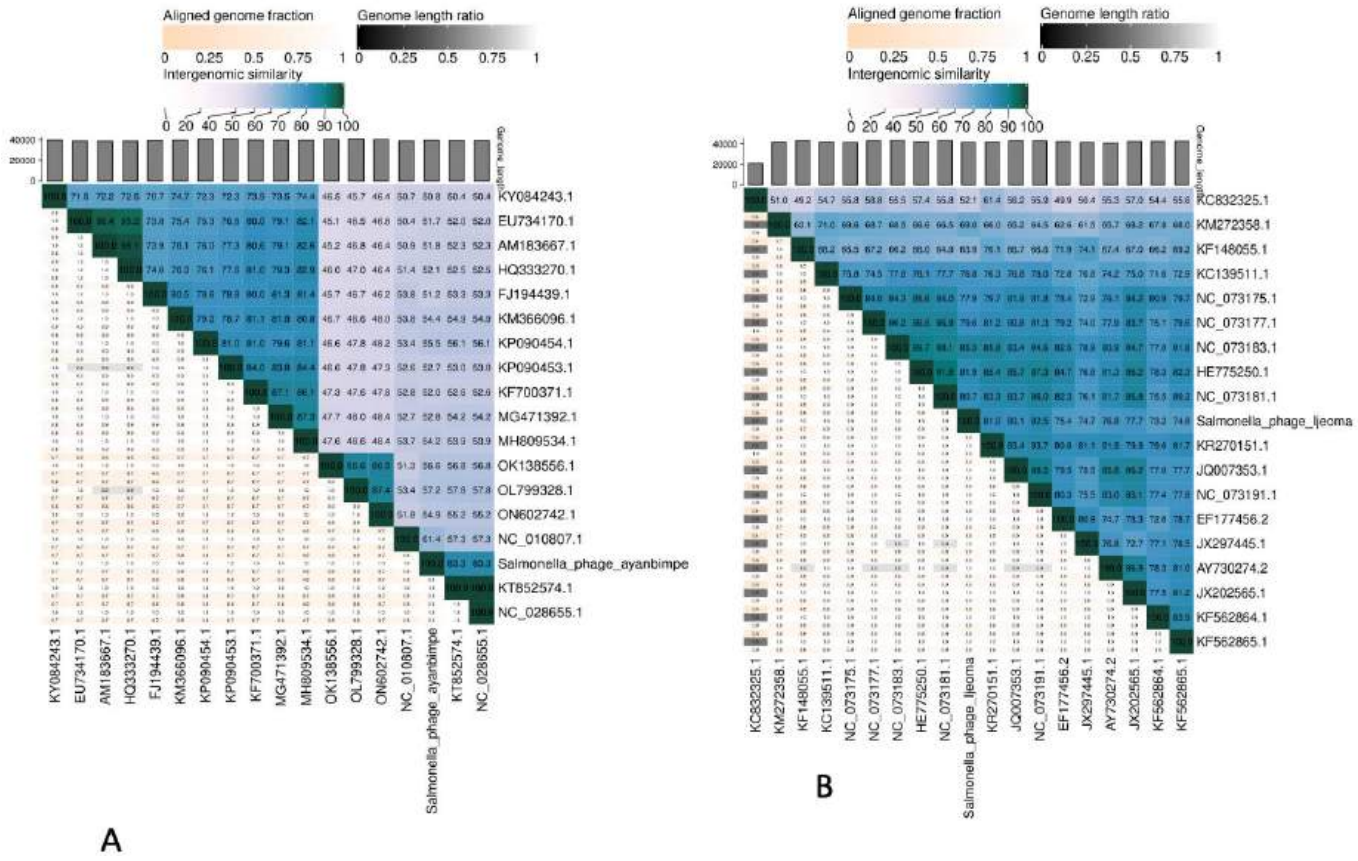

Fig 4 Taxonomic classification of *Apdecimavirus* Ayanbimpe (A) and *Jerseyvirus* Ijeoma (B) using VIRIDIC

Phage stability assay:

The stability of the phages at various abiotic factors (Figs 5,6,7) shows that *Jerseyvirus* Ijeoma exhibited consistent growth with an increase in pH, reaching an optimal growth of 7 log10 PFU/ml. The minimal growth of 2 log10 PFU/ml was observed at pH 2. Conversely, *Apdecimavirus* Ayanbimpe displayed its highest growth at pH 8, reaching about 9 log 10 PFU/ml, and the lowest growth at pH 2, with 5 log10 PFU/ml (Fig 5).

When subjected to varying salt concentrations (Fig 6), *Jerseyvirus* Ijeoma demonstrated its highest growth rate of 9 log 10 PFU/ ml at 0.5% NaCl, 7 log10 PFU/ml at 5% NaCl, and the lowest growth of 3 log10 PFU/ml at 15% NaCl. Similarly, *Apdecimavirus* Ayanbimpe exhibited a parallel pattern, achieving the highest titre of 9 log10 PFU/ml at 0.5% NaCl, 7 log10 PFU/ml at 5% NaCl and the lowest titre of 4 log10 PFU/ml at 15% NaCl. Both phages maintained a high titre at 45°C but experienced a decline at 55°C (Fig 7).

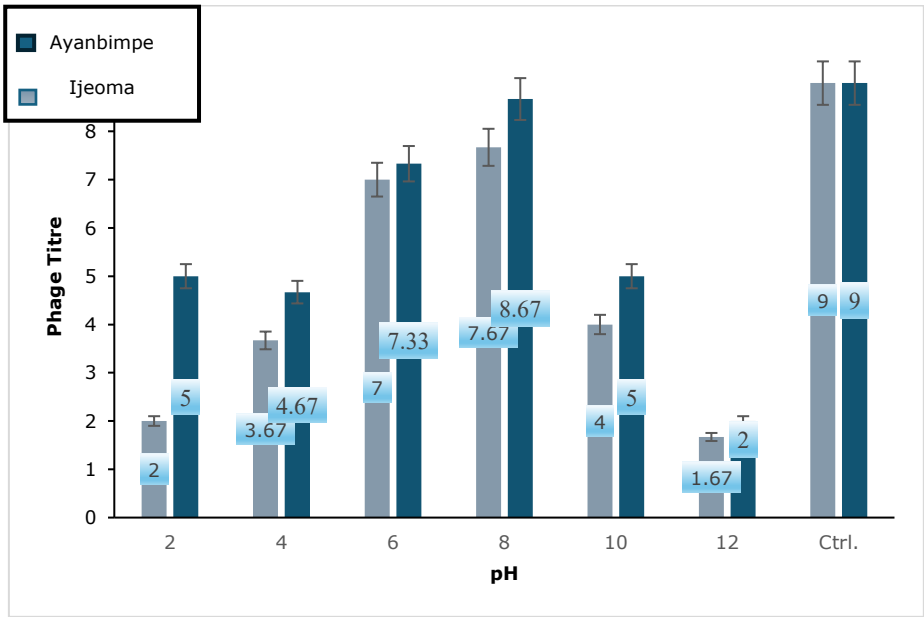

Fig 5: Stability of phages at different pH

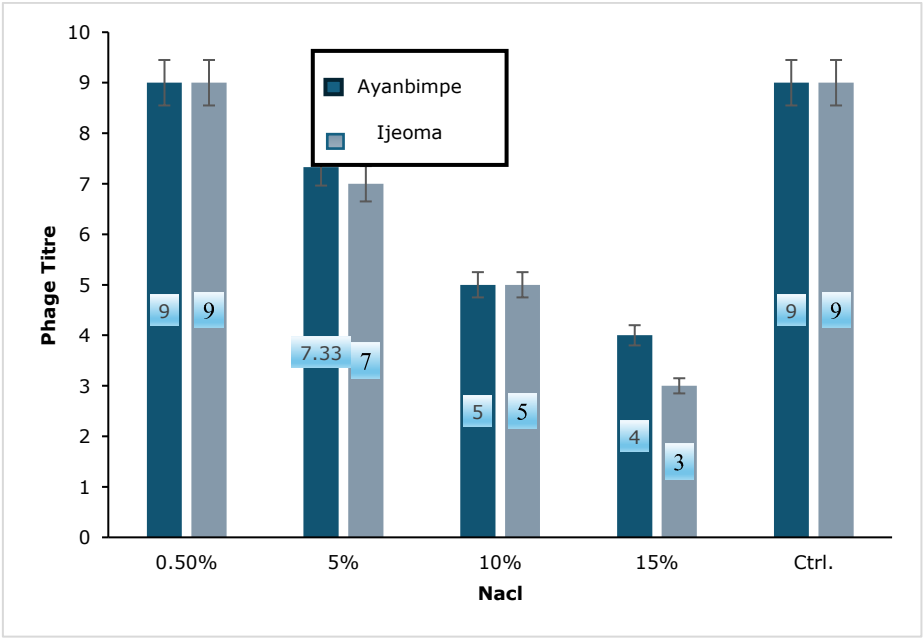

Fig 6: Stability of phages at different NaCl concentrations

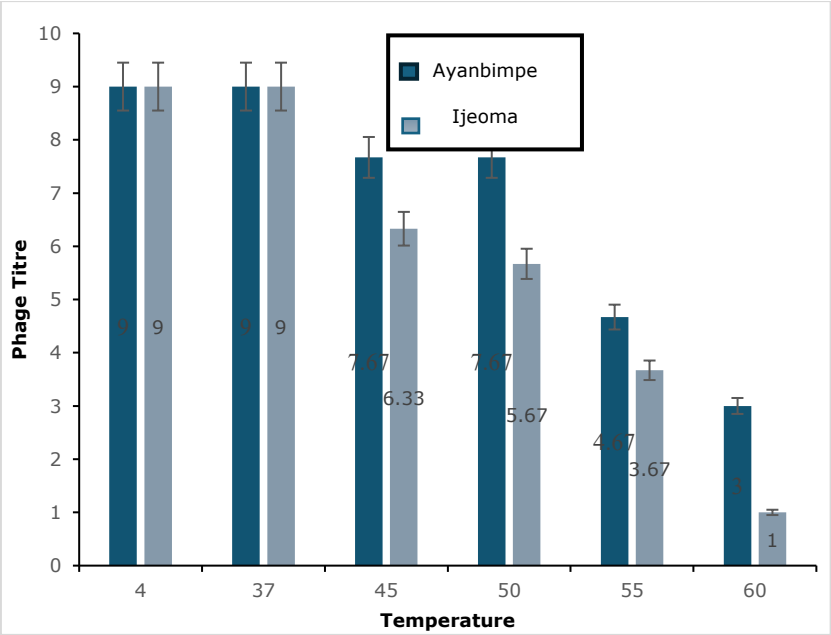

Fig 7: Thermal stability of phages

Discussion:

The two phages were isolated from sewage samples in Jos, Nigeria and were characterized and named as *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe according to the binomial nomenclature for phages (25). These phages can be used as a surveillance tool (26). Although the phages are not novel, the predicted life cycle of the phages showed that they are lytic phages which makes them a good potential antibacterial agent and suitable for phage therapy use. In Africa and Asia, the effects of invasive non-typhoidal and typhoidal *Salmonella* infections on public health are especially notable, as they have a major impact on morbidity and death (27).

The burden of typhoid fever is high in low- and middle-income countries (LMICs), including those in these regions, where an estimated 17.8 million cases are recorded annually (28). Particularly in Sub-Saharan Africa, typhoid fever poses a serious threat, with an estimated yearly incidence of more than 100 cases per 100,000 people and a 1% fatality rate (29). The presence of drug resistance *S.* Typhi in Africa makes the need for increased research in phage therapy. Mondal, et al., (8) in their study showed that *S.* Typhi phages increased the survivability of infected mice and effectively reduced bacterial cell numbers, cell culture density, and biofilm formation *in vitro* (30). The stability of phages is significant because it influences the killing activity, and the suitable application can be determined based on their stability (31).

The two phages in our study exhibited good tolerance to alkaline and high-salt conditions, and high stability in low salt concentration (0.5–5%) but increasing the concentration (10-15%) led to gradual reduction in phage activity. Their resilience in alkaline environments enhances their potential as effective biocontrol agents in food and processing settings. In the food industry, phages serve significant roles not only in "starter culture mixes" but also as "in-process agents of concern," particularly in fermentation products (32). Saline stability was observed in studies conducted by Lu and Breidt (33) where high levels of salt, in some cases did not affect the phage titre. Additionally, the phages maintained reasonable titres even at elevated temperatures, their thermal stability qualifies them for growth in elevated temperatures, further expanding their applicability in conditions associated with feverish environments.

Genome analysis helps to determine phage suitability and safety for phage therapy (34) or if it harbors any genes that encode for antimicrobial resistance and virulence genes. Some concerns regarding using phage with harmful genes can be solved by complete genomic characterization of the phage with genome sequencing, annotation of the genome and determining the functions of genes. The knowledge of the functions of the genes will determine the suitability of phage for therapy. In this study, the two phages isolated showed no lysogenic genes or genes that encode virulence factors or antibiotic resistance. This indicates that the isolated phages could be considered genomically safe for further investigations in the control of *S.* Typhi.

Conclusion:

Two lytic *Salmonella* phages from the

Apdecimavirus and *Jerseyvirus* families, respectively, were isolated and examined in this study, and were named *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe. These phages showed remarkable stability and tolerance over a broad range of pH and temperature settings, but they lacked virulence, solubility, and antibiotic resistance genes. These results suggest that both phages have potential applications in phage treatment and as tools for the biocontrol of *Salmonella* proliferation. Further studies are recommended to validate the phage efficacy in biological systems *in vitro* and *in vivo* using mice model.

Availability of nucleotides sequences of the phages:

The nucleotide sequences of the genomes of the two phages have been deposited at NCBI GenBank with accession numbers *Apdecimavirus* Ayanbimpe PQ139365 and *Jerseyvirus* Ijeoma PQ139366

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Contributions of authors:

OOO, NEN, LN, and GMA were involved in the study conceptualization; OOO and NEN were involved in the study methodology, and writing/original draft preparation; GMA and LN were involved in the study supervision. All authors read and approved the submitted version of the manuscript.

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Conflicts of interest:

Authors declare no conflict of interest.

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Original Article **Open Access**

Rotavirus serotypes/genotypes distribution and their implications in children with diarrhoea admitted to the Charles de Gaulle University Paediatric Hospital, Ouagadougou, Burkina Faso

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Abstract:

Background: Diarrhoeal diseases are a source of public health problems in low-income countries such as Burkina Faso, where the aetiology of rotavirus plays an important role. This led to the introduction of surveillance since the introduction of the rotavirus vaccine. The aim of this study is to take stock of rotavirus diarrhoea since the introduction of the vaccine.
Methodology: This was a retrospective cross-sectional study of children aged 0-56 months with diarrhoea admitted to the Charles de Gaulle Paediatric University Teaching Hospital, Ouagadougou between December 2013 and January 2021. Stool samples were collected for rapid diagnostic test for rotavirus, which was confirmed by ELISA for rotavirus group A VP6 antigen. ELISA positive samples were genotyped (for genotype G and P) using multiplex real-time RT-PCR.
Results: Of all the stool samples from 1223 children with suspected rotavirus diarrhoea, 401 were confirmed by ELISA, representing an average prevalence of 32.8%. The distribution of confirmed cases by year showed a considerable drop in positive cases between 2014 and 2015, with 61.7% and 17.6% prevalence rate respectively. This prevalence remained low in 2017 and increased in the other years without exceeding the 50.0% observed before the introduction of the vaccine. Genotyping identified P6(59.6%) and P8(40.4%) types for genotype P, and G12 (66.0%) for genotype G.
Conclusion: This study shows a considerable decrease in the prevalence of rotavirus diarrhoea which could be linked to the introduction of rotavirus vaccine in the Extended Vaccination Program (EVP) in Burkina Faso in 2013, although rotavirus diarrhoea cases are still considerably occurring.

Key words: Rotavirus, diarrhoea, vaccine, genotype, paediatrics, Burkina-Faso

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Distribution des sérotypes/génotypes du rotavirus et leurs implications chez les enfants atteints de diarrhée admis à l'hôpital pédiatrique universitaire Charles de Gaulle de Ouagadougou, Burkina Faso

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Résumé:

Contexte: Les maladies diarrhéiques constituent un problème de santé publique majeur dans les pays à faible revenu comme le Burkina Faso, où l'étiologie du rotavirus joue un rôle important. Ceci a conduit à la mise en place d'une surveillance depuis l'introduction du vaccin contre le rotavirus. L'objectif de cette étude est de dresser un bilan des cas de diarrhée à rotavirus depuis l'introduction de ce vaccin.
Méthodologie: Il s'agit d'une étude transversale rétrospective menée auprès d'enfants âgés de 0 à 56 mois, hospitalisés pour diarrhée au CHU Charles de Gaulle de Ouagadougou entre décembre 2013 et janvier 2021. Des échantillons de selles ont été prélevés pour un test de diagnostic rapide du rotavirus, confirmé par un test ELISA de l'antigène VP6 du rotavirus du groupe A. Les échantillons positifs à l'ELISA ont été génotypés (génotypes G et P) par RT-PCR multiplex en temps réel.
Résultats: Sur l'ensemble des échantillons de selles prélevés chez 1223 enfants présentant une suspicion de diarrhée à rotavirus, 401 ont été confirmés par ELISA, soit une prévalence moyenne de 32,8%. La répartition des cas confirmés par année a montré une baisse considérable du nombre de cas positifs entre 2014 et 2015, avec des taux de prévalence respectifs de 61,7% et 17,6%. Cette prévalence est restée faible en 2017 et a augmenté les années suivantes sans toutefois dépasser les 50,0% observés avant l'introduction du vaccin. Le génotypage a identifié les types P6 (59,6%) et P8 (40,4%) pour le génotype P, et G12 (66,0%) pour le génotype G.
Conclusion: Cette étude met en évidence une diminution considérable de la prévalence de la diarrhée à rotavirus, qui pourrait être liée à l'introduction du vaccin contre le rotavirus dans le Programme élargi de vaccination (PEV) au Burkina Faso en 2013, même si des cas de diarrhée à rotavirus persistent.

Mots clés: Rotavirus, diarrhée, vaccin, génotype, pédiatrie, Burkina Faso

Introduction:

Infant mortality has been a public health challenge for many years, and one of the most frequently cited causes is infection. In 2010, of the 6 to 7 million children who died before their fifth birthday, about two-thirds died of infectious causes, most of which are preventable (1). Among infectious diseases, diarrhoeal illnesses, in addition to pneumonia, remain top of the list of causes of death in children under 5 years of age (2,3). In low-income countries, the situation is worse. The sub-Saharan Africa countries such as Burkina Faso, Mali, Niger and Nigeria are among the 10 countries most affected by diarrhoeal diseases worldwide (3).

In Burkina Faso, the number of deaths due to diarrhoeal diseases in children under 5 years of age remains high, with a large number attributable to rotavirus (4,5). Rotavirus diarrhoea causes around 185,390 deaths in children under 5 years of age worldwide, with more than 80% of deaths attributed to sub-Saharan Africa, including Burkina Faso, which is one of the 8 countries most affected (6). Rotavirus diarrhoea has been the subject of epidemiological surveillance in Burkina Faso since 2010 at a single sentinel site, which has been extended to 4 sites comprising 4 health centres since the introduction of the Rotateq® vaccine in 2013, with a laboratory chosen as the national reference for case confirmation (7).

This study aims at reviewing cases of rotavirus diarrhoea at the Charles de Gaulle Paediatric University Teaching Hospital in Ouagadougou since surveillance was set up in 2013, which is one of the sentinel surveillance sites.

Materials and method:

Study setting and design:

This was a retrospective study of children aged 0-56 months between December 2013 and January 2021 admitted for diarrhoea illnesses to the Charles de Gaulle Paediatric University Teaching Hospital in Ouagadougou, Burkina Faso.

Study participants and data collection:

A total of 1223 children were studied over 8 years (December 2014 and January 2021). After a clinical examination and when infectious diarrhoea was suspected in a child, stool sample was taken and accompanied by a notification form. This notification form included socio-demographic data, medical history, vaccination status, and other relevant clinical information. The stool sample was collected from each patient into a sterile jar and transported to the laboratory for analysis.

Laboratory analysis:

***Rotavirus antigen detection test on stool samples*:**

Once in the laboratory, the faeces were first examined by a rotavirus rapid diagnostic test and the results were confirmed by an ELISA test. ELISA testing was performed using the ProSpecT™ Rotavirus Microplate Assay Kit (www.thermofisher.com/order/catalog/product/R240396) which detects group A rotavirus antigen, i. e. VP6 capsular antigen. The positive control was considered valid when the optical density (O.D.) was >0.500, the nega-

tive control when the O.D. was < 0.150. The results were interpreted on the basis of the same O.D. threshold as the positive and negative controls.

Rotavirus genotyping by multiplex real-time PCR assay:

The rotavirus A positive samples were genotyped by. amplifying the VP7 and VP4 protein genes, which determine the G (glycoprotein) and P (protease-sensitive) genotypes respectively, using multiplex real-time RT-PCR. This amplification was carried out after DNA extraction using the Qiagen® kit. A total of 8 primers were used for the G genotypes and 7 for the P genotypes. For genotypes G, the primers were aBT1 (G1), aCT2 (G2), G3 (G3), aDT4 (G4), aAT8 (G8), G9 (G9), G10 (G10), and the VP7 primer, and for P genotypes the primers for the genes were 2T1 (P[4]), 3T1 (P[6]), 1T1D (P[8]), 4T1 (P[9]), 5T1 (P[10]), P[11] (P[11]) and the VP4 gene primers (8–12).

The concentration of primers for genotyping the G and P genotypes was 20µM for each primer with a volume of 5µL of 5X Buffer, 1µL of dNTP mix (10µM), 1µL of enzyme, 5µL of sample and RNAse-free water in sufficient quantity to have 25µL of final volume of reaction mixture. The thermal profile and amplification cycles were as follows: 1 cycle of 50°C for 30 minutes and 95°C for 15 minutes, followed by 35 cycles each consisting of 94°C for 1 minute, 42°C for 1 minute, 72°C for 1 minute and finally 1 cycle of 72°C for 10 minutes.

Statistical analysis:

Data was entered on a computer using Microsoft Excel 2021, analyzed by EPI INFO version 3.5.4 software and the Chi-square test was used to compare qualitative variables.

Results:

Prevalence of rotavirus diarrhoea in the children:

Of the stool samples analysed for 1223 children, 726 were from male patients and 497 from female patients, representing a sex ratio (M/F) of 1.46 (Table 1). There were 401 ELISA positive samples, representing rotavirus positivity rate of 32.8% (Table 1). There were 245 positive samples in male patients (33.7%, n=245/726) and 156 in female patients (31.4%, n=156/497), representing a male/female ratio of 1.57 (OR=1.113, 95% CI=0.8722-1.421, p=0.4232) (Table 1).

The age group analysis shows that neonates (0-1 month of age) had significantly higher prevalence of rotavirus diarrhoea (58.8%, n=47/80) than infants (2-30 months of age) with 31.3% (n=344/1099) and children (31-56 months of age) with 28.6% (n=12/42) (x^2=25.786, p<0.0001). (Table 1).

Trend in rotavirus diarrhoea cases by months during the 8-year study period:

Stool samples were analysed for most patients in 2017 followed by 2021 (Fig 1). However, in terms of the positivity rate, 2014 was the year with the most cases, with positivity rate of 61.7% (n=142/230), 17.6% (n=21/119) in 2015, 27.8% in 2016 (n=32/115), 18.2% in 2017 (n=45/248), 29.3% in 2018 (n=48/164), 35.3% in 2019 (n=12/34), 11.1% in 2020 (n=15/135) and 48.3% in 2021 (n=86/178) (x^2=174.55, p<0.0001).

As regards the distribution by month of the year, the number of cases begins to rise in December, peaks in January, then falls, reaching its lowest level in May (Fig 2).

Table 1: Prevalence of rotavirus diarrhoea in the children over the 8-year period of study with respect to sex and age group

Characteristics	Number of samples	Number of positive samples (%)	Chi square	OR (95% CI)	*p* value
Sex					
Male	726	245 (33.7)		1.113	
Female	497	156 (31.4)	0.6414	(0.8722-1.421)	0.4232
M : F ratio	1.46	1.57			
Age in months					
0-1	80	47 (58.8)			
2-30	1099	344 (31.3)	25.786	NA	<0.0001*
31-56	42	12 (28.6)			
Total	1223	401 (32.8)			

* = statistically significant at p<0.05; NA = Not applicable; OR=Odd ratio; CI=Confidence interval

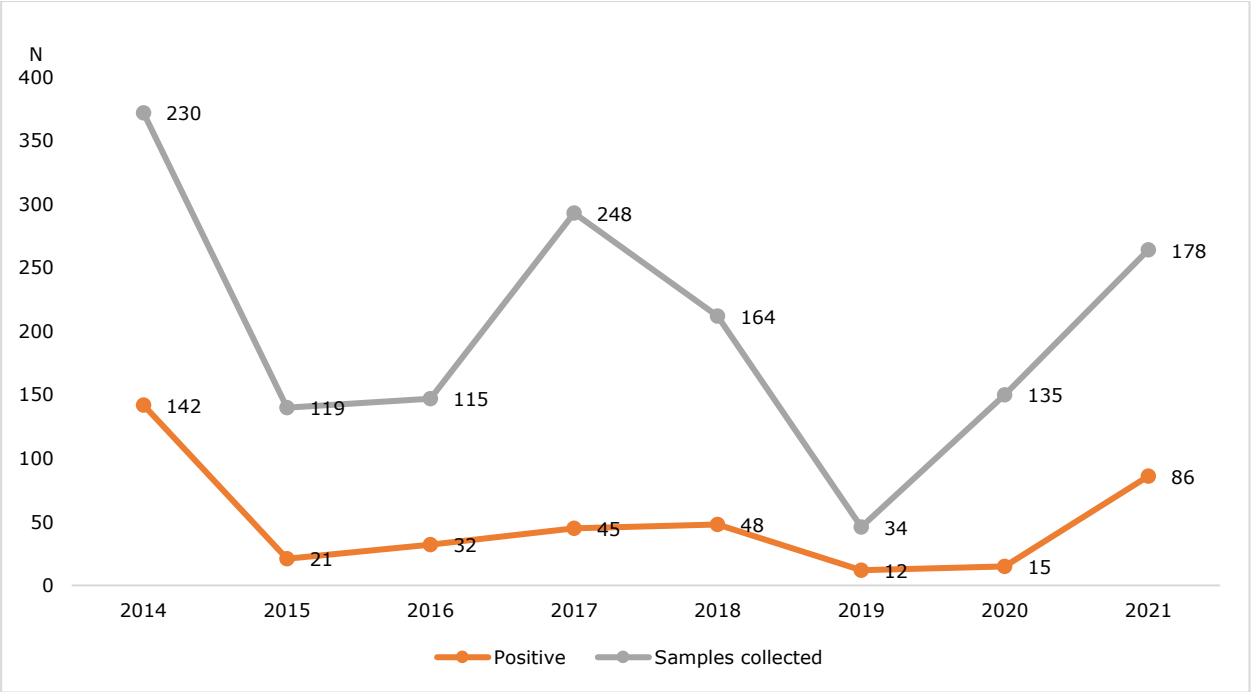

Fig 1: Number of enrolled and positive cases of rotavirus diarrhoea over the 8 years period of study (x^2=174.55, p<0.0001*)

Rotavirus diarrhoea cases by vaccination status:

The distribution of cases according to vaccination status shows that 35.7% of the 401 (n=143) unvaccinated patients, and 69.5% of 177 (n=123) patients with unknown vaccination status were rotavirus positive, compared with 20.9% of the 645 (n=135) vaccinated patients (Fig 3) (x^2=150.85, p<0.0001) .

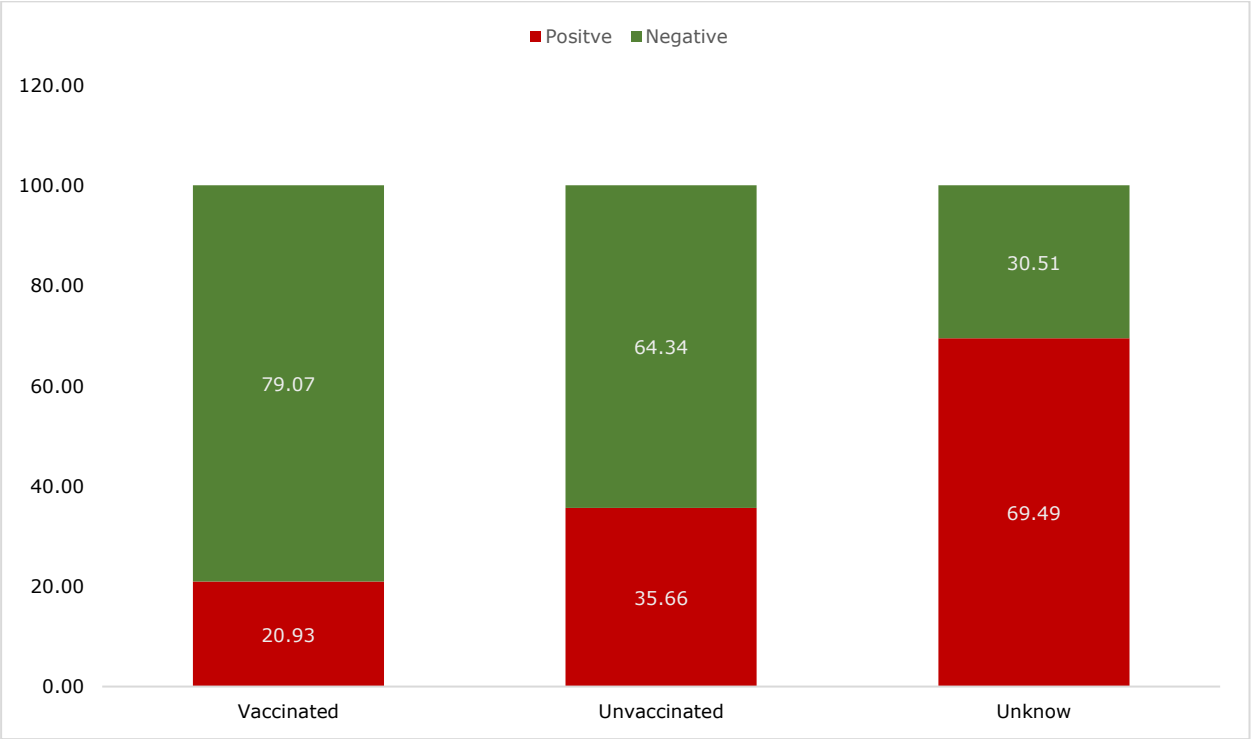

Fig 3: Distribution of rotavirus cases by vaccination status (x^2=150.85, p<0.0001*)

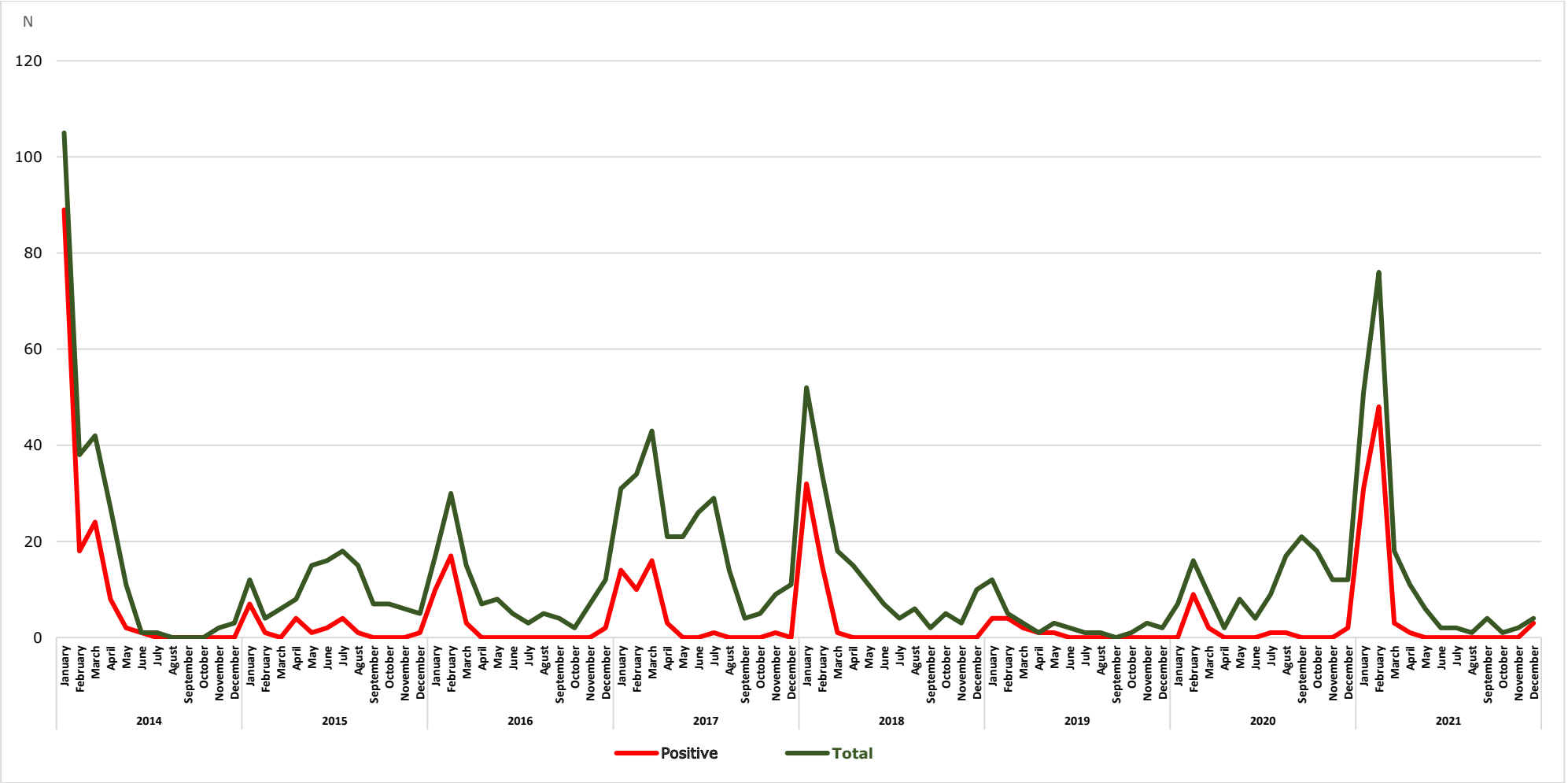

Fig 2: Number of enrolled and positive cases of rotavirus diarrhoea with respect to months during the 8-year period of study

Table 2: Frequency of G (VP7) and P (VP4) rotavirus serotypes and different combinations from RT-PCR assay of stool samples of children with rotavirus diarrhoea

Types	Frequency	Percent
VP7 (n = 47)		
G1	2	4.3
G2	1	2.1
G3	11	23.4
G9	2	4.3
G12	31	66.0
VP4 (n = 47)		
P6	28	59.6
P8	19	40.4
Combination genotypes (n = 7)		
G1P[8]	2	4.3
G2P[6]	1	2.1
G3P[6]	5	10.6
G3P[8]	6	12.8
G9P[8]	2	4.3
G12P[6]	22	46.8
G12P[8]	9	19.2

Results of RT-PCR genotyping:

A total of 47 strains were genotyped by RT-PCR. Within the G genotype, G1, G2, G3, G9 and G12 genotypes were identified in proportions of 4.3%, 2.1%, 23.4%, 4.3% and 66,0% respectively (Table 2). For genotype P, P6 and P8 were identified in 59.6% and 40.4% of cases respectively (Table 2). The genotype combinations identified were G1P[8] (4.3%), G2P[6] (2.1%), G3P[6] (10.6%), G3P[8] (12.8%), G9P[8] (4.3%), G12P6 (46.8%) and G12P[8] (19.2%) (Table 2).

Discussion:

This study showed a prevalence rate of 32.7% for rotavirus infection in children with gastroenteritis over the 8 years of the study. Other studies in Burkina Faso prior to the introduction of the vaccine found an approximate prevalence rate of 33.8% between 2008 and 2010 (13), and a much higher rate of 63.5% between 2011 and 2012 (14). This high prevalence of 63.5% corroborates the prevalence of 61.7% in 2014 in our study, which fell to 17.6% in 2015. This rate remained low in 2017 (18.2%) and 2020 (11.1%), in contrast to 2016 (27.8%), 2018 (29.3%), 2019 (35.3%) and 2021 (48.3%), the year in which the highest rate since the introduction of the vaccine was reported. However, all the rates in our study with the exception of 2014, are lower than the rates before the year in which the vaccine was introduced. This drop in the proportion of positive cases of rotavirus infection can be seen in certain African countries such as Mauritania, which saw a 24% drop in cases of hospitalisation, with a sharp fall (74%) in children aged 2 to 5 years (15).

The drop in the number of Rotavirus diarrhoea illnesses between 2014 and 2015 is probably linked to the introduction of the vaccine on 31 October 2013 in Burkina Faso. This drop is estimated to be 73.8% effective against rotavirus gastroenteritis, but could fall to around 60% in high-mortality regions (7,16–18). In 2016, Burkina Faso achieved an estimated vaccination coverage rate of 91% throughout the country, which could contribute to this drop in the positivity rate (5). The reduction in cases due to the vaccine is confirmed by the results according to vaccination status that we observed in this study.

In fact, 64.3% of stool samples from unvaccinated children were negative for rotavirus antigen, compared to 79.1% of samples from vaccinated children. Although the negativity rate in vaccinated children was significantly higher than in unvaccinated children (x^2=150.85, p<0.0001), 20.9% of rotavirus infections were reported despite vaccination. Rotavirus infections despite vaccination have been noted in certain countries where a weak protective effect was reported in low-income countries compared with industrialized countries (19). The reason for this weak protective effect could be linked to various co-morbidities such as malnutrition, variations in the genotypes circulating in different countries and individual variations in the vaccine response (6,20). Examples of inter-individual variations

in vaccine response include variations in human blood group antigens, which are antigens that bind the VP4 protein of the P genotype. It has been shown that this variation could be the cause of reduced vaccine sensitivity in Lewis-negative children, a very common group in Africa (21).

Another factor that could favor rotavirus infection is the seasonal cycle of the year. In this study, the number of cases increases between December and April, peaking between January and February. The incidence of gastroenteritis increases during the dry, cold period of the year, and in Burkina Faso it could be said that the number of cases increases in December and declines from April onwards, corresponding to the dry, cold season (13,22,23). Climate is certainly a factor in the outbreak of cases, but there are also factors linked to the virus, such as the VP6 protein, which defines the different groups of rotaviruses, of which group A is the main group responsible for more than 90% of cases of gastroenteritis in humans (24). Epidemics linked to certain group B viruses have been observed in certain countries such as China and India, and group C viruses have been sporadically isolated on almost every continent. In this study, the diagnosis of diarrhoea on suspicion of rotavirus concerned the group A virus.

Of the P (VP4) genotypes, P6 predominated, accounting for 59.6% of cases, and P8 for 40.4%. These two genotypes are the majority circulating worldwide, as is the P4 genotype, which was not identified in this study (25,26). In genotype G (VP7), G12 was the majority genotype identified with 66%, followed by G3 genotype with 23.4%. Genotypes G12 and G3, along with genotypes G1, G2 and G9, which were only identified in a minority of cases in this study, are the most frequently incriminated genotypes in humans (25). We did not identify G4 genotypes, which declined dramatically since 2003, or G8 and G6 genotypes, which were reported in other studies in Burkina Faso and other African countries (27–29).

This predominance of G12 corroborates the results of other studies in other African countries (28). The G12 genotype was first identified in sub-Saharan Africa in 2004, and since then it has emerged as the most important phenotype in several African countries, even after the introduction of the vaccine (28, 30). Among the genotype combinations notified, G12P[8] accounts for almost half of all genotype combinations, followed by G12P[6]. These two combinations are the most commonly encountered in Africa, but previous studies showed the emergence of the G6P[6] genotype in Burkina Faso and other African countries (27,31), but in our study we did not identify this genotype.

Conclusion:

This study highlighted the involvement of a variety of group A rotavirus genotypes in diarrhoea in children aged 0-5 years. The P genotypes implicated in this study are P6 and P8, and the G genotypes implicated are G1, G2, G3, G9 and G12. The genotypic combinations are dominated by G12P[6] followed by G12P[8].

What is remarkable is the considerable drop in the proportion of positive cases in the year following the introduction of the vaccine into the EPI programme in Burkina Faso on 31 October 2013. However, cases are still being recorded, even though the proportions remain lower than before the vaccine was introduced, thus demonstrating the efforts that still need to be made in fighting against rotavirus diarrhoea in children.

Contributions of authors:

IT conceptualized the study and was involved in data curation, formal analysis, and writing of original manuscript draft; DCD was involved in data curation; HS, AKB, JTD, MT, MK, MS, ASO, IS were involved in review and editing of manuscript; and ROT was involved in review/editing of the manuscript and supervision of the project.

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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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Original Article **Open Access**

Comparative performance of Cobas 4800 and 5800 assays with Cobas 6800 assay for detecting/genotyping high-risk HPV in cervical samples: An interlaboratory evaluation in Maputo, Mozambique

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Abstract:

Background: High-risk (hr) human papillomaviruses (HPV) testing is immensely important in elucidating molecular epidemiology of HPV infections and has become an essential part of current clinical practice in the management of the precancerous lesions and cervical cancer risks. Evaluation of HPV testing systems suitable for large-scale organized cervical screening programs is required. Very little is known about the diagnostic performance of the Cobas assays in resource-limited settings, particularly in Mozambique. We evaluated the concordance of the Cobas 4800 and Cobas 5800 assays with the Cobas 6800 assay for detecting high-risk HPV genotypes in clinician-collected cervical samples.
Methodology: In Mozambique, HPV-based cervical cancer screening is being regionally implemented and is currently well established in Maputo using the Cobas 4800, Cobas 5800 and Cobas 6800 assays. A random subset of 124 samples from women older than 18 years attending organized cervical cancer screening and previously tested for hrHPV-DNA using Cobas 4800 and Cobas 5800, were used for a subsequent analysis on Cobas 6800.
Results: Our results showed a good-to-high overall concordance of the Cobas 4800 and Cobas 5800 tests with Cobas 6800 test, showing strong Cohen's kappa values (95%, κ=0.89, 95% CI: 0.80–0.98, for Cobas 4800 versus Cobas 6800; and 100%, κ=1.00, 95% CI: 1.00–1.00 for Cobas 5800 versus Cobas 6800). Similar performance to detect HPV-16, HPV-18, and the 12 other hrHPV genotypes was observed.
Conclusion: The global performance to detect the 14 hrHPV genotypes was not significantly different between the three Cobas assays, which supports their suitability for use in large centralized laboratories included within population-based cervical cancer screening programs in resource-limited settings.

Keywords. Cervical cancer screening, HPV testing; Cobas assays; Performance; Interlaboratory comparison

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Performances comparatives des tests Cobas 4800 et 5800 avec le test Cobas 6800 pour la détection/génotypage des HPV à haut risque dans des échantillons cervicaux: Une évaluation interlaboratoires à Maputo, Mozambique

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Résumé:

Contexte: Le test des papillomavirus humains à haut risque (hrHPV) est d'une importance majeure pour élucider l'épidémiologie moléculaire des infections à HPV. Il est devenu un élément essentiel de la pratique clinique actuelle pour la prise en charge des lésions précancéreuses et des risques de cancer du col de l'utérus. L'évaluation des systèmes de dépistage du HPV adaptés aux programmes de dépistage cervical organisé à grande échelle est nécessaire. Très peu de données existent concernant la performance diagnostique des tests Cobas dans les contextes à ressources limitées, en particulier au Mozambique. Nous avons évalué la concordance des tests Cobas 4800 et Cobas 5800 avec le test Cobas 6800 pour la détection des génotypes hrHPV dans des échantillons cervicaux prélevés par des cliniciens.
Méthodologie: Au Mozambique, le dépistage du cancer du col de l'utérus basé sur le HPV est mis en œuvre à l'échelle régionale et est actuellement bien établi à Maputo grâce aux tests Cobas 4800, Cobas 5800 et Cobas 6800. Un sous-ensemble aléatoire de 124 échantillons de femmes âgées de plus de 18 ans, participant à un programme organisé de dépistage du cancer du col de l'utérus et préalablement testées pour l'ADN hrHPV avec les tests Cobas 4800 et Cobas 5800, a été réanalysé avec le test Cobas 6800.
Résultats: Nos résultats ont montré une concordance globale bonne à élevée des tests Cobas 4800 et Cobas 5800 avec le test Cobas 6800, avec des valeurs de kappa de Cohen élevées (95%, κ= 0,89, IC 95%: 0,80–0,98, pour Cobas 4800 versus Cobas 6800; et 100%, κ=1,00, IC 95%: 1,00–1,00 pour Cobas 5800 versus Cobas 6800). Des performances similaires ont été observées pour la détection du HPV-16, du HPV-18 et des 12 autres génotypes hrHPV.
Conclusion: Les performances globales pour la détection des 14 génotypes hrHPV n'ont pas montré de différences significatives entre les trois tests Cobas, ce qui confirme leur pertinence pour une utilisation dans les laboratoires centralisés de grande envergure intégrés aux programmes de dépistage du cancer du col de l'utérus dans les contextes à ressources limitées.

Mots-clés: Lésions cervicales; Dépistage HPV; Tests Cobas; Performance; Comparaison interlaboratoires

Introduction:

Human papillomavirus (HPV) is one of the most common sexually transmitted viruses worldwide and accounts for approximately 7.7% of all cancers in developing countries, predominantly cervical cancer, resulting in over 75,000 new cases and 50,000 deaths annually in sub-Saharan Africa (1,2). Infection with oncogenic types of HPV is the causative factor of nearly all (>99%) cases of cervical cancer (1,3). Fifteen genotypes (including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) are classified as oncogenic types of HPV, well known as high-risk HPV (hrHPV), and three genotypes (HPV 26, 53 and 66) are considered probably carcinogenic (4, 5).

Mozambique is among the countries with the highest burden of HPV infection, HIV and cervical cancer. Each year, over 5,300 new cases of cervical cancer and more than 3,800 deaths are registered (6), disproportionally affecting women living with HIV/AIDS (7,8). HPV infection is common among Mozambican women, with data indicating considerable prevalence rates of hrHPV (HPV 16, 18, 31, 33, 35, 45, 51, 52, and 58), varying from 26.3% to 75.9%, according to studies (9–13).

After the WHO Global strategy for the elimination of cervical cancer was launched (14), hrHPV-based tests have been increasingly adopted, as primary or co-test for cervical cancer screening, and for monitoring of the effectiveness of HPV vaccination (15,16). The key feature of hrHPV-based tests for cervical cancer screening is their high sensitivity and accuracy for screening women at risk for cervical lesions, as well as for detecting high-grade cervical intraepithelial neoplasia (CIN2+) and cervical cancer (CC), particularly in women above 30 years of age (16–18).

In Mozambique, the National Cancer Control Program, Ministry of Health has recently introduced (in Maputo) the pilot implementation of hrHPV testing in primary cervical screening to comply with the WHO guidelines. Although there have been many pilot projects and studies using several HPV tests (12,13, 19), the assay actually in use within the government healthcare system is the recently developed Cobas 5800/6800/8800 HPV DNA for use on the Cobas 5800/6800/8800 systems (20). The Cobas 4800 HPV DNA test which has been successfully deployed for primary HPV-based screening (21,22), was introduced for hrHPV screening in a cervical screening service at the DREAM program, Sant'Egídio (12). This test is for use on the Cobas 4800 system; an automated system that has been a reference standard for HPV screening programs due to its high sensitivity, reliability and ability to predict CIN2+ or worse (23–25).

Current guidelines recommend that, in order to enable testing in large centralized laboratories and ensure diagnostic accuracy, clinically validated HPV assays should be used (14,17). The aim of this study was to evaluate

the performance concordance of Cobas 4800 and Cobas 5800 tests with Cobas 6800 test for the detection and genotyping of hrHPV, using an interlaboratory comparative approach. Yet, there is no information on the performance concordance of these tests, nor on their clinical validation in the Mozambican context.

Materials and method:

Study design, population and ethical considerations:

This is a cross-sectional retrospective study, under the scope of activities of laboratory quality management in 2023 and 2024, of two molecular laboratories (DREAM Sant' Egidio laboratory and laboratory of Mavalane General Hospital), in Maputo, Mozambique. The DREAM Laboratory is accredited by the Portuguese Institute for Accreditation (IPAC) in accordance with ISO 15189 standards.

This study used samples from volunteer women aged >18 years, recruited at the DREAM Health Center in Zimpeto between July 2021 and May 2023 as part of a larger hrHPV screening study (HPV-ISI). The parent study evaluated the feasibility of hrHPV testing as a primary screening method, compared with the visual inspection with acetic acid (VIA) approach (12), and was conducted within the context of a routine cervical cancer screening program.

The HPV-ISI study was approved by the Mozambican national ethical committee (ref. 688/CNBS/20; amendment nº176/CNBS/22). Furthermore, due to the need to use the collected data for academic purposes, additional approval was obtained from the Institutional Review Board of the Eduardo Mondlane University–Faculty of Medicine and Maputo Central Hospital, Mozambique (ref. CIBSFM & HCM/01/2024). Informed written consents were obtained from all the participants, and all the samples and data were anonymized.

Samples and HPV testing:

A random subset of 124 samples was selected from a larger cohort of 1,323 previously tested for hrHPV-DNA at the DREAM Sant'Egidio laboratory using the Cobas 4800 system (Roche Diagnostics), from 2021-2023 (12) and Cobas 5800 in 2023. Only samples with valid hrHPV-DNA test results on both Cobas 4800 and Cobas 5800 were selected and included in the subset for the interlaboratory evaluation by subsequent analysis on Cobas 6800. Limited laboratory test resources were the primary reason for the choice of the sample size. Samples without age data and those from participants aged under 18 years were excluded.

The samples were collected by a trained health professional using cervical brush into a 20 mL liquid-based cytology media (Roche Cell Collection Medium), then followed by hrHPV DNA test using Cobas 4800/5800 assays. After testing, samples were stored at –20°C, until sent to the laboratory of Mavalane General Hospital, for a second/interlaboratory testing, using the Cobas 6800 HPV assay (Roche Diagnostics/Molecular Systems).

The Cobas 4800/5800/6800 tests are a real-time PCR based assays targeting hrHPV L1 gene and human β-globin gene as an internal control. All three tests allow the detection of 14 hrHPV types, genotyping HPV16 and HPV18 and reporting other 12 hrHPVs (including HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) as a "pooled" result in the same channel. The testing was carried out according to the manufacturer's instructions (20,26). If internal control was not amplified, samples were referred as invalid.

Statistical analysis:

Statistical analysis was performed using SPSS 30 software (IBM Corp., Armonk, New York, USA) and GraphPad QuickCalcs (https://www.graphpad.com/quickcalcs/). Quantitative variables were presented as median with interquartile range (IQR), and categorical variables were summarized with the number and percentage of participants in each category. A 2-tailed *p* value of <0.05 was considered significant.

McNemar's test and Cohen's kappa (κ) statistics were calculated to, respectively, assess differences in the proportion of positive samples and the level of agreement between the Cobas tests for detecting the 14 hrHPV types. The strength of agreement was classified according to the criteria described by Landis & Koch (27). Simple agreement was calculated by adding the agreement of the positive and negative results for both techniques; complete and partial discordant results were evaluated.

Results:

Of the 124 samples selected and included in the subset for the interlaboratory evaluation using Cobas 6800 system at the laboratory of Mavalane General Hospital, 94 samples (47 hrHPV-positive and 47 hrHPV-negative) had been previously tested with the Cobas 4800, while 30 samples (15 hrHPV-positive and 15 hrHPV-negative) had been tested with the Cobas 5800.

The median (IQR) age of the sampled participants was 42 (35–48) years, of whom the majority (61.3%) belonged to women aged 30-45 years, followed by 31.5% for >45 years, and 4.8% for 18-30 years. Information on age were missing for 3 (2.4%) participants. The median interval between sample collection

and testing was 5 days (IQR: 2–6 days) for Cobas 4800 and 10 days (IQR: 8–12 days) for Cobas 5800 at the DREAM Sant'Egidio laboratory, whereas for the testing with Cobas 6800 at the Mavalane General Hospital laboratory, it took 76 days (IQR: 59–202 days) for samples tested with Cobas 4800, and 17.5 days (IQR: 16–19 days) for those tested with Cobas 5800.

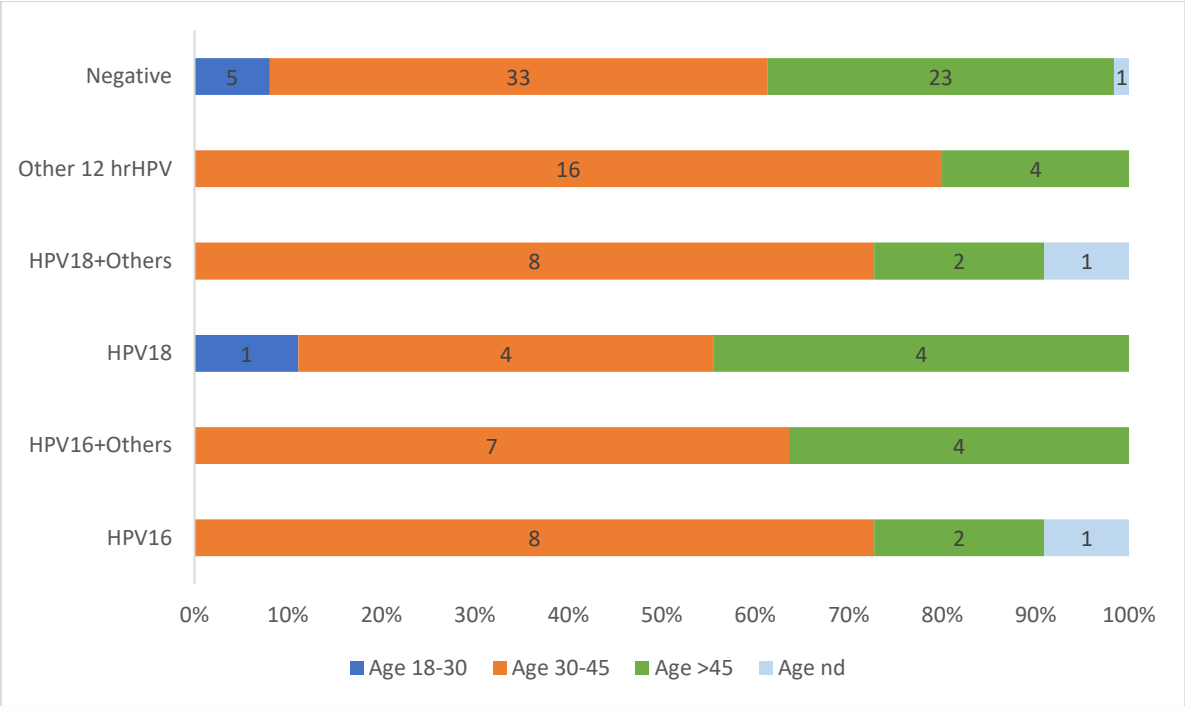
All 124 samples were tested with the three Cobas HPV DNA tests (4800, 5800 and 6800), and none of the samples failed for hr-HPV testing. Among the 94 samples tested with Cobas 4800 versus Cobas 6800, the prevalence of HPV16 alone, HPV18 alone, HPV16 with 'Other hrHPV', HPV18 with 'Other hrHPV', and 'Other hrHPV' types was not significantly different between the 2 tests: 11% versus 12% (p=0.82), 9.6% versus 8.5% (p=0.80), 8.5% versus 8.5% (p=1.00), 9.6% versus 10.6% (p=0.81), and 10.6% versus 10.6% (p=1.00), respectively.

A similar observation was found for the 30 samples tested with Cobas 5800 versus Cobas 6800, with prevalence of HPV16 with 'Other hrHPV', HPV18 with 'Other hrHPV', and 'Other hrHPV', accounting for 10%, 6.7%, and 50.0%, respectively, in both tests. HPV16 alone and HPV18 alone were not detected (Table 1). Fig 1 presents the overall distribution of hrHPV by age group, showing a considerable positivity rate among women aged 30-45 years.

Table 1: Prevalence of human papillomavirus (HPV) types 16 and 18 and other 12 high-risk HPV types found between Cobas 4800 and Cobas 6800 tests, and between Cobas 5800 and Cobas 6800 tests

hrHPV Type		**Cobas 4800 vs Cobas 6800 (n=94)**			**Cobas 5800 vs Cobas 6800 (n=30)**		
		c4800 n (%)	**c6800 n (%)**	***p* value[a]**	**c5800 n (%)**	**c6800 n (%)**	***p* value[b]**
All hrHPV							
	Negative	47 (50.0)	46 (48.9)	0.88	15 (50.0)	15 (50.0)	1.00
	Positive	47 (50.0)	48 (51.1)		15 (50.0)	15 (50.0)	
HPV16 alone							
	Negative	83 (88.3)	82 (87.2)	0.82	30 (100)	30 (100)	-
	Positive	11 (11.7)	12 (12.8)		0 (0)	0 (0)	
HPV18 alone							
	Negative	85 (90.4)	86 (91.5)	0.80	30 (100)	30 (100)	-
	Positive	9 (9.6)	8 (8.5)		0 (0)	0 (0)	
HPV16 + other hrHPV[c]							
	Negative	86 (91.5)	86 (91.5)	1.00	27 (90.0)	27 (90.0)	1.00
	Positive	8 (8.5)	8 (8.5)		3 (10.0)	3 (10.0)	
HPV18 + other hrHPV[c]							
	Negative	85 (90.4)	84 (89.4)	0.81	28 (93.3)	28 (93.3)	1.00
	Positive	9 (9.6)	10 (10.6)		2 (6.7)	2 (6.7)	
Other hrHPV types[c]							
	Negative	84 (89.4)	84 (89.4)	1.00	15 (50.0)	15 (50.0)	1.00
	Positive	10 (10.6)	10 (10.6)		15 (50.0)	15 (50.0)	

HPV: human papillomavirus; hrHPV: high-risk human papillomavirus; (-): not Applicable
[a]Calculated by Chi-square test but using Fisher exact test (given the relatively small total sample size: n=94 per group) a p value of 1.00 was observed for all comparisons
[b]Calculated by Fisher exact test (given the relatively small total sample size: n=30 per group)
[c]12 HPV types: 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68, "pooled" in the same channel



Other (s) 12 HPV types include 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68, "pooled" in the same channel; HPV= human papillomavirus; hrHPV = high-risk human papillomavirus; nd = not determined/available

Fig 1: Overall distribution of hrHPV positivity by age interval

Further statistical analyses for method comparison were also performed on all samples; separately for Cobas 4800 versus Cobas 6800, and Cobas 5800 versus Cobas 6800, with an overall concordance of detecting any hrHPV of 94.68% (agreement on 89/94 samples, Kappa=0.89; 95% confidence interval: 0.80–0.98), and 100.0% (agreement on all 30 samples, Kappa=1.00), respectively. The concordance of the results between Cobas 4800 versus Cobas 6800 for detection of HPV16 alone, HPV18 alone, HPV16 with 'Other hrHPV', HPV18 with 'Other hrHPV', and 'Other hrHPV' types was 98.9%, 98.9%, 100%, 98.9%, and 95.7%, respectively (Table 2).

Five samples were discordant; (i) 1 for HPV16 positivity and 2 for 'Other hrHPV' positivity were missed by Cobas 4800 and detected by Cobas 6800; and (ii) 2 for 'Other hrHPV' positivity, detected by Cobas 4800, were missed by Cobas 6800. Additionally, one sample was partially discordant; Cobas 4800 detected only HPV18 while Cobas 6800 detected HPV18 with 'Other hrHPV' (Table 3). The 'Other hrHPV' was the most common type missed by the 2 methods (5/6 cases). In contrast, for Cobas 5800 versus Cobas 6800, all results were 100.0% concordant (absence of discordance); same agreement index were observed for genotyping HPV16+ 'Other hrHPV' and HPV 18+ 'Other hrHPV' (Table 2).

The agreement (Cohen kappa) between Cobas 4800 versus Cobas 6800 for detection of HPV16 alone, HPV18 alone, HPV16 with 'Other hrHPV', HPV18 with 'Other hrHPV', and 'Other hrHPV' types was 0.95 (CI: 0.85–1.00), 0.94 (CI: 0.81–1.00), 1.00 (CI: 1.00–1.00), 0.94 (CI: 0.83–1.00) and 0.78 (CI: 0.57–0.99), respectively. The performance to positively detect HPV-16 (alone/with 'Other hrHPV'), HPV-18 (alone/with 'Other hrHPV'), and 'Other hrHPV' were not significantly different between the 2 tests (McNemar test, $p>0.05$) (Table 2). In total, the global performance to detect any of the 14 hrHPV types was not significantly different (McNemar test, $p=1.00$).

Discussion:

This study aimed to evaluate, within an interlaboratory quality assessment process, the concordance of Cobas 4800 and Cobas 5800 tests with Cobas 6800 test in detecting and genotyping high-risk human papillomavirus (hrHPV) in 124 cervical samples (94 for Cobas 4800 *versus* Cobas 6800; and 30 for Cobas 5800 *versus* Cobas 6800), from women aged 18 years or older, screened for hrHPV in a cervical cancer screening service. To the best of our knowledge, this is the first study evaluating the diagnostic performance of the Cobas tests within the recently introduced pilot implementation of hrHPV testing in primary cervical screening in Maputo, Mozambique.

The results demonstrate a high level of agreement between Cobas 4800 and Cobas

Table 2: Agreement and performance between Cobas 4800 and Cobas 6800 tests, and between Cobas 5800 and Cobas 6800 tests in detecting Human Papillomavirus (HPV) types 16 and 18 and other 12 high-risk types

hrHPV Type		Number (%)	Cohen Kappa Test (95% CI)	McNemar Test
Cobas 4800 vs Cobas 6800 (n=94)				
All hrHPV				
	Concordant	89 (94.7)	0.89 (0.80–0.984)	1.00
	Discordant	5 (5.3)		
HPV16 alone, n (%)				
	Concordant	93 (98.9)	0.95 (0.85–1.00)	1.00
	Discordant	1 (1.1)		
HPV18 alone, n (%)				
	Concordant	93 (98.9)	0.94 (0.81–1.00)	1.00
	Discordant	1 (1.1)		
HPV16 + other hrHPV[a], n (%)				
	Concordant	94 (100)	1.00 (1.00–1.00)	-
	Discordant	0 (0)		
HPV18 + other hrHPV[a], n (%)				
	Concordant	93 (98.9)	0.94 (0.83–1.00)	1.00
	Discordant	1 (1.1)		
Other hrHPV types[a], n (%)				
	Concordant	90 (95.7)	0.78 (0.57–0.99)	1.00
	Discordant	4 (4.3)		
Cobas 5800 vs Cobas 6800 (n=30)				
All hrHPV[b]				
	Concordant	30 (100)	1.00 (1.00–1.00)	-
	Discordant	0 (0)		

Cohen Kappa test and McNemar test were performed to compare the agreement and the performance of the 2 methods to detect HPV-16, HPV-18, the other hrHPV types, and the 12 hrHPV types "pooled" in the same channel.
HPV: human papillomavirus; hrHPV: high-risk human papillomavirus; CI: Confidence interval; (-): not Applicable
[a]12 HPV types: 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68, "pooled" in the same channel.
[b]Including for genotypes detection; All results between Cobas 5800 and Cobas 6800 tests were 100% concordant (absence of discordance)
–: same values of Cohen Kappa index and McNemar test were observed for hrHPV genotyping (HPV16 + other hrHPV and HPV18 + other hrHPV)

Table 3: Discordant and partial discordant results in the comparison of results between Cobas 4800 and Cobas 6800 tests*

Sample ID	Any hrHPV		hrHPV types		Result classification
	Cobas 4800	Cobas 6800	Cobas 4800	Cobas 6800	
11012	Negative	Positive	-	HPV16	Discordant
10891	Negative	Positive	-	Other hrHPV[a]	Discordant
1883	Negative	Positive	-	Other hrHPV[a]	Discordant
9839	Positive	Negative	Other hrHPV[a]	-	Discordant
9911	Positive	Negative	Other hrHPV[a]	-	Discordant
3193	Positive	Positive	HPV18	HPV18+Others[a]	partial discordant

HPV: human papillomavirus; hrHPV: high-risk human papillomavirus.
[a]12 HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68, "pooled" in the same channel.
(*): All results between Cobas 5800 and Cobas 6800 tests were 100% concordant (absence of discordance)

5800 tests with the Cobas 6800 test across both laboratories, showing an overall concordance (95%, for Cobas 4800 *versus* Cobas 6800; and 100%, for Cobas 5800 *versus* Cobas 6800), which indicates an almost perfect agreement (27,28). These findings support the interchangeability of the three platforms in clinical and epidemiological hrHPV screening and surveillance programs. It is widely accepted that, to ensure a robust and highly reliable

test performance in clinical settings, the candidate assay must demonstrate inter-laboratory agreement with a lower confidence limit of no less than 87% (17).

The distribution of hrHPV types, including HPV 16, HPV 18, and Other hrHPV types, was largely comparable between both methods, with no statistically significant differences observed in their detection frequencies. Notably, the detection concordance for HPV16 and HPV-18, the two most oncogenic HPV genotypes, reached 98.9%, underscoring both platforms reliability in identifying these high-risk types. The complete agreement observed in cases with co-infection of HPV16 and Other hrHPV further reinforces the robustness of both assays in multi-type detection scenarios.

The discrepancies between the assays were minimal. Only five samples showed discordant results, with the Cobas 6800 slightly outperforming the Cobas 4800 by detecting three additional positive cases (one HPV16 and two Other hrHPV). Two samples were detected exclusively by the Cobas 4800, and one sample was partially discordant with differing co-infection profiles. The marginally higher detection of Other hrHPV by the Cobas 6800 could be attributed to its technological enhancements over the 4800 system, including analytical sensitivity and automation design, which allows for improved nucleic acid extraction and amplification (17,20,29). However, the difference was not statistically significant, and the kappa agreement for Other hrHPV was slightly lower compared to other genotype categories, suggesting a modest variation in detecting non-HPV16/18 genotypes, which are often present at lower viral loads (30). Similar discrepancies were also reported in other studies (23,24).

The age distribution of hrHPV positivity aligns with epidemiological patterns, where the highest burden of hrHPV was observed among women aged 30–45 years. This reinforces the importance of targeting this age group for primary HPV screening, especially in low- and middle-income settings where cervical cancer remains a leading cause of mortality among women (31,32).

One important procedural difference between the two laboratories involved in this study was the storage time between sample collection and testing. Samples processed at the Mavalane General Hospital laboratory had a significantly longer storage interval (median of 76 days for samples previously tested with Cobas 4800, and 17.5 days for those tested with Cobas 5800) compared to those at the DREAM Sant'Egidio (median of 5 days for Cobas 4800 and 10 days for Cobas 5800). Despite this, there was no impact on assay performance, underscoring the stability of cervical specimens in liquid-based cytology collection media under appropriate storage conditions, as previously reported (33–35).

Our findings support previous studies that have shown strong agreement between Cobas platforms (20,23,24,36), emphasizing their validity in clinical practice. Importantly, while the Cobas 4800 system is a medium throughput (384 samples/day), the Cobas 5800 and Cobas 6800 offers improved workflow efficiency, higher throughput (528 and >1500 samples/day, respectively), and reduced hands-on time, making it particularly suitable for large-scale screening programs and molecular testing for other viruses (20,26). Similar conclusions were also reached by studies that clinically evaluated the performance according to the Meijer criteria, and observed high agreement (>98%) for the two assays, and that clinical sensitivity and specificity met the non-inferiority criteria defined to consider an HPV assay as suitable for large-scale laboratory use (23,24,36).

This study has some limitations. The relatively small sample size, due to limited laboratory test resources, archival sample-based testing and the single-country setting may limit generalizability. Additionally, sequencing confirmation of discordant samples was not performed, which could have helped clarify the true-positive status. Nonetheless, the high concordance metrics and low number of discrepancies suggest these limitations did not significantly impact the conclusions.

Conclusion:

Based on the results of this study, all three Cobas platforms – 4800, 5800, and 6800 – demonstrated high concordance in detecting and genotyping high-risk HPV in cervical samples. These findings support the suitability of each platform for use in routine hrHPV screening programs and underscore the Cobas 6800 as a reliable, high-throughput option particularly well-suited for scaling up cervical cancer prevention efforts.

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Contribution of authors:

AS, TC and JS conceptualized and designed the study, acquired, analyzed and interpreted the data. All the authors drafted, revised, read, provided critical feedback and approved the manuscript.

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Data availability:

The data that support the findings made in this study can be made available from the corresponding author on request.

Disclaimer:

The views expressed in this study are those of the authors and are not an official position of the laboratories administrative; DREAM Program, Community of Sant'Egidio and Mavalane General Hospital.

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Original Article **Open Access**

Assessment of genetic diversity among environmental *Cryptococcus* species using RAPD analysis

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Abstract:

Background: *Cryptococcus* species, including *C. neoformans* and *C. gattii*, are environmental yeasts of clinical importance and major agents of cryptococcosis. Environmental reservoir represents the primary source of human and animal infections therefore, characterization of genetic diversity is essential for epidemiological surveillance and understanding the pool of genotypes from which pathogenic strains may arise.
Methodology: This study evaluated the genetic diversity of 18 environmental *Cryptococcus* isolates recovered from soil samples using random amplified polymorphic DNA (RAPD) analysis. Seven primer combinations (three 20–22 mer and four 12-mer) were employed to generate reproducible banding patterns, and genetic diversity indices were calculated based on RAPD polymorphic loci.
Results: A total of 214 bands, ranging from 330-3800 bp, were amplified from 17 isolates with 10-13 polymorphic bands per isolate; one isolate failed to amplify with all primers and was excluded from further analysis. The isolates were resolved into five haplotypes (A-E), with haplotype B being most prevalent (61.1%). Primer-specific bands enabled diagnostic differentiation of certain haplotypes, demonstrating the discriminating capacity of the selected RAPD primers. Diversity indices indicated high genetic variability among the soil isolates, with a Shannon–Wiener index (H') of 2.83, Simpson's index of diversity (1-D) of 0.941, and evenness (E) of 0.999.
Conclusion: These results demonstrate substantial genetic heterogeneity among environmental *Cryptococcus* isolates, even within a restricted locale. Although no direct comparisons were made with clinical or animal isolates, the observed diversity highlights the complexity of environmental reservoirs that serve as potential sources of infection. This study underscores the utility of RAPD markers for preliminary molecular characterization and epidemiological studies, providing baseline data relevant to environmental and public health surveillance in Nigeria.

Keywords: *Cryptococcus* Species; Genetic Diversity; RAPD; Polymorphic bands

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Évaluation de la diversité génétique des espèces de *Cryptococcus* environnementales par analyse RAPD

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Résumé:

Contexte: Les espèces de *Cryptococcus*, notamment C. *neoformans* et *C. gattii*, sont des levures environnementales d'importance clinique et des agents majeurs de la cryptococcose. Le réservoir environnemental représente la principale source d'infections humaines et animales. Par conséquent, la caractérisation de la diversité génétique est essentielle à la surveillance épidémiologique et à la compréhension du pool de génotypes à partir duquel des souches pathogènes peuvent émerger.
Méthodologie: Cette étude a évalué la diversité génétique de 18 isolats environnementaux de *Cryptococcus*, obtenus à partir d'échantillons de sol, par analyse RAPD (ADN polymorphe amplifié aléatoirement). Sept combinaisons d'amorces (trois de 20 à 22 nucléotides et quatre de 12 nucléotides) ont été utilisées pour générer des profils de bandes reproductibles, et les indices de diversité génétique ont été calculés à partir des loci

polymorphes RAPD.
Résultats: Au total, 214 bandes, de 330 à 3 800 pb, ont été amplifiées à partir de 17 isolats, avec 10 à 13 bandes polymorphes par isolat. Un isolat n'a pas pu être amplifié avec toutes les amorces et a été exclu des analyses ultérieures. Les isolats ont été classés en cinq haplotypes (A à E), l'haplotype B étant le plus fréquent (61,1 %). Les bandes spécifiques aux amorces ont permis la différenciation diagnostique de certains haplotypes, démontrant ainsi le pouvoir discriminant des amorces RAPD sélectionnées. Les indices de diversité ont révélé une forte variabilité génétique parmi les isolats de sol, avec un indice de Shannon-Wiener (H') de 2,83, un indice de diversité de Simpson (1-D) de 0,941 et un indice d'équitabilité (E) de 0,999.
Conclusion: Ces résultats démontrent une hétérogénéité génétique substantielle parmi les isolats environnementaux de *Cryptococcus,* même au sein d'une zone géographique restreinte. Bien qu'aucune comparaison directe n'ait été effectuée avec des isolats cliniques ou animaux, la diversité observée souligne la complexité des réservoirs environnementaux qui constituent des sources potentielles d'infection. Cette étude met en évidence l'utilité des marqueurs RAPD pour la caractérisation moléculaire préliminaire et les études épidémiologiques, fournissant des données de référence pertinentes pour la surveillance environnementale et de santé publique au Nigéria.

Mots-clés: Espèces de *Cryptococcus*; Diversité génétique; RAPD; Bandes polymorphes

Introduction:

The genus *Cryptococcus* comprises a group of encapsulated, basidiomycetous yeasts of significant medical importance, particularly *Cryptococcus neoformans* and *Cryptococcus gattii* (1,2), which are recognized globally as major etiological agents of cryptococcosis (3, 4). As a consequence of their pathogenic potential and environmental persistence, infections caused by these species manifest clinically as cryptococcosis, a life-threatening systemic fungal disease that primarily affects immuno-compromised individuals, especially patients with HIV/AIDS, organ transplant recipients, and those receiving immunosuppressive therapy (5,6). The disease burden is especially high in sub-Saharan Africa, where cryptococcal meningitis remains a leading cause of AIDS-related mortality (5,7).

Generally, *Cryptococcus* species exhibit remarkable genetic and phenotypic diversity, which contributes to variations in virulence, antifungal susceptibility, environmental adaptation, and geographic distribution (8,9,10). Thus, molecular epidemiological studies have revealed that *C. neoformans* and *C. gattii* are species complexes composed of multiple molecular types and genotypes, and reflect extensive evolutionary divergence (11,12). This pronounced genetic heterogeneity underscores the importance of understanding the population structure, genetic diversity, and evolutionary relationships within and between these species complexes as a basis for epidemiological and ecological investigations rather than direct inference of transmission pathways or outbreak dynamics which is beyond the scope of the present study (13,14).

Traditional phenotypic identification methods, including capsule visualization, biochemical profiling, and serotyping, are limited in their ability to discriminate among closely related *Cryptococcus* strains (3). Consequently, molecular techniques have become indispensable tools for strain differentiation and genetic characterization. Among these techniques, random amplified polymorphic DNA (RAPD) analysis has been widely employed due to its simplicity, cost-effectiveness, and ability to detect genome-wide polymorphisms without prior sequence information (15,16, 17). RAPD analysis relies on the amplification of random DNA segments using short, arbitrary primers under low stringency conditions, generating unique banding patterns that reflect genetic variation among isolates (17,18). In *Cryptococcus*, RAPD has been successfully used to assess genetic diversity, differentiate strains, and infer phylogenetic relationships across clinical, veterinary, and environmental isolates (19,20,21), while this study is providing a comparative and preliminary level of genetic resolution.

The technique has proven particularly useful in resource-limited settings, where access to more advanced genotyping platforms such as multilocus sequence typing (MLST) or whole-genome sequencing may be constrained. Several studies have demonstrated significant genetic heterogeneity among *Cryptococcus* isolates using RAPD markers, revealing correlations between RAPD profiles and serotypes, molecular types, geographic origin, and ecological niches (22,23,24). This genetic variability has important epidemiological and clinical implications, as certain genotypes have been associated with increased virulence, altered host specificity, and differences in antifungal response (25,26). However, such clinical associations are derived from studies incorporating non-environmental isolates and were not directly evaluated in the present investigation. Despite the availability of newer high-resolution molecular tools, RAPD remains a valuable preliminary and comparative method for genetic diversity studies, particularly in developing regions where cryptococcosis poses a substantial public health challenge. Its application provides essential baseline data that can support surveillance programs, inform infection control strategies, and guide further molecular investigations.

Therefore, this study aims to determine the genetic diversity among *Cryptococ-*

cus species recovered from soil samples. Using RAPD analysis, thereby contributing to a deeper understanding of their population structure and genetic variability. Such insights are relevant for characterizing environmental, clinical and animal isolates useful and for diagnostic resolution, and advancing knowledge of the evolutionary dynamics of fungal pathogens of public health significance.

Materials and method:

Source of *Cryptococcus* strains:

The *Cryptococcus* strains used in this study were isolated from soil samples as part of an ongoing PhD research project (Coker, unpublished data). Soil sampling and primary isolation were conducted using standard mycological procedures and the isolates were subsequently sub-cultured on Sabouraud Dextrose agar (SDA; HiMedia, India) and incubated at 37°C for 48 h to ensure viability and purity prior to downstream analyses.

Random amplified polymorphic DNA analysis

DNA extraction:

Genomic DNA was extracted from *Cryptococcus* isolates using modified alkaline lysis-silica binding protocol adapted from Ruma et al., (22). Approximately 250–500 mg (wet weight) of *Cryptococcal* cells were harvested and suspended in lysis buffer containing 0.2 M NaOH, 1% (w/v) sodium dodecyl sulfate (SDS), and 10 mM EDTA. The suspension was incubated in a boiling water bath for 30 min to facilitate disruption of the rigid cryptococcal cell wall and polysaccharide capsule, followed by centrifugation at 12,000 × g for 5 min.

The supernatant was neutralized by the addition of potassium acetate–acetic acid solution, prepared to yield final concentrations of 3 M potassium acetate and 2 M acetic acid, and centrifuged under the same conditions to remove cellular debris. The clarified supernatant was subsequently incubated with 10% (w/v) Celite 545 resin (Fluka Chemical Corp., USA), pre-equilibrated in guanidine hydrochloride (GuHCl) binding buffer (pH 5.5), at 60°C for 60 min on a rotary mixer to allow adsorption of DNA to the silica matrix.

The DNA–resin mixture was transferred into a column and washed three times with wash buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.6), and 2.5 mM EDTA in 50% (v/v) ethanol. The wash solution was allowed to drain by gravity, after which the column was centrifuged briefly to remove residual wash buffer. Genomic DNA was eluted twice using preheated Tris–EDTA buffer (10 mM Tris-HCl, pH 7.6; 1 mM EDTA) at 55°C. DNA concentration and purity were determined spectrophotometrically by measuring A260/A280 ratios, and DNA preparations were stored at −20 °C until further analysis.

Primer screening and PCR amplification:

Seven primer combinations were selected for RAPD analysis following preliminary optimization for reproducibility and polymerphism among the *Cryptococcus* isolates (22). Three 20–22 mer primers viz; CN1 (5′-TACC CCCGCCCATATTCCAT-3′), MYC1 (5′-GAGGAA GGTGGGGATGACGT-3′), and 5SOR (5′-ATGG GAATACGACGTGCTGTAG-3′) were obtained from laboratory stocks and used in paired combinations. In addition, six commercially synthesized 12-mer primers from FPK1-FAPD kit (Bresatec, Thebarton, SA, Australia) — FPK1-01 (5′-ACACGGACGTCA-3′), FPK1-05 (5′-ACTTG GCGGCCT-3′), FPK1-07 (5′-ACCCTGCTCATC-3′), FPK1-08 (5′-CAGGCGAAGGTT-3′), FPK1-13 (5′-ACGGCTGGTTCC-3′), and FPK1-20 (5′-CAACAGCCCCCA-3′) were screened individually and in paired combinations to evaluate their discriminatory potential across isolates. Based on reproducibility, clarity, and polymerphism of amplification profiles, four 12-mer primer combinations (FPK1-01–FPK1-05, FPK1-05–FPK1-07, FPK1-05–FPK1-13, and FPK1-08–FPK1-20) and three 20–22 mer combinations were selected for further analysis as they produced the most consistent and informative RAPD patterns.

PCR amplification was performed in 25 µL reaction volumes containing 10 ng of genomic DNA, 200 µM of each deoxynucleoside triphosphate, 0.2 µM of each 12-mer primer or 0.4 µM of each 20–22 mer primer, 0.8 U of Taq DNA polymerase, and $MgCl_2$ at a final concentration of 3 mM for 12-mer primers or 6 mM for 20–22 mer primers, under the manufacturer's recommended buffer conditions. Amplifications were carried out in a thermal cycler (Bio-Rad, Hercules, CA, USA) with an initial denaturation at 93°C for 3 min, followed by 10 low-stringency cycles (denaturation at 93°C for 1 min, annealing at 35°C for 1 min, extension at 72°C for 1 min), and then 20 high-stringency cycles (denaturation at 93°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min), with a final extension at 72 °C for 5 min. For the primer pair 5SOR–CN1, the annealing temperature during the initial 10 cycles was increased to 40°C, and the number of high-stringency cycles was increased to 30.

Amplification products were resolved on 8% polyacrylamide gels and visualized by silver staining using a modified Bassam and Caetano-Anollés (27) method, including 0.6 mg/mL sodium thiosulfate in the development step. RAPD assays were repeated at least once per DNA preparation, and DNA extracted from two independent cultures of each isolate was analyzed in parallel to ensure reproducibility.

Amplified bands were scored manually based on presence (+) or absence (−) irrespective of intensity. RAPD profiles were considered distinct and classified into major haplotypes only when reproducible, polymorphic banding patterns were obtained with all the seven primer combinations.

Genetic diversity analysis:

The genetic diversity of the *Cryptococcus* isolates was assessed using the number of polymorphic bands generated by RAPD profiling as a measure of genomic variation among isolates. Several diversity indices were calculated, including species richness (S), Simpson's index (D), index of similarity (1 − D), reciprocal index (1/D), Shannon–Wiener index (H), and evenness (E) to comprehensively describe population structure and diversity. Calculations were performed using the online biodiversity calculator available at https://www.virtue.gmbl.se/english-content/biodiversity-calculator.

RAPD-derived genetic diversity indices:

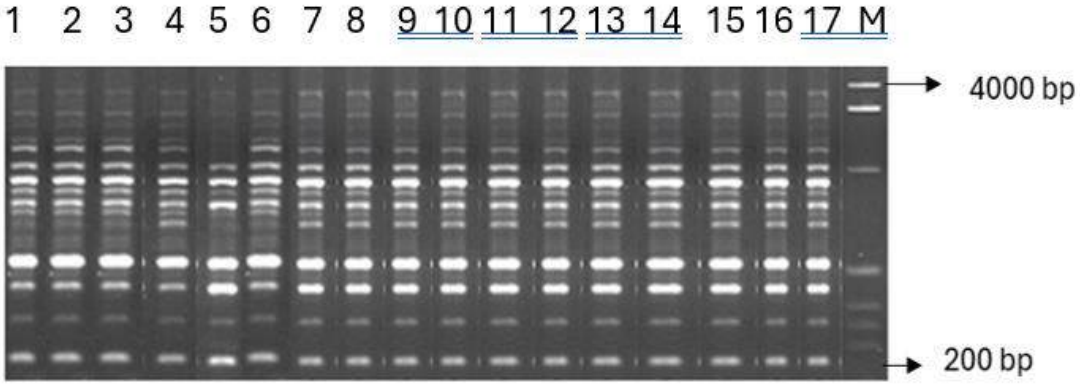
RAPD banding patterns were scored as binary data and used to calculate diversity indices based on band frequency distributions. Species richness (S), Simpson's index (D), Simpson's index of diversity (1−D), Simpson's reciprocal index (1/D), Shannon–Wiener index (H), and evenness (E) were employed to comprehensively assess genome-wide genetic diversity among *Cryptococcus* isolates. These indices collectively capture the extent of polymorphism, dominance of common RAPD band, effective diversity, and distribution balance, and are well suited for dominant RAPD markers where allele-based estimates are unreliable.

Results:

RAPD profile and genetic diversity of *Cryptococcus* isolates:

The RAPD profiles of the eighteen (18) *Cryptococcus* isolates analysed revealed clear and reproducible amplification patterns across multiple loci, indicating substantial polymorphism and genetic diversity within the population (Table 1, Plate 1). Most isolates produced 10–13 bands, with RAPD loci ranging from 330 to 3800 bp, whereas isolate 18 failed to amplify with all primer combinations and was excluded from further analysis. The banding pat terns demonstrated that not all isolates shared identical profiles, reflecting significant genetic variation. For example, the 330 bp locus was present only in isolates 5 and 7, whereas loci ranging from 1332 bp to 3800 bp were conserved across most isolates. Other loci, including 333, 932, 934, 2031, and 2329 bp, exhibited greater variability among isolates.

Based on the presence or absence of DNA fragments across 17 polymorphic loci, the isolates were classified into five haplotypes (A–E) (Table 1). Haplotype B was the most prevalent, encompassing 11 isolates (61.1%), whereas isolates 5, 6, and 7 displayed unique banding patterns and were assigned to haplotypes C, D, and E, respectively. Haplotype A included isolates 1–3, which shared identical profiles across all loci. Overall, the RAPD analysis demonstrates both conserved and variable genomic regions, reflecting the genetic heterogeneity of the *Cryptococcus* population.



M – 200 bp molecular marker; 1: SB6 = *C. neoformans*; 2: SD1 = *C. neoformans*; 3: SD2 = *C. neoformans*; 4: SE3 = *C. neoformans*; 5: SG1 = *C. neoformans*; 6: SG3 = *C. neoformans*; 7: SG7 = *C. neoformans*; 8: SG8 = *C. neoformans*; 9: SH1 = *C. neoformans*; 10: SH2 = *C. gattii*; 11: SJ1 = *C. neoformans*; 12: SL1 = *C. neoformans*; 13: SM1 = *C. neoformans*; 14: SP1 = *C. neoformans*; 15: SQ1 = *C. neoformans*; 16: SQ2 = *C. gattii*; 17: SR1 = *C. neoformans*; 18: SR2 = *C. neoformans*.

Plate 1: RAPD profile of *Cryptococcus* isolates

Table 1: RAPD profile and haplotype assignment of *Cryptococcus* isolates

RAPD Loci (bp)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
330	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
333	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	-
932	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-
934	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
1332	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
1665	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
2031	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-
2198	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
2329	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
2331	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
2531	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
2664	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
2797	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
3064	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
3400	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
3800	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Haplotype	**A**	**A**	**A**	**B**	**C**	**D**	**E**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**-**

+ = presence, – = absence; Haplotypes A–E correspond to distinct RAPD profiles. 1: SB6 = *C. neoformans*; 2: SD1 = *C. neoformans*; 3: SD2 = *C. neoformans*; 4: SE3 = *C. neoformans*; 5: SG1 = *C. neoformans*; 6: SG3 = *C. neoformans*; 7: SG7 = *C. neoformans*; 8: SG8 = *C. neoformans*; 9: SH1 = *C. neoformans*; 10: SH2 = *C. gattii*; 11: SJ1 = *C. neoformans*; 12: SL1 = *C. neoformans*; 13: SM1 = *C. neoformans*; 14: SP1 = *C. neoformans*; 15: SQ1 = *C. neoformans*; 16: SQ2 = *C. gattii*; 17: SR1 = *C. neoformans*; 18: SR2 = *C. neoformans*.

Table 2: Population-level genetic diversity of *Cryptococcus* isolates

Diversity index	Value
Total polymorphic bands (TNOPB)	214
Species richness (S)	17
Simpson's index (D)	0.059
Simpson's index of diversity (1−D)	0.941
Simpson's reciprocal index (1/D)	16.95
Shannon–Wiener index (H')	2.83
Evenness (E)	0.999

NOPB- number of polymorphic bands; TNOPB- total number of polymorphic bands; G.D- genetic diversity; S- species richness; D- Simpson's index; 1-D- Simpson's index of diversity; 1/D- Simpson's reciprocal index; H- Shannon Weiner index; E- evenness;

The genetic diversity of the isolates was further quantified using multiple diversity indices (Table 2). Across the population, a total of 214 polymorphic bands were identified. Most isolates (n=12, 66.7%) exhibited 13 polymorphic bands each, isolate 5 produced 10 bands, and one isolate (isolate 18) did not yield amplification products. The species richness (S) was 17, indicating the number of distinct genetic variants observed among the analysed isolates.

The Simpson's index (D) was 0.059, reflecting a low probability that two randomly selected isolates share the same genotype, while the Simpson's index of diversity (1 − D) was 0.941, underscoring high genetic variability. The Simpson's reciprocal index (1/D) was 16.95, supporting the presence of multiple genetically distinct individuals.

The Shannon–Wiener diversity index (H') was 2.83, indicating moderate-to-high genetic variation, and the evenness value (E) was 0.999, suggesting an almost uniform distribution of genetic variants across isolates. Collectively, these indices confirm the high *g*enetic diversity of the *Cryptococcus* population examined in this study and the broad distribution of polymorphic loci.

Primer-specific RAPD polymorphism:

The RAPD analysis of the *Cryptococcus* isolates revealed substantial genetic polymorphism across the primer combinations tested (Table 3). A total of 20 to 40 polymorphic bands were amplified per primer pair, with band sizes ranging from 330 to 3800 bp, indicating high variability within the population analysed. Several primer combinations demonstrated both resolving and diagnostic capacity within the analysed dataset. For example, the CN1–5SOR primer pair produced a 934 bp band that was diagnostic for haplotype D, while FPK1-05–FPK1-07 amplified bands that were diagnostic for haplotypes B and D. Other primer combinations, such as CN1–MYC1 and MYC1–5SOR, resolved multiple haplotypes, distinguishing groups such as SG1, SG3, SH2, and others, although no single band was uniquely diagnostic across all isolates.

The FPK1-01–FPK1-05 combination amplified the highest number of polymorphic loci, facilitating the differentiation of closely related haplotypes SB6, SD1, and SD2. Simi-

larly, FPK1-08–FPK1-20 produced distinct bands that aided in differentiating SQ1, SQ2, and SR1 isolates. These results collectively indicate that the selected RAPD primers are effective both in distinguishing most *C. neoformans* isolates and in identifying specific haplotypes through diagnostic bands, highlighting the utility of RAPD profiling for molecular characterization of environmental isolates rather than direct inference of clinical or epidemiological relationships.

Discussion:

Random amplified polymorphic DNA (RAPD) markers are widely recognized as effective tools for assessing genetic diversity and population structure in fungal pathogens, including *Cryptococcus* species (17,23,24,28). In the present study, RAPD analysis of 17 *Cryptococcus* isolates generated a total of 214 bands, with an average of 10–13 polymorphic bands per isolate. Each band represents a putative genetic locus that may be present or absent, and the resulting patterns revealed substantial genetic heterogeneity, resolving the isolates into five distinct haplotypes (A–E). Haplotype B was the most prevalent, encompassing 11 isolates (61.1%), while isolates 5, 6, and 7 were assigned to unique haplotypes C, D, and E, respectively, and haplotype A included isolates 1–3. The high proportion of polymorphic loci, even though all isolates originating from a single environmental source underscores the ability of RAPD markers to uncover fine-scale genetic variation within apparently homogeneous populations.

The primer-specific RAPD profiles also contributed significantly to resolving this diversity. For example, the CN1–MYC1 primer pair (20–22 mer) produced 30 polymorphic bands (330–3063 bp), differentiating haplotypes A, B, and C. The CN1–5SOR combination (20–22 mer) amplified a 934 bp band that was diagnostic for haplotype D, while FPK1-05–FPK1-07 (12-mer) produced bands at 2197.8 and 2530.8 bp that were diagnostic for haplotypes B and D. Other primer combinations, such as MYC1–5SOR and FPK1-05–FPK1-13, resolved multiple haplotypes, including SG1, SG3, SH2, and SG7, whereas FPK1-01–FPK1-05 amplified the greatest number of polymorphic loci, thereby facilitating discrimination of closely related isolates (SB6, SD1, SD2). FPK1-08–FPK1-20 further aided differentiation of SQ1, SQ2, and SR1 isolates.

These results indicate that the selected primers were consistently effective in capturing both intra- and inter-haplotype variation with certain bands serving as diagnostic markers for specific haplotypes.

The quantitative diversity indices further support the high level of genetic variability observed. The Shannon–Wiener index (H' = 2.83) indicated moderate-to-high genotype diversity, reflecting both the number of haplotypes and their distribution among isolates (29). Simpson's index (D=0.059) and its complement (1-D=0.941) indicate a low probability that two randomly selected isolates share the same genotype (30), while the reciprocal index (1/D=16.95) confirms the presence of multiple genetically distinct individuals. The evenness value (E=0.999) suggests an almost uniform distribution of haplotypes across the population. Collectively, these indices corroborate the RAPD patterns, demonstrating that genetic diversity is widely distributed rather than dominated by a single genotype (31,32).

The observed diversity aligns with previous RAPD-based studies of *Cryptococcus* (23,24,33,34,35), which report stable and reproducible RAPD profiles that reflect genuine genetic differences rather than methodological artifacts. Unlike some studies in which isolates cluster strictly by clinical or environmental origin (23,33), the recovery of multiple haplotypes and the absence of strong clustering in this study suggest that microenvironmental complexity within the sampling site may drive diversification, even in geographically restricted niches. Such localized ecological pressures likely contribute to the extensive polymorphism observed, reinforcing the idea that *Crypto coccus* populations can be genetically heterogeneous within small environmental regions.

At a broader scale, these findings are consistent with global trends in *Cryptococcus* diversity. Strains from sub-Saharan Africa, particularly southern Africa, exhibit remarkable genetic heterogeneity (36), with populations described as geographically restricted yet genetically diverse, likely due to long-term endemicity, complex environmental reservoirs and historical recombination events. Genome-based analyses further support this, showing higher nucleotide diversity in African isolates relative to other populations (37). Although RAPD markers offer lower resolution than whole-genome sequencing, the diversity observed in this study mirrors these global patterns, suggesting that the isolates analyzed represent part of a broader, genetically diverse regional gene pool. From biological and clinical perspectives, this variability is significant because it underpins microevolutionary processes that can generate phenotypic differences.

Table 3: Primer-wise RAPD bands and haplotype resolution in *Cryptococcus* isolates

Primer combination	**Primer type**	**Number of polymorphic bands**	**Band size range (bp)**	**Representative bands (bp)**	**Key haplotypes resolved**	**Haplotype differentiation**
CN1–MYC1	20–22 mer	30	330–3063	330, 333, 932.4, 1665, 2530.8	A, B, C	Differentiates most *C. neoformans* isolates
CN1–5SOR	20–22 mer	20	334–934	334, 934	B, D	Band at 934 bp diagnostic for haplotype D
MYC1–5SOR	20–22 mer	26	1332–2031	1332, 2031.3	C, E	Resolves SG1, SG3, SH2
FPK1-01–FPK1-05	12-mer	40	330–3800	330, 333, 932.4, 3400, 3800	A	Highest number of polymorphic loci; distinguishes SB6, SD1, SD2
FPK1-05–FPK1-07	12-mer	35	2197–2531	2197.8, 2530.8	B, D	Diagnostic bands for haplotypes B and D
FPK1-05–FPK1-13	12-mer	35	932–2797	932.4, 2797.2	C, E	Resolves SG3, SG7, SH2
FPK1-08–FPK1-20	12-mer	28	2664–3800	2664, 2797.2, 3800	B, C	Distinct bands facilitate differentiation of SQ1, SQ2, SR1

In this study, no clear correlation was observed between RAPD genotypes and phenotypic traits, consistent with previous reports (23,38). Nevertheless, the extensive genetic heterogeneity suggests the presence of underlying variation that may influence virulence, antifungal susceptibility, and environmental persistence. Previous studies have demonstrated that genetic diversity within *Cryptococcus* populations can affect disease severity, therapeutic outcomes, and even the coexistence of multiple species within a single host (39,40).

Finally, it is plausible that the observed diversity reflects not only nucleotide-level polymorphisms, but it is reasonable to hypothesize that the observed diversity could also be influenced by epigenetic mechanisms. Processes such as DNA methylation, histone modification, and non-coding RNA regulation are known to modulate gene expression without changing the DNA sequence (41,42). Previous studies in *C. neoformans* and *C. gattii* have linked epigenetic variation to capsule production and melanin synthesis (43,44,45), however confirming such contributions in this context will require targeted epigenetic analysis.

Conclusion:

In conclusion, RAPD analysis of environmental *Cryptococcus* isolates showed substantial genetic diversity and multiple distinct haplotypes, demonstrating the utility of RAPD markers in capturing population-level variation. Primer-specific amplification patterns highlighted diagnostic bands that distinguish key haplotypes, while quantitative diversity indices confirmed high heterogeneity across the population. These findings underscore the ecological, biological, and potential clinical significance of *Cryptococcus* genetic diversity within restricted environmental niches.

Contributions of authors:

MOC and BTT were involved in the study conceptualization and design; MOC, BTT and ODP were involved in the methodology and data analysis; MOC wrote the original draft; BTT, ODP and MOE reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest:

No conflict of interest is declared.

Availability of data and materials:

All data generated or analysed during this study are included in this manuscript.

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Original Article **Open Access**

Profile of multidrug-resistant bacteria in neonatal nosocomial infections at the Brazzaville University Hospital, Congo

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Abstract:

Background: Antibiotic resistance represents a major threat in neonatology, particularly in sub-Saharan Africa where local data remain limited. The present study aims to establish the profile of multidrug-resistant bacteria isolated from cases of neonatal nosocomial infections at the Brazzaville University Hospital, Congo.
Methodology: This analytical cross-sectional study, conducted at the Brazzaville University Hospital on newborns with nosocomial infections between May and September 2023, characterized the bacterial organisms and their antimicrobial resistance profiles. Blood samples were collected from the neonates and cultured for bacterial isolation using conventional bacteriology methods. Antibiotic susceptibility test of each isolated bacterium was determined by the disc diffusion method and interpreted according to CA-SFM 2023 standards.
Results: Of 542 hospitalized patients during the period of study, 97 were neonates, 33 of whom had nosocomial infections, giving a prevalence of 34.0%. Bacteremia was the most common nosocomial infection with 78.8%, pneumonia (12.2%), urinary catheter infection (6.0%) and omphalitis (3.0%). Enterobacteriaceae, notably *Klebsiella pneumoniae* and *Escherichia coli*, dominated the infectious landscape, with marked multi-drug resistance (MDR) profile, nearly 80.0% of which is due to presumptive carbapenemase production. Gram-positive cocci and non-fermentative bacilli also present high resistance profiles. Factors significantly associated with nosocomial neonatal infection on multivariate analysis are low birthweight (<2500g) (OR=5.27, p=0.014) and requirement for oxygen (OR=8.12, p=0.003). But factors such as prematurity and the place of care of the newborns were not significantly associated with neonatal nosocomial infection on multivariate analysis (p>0.05).
Conclusion: These results highlight the vulnerability of newborns with low birth weight to nosocomial infection caused by MDR bacterial pathogen. There is need to strengthen antibiotic stewardship and integrate local data into hospital surveillance and infection prevention strategies.

Keywords: Nosocomial; Neonate; Infection; Multidrug resistance; Brazzaville-Congo

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Profil des bactéries multirésistantes dans les infections nosocomiales néonatales au CHU de Brazzaville, Congo

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Résumé:

Contexte: La résistance aux antibiotiques représente une menace majeure en néonatologie, particulièrement en Afrique subsaharienne où les données locales restent limitées. La présente étude vise à établir le profil des bactéries multirésistantes isolées chez des nouveau-nés atteints d'infections nosocomiales au CHU de Brazzaville, Congo.
Méthodologie: Cette étude transversale analytique, menée au CHU de Brazzaville auprès de nouveau-nés présentant des infections nosocomiales entre mai et septembre 2023, a caractérisé les bactéries isolées et leurs profils de résistance aux antimicrobiens. Des prélèvements sanguins ont été effectués chez les nouveau-nés et mis en culture pour l'isolement bactérien selon les méthodes bactériologiques conventionnelles. La sensibilité aux antibiotiques de chaque bactérie isolée a été déterminée par la méthode de diffusion sur disque et interprétée selon les normes CA-SFM 2023.
Résultats: Sur 542 patients hospitalisés pendant la période d'étude, 97 étaient des nouveau-nés, dont 33 présentaient une infection nosocomiale, soit une prévalence de 34,0%. La bactériémie était l'infection nosocomiale la plus fréquente (78,8%), suivie de la pneumonie (12,2%), de l'infection liée à une sonde urinaire (6,0%) et de l'omphalite (3,0%). Les entérobactéries, notamment *Klebsiella pneumoniae* et *Escherichia coli,* ont dominé le paysage infectieux, présentant un profil de multirésistance (MDR) marqué, dont près de 80.0% est dû à une production présumée de carbapénémases. Les cocci Gram positifs et les bacilles non fermentaires présentent également des profils de résistance élevés. Les facteurs significativement associés à l'infection néonatale nosocomiale en analyse multivariée sont un faible poids de naissance (<2500 g) (OR=5,27, p=0,014) et un besoin en oxygène (OR=8,12, p=0,003). En revanche, des facteurs tels que la prématurité et le lieu de prise en charge des nouveau-nés n'étaient pas significativement associés à l'infection néonatale nosocomiale en analyse multivariée (p>0,05).
Conclusion: Ces résultats soulignent la vulnérabilité des nouveau-nés de faible poids de naissance aux infections nosocomiales causées par des bactéries multirésistantes. Il est nécessaire de renforcer la gestion des antibiotiques et d'intégrer les données locales dans la surveillance hospitalière et les stratégies de prévention des infections.

Mots-clés: Infection nosocomiale; Nouveau-né; Infection; Multirésistance aux antibiotiques; Brazzaville-Congo

Introduction:

Antimicrobial resistance (AMR) is one of the most formidable challenges in global public health today. It is a complex phenomenon in which antimicrobial agents lose their effectiveness against microorganisms that have acquired defense mechanisms, often of genetic origin (1). Described as a "silent pandemic" by the World Health Organization (WHO), it was responsible for 1.27 million deaths worldwide in 2019, illustrating its devastating impact (2). In Europe, there are more than 33,000 deaths annually attributable to multidrug-resistant (MDR) bacteria (3), while in sub-Saharan Africa, this scourge is worsening in a context of limited resources, with an estimated mortality rate of 27.3 deaths per 100,000 inhabitants (4).

The hospital settings, particularly neonatal intensive care units, constitute high-risk environments for the emergence and spread of these resistant pathogens. Neonatal nosocomial infections (NNIs) refer to all infections acquired within a healthcare facility by newborns after admission. Their frequency depends on various factors such as early diagnosis, nature of invasive care, quality of microbiological control and patient immune profile. Although widely reported in both industrialized and developing countries, the incidence of NNIs varies significantly depending on the context. Estimated at 2.5% of hospitalized newborns in Europe (5), it reaches 15.8% in North Africa (6) and varies between 10% and 60% in sub-Saharan Africa (7).

In Congo, a study conducted at Brazzaville University Hospital reported a frequency of 1% of nosocomial neonatal bacterial infections among hospitalized newborns (8). Beyond their frequency, NNIs are associated with high morbidity and mortality, prolonged hospital stays, and significant treatment costs (9,10). The role of MDRs in these infections is worrying, with a distribution and resistance profile varying between units and regions. International data highlight this heterogeneity, in Iran, Rafati et al., (11) observed high resistance of Gram-negative bacilli to ceftazidime (72.7%), cefotaxime (54.5%), and ceftriaxone (60%); in Brazil, Limal et al., (12) reported that 14.9% of NNIs were attributable to MDRs.

At the Brazzaville University Hospital, the most frequently isolated bacterial pathogens in newborns are Gram-negative bacilli, including MDR *K. pneumoniae* (8), but local data remain incomplete regarding the resistance spectrum of the other pathogens involved. In this context, the present study aims to establish the profile of MDR bacteria isolated in cases of neonatal nosocomial infections at the Brazzaville University Hospital. This is to provide updated and contextualized data in order to strengthen therapeutic management, optimize antibiotic protocols, and contribute to the implementation of targeted surveillance and prevention strategies.

Materials and method:

Study design:

This analytical cross-sectional study was conducted at the neonatology and bacteriology-

immunology-virology departments of the University Hospital of Brazzaville, Congo, over a 4-month period (May–September 2023).

Ethical consideration:

The study respected the principles of confidentiality by anonymizing the data. Administrative authorizations were obtained from the Brazzaville University Hospital and the Research Ethics Committee (decision no. 054-40/MERSIT/DGRST/CERSSA/-23). Informed consent of the parents was systematically obtaimed. No medical risks were associated with participation.

Study population, participants and clinical data:

The study population included all neonates hospitalized during the period of study (n=97) from whom 33 neonates who have been hospitalized for more than 48 hours, with no documented infection at admission (CRP <20 mg/L and negative blood culture), and whose parents provided informed consent were included as study participants. Neonates with pre-analytical abnormalities or pre-existing neonatal infections were excluded.

Exhaustive blood sampling of each neonate was performed throughout the study period. A nosocomial infection was considered if the CRP exceeded 20 mg/L after 48 hours of hospitalization, associated with a positive blood culture (Fig 1).

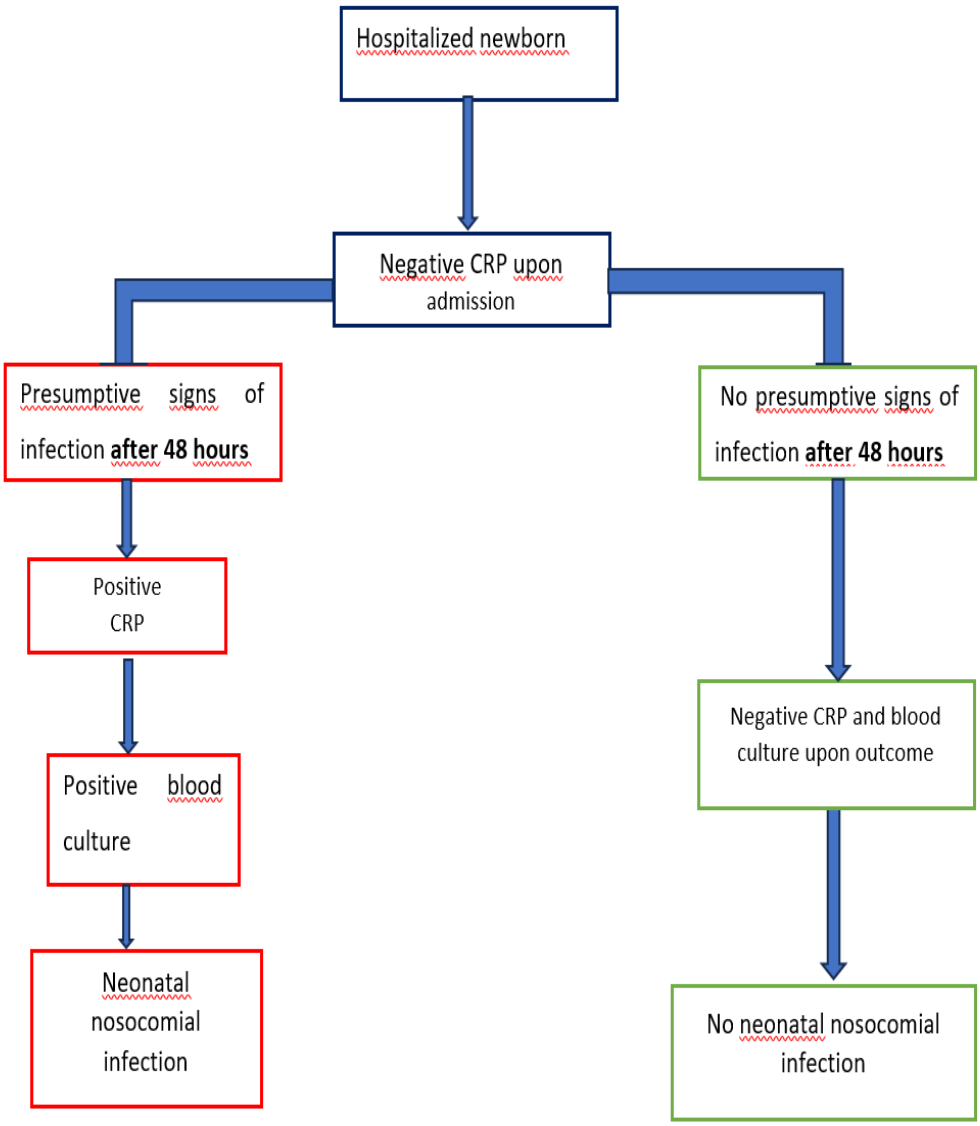


Fig 1: Diagnostic algorithm for neonatal nosocomial infections

Bacteriological analysis:

Blood culture:

For each neonate, 1-2 ml of whole blood was collected by peripheral venipuncture before antibiotic administration. Sampling was performed according to a strict protocol validated by the Hospital Infection Control Committee with the use of surgical mask, hand hygiene with an alcohol-based antiseptic solution, sterile gloves, disinfection of the puncture site (70% alcohol followed by an iodine-based product) and disinfection of the bottle septum.

The blood was inoculated into PF Plus bottles (bioMérieux) containing liquid medium that supports the growth of strict aerobic and facultative anaerobic bacteria. These bottles, equipped with a perforable rubber septum, were immediately transported to the laboratory. Blood culture bottles were incubated in a conventional incubator at 37°C for 5 days. Subcultures from the broth were performed on Mueller-Hinton agar at day 1, day 3, and day 5. Cultures remaining negative after 5 days were declared sterile; any culture positive at least once was retained for identification.

Bacterial identification and antibiotic Susceptibility:

Positive blood culture bottles were subcultured on solid culture media (Blood and MacConkey agar) and bacterial identification was performed using conventional methods (morphology, Gram staining, manual biochemical tests, Biomérieux® API galleries), in accordance with the laboratory's technical capabilities.

A standardized antibiotic susceptibity test (AST) was performed in Mueller-Hinton agar medium by the disc diffusion methd according to the CA-SFM 2023 recommendations. Selected antibiotic discs were tested in different classes; beta lactams, aminoglycosides, quinolones, nitrofurans, and trimethoprim-sulfamethoxazole. Inhibition zones were measured and interpreted to classify strains as sensitive, intermediate, or resistant in line with the CA-SFM 2023 guideline.

Phenotypic resistance detection:

Phenotypic detection of ESBLs was performed by double disc synergy testing with amoxicillin-clavulanic acid and 3rd generation cephalosporin discs. Carbapenemases and AmpC-type cephalosporinases were infered from the AST but not confirmed by specific or molecular tests due to lack of technical resources. Multi-drug-resistant in bacteria isolate was defined as resistance to ≥ 3 classes of antibiotics.

Statistical analysis:

Data were entered into CSPro 7.4 labtop computer and analyzed using SPSS version 24.0 and R software. Given the small sample size and the categorical nature of the variables studied, only Fisher's exact test was used to compare proportions between groups. The significance threshold was set at $p<0.05$.

Results:

Sociodemographic characteristics of newborns with nosocomial infection:

During the study period, 97 out of 542 hospitalized patients were included (54 females and 43 males). Among the 97 patients, 33 had nosocomial infection, giving a prevalence of 34.0%. Eighteen of the 54 (33.3%) females and 15 (34.9%) of the 43 males had nosocomial infection, but there was no significant difference in the prevalence of nosocomial infection with respect to sex (OR=0.9333, 95% CI=0.4011-2.1720), p=0.8728). Bacteremia was the most common nosocomial infection with 78.8% (n=26) followed by pneumonia (12.2%, n=4), catheter infection (6.0%, n=2) and omphalitis (3.0%, n=1) (Fig 2).

Factors associated wth neonatal nosocomial infections:

The prevalence of nosocomaial neonatal infections was significantly higher in neonates with birth weight <2500g (45.6%, n=26) than neonates with birth weigh >2.5g (21.9%, n=7) in both univariate (OR 3.00, p=0.029) and multivariate analyses (OR 5.27, p=0.014). The prevalence of nosocomaial neonatal infections was also significantly higher in neonates on oxygen therapy (48.3%, n=29) compared to those not on oxygen therapy (13.8%, n=4) in both univariate (OR 5.85, p=0.003) and multivariate analyses (OR 8.12, p=0.003. However, However, factors such as prematurity and the place of care of the newborns were not significantly associated with prevalence of neonatal nosocomial infection (Table 1).

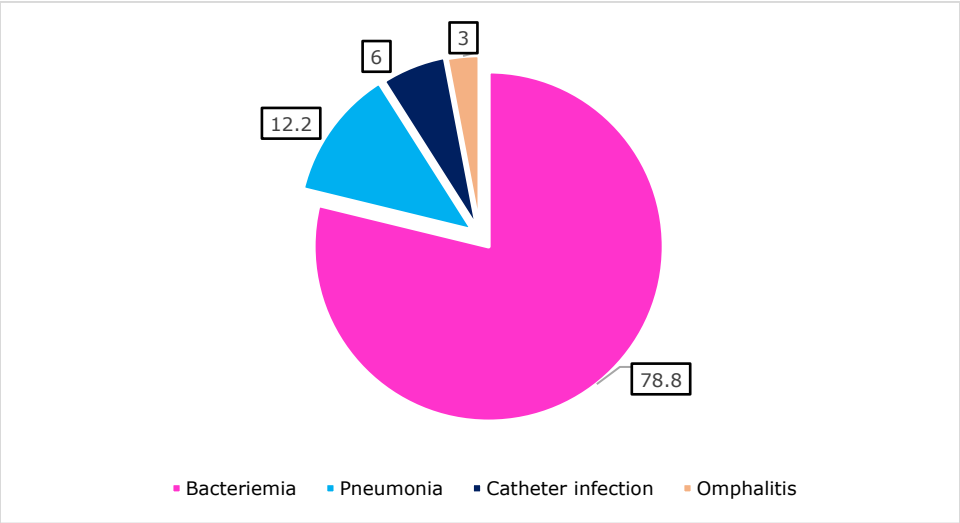


Fig 2: Types of neonatal nosocomial infections

Table 1: Univariate and multivariate analysis of risk factors for neonatal nosocomial infections

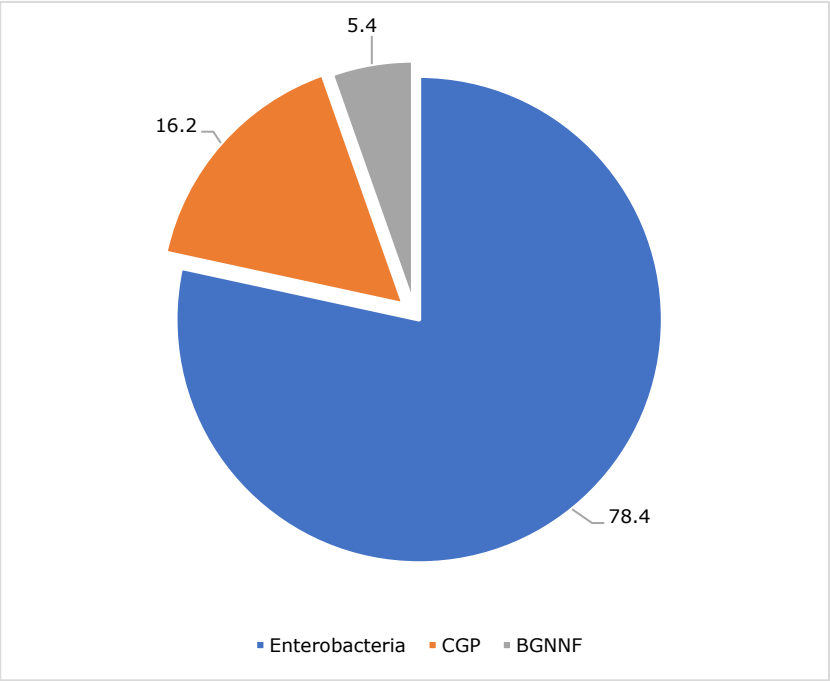
Risk factors	Nosocomial infection		OR (95% CI) *p*-value (univariable)	OR (95% CI) *p*-value (multivariable)
	Yes (n=33, 34.0%)	No (n=64, 66.0%)		
Birth weight (g)				
> 2500	7 (21.9)	25 (78.1)	3.00 (1.16-8.52) (*p*=0.029*)	5.27 (1.50-22.07) (*p*=0.014*)
< 2500	26 (45.6)	31 (54.4)		
Oxygen therapy				
No	4 (13.8)	25 (86.2)	5.85 (1.98-21.68) (*p*=0.003*)	8.12 (2.20-38.37) (*p*=0.003*
Yes	29 (48.3)	31 (51.7)		
Premature birth				
No	10 (27.0)	27 (73.0)	2.14 (0.88-5.48) (*p*=0.101)	0.59 (0.10-2.97) (*p*=0.526)
Yes	23 (44.2)	29 (55.8)		
Place of care				
Crib	10 (26.3)	28 (73.7)	2.48 (1.01-6.38) (*p*=0.05)	1.00 (0.19-5.34) (*p*=0.998)
Incubator	23 (46.9)	26 (53.1)		

* = Statistically significant at $p<0.05$

Frequency distribution of bacteria isolates of neonatal nosocomial infections:

A total of 37 bacteria were isolated from the 33 cases of neonatal nosocomial infections at the Brazzaville University Hospital and this is dominated by Enterobacteriaceae (78.4%, n= 29). Gram-positive cocci represent 16.2% (n=6) of isolates, while Non-Fermentative Gram-negative bacilli (NFGNB) are rare (5.4%, n=2) (Fig 3).

The most frequent members of the family Enterobacteriaceae, were *Klebsiella pneumoniae* (27%, n=10), *Escherichia coli* (16.3%, n=6), and *Enterobacter aerogenes* (10.8%, n=4) (Table 2). The distribution according to multi-drug resistant (MDR) bacteria strains is shown in Table 3, with 78.4 % (n=28) of the isolates being MDR.



NFGNB: Non-fermenting Gram-negative bacilli; GPC: Gram-positive cocci

Fig 3: Frequency distribution of bacterial isolates of neonatal nosocomial infections

Table 2: Distribution of bacterial isolates responsible for nosocomial infections

Bacterial isolates	**Frequency**	**Percentage (%)**
Enterobacteriaceae		
Klebsiella pneumoniae	10	27.0
Escherichia coli	6	16.3
Enterobacter aerogenes	4	10.8
Klebsiella spp	2	5.4
Serretia liquefasciens	2	5.4
Pantoea spp	2	5.4
Enterobacter cloacae	1	2.7
Raoutella terrigena	1	2.7
Raoutella ornithinolytica	1	2.7
NFGNB*		
Pseudomonas luteola	1	2.7
Pseudomonas aeruginosa	1	2.7
GPC**		
Staphylococcus spp	4	10.8
Staphylococcus aureus	1	2.7
Enterococcus spp	1	2.7
Total	**37**	**100.0**

NFGNB: Non-fermenting Gram-negative bacilli; GPC: Gram-positive cocci

Table 3: Distribution of multidrug-resistant bacteria (MDR) isolated from neonates with nosocomial infections at the Brazzaville University Hospital, Congo

Bacterial species	Total number of isolates	Number of MDR isolates	Percentage of MDR isolates
Klebsiella pneumoniae	10	10	100.0
Escherichia coli	6	6	100.0
Enterobacter aerogenes	4	4	100.0
Staphylococcus spp	4	3	75.0
Pantoea spp	2	2	100.0
Serratia liquefaciens	2	1	50.0
Raoultella terrigena	1	1	100.0
Staphylococcus aureus	1	1	100.0
Pseudomonas aeruginosa	1	1	100
Pseudomonas luteola	1	0	0
Enterococcus spp	1	0	0
Klebsiella spp	2	0	0
Raoutella terrigena	1	0	0
Raoutella ornithinolytica	1	0	0
Total	**37**	**29**	**78.4**

MDR: Multi-resistant bacteria (resistance to at least three families of antibiotics). Resistance involved the following main agents: ceftriaxone, ceftazidime, imipenem, tobramycin, ciprofloxacin, and pefloxacin.

Phenotype of bacterial resistance to β-lactam antibiotics:

Among the 23 multidrug-resistant Enterobacteriaceae identified, 18 (78.3%) expressed carbapenemase phenotype, indicating worrying resistance to carbapenems. High-level cephalosporinases were detected in 4 (17.4%) cases, while ESBLs was seen in only 1 (4.3%) strain. No wild-type phenotypes or single-penicillinase producers was observed.

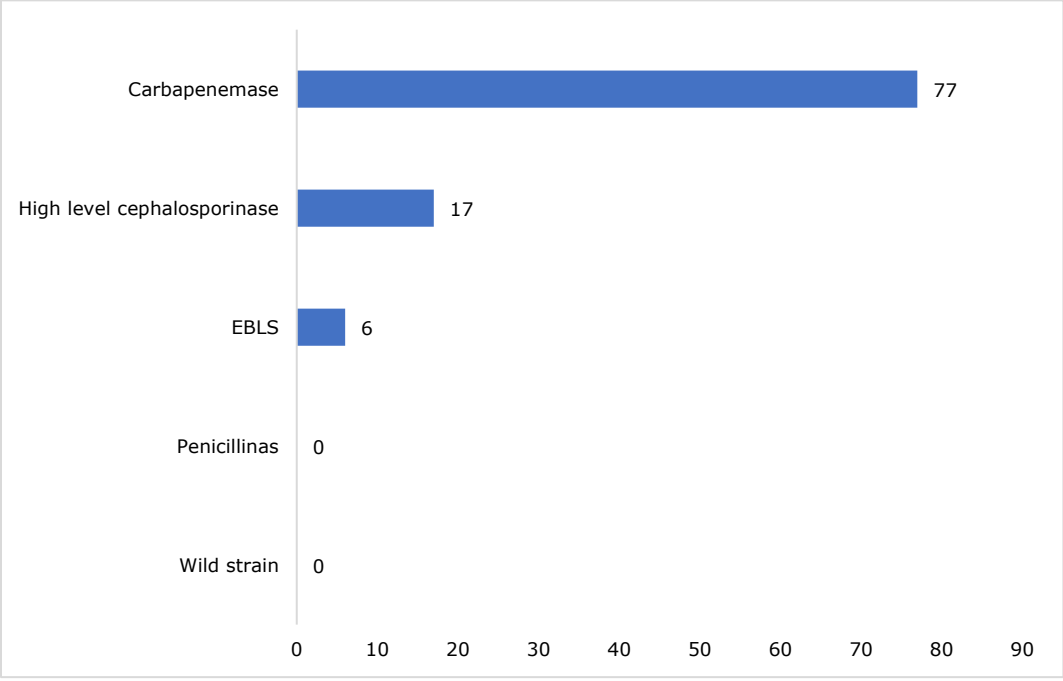


Fig 3: Phenotype of resistance of enterobacteria to beta-lactams

Discussion:

The frequency of neonatal nosocomial infection (NNI) in our study was 33.7%, similar to 33.0% reported by Lasme-Guillao et al., (13) in Abidjan. However, lower frequencies than ours have been reported in the United States and Europe, with rates ranging from 3.58% to 11.1% and 1.6% to 13.2%, respectively (14), and also in Africa, particularly in the Democratic Republic of Congo, with an incidence of 22.3%. These variations in frequency may be due to the criteria for defining NNI, the inclusion criteria, the sample size, and the study duration, which varied from one study to another, but also due to better hygiene conditions in Western countries.

We noted a slight female predominance in the study, with a sex ratio of 0.8. Chabni et al., (15) reported a sex ratio of 0.9, Bukasa et al., (14) also found a female predominance. The risk of developing NNI was higher in low birth weight newborns (p=0.029) which is identical to the findings of Auriti et al., (16) in Italy and Rangelova et al., (17) in Bulgaria. Indeed, low birth weight is characteristic of premature newborns, as these infants have immature immune system, making them more vulnerable to infection (18). The main site of nosocomial infections was blood with bacteremia in 78.8%, followed by pneumonia (12.2%). Our results are similar to those reported in the literature (18). Other localizations such as urinary tract infection, are very rarely diagnosed in our setting due to the low prevalence of urine cultures usually performed in neonatology.

Regarding factors associated with the occurrence of NNI, this study identified low birth weight, the use of oxygen therapy, and living in an incubator as factors associated with the occurrence of NNI. Newborns with a birth weight <2500g had a 3-fold increased risk of developing the infection in univariate analysis (OR= 3.00; p=0.029) and a 5-fold in multivariate analysis (OR=5.27; p=0.014). Those who were on oxygen therapy had a 6 times greater risk (OR =5.85; p=0.003) in univariate analysis and 8 times in multivariate analysis (OR=8.12; p= 0.003) of being infected by nosocomial bacterial pathogens. Newborns placed in an incubator also had a greater risk of developing a nosocomial infection in univariate analysis (OR=2.48; p=0.05) but not in multivariate analysis (OR= 1.00; p=0.998). The use of an incubator was also found to be a factor associated with the occurrence of infection in the study carried out in Madagascar on nosocomial bacteremia in neonatology (OR=6.78; 95% CI: 1.66-27.72; p=0.003) (19). Li Wang et al., (18) reported that low birth weight is a risk factor (OR=3.44, 95% CI: 2.31–5.11) among other factors such as use of mechanical ventilation (OR=3.16, 95% CI: 2.21–4.50) and gestational age of infection (OR=2.58).

The main bacterial pathogens isolated in our study were Gram-negative bacilli, particularly Enterobacteriaceae, dominated by *K. pneumoniae* (27.0%), followed by *E. coli* (17.0%) and *Enterobacter aerogenes* (10.0%). Gram-positive cocci were also isolated notably *Staphylococcus* spp in 10% of cases. Chemsi et al., (20) in Morocco, Ngakengni et al., (8) in Congo also reported *K. pneumoniae* as the main bacteria responsible for NNIs with respective frequencies of 37% and 50%. Among the isolated bacterial pathogens, none was found in its wild form; all the bacteria were therefore resistant. This resistance concerned the main antimicrobial molecules used in current practice in hospitals, such as ceftriaxone, ceftazidime, imipenem, tobramycin, ciprofloxacin, and pefloxacin but good activity was noted with gentamicin. These data thus call into question current standard therapeutic maanagement and shows the value of rapid blood culture with antibiotic sensitivity of isolated bacteria, with a view to better therapeutic management of patients with bacteremia.

The resistance to numerous antimicrobial molecules explains the prevalence of MDR bacteria in our study. These bacteria were MDR in 78.4 % of cases. All strains of *K. pneumoniae*, *E. coli* and *E. aerogenes* were MDR. The emergence and spread of antibiotic resistance represent a real threat to public health worldwide. Recent data in the literature report numerous cases of MDR or even totally-drug resistant (TDR) or pandrug resistant (PDR) bacteria to antibiotics, the number of which continues to increase both in industrialized and developing countries (20,21). The situation is alarming in resource-limited countries where infectious diseases, poverty, and malnutrition are endemic.

Regarding the phenotypes of resistance of Enterobacteriaceae to beta-lactams, they were dominated by the carbapenemase profile in 77.0% followed by the high-level cephalosporinase profile in 17.0% and the ESBL profile in 6.0% but we could not detect enzymes encoded by plasmid genes such as bla_{KPC}, bla_{NDM}, bla_{OXA-48}, which confer resistance to carbapenems (22,23) in our study due to lack of resources. This high prevalence of resistance profile is particularly worrying in neonatology, where therapeutic options are limited. However, the low prevalence of ESBL (4.3%) contrasts with the usual regional trends and could reflect a local evolution of the resistance profile or increased selective pressure linked to the intensive

use of carbapenems. This difference could be related to the small size of our sample but shows a worrying presence of MDR bacteria resistant to carbapenems. The presence of high-level cephalosporinases (17.4%) reinforces the therapeutic complexity, these enzymes being often inducible and not inhibited by clavulanic acid, which limits the effectiveness of classic combinations. In Congo Brazzaville, no study on the determination of carbapenemase genes has been carried out to date to highlight the different types.

With regard to these MDR strains, the therapeutic arsenal is extremely limited, particularly in pediatrics, requiring the administration of combinations of antibiotics which use in children is still poorly documented. Nevertheless, the present study allows us to propose a therapeutic protocol based on the bacterial ecology encountered in the neonatology department of CHU-B and the resistance phenotypes. However, it would be important to carry out the analysis of blood cultures and associate it with antibiotic susceptibility test results to confirm the detection of resistance. Thus, the use of therapeutic combinations such as ciprofloxacin and amikacin in cases of ESBL-producing bacteria and imipenem-relebactam/amikacin or meropenem-varobactam/amikacin could be used in case of MDR bacteria. However, this use should be better regulated, and blood cultures with antibiotic sensitivity tests are essential for diagnosis of MDR bacteria.

Conclusion:

This study highlights the high prevalence of MDR Enterobacteriaceae in neonatal nosocomial infections at the Brazzaville University Hospital, with a worrying predominance of carbapenemases as a resistance mechanism. Sociodemographic data confirm the increased vulnerability of premature and low birth weight neonates, highlighting the need for targeted prevention strategies.

The diversity of bacterial species isolated, including opportunistic and classic hospital pathogens, reflects a complex and dynamic ecology influenced by care practices, the hospital environment, and antibiotic pressure. The observed resistance profiles, particularly to β-lactams, fluoroquinolones, and macrolides, limit therapeutic options and call for a revision of empirical antibiotic protocols.

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Contributions of authors:

ENON conceived and designed the study, supervised data collection, and critically revised the manuscript; TM drafted the manuscript, performed data analysis, and coordinated the revision process; LM conducted bacteriological analyses and contributed to laboratory data interpretation; NN assisted in clinical data collection in neonatology and contributed to manuscript revision; OEN participated in data acquisition and contributed to statistical analysis; and GE provided clinical expertise in neonatology, validated results, and contributed to the discussion section. All authors approved the manuscript submit for publication.

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No confloict of interest is declared.

Data availability:

The dataset of this study is available from the corresponding author on reasonable request.

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Original Article **Open Access**

Bacteriological profile of *Staphylococcus aureus* isolates at the Bogodogo University Hospital Center, Ouagadougou, Burkina Faso

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Abstract:

Background: *Staphylococcus aureus* is a common major human pathogen involved in a wide variety of infections. The objective of this study is to investigate the bacteriological profile of *S. aureus* at the Bogodogo University Hospital Center, Ouagadougou, Burkina Faso.
Methodology: This was a descriptive cross-sectional study conducted from January 1 to December 31, 2024 at the bacteriology-virology department of Bogodogo University Hospital. All biological samples (pus, blood, vaginal swabs, and effusion fluid) received in the laboratory were cultured on mannitol salt agar for isolation of *S. aureus.* The identification of *S. aureus* was based on morphological characteristics of the colonies described as round, smooth, convex colonies, well defined with a smooth, shiny surface, sometimes slightly oily, appearing in irregular clusters resembling "grapes" under the microscope, standard biochemical tests (catalase positive, coagulase positive, and oxidase negative), and the use of an API Staph® panel. Antibiotic susceptibility was determined on Müller-Hinton agar by the Kirby Bauer diffusion method, with inhibition zone diameters read and interpreted according to CASFM/EUCAST recommendations.
Results: A total of 1,530 bacteria were isolated from pathological samples of patients with infections at the hospital during the study period, of which 184 (12.0%) were *S. aureus*. Pus was the most common sample type, accounting for 98 isolates (53.2%). The mean age of the 184 patients is 30±19 years, with a range of 1 to 78 years, and a predominance of females (sex ratio = 0.73). The trauma department contributed the most isolates with 37 (20.1%). The isolates showed high resistance to penicillin G (96.0%, n=178) and erythromycin (42.3%, n=78). In terms of resistance mechanisms, 58.0% of the isolates produced penicillinase and 38.0% were methicillin-resistant *S. aureus* (MRSA) isolates.
Conclusion: This study highlights a high prevalence of *S. aureus* in biological samples of patients with infections (12.0%) at Bogodogo University Hospital, as well as high resistance to beta-lactam antibiotics. These results underscore the need to strengthen surveillance strategies and promote the judicious use of antibiotics in hospitals.

Keywords: *Staphylococcus aureus*; MRSA; Penicillinase; Bogodogo University Hospital; Burkina Faso.

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Profil bactériologique des isolats de *Staphylococcus aureus* au Centre Hospitalier Universitaire Bogodogo, Ouagadougou, Burkina Faso

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Résumé:

Contexte: *Staphylococcus aureus* est un pathogène humain majeur fréquent, impliqué dans une grande variété d'infections. L'objectif de cette étude est d'étudier le profil bactériologique de *S. aureus* au Centre Hospitalier Universitaire de Bobo-Dioulasso, à Ouagadougou, au Burkina Faso.
Méthodologie: Il s'agit d'une étude descriptive transversale menée du 1er janvier au 31 Décembre 2024 au sein du service de bactériologie-virologie du CHU de Bogodogo. Tous les prélèvements biologiques (pus, sang, écouvillons vaginaux et liquide d'épanchement) reçus au laboratoire ont été mis en culture sur gélose au mannitol et au sel pour l'isolement de *S. aureus*. L'identification de *S. aureus* s'est basée sur les caractéristiques morphologiques des colonies; colonies rondes, lisses, convexes, bien délimitées, à surface lisse et brillante, parfois légèrement huileuse, apparaissant en amas irréguliers évoquant des «grains de raisin» au microscope; sur les tests biochimiques standards (catalase positive, coagulase positive et oxydase négative); et sur l'utilisation du panel API Staph®. La sensibilité aux antibiotiques a été déterminée sur gélose Müller-Hinton par la méthode de diffusion de Kirby-Bauer, les diamètres des zones d'inhibition étant mesurés et interprétés selon les recommandations CASFM/EUCAST.
Résultats: Au total, 1530 bactéries ont été isolées à partir d'échantillons pathologiques de patients hospitalisés pour infection durant la période d'étude, dont 184 (12,0%) étaient des *S. aureus*. Le pus était le type d'échantillon le plus fréquent, représentant 98 isolats (53,2%). L'âge moyen des 184 patients était de 30±19 ans (de 1 à 78 ans), avec une prédominance féminine (sex-ratio = 0,73). Le service des urgences a fourni le plus grand nombre d'isolats, soit 37 (20,1%). Les isolats présentaient une forte résistance à la pénicilline G (96,0%, n=178) et à l'érythromycine (42,3%, n=78). Concernant les mécanismes de résistance, 58,0% des isolats produisaient de la pénicillinase et 38,0% étaient des Staphylococcus aureus résistants à la méthicilline (SARM).
Conclusion: Cette étude met en évidence une forte prévalence de Staphylococcus aureus dans les prélèvements biologiques de patients présentant des infections (12,0%) au CHU de Bogodogo, ainsi qu'une forte résistance aux bêta-lactamines. Ces résultats soulignent la nécessité de renforcer les stratégies de surveillance et de promouvoir un usage judicieux des antibiotiques en milieu hospitalier.

Mots-clés: *Staphylococcus aureus*; SARM; pénicillinase; CHU de Bogodogo; Burkina Faso

Introduction:

Staphylococcus aureus are Gram-positive cocci, highly resistant in the external environment and non-fastidious in culture. It is a major human pathogen capable of causing a wide range of infections, including skin infections, septicemia, pneumonia, endocarditis, postoperative infections, and foodborne infections. *S. aureus* has a strong ability to adapt, which allows it to develop various mechanisms of antibiotic resistance, including the production of penicillinase (β-lactamase), and the carriage of *mecA* and *mecC* genes, which confer resistance to β-lactams (1). Staphylococcal infections are observed in many clinical situations, both in community-acquired and nosocomial settings, and *S. aureus* is the pathogen most often implicated in nosocomial infections (2).

Resistance to certain antibiotics has emerged in some strains of *S. aureus* isolated from many biological samples such as methicillin or oxacillin, clindamycin, fluoroquinolones, vancomycin, and erythromycin (3). As soon as penicillin G was introduced into therapy, staphylococci capable of destroying the antibiotic by producing penicillinase were discovered. Studies have shown that more than 90% of *S. aureus* strains are resistant to penicillin G (4). *S. aureus* infection has become a growing concern for global public heath due to the emergence of methicillin-resistant strains (MRSA) in both healthcare facilities and the community (5). These staphylococci have acquired the *mec* gene, which enables the synthesis of an enzyme (PBP2a or PBP2') with very low affinity for β-lactams, preventing them from exerting their inhibitory action (6).

In France, according to the latest data from the National Reference Center for Staphylococci, approximately 30% of hospital isolates of *S. aureus* are resistant to methicillin (7). In the United States, MRSA causes approximately 75,000 cases of infection and nearly 10,000 deaths per year (8). In Africa, *S. aureus* infection is also a major public health problem, particularly due to MRSA strains. Several studies conducted in different countries on the continent have shown an average MRSA prevalence of approximately 32.0%, highlighting the extent of resistance in the region (9). In Burkina Faso, Ba et al., (10) in 2019 reported a MRSA prevalence of 32.0%

This study aims to investigate the antibiotic profiles of *S, aureus* isolates responsible for bacterial infections at Bogodogo University Hospital Center, Ouagadougou, Burkina Faso, in order to provide updated epidemiological data to optimize strategies to combat this growing threat to public health and improve patient care.

Materials and method:

Study setting, design and duration:

This was a descriptive cross-sectional study conducted over a 12-month period (January 1 to December 31, 2024) at the Bogodogo University Hospital Center (CHU-B) in Ouagadougou, Burkina Faso.

Ethical considerations:

The study protocol was submitted for approval to the CHU-B management and the Health Research Ethics Committee under reference number 2024-10-318. Patient anonymity and data confidentiality were strictly ensured, in accordance with current ethical principles. The data collected were used exclusively for scientific purposes.

Study population and inclusion criteria.

The study population included all patients with clinical infections in the hospital from whom biological samples (pus, blood, vaginal swabs, and effusion fluid) were collected and received by the bacteriology-virology unit at CHU-B for *Staphylococcus aureus* isolation on culture.

Laboratory analysis:

Clinical samples were cultured on Mannitol salt agar and incubated aerobically for 24 hours to isolate *S. aureus,* which was identified on the culture media based on the morphological characteristics of the colonies (round, smooth, convex colonies with well-defined, smooth, shiny surfaces, sometimes slightly oily, appearing as irregular clusters resembling "grapes" under the microscope), standard biochemical tests (catalase positive, coagulase positive, and oxidase negative), and the use of an API Staph® panel.

Antibiotic sensitivity was determined by the Kirby Bauer disc diffusion method on Müller-Hinton (MH) agar with inhibition diameters read and interpreted as sensitive or resistant according to CASFM/EUCAST recommendations.

To detect penicillinase production, penicillin G (1 U) was used to assess the intrinsic sensitivity of the pathogen. Cefoxitin (30 µg) was used as a substitute marker to detect methicillin-resistant strains (MRSA), replacing oxacillin in agar diffusion tests, in accordance with current CA-SFM/EUCAST recommendations.

Data collection:

The data collected include sociodemographic data (age, sex, patient's department of origin) and bacteriological data (type of sample, antibiotic resistance profile, and resistance phenotype identified). The data were extracted from laboratory notebooks, laboratory records, and biological analysis reports. A standardized data collection form was designed for systematic entry of information.

Data analysis:

Data entry and analysis were performed using Stata software.

Results:

During the period of the study, a total of 1,530 bacterial strains were isolated from patients with infections in the hospital, of which 184 (12.0%) were *S. aureus*. Of these, 97 (53.0%) were isolated from pus samples, and 38 (21.0%) from blood cultures (Fig 1). Of the 184 patients infected with *S. aureus*, females predominated with 106 (58.0%) compared to 78 males (42.0%), giving a sex ratio (M:F ratio) of 0.73. The average age of the patients was 30±19 years, with a range of 1 to 78 years (Table 1). Majority of *S. aureus*-positive cultures were from the trauma department (20.1%, n=37/184) while gynaecology-obstetrics department was second with, 33 (17.9%), followed by the rheumatology department with 25 (13.6%) (Fig 2).

Table 1: Sociodemographic characteristics of patients with *Staphylococcus aureus* infection in the study

Variable	Number	Percentage
Age group (years)		
0-20	48	26.1
21-40	88	47.8
41-60	17	9.2
61-80	31	16.8
Sex		
Male	78	42.0
Female	106	58.0
M: F ratio	0.73	
Total	**184**	**100**

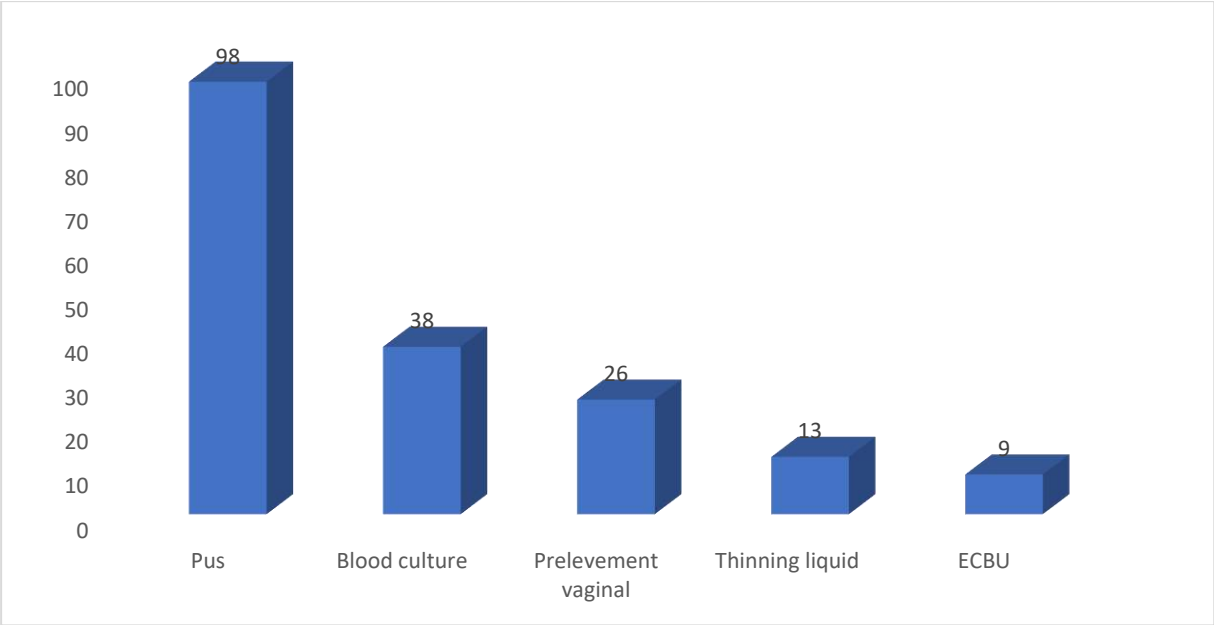

Fig 1: Frequency distribution of pathological samples from patients with *Staphylococcus aureus* infections

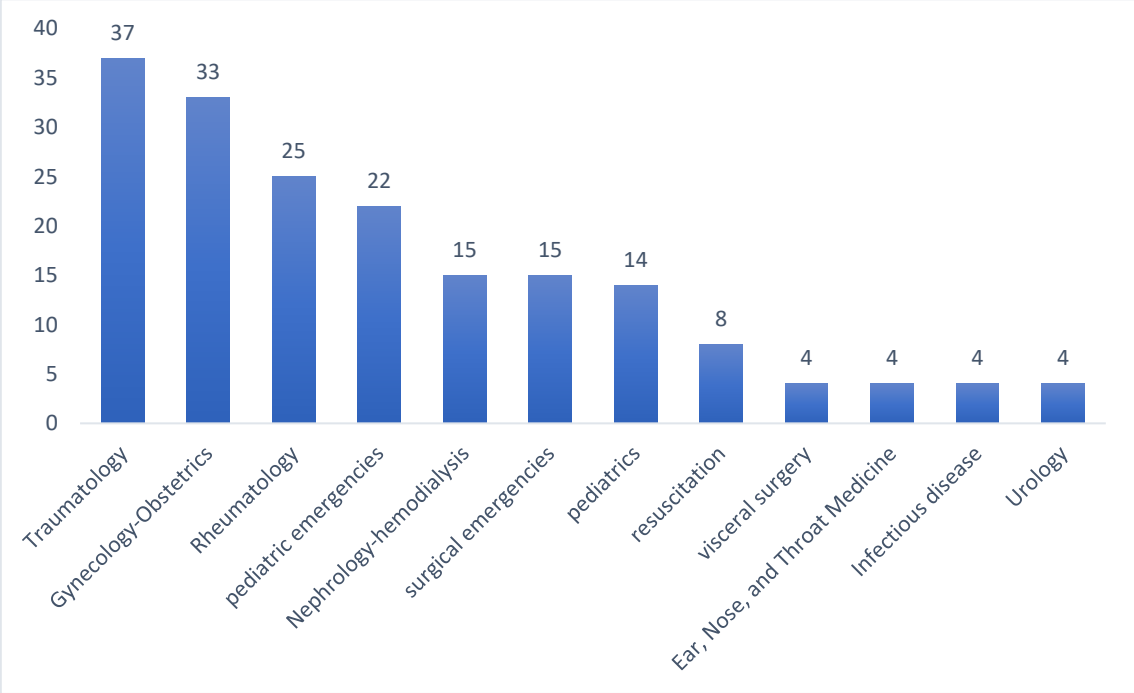

Fig 2: Frequency distribution of pathological samples from patients with *Staphylococcus aureus* infections by clinical department

The results of the antibiotic susceptibility test show that *S. aureus* isolates were resistant to penicillin G (96.0%, n=178) and cefoxitin (38.0%, n=70) indicating phenotypic methicillin-resistant strains (MRSA). For aminoglycosides, 72.8% (n=134) of the isolates were resistant to gentamicin and 23.9% (n=44) to kanamycin. For macrolides and related compounds, 42.3% (n=78) of the isolates were resistant to erythromycin, while 20 (10.8%) were resistant to clindamycin.

For fluoroquinolones, 70.6% (n=130) were resistant to ciprofloxacin and 47.8% (n=88) to norfloxacin. For cotrimoxazole, resistance was observed in 45.6% (n=84) of the isolates. In contrast, resistance to fusidic acid and linezolid was low and seen in only 8.1% (n=15) and 3.8% (n=7) of the isolates respectively, as shown in Table 2.

Table 2: Antibiotic resistance of *Staphylococcus aureus* isolates

Antibiotics	Number of resistant isolates	Percentage (%)
β-lactams		
Penicillin G	178	96.7
Cefoxitin	70	38.0
Aminoglycosides		
Gentamicin	134	72.8
Tobramycin	42	22.8
Kanamycin	44	23.9
Macrolides		
Erythromycin	78	42.3
Clindamycin	20	10.8
Fluoroquinolones		
Ciprofloxacin	130	70.6
Nofloxacin	88	47.8
Other class		
Tetracycline	86	46.7
Cotrimoxazole	84	45.6
Chloramphenicol	86	46.7
Fusidic acid	15	8.1
Linezolid	7	3.8
Fosfomycin	56	30.4

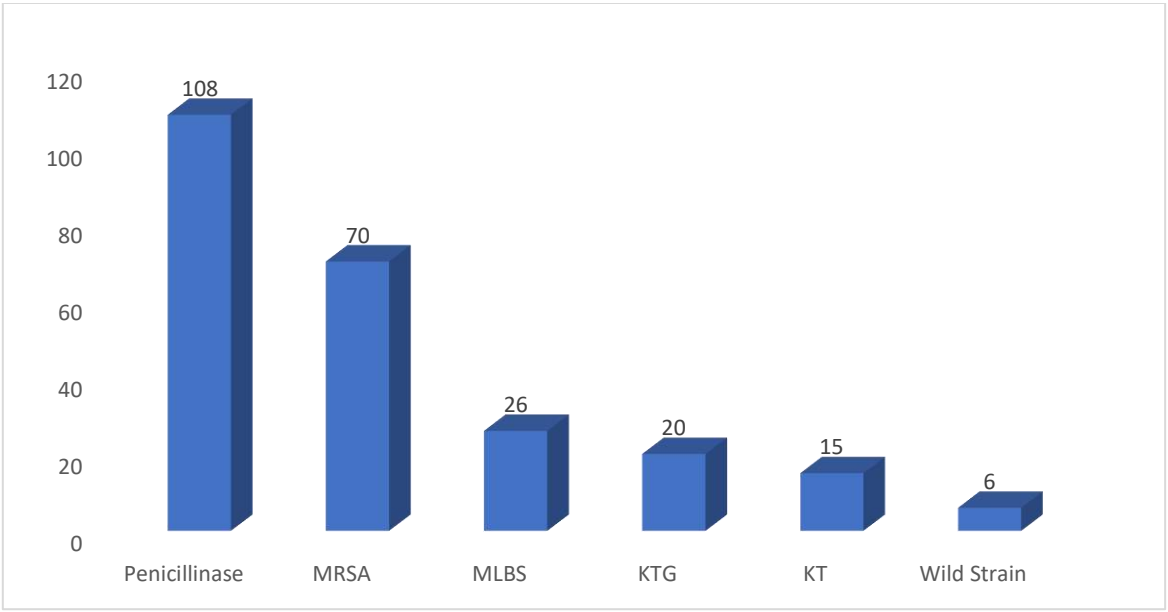

Fig 3: Frequency of antibiotic resistance mechanism and phenotypes in the *Staphylococcus aureus* isolates

Analysis of resistance mechanisms and phenotypes showed penicillinase production in 58.7% (n=108) of the isolates, while 38.0% (n=70) of the isolates were identified as MRSA. Specific resistance phenotypes also identified include 26 (14.1%) isolates with macrolide-lincosamide-streptogramin (MLS_B) phenotype, 20 (10.9%) isolates with kanamycin-tobramycin-gentamicin (KTG) phenotype, and 15 (8.2%) isolates with kanamycin-tobramycin (KT) phenotype as shown in Fig 3.

Discussion:

The 12.0% prevalence of *S. aureus* infection in this study is significant but the prevalence is known to vary according to geographical context, characteristics of the populations studied, and types of samples analyzed. This prevalence is comparable to that reported by Diallo et al., (11) in 2021 at the Dakar University Hospital, with *S. aureus* isolation rate of 11.5% in both community-acquired and

nosocomial infections. In contrast, Faye et al., (12) in a study conducted in 2018 at the Ouagadougou University Hospital, reported a higher rate of 18.7%, a result that could be explained by the high proportion of skin infections and abscesses in their cohort. The frequency reported in these various studies confirms the major role of *S. aureus* as an opportunistic pathogen involved in many suppurative infections, particularly skin, bone, respiratory, and blood infections. This high prevalence justifies the need for continuous microbiological surveillance, which is all the more important given the growing emergence of resistant strains, particularly MRSA.

In this study, pus was the most frequent sample collected for isolation of *S. aureus* from infected patients, with 98, representing 53.2% of all samples collected. This result is consistent with the well-known cutaneous and suppurative infections usually caused by *S. aureus*, which is often involved in both community- and hospital-acquired infections. Our finding is consistent with that reported by Traoré et al., (14) in 2019 at the Bamako University Hospital, where *S. aureus* was isolated in 58.0% of pus samples analyzed in a hospital setting, making this type of sample the main source of isolation of the *S. aureus*. Similarly, Ndiaye et al., (15) in 2020 at Aristide Le Dantec Hospital in Dakar reported a similar frequency of 54.0% in skin suppurations, highlighting the high prevalence of this pathogen in skin and soft tissue infections. In comparison, Kouadio et al., (16) in Côte d'Ivoire reported a slightly lower frequency (47.0%) in pus, probably due to a greater diversity of pathogens in nosocomial wound infections. It should also be noted that the high proportion of *S. aureus* in purulent samples in our series may reflect a delay in the management of skin infections, promoting suppuration, or even poor hygiene in the community.

Analysis of the distribution of *S. aureus* cases by hospital department showed a marked predominance in the trauma department, which accounted for 20.10% of the isolates. This data suggests that *S. aureus* infections are common in trauma patients, who often have open wounds, exposed fractures, or have undergone surgery, all of which are factors that promote colonization or infection by this pyogenic pathogen. However, our results contrast with that reported by Aidoun et al., (17) in Algeria, where the distribution of cases differed significantly with 34.48% of strains from intensive care unit, 13.79% from pediatrics department, and 13.79% from general surgery department. This discrepancy can be explained by several factors including differences in hospital organization (e. g., presence or absence of a separate trauma department), profile of patients admitted to each department (age, immune status, severity of conditions), and the infection control and prevention protocols specific to each institution. Furthermore, in a study conducted in Abidjan by Kacou et al., (18) surgery ranked first (27.0%) in terms of *S. aureus* isolation, followed by trauma (18.0%) and pediatrics (12.0 %). This shows that the frequency of *S. aureus* infections is closely linked to invasive procedures, the presence of wounds, and skin colonization, which are typical of surgical and traumas patients.

It should also be noted that the risk of nosocomial *S. aureus* infection is higher in departments such as intensive care, due to the proliferation of invasive devices (catheters, probes, ventilators), the immunocompromised state of patients, and prolonged hospitalization. These conditions are conducive for the emergence of multidrug-resistant strains, particularly MRSA which warrants specific analysis in a future study. These results confirm that the trauma department is a significant reservoir of S. *aureus* infections in our hospital, calling for increased vigilance in the prevention of healthcare-associated infections (HAIs) in this department.

In this study, *S. aureus* showed a worrying resistance profile, particularly to certain commonly used antibiotics. Resistance was very high resistance to penicillin G (96.0%) confirming the almost universal loss of efficacy of this antibiotic against *S. aureus* strains. This rate is consistent with data in the literature, as numerous studies report resistance to penicillin G of over 90.0%, due to the production of penicillinases (β-lactamases) by majority of strains. For example, Traoré et al., (14) in Mali reported a penicillin G resistance rate of 94.3%, while in Algeria, Aidoun et al., (17) reported a similar rate of 95.5%.

Regarding erythromycin, our study showed a resistance rate of 42.48%, which remains high. This upward trend in macrolide resistance has been observed in several countries. By way of comparison, Faye et al., (20) in Senegal reported resistance of 39.2%, while Kouadio et al., (19) in Côte d'Ivoire reported a rate of 45.8%, reflecting a gradual decline in the effectiveness of this molecule. In contrast, we noted good activity of fusidic acid and linezolid, with low resistance rates of 7.9% and 3.92%, respectively. These results are consistent with those of the ONERBA multicenter study in France in 2020, where resistance to linezolid in *S. aureus* remained below 2.0% and resistance to fusidic acid around 5.0% (13). These antibiotic molecules therefore remain valuable against multidrug-resistant strains, particularly in the treatment of severe or invasive skin infections.

However, it is important to emphasize that the use of these antibiotics must be restricted and supervised, particularly linezolid, which is a 'last-resort' antibiotic, in order to

prevent the emergence of resistant strains, as has already been reported sporadically in certain regions. These data highlight the need for regular monitoring of the sensitivity profiles of *S. aureus* strains and reinforce the call for rational use of antibiotics, adapted to the results of the antibiogram.

In this study, 58.0% of the *S. aureus* isolates produced penicillinase, while 38.0% were methicillin-resistant (MRSA) strains. This dual mechanism of resistance, enzymatic and structural, highlights the remarkable adaptability of this pathogen to β-lactam antibiotics. The production of penicillinase, responsible for resistance to penicillin G, remains one of the primary mechanisms of resistance developed by *S. aureus*. Although most hospital strains have already acquired this trait, the 58.0% rate observed in our study indicates that more than half of the isolates remain resistant to natural penicillins. This result is slightly lower than that reported by Aidoun et al., (17) in Algeria, where 74.0% of strains expressed penicillinase. On the other hand, Faye et al., (20) in Senegal found a rate closer to ours, at 61.5%. This variability can be explained by differences in antibiotic pressure, prescribing practices, and prophylactic antibiotic therapy protocols between institutions

The MRSA rate of 38.0% in our study, represents a moderate to high prevalence but is still a cause for concern. This rate is comparable to that of Kouadio et al., (19) in Côte d'Ivoire (41.0%), but remains lower than the rate reported in some Maghreb countries, such as Tunisia, where Ben Abdallah et al., (21) reported a MRSA rate of 50 to 60% in hospitals. Internationally, the CDC (USA) estimated in 2022 an average MRSA prevalence of 30 to 40% in resource-limited hospitals, with peaks in intensive care units (22). This phenomenon highlights the need for effective strategies to prevent resistance to *S. aureus* infections

Conclusion:

This study, conducted at the Bogodogo University Hospital Center (CHU-B) in Ouagadougou Burkina Faso, showed a relatively high frequency of *S. aureus* infections and a high prevalence of MRSA in the *S. aureus* population. MRSA isolates are particularly resistant to other antibiotics, with the exception of linezolid and fusidic acid, which remain active. It is essential to understand the epidemiological profiles of pathogens and their sensitivity to antibiotics in order to rationalize the use of these antimicrobial agents and limit the development of multidrug-resistant bacteria.

Contributions of authors:

All authors were involved in the study conceptualization, execution, and writing of the manuscript, and all read and approved the final version of the manuscript submitted for publication.

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Conflict of interest:

The authors declare that they have no conflicts of interest in relation to the content of this article

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Original Article **Open Access**

Prevalence of New Delhi Metallo-β-lactamase gene and antibiotic susceptibility of clinical *Klebsiella pneumoniae* isolates from selected hospitals in Calabar, Nigeria

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Abstract:

Background: The global spread of extended spectrum beta-lactamases (ESBL) and carbapenemase-producing strains of *Klebsiella pneumoniae,* is considered a major public health threat to humans. Antimicrobial resistance mediated by New Delhi Metallo-β-lactamase (NDM) enzyme has become a major threat to treatment outcome. This study is aimed at investigating the carriage rate of bla_{NDM} gene among multidrug resistant (MDR) clinical *K. pneumoniae* isolates from four selected health facilities in Calabar, Nigeria.
Methodology: This was a laboratory-based study of 70 confirmed *K. pneumoniae* isolates recovered from various clinical sources at the University of Calabar Teaching Hospital (UCTH), General Hospital Calabar (GHC), Faith Foundation Specialist Clinic (FFSC) and Bakor Medical Centre Calabar (BMCC) using the Vitek® 2 Compact system, with antibiotic susceptibility testing performed by the Kirby-Bauer disc diffusion method. Molecular detection of bla_{NDM} gene in 15 selected MDR isolates was carried out by conventional PCR assay and agarose gel electrophoresis detection of the gene amplicon.
Results: The distribution of the confirmed isolates according to healthcare facilities showed that the highest frequency of *K. pneumoniae* isolates was obtained from UCTH (52.9%, n=37), followed by GHC (17.1%, n=12), BMCC (15.7%, n=11) and FFSC (14.3%, n=10). Urine sample accounted for the highest frequency of the isolates (40.0%, n=28), followed by sputum (31.4%, n=22), wound swabs (8.6%, n=6), and blood (5.7%, n=4) while the frequency of isolates was higher among inpatients (57.1%, n=40) than outpatients (42.9%, n=30). The susceptibility of the isolates to meropenem, ertapenem, piperacillin/tazobactam, amoxicillin/clavulanate, ciprofloxacin, gentamicin and ceftazidime was 57.1% (n=40), 54.3% (n=38), 51.4% (n=36), 50.0% (n=35), 48.6% (n=34), 47.1% (n=33) and 17.1% (n=12) respectively. Eighteen (25.7%) isolates were MDR *K. pneumoniae* strains while bla_{NDM} gene was detected in 9 (60.0%) of 15 MDR isolates tested.
Conclusion: The high prevalence of the resistance gene in this study setting underscores the urgent need for an informed and discrete use of antimicrobial drugs, particularly β-lactam antibiotics, for management of bacterial infections to prevent further spread of drug-resistant *K. pneumoniae* strains.

Keywords: *Klebsiella pneumoniae*, Multidrug Resistance, Multiple antibiotic resistance index, NDM gene

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Prévalence du gène de la métallo-β-lactamase de New Delhi et sensibilité aux antibiotiques des isolats cliniques de *Klebsiella pneumoniae* provenant d'hôpitaux sélectionnés de Calabar, au Nigéria

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Résumé:

Contexte: La propagation mondiale des souches de *Klebsiella pneumoniae* productrices de bêta-lactamases à spectre étendu (BLSE) et de carbapénémases constitue une menace majeure pour la santé publique. La résistance aux antimicrobiens médiée par l'enzyme New Delhi Métallo-β-lactamase (NDM) représente un obstacle majeur à l'efficacité des traitements. Cette étude vise à déterminer la fréquence de portage du gène *bla*$_{NDM}$ parmi les isolats cliniques de *K. pneumoniae* multirésistants (MDR) provenant de quatre établissements de santé sélectionnés à Calabar, au Nigéria.
Méthodologie: Cette étude en laboratoire a porté sur 70 isolats confirmés de *K. pneumoniae* provenant de différents établissements cliniques du Centre Hospitalier Universitaire de Calabar (UCTH), de l'Hôpital Général de Calabar (GHC), de la Clinique Spécialisée de la Fondation Faith (FFSC) et du Centre Médical Bakor de Calabar (BMCC). Les prélèvements ont été effectués à l'aide du système Vitek® 2 Compact, et la sensibilité aux antibiotiques a été testée par la méthode de diffusion sur disque de Kirby-Bauer. La détection moléculaire du gène *bla*$_{NDM}$ dans 15 isolats multirésistants sélectionnés a été réalisée par PCR conventionnelle et électrophorèse sur gel d'agarose pour la détection de l'amplicon du gène.
Résultats: La répartition des isolats confirmés selon les établissements de santé a montré que la fréquence la plus élevée d'isolats de *K. pneumoniae* provenait de l'UCTH (52,9%, n=37), suivie du GHC (17,1%, n=12), du BMCC (15,7%, n=11) et du FFSC (14,3%, n=10). L'échantillon d'urine représentait la fréquence la plus élevée des isolats (40,0%, n=28), suivi par les expectorations (31,4%, n=22), les écouvillons de plaies (8,6%, n=6) et le sang (5,7%, n=4), tandis que la fréquence des isolats était plus élevée chez les patients hospitalisés (57,1%, n=40) que chez les patients ambulatoires (42,9%, n=30). La sensibilité des isolats au méropénem, à l'ertapénem, à la pipéracilline/tazobactam, à l'amoxicilline/acide clavulanique, à la ciprofloxacine, à la gentamicine et à la ceftazidime était respectivement de 57,1% (n=40), 54,3% (n=38), 51,4% (n=36), 50,0% (n=35), 48,6% (n=34), 47,1% (n=33) et 17,1% (n=12). Dix-huit isolats (25,7%) étaient des souches de *K. pneumoniae* multirésistantes, tandis que le gène *bla*$_{NDM}$ a été détecté chez 9 des 15 isolats multirésistants testés (60,0%).
Conclusion: La forte prévalence du gène de résistance observée dans cette étude souligne l'urgence d'un usage raisonné et ciblé des antibiotiques, notamment des bêta-lactamines, dans la prise en charge des infections bactériennes afin de prévenir la propagation des souches de *K. pneumoniae* résistantes aux médicaments.

Mots-clés: *Klebsiella pneumoniae*, multirésistance, indice de résistance aux antibiotiques multiples, gène NDM

Introduction:

Klebsiella pneumoniae is one of several bacteria that have expressed dramatic increase in antibiotic resistance in the past decades. Several mechanisms of antibiotic resistance are found in *K. pneumoniae*, with resistance to β-lactams having the greatest impact on effective treatment. Colonization with antibiotic-resistant *K. pneumoniae* has been associated with subsequent infection in hospitalized patients. Although the progression from colonization to infection is not completely understood, the accessory genome is central to antibiotic resistance in *K. pneumoniae*, with plasmid-based resistance genes rapidly reducing the armamentarium of effective antibiotics against this pathogen.

Extended-spectrum β-lactamases (EsβLs) are plasmid-borne resistance mechanisms that are part of the accessory genome. ESβL-producing *K. pneumoniae* were first identified in Europe in 1983 (1) and in the United States in 1989 (2). ESβLs are able to hydrolyze oxyimino-cephalosporins such as third-generation cephalosporins and aztreonam but are inhibited by clavulanic acid (3). Frequently, plasmids encoding β-lactamases also possess resistance genes to other antibiotics as well as heavy metals (4). Therefore, ESβLs are tightly linked to other accessory genes that could improve fitness of the strain harboring it, and plasmid-based resistance mechanisms are associated with certain lineages that may have a characteristic accessory genome.

Carbapenems have typically been the drug of choice to treat severe infections caused by ESβL-producing bacteria, however, carbapenem resistance in *K. pneumoniae* has emerged causing substantial clinical problems and further complicating treatment (5) The New Delhi metallo-β-lactamase-1 (NDM-1) in *K. pneumoniae* have been reported in Europe, Middle East, North Africa, Asia and North America, mostly from patients who have a history of hospitalization in the Indian subcontinent (6-7). OXA-48 in *K. pneumoniae* was first isolated from Turkey in 2004 and is now endemic in Turkey and countries around the Mediterranean and rapidly spreading into other countries in Europe (8).

Of the three main carbapenemases reported in *K. pneumoniae*, KPC producers are still mostly identified in nosocomial isolates while the NDM and OXA-48 are extensively identified in both nosocomial and community-acquired strains. Genetic analyses have indicated that the gene encoding OXA-48 has the propensity to spread among the Enterobacterial species at a much higher rate than the KPC and NDM genes (9).

After 30 years since the emergence of ESβLs, the occurrence of ESβL-producing *K. pneumoniae* is increasing worldwide, reaching an epidemic proportion in many countries (10). Although the prevalence and ESβL gene

carriage varies between different geographical regions, according to the World Health Organization (WHO), the occurrence of ESβL-producing *K. pneumoniae* has reached an endemic rate of up to 50% in many parts of the world and up to 30% resistant rate in the community, demonstrating the widespread nature of this resistance (11). This study was thus aimed at investigating the extent of spread of specific carbapenemase encoded by the NDM gene, which confers resistance to commonly used antimicrobial drugs in this study setting.

Materials and method:

Study area and design:

This descriptive laboratory-based study was carried out in four health facilities in Calabar metropolis including University of Calabar Teaching Hospital (UCTH), General Hospital Calabar (GHC), Faith Foundation Specialist Clinic (FFSC) and Bakor Medical Centre Calabar (BMCC) within the Calabar metropolis, Nigeria.

Ethical consideration:

Ethical approval for the research work was fully granted by the University of Calabar Teaching Hospital Health Research Ethics Committee (UCTH NHREC NO: NHREC/07/10/2012) and Cross River State Health Research Ethics Committee (REG. NO: CRSMOH/HRP/REC/2023/401).

Phenotypic identification of *K. pneumoniae* isolates:

A total of 78 suspected *K. pneumoniae* isolated from urine, sputum, wound swab and blood samples of patients in the routine microbiology laboratories of the healthcare facilities were initially cultured on MacConkey agar media. A total of 70 isolates were confirmed as *K. pneumoniae* by the Vitek® 2 Compact system (BioMérieux; Marcy L'Étoile, France). The confirmed isolates were sub-cultured on nutrient agar slant for antibiotic susceptibility testing.

Antibiotic susceptibility testing (AST):

Antibiotic susceptibility of the 70 confirmed *K. pneumoniae* isolates was performed against 6 classes of antibiotics including gentamicin (CN-10µg), ciprofloxacin (CIP-5µg), ertapenem (ETP-10µg), meropenem (MEM-10µg) amoxicillin/clavulanate (AUG-30µg), piperacillin/tazobactam (TZP-30µg) and ceftazidime (CAZ-30µg), using the Kirby-Bauer disc diffusion technique. Sterile swabs were used to inoculate sterile Mueller Hinton (MH) agar plates with inoculum of the isolates that was prepared in tryptone soy broth and standardized to 0.5 McFarland turbidity standard. Antibiotic discs were applied to the inoculated MH agar plates and incubated aerobically at 37^0C for 24 hours. The zone diameters of inhibition were measured with a ruler and results were recorded for each isolate as sensitive, intermediate or resistant using the interpretative criteria of the Clinical and Laboratory Standards Institute (12).

Multi-drug resistance (MDR)

Multidrug resistance was defined as resistance of *K. pneumoniae* isolate to three or more classes of antibiotics tested against the isolate (13).

Multiple antibiotic resistance (MAR) index:

The MAR index was determined for *K. pneumoniae* isolate by dividing the number of antibiotics to which the isolate was resistant to, by the total number of antibiotics tested against it (14).

Molecular detection of NDM genes by conventional PCR:

Genomic DNA extraction using boiling method:

Five ml of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 12,000rpm for 3min. The cells were re-suspended in 500µl of normal saline and heated at 95^0C for 15min. The heated bacterial suspension was cooled on ice for 10 min and spun for 3min at 12000rpm, 200µl of the supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20°C for other downstream tests.

DNA quantification:

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2µl of sterile distilled water and blanked using normal saline. About 2µl of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

PCR amplification of NDM genes:

The NDM genes from 15 selected MDR isolates were amplified by conventional PCR assay using the NDM F: 5-GGTTTGGCGATCTG GTTTTC-3' and NDM R: 5'-CGGAATGGCTCATC ACGATC-3' primers on ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 µl for 35 cycles. The PCR mix included X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, dNTPs, $MgCl_2$), primers at a concentration of 0.4M and 50ng of the extracted DNA as template.

The PCR conditions were as follows; initial denaturation at 95ºC for 5min, denaturation at 95ºC for 30 sec, annealing at 56ºC for 45 sec, extension at 72ºC for 45 sec for 35 cycles, and final extension at 72ºC for 5 min. The product was resolved on a 1% agarose gel

at 200V for 15 minutes and visualized on blue light imaging system for a 380bp product size.

Statistical analyses:

Data generated in the study were analyzed using SPSS statistical software, version 20.0 (IBM Corporation, Armonk, New York, USA) for test of significance. Proportions were presented as frequency and percentages. Pearson's Chi square test was used to measure dependence between study variables, while *p*-value ≤0.05 was considered statistically significant.

Results:

The highest frequency of *K. pneumoniae* isolates (52.9%, n=37) was obtained from UCTH, followed by GHC (15.3%, n=12), BMCC (15.7%, n=11) and FFSC (14.3%, n= 10). The distribution of *K. pneumoniae* isolates by clinical source showed that 42.9% (n=30) were isolated from urine samples, followed by sputum samples (35.7%, n=25) while 14.3% (n=10) and 7.1% (n=5) were isolated from wound swab and blood samples respectively. A total of 40 (57.1%) *K. pneumoniae* isolates were recovered from inpatients compared to 30 (42.9%) from outpatients (Table 2).

Table 1: Distribution of *Klebsiella pneumoniae* according to health facility

Hospital	Isolates	Percentage
UCTH	37	52.9
GHC	12	17.1
FFSC	10	14.3
BMCC	11	15.7
Total	**70**	**100.0**

UCTH=University of Calabar Teaching Hospital; GHC=General Hospital Calabar; FFSC=Faith Foundation Specialist Clinic; BMCC=Bakor Medical Centre Calabar

The result of the antibiotic sensitivity testing of *K. pneumoniae* isolates in Table 3 showed that the isolates were susceptible to meropenem (n=40, 57.1%) at inhibition zone size (IZ) of 28-40mm and mean value of 34 mm, closely followed by ertapenem (n=38, 54.3%), piperacillin/tazobactam (n=36, 51. 4%; IZ: 24-30; mean: 27), amoxicillin/clavulanate (n=35, 50.0%; IZ: 18-20; mean: 19), ciprofloxacin (n=34, 48.6%; IZ: 16-22; mean: 29) and gentamicin (n=33, 47.1%; IZ: 16-22; mean: 19) while the isolates were least susceptible (highly resistant) to ceftazidime (n= 12, 17.1%; IZ: 24-34; mean: 29).

Table 2: Distribution of *Klebsiella pneumoniae* isolates by clinical source

Source	Isolates (%)	Inpatients (%)	Outpatients (%)
Urine	30 (42.9)	19 (27.1)	11 (15.7)
Sputum	25 (35.7)	12 (17.1)	13 (18.6)
Wound swab	10 (14.3)	6 (8.6)	4 (5.7)
Blood	5 (7.1)	3 (4.3)	2 (2.9)
Total	**70 (100.0)**	**40 (57.1)**	**30 (42.9)**

$p = 0.1967$

Of the 70 *K. pneumoniae* isolates, 16 (22.9%) isolates were resistant to 4 antibiotics with MAR index of 0.6, followed by 14 (20. 9%) isolates to 3 antibiotics with MAR index of 0.4, 13 (18.6%) to 5 antibiotics with MARI of 0.7, 11 (15.7%) isolates to 2 antibiotics with MARI of 0.3, 6 (8.6%) isolates with MARI of 0.9, 5 (7.1%) isolates with MARI of 0.1 and 5 (7.1%) isolates with MARI of 1.0 (Table 4).

Eighteen (25.7%) isolates were multidrug resistant, out of which 11 (15.7%) were from UCTH, followed by BMCC with 3 (4.3%) while only 2 (2.9%) were obtained each from GHC and FFSC. Majority of the MDR isolates were resistant to three classes of antibiotics (n=8, 44.4%), followed by four classes of antibiotics (n=4, 22.2%) and five classes of antibiotics (n=3, 16.7%) (Table 5). Of 15 MDR isolates subjected to PCR and agarose gel electrophoresis, NDM gene was detected in 9 (60.0%).

Table 3: Antibiotic susceptibility of *Klebsiella pneumoniae* isolates to six selected antibiotics tested

Status	Number of *Klebsiella pneumoniae* isolates (%)						
	TZP	CIP	CAZ	CN	AUG	MEM	ETP
Sensitive	36 (51.4)	34 (48.6)	12 (17.1)	33 (47.1)	35 (50.0)	40 (57.1)	38 (54.3)
Intermediate	16 (22.9)	10 (14.3)	13 (18.6)	13 (18.6)	15 (21.4)	15 (21.4)	16 (22.9)
Resistant	18 (25.7)	26 (37.1)	45 (64.3)	24 (34.3)	20 (28.6)	15 (21.4)	16 (22.9)

TZP=Piperacillin/tazobactam; CIP=Ciprofloxacin; CAZ=Ceftazidime; CN=Gentamicin; AUG=Amoxicillin/clavulanate; MEM=Meropenem; ETP=Ertapenem

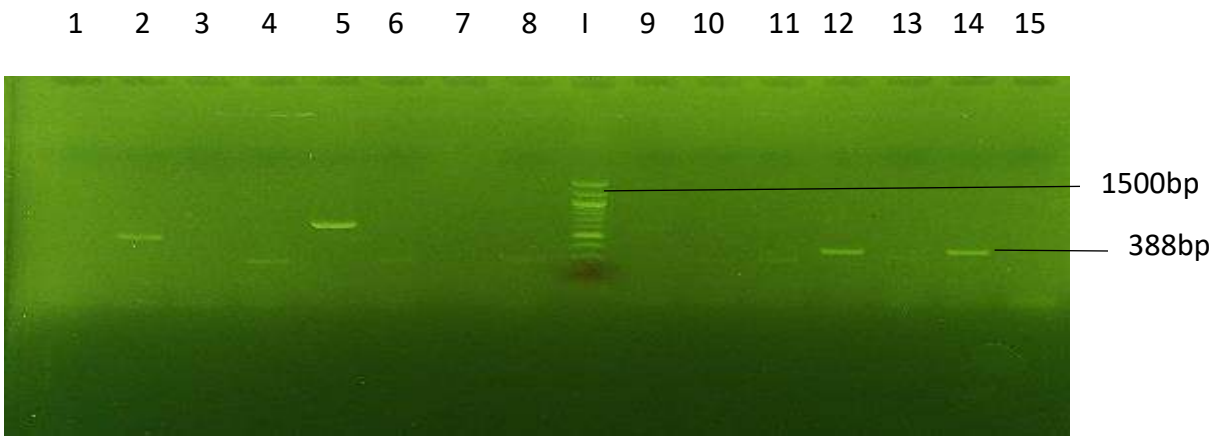
Table 4: Multiple antibiotic resistance index (MARI) of *Klebsiella pneumoniae* isolates

Number of antibiotics	Number of resistant isolates	MARI	MARI (%)
1	5 (7.1)	1/7	0.1
2	11 (15.7)	2/7	0.3
3	14 (20.0)	3/7	0.4
4	16 (22.9)	4/7	0.6
5	13 (18.6)	5/7	0.7
6	6 (8.6)	6/7	0.9
7	5 (7.1)	7/7	1.0

Table 5: Multidrug resistance pattern of *Klebsiella pneumoniae* isolates to the classes of antibiotics

Antibiotics	Number of resistant isolates	Drug resistance status
CAZ, CN, CIP, TZP, MEM	3 (16.7)	MDR
CAZ, AUG, CIP, TZP, ETP	3 (16.7)	MDR
CAZ, AUG, MEM, TZP	4 (22.2)	MDR
CAZ, CIP, ETP	8 (44.4)	MDR
Total	**18 (100.0)**	**MDR**

TZP=Piperacillin/tazobactam; CIP=Ciprofloxacin; CAZ=Ceftazidime; CN=Gentamicin; AUG=Amoxicillin/clavulanate; MEM=Meropenem; ETP=Ertapenem; MDR=Multi-drug resistance



Lanes I represent 100bp molecular ladder of 1500bp. Lanes 2,4,5,6,8,11,12,13 and 14 represent the NDM gene band (388bp).

Plate: Agarose gel electrophoresis of NDM gene of some *Klebsiella pneumoniae* strains

Discussion:

Klebsiella pneumoniae isolates were most frequently recovered from urine, followed by sputum sample, while wound swab and blood samples had the least number of *K. pneumoniae* isolates in the study. Although, similar result was obtained in other part of Nigeria, study in New York city by Parrott et al., (15) and the report of Palmeiro et al., (16) identified *K. pneumoniae* isolates as the major causal agent of blood and wound infections, while Sedighi et al., (17) identified throat, urine, and tracheal region as the major sites affected by this bacterium. This remarkable difference in incidence of *K. pneumoniae* according to sample types across the world underscores the significant impact of the immune status of individual and lifestyle to the epidemiology of the infection.

The study identified only 12 (17.1%) *K. pneumoniae* from sputum samples of in-patients with probable hospital acquired pneumonia compared to 13 (18.6%) from out-patients with report from Chen et al., (18) showing increased prevalence of hospital-acquired pneumonia across the world, but there was no significant difference in the prevalence of *Kleb-*

siella infection between hospital and community settings (p=0.1967) in our study.

The antibiotic sensitivity of *K. pneumoniae* isolates showed higher sensitivity to meropenem (57.1%) and ertapenem (54.3%) than other antibiotics tested in the study. The susceptibility of these clinical isolates to carbapenems is higher compared to the study in Ebonyi State Nigeria, where Ejikeugwu et al., (19) reported about 43.0% meropenem-resistant *K. pneumoniae* strains. Surprisingly, *in vitro* sensitivity testing of carbapenems reported by Muller-Schulte et al., (20) showed that, all *K. pneumoniae* isolates tested were 100% sensitive to carbapenems in Central Cote d'Ivoire, a development which could be connected to some regulations on the use of carbapenems for treatment in the region. However, finding from Northern Province of China by Wang et al., (21) contrastingly reported highly resistant *K. pneumoniae* isolates to meropenem at 93.2%. The susceptibility to other commonly used antibiotics in the study, showed varying degrees of *in vitro* antimicrobial activity against *K. pneumoniae* isolates with piperacillin/tazobactam (51.4%), amoxicillin/clavulanate (50.0%), but sensitivity to ceftazidime was the least by the clinical isolates with only 17.1%.

Furthermore, 25.7% of the *K. pneumoniae* isolates were multidrug resistant strains, and higher number of these strains (61.1%) were recovered from patients on hospital admission compared to 38.9% from out-patients who visited the hospital for medical treatment. The prevalence MDR-*K. pneumoniae* strain in the study is relatively low compared to recent study from South-Eastern Asia by Salawudeen et al., (22) who reported higher prevalence of 55.0% for MDR-*K. pneumoniae* in the clinical setting. Generally, infections caused by MDR *K. pneumoniae* are associated with high mortality which is often linked to inadequate and inappropriate antimicrobial therapy.

The bla_{NDM} gene was detected in urine and sputum samples of 9 (60.0%) of the 15 patients with *K. pneumoniae* infections across the four selected healthcare facilities. Six of the bla_{NDM} gene were detected among those on hospital admission while 3 were detected among out-patients. Earlier report by Asuquo et al., (23) reported bla_{NDM} gene in two *K. pneumoniae* isolates in the same population, however, the presence of bla_{NDM} gene in nine *K. pneumoniae* strains in our study, suggest an increased spread of this resistant gene in Calabar, Nigeria. Ogbolu and Webber (24) also reported low prevalence (5.5%) of this carbapenemase-producing isolates in some Nigerian tertiary hospitals. Wailan and Peterson (6) had earlier linked NDM to medical tourism in India, however, some of the patients with *K. pneumoniae* that harbored this gene had no travel history outside of Nigeria, suggesting increased local spread of the resistant organisms in the study population.

A study in other part of Nigeria detected bla_{NDM} gene in patients with no history of travel to India (25). Initially, this bla_{NDM} gene in Africa was first identified in Kenya among seven clonally related *K. pneumoniae* isolates from urine and urethral pus of adult patients hospitalized in different wards of the Aga Khan University Hospital (26), and since then, has spread across various countries in the African continent, including South Africa, Benin, Angola and Algeria (27).

Conclusion:

In this study, the prevalence of MDR among *K. pneumoniae* isolates was 25.7%, with high resistance to ceftazidime. High carriage (60.0%) of NDM gene encoding carbapenemase production was detected among MDR strains. The high prevalence of the resistance gene in the study population underscores the urgent need for regulations on use of antibiotics especially the β-lactams for treatment of infections, to prevent further spread of difficult to-treat infections.

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Contributions of authors:

IIU and NOM were involved in the conceptualization, design and writing of the manuscript; IIU and SAB were involved in data collection and analysis of results; Ethical approval of the study was facilitated by SOI. NOM and OMO supervised the research work. JCU and BMW edited the manuscript. All authors read and approved the manuscript for publication.

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Conflict of interest:

No conflict of interest is declared.

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Original Article **Open Access**

Prevalence of extended spectrum β-lactamase genes among multi-drug resistant *Klebsiella pneumoniae* isolates from clinical specimens in Calabar, Nigeria

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Abstract:

Background: The global spread of the extended spectrum beta-lactamases (ESβL) and carbapenemases producing strains of *Klebsiella pneumoniae* constitutes a public health threat to both humans and animals. This study investigated the prevalence of ESβLs genes among multidrug resistant (MDR) *K. pneumoniae* isolates from clinical specimens of patients attending selected hospitals in Calabar, Nigeria.
Methodology: This was a laboratory-based study of 80 *K. pneumoniae* isolates preliminarily identified at the routine microbiology laboratories of four healthcare facilities in Calabar metropolis, with 60 *K pneumoniae* isolates confirmed using Vitek^{R} 2 Compact system (BioMérieux; Marcy L'Étoile, France). Antibiotic susceptibility testing of the confirmed isolates to selected antibiotics was performed by the Kirby Bauer disc diffusion method and result interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines. MDR was defined as resistance to three or more antibiotic classes. ESBL (bla_{CTM-M} and bla_{KPC}) genes were detected among the MDR strains by conventional PCR assay and agarose gel electrophoresis of the gene amplicons. Data of patients from whom the organism were recovered, were extracted from the laboratory records. Pearson's Chi square test was used to measure association between categorical variables, and $p \leq 0.05$ was considered statistically significant.
Results: Of the 60 confirmed *K. pneumoniae* isolates, the highest frequency of the isolate recovery was from patients aged >60 years (25.0%, n=15) and 50-60 years (21.7%, n=13), while the least frequency was from patients aged 1-10 years (5.0%, n=3). Recovery rate was higher in females (n=35, 58.3%) compared to males (n=25, 41.7%). The frequency of resistance of the isolates to meropenem, piperacillin/tazobactam, amoxicillin/clavulanate, ciprofloxacin, gentamicin and ceftriaxone was 25.0% (n=15), 31.7% (n=19), 36.7% (n=22), 48.3% (n=29), 48.3% (n=29) and 76.7% (n=46) respectively. Only 15 (25.0%) of the 60 isolates were MDR strains. Age was significantly associated with MDR strains, with higher frequency in age groups 51-60 years (38.5%, n=5/13) and >60 years (40.0%, n=6/15) compared to other age groups (x^2=13.733, p=0.035). Carriage rate of bla_{CTX-M} gene among the MDR strains was 100% (n=15) while only 26.7% (n=4) carried the bla_{KPC} genes.
Conclusion: The relatively high prevalence of ESβLs encoding genes among *K. pneumoniae* clinical isolates in this study may represent a grim prospect for successful treatment and management of infections caused by this pathogen in the population. Informed and discrete use of antimicrobial drugs, particularly β-lactam antibiotics, for management of bacterial infections will prevent further escalation of drug-resistance in this epidemiological setting.

Keywords: Prevalence; *Klebsiella pneumoniae*; Multidrug resistance, ESBL, bla_{CTX-M}; bla_{KPC}

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Prévalence des gènes de β-lactamases à spectre étendu parmi les isolats de *Klebsiella pneumoniae* multirésistants provenant d'échantillons cliniques à Calabar, au Nigéria

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Résumé:

Contexte: La propagation mondiale des souches de *Klebsiella pneumoniae* productrices de bêta-lactamases à spectre étendu (BLSE) et de carbapénémases constitue une menace pour la santé publique humaine et animale. Cette étude a examiné la prévalence des gènes BLSE parmi les isolats de *K. pneumoniae* multirésistants (MDR) provenant d'échantillons cliniques de patients fréquentant certains hôpitaux de Calabar, au Nigéria.
Méthodologie: Cette étude en laboratoire a porté sur 80 isolats de *K. pneumoniae*, identifiés préliminairement dans les laboratoires de microbiologie de routine de quatre établissements de santé de la métropole de Calabar. Parmi ces isolats, 60 ont été confirmés par le système Vitek® 2 Compact (BioMérieux; Marcy-l'Étoile, France). La sensibilité aux antibiotiques des isolats confirmés a été testée par la méthode de diffusion sur disque de Kirby-Bauer et les résultats interprétés selon les recommandations du CLSI (Institut des Normes Cliniques et de Laboratoire). La multirésistance (MDR) a été définie comme une résistance à trois classes d'antibiotiques ou plus. Les gènes ESBL (bla_{CTM-M} et bla_{KPC}) ont été détectés parmi les souches MDR par PCR conventionnelle et électrophorèse sur gel d'agarose des produits d'amplification. Les données des patients chez lesquels l'organisme a été isolé ont été extraites des dossiers de laboratoire. Le test du χ^2 de Pearson a été utilisé pour évaluer l'association entre les variables catégorielles, et un seuil de signification statistique de $p \leq 0,05$ a été retenu.
Résultats: Parmi les 60 isolats de *K. pneumoniae* confirmés, la fréquence la plus élevée a été observée chez les patients âgés de plus de 60 ans (25,0%, n=15) et de 50 à 60 ans (21,7%, n=13), tandis que la fréquence la plus faible a été constatée chez les patients âgés de 1 à 10 ans (5,0%, n=3). Le taux d'isolement était plus élevé chez les femmes (n=35, 58,3%) que chez les hommes (n=25, 41,7%). La fréquence de résistance des isolats au méropénem, à la pipéracilline/tazobactam, à l'amoxicilline/acide clavulanique, à la ciprofloxacine, à la gentamicine et à la ceftriaxone était respectivement de 25,0% (n=15), 31,7% (n=19), 36,7% (n=22), 48,3% (n=29), 48,3% (n=29) et 76,7% (n=46). Seuls 15 (25,0%) des 60 isolats étaient des souches multirésistantes (MDR). L'âge était significativement associé à la présence de souches MDR, avec une fréquence plus élevée dans les groupes d'âge de 51 à 60 ans (38,5%, n=5/13) et de plus de 60 ans (40,0%, n=6/15) comparativement aux autres groupes d'âge (χ^2 =13,733, p=0,035). Le taux de portage du gène bla_{CTX-M} parmi les souches multirésistantes était de 100.0% (n=15), tandis que seulement 26,7% (n=4) portaient le gène bla_{KPC}.
Conclusion: La prévalence relativement élevée des gènes codant pour des β-lactamines à spectre étendu (BLSE) parmi les isolats cliniques de *K. pneumoniae* dans cette étude pourrait compromettre le succès du traitement et de la prise en charge des infections causées par ce pathogène au sein de la population. Un usage raisonné et judicieux des antibiotiques, en particulier des β-lactamines, pour la prise en charge des infections bactériennes permettra de prévenir l'escalade de la résistance aux antibiotiques dans ce contexte épidémiologique.

Mots-clés: Prévalence; *Klebsiella pneumoniae*; Multirésistance; BLSE; bla_{CTX-M}; bla_{KPC}

Introduction:

Klebsiella pneumoniae is a Gram negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium that is associated with both community-acquired and nosocomial infections including pneumonia, urinary tract infections, septicaemia and wound infections (1). The ability of this bacterium to evade, survive and actively suppress many components of the immune system and grow at many sites in the host, makes it even more life threatening, particularly in patients with underlying medical problems such as alcoholism, diabetes and chronic pulmonary diseases. Most often, *K. pneumoniae* infections occur in association with invasive treatment or long-term care in hospitals and other community settings (1).

Although found as a normal flora of the mouth, skin, and intestines, *K. pneumoniae* can cause destructive changes to human and animal lungs if aspirated, specifically to the alveoli resulting in bloody, brownish or yellow colored jelly-like sputum (2), and it is the most significant member of the genus *Klebsiella* of Enterobacteriaceae family.

The increasing prevalence of multi-drug resistant (MDR) *K. pneumoniae* strains in both hospital and community settings pose serious therapeutic challenges that heighten treatment costs, morbidity and mortality globally. Studies have shown that *K. pneumoniae* can develop resistance to antibiotics faster than other bacteria and is often resistant to multiple antibiotics (3). Current evidence implicates plasmids as the primary source of the resistance genes (4). *K. pneumoniae* is resistant to virtually all beta-lactam antibiotics including carbapenems, due to its ability to produce extended-spectrum beta-lactamases (ESβLs) such as Cefotaximase-Munich (CTX-M). This beta-lactamase, which was initially reported in the late 1980s, became recognized as the most common ESβL group in early 2000, replacing TEM and SHV as the dominant ESβL type (5). Since then, CTX-M variants have been reported among several members of the Enterobacterales and in *Pseudomonas aeruginosa* and *Acinetobacter* (6-8).

Similarly, carbapenems which are the preferred agents for the treatment of serious

infections caused by ESβL-producing *K. pneumoniae*, were considered highly stable to *β*-lactamase hydrolysis, and have retained activity against ESβL producers (9). However, carbapenem resistance in *K. pneumoniae*, which is mainly attributed to the production of *K. pneumoniae* carbapenemase (KPC), has emerged causing substantial clinical challenges, further complicating treatment (10). At present, carbapenem-resistant *K. pneumoniae* (CRKP), listed by the WHO as a critical priority pathogen, has become a global threat to human health due to high morbidity and mortality associated with infections caused by this strain (11). The resistant gene in this strain, which belongs to Class A Ambler classification system, has become the more frequently reported carbapenemase gene in the United States of America (USA) and across the world including Nigeria due to international travels and medical tourism (12).

This study was therefore aimed to determine the prevalence of CTX-M and KPC genes among multidrug resistant strains of *K. pneumoniae* obtained from clinical specimens of patients attending selected healthcare facilities in Calabar, Nigeria.

Materials and method:

Study area:

This study was carried out in four health facilities in Calabar metropolis including University of Calabar Teaching Hospital (UCTH), Nigerian Navy Hospital Calabar (NNHC), General Hospital Calabar (GHC) and Arubah Specialist Clinic Calabar (ASCC) within the Calabar metropolis, Cross River State, Nigeria.

Ethical consideration:

Ethical approval for this research work was fully granted by the University of Calabar Teaching Hospital Health Research Ethics Committee (UCTH NHREC NO: NHREC/07/10/2012) and Cross River State Health Research Ethics Committee (REG. NO: CRSMOH/HRP/REC/2023/401).

Collection of isolates:

A total of 80 microbial isolates suspected to be *K. pneumoniae* were obtained from medical laboratories across four healthcare facilities in Calabar. Isolates were initially subcultured on both MacConkey agar and Cysteine-Lactose-Electrolyte-Deficient (CLED) agar media. Vitek® 2 Compact system (BioMérieux; Marcy L'Étoile, France) was used to confirm 60 *K. pneumoniae* isolates.

Antibiotic susceptibility testing (AST):

Antimicrobial susceptibility of confirmed *K. pneumoniae* isolates was performed to six classes of antibiotics including gentamicin (10µg), ciprofloxacin (5µg), meropenem (10 µg), amoxicillin/clavulanate (30µg), piperacillin/tazobactam (30µg) and ceftriaxone (10µg), using the Kirby-Bauer disc diffusion technique. Sterile swabs were used to inoculate Mueller Hinton (MH) agar plates with inoculum of isolates grown in tryptone soy broth, with comparable turbidity with 0.5 McFarland standard. Antibiotic discs were applied onto inoculated MH agar plates and the plates incubated at 37^0C for 24 hours. The zone diameters of growth inhibition were measured with a ruler and results were recorded as sensitive (S) or resistant (R) using the interpretative criteria of the Clinical and Laboratory Standard Institute (13).

Bacterial genomic DNA extraction:

Genomic DNA of each confirmed bacterial isolate was extracted using the boiling method. Five millilitres of an overnight broth culture of the confirmed bacterial isolate in Luria Bertani (LB) were spun at 12000rpm for 3 min. The cells were re-suspended in 500µl of normal saline and heated at 95^0C for 15 min. The heated bacterial suspension was cooled on ice for 10 minutes and spun for 3 min at 12000 rpm, 200µl of the supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20°C for other downstream reactions.

PCR amplification and agarose gel electrophoresis:

The bla_{CTX-M} gene in the extracted DNA from the isolates was amplified using the CTX-M F: 5- CGCTTTGCGATGTGCAG -3' and CTX-M R: 5'-ACCGCGATATCGTTGGT -3' primers (Inqaba, South Africa) in an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles, and annealing temperature of 52^0C. Similarly, bla_{KPC} gene was amplified using the KPC F: 5-GCTCAGGC GCAACTGTAAG-3' and KPC R: 5'-AGCACAGCG GCAGCAAGAAAG-3' primers (Inqaba, South Africa) at annealing temperature of 58^0C. The products were resolved on a 1% agarose gel at 200V for 15 minutes and visualized on blue light imaging system for a 550 bp product size.

Data analysis:

Data were presented as frequency and percentages. Pearson's Chi square test was used to measure association between categorical variables, while *p*-value less than or equal to 0.05 ($p \leq 0.05$) was considered statistically significant.

Results:

Of the 60 *K. pneumoniae* isolates confirmed by VITEK® 2 COMPACT system, 38 (63.3%) were hospital-acquired strain while 22 (36.7%) were community-acquired strain.

Fifteen (25.0%) of the 60 *K. pneumoniae* isolates were MDR (resistant to at least 3 different class of antibiotics tested), 11 (28.9%) were hospital-acquired strain while 4 (18.2%) were community-acquired strain (OR=1.833, 95% CI=0.5043-6.665, p=0.5373) (Table 2). Age was significantly associated with MDR *K. pneumoniae* strains, with significantly higher frequency of isolation in age groups 51-60 years (38.5%, n=5/13) and >60 years (40%, n=6/15) compared to other age groups (x^2= 13.733, p=0.035). However, there was no significant association with respect to gender (x^2=1.667, p=0.197) (Table 1).

Antimicrobial susceptibility testing of the isolates against selected antibiotics showed that hospital-acquired *K. pneumoniae* strains (n=38) exhibited low resistance to meropenem (n=9, 23.7%), followed by piperacillin/tazobactam (n=12, 31.6%), amoxicillin/clavulanate (n=13, 34.2%), ciprofloxacin (n= 17, 44.7%), and gentamicin (n=17, 44.7%), but high resistance to ceftriaxone (n=30, 78.9 %). Similarly, community-acquired *K. pneumoniae* strains (n=22) exhibited low resistance to meropenem (n=6, 18.2%), piperacillin/tazobactam (n=7, 31.8%), amoxicillin/clavulanate (n=9, 40.9%), ciprofloxacin (n=12, 54.5%), and gentamicin (n=12, 54.5%) while the strains showed high resistance to ceftriaxone (n=16, 72.7%) (Table 3).

Overall, meropenem showed high antimicrobial activity against *K. pneumoniae* strains with low resistant rate of 25.0% (n=15), followed by piperacillin/tazobactam (n=19, 31.7%), amoxicillin/clavulanate (n=22, 36.7 %), ciprofloxacin and gentamicin (n=29, 48.3 %), while the isolates showed high resistance to ceftriaxone (n=46, 76.7%) (Table 3). A high frequency of the isolates (n=15, 25.0%) were resistant to 4 antibiotics with multiple antibiotic resistance index (MARI) of 0.7, followed by MARI of 0.5 (n=13, 21.7%), MARI of 0.8 (20.0%), MARI of 0.3 (16.7%), MARI of 1.0 (8.3%) and MARI of 0.2 (8.3%) (Table 4), while the AST of the MDR-*K. pneumoniae* strains identified R3 (resistance to 3 antibiotics) as the most frequent profile (n=9, 60%) followed by R4 (n=3, 20.0%) and R5 (n=3, 20.0%) (Table 5).

Agarose gel electrophoresis of $bla_{\text{CTX-M}}$ and bla_{KPC} genes (Plates 1 and 2) showed that all the 15 MDR *K. pneumoniae* strains carried $bla_{\text{CTX-M}}$ gene (25.0% carriage rate prevalence in the study) while only 4 isolates carried bla_{KPC} gene (6.7% carriage rate in the study).

Table 1: Frequency distribution of multidrug-resistant *Klebsiella pneumoniae* by age and gender of patients from whom they were isolated

Variables	No of isolates (%)	No of MDR isolates (%)	x^2	p value
Age group (years)				
1-10	3 (5.0)	0	13.733	0.035*
11-20	4 (6.7)	1 (25.0)		
21-30	7 (11.7)	0		
31-40	8 (13.3)	1 (12.5)		
41-50	10 (16.7)	2 (20.0)		
51-60	13 (21.7)	5 (38.5)		
>60	15 (25.0)	6 (40.0)		
Sex				
Male	25 (41.7)	9 (36.0)	1.667	0.197
Female	35 (58.3)	6 (17.1)		
Total	**60 (100.0)**	**15 (25.0)**		

*=statistically significant at p<0.05; MDR=Multidrug resistant

Table 2: Distribution of *Klebsiella pneumoniae* and multidrug-resistant strains by setting

Strain	Number of isolates	Number of MDR isolates	x^2	OR (95% CI)	p value
HA-KP	38 (63.3)	11 (28.9)	0.3828	1.833 (0.5043-6.665)	0.5373
CA-HP	22 (36.7)	4 (18.2)			
Total	**60 (100.0)**	**15 (25.0)**			

HA-Hospital acquired; CA-Community acquired; MDR-Multi-drug resistant; x^2-Chi square; OR-Odd ratio; CI-Confidence interval

Table 3: Antibiotic susceptibility testing of *Klebsiella pneumoniae* isolates to routinely used antibiotics drugs according to setting

Isolates	Status	TZP	CIP	CRO	CN	AMC	MEM
HA-KP (n=38)	S	26 (68.4)	21 (55.3)	8 (21.1)	21 (55.3)	25 (65.8)	29 (76.3)
	R	12 (31.6)	17 (44.7)	30 (78.9)	17 (44.7)	13 (34.2)	9 (23.7)
CA-KP (n=22)	S	15 (68.2)	12 (54.5)	6 (27.3)	12 (54.5)	13 (59.1)	16 (72.7)
	R	7 (31.8)	10 (45.5)	16 (72.7)	10 (45.5)	9 (40.9)	6 (18.3)
Total (n=60)	S	41 (68.3)	31 (51.7)	14 (23.3)	31 (51.7)	38 (63.3)	45 (75.0)
	R	19 (31.7)	29 (48.3)	46 (76.7)	29 (48.3)	22 (36.7)	15 (25.0)

HA-KP: Hospital-acquired *Klebsiella pneumoniae*; CA-KP: Community-acquired *Klebsiella pneumoniae*; TZP: Piperacillin-tazobactam; CIP: Ciprofloxacin; CRO: Ceftriaxone; CN: Gentamicin; AMC: Amoxicillin-clavulanic acid; MEM: Meropenem

Table 4: Multiple antibiotic resistance index of *Klebsiella pneumoniae* isolates

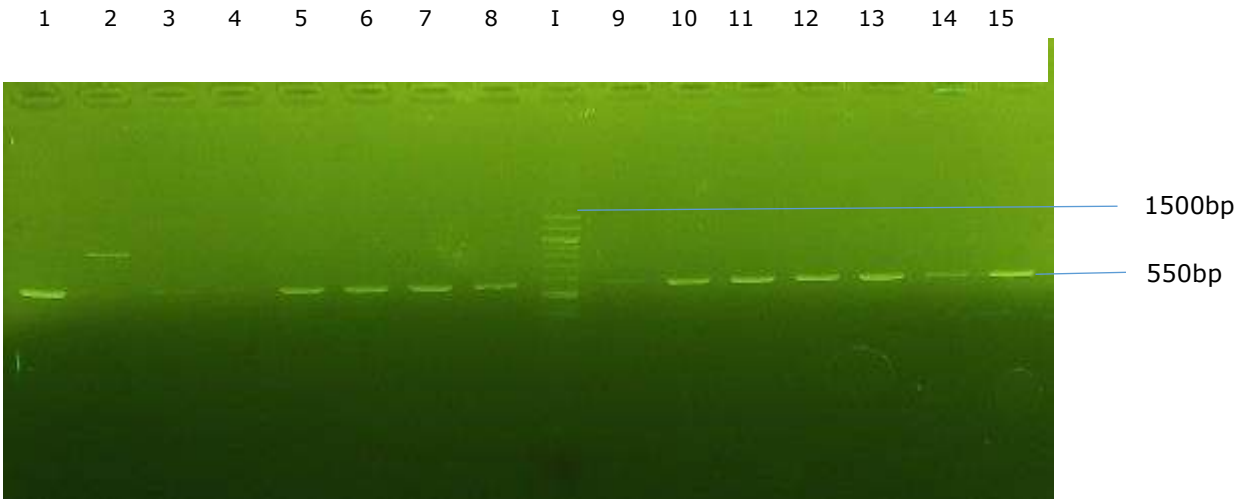
No of antibiotics	No of isolates (%)	HA-KP (%)	CA-KP (%)	MARI
1	5 (8.3)	3 (5.0)	2 (3.3)	0.2
2	10 (16.7)	6 (10.0)	3 (6.7)	0.3
3	13 (21.7)	8 (13.3)	5 (8.3)	0.5
4	15 (25.0)	9 (15.0)	6 (10.0)	0.7
5	12 (20.0)	7 (11.5)	5 (8.3)	0.8
6	5 (8.3)	2 (3.3)	3 (5.0)	1.0

HA-KP: Hospital-acquired *Klebsiella pneumoniae*; CA-KP: Community-acquired *Klebsiella pneumoniae*

Table 5: Resistance pattern of MDR *Klebsiella pneumoniae* strains to the classes of antibiotics tested

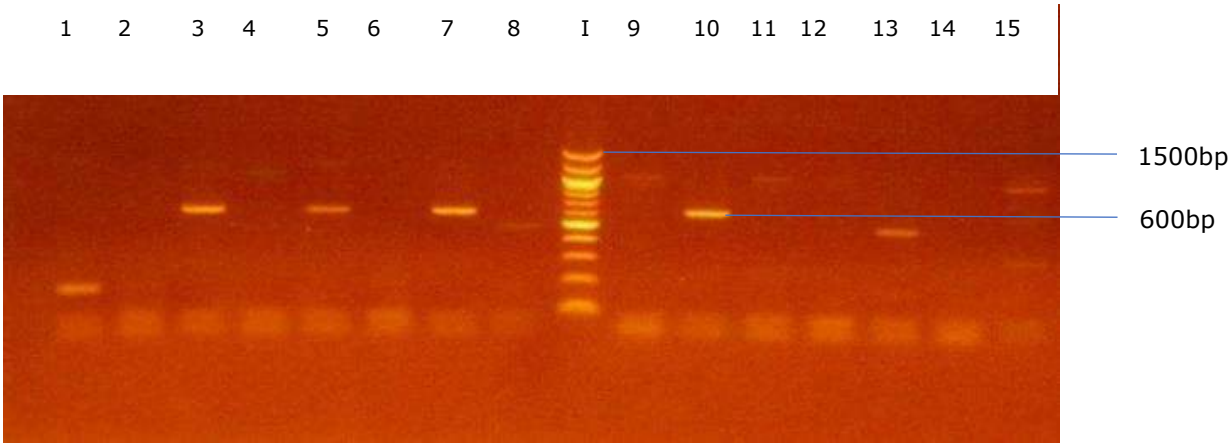
Class of antibiotics	HA-KP (%)	CA-KP (%)	Total	Prevalence (%)
R5	2 (13.3)	1 (6.7)	3	20.0
R4	1 (6.7)	2 (13.3)	3	20.0
R3	5 (33.3)	4 (26.7)	9	60.0
Total	**8 (53.3)**	**7 (46.7)**	**15**	

HA-KP: Hospital-acquired *Klebsiella pneumoniae*; CA-KP: Community-acquired *Klebsiella pneumoniae*; R=Resistance



Lanes 1-15 represent CTX-M gene bands (550bp); Lane I represents 100bp molecular ladder of 1500bp

Plate 1: Agarose gel electrophoresis of CTX-M gene of multidrug-resistant *Klebsiella pneumoniae* strains



Lane 3, 5, 7 and 10 represent KPC gene band (600bp); Lane I represents 100bp molecular ladder of 1500bp

Plate 2: Agarose gel electrophoresis of KPC gene of some multidrug-resistant *Klebsiella pneumoniae* strains

Table 6: Prevalence of ESβL genes among multidrug-resistant *Klebsiella pneumoniae* strains

Genes	No of MDR strain (%)	Prevalence (%)
bla_{CTX-M}	15 (100.0)	25.0
bla_{KPC}	4 (26.7)	6.7
bla_{CTX-M} & bla_{KPC}	4 (26.7)	6.7

MDR=Multidrug resistant

Discussion:

The frequency distribution of the *K. pneumoniae* isolates by age showed that patients in the age group >60 years had the highest number of *K. pneumoniae* recovered (25.0%, n=15), compared to patients in the age group 1-10 years (5.0%, n=3) in the study. This agrees with the study of Wang et al., (14) who reported prevalence of 53.6% among the elderly compared to 4.8% among minors in their region of study. The significant difference in frequency of *K. pneumoniae* recovery from focus of infection by age suggest a growing health need of the elderly population due to underlying ailments and changes in immune responses to invading pathogens. The frequency of *K. pneumoniae* isolation/infection was higher among patients on hospital admission (n=35, 58.3%) compared to those acquired from community (n=25, 41.7%). Although there was no significant difference in the frequency of *K. pneumoniae* isolation/infection between hospitalized and non-hospitalized patients (p=0.197), there are sufficient evidence that linked *K. pneumoniae* infection to hospital environments (15-17), however, the study revealed the extent to which the infection cause by this pathogen is fast spreading in community setting within the study population.

Antibiotic sensitivity testing results showed highest sensitivity of the isolates to meropenem (n=45, 75.0%), with 25.0% resistance rate. Resistance of these *K. pneumoniae* isolates to the carbapenems was lower compared to the finding from Ebonyi State, where Ejikeugwu et al., (18) reported 43.0% meropenem-resistant *K. pneumoniae* isolates (18). Similarly, high antimicrobial activity was observed with piperacillin/tazobactam against the bacterium. Previous research by Muhammed et al., (19) and Rodrıguez-Bano et al., (20) corroborates our finding. The use of piperacillin/tazobactam combination therapy as a choice drug for treatment of MDR *K. pneumoniae* could spare the carbapenems as the last line of drug against MDR-Enterobacteriaceae. Ceftriaxone on the other hand, showed the least antimicrobial activity against the isolates suggesting overuse/misuse of this antibiotic due to over-the-counter access of the drug in the population. There was however, no significant difference in the antimicrobial susceptibility pattern of the *K. pneumoniae* isolates with respect to hospital or community setting, underscoring the antibiotic 'selection pressure' present in both settings.

Fifteen (25.0%) of *K. pneumoniae* isolates were multidrug resistant. Majority of the MDR isolates were recovered among hospitalized individuals, however, the MDR rate was relatively low compared to the finding of Akinyemi et al., (21) in Lagos, Nigeria who reported 62.8% prevalence, but agrees with the finding of Tesfa et al., (22) who put the global prevalence of multidrug-resistant *K. pneumoniae* colonization in the range of 0.13% to 22%.Infections caused by MDR *K. pneumoniae* is associated with high mortality, often linked to inadequate or inappropriate antibiotic treatment.

The bla_{CTX-M} gene was detected in all the MDR *K. pneumoniae* strains, however, only 4 isolates carried the bla_{KPC} gene and were detected in the isolates recovered from hospitalized patients above 50 years of age in UCTH

and NNHC. Four of the strains co-habored *bla*$_{CTX-M}$ and *bla*$_{KPC}$ genes while the other 11 MDR strains harbored only the *bla*$_{CTX-M}$ gene. Although, the predominance of CTX-M gene in the population is unclear, however, it is thought that this resistant gene in the population, may have a link to frequent international travels of patients to high-prone areas for advance medical services. The report by Asir et al., (23) in India, corroborates the 100% distribution of the CTX-M gene in the population. Furthermore, in other part of Nigeria, low prevalence of the ESβL gene was reported (24) but in others such as Port Harcourt, Sokoto State, and in Southwestern Nigeria the prevalence of *bla*$_{CTX-M}$ gene is put at 41.67%, 35.71% and 32% respectively (25-27).

Additionally, 4 *K. pneumoniae* isolates recovered from sputum and urine samples of adult males and females habored *bla*$_{KPC}$ gene, and medical records showed that one of patients had visited the USA for medical treatment while the other three individuals received medical care in Nigeria, suggesting that this rare type of carbapenemase in Calabar, may also have a link to international travels for medical treatments. The study by Kabiru et al., (28) in four medical centres in Lagos Nigeria, identified 7.0% of carbapenemase-producing *K. pneumoniae* strains from similar sample types, while Oshun and Ogunsola (29) reported only 2.6% carbapenemase producing *K. pneumoniae* in one of the teaching hospitals in Lagos (29). In other parts of Africa, specifically in Sudanese and Egyptian University Hospitals, low prevalence of *bla*$_{KPC}$ gene was also detected in the study population (30-31).

Conclusion:

The MDR prevalence of 25.0% was seen among *K. pneumoniae* isolates with high resistance to the third-generation cephalosporin antibiotic tested in this study. The commonest ESβLs-encoding gene detected among the MDR strains was *bla*$_{CTX-M}$ gene. Generally, an informed and appropriate use of antimicrobial drugs, particularly the beta-lactam antibiotics, for management of bacterial infections, will prevent further escalation of drug-resistant *K. pneumoniae* strains in this epidemiological setting.

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Contributions of authors:

IIU and NOU were involved in the conceptualization, design and writing of the manuscript; IIU and SOI were involved in data collection and analysis of results; Ethical approval of the study was facilitated by BCO and VUA. NOU supervised the research work. AAU, FBU and JIU edited the manuscript. All authors read and approved the manuscript for publication.

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Original Article **Open Access**

Bacterial isolates from toilet seats in a tertiary institution and their susceptibility to antibiotics and selected toilet cleaning agents marketed in Nigeria

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Abstract:

Background: Infections associated with the use of contaminated public toilets and the control of such infections are a major challenge especially with the high prevalence of substandard toilet cleaning agents in the Nigerian market. The study aimed to determine bacterial isolates contaminating public toilet seats and their susceptibility to commonly used antibiotics, and to assess *in vitro* antimicrobial activity of selected toilet cleaning agents commercially available in the Nigerian market.
Methodology: Swab samples were collected from 50 randomly selected toilet seats from three designated areas in Obafemi Awolowo University campus, Ile-Ife, Nigeria. Bacteria were isolated and identified using conventional culture and biochemical identification test scheme. Susceptibility of the isolates to conventional antibiotics was determined by the Kirby Bauer disk diffusion method and interpreted as resistant, intermediate or susceptible using the Clinical and Laboratory Standard Institute guidelines. The *in vitro* antimicrobial activity of selected toilet cleaning agents was evaluated using the agar well diffusion technique, with 1% cetrimide as the standard positive control.
Results: A total of 90 bacteria were isolated from the 50 toilet seats with a frequency of *Bacillus* spp (33.3%, n=30), *Escherichia coli* (23.3%, n=21), *Staphylococcus aureus* (15. 6%, n=14), *Klebsiella pneumoniae* (8. 9%, n=8), *Micrococcus luteus* (3.3%, n=3), *Enterococcus faecalis* (3.3%, n=3), *Salmonella typhi* (2.2%, n=2), *Proteus vulgaris* (2.2%, n=2), *Staphylococcus epidermidis* (2.2%, n=2), *Shigella sonnei* (1.1%, n=1), *Enterobacter aerogenes* (1.1%, n=1), *Staphylococcus intermedius* (1.1%, n=1), *Proteus mirabilis* (1.1%, n=1), and *Serratia marcescens* (1.1%, n=1). Majority of the isolates were resistant to multiple antibiotics. Susceptibility to ciprofloxacin antibiotic was the highest, while all the isolates were susceptible to all the toilet cleaning agents evaluated when compared to 1% cetrimide used as the standard positive control.
Conclusion: The study showed that toilet seats harbor pathogenic bacteria with varying susceptibilities to antibiotics and toilet cleaning agents.

Keywords: Toilet seats; Toilet cleaning agents; Bacteria; Antimicrobial activity; Susceptibility

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Isolats bactériens prélevés sur des sièges de toilettes dans un établissement d'enseignement supérieur et leur sensibilité aux antibiotiques et à certains produits d'entretien commercialisés au Nigéria

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Résumé:

Contexte: Les infections liées à l'utilisation de toilettes publiques contaminées et leur contrôle constituent un défi majeur, notamment en raison de la forte prévalence de produits d'entretien de qualité inférieure sur le marché nigérian. Cette étude visait à identifier les isolats bactériens contaminant les sièges de toilettes publiques et leur sensibilité aux antibiotiques couramment utilisés, ainsi qu'à évaluer l'activité antimicrobienne in vitro de certains produits d'entretien disponibles dans le commerce au Nigéria.
Méthodologie: Des prélèvements ont été effectués sur 50 sièges de toilettes sélectionnés aléatoirement dans

trois zones désignées du campus de l'Université Obafemi Awolowo, à Ile-Ife, au Nigéria. Les bactéries ont été isolées et identifiées par culture conventionnelle et tests biochimiques. La sensibilité des isolats aux antibiotiques conventionnels a été déterminée par la méthode de diffusion sur disque de Kirby-Bauer et interprétée comme résistante, intermédiaire ou sensible selon les recommandations du CLSI (Institut des Normes Cliniques et de Laboratoire). L'activité antimicrobienne *in vitro* de certains produits de nettoyage pour toilettes a été évaluée par la technique de diffusion en puits sur gélose, avec du cétrimide à 1 % comme témoin positif.
Résultats: Au total, 90 bactéries ont été isolées des 50 sièges de toilettes, avec les fréquences suivantes: *Bacillus* spp (33,3%, n=30), *Escherichia coli* (23,3%, n=21), *Staphylococcus aureus* (15,6%, n=14), *Klebsiella pneumoniae* (8,9%, n=8), *Micrococcus luteus* (3,3%, n=3), *Enterococcus faecalis* (3,3%, n=3), *Salmonella* Typhi (2,2 %, n=2), *Proteus vulgaris* (2,2%, n=2), *Staphylococcus epidermidis* (2,2%, n=2), *Shigella sonnei* (1,1%, n=1), *Enterobacter aerogenes* (1,1%, n=1), *Staphylococcus intermedius* (1,1%, n=1) et *Proteus mirabilis* (1,1%, n=1) et *Serratia marcescens* (1,1%, n=1). La majorité des isolats étaient résistants à plusieurs antibiotiques. La sensibilité à la ciprofloxacine était la plus élevée, tandis que tous les isolats étaient sensibles à tous les produits de nettoyage pour toilettes évalués, comparativement au cétrimide à 1% utilisé comme témoin positif. **Conclusion:** Cette étude a montré que les sièges de toilettes abritent des bactéries pathogènes présentant une sensibilité variable aux antibiotiques et aux produits de nettoyage pour toilettes.

Mots-clés: Sièges de toilettes; Produits de nettoyage pour toilettes; Bactéries; Activité antimicrobienne; Sensibilité

Introduction:

A toilet is a device that allows one to urinate or defecate. It usually has a flushing mechanism, a lid that can be removed, and a seat that can be pivoted (1). However, public toilets are facilities where individuals can address their hygienic needs. These can be found in all manner of places including markets, transport centers, schools, eateries, hostels, offices, factories, cinemas, bars, museum and more (1). Public restrooms, on the other hand, are any modest room or building with one or more toilets (2).

According to the World Health Organization (WHO), over 1.5 billion people of the global population still do not have basic sanitation services, such as private toilets or latrines of which 419 million still defecate in the open, for example in street gutters, behind bushes or into open bodies of water (3). Access to toilets has been recognized by the United Nations as a human right that provides privacy and ensures dignity. Public health depends on toilets and except that everyone in a community has a safe toilet, the health of everyone is threatened (4).

Unhygienic use of public toilets can lead to urine and faecal residues remaining on surfaces after use, potentially serving as significant reservoirs or sources of human pathogens (5). Public toilets exhibit microbial diversity due to the high activity levels of individuals with varying hygienic practices (6). Bacterial pathogens that have been reportedly associated with toilets include *Escherichia coli*, *Klebsiella*, *Pseudomonas, Salmonella, Proteus vulgaris, Enterococcus faecalis, Staphylococcus epidermidis, Staphylococcus aureus, Citrobacter* spp and *Bacillus* spp (7-9).

The presence of pathogenic organisms in toilets implies that toilets can serve as a source of infections aside their aiding the transmission of infections. Poor and unhygienic use of toilets has been linked to the transmission of diarrheal diseases such as cholera and dysentery, as well as typhoid, intestinal worm infections, and polio (10-12). Moreover, outbreaks of infectious diseases arising from toilets have been documented, largely from improper cleaning and disinfection of restroom facilities (13,14). However, despite that hazards associated with toilet seats have been reported by researchers, less attention has been given to toilet seats as inanimate objects which could harbor and transmit infectious agents (15).

One of the ways by which infections associated with toilet seats can be curtailed is through the use of toilet cleaning agents which are liquid solutions used in disinfecting the toilet bowl and even the seat. They are cleaning products specifically designed to sanitize and clean the toilet. They help remove germs, stains, and odors (16). They contain different active agents such as hydrochloric acid, sodium hypochlorite, sodium lauryl ether sulfate, sodium hydroxide, quaternary ammonium compounds and borax (17).

One of the factors militating against the increasing awareness on the need to maintain good toilet hygiene is the proliferation and preponderance of adulterated, substandard and fake toilet cleaning agents in Nigeria markets (18). This has become a national embarrassment and a public health concern. This study therefore aimed at evaluating some toilet cleaning agents in Nigeria markets for their antibacterial activity against bacterial isolates associated with toilet seats.

Materials and method:

Study area:

The study was carried out at the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Obafemi Awolowo University (O.A.U), Ile-Ife, Nigeria.

Collection of samples:

Samples were collected from 50 randomly selected toilet seats at 3 designated areas of the University; Faculty of Pharmacy (male and female toilets), a male hostel and a

female hostel using the swab-rinse technique. Briefly, sterile swab sticks pre-moistened with normal saline were used to swab the toilet seats, and then pre-incubated for 30 seconds in normal saline.

Bacteria isolation by conventional culture:

Samples were cultured on *Salmonella-Shigella*, MacConkey, Cetrimide, Mannitol salt and Blood agar plates. Following a 24-h incubation at 37^{o}C, distinct colonies were sub-cultured and purified for identification using conventional biochemical test schemes (19, 20).

Antibiotic susceptibility test of isolates:

Antimicrobial susceptibility testing of each bacterial isolate was determined using the Kirby Bauer disc diffusion technique (21). Briefly, colonies of each bacterial isolate were dissolved in 10ml sterile distilled water in McCartney bottle and standardized to 0.5 MacFarland standards using sterile distilled water. The resulting bacterial suspension was uniformly spread on the surface of Mueller-Hinton (MH) agar plate using a sterile swab stick.

Antibiotic discs (cefuroxime 30µg, ceftriaxone 30µg, cefotaxime 30µg, ceftazidime 30µg, gentamicin 10µg, amikacin 30µg, tetracycline 10µg, cotrimoxazole 25µg, chloramphenicol 10µg, ciprofloxacin 5µg, and meropenem 10µg) were placed on the agar plates. The plates were incubated at 37^{o}C for 24 hours after which the inhibition zone diameters were measured. The results were interpreted as susceptible, intermediate or resistant using the Clinical and Laboratory Standard Institute (CLSI) guidelines (22).

***In vitro* antibacterial activity of toilet cleaning agents:**

The toilet cleaning agents used were purchased from various supermarkets in Lagos and their containers disclosures are shown in Table 1. *In vitro* antibacterial activity of the cleaning agents was determined using the agar well diffusion method. Overnight grown culture of the bacterial isolates in Mueller-Hinton broth (MHB) was standardized to 0.5 McFarland standard (an equivalent of 1.5 x 10^8 CFU/ml) using sterile Mueller-Hinton broth. One in hundred (1 in 100) dilution of the standardized bacterial culture was made by adding 0.1ml to 10ml MHB to give 1.5 x 10^6 CFU/ml. A further 1 in 100 dilution was made by seeding 0.2ml in 20ml melted and cooled Mueller-Hinton agar in McCartney bottle, poured into Petri dish and allowed to set. This gave a final inoculum of 1.5 x 10^4 CFU/ml.

Wells were then bore into the MH agar using a sterile cork-borer. Exactly 4 drops of each of the toilet cleaning agents were put into each of the wells while 4 drops of 1% cetrimide solution was used as standard positive control. The plates were allowed to stand for one hour for diffusion of the toilet cleaning agent and then incubated at 37^{o}C for 24 hours. The antibacterial activity of each of the toilet cleaning agent was evaluated by measuring the diameter of the zones of inhibition. All the plates were made in duplicates.

Results:

The frequency distribution of 90 bacterial isolates from the 50 toilet seats is shown in Table 2, with 53 (58.9%) Gram-positive and 37 (41.1%) Gram-negative isolates. While *Bacillus* spp had the highest frequency of 33.3% among the Gram-positive isolates, *Escherichia coli* has the highest frequency of 23.3% among the Gram-negative isolates.

The detail susceptibility and resistance profiles of the bacterial isolates to selected conventional antibiotics are displayed on Table 3. Majority of the isolates were multiply-resistant to the antibiotics, with all *Bacillus* spp resistant to at least 4 antibiotics, and all *E. coli* and *S. aureus* resistant to at least 5 antibiotics. The percentage susceptibility of the isolates to the selected antibiotics is shown in Table 4, with majority of the isolates being most susceptible to ciprofloxacin but resistant to all the cephalosporins (cefotaxime, ceftazidime, ceftriaxone and cefuroxime) and meropenem.

The *in vitro* activity of selected toilet cleaning agents marketed and distributed in Nigerian markets on the bacterial isolates are presented in Table 5. Despite the fact that majority of the isolates were resistant to multiple antibiotics, all the toilet cleaning agents had high *in vitro* antibacterial activity against the bacterial isolates, higher than the 1% cetrimide used as the standard positive control agent.

Table 1: Detail of the contents of selected toilet cleaners marketed in Nigeria

S/N	Name of toilet washer	MFD	Expiry Date	Manufacturer's Address	Ingredients	Batch Number	NAFDAC Number
1.	Izal Germicide	03/24	02/26	GONGONI CO. LTD. 89A Sharada Industrial Estate, Phase III, Kano	Active Ingredient: Tar Acid Phenol 7%; Cresylic Creosote 2%	051	04-1130
2.	Jenic Power Plus Ceramic/toilet/floor Cleaner	2024	2027	Jenic Merchandise Industries Nig. LTD. H2 Plot 492 Signal Barracks Mile 2, Lagos. 08065378814	Active Ingredient: Hydrochloric acid	NA	05902
3.	Harpic Original	02/24	02/26	Reckitt Benckiser Nig LTD. Plot C2/3 Agbara Industrial Estate, Ogun State, Nigeria. 0902626262	Active Ingredient: 10%w/v Hydrochloric acid, 0.63%w/v Hexadecetrimethyl ammonium chloride	0352	02-2018
4.	Spark Toilet cleaner	2023	2025	Betholite Trust Int'l Venture, Km 42, Lagos- Ibadan Expressway, Pakoro, Ogun State. 08036740626	Deionised water, anionic surfactant, foam agent, Fragrance. Active Ingredient: Sodium Hydroxide, Hydrogen peroxide	NA	02-4971
5.	Squick Multiactive Ceramic/tiles/toilet cleaner	05/24	04/26	E-Summit multi-Global Technologies. 01 Orji Lane, Ojo, Lagos. +2347063584447	Aqua, surfactant, emulsifier, dye, fragrance, preservative Active Ingredients: Hydrochloric acid Disinfectant	0062	A2-1184
6.	Hypo Toilet Cleaner	09/23	09/25	Hypo Homecare Products LTD, Km 5, Itokin Rd, Ikorodu, Lagos. 07000004976	De-mineralised water, amine ethoxylate and color Active Ingredients: 10.5%w/v Hydrochloric acid	15 I	A2-1009
7.	Luvic Power Plus ceramic/toilet/ tiles cleaner	02/22	02/26	Luvic Nig ltd. 250, Old Ojo road, Agboju Amuwo odofin, Lagos State. 08100191319	Active Ingredient: Hydrochloric acid 85% Sulphuric acid 75%	NA	02-3982

Table 2: Frequency distribution of bacterial isolates from toilet seats

Bacterial isolates	Number	Percentage
Gram-positive bacteria		
Bacillus spp	30	33.3
Staphylococcus aureus	14	15.6
Micrococcus luteus	3	3.3
Enterococcus faecalis	3	3.3
Staphylococcus epidermidis	2	2.2
Staphylococcus intermedius	1	1.1
Gram-negative bacteria		
Escherichia coli	21	23.3
Klebsiella pneumoniae	8	8.9
Salmonella Typhi	2	2.2
Proteus vulgaris	2	2.2
Proteus mirabilis	1	1.1
Shigella sonnei	1	1.1
Enterobacter aerogenes	1	1.1
Serratia marcescens	1	1.1
Total	**90**	**100.0**

Table 3: Detailed antibiotic susceptibility and resistance profiles of bacterial isolates of selected toilet seats in Obafemi Awolowo University, Ile -Ife

Code	Bacterial species	AMK	CHL	CIP	COT	CPZ	CRX	CTR	CTX	GEN	MEM	TET	MAR
	Gram-positive isolates												
	Bacillus												
F16b	*Bacillus* spp	S	S	S	R	S	R	S	S	R	S	R	4
F17a	*Bacillus* spp	S	S	S	I	R	R	R	R	S	R	S	5
F19b	*Bacillus* spp	S	S	S	S	R	R	R	R	S	R	S	5
F20	*Bacillus* spp	S	S	S	R	R	R	S	R	S	R	S	5
FOB2b	*Bacillus* spp	S	S	S	S	R	R	R	R	S	R	S	5
FOB5a	*Bacillus* spp	S	I	S	S	R	R	R	R	S	R	S	5
M14b	*Bacillus* spp	S	S	S	I	R	R	R	R	S	R	R	6
F6	*Bacillus* spp	S	S	S	R	R	R	R	R	S	R	S	6
F18	*Bacillus* spp	S	S	S	R	R	R	R	R	S	R	S	6
F21	*Bacillus* spp	S	S	S	R	R	R	R	R	S	R	I	6
FOB3a	*Bacillus* spp	R	S	S	S	R	R	R	R	S	R	S	6
FOB3b	*Bacillus* spp	S	S	S	S	R	R	R	R	S	R	R	6
S1	*Bacillus* spp	S	S	I	R	R	R	R	R	S	R	R	7
F2b	*Bacillus* spp	S	S	S	R	R	R	R	R	R	R	S	7
F3b	*Bacillus* spp	S	S	S	R	R	R	R	R	R	R	I	7
F5b	*Bacillus* spp	S	S	S	R	R	R	R	R	I	R	R	7
F8a	*Bacillus* spp	S	S	S	R	R	R	R	R	R	R	I	7
F16a	*Bacillus* spp	S	S	I	I	R	R	R	R	R	R	R	7
MOB3a	*Bacillus* spp	S	S	S	R	R	R	R	R	S	R	R	7
FOB4a	*Bacillus* spp	S	S	S	I	R	R	R	R	R	R	R	7
M15a	*Bacillus* spp	S	S	S	R	R	R	R	R	R	R	R	8
M15b	*Bacillus* spp	S	S	S	R	R	R	R	R	R	R	R	8
F7a	*Bacillus* spp	S	S	R	R	R	R	R	R	S	R	R	8
F10	*Bacillus* spp	S	S	S	R	R	R	R	R	R	R	R	8
FOB5b	*Bacillus* spp	S	S	S	R	R	R	R	R	R	R	R	8
M5b	*Bacillus* spp	R	S	S	R	R	R	R	R	R	R	R	9
M7bi	*Bacillus* spp	S	R	S	R	R	R	R	R	R	R	R	9
M9a	*Bacillus* spp	R	S	S	R	R	R	R	R	R	R	R	9
FOB4b	*Bacillus* spp	S	R	S	R	R	R	R	R	R	R	R	9
F14a	*Bacillus* spp	R	R	S	R	R	R	R	R	R	R	R	10
	Staphylococcus												
F17b	*Staphylococcus aureus*	S	S	S	S	R	R	R	R	S	R	S	5
M1c	*Staphylococcus aureus*	S	I	I	S	R	R	R	R	R	R	R	7
M1b	*Staphylococcus aureus*	R	S	R	S	R	R	R	R	R	R	S	8
M4b	*Staphylococcus aureus*	S	I	S	R	R	R	R	R	R	R	R	8
F1b	*Staphylococcus aureus*	S	S	S	R	R	R	R	R	R	R	R	8
F8c	*Staphylococcus aureus*	S	S	S	R	R	R	R	R	R	R	R	8
M3b	*Staphylococcus aureus*	S	S	R	R	R	R	R	R	R	R	R	9
M4a	*Staphylococcus aureus*	R	S	S	R	R	R	R	R	R	R	R	9
M6a	*Staphylococcus aureus*	R	S	I	R	R	R	R	R	R	R	R	9
F8b	*Staphylococcus aureus*	S	R	S	R	R	R	R	R	R	R	R	9
F12	*Staphylococcus aureus*	S	R	S	R	R	R	R	R	R	R	R	9
F13	*Staphylococcus aureus*	S	R	S	R	R	R	R	R	R	R	R	9
FNB3	*Staphylococcus aureus*	R	R	I	R	R	R	R	R	S	R	R	9
M3a	*Staphylococcus aureus*	S	R	R	R	R	R	R	R	R	R	R	10
MOB2a	*Staphylococcus epidermidis*	S	S	R	I	R	R	R	R	R	R	R	8
M12b	*Staphylococcus epidermidis*	S	R	S	R	R	R	R	R	R	R	R	9
F19a	*Staphylococcus intermedius*	R	S	S	S	R	R	R	R	I	R	S	6
	Micrococcus												
F4a	*Micrococcus luteus*	S	S	S	R	R	R	R	R	S	R	S	6

F4b	*Micrococcus luteus*	S	S	S	R	R	R	R	R	R	R	R	8
MOB1b	*Micrococcus luteus*	S	R	R	R	R	R	R	R	I	R	R	9
	Enterococcus												
F2a	*Enterococcus faecalis*	S	S	I	R	R	R	R	R	S	R	R	7
M11a	*Enterococcus faecalis*	S	R	S	S	R	R	R	R	I	R	R	7
M2bii	*Enterococcus faecalis*	S	S	R	R	R	R	R	R	R	R	R	9
	Gram-negative isolates												
	Escherichia												
MOB2b	*Escherichia coli*	S	S	S	S	R	R	R	R	S	R	S	5
M8ai	*Escherichia coli*	S	S	S	R	R	R	R	R	I	R	R	7
M12a	*Escherichia coli*	S	S	S	R	R	R	R	R	S	R	R	7
M14a	*Escherichia coli*	R	S	S	S	R	R	R	R	I	R	R	7
F5a	*Escherichia coli*	S	S	S	R	R	R	R	R	S	R	R	7
FOB1b	*Escherichia coli*	S	S	S	R	R	R	R	R	S	R	R	7
FOB2a	*Escherichia coli*	R	S	I	S	R	R	R	R	R	R	S	7
M8b	*Escherichia coli*	S	S	I	R	R	R	R	R	R	R	R	8
F3a	*Escherichia coli*	S	S	I	R	R	R	R	R	R	R	R	8
F9	*Escherichia coli*	S	S	S	R	R	R	R	R	R	R	R	8
FNB4	*Escherichia coli*	R	S	S	S	R	R	R	R	R	R	R	8
M6bii	*Escherichia coli*	S	S	R	R	R	R	R	R	R	R	R	9
M9b	*Escherichia coli*	R	R	S	R	R	R	R	R	S	R	R	9
F14b	*Escherichia coli*	S	R	S	R	R	R	R	R	R	R	R	9
FOB1a	*Escherichia coli*	S	R	S	R	R	R	R	R	R	R	R	9
M5a	*Escherichia coli*	S	R	R	R	R	R	R	R	R	R	R	10
M10a	*Escherichia coli*	R	R	S	R	R	R	R	R	R	R	R	10
M10b	*Escherichia coli*	R	R	S	R	R	R	R	R	R	R	R	10
F15	*Escherichia coli*	R	R	S	R	R	R	R	R	R	R	R	10
FNB1b	*Escherichia coli*	S	R	R	R	R	R	R	R	R	R	R	10
M6bi	*Escherichia coli*	R	R	R	R	R	R	R	R	R	R	R	11
	Klebsiella												
M13	*Klebsiella pneumoniae*	S	S	S	S	R	R	R	R	S	R	R	6
FNB2	*Klebsiella pneumoniae*	R	S	S	S	R	R	R	R	S	R	R	7
M7bii	*Klebsiella pneumoniae*	S	S	S	R	R	R	R	R	R	R	R	8
F11a	*Klebsiella pneumoniae*	R	S	S	R	R	R	R	R	R	R	I	8
F1a	*Klebsiella pneumoniae*	S	S	R	R	R	R	R	R	R	R	R	9
F7b	*Klebsiella pneumoniae*	S	S	R	R	R	R	R	R	R	R	R	9
F11b	*Klebsiella pneumoniae*	S	R	S	R	R	R	R	R	R	R	R	9
MOB3b	*Klebsiella pneumoniae*	S	R	S	R	R	R	R	R	R	R	R	9
	Salmonella												
M7a	*Salmonella* Typhi	S	R	S	R	R	R	R	R	R	R	R	9
M11b	Salmonella Typhi	S	R	S	R	R	R	R	R	R	R	R	10
	Proteus												
FNB5	*Proteus vulgaris*	S	S	S	R	R	R	R	R	R	R	R	8
M1a	*Proteus vulgaris*	S	R	R	R	R	R	R	R	R	R	R	10
MOB1a	*Proteus mirabilis*	S	R	I	R	R	R	R	R	R	R	R	9
	Shigella												
M2bi	*Shigella sonnei*	S	S	S	S	R	R	R	R	S	R	S	5
	Enterobacter												
M8aii	*Enterobacter aerogenes*	R	R	S	R	R	R	R	R	S	R	R	9
	Serratia												
FNB1a	*Serratia marcescens*	S	S	S	S	R	R	R	R	S	R	R	6

TET: Tetracycline 10µg; COT: Cotrimoxazole 25µg; GEN: Gentamicin 10µg; CRX: Cefuroxime 30µg; CHL: Chloramphenicol 10µg; CTR: Ceftriaxone 30µg; CTX: Cefotaxime 30µg; CIP: Ciprofloxacin 5µg; AMK: Amikacin 30µg; VAN: Vancomycin 30µg; CPZ: Ceftazidime 30µg; MEM: Meropenem 10µg; R: Resistant; S: Sensitive; I: Intermediate; MAR: Multiple Antibiotic Resistance

Table 4: Susceptibility of bacterial isolates to selected antibiotics

Bacterial isolates	AMK (%)	CHL (%)	CIP (%)	COT (%)	CPZ (%)	CRX (%)	CTR (%)	CTX (%)	GEN (%)	MEM (%)	TET (%)
Gram-positives											
Staphylococcus aureus	11 (78.6)	9 (64.3)	12 (85.7)	4(28.6)	0	0	0	0	3 (21.4)	0	2 (14.3)
Staphylococcus epidermidis	2 (100)	1 (50)	1 (50)	1 (50)	0	0	0	0	0	0	0
Staphylococcus intermedius	0	1 (100)	1 (100)	1 (100)	0	0	0	0	1 (100)	0	1 (100)
Enterococcus faecalis	3 (100)	2 (66.7)	2 (66.7)	1 (33.3)	0	0	0	0	2 (66.7)	0	0
Micrococcus luteus	3 (100)	2 (66.7)	2 (66.7)	0	0	0	0	0	2 (66.7)	0	1 (33.4)
Bacillus spp	26 (86.7)	27 (90)	29 (96.7)	9 (30)	1 (3.3)	0	2 (6.7)	1 (3.3)	14 (46.7)	1 (3.3)	11 (36.7)
Gram-negatives											
Escherichia coli	13 (61.9)	12 (57.1)	19 (90.5)	3 (14.3)	0	0	0	0	7 (33.3)	0	2 (9.5)
Klebsiella pneumoniae	6 (75.0)	6 (75.0)	6 (75.0)	2 (25.0)	0	0	0	0	2 (25.0)	0	1 (12.5)
Salmonella Typhi	2 (100)	0	1 (50)	0	0	0	0	0	0	0	0
Proteus vulgaris	2 (100)	1 (50)	1 (50)	0	0	0	0	0	0	0	0
Shigella sonnei	1 (100)	1 (100)	1 (100)	1 (100)	0	0	0	0	1 (100)	0	1 (100)
Enterobacter aerogenes	0	0	1 (100)	0	0	0	0	0	1 (100)	0	0
Proteus mirabilis	1 (100)	0	1(100)	0	0	0	0	0	0	0	0
Serratia marcescens	1 (100)	1 (100)	1 (100)	1 (100)	0	0	0	0	1 (100)	0	0

TET: Tetracycline 10µg; COT: Cotrimoxazole 25µg; GEN: Gentamicin 10µg; CRX: Cefuroxime 30µg; CHL: Chloramphenicol 10µg; CTR: Ceftriaxone 30µg; CTX: Cefotaxime 30µg; CIP: Ciprofloxacin 5µg; AMK: Amikacin 30µg; VAN: Vancomycin 30µg; CPZ: Ceftazidime 30µg; MEM: Meropenem 10µg

Table 5: *In vitro* antimicrobial activity (average zone diameter of inhibition in mm) of bacterial species isolated from toilet seats to selected toilet cleaning agents

Bacterial isolates	Mean zone diameter of inhibition (mm)							
	IZAL	JENIC	HARPIC	SPARK	SQUICK	HYPO	LUVIC	1% CETRIMIDE
Gram-positives								
Staphylococcus aureus (n=17)	23	19	30	25	20	26	21	13
Micrococcus luteus (n=3)	22	22	32	26	22	28	20	14
Enterococcus faecalis (n=3)	25	22	30	29	23	29	25	17
Bacillus spp (n=30)	24	21	34	28	23	29	22	15
Gram-negatives								
Escherichia coli (n=21)	24	20	32	26	20	28	21	14
Klebsiella pneumoniae (n=8)	24	21	30	27	23	29	24	16
Salmonella Typhi (n=2)	23	22	35	28	22	30	23	13
Proteus mirabilis (n=3)	24	22	31	28	24	29	24	15
Shigella sonnei (n=1)	30	18	40	25	23	35	30	18
Enterobacter aerogenes (n=1)	25	16	35	25	25	25	20	10
Serratia marcescens (n=1)	21	18	45	35	21	25	22	12

Discussion:

Public toilets are required when there is a need to eliminate bodily waste in public settings. Safe, private and purposeful visits of public toilets are guided by factors as accessibility, availability and adaptability (23). Infections, which may be bacterial, fungal or viral in origin, are one of the hazards associated with public toilet use. All the pathogens responsible for toilet-associated infections may be harbored in any part of the toilet. However, less attention has been given to toilet seats as inanimate objects which could harbor and serve as a vehicle for transmission of infectious agents despite that hazards associated with toilet seats have been reported by researchers.

In this study, several species of bacteria were isolated and identified from the toilet seats. The finding in this study agrees with the report of Juliene et al., (24) on identification of bacteria from public toilets in Rwanda. Many of the isolates in our study have been reported by many researchers (1,7-9). The presence of these pathogens on toilet seats is an indication of unhygienic toilet use. For instance, the presence of *Bacillus* spp is an indication of a long-time settlement of dust on toilet seats without being cleaned, *Bacillus* spp being a spore-forming Gram-positive bacterium found in soil but also in the gastrointestinal tract of humans. The pathogenesis of the *Bacillus* species depends upon the survival of spores in the non-host environment, as dormant spores are the main infectious form of the bacterium (25). The presence of *E. coli* and *K. pneumoniae* indicates faecal contamination as well as human vectors involvement. These two species of bacteria are the leading causes of urinary tract infections. *Staphylococcus aureus* is a major component of the normal flora of the skin and nose, which can easily be discharged by several human activities.

Treatment of infections acquired from toilet seats often involves the use of antibiotics, the choice of which depends on the susceptibility of the organism responsible for the infection. In this study, the bacterial isolates displayed varying degrees of susceptibilities with majority of the isolates being multiple antibiotic-resistant. However, the presence of drug-resistant *Shigella* spp and *Salmonella*

Typhi in this study poses a threat to human health and requires a serious health control intervention. Although majority of the isolates were resistant to multiple antibiotics, they were however susceptible to ciprofloxacin, a fluoroquinolone antibiotic. This finding also corroborates the study of Lincy et al., (26) who reported high susceptibility of bacterial isolates from public toilets to ciprofloxacin.

Acquisition of infections as well as the potential for pathogen transmission through toilet seats can be curtailed through good toilet hygiene which can be achieved through the use of toilet cleaning agents (toilet sanitizers/cleaners). Toilet cleaners differ in their compositions and actions. They can be classified into cleaners containing acids for removing calcium or metal salts and cleaners containing a bleaching system (27). In this study, all the toilet cleaners demonstrated high *in vitro* antibacterial activity against all the bacterial isolates, compared to 1% cetrimide used as the standard positive control and irrespective of the resistance of the isolates to antibiotics.

Conclusion:

This study showed that toilet seats harbor pathogenic bacteria that are resistant to multiple antibiotics while all the brands of toilet cleaners marketed in Nigeria and tested in this study exhibited high *in vitro* antimicrobial activity against the isolates. However, toilets should be shared with caution among students of tertiary institutions since all the bacteria isolated in the study are known to be associated with various forms of infection in human.

Contributions of authors:

MOO designed the study and was involved in collation, analysis and interpretation of the data, and writing of the manuscript. OS-RO was involved in culture isolation, identification and antibiotic susceptibility testings of bacterial isolates. FOA was involved in culture isolation, identification, antibiotic susceptibility testings of bacterial isolates, collation, and analysis of data. DIO was involved in antibiotic susceptibility testings of bacterial isolates, collation and analysis of data.

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Conflict of interest:

Authors declare no conflict of interest.

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Original Article **Open Access**

Prevalence of parasitic contamination of wastewater samples from selected slaughter houses in Benin City, Edo State, Nigeria

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Abstract:

Background: Wastewater from slaughterhouses can contain a variety of parasites, including protozoa and helminths. This study was designed to systematically examine wastewater samples from selected slaughter houses in Benin-City, Edo State, and determine the prevalence parasite contamination and spatial distribution of these parasites, to inform future public health and environmental protection strategies.
Methodology: Wastewater samples (n=120) collected from four randomly selected slaughterhouses (Ikpoba Hill, New-Benin, Oluku, and Ugbowo) in Benin-City, Edo State, Nigeria, were examined for the presence of parasites. The samples were processed using standard parasitological techniques, involving saline and iodine wet preparations on the slides, which were then examined under compound light microscope at 10x and 40x magnifications. The modified Ziehl-Neelsen staining procedure was performed for detection of *Cryptosporidium* ova.
Results: Of the 120 wastewater samples examined, 118 tested positive for parasites, giving a parasitic contamination rate of 98.3%, with a total of 360 parasites detected. *Ascaris lumbricoides* was the most prevalent (n=85, 23.6%), followed by *Taenia* spp (n=51, 14.2%) and *Balantidium coli* (n=48, 13.3%). Other notable parasites detected were *Fasciola hepatica* (n=37, 10.3%) and *Strongyloides stercoralis* (n=32, 8.9%). Conversely, less prevalent parasites included *Chydonis spherieus* (n=1, 0.3%), *Amoeba* spp (n=2, 0.6%), Hookworm (n=2, 0.6%), and Pin worm (n=2, 0.6%). There was no significant difference in the prevalence of parasites across the four locations (p=0.545).
Conclusion: The results of this study provide a comprehensive assessment of parasitic contamination in abattoir wastewater in Benin-City, highlighting substantial public health risks associated with the effluents.

Keywords: Parasites, Wastewater, Slaughterhouses, Public Health

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Prévalence de la contamination parasitaire des eaux usées provenant d'abattoirs sélectionnés à Benin City, État d'Edo, Nigéria

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Résumé:

Contexte: Les eaux usées des abattoirs peuvent contenir divers parasites, notamment des protozoaires et des helminthes. Cette étude visait à examiner systématiquement des échantillons d'eaux usées provenant d'abattoirs sélectionnés à Benin City, dans l'État d'Edo, afin de déterminer la prévalence de la contamination parasitaire et la distribution spatiale de ces parasites, dans le but d'éclairer les futures stratégies de santé publique et de protection de l'environnement.
Méthodologie: Cent vingt échantillons d'eaux usées (n=120) prélevés dans quatre abattoirs sélectionnés aléatoirement (Ikpoba Hill, New-Benin, Oluku et Ugbowo) à Benin City, État d'Edo, Nigéria, ont été analysés afin de détecter la présence de parasites. Les échantillons ont été traités selon les techniques parasitologiques standard, notamment par préparation humide à l'iode et au sérum physiologique, puis examinés au microscope optique à des grossissements de 10x et 40x. La coloration de Ziehl-Neelsen modifiée a été utilisée pour la

détection des œufs de *Cryptosporidium*.
Résultats: Sur les 120 échantillons d'eaux usées analysés, 118 se sont révélés positifs pour les parasites, soit un taux de contamination parasitaire de 98,3%, pour un total de 360 parasites détectés. *Ascaris lumbricoides* était le parasite le plus fréquent (n=85, 23,6%), suivi de *Taenia* spp (n=51, 14,2%) et de *Balantidium coli* (n=48, 13,3%). Parmi les autres parasites notables détectés figuraient *Fasciola hepatica* (n=37, 10,3%) et *Strongyloides stercoralis* (n=32, 8,9%). À l'inverse, les parasites moins fréquents comprenaient *Chydonis sphereius* (n=1, 0,3%), *Amoeba* spp (n=2, 0,6%), les ankylostomes (n=2, 0,6%) et les oxyures (n=2, 0,6%). Aucune différence significative n'a été observée dans la prévalence des parasites entre les quatre sites (p=0,545).
Conclusion: Les résultats de cette étude fournissent une évaluation complète de la contamination parasitaire des eaux usées d'abattoirs à Benin City, soulignant les risques importants pour la santé publique associés à ces effluents.

Mots-clés: Parasites, eaux usées, abattoirs, santé publique

Introduction:

The slaughterhouse industry stands as a critical pillar of the global food supply chain, providing meat and other animal products to a vast and ever-growing human population. However, this indispensable industry also generates a significant volume of wastewater, a by-product that can harbour a wide array of pollutants, including a complex community of parasites (1). The presence of these organisms in abattoir effluents is a matter of profound concern, as many, such as the helminth *Ascaris lumbricoides* and the tapeworm *Taenia saginata*, are well-documented to cause severe health problems in humans, ranging from intestinal blockage and malnutrition to other life-threatening complications (2). These parasites find their way into the wastewater stream through multiple pathways, including the slaughtering process itself, the improper handling and storage of animal waste, and the use of contaminated water for cleaning and processing activities (3).

The risk posed by parasitic contamination is particularly acute in developing nations like Nigeria, where the slaughterhouse industry serves as a crucial component of the national economy and a primary source of protein for millions of people (4,5). Despite its economic importance, the sector is plagued by systemic challenges, most notably the widespread absence of proper wastewater treatment and disposal infrastructure (6). This deficiency allows untreated effluents to be discharged directly into the environment, contaminating surface and groundwater sources and posing a direct threat to both aquatic life and human populations. Several studies conducted across Nigeria have corroborated this problem. For instance, a study in Ibadan revealed that a staggering 75% of wastewater samples from local slaughterhouses were contaminated with parasites, including the highly pathogenic *A. lumbricoides* and *T. saginata* (7).

Parasitic particles, which include the resilient helminth ova and protozoan oocysts, are of particular concern due to their robust nature. They are not only found more frequently in wastewater than in other surface waters but are also notoriously resistant to conventional treatment methods such as chlorination or ozonation, which are commonly used in water and wastewater treatment systems (8). The pathogenic protozoa, in particular, are major causes of human gastroenteritis and pose significant global public health challenges. Numerous water-borne disease outbreaks of giardiasis, amoebiasis, cryptosporidiosis, and balantidiosis have been reported worldwide, highlighting the global scale of the problem (9,10).

In Nigeria, common parasites found in wastewater, such as *A. lumbricoides*, *Trichuris trichiura*, *Ancylostoma duodenale*, and various protozoan like *Entamoeba histolytica* and *Giardia lamblia*, are known contributors to a range of health issues, including stunted growth, impaired nutrient absorption, cognitive deficits, and anaemia (11). The primary mode of transmission for these infections is the faeco-oral route, typically through the consumption of contaminated water or food. Effective control, therefore, hinges on a multifaceted approach that includes improving environmental conditions, implementing proper wastewater treatment, promoting good personal hygiene, and addressing underlying issues of poor sanitation, poverty, and a lack of public health education (12).

In Benin-City, Edo State, the disposal of untreated wastewater from slaughterhouses constitutes a significant environmental and public health crisis. Abattoir effluents are rich in organic waste, fecal matter, and a high concentration of parasites that can survive for extended periods, posing serious health risks to local communities, especially through the contamination of essential water sources (13). Despite the clear and present danger, there is a notable lack of specific, localized data regarding the examination of parasites in wastewater from Benin-City's slaughterhouses.

This knowledge gap is a major hindrance, as it prevents the development of targeted and effective health interventions and public awareness programs tailored to the

specific context of the city. Consequently, this study was designed to bridge this gap by systematically examining the presence of parasites in wastewater from selected slaughter houses in Benin-City, Edo State, and evaluating the prevalence and spatial distribution of these parasites to inform future public health and environmental protection strategies.

Materials and method:

Study area:

The study was conducted at four selected abattoirs in Benin-City, Edo State, Nigeria. The chosen locations were Ikpoba Hill slaughterhouse, situated in Ikpoba-Okha Local Government Area (LGA); New-Benin slaughterhouse located in Oredo LGA; and Oluku and Ugbowo slaughterhouses, both situated in Ovia North-east LGA. The geographical coordinates of the study area are approximately latitude 6.3379° N and longitude 5.6123° E.

Study design:

A descriptive cross-sectional study design was employed to examine the presence of parasites in wastewater. The study involved random selection of four slaughter houses within Benin-City for sample collection and subsequent laboratory analysis.

Ethical approval:

Ethical approval for this study was obtained from the Research and Ethics Committee, Ministry of Health, Edo State, Nigeria. A verbal consent was secured from the management and workers of the participating slaughterhouses prior to the collection of samples to indicate their approval of the study.

Sample collection and transportation:

Twenty-four hours prior to sample collection, 1ml of formal saline was prepared and added to 20ml sterile universal containers. Fresh wastewater samples were collected in the morning from the abattoirs shortly after the slaughtering process using the clean containers. A total of 120 samples were collected (30 samples in each of the four selected abattoirs).

The wastewater samples were carefully transferred into pre-labelled, sterile universal containers, and transported to the microbiology laboratory of the Department of Medical Laboratory Science, University of Benin, within two hours of collection for analysis using standard parasitological methods.

Laboratory analysis of wastewater samples:

Standard parasitological technique used for the examination of parasites in samples was the Bailenger method, as modified by Ayres and Mara (14). The samples were first homogenized, and the suspension was then filtered into conical cups using surgical gauze and allowed to rest for 60 minutes. After settling, the clear supernatant was carefully discarded, and 4 ml of the filtered samples were transferred into 10 ml test tubes. These samples were then centrifuged at 2000rpm for 3 minutes, following which the cloudy supernatant was discarded, leaving only the pellet for further analysis.

The wastewater sediment from the test tubes was examined using saline and iodine mounts prepared on clean grease-free slides. The slides were examined under the light microscope and the parasites observed were recorded. The modified Ziehl-Neelsen (ZN) staining technique was performed for *Cryptosporidium* detection. The slide was observed under the light microscope for presence of *Cryptosporidium* oocysts by placing a drop of immersion oil on the smear and viewed with oil immersion objective lens.

Statistical analysis of data:

Data obtained were analyzed using the IBM SPSS version 27.0 software. Descriptive statistics, including percentage and frequency distributions, were computed. The difference in the parasite prevalence between the four locations was determined using the Chi square test. A *p* value of less than 0.05 was considered statistical significance.

Results:

Of the 120 wastewater samples examined, parasites were detected in 118 samples (98.3%), while only 2 samples (1.7%) were parasite-free (Fig 1). A total of 360 parasites were identified in the wastewater samples with *A. lumbricoides* being the most prevalent, accounting for 23.6% (n=85), followed by *Taenia* spp (14.2%, n=51) and *B. coli* (13.3%, n=48). Other notable parasites included *F. hepatica* (10.3%, n=37) and *S. stercoralis* (8.9%, n=32). Conversely, less prevalent parasites included *Chydonis sphe-rieus* (0.3%, n=1), *Amoeba* spp (0.6%, n=2), hookworm (0.6%, n=2), and pinworm (0.6%, n=2) (Fig 2 & Table 1).

Fig 3 shows that the highest frequency of parasites from the wastewater was recorded in New Benin (31.4%), followed by Oluku (25.3%), Ugbowo (23.1%), and Ikpoba Hill (20.3%). However, there was no significant difference in the frequency of parasite detected across the four locations (p= 0.545). The highlights of the variation in parasite frequency across different locations revealed that *A. lumbricoides* was the most frequent parasite overall with 23.6% (n= 85/360), with the highest frequency of detection in Ugbowo (32.5%, n=27/83), followed by Ikpoba Hill (21.9%, n=16/73) and

Oluku (21.9%, n=20/91), and the least frequency in New Benin (19.5%, n=22/113), but this was not statistically significant (x^2= 4.986, p=0.1728).

Similarly, *B. coli* was most frequently identified in Ikpoba Hill (39.7%, n=29/73), with statistically significant lower frequencies of detection in New Benin (10.6%, n=12/113), Oluku (4.4%, n=4/91), and Ugbowo (3.6%, n=3/83) (x^2=57.8, p<0.0001). Conversely, certain parasites, such as *Ascaris suum* were detected exclusively in Oluku, *C. spherieus* exclusively in Ugbowo, and hookworm and roundworm exclusively in Ikpoba Hill (Table 1).

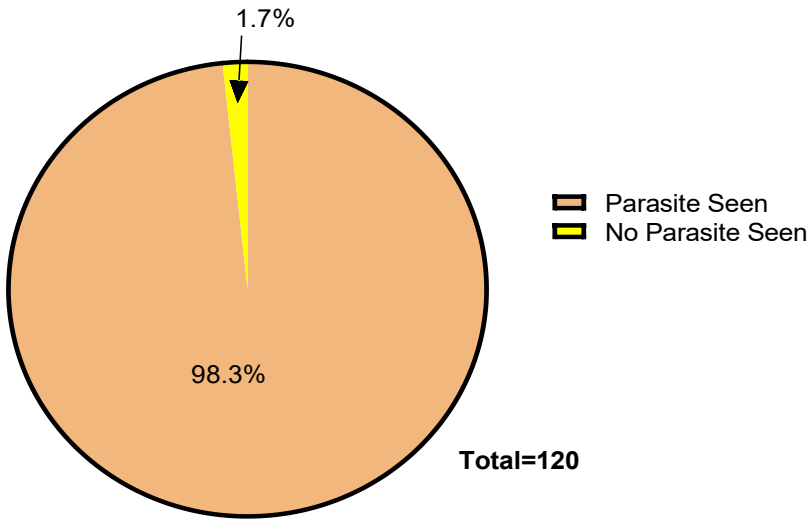

Fig 1: Prevalence of parasites in wastewater samples from slaughter houses in Benin-City, Nigeria

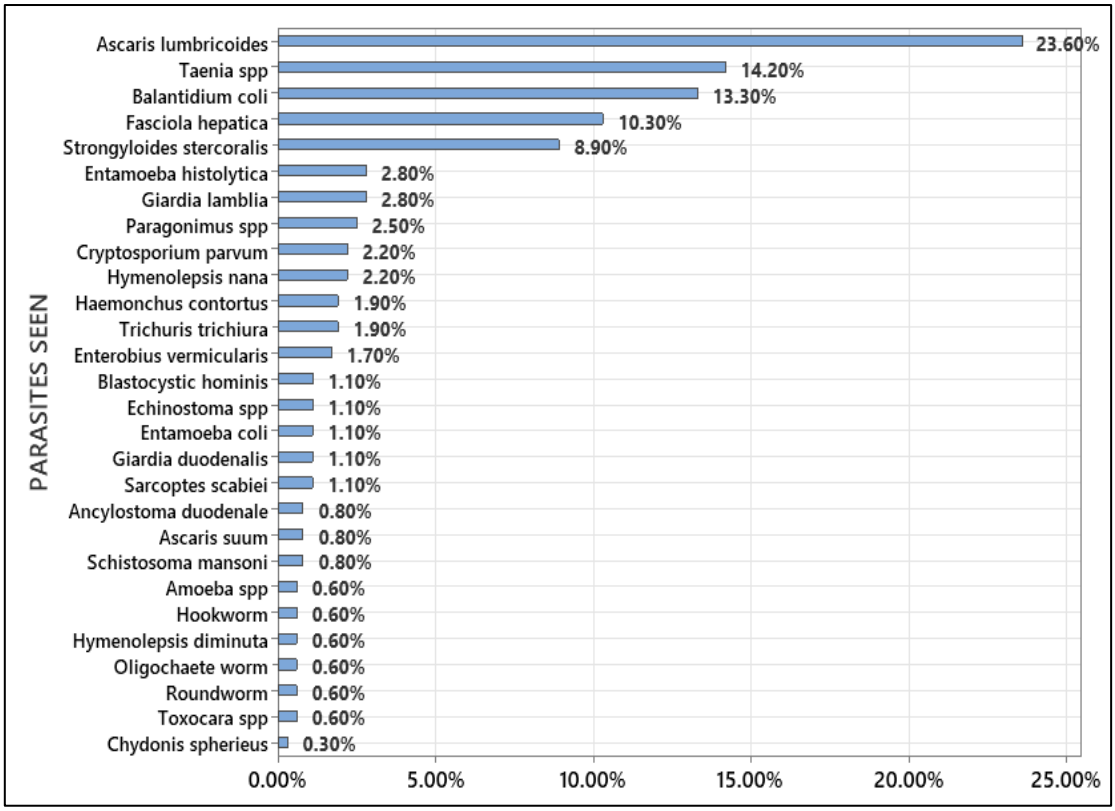

Fig 2: Frequency distribution of parasites from wastewater samples from slaughter houses in Benin City, Nigeria

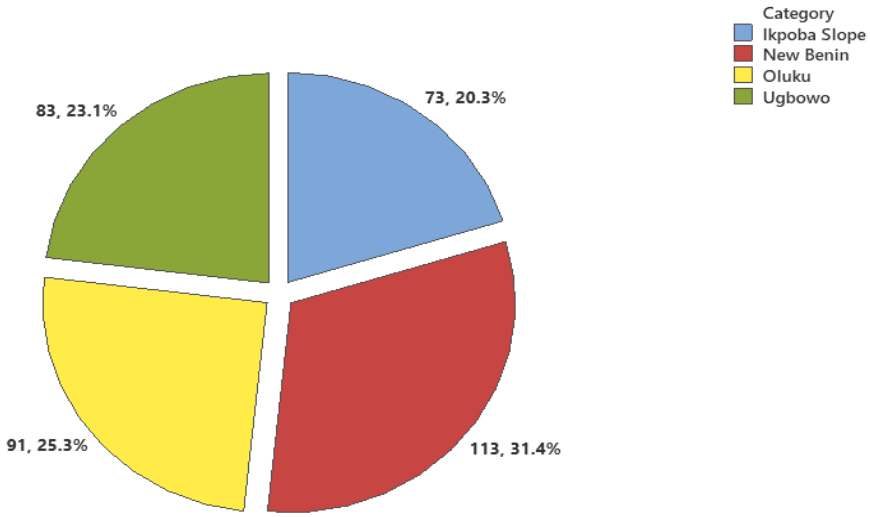

Fig 3: Prevalence of parasites in wastewater samples from slaughter houses according to locations in Benin City, Nigeria

Table 1: Frequency distribution of parasites in wastewater samples from slaughter houses in four locations in Benin City

Parasite	Ikpoba Hill (%)	New Benin (%)	Oluku (%)	Ugbowo (%)	Total (%)
Amoeba spp	0	1 (0.9)	1 (1.1)	0	2 (0.6)
Ancylostoma duodenale	0	1 (0.9)	2 (2.2)	0	3 (0.8)
Ascaris lumbricoides	16 (21.9)	22 (19.5)	20 (21.9)	27 (32.5)	85 (23.6)
Ascaris suum	0	0	3 (3.3)	0	3 (0.8)
Balantidium coli	29 (39.7)	12 (10.6)	4 (4.4)	3 (3.6)	48 (13.3)
Blastocystis hominis	2 (2.7)	0	1 (0.9)	1 (1.2)	4 (1.1)
Chydonis spherieus	0	0	0	1 (1.2)	1 (0.3)
Cryptosporidium parvum	0	2 (1.8)	3 (3.3)	3 (3.6)	8 (2.2)
Echinostoma spp	0	2 (1.8)	0	2 (2.4)	4 (1.1)
Entamoeba coli	1 (1.4)	1 (0.9)	1 (0.9)	1 (1.2)	4 (1.1)
Entamoeba histolytica	3 (4.1)	2 (1.8)	3 (3.3)	2 (2.4)	10 (2.8)
Enterobius vermicularis	1 (1.4)	2 (1.8)	1 (0.9)	2 (2.4)	6 (1.7)
Fasciola hepatica	6 (8.2)	23 (20.4)	5 (5.5)	3 (3.6)	37 (10.3)
Giardia duodenales	1 (1.4)	1 (0.9)	2 (2.2)	0	4 (1.1)
Giardia lamblia	1 (1.4)	3 (2.7)	4 (4.4)	2 (2.4)	10 (2.8)
Haemonchus contortus	1 (1.4)	3 (2.7)	3 (3.3)	0	7 (1.9)
Hookworm	2 (2.7)	0	0	0	2 (0.6)
Hymenolepsis diminuta	0	1 (0.9)	0	1 (1.2)	2 (0.6)
Hymenolepsis nana	1 (1.4)	2 (1.8)	5 (5.5)	0	8 (2.2)
Oligochaete worm	1 (1.4)	0	0	1 (1.2)	2 (0.6)
Paragonimus spp	0	2 (1.8)	2 (2.2)	5 (6.0)	9 (2.5)
Roundworm	2 (2.7)	0	0	0	2 (0.6)
Sarcoptes scabiei	1 (1.4)	1 (0.9)	2 (2.2)	0	4 (1.1)
Schistosoma mansoni	1 (1.4)	1 (0.9)	1 (1.1)	0	3 (0.8)
Strongyloides stercoralis	0	16 (14.2)	9 (9.9)	7 (8.4)	32 (8.9)
Taenia spp	2 (2.7)	13 (11.5)	15 (16.5)	21 (25.3)	51 (14.2)
Toxocara spp	0	1 (0.0)	1 (1.1)	0	2 (0.6)
Trichuris trichiura	2 (2.7)	1 (0.9)	3 (3.3)	1 (1.2)	7 (1.9)
Total	**73 (20.3)**	**113 (31.4)**	**91 (25.3)**	**83 (23.1)**	**360 (100.0)**

Discussion:

The results of our study indicate a remarkably high prevalence of parasitic contamination, with 98.3% of the wastewater samples testing positive for parasites, emphasizing the substantial public health concerns linked to abattoir wastewater. This prevalence differs from 54.5% of parasite contamination rate reported in Jos Metropolis (15) and the study by Bolaji et al., (16) who reported 68.5% contamination rate in abattoir effluents in Oyo State, Nigeria. The variation in contamination rates may be attributed to differences in the types of livestock slaughtered, the volume of wastewater produced, and the specific waste management practices at each location (17).

The array of parasites identified in the wastewater highlights the complexity of contamination dynamics within the slaughterhouses. *Ascaris lumbricoides*, the most prevalent parasite (23.6%), is known for its robust environmental resilience and ability to endure harsh conditions, corroborating our findings (18). The high prevalence of *Ascaris* spp in abattoir wastewater has been consistently reported in other studies as a common contaminant in Minna, Niger State, Nigeria (19). Other commonly identified parasites such as *Taenia* spp (14.2%) and *B. coli* (13.3%) suggest a significant zoonotic potential as these species are frequently linked to livestock (20). The presence of these parasites underscores the public health risk posed to both abattoir workers and residents of adjacent communities through direct contact or the consumption of contaminated water (21). The detection of other protozoan species, such as *E. histolytica* and *G. lamblia*, aligns with findings from similar studies of abattoirs in Minna Metropolis, Niger State, Nigeria (19).

The identification of parasites in the samples from the abattoirs strongly suggests that deficient wastewater management practices and poor sanitation are significant contributors to the contamination levels observed at these sites (22). Additionally, the high frequency in New Benin (31.4%) further supports this hypothesis. Despite these high frequencies in New Benin, there was no statistically significant difference in the frequency of parasite detection across the locations (p= 0.545), indicating that parasitic contamination is a widespread issue in the abattoirs studied, and not confined to specific geographic areas. This suggests that inefficient waste management practices and limited regulatory oversight are systemic problems throughout the study sites (23).

Certain parasites displayed location-specific distribution patterns such as the exclusive presence of *Ascaris suum* in Oluku and the sole detection of hookworm and pinworm in Ikpoba Hill. These findings imply that localized variations in livestock management, types of animals slaughtered, and waste disposal practices may significantly influence parasite prevalence (24). For instance, the high frequency of *F. hepatica* identification in New Benin abattoir (20.4%, n=23/113) could be linked to the types of livestock processed at that particular facility, as different animal species serve as hosts for specific parasitic organisms. This observation points to the influence of local factors on the distribution of parasitic contamination, even within a single urban area (25).

The high prevalence of parasitic contamination in abattoir wastewater presents significant risks to public health, especially in urban and peri-urban areas where untreated effluent could contaminate water supplies and agricultural land (26). The prominence of helminths like *A. lumbricoides* suggests that improper waste disposal could lead to widespread soil-transmitted infections, further exacerbating public health challenges. The presence of zoonotic parasites such as *Taenia* spp and *B. coli* elevates the risk of both direct and indirect transmission to humans, particularly those working in abattoirs and residents in adjacent communities (27). These findings underscore the urgent need for the enforcement of more stringent wastewater treatment regulations and the implementation of enhanced sanitation protocols in slaughterhouses. The persistence of parasites like *A. lumbricoides*, known for its ability to withstand harsh environmental conditions, further compounds the risks associated with wastewater contamination (28).

This study highlights the significant public health risks posed by parasitic contamination in abattoir wastewater, which has far-reaching implications for water safety and the transmission of zoonotic diseases. The findings emphasize the necessity for a multifaceted approach to mitigate these risks, including the implementation of advanced wastewater treatment technologies, stricter regulatory enforcement, and targeted public health education for both abattoir workers and nearby communities (29).

Conclusion:

This study provides a comprehensive assessment of parasitic contamination in wastewater from slaughterhouses in Benin-City, highlighting the substantial public health risks associated with abattoir wastewater. The findings indicate a remarkably high prevalence, with 98.3% of wastewater samples testing positive for parasites, confirming the wide-

spread nature of parasitic contamination in the study area. The presence of a diverse array of parasites, including *A. lumbricoides*, *Taenia* spp, and *B. coli*, demonstrates the complexity of the contamination dynamics and the zoonotic potential of these parasites.

The findings of this study emphasize the urgent need for improved wastewater management practices in abattoirs, especially given the implications for public health, water safety, and zoonotic disease transmission. The high prevalence of helminths, in particular, underscores the need for more stringent regulatory measures to mitigate the risks of soil-transmitted infections, especially in urban and peri-urban settings. The localized variations in parasite types suggest that factors such as livestock health and waste disposal practices play a role in shaping parasite distribution, further necessitating targeted interventions.

Based on the findings of this study, several recommendations are proposed to mitigate the public health risks posed by parasitic contamination in abattoir wastewater, and these include improved wastewater management, stricter regulatory enforcement by local authorities, public health education of abattoir workers and residents on risk of zoonotic infection and hygiene practices, and local interventions such as improving livestock management practices, enhancing waste disposal protocols, and addressing environmental conditions that favour parasite survival. Further studies should explore the molecular characterization of the parasites identified in this study to better understand their transmission dynamics. Additionally, research into the environmental factors influencing parasitic contamination could provide valuable information for developing more effective control measures

Contributions of authors:

ZO designed and directed the study; ZO and OED performed the laboratory analysis; OED performed statistical analysis of data; and ZO and OED wrote the manuscript and approved the final version submitted for publication.

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Conflict of interest:

Authors declare no conflict of interest

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Original Article **Open Access**

Evaluation of α-fetoprotein and selected inflammatory enzyme markers in chronic hepatitis B patients seropositive and seronegative for hepatitis B envelope antibody in UITH, Ilorin, Nigeria

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Abstract:

Background: Hepatitis B virus (HBV) can cause both acute and chronic hepatitis and is a leading contributor to hepatic cirrhosis and hepatocellular carcinoma (HCC), imposing a substantial burden on healthcare systems due to its high morbidity, mortality, and treatment costs. HBV infection remains a major public health concern, particularly in sub-Saharan Africa. Investigating the serum levels of alpha-fetoprotein (AFP) and selected inflammatory markers in individuals seropositive for hepatitis B envelope antibody (HBeAb) provides valuable insights into disease progression, early detection of HCC, and monitoring treatment efficacy. This study assessed serum levels of AFP and selected inflammatory markers such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, albumin, and globulin among chronic HBV patients seropositive and seronegative for HBeAb at the University of Ilorin Teaching Hospital (UITH), Ilorin, Nigeria.
Methodology: A comparative cross-sectional study was conducted among 70 chronic HBV patients at UITH, Ilorin, Nigeria, including 30 patients seropositive and 40 patients seronegative for HBeAb. Sociodemographic information of the patients was collected using a designed form. Serum levels of AFP, ALT, AST, total protein, albumin, and globulin were determined using ELISA and spectrophotometry. Data were analyzed using the Mann-Whitney U test and Spearman's correlation, with $p \leq 0.05$ considered statistically significant.
Results: Serum AST and ALT levels were significantly lower in patients seropositive for HBeAb (17.43±33.90 IU/L and 11.13±10.95 IU/L) than patients seronegative for HBeAb (34.90±22.95 IU/L and 23.00±8.68 IU/L; $p=0.040$ and $p=0.000$ respectively). AFP level was also significantly lower in patients seropositive (17.59± 44.32 ng/mL) compared to patients seronegative (44.31±14.80ng/mL) for HBeAb ($p=0.003$). Serum total protein and globulin were significantly higher in patients seropositive for HBeAb (75.97±11.89g/L and 29.70 ±4.17g/L; $p=0.001$ and $p=0.000$ respectively). Serum albumin was higher in patients seropositive for HBeAb (46.33±9.54 g/L), but this was not statistically significant from patients seronegative (41.95 ±7.80g/L) for HBeAb ($p=0.081$).
Conclusion: HBeAb seroconversion is associated with improved liver function, reduced hepatic inflammation, and decreased HCC risk. Monitoring AFP and liver enzyme markers can aid the management of chronic HBV infected patients in resource-limited settings.

Keywords: HBV, HBeAb, HBsAg, AFP, liver enzymes, hepatocellular carcinoma

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Évaluation de l'α-fœtoprotéine et de certains marqueurs enzymatiques inflammatoires chez des patients atteints d'hépatite B chronique, séropositifs et séronégatifs pour l'anticorps anti-HBs, à l'UITH d'Ilorin, au Nigéria.

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Résumé:

Contexte: Le virus de l'hépatite B (VHB) peut provoquer des hépatites aiguës et chroniques et contribue fortement à la cirrhose hépatique et au carcinome hépatocellulaire (CHC), imposant un fardeau considérable aux

systèmes de santé en raison de sa morbidité et de sa mortalité élevée, ainsi que du coût important des traitements. L'infection par le VHB demeure un problème majeur de santé publique, notamment en Afrique subsaharienne. L'analyse des taux sériques d'alpha-fœtoprotéine (AFP) et de certains marqueurs inflammatoires chez les personnes séropositives pour l'anticorps anti-HBe (HBeAb) apporte des informations précieuses sur la progression de la maladie, le dépistage précoce du carcinome hépatocellulaire (CHC) et le suivi de l'efficacité du traitement. Cette étude a évalué les taux sériques d'AFP et de certains marqueurs inflammatoires, tels que l'aspartate aminotransférase (AST), l'alanine aminotransférase (ALT), les protéines totales, l'albumine et les globulines, chez des patients atteints d'hépatite B chronique, séropositifs et séronégatifs pour l'HBeAb, au Centre hospitalier universitaire d'Ilorin (UITH), à Ilorin, au Nigéria.

Méthodologie: Une étude transversale comparative a été menée auprès de 70 patients atteints d'hépatite B chronique à l'UITH, à Ilorin, au Nigéria, dont 30 patients séropositifs et 40 patients séronégatifs pour l'HBeAb. Les données sociodémographiques des patients ont été recueillies à l'aide d'un formulaire spécifique. Les concentrations sériques d'AFP, d'ALT, d'AST, de protéines totales, d'albumine et de globulines ont été déterminées par ELISA et spectrophotométrie. Les données ont été analysées par le test U de Mann-Whitney et la corrélation de Spearman, un seuil de signification statistique étant fixé à $p \leq 0,05$.

Résultats: Les concentrations sériques d'AST et d'ALT étaient significativement plus faibles chez les patients séropositifs pour les anticorps anti-HBe (17,43±33,90 UI/L et 11,13±10,95 UI/L) que chez les patients séronégatifs (34,90±22,95 UI/L et 23,00±8,68 UI/L; $p=0,040$ et $p=0,000$ respectivement). Le taux d'AFP était significativement plus faible chez les patients séropositifs (17,59±44,32 ng/mL) que chez les patients séronégatifs (44,31±14,80 ng/mL) pour les anticorps anti-HBe ($p=0,003$). Les taux de protéines totales et de globulines sériques étaient significativement plus élevés chez les patients séropositifs pour les anticorps anti-HBe (75,97±11,89 g/L et 29,70±4,17 g/L; $p=0,001$ et $p=0,000$ respectivement). L'albuminémie était plus élevée chez les patients séropositifs pour les anticorps anti-HBe (46,33±9,54 g/L), mais cette différence n'était pas statistiquement significative par rapport aux patients séronégatifs (41,95±7,80 g/L) pour ces mêmes anticorps ($p=0,081$).

Conclusion: La séroconversion des anticorps anti-HBe est associée à une amélioration de la fonction hépatique, une réduction de l'inflammation hépatique et une diminution du risque de carcinome hépatocellulaire. Le suivi de l'AFP et des marqueurs enzymatiques hépatiques peut faciliter la prise en charge des patients atteints d'une infection chronique par le VHB dans les contextes aux ressources limitées.

Mots-clés: VHB, anticorps anti-HBe, AgHBs, AFP, enzymes hépatiques, carcinome hépatocellulaire

Introduction:

Hepatitis B virus (HBV) infection remains one of the most prevalent and serious infectious diseases globally, particularly in sub-Saharan Africa and the Western Pacific, where it is highly endemic. According to the World Health Organization (WHO), over 296 million people were chronically infected with HBV in 2019, with more than 800,000 annual deaths resulting from complications such as cirrhosis and hepatocellular carcinoma (1,2). Despite the availability of effective vaccines and antiviral therapies, HBV diagnosis rates in low- and middle-income countries (LMICs) remain disproportionately low compared to high-income countries, estimated at only 0.8% versus 18% respectively (3). As a result, large portion of HBV-infected individuals remain undiagnosed and untreated, facilitating continued transmission and disease progression (4).

HBV is a partially double-stranded DNA virus with a strong tropism for hepatocytes (5). It is transmitted through perinatal routes, sexual contact, blood transfusions, and other exposures to infected body fluids (1,6). The course of HBV infection is marked by dynamic phases characterized by changes in viral replication and host immune response. Serological markers such as hepatitis B envelope antigen (HBeAg) and its corresponding antibody, hepatitis B envelope antibody (HBeAb), are central to monitoring disease progression.

HBeAg is typically associated with active viral replication and high infectivity, while the appearance of HBeAb marks a transition to a less replicative state, often indicating immune control over the virus and improved clinical prognosis (7,8). HBeAb is not only valuable diagnostically but also prognostically. It is often used to guide treatment decisions and evaluate the likelihood of favorable treatment outcomes (9). Patients who seroconvert to HBeAb tend to exhibit lower viral loads, reduced liver inflammation, and improved liver enzyme profiles (10). Moreover, seroconversion has been associated with decreased risk of progression to cirrhosis and hepatocellular carcinoma (11,12). As such, evaluating the biochemical changes associated with HBeAb status is clinically relevant in assessing disease activity and therapeutic response.

Liver enzymes, particularly alanine aminotransferase (ALT) and aspartate aminotransferase (AST), are widely used indicators of hepatocellular injury. Elevated levels of ALT and AST are commonly seen in patients with active HBV infection and correlate with the degree of hepatic inflammation and necrosis (13,14). ALT is considered a more specific marker of liver damage than AST due to its predominance in hepatocytes (15,16). Therefore, declining enzyme levels in HBeAb-positive individuals often reflect disease remission or therapeutic success.

Other important indicators of liver function include serum albumin, globulin, and total protein. Albumin, synthesized in the liver, plays a key role in maintaining oncotic pressure and transporting hormones and drugs

(17). Decreased albumin levels are associated with chronic liver diseases, especially cirrhosis (18). Globulins, which include immunoglobulins, are elevated in response to viral infections and immune activation (19). Elevated total protein and globulin levels in HBeAb-positive patients may thus reflect an enhanced immune response and restoration of liver synthetic function (20).

Alpha-fetoprotein (AFP) is another clinically significant biomarker, particularly for hepatocellular carcinoma (HCC). In healthy adults, AFP levels are typically undetectable; however, they can rise markedly in the context of chronic HBV infection and liver cancer (21, 22). The clinical utility of AFP in monitoring HBV patients lies in its role as a non-invasive screening tool for early detection of HCC, especially when interpreted alongside imaging studies and liver function tests (23,24). Studies have shown that AFP levels are often significantly higher in HBeAg-positive individuals compared to those who have seroconverted to HBeAb (25,26).

In Nigeria, HBV is considered hyperendemic, with prevalence estimated among the highest in sub-Saharan Africa (15,27). The combination of high disease burden, low vaccination coverage, and inadequate access to early diagnostic services makes HBV a critical public health threat in the country. Given these challenges, the use of readily available biochemical markers such as ALT, AST, AFP, albumin, and globulin can be instrumental in guiding early diagnosis, staging, and treatment decisions.

This study, therefore aimed to evaluate the serum levels of alpha-fetoprotein and selected liver-related biochemical parameters among chronic HBV patients seropositive and seronegative for HBeAb at the University of Ilorin Teaching Hospital (UITH), Ilorin, Kwara State, Nigeria. By examining these markers across these two groups, the study aims to provide insight into the clinical significance of HBeAb seroconversion and its association with improved liver function and reduced HCC risk. These have potential implications for enhancing HBV monitoring and management protocols in Nigeria and other resource-limited settings.

Materials and method:

Study site:

This study was carried out in the General-Out-Patient clinic of the University of Ilorin Teaching Hospital (UITH), a major tertiary healthcare facility located in Ilorin, the capital city of Kwara State in Nigeria. The hospital is affiliated to the University of Ilorin and provides a wide range of healthcare services to patients from Ilorin and its surrounding areas.

Study design and population:

This was a comparative cross-sectional study of patients with diagnosis of chronic HBV infections attending the outpatient clinic of UITH, Ilorin, which formed the study population. Diagnosis of HBV infection was made based on the detection of hepatitis B surface antigen (HBsAg) in the serum of patients using standard serological testing methods.

Ethical approval:

Ethical approval was obtained from the Ethical Review Committee of UITH Ilorin, and from Al-Hikmah University, Kwara State, Nigeria. Informed consent was obtained from all participants before collection of data.

Study participants, inclusion and exclusion criteria:

The study participants included 70 chronic HBV patients that were seropositive (n=30) and seronegative (n=40) for hepatitis B envelope antibody (HBeAb). Participants who have received HBV vaccination, those with other liver diseases such as hepatitis C, autoimmune liver disease, liver cirrhosis, pregnant women, patients with unstable medical conditions such as uncontrolled hypertension or diabetes and those with history of liver transplantation, were excluded from the study.

Data and sample collection/handling:

Participants were consecutively recruited at the clinic. Sociodemographic data were collected using a designed form. Approximately 5ml of blood was collected aseptically by venipuncture from each selected participant into sterile plain test tube and transported immediately from the clinic to the laboratory for analysis.

Laboratory analyses

All laboratory analyses were carried out at the Chemical Pathology laboratory, UITH, Ilorin. The blood was allowed to clot in the test tube, and the serum was separated by centrifugation at 3000 rpm for 5 minutes. The serum specimen was transferred separately to different vials and preserved at -2^{0}C until analysis.

Alpha-fetoprotein (AFP) assay:

Serum level of alpha-fetoprotein was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (ACCUlite®). The method is based on a sandwich ELISA principle, wherein serum AFP binds to immobilized anti-AFP antibodies on microtiter wells, followed by the addition of an enzyme-conjugated secondary antibody. The bound complex was visualized with a tetramethyl benzidine (TMB) substrate, and absorbance was measured at 450 nm using a microplate reader. Concentrations were extrapolated from a standard curve generated with known AFP standards.

Alanine aminotransferase (ALT) assay:

ALT activity was measured using the kinetic UV method with the Randox® ALT reagent kit. The assay monitors the rate of decrease in NADH absorbance at 340 nm as ALT catalyzes the conversion of alanine and α-ketoglutarate to pyruvate and glutamate. Pyruvate is subsequently reduced to lactate, by lactate dehydrogenase, resulting in oxidation of NADH to NAD+. The change in absorbance per minute was used to calculate enzyme activity in IU/L.

Aspartate aminotransferase (AST) assay:

AST activity was assessed using the Randox® AST reagent kit and kinetic UV method. The assay measures the conversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate, with oxaloacetate being reduced to malate, by malate dehydragenase, accompanied by the oxidation of NADH. The rate of NADH consumption was measured at 340 nm, and AST activity was calculated in IU/L.

Total protein determination:

Total serum protein was quantified using the Biuret method (Agappe® total protein kit). Proteins in the serum react with copper ions in an alkaline medium to form a violet-colored complex. The intensity of the color, which is directly proportional to protein concentration, was measured spectrophotometrically at 540 nm. Protein concentration was calculated using a known protein standard.

Albumin assay:

Serum albumin was estimated using the bromocresol green (BCG) method (Agappe® albumin kit). At acidic pH, albumin binds to BCG to form a blue-green complex, which was measured spectrophotometrically at 630 nm. Albumin concentration was determined by comparing absorbance to that of a standard solution.

Globulin calculation:

Serum globulin levels were derived by subtracting the measured albumin concentration from the total protein concentration for each sample: Globulin (g/L) = Total Protein – Albumin

Data analysis:

All data were carefully entered into Microsoft Excel spreadsheet and assessed for accuracy and completion before statistical analysis using the Statistical Package for the Social Sciences (SPSS) version 27.0. Before analysis, the data were assessed for Gaussian distribution and thereafter, appropriate statistical analysis tests was applied.

The Student's *t*-test was used to determine differences in means of the variables with Gaussian distribution. Mann-Whitney U test was used to determine differences in medians of the variables without Gaussian distribution. Correlation between variables was determined using Spearman's Rho correlation. *P*-values less than 0.05 (2-tailed) were considered as statistically significant.

Results:

Socio-demographic characteristics of study participants:

As shown in Table 1, the age of participants ranged from 15 to 42 years. Of the 70 participants, 13% were between the age of 15-24 years, 53% were between 25-34 years while 34% were between 34-44 years of age. The frequency of male participants (68.5%) was higher than the female participants (31.5%), and 63% were single while 37% were married.

Table 1: Socio-demographic characteristics of patients

Characteristics	Number	%
Age group (years)		
15-24	9	13
25-34	37	53
34-44	24	34
Total	70	100
Marital status		
Married	26	37
Single	44	63
Total	70	100
Sex		
Male	48	68.5
Female	22	31.5
Total	70	100
Education level		
Primary	5	7
Secondary	26	37
Tertiary	39	56
Total	70	100
HBeAb		
HBeAb seronegative	40	57.1
HBeAb seropositive	30	42.9
Total	70	100

Table 2: Comparative levels of alpha-feto protein and liver enzymes in chronic HBV patients seropositive and seronegative for hepatitis B envelope antibody

Parameters	HBeAb positive (n=30)	HBeAb negative (n=40)	*t*-value	*p*-value
AST (IU/L)	17.43±33.90	34.90±22.95	2.106	0.040**
ALT (IU/L)	11.13±10.95	23.00±8.68	4.246	0.000**
AFP (ng/mL)	17.59±44.32	44.31±14.80	3.174	0.003**

(**) means statistically significance at $p<0.05$

Table 3: Comparative levels of serum total protein, albumin and globulin in chronic HBV patients seropositive and seronegative for hepatitis B envelope antibody

Parameters	HBeAb positive (n=30)	HBeAb negative (n=40)	*t*-value	*p*-value
Total protein (g/L)	75.97±11.89	67.20±9.39	2.892	0.001**
Albumin (g/L)	46.33±9.54	41.95±7.80	1.781	0.081
Globulin (g/L)	29.70±4.17	25.25±2.00	4.642	0.000**

(**) means statistically significance at $p<0.05$

Results of AFP and liver enzymes in the study participants:

As shown in Table 2, AST levels were significantly lower in patients seropositive for HBeAb compared to patients seronegative for HBeAb (p=0.040). ALT levels were also significantly lower in patients seropositive for HBeAb compared to patients seronegative for HBeAb (p=0.000). Serum alpha-fetoprotein level was significantly lower in patients seropositive for HBeAb compared to patients seronegative for HBeAb (p=0.003).

Results of serum total protein, albumin and globulin in the study participants:

As shown in Table 3, serum total protein level was significantly higher in patients seropositive for HBeAb compared to patients seronegative for HBeAb (p=0.001). Serum albumin level was not significantly different in patients seropositive for HBeAb compared to patients seronegative for HBeAb (p=0.081) but serum globulin level was significantly higher in patients seropositive for HBeAb compared to patients seronegative for HBeAb (p=0.000).

Discussion:

The study indicates that chronic HBV patients seropositive for HBeAb have significantly lower serum AST and ALT levels compared to patients seronegative for HBeAb. This reduction in liver enzyme levels suggests reduced liver damage in HBeAb-positive individuals. This finding aligns with previous research. Patients with resolved HBV infection, often associated with the presence of HBeAb, have been reported to exhibit significantly lower ALT levels, indicating improved liver health (28). Similarly, individuals seropositive for HBeAb have been shown to have lower viral loads, consistent with a reduced risk of liver damage (29). The observed differences in liver enzyme levels between patients seropositive and seronegative for HBeAb may also reflect the impact of therapeutic interventions. Antiviral therapy plays a pivotal role in the clinical management of chronic HBV infection (12).

Our study revealed a significant increase in serum total protein in patients seropositive for HBeAb, indicating an overall elevation in protein content in their blood. This finding aligns with the study by Chan et al., (29), which reported that HBeAb seroconversion was associated with improved liver function, including increased total protein levels, reflecting the restoration of liver synthetic capacity. Although our study reports an insignificant increase in albumin levels in patients seropositive for HBeAb. It is important to note that albumin is a major protein synthesized by the liver. In hepatitis, liver inflammation can lead to reduced albumin production. A study by Hu et al., (30) had highlighted the correlation between HBeAb positivity and stabilized albumin levels, suggesting better liver function.

The significant increase in serum globulin levels in patients seropositive for HBeAb aligns with the idea that globulins consist of various antibodies and enzymes, reflecting an enhanced immune response. This agrees with the study by Wang et al., (24), which reported that patients seropositive for HBeAb had higher levels of certain immunoglobulins, contributing to improved viral control. The presence of HBeAb suggests an immune response against HBV, which could lead to reduced liver inflammation and improved liver function. This finding also agrees with the study by Yuen et al., (31) which highlighted the importance of HBeAb seroconversion in predicting favorable outcomes in chronic hepatitis B.

The observation in our study of an enhanced immunological response, as reflected in high serum total protein and globulin levels, is consistent with the attempt by the liver to combat HBV infection. The results indicate that patients seropositive for HBeAb have a more favorable protein profile, suggesting better liver function and potentially less severe liver disease. This is supported by a systematic review by Martinot-Peignoux et al., (32), which emphasized the importance of monitoring serum protein parameters in assessing liver health and the effectiveness of antiviral treatment in hepatitis B.

The significant decrease in AFP levels in patients seropositive for HBeAb is a notable finding in our study. Elevated AFP is widely recognized as a potential marker for hepatocellular carcinoma (HCC) in patients with chronic liver diseases, including hepatitis B. This observation agrees with the study by Zhang et al., (33), which emphasized that higher AFP levels were associated with an increased risk of HCC, particularly in hepatitis B patients. The decreased AFP levels in patients seropositive for HBeAb may indeed reflect improved liver health (34). The results underscore the importance of monitoring AFP levels in hepatitis B patients, especially those with different serological markers such as HBeAb. This aligns with guidelines proposed by the American Association for the Study of Liver Diseases (AASLD), which recommend regular surveillance for HCC in hepatitis B patients, including AFP measurements. Monitoring AFP can assist healthcare professionals in identifying patients at higher risk of liver cancer.

The reduced AFP levels in patients seropositive for HBeAb can be attributed to the ability of the immune system to suppress viral replication and limit liver cell damage. This aligns with a study by Chen et al., (26) which highlighted the immunological control of hepatitis B as a significant factor in liver health and cancer risk reduction.

Conclusion:

The comprehensive analysis of various biochemical parameters in patients with chronic HBV patients seropositive for HBeAb compared to their seronegative counterparts, provides valuable insights into the complex dynamics of HBV infection and its impact on liver health. The results showed that patients seropositive for HBeAb exhibit significantly improved liver health, as evidenced by lower AST and ALT levels, indicative of reduced liver cell damage and inflammation. This improvement can be attributed to the successful resolution or control of the HBV infection, as reflected in their HBeAb-positive status. Additionally, the elevation in serum total protein and globulin levels in patients seropositive for HBeAb suggests a strengthened immune response and better liver function, further highlighting the positive influence of HBeAb seroconversion on overall health. Furthermore, the notable decrease in alpha-fetoprotein levels in patients seropositive for HBeAb indicates a decreased risk of hepatocellular carcinoma, underscoring the clinical significance of these findings in cancer prevention and liver care.

These results collectively emphasize the importance of regularly monitoring these serum markers in hepatitis B patients to tailor appropriate treatment strategies and cancer screening protocols, ultimately contributing to improved patient outcomes. Healthcare providers are encouraged to implement routine monitoring of key serum biomarkers such as AST and ALT in hepatitis B patients. Furthermore, AFP should be acknowledged as a vital marker in the early detection and monitoring of hepatocellular carcinoma risk.

Contributions of authors:

MAN and SAY conceived and designed the study; MAN and OJO conducted participant recruitment, sample collection, and laboratory assays; SAY and OJO performed statistical analyses and data interpretation; MAN drafted the initial manuscript; SAY and OJO critically revised the manuscript for important intellectual content; and SAY supervised the overall research activity. All authors reviewed and approved the final manuscript.

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Conflict of interest

Authors declare no conflict of interest.

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Case Series **Open Access**

Bloodstream infection caused by *Kocuria* species in a tertiary hospital in Lagos, Nigeria: A case series

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Abstract

Kocuria species are emerging opportunistic pathogens commonly misidentified as coagulase-negative staphylococcus. Improvement in laboratory diagnostic techniques and the expansion in the number of patients with immunocompromised diseases has led to the rising number of *Kocuria* infections being reported. We report cases of *Kocuria* bacteraemia in three immunocompromised patients. The first case was an 11-year-old with metastatic nasopharyngeal carcinoma and *Kocuria kristinae* isolated from the second blood culture, but due to discharge of the patient against medical advice, the role of *K. kristinae* and outcome of the case could not be ascertained. The second case was a 30-year-old with acute lymphoblastic leukaemia, with fever and diarrhoea, and *Kocuria* species isolated from the second blood culture, but despite treatment with antibiotic with *invitro* activity against the organism, the outcome was fatal. The third case was a very low birth weight premature male neonate who was delivered by emergency cesarean at 31 weeks gestation, due to maternal chronic hypertension and superimposed preeclampsia. Although, *Kocuria rosea* was isolated from the third blood culture of the case, it was most likely a colonizer, as the clinical condition of the patient had improved before isolating the organism. This report may be the first case of bacteraemia caused by *Kocuria* from Nigeria, indicating that this organism should not ignored as a possible cause of bacteraemia because advancement in laboratory diagnostics will aid correct identification.

Keywords: *Kocuria* species; bloodstream infection; opportunistic pathogen; immunocompromised

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Infection sanguine à *Kocuria* spp dans un hôpital universitaire de Lagos, au Nigéria: une série de cas

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Résumé:

Les espèces de *Kocuria* sont des pathogènes opportunistes émergents souvent confondus avec des staphylocoques à coagulase négative. L'amélioration des techniques de diagnostic en laboratoire et l'augmentation du nombre de patients immunodéprimés ont entraîné une hausse des infections à *Kocuria* rapportées. Nous présentons ici trois cas de bactériémie à *Kocuria* chez des patients immunodéprimés. Le premier cas concernait un enfant de 11 ans atteint d'un carcinome nasopharyngé métastatique chez lequel *Kocuria kristinae* a été isolée lors de la seconde hémoculture. Cependant, en raison de sa sortie contre avis médical, le rôle de *K. kristinae* et l'évolution de la maladie n'ont pu être déterminés. Le deuxième cas concernait un homme de 30 ans atteint de

leucémie lymphoblastique aiguë, présentant fièvre et diarrhée. Une espèce de *Kocuria* a été isolée lors de la seconde hémoculture. Malgré un traitement antibiotique actif in vitro contre cette bactérie, l'issue a été fatale. Le troisième cas était celui d'un nouveau-né prématuré de sexe masculin, de très faible poids de naissance, né par césarienne en urgence à 31 semaines d'aménorrhée en raison d'une hypertension artérielle chronique maternelle et d'une prééclampsie surajoutée. Bien que *Kocuria rosea* ait été isolée lors de la troisième hémoculture de ce patient, il s'agissait très probablement d'une bactérie colonisatrice, l'état clinique du patient s'étant amélioré avant l'isolement de cet organisme. Ce cas pourrait être le premier cas de bactériémie à *Kocuria* rapporté au Nigéria, ce qui souligne l'importance de ne pas négliger cet organisme comme cause possible de bactériémie, les progrès des techniques de diagnostic en laboratoire facilitant son identification.

Mots-clés: *Kocuria* spp; infection sanguine; pathogène opportuniste; immunodéprimé

Introduction:

Kocuria species are opportunistic pathogens that affect mostly the immune compromised (1). Phylogenetic and chemotaxonomic analyses have reclassified *Kocuria* species from the family Micrococcacae to the family Kocuria (2). They are normal flora of skin and mucus membrane, strict aerobes except for *Kocuria kristinae,* that is a facultative anaerobe (3). They are catalase-positive, coagulase-negative Gram-positive cocci arranged in tetrads, which often to misidentification and misinterpretation as coagulase-negative staphylococci in the clinical laboratory (3).

Five *Kocuria* species namely *K. Kristinae, K. rosea, K. varians, K. marinia*, and *K. rhizophila* (*4*) are reported to cause a wide range of infections in the immunocompromised and immunocompetent host (3) worldwide and have been described to cause bacteraemia, septicaemia, urosepsis (5), endocarditis (6), cholecystitis (7), peritonitis (8), and arthritis (5). However, the risk of infection increases with indwelling medical devices (central venous catheter, ventilator, urethral catheterization, total parenteral nutrition), haemodialysis or peritoneal dialysis (8), and malignancy.

Advancement in laboratory diagnostics has made possible the accurate diagnoses of infection caused by this pathogen as we reported in these case series from a tertiary hospital in Lagos, Nigeria. To the best of our knowledge, this is the first report of *Kocuria* bloodstream infections from Nigeria.

Case Series:

Case 1:

An 11-year-old boy with metastatic nasopharyngeal carcinoma was referred to the Lagos University Teaching Hospital (LUTH) on 3rd of June 2021, with a history of progressive swelling on the back of the head of 2 months duration, high-grade fever, and pain in the right hip of 3 weeks duration. There was associated generalized recurrent throbbing headache with blurring of vision, but no associated vomiting, cough, trauma or loss of consciousness, memory, seizure, or sphincteric control. Because his condition persisted, he was admitted to a private facility for 2 weeks, where he received analgesics to relieve pain and transfused with 2 units of blood on account of anaemia. Brain and cervical computerized tomography (CT) scans were suggestive of metastatic lesions to the scalp, occipital bone and lobe. Abdominopelvic ultrasound scan showed liver metastasis. Because the mother declined further treatment at the private facility, the patient was referred to LUTH. Two years prior to the present admission of the patient, he was diagnosed with nasopharyngeal carcinoma and had undergone chemotherapy, with a course of radiotherapy, but defaulted from the paediatric oncology clinic and taken to his hometown for an undisclosed reason.

Physical examination on admission revealed a young boy that was febrile (temperature: 38°C), mildly pale, acyanosed, anicteric, not in obvious respiratory distress, not dehydrated, and no pedal oedema. There was an occipital swelling that measured 10cm by 10cm, firm and non-tender. He was conscious, and oriented in time, place, and person, with no other craniofacial abnormalities. Systemic examinations revealed no significant findings. He was commenced empirically on intravenous (IV) ceftriaxone 500 mg daily in line with the oncology department protocol of placing immunocompromised patients with recent hospitalisation on empirical antibiotic with or without evidence of infection.

Blood culture, serology for viral markers (hepatitis B, hepatitis C, and human immunodeficiency virus) and blood film for malaria parasites, were all negative. Full blood count (FBC) showed a white cell count (WBC) of 8,260/mm^3 (neutrophil 78%, lymphocyte 12%), packed cell volume (PCV) 38.2%, and platelet count of 547,000/mm^3. Erythrocyte sedimentation rate (ESR) was elevated (85 mm/hour). Electrolyte analysis (E, U, Cr) showed hyponatraemia with metabolic acidosis (Na^+ 130m mol/l, K^+ 3.18 mmol/l, HCO_3^- 17 (22-30mmol/l) that was appropriately corrected.

On day 15 of admission, a mini craniectomy was performed, and a biopsy of the sub-galeal mass and extradural tumor was taken for histology, the result of which was suggestive of metastatic adenocarcinoma a

week later. Patient then developed fever and non-productive cough two weeks after craniectomy, necessitating an increase in the dose of empirical IV ceftriaxone to 1 g 12 hourly. Despite this, fever persisted, and the antibiotic was changed to meropenem. A repeat blood culture and FBC analysis were performed with FBC result showing neutropenic leukocytosis (WBC 12,600/mm^3, neutrophil 11%), anaemia (PCV 28%) but normal platelet counts of 539,000/mm^3. The patient was optimized with blood products. The repeat blood culture incubated in Bact/Alert (BioMérieux) equipment flagged positive on day 3 of incubation, with Gram smear showing Gram-positive cocci that was identified by Vitek 2 version 8.0 as *Kocuria kristinae*, sensitive to linezolid, erythromycin, clindamycin, minocycline, levofloxacin, ciprofloxacin, and sulfamethoxazole.

Fever subsided upon commencement of meropenem and before the result of blood culture positive for *K. kristinae* was available. His craniectomy wound healing was favorable, and anti-cancer chemotherapy was commenced, for which the patient received a dose before the mother declined further chemotherapy, and requested for the patient discharge against medical advice (DAMA). Further clinical condition of the patient remained unknown to us after DAMA.

Case 2:

A 30-year-old woman known acute lymphoblastic leukaemia (ALL) BCR-ABL positive was admitted on 23rd March 2022 with elevated white cell counts of 458,000/mm^3 and associated history of blurred vision, vomiting, and difficulty in swallowing and walking of 2 days duration. There was an associated history of recurrent headaches, difficulty in urinating, and deviation of the mouth to the left. There was no fever, night sweats, neck pain, eye pain, loss of consciousness, or seizure. She was treated for COVID-19 in 2020 and diagnosed with adult-onset ALL in October 2021. She had haematological remission, and subsequently discharged home for follow-up in the outpatient clinic. However, the patient defaulted only to present with the present complaints. She was not a known hypertensive or diabetic, and does not smoke or take alcohol.

On examination at admission, she was chronically ill-looking, pale, conscious and afebrile (temperature: 36.9°C). There was bilateral ptosis, ophthalmoplegia, bilateral cranial nerve (CN) 6, right CN 7, and CN 12 palsies. There was also a deviation of the mouth to the left, however, she had no neck stiffness, and the muscle tones were normotonic globally. Abdominal examination showed that liver was about 10 cm below the right coastal margin, non-tender, with a smooth surface, while the chest and cardiovascular examinations were essentially normal. A diagnosis of relapsed adult ALL with possible metastatic lesions to the CNS was made. Exchange blood transfusion (EBT) and pre-induction chemotherapy with oral prednisolone (60 mg daily) and intravenous vincristine (2 mg) were administered, in addition to prophylactic antibacterial (trimethoprim-sulfamethoxazole 960 mg daily), antivirals (acyclovir 400 mg and itraconazole 200 mg daily) and antimalarial (proguanil/ paludrine 100 mg daily).

Baseline investigations consisting of FBC showed anaemia (PCV 28%), leucocytosis (WBC 309,013/mm^3 and absolute neutrophil count 13,850/mm$^{3)}$), and thrombocytopaenia (platelets 52,000/mm^3). Electrolytes were deranged (Na^+ 147 mmol/l, K^+ 2.47 mmol/ l, Cl^- 109 mmol/l, bicarbonate 28 mmol/l, blood urea nitrogen 7.5 mmol/l, creatinine 55 µmol/ l) and hypokalaemia was appropriately corrected. The clotting profile was normal (prothrombin time PT 11.6 seconds, PT control 12.5 seconds, INR 1.00, partial thromboplastin time in kaolin (PTTK) 29.6 seconds, PTTK control 27.6 seconds) and optimization with 2 units of packed cells and 3 units of platelet concentrates were done. On day 3, brain MRI revealed no intracranial extensions, and induction chemotherapy with hypercvad-methcytarabine was commenced.

There was a gradual clinical improvement until patient complained of a productive cough with non-radiating chest pain, sore throat, orthopnoea and diarrhoea on day 13. On chest auscultation, bi-basal crepitations were heard. Consequently, urine and blood cultures, urinalysis, and FBC investigations were ordered. The results of FBC showed pancytopaenia (Hb 9.8 g/dl, PCV 27%, WBC 0.1 x 10^9/L with ANC of 0.06, and platelets 31,000/ mm^3), but urine culture and urinalysis results were negative. Blood culture incubated in Bact/Alert machine grew *Enterobacter cloacae* sensitive to ciprofloxacin, ertapenem, imipenem, levofloxacin, cefoxitin, cefepime, but resistant to meropenem, amikacin, gentamicin, trimethoprim-sulfamethoxazole, and tobramycin, necessitating antibiotic change from trimethoprim-sulfamethoxazole to levofloxacin 500 mg daily, with optimization of her blood level. By day 22, patient was able to swallow food and saliva, ambulating slowly, and fever had subsided. However, by day 29, patient developed fever associated with diarrhoea, and fluconazole (200 mg), vancomycin (500 mg), metronidazole (500 mg 8 hourly), and subcutaneous filgrastim (neupogen) 300 µg daily were added to the treatment.

The patient's condition however continued to deteriorate, and a repeat blood culture in BACTEC equipment, which flagged po-

sitive after 48 hours of incubation showed Gram-positive cocci on direct Gram stain, and was identified as *Kocuria* species using Vitek 2 version 8.0 system, sensitive to linezolid, clindamycin, ciprofloxacin, levofloxacin, and moxi floxacin. Despite continuous treatment with levofloxacin, patient had cardiopulmonary arrest, and after unsuccessful active resuscitation, was certified dead by 2.10 a.m. on May 2022 (day 41 of admission).

Case 3:

This case was a preterm very low birth weight male neonate delivered on 15th March 2022 via an emergency cesarean section at 31 weeks gestation on the indication of superimposed preeclampsia secondary to chronic hypertension. Examinations revealed a conscious neonate with a birth weight of 1260g, occipitofrontal circumference of 28.4cm and length of 39cm, in obvious respiratory distress evidenced by respiratory rate of 50 cycles per minute. Other examinations were essentially normal. He was commenced on continuous positive airway pressure (CPAP) at 3L oxygen flow from the theatre with SPO_2 of 98% and was admitted directly to the neonatal intensive care unit (NICU) on account of prematurity.

Baseline laboratory investigations at NICU consisting of FBC, ESR, serum bilirubin, and random plasma glucose (RPG) were essentially within reference intervals except for the low random plasma glucose (32.4 mg/dl) that was corrected. In addition, a blood culture sample was collected and incubated in Bact/Alert (BioMérieux) equipment in the Medical Microbiology laboratory before commencing empirical antimicrobials which included cefotaxime 50 mg/kg and fluconazole 5 mg/kg stat, then 3 mg/kg every 72 hours.

He was also placed on therapeutic phototherapy at 21 hours of age and became warm to touch (temperature: 36.3-37.7°C), cyanosed, icteric, dyspneic with subcoastal resection (RR-64cpm) at 48 hours of age. The abdomen also becomes scaphoid with bronchovesicular sound and crepitations on chest auscultation. Amikacin 5 mg/kg/day was added to the treatment and CPAP was increased to 8L oxygen flow/minute. Serum bilirubin was elevated (total bilirubin was 100.2 µmol/l, conjugated bilirubin 13.7 µmol/l, unconjugated bilirubin 86.50 µmol/l). Electrolytes result suggested metabolic acidosis with hyponatremia (Na^+ 131 mmol/l (135-145 mmol/l), K^+ 5.19 mmol/l (3.5-5.1 mmol/l), Cl^- 98 mmol/l (98-110 mmol/l), HCO_3^- 17 (22-30mmol/l)), which was appropriately corrected.

At 96 hours of age, the patient condition began to deteriorate with development of generalised sclerema, with FBC result showing thrombocytopaenia (which was optimized) and serum procalcitonin of 7.77 ng/dl. Blood culture result showed no bacteria growth on day 5. Despite changing the empirical antibiotics from cefotaxime to levofloxacin, the patient developed abdominal distension on day 9, with periumbilical erythema and scrotal oedema, prompting insertion of a nasogastric tube for abdominal decompression. A second blood culture sample was collected. Babygram showed dilated bowel loops with no intraluminal and extraluminal gas, suggestive of necrotizing enterocolitis. The electrolytes were essentially within the reference interval.

On 13th day of life, the second blood culture grew extended-spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae* sensitive to amikacin, ertapenem, imipenem, and levofloxacin with intermediate sensitivity to cefotaxime and meropenem. Transfontanelle ultrasound scan (USS) and Chest x-ray findings were normal. Levofloxacin was discontinued, and tigecycline commenced on day 15 of life. The fever and abdominal distension however persisted, and by day 23, the patient developed respiratory distress (RR: 48 cycle per minute) and right upper limb swelling. There was laboratory evidence of hypokalemia (K^+ 3.13 mmol/l), anaemia with thrombocytopaenia (PCV 25.8%, WBC 8000/mm^3, ANC 1,920/mm^3, and platelets 6,000/mm^3), which were appropriately corrected.

A third blood culture collected on day 25 flagged positive and Gram stain of the culture showed Gram-positive cocci that was identified by Vitek 2 version 8.0 as *Kocuria rosea*, sensitive to clindamycin, moxifloxacin, gentamicin, erythromycin, and ciprofloxacin. By the time of isolation of *K. rosea* from the blood, the clinical condition of the patient had improved significantly and was continued on tigecycline until day 43, with serum procalcitonin level dropping to <0.5 ng/ml. Patient was discharged home alive on day 46 of life.

Discussion:

Kocuria species are now regarded as potential true pathogen because infections caused by them in transplant recipients, premature infants, patients with malignancy, diabetes mellitus, sickle cell and chronic renal diseases, pregnant women, and immunocompetent hosts, have been well-documented in the literature (1,7,9,10,11). Interestingly, the changes in climatic conditions and lifestyles have altered the evolution of microorganisms in a way that new pathogens are emerging (12).

Our case series were diagnosed clinically as sepsis and microbiologically confirmed as bacteraemia from blood culture specimens. Identifications were carried out with Vitek 2

automated system, thus avoiding issues of misidentification. Although the three cases had different clinical presentations and differing co-morbid conditions, fever unresponsive to empirical antibiotics was common to all. Cases 1 and 2 were patients with background malignancies who received cancer chemotherapy on admission, while case 3 was a premature, very low birth weight neonate who had the propensity to develop sepsis because of the immature immune cells (3,18). Isolating *Kocuria* species from the blood cultures of these patients was not surprising as invasive devices used, broad-spectrum antibiotics administered and potential immunosuppression in the neonate were predisposing risks for *Kocuria* infections. *Kocuria* species are normal flora of the skin and mucous membrane, and may have gained entry into the bloodstream during the insertion of the invasive devices.

All the three cases were on admission for more than 72 hours before *Kocuria* species were isolated. Also, the first blood culture was negative for cases 1 and 3, while *Enterobacter cloacae* was isolated in the first blood culture in case 2. This case 2 did not respond to the various antibiotics administered, which warranted a repeat blood culture that grew *Kocuria* species. This *Kocuria* may be a true pathogen contributing to the patient's clinical illness, but despite the use of antibiotic (levofloxacin) that showed good *invitro* activity against the organism, the clinical outcome was poor. However, *K. rosea* isolated in case 3 may be a coloniser that contaminated the sample during collection while the role of *K. kristinae* in case 1 remains uncertain because the patient voluntarily discharged self against medical advice and failed to return for further follow-up.

Recently, there have been growing reports of infections caused by *Kocuria* species in the literature, with some patients recovering with antibiotic treatment or the removal of the invasive devices. In Italy, a woman with ovarian cancer recovered from recurrent *K. Kristinae* catheter-associated bacteraemia when the central line was removed (4). Similarly, two paediatric patients with *K. kristinae* and *K. varians* were reported by Bhavar et al., (15), one was a 3-year-old child on chemotherapy following nephrectomy for bilateral Wilms tumour and the second was a 7-month-old child with shotgun syndrome on total parenteral nutrition, both recovered with combination of anti biotic treatment and invasive catheter removal.

Kocuria kristinae is a potential pathogen (13) and other rare *Kocuria* species cause infection in immunocompromised patients (14). Several cases of *K. kristinae* infections have been reported from various countries in diverse patient populations and clinical conditions (3,16,17). Siravaman et al., (18) reported a case of umbilical sepsis due to *K. kristinae* is a term male neonate. In another report from Greece, *K. rosea* peritonitis was reported in an 8-year-old girl with dysplastic kidney on peritoneal dialysis (19). The second case of *K. rosea* in paediatric was reported from Brazil in a 10-year-old with endocarditis post-surgery for congenital heart disease (20). Also, endocarditis and bacteremia due to *K. rosea* in a 10-year-old boy following heart valve replacement for ventricular septal defect and mitral valve incompetence have been reported (21). A prospective study in Iraq reported 7 *Kocuria* bacteraemia from 261 positive cultures, 5 of these patients had *K. kristinae* infection while the remaining 2 patients had *K. rosea* and *K rhizophilia* infections respectively (22). However, in case 3 of our report, *K. rosea* was recovered from the blood culture, but may have been a colonizer because the patient's condition had improved before it was isolated from the third blood culture, rather the ESBL-producing *K. pneumoniae* isolated from the second blood culture was likely the pathogen responsible for sepsis in this patient, and which was responsive to empirical tigecycline administered.

All the patients in this case series were exposed to various antibiotics. The use of antibiotics promotes resistance development, leading to treatment failure. Despite the patients' prior antibiotic exposure, all *Kocuria* species isolated in our cases were sensitive to fluoroquinolones, lincosamides, and macrolides. This agrees with what has been reported in the literature, in which *Kocuria* species are sensitive to a broad spectrum of antibiotics (17,18), but contrast the case described by Siravaman, in which the isolated *Kocuria* was resistant to multiple antibiotics (18).

Conclusion:

Uncommon pathogens such as *Kocuria* species that rarely cause human infection are emerging and increasingly recognized worldwide because of the introduction of better identification techniques. Therefore, their isolation in the laboratory should not be considered as laboratory contaminants in all cases but must be correlated with clinical findings to guide proper treatment of bloodstream infections and good clinical outcomes.

Contributions of authors:

OCS conceptualized the study, participated in the literature review and writing of the manuscript; DAI was involved in reviewing

the literature and writing the manuscript; ROO participated in the literature review and performed a critical review of the manuscript. All the authors reviewed and accepted the final version of the manuscript submitted for publication.

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Authors declare no conflict of interest.

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Short Communication **Open Access**

Antibiotic susceptibility of bacteria isolated from suppurative acute otitis media in children in Ouagadougou, Burkina Faso

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Abstract:

Background: Acute otitis media (AOM) is one of the most common bacterial infections in children. The emergence of antimicrobial resistance (AMR) poses a threat to the management of these infections. The objective of this study was to identify and describe the sensitivity profile of bacterial agents isolated in AOM to commonly used antibiotics in the city of Ouagadougou, Burkina Faso.
Methodology: This was a descriptive cross-sectional study conducted between September 2023 and February 2024 in children aged 0-15 years with clinical features of suppurative AOM seen at the otorhinolaryngology (ORL) departments of four health facilities in Ouagadougou. Ear discharge was collected using sterile swabs and cultured on Chocolate agar media, incubated at 37°C for 24-48 hours in 5% CO_2. The sensitivity of the isolated bacteria to antibiotics was determined using the Kirby-Bauer disc diffusion method and interpreted according to the 2021 guideline of the Antibiogram Committee of the French Society of Microbiology (CA-SFM).
Results: A total of 61 children with AOM were recruited and bacteriological culture examination was performed on the ear swabs obtained from them. All but two cultures were positive. A total of 59 bacterial pathogens were isolated with Gram-negative bacilli (GNB) more commonly isolated (67.8%, n=40) in the children, dominated by *Klebsiella pneumoniae* (18.6%, n=11) and *Pseudomonas aeruginosa* (18.6%, n=11). Aztreonam and imipenem were the most active antibiotics against GNB, but majority of the GNB were sensitive to gentamicin and ciprofloxacin.
Conclusion: Acute otitis media in children, combined with the growing resistance of pathogens to antibiotics, is a real public health challenge. It is necessary to strengthen regular microbiological monitoring and the development of appropriate therapeutic strategies in order to limit the emergence and spread of resistant strains.

Keywords: Acute otitis media; AMR; Antibiotics; Bacteria; Burkina Faso

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Sensibilité aux antibiotiques des bactéries isolées d'otites moyennes aiguës suppuratives chez l'enfant à Ouagadougou, Burkina Faso

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Résumé:

Contexte: L'otite moyenne aiguë (OMA) est l'une des infections bactériennes les plus fréquentes chez l'enfant. L'émergence de la résistance aux antimicrobiens (RAM) constitue une menace pour la prise en charge de ces infections. L'objectif de cette étude était d'identifier et de décrire le profil de sensibilité des agents bactériens isolés dans les cas d'OMA aux antibiotiques couramment utilisés dans la ville de Ouagadougou, au Burkina Faso.
Méthodologie: Il s'agit d'une étude descriptive transversale menée entre septembre 2023 et février 2024 auprès d'enfants âgés de 0 à 15 ans présentant des signes cliniques d'otite moyenne aiguë suppurée (OMA) et suivis dans les services d'oto-rhino-laryngologie (ORL) de quatre établissements de santé à Ouagadougou. Les sécrétions auriculaires ont été prélevées à l'aide d'écouvillons stériles et mises en culture sur gélose chocolatée, incubée à 37°C pendant 24 à 48 heures sous 5% de CO_2. La sensibilité des bactéries isolées aux antibiotiques a été déterminée par la méthode de diffusion sur disque de Kirby-Bauer et interprétée selon les recommandations 2021 du Comité d'Antibiogramme de la Société Française de Microbiologie (CA-SFM).
Résultats: Au total, 61 enfants atteints d'OMA ont été inclus et un examen bactériologique a été réalisé sur les prélèvements auriculaires. Toutes les cultures, sauf deux, étaient positives. Au total, 59 agents pathogènes bactériens ont été isolés, les bacilles Gram négatif (BGN) étant les plus fréquemment isolés (67,8%, n=40) chez les enfants, avec une prédominance de *Klebsiella pneumoniae* (18,6%, n=11) et de *Pseudomonas aeruginosa* (18,6%, n=11). L'aztréonam et l'imipénem se sont révélés les antibiotiques les plus actifs contre les BGN, mais la majorité de ces derniers étaient sensibles à la gentamicine et à la ciprofloxacine.
Conclusion: L'otite moyenne aiguë chez l'enfant, associée à la résistance croissante des agents pathogènes aux antibiotiques, constitue un véritable enjeu de santé publique. Il est nécessaire de renforcer la surveillance microbiologique régulière et le développement de stratégies thérapeutiques appropriées afin de limiter l'émergence et la propagation des souches résistantes.

Mots-clés: Otite moyenne aiguë; résistance aux antimicrobiens; antibiotiques; bactéries; Burkina Faso

Introduction:

Acute otitis media (AOM) is a common ear infection in children, and a major reason for consultation in the ear nose and throat (ENT) and a significant cause of school absenteeism. They affect the middle ear and occur frequently in children aged 0 to 15 years, linked to immune immaturity and the anatomical particularities of the ear in young children (1). In children under five years of age, AOM is the most common infectious disease, ranking fifth among global causes of morbidity and remains more common in developing regions, such as sub-Saharan Africa and South Asia (2). It mainly affects the pediatric population, with a maximum incidence observed between the ages of 6 months and 2 years (3).

An epidemiological survey carried out in Nigeria in 2005 among 600 children aged 0 to 12 years reported a prevalence of AOM estimated at 11.8% (4). In Burkina Faso, a developing country, AOM remains a very common pediatric condition, ranking among the top ten reasons for seeking care in health facilities. According to data from the statistical yearbook of the country's Ministry of Health and Public Hygiene, AOM was the cause of 38,807 cases of hospitalization during the year 2020 (5).

Acute otitis media results from viral and/or bacterial infections. The most frequently isolated bacteria are *Haemophilus influenzae* and *Streptococcus pneumoniae* (6). However, the involvement and emergence of other bacterial pathogens such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus* have been reported in recent years (2). Acute otitis media is an otolaryngological emergency, jeopardizing functional prognosis with an increased risk of deafness and facial paralysis (7).

In current practice, antibiotic treatment is carried out without biological confirmation and without antibiotic susceptibility tests to guide the choice of antibiotics. The most frequently prescribed empirical antibiotic is the combination of amoxicillin and clavulanic acid (8). These practices constitute factors favoring the emergence and spread of antimicrobial resistance (AMR), particularly to antibiotics through selection of mutant strains which constitute a major public health issue. To optimize the prescription and limit the inappropriate use of antimicrobials, it is essential to have updated data on the bacterial etiology of AOM and the sensitivity profile of pathogens to antibiotics.

This study aimed to characterize the bacterial agents involved in AOM in children in the city of Ouagadougou and to describe their sensitivity to common antibiotics.

Materials and method:

Study sites:

The study was conducted in the city of Ouagadougou, capital of Burkina Faso, in four health facilities of different levels and geographical locations. The four sites were the ENT departments of the University Hospital Center (CHU) Yalgado Ouedraogo (CHU YO) in the city Center, the University Hospital Center Pediatric Charles de Gaulle (CHU-P-CDG) in the East, the Medical Center with Surgical Antenna (CMA) of Pissy in the West, and the CMA of Kossodo in the North

of the city. Cytobacteriological analyses were carried out at the CHU-P-CDG laboratory.

Ethical considerations:

The study protocol was submitted for approval to the Ministry of Health's Ethics Committee and received authorization from the administrative managers of the various structures involved in the study.

Study design and period:

This was a descriptive cross-sectional study conducted over a six-month period from September 2023 to February 2024. This period corresponds to the end of the rainy season and the school year for children. It is a period of population movement, which is more significant during the month of December for vacationers. The study ended with a dry period in February.

Study population and participants:

The study population consisted of children aged 0 to 15 years seen in the ENT departments of the four selected sites and who presented with AOM upon clinical examination. The study participants were recruited based on defined criteria, which primarily included age 0 to 15 years.

The clinical diagnosis of acute otitis media (AOM) was made based on the sudden onset of fever associated with ear pain, or on otoscopic examination revealing a congested, opaque, bulging, or perforated eardrum with purulent otorrhea.

Children currently undergoing antibiotic treatment or who had recently used herbal remedies or other local treatments likely to alter the bacterial flora were excluded from the study.

Sample collection:

A bacteriological sample was taken after obtaining free and informed consent of a parent or legal guardian. Samples of ear discharge were collected using sterile swabs.

Laboratory analysis procedure:

The cytobacteriological analysis of the samples involved macroscopic examination of the sample (appearance, color, consistency); direct microscopic examination after Gram staining, allowing evaluation of the morphology and arrangement of the bacteria; culture of swabs on standard and enriched media, in particular chocolate agar enriched with polyvitamins. The isolated bacteria were identified based on cultural, morphological, biochemical, and antigenic characteristics. Pure cultures were obtained by subculture for the purpose of performing antibiotic sensitivity testing.

The antibiotic sensitivity of the isolated strains was evaluated using the Kirby-Bauer agar diffusion method on Mueller-Hinton agar. The results were interpreted in accordance with the recommendations of the 2021 Antibiogram Committee of the French Society of Microbiology (CA-SFM (9).

Data analysis:

The data obtained were entered using Excel software and analyzed using Epi info version 7.2 and Stata version 15.1.

Results:

Sociodemographic characteristics of the study participants:

A total of 61 cases of AOM were identified during the study period. The mean age of affected children was 28±29 months, with infants and young children being the most affected, ranging from 1 month to 13 years. Males predominated (55.7%), with a sex ratio of 1.25 (Table 1).

Table 1: Distribution of children with acute otitis media by age group and sex in Ouagadougou, Burkina Faso

Characteristics	Number	Percentage (%)
Age group (months)		
1-20	39	63.9
21-40	11	18.0
> 40	11	18.0
Sex		
Males	34	55.7
Females	27	44.3
Sex ratio	1.25	
Total	**61**	**100.0**

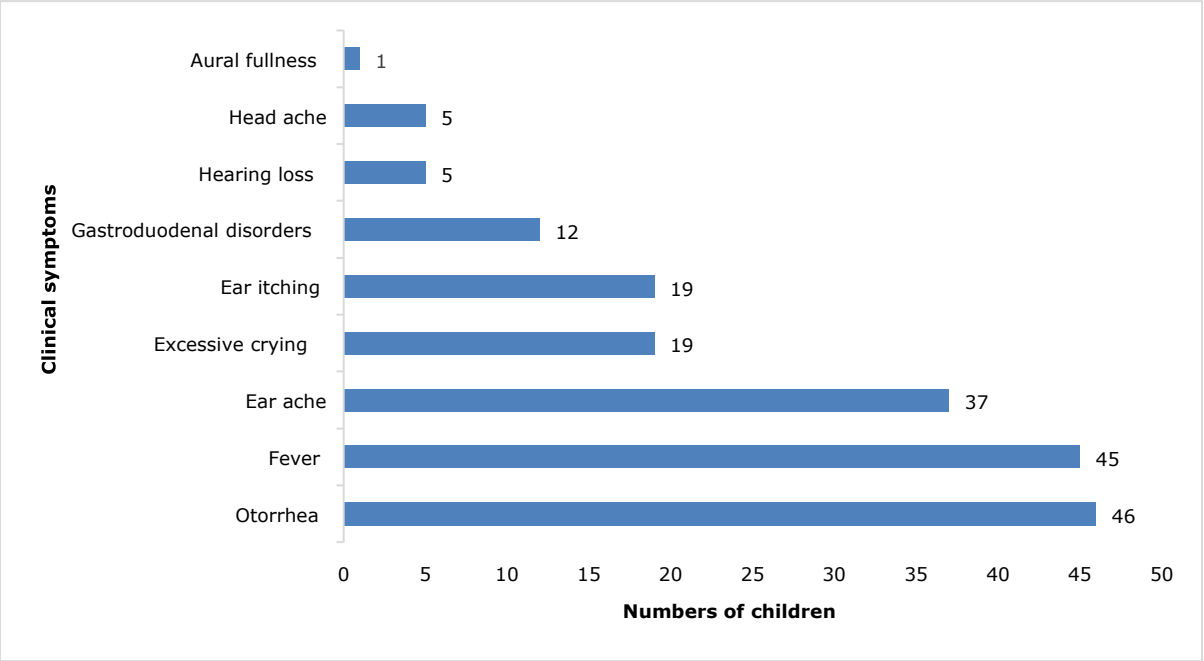

Fig 1: Distribution of clinical symptoms of AOM in children in Ouagadougou, Burkina Faso

Clinical characteristics of study participants: The clinical manifestations observed during AOM were dominated by otorrhea (75.4%), frequently associated with fever (73.8%) and ear ache (60.7%). This symptomatic triad thus constituted the typical clinical picture suggestive of AOM (Fig 1).

Bacteriological profile of isolated organisms: Bacteriological analysis showed that a total of 59 bacterial pathogens were isolated from the participants with a predominance of GNB (67.8%, n=40), dominated by *K. pneumoniae* (18.6%, n=11) and *P. aeruginosa* (18.6%, n=11). Gram-positive cocci (GPC) constituted 32.2% (n=19) of the isolates, dominated by *S. aureus* (11.9%, n=7) and *Staphylococcus epidermidis* (11.9%, n=7) (Table 2).

Table 2: Frequency of isolated bacteria isolates in children with acute otitis media in Ouagadougou, Burkina Faso

Isolated bacteria	Numbers	Percentage (%)
Gram-negative bacilli	40	67.8
Klebsiella pneumoniae	11	18.6
Pseudomonas aeruginosa	11	18.6
Escherichia coli	10	16.9
Proteus mirabilis	5	8.6
Acinetobacter spp	2	3.4
Citrobacter spp	1	1.7
Gram-positive cocci	19	32.2
Staphylococcus aureus	7	11.9
Staphylococcus epidermidis	7	11.9
Staphylococcus saprophyticus	5	8.5
Total	59	**100.0**

Table 3: Susceptibility of selected Gram-negative bacilli to antibiotics

Antibiotics	***Escherichia coli* (%) (n=10)**	***Klebsiella pneumoniae* (%) (n=11)**
Amoxicillin-clavulanic acid	4 (40.0)	5 (45.5)
Ceftriaxone	5 (50.0)	5 (45.4)
Ceftazidime	6 (60.0)	6 (54.5)
Aztreonam	7 (70.0)	9 (81.8)
Imipenem	10 (100.0)	7 (63.6)
Ciprofloxacin	7 (70.0)	4 (36.4)
Cotrimoxazole	7 (70.0)	5 (45.5)
Gentamicin	8 (80.0)	4 (36.4)

Table 4: Susceptibility of selected Gram-positive cocci to antibiotics

Antibiotics	***Staphylococcus aureus* (%) (n=7)**	***Staphylococcus saprophyticus* (%) (n=5)**
Cefoxitin	4 (57.1)	3 (60.0)
Penicillin G	2 (28.5)	0
Erythromycin	5 (71.4)	3 (60.0)
Lincomycin	5 (71.4)	1 (25.0)
Gentamicin	6 (85.7)	2 (40.0)
Tobramycin	6 (85.7)	1 (25.0)
Kanamycin	5 (71.4)	1 (25.0)
Ciprofloxacin	6 (85.7)	1 (25.0)
Cotrimoxazole	3 (42.8)	2 (40.0)

Antibiotic susceptibility profile of isolated pathogens:

With regard to the sensitivity profiles of GNB, *K. pneumoniae* were sensitive to aztreonam (81.8%, n=9/11) and imipenem (63.6%, n=7/11). *Escherichia coli* isolates were sensitive to gentamicin (80.0%, n=8/10) and cotrimoxazole (70.0%, n=7/10) (Table 3). For the GPC, *S. aureus* isolates were sensitive to erythromycin, cefoxitin, and cotrimoxazole with respective proportions of 85.7% (n=6/7), 57.1% (n=4/7), and 42.8% (n=3/7) (Table 4).

Discussion:

This study provided data on the sensitivity of isolated bacterial pathogens to antibiotics commonly used for treatment of acute otitis media in children in Burkina Faso. The mean age of children with AOM was 28±29 months, with a strong predominance of infants and young children. This population is more exposed to AOM due to the immaturity of the immune system, in particular, the humoral response, as well as the horizontal and short morphology of the eustachian tube, favoring stasis of secretions and bacterial colonization (10).

The most suggestive clinical sign of AOM was otorrhea. This symptom is common in cases of suppurative AOM with a close relationship between the duration of discharge and the risk of hearing loss (11). The occurrence of this suppuration during the course of AOM could be explained by tympanic perforations associated with post-infectious purulent discharge, by the progression

towards chronicity, but also by exposure to risk factors such as high humidity, household dust and fumes, and inappropriate ear hygiene practices.

Gram-negative bacilli were the predominant bacterial group involved in AOM in this study. This observation had been made by several authors (12-14). Conversely, Wassef et al., (15) found a predominance of *S. aureus* in AOM. This epidemiological divergence could be explained by several factors including the clinical phenotype (series dominated by *S. aureus* often concern non-perforated AOM), sampling methods (middle ear aspiration versus otorrhea logorrheic discharge), prior exposure to antibiotics and the hospital setting. In this present study, the isolation of GNB was probably favored by the frequency of suppurative or perforated forms, and by samples mainly taken from discharges.

In terms of antimicrobial susceptibility, we observed a worrying 40% susceptibility rate of *E. coli* to amoxicillin–clavulanic acid. Although *E. coli* is not a pathogen classically implicated in AOM, this low susceptibility rate should prompt monitoring, as it is part of a documented regional trend. In Ethiopia, several studies have reported very high resistance rates of GNB isolated from otitis media to amoxicillin–clavulanic acid, reaching 66–90% depending on the species and infection sites (16). A study conducted in Ghana in children with suppurative otitis media isolated extended spectrum β-lactamase (ESBL)-producing strains of *E. coli* and *K. pneumoniae* (17). This enzymatic mechanism, responsible for the hydrolysis of penicillins, cephalosporins, and sometimes monobactams, can negate the clinical efficacy of amoxicillin-clavulanic acid and reflects a selection pressure maintained by repeated empirical prescriptions.

In general, African data converge towards a limited efficacy of amoxicillin-clavulanic acid combinations in suppurative otitis, contrasting with better sensitivities to aminoglycosides, fluoroquinolones and certain cephalosporins. These alternatives must be handled with caution in children and taking in to account the administration methods. In our context, the most frequently prescribed antibiotic is amoxicillin-clavulanic acid. The observed resistance of certain bacterial agents to this molecule suggests particular attention during the treatment of AOM not guided by bacteriological data, which may be responsible for therapeutic failures (8).

Conclusion:

Acute otitis media in children and the growing resistance of pathogens to antibiotics constitute a real public health challenge. The results of this study highlight a predominance of GNB in suppurative infections, highlighting the need for regular microbiological monitoring and the development of appropriate therapeutic strategies, in order to limit the emergence and spread of resistant strains.

Contributions of authors:

All authors contributed to the conception, execution, and writing of the manuscript. All authors have read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that they have no conflicts of interest in relation to the content of this article.

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Short Communication **Open Access**

Evaluation of microbial quality of grilled foods sold at roadside in Calabar metropolis, Cross River State, Nigeria

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Abstract:

Background: Roadside foods are central parts of culinary custom in many African countries. In addition to their dietary importance, they contribute immensely to the national and economic values of a nation. However, the growing incidence of food-borne illnesses in relation to the roadside foods is a great concern. Hence, this study assessed the microbial quality of grilled foods sold at roadside in Calabar metropolis, Cross River State, Nigeria.
Methodology: A total of thirty grilled food (roasted ripe or unripe plantain with grilled mackerel fish) samples were collected from the three busiest areas (Marian, Etagbor and Murtala Muhammed Highway) in Calabar metropolis. Isolation and enumeration of bacteria and fungi were done using standard techniques to obtain colony counts that were correspondingly employed in the determination of total bacteria count (TBC) and total fungi count (TFC).
Results: Grilled ripe plantain from Marian axis recorded the highest TBC ($4.0x10^6$ CFU/g) while grilled unripe plantain from Etagbor location had the least ($8.0x10^3$ CFU/g) TBC. *Streptococcus* spp., *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis* were isolated from the samples, with *E. coli* as the most predominant. Grilled food from Marian location had the highest ($2.8x10^4$ CFU/g) TFC while samples from Etagbor axis had the lowest ($3.6x10^3$ CFU/g) TFC, with *Aspergillus flavus* and *Penicillium digitatum* as the most predominant fungal isolates.
Conclusion: The level of microbial loads detected was above the safe limit, thus signifying lack of proper food handling and sanitary practices among the grilled food vendors.

Keywords: Grilled foods, Microbial quality, Roadside, Vendor, Calabar metropolis

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Évaluation de la qualité microbiologique des aliments grillés vendus en bord de route dans la métropole de Calabar, État de Cross River, Nigéria

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Résumé:

Contexte: La restauration de rue occupe une place centrale dans les traditions culinaires de nombreux pays africains. Outre son importance nutritionnelle, elle contribue largement au patrimoine national et économique. Cependant, l'incidence croissante des maladies d'origine alimentaire liées à cette restauration est très préoccupante. Cette étude a donc évalué la qualité microbiologique des aliments grillés vendus en bord de route dans l'agglomération de Calabar, dans l'État de Cross River, au Nigéria.
Méthodologie: Trente échantillons d'aliments grillés (bananes plantains mûres ou vertes rôties accompagnées de maquereau grillé) ont été prélevés dans les trois zones les plus fréquentées de l'agglomération de Calabar (Marian, Etagbor et l'autoroute Murtala Muhammed). L'isolement et le dénombrement des bactéries et des

champignons ont été réalisés selon des techniques standard afin d'obtenir le nombre de colonies, lequel a ensuite été utilisé pour déterminer le nombre total de bactéries (NTB) et le nombre total de champignons (NTC).
Résultats: Les bananes plantains mûres grillées de la zone de Marian présentaient le NTB le plus élevé ($4,0\times10^6$ UFC/g), tandis que les bananes plantains vertes grillées d'Etagbor présentaient le NTB le plus faible ($8,0\times10^3$ UFC/g). Des *Streptococcus* spp., *Staphylococcus aureus*, *Escherichia coli* et *Bacillus subtilis* ont été isolés des échantillons, *E. coli* étant l'espèce prédominante. Les aliments grillés provenant de la zone de Marian présentaient la charge microbienne totale la plus élevée ($2,8x10^4$ UFC/g), tandis que ceux provenant de l'axe Etagbor affichaient la plus faible ($3,6x10^3$ UFC/g). *Aspergillus flavus* et *Penicillium digitatum* étaient les isolats fongiques les plus prédominants.
Conclusion: Le niveau de charge microbienne détecté était supérieur au seuil de sécurité, ce qui indique un manque de bonnes pratiques de manipulation et d'hygiène des aliments chez les vendeurs d'aliments grillés.

Mots-clés: Aliments grillés, Qualité microbiologique, Bord de route, Vendeur, Métropole de Calabar

Introduction:

The availability and consumption of roadsides foods have tremendously increased recently in many parts of the globe, serving both as a source of livelihood and dietary nutrients to the growing populace. In Nigeria, roadside foods fall under the category of ready-to-eat street foods, as they are always consumed immediately after purchased without further processing, thereby posing substantial public health threats as the food can be easily contaminated (1).

According to Food and Agriculture Organization (FAO), street foods are defined as ready-to-eat foods and drinks made and/ or sold by food merchants especially in an open space along major roads and public places (2). These roadside ready-to-eat foods including, but not limited to suya and pork, roasted plantains, grilled meats, fishes and chicken gratify indigenous cravings, in addition to posing exceptional ethnic values for citizens and tourists. Grilled foods are meals cooked using dry-heat cooking methods, mostly over a heat source, such as charcoal, wood, gas, hot plate or electric grill. Meat (e.g. steak, chicken, pork, sausages, and burgers), seafood (e. g. fish, shrimps, and oysters), vegetables (e. g. corn, peppers, zucchini, and onions), fruits (e.g. pineapple, peaches, and strawberries), pizza, flatbreads, and others are among the various types of grilled foods

Recently, food hawking has grown into a norm in the metropolitan of Cross River State, with a good number of residents and tourists eating meals from food sellers, citing time-saving, lower costs, and easy accessibility as major reasons for such practices (3). An overall scrutiny of the social order in Calabar metropolis has placed the area as a centre for tourism, with urban settlement, travelling workforces, and less household focused activities, leading to complete dependent on the ready-to-eat roadside food by high number of the populace. These have shifted the burden of food hygiene and handling practices from households to the food sellers who may not adequately comply with the necessary food processing principles (4).

The quality and safety of these roadside foods is therefore a universal challenge, as these foods are basically prepared in open-air surroundings, behind dirty and smelly drainages, with limited access to adequate cooking utensils, thereby suggesting possibility of microbial contamination at the various processing and packing points (5).

Previous studies on microbiological assessment of roadside foods in different countries have reported microbial contamination and high incidence of food-borne bacterial pathogens (6). For instance, in India, Rath and Patra (7), and Suneetha et al., (8) reported high loads of bacterial pathogens on common street foods in various areas of the country. In Africa, El-Shenawy et al., (9) reported high loads of *Bacillus cereus, Staphylococcus aureus, Shigella sonnei, Escherichia coli,* and *Salmonella arizonae* in roadside foods in Egypt. In Nigeria, Ahmad et al., (10) reported various antibiotics bacterial pathogens in roadside foods sold in Katsina metropolis. Also, Oranusi and Braide (11) reported the presence of *Salmonella* spp*, S. aureus, E. coli, B. cereus, Shigella* spp*, Enterococcus, Pseudomonas* and *Aspergillus niger* in grilled foods sold in Onitsha-Owerri metropolis. In addition, Oyet et al., (5) reported the presence of microbial pathogens in grilled foods in Port-Harcourt metropolis, Rivers State, Nig eria. Unfortunately, most of these pathogens recovered from grilled foods have been implicated in food poisoning, intoxication as well as foodborne diseases (4).

Foodborne infections are described as one of the utmost prevalent diseases, bothering the healthcare system, responsible for the preponderance of infections, mortality and morbidity across all categories of individuals globally, mostly in the developing countries (12). Although numerous risk factors of foodborne illnesses, including polluted water and environment, inadequate and non-compliance to food safety regulations, poverty and ignorance, have been reported; poor knowledge, attitude and practices of food safety by food dealers have been regarded as the paramount factor in the progress of the disease (13). This may be due to the fact that most

of these food vendors are not licensed and their activities are not regulated by appropriate food safety bodies. More worrying is the great demand and consumption of roadside grilled foods in Calabar, Cross River State, as many consumers are ignorant of the health implication of eating these foods.

Although there are published studies in the literature on nutritional composition and microbial assessment of some roadside fruits and fruit salads, soups, and foods in Calabar metropolis, there is currently no information on the microbial evaluation of grilled foods (roasted ripe or unripe plantain with sausage, commonly called "bole") in this setting despite their high demand and consumption. Therefore, the present study was aimed at evaluating the microbial quality of roadside grilled foods sold in Calabar metropolis, Cross River State, Nigeria.

Materials and method:

Study setting and sample collection:

Grilled plantain (ripe and unripe) with mackerel fish were collected randomly from three busiest locations (Marian, Murtala Mohammed Highway and Etagbor) in Calabar metropolis, Cross River State, Nigeria. Five samples of each of roasted ripe and unripe plantain with grilled mackerel fish were purchased directly from roadside vendors in 3 randomly selected locations, making a total of 30 samples. The samples were put in different sterile aluminum foils, and immediately transported to the laboratory in the Department of Biochemistry, University of Calabar for analysis.

Sample preparation and microbial analysis:

Ten grams of each grilled sample were homogenized by blending in 90 ml of nutrient broth (Biotec, UK) and serial dilutions of each grilled sample homogenate were made to 10^{-5} dilutions. Precisely, 0.1 ml of the appropriate dilutions were spread using pour plate methods onto duplicate sterile nutrient agar and MacConkey agar, and Sabouraud Dextrose agar (SDA) plates for bacterial and fungal isolation respectively.

The nutrient and MacConkey agar plates were incubated for 24 hours at 37° C, while the SDA plate was incubated for 96 hours at 30° C. The agar plates were examined after incubation for growth of pathogens and the colonies were counted using colony counter (Gallenkamp, England) and expressed as colony forming unit/gram (CFU/g) of sample homogenate.

Identification of isolates:

Pure colonies of each isolate were kept on agar slants at 4° C for subsequent identification by the methods described by Oranusi et al., (14) and Fawole and Oso (15) for bacterial and fungal isolates respectively. However, the identities of other bacterial isolates were done by comparing their appearances with the descriptions in the Bergey's Manual of Determinative Bacteriology (16).

Statistical analysis:

Data obtained for total bacterial and fungal counts were calculated and all CFU/g values were converted to log10. Foods were then classified according to the values obtained after conversion with reference to International Commission on Microbiological Specifications.

Results:

Table 1 shows the total bacterial counts of grilled foods sold at roadside in 3 major locations (Etagbor, Marian and Murtala Mohammed Highway) in Calabar metropolis, Cross River State, Nigeria. The result indicated that, the highest total bacterial counts were found in the roasted ripe plantain grilled mackerel fish from Marian axis with $4.0x10^6$ CFU/g, while the lowest value was found the in roasted unripe plantain with grilled mackerel fish from Etagbor axis with $8.0x10^3$ CFU/g. The results of bacterial identification revealed the presence of *Streptococcus* spp, *S. aureus, E. coli* and *B. subtilis* in the samples, with *E. coli* as the most prevalent.

Table 2 showed the total fungi counts of the grilled foods sold at roadside from 3 major locations in Calabar metropolis, Cross River State, Nigeria. The result indicates that the highest total fungal counts were found in roasted unripe plantain with grilled mackerel fish from Marian axis, with 2.8×10^4 CFU/g, while lowest value was found in roasted ripe plantain with grilled mackerel fish from Etagbor axis with $3.6x10^3$ CFU/g. The results of fungal identification revealed the presence of *Aspergillus* spp*, Penicillium* spp*, Rhizopus* spp and *Saccharomyces cerevisiae* in the samples with *Aspergillus* spp and *Penicillium* spp as most abundant fungi isolates in the samples.

Table 1: Bacterial load of grilled foods sold at roadside in Calabar metropolis, Cross River State, Nigeria

Samples/location	Total number of samples	Total bacterial count (CFU/g)	Identified bacterial species
Roasted ripe plantain with grilled mackerel fish from Etagbor	5	1.0×10^4	*Streptococcus* spp, *Escherichia coli,* and *Staphylococcus aureus*
Roasted ripe plantain with grilled mackerel fish from Marian	5	4.0×10^6	*Bacillus subtilis, Escherichia coli* and *Staphylococcus aureus*
Roasted ripe plantain with grilled mackerel fish from Highway	5	1.0×10^4	*Bacillus subtilis, Streptococcus* spp, *Escherichia coli* and *Staphylococcus aureus*
Roasted unripe plantain with grilled mackerel fish from Etagbor	5	8.0×10^3	*Streptococcus* spp*, Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis*
Roasted unripe plantain with grilled mackerel fish from Marian	5	4.4×10^4	*Bacillus subtilis, Streptococcus* spp and *Escherichia coli*
Roasted unripe plantain with grilled mackerel fish from Highway	5	3.5×10^4	*Escherichia coli, Streptococcus* spp*, Staphylococcus aureus.* and *Bacillus subtilis*

Table 2: Fungal load of grilled foods sold at roadside in Calabar Metropolis, Cross River State, Nigeria

Samples/location	Total number samples	Total fungal count (CFU/g)	Identified fungal species
Roasted ripe plantain with grilled mackerel fish from Etagbor	5	3.6×10^3	*Rhizopus* spp*., Penicillium* spp. and *Mucor* spp*.*
Roasted ripe plantain with grilled mackerel fish from Marian	5	2.1×10^4	*Aspergillus* spp*., Penicillium* spp. and *Rhizopus* spp.
Roasted ripe plantain with grilled mackerel fish from Highway	5	4.6×10^3	*Penicillium* spp*., Aspergillus* spp. *and Saccharomyces cerevisiae*
Roasted unripe plantain with grilled mackerel fish from Etagbor	5	2.4×10^4	*Penicillium* spp*., Aspergillus* spp. and *Rhizopus* spp.
Roasted unripe plantain with grilled mackerel fish from Marian	5	2.8×10^4	*Penicillium* spp*., Aspergillus* spp. and *Rhizopus* spp*.*
Roasted unripe plantain with grilled mackerel fish from Highway	5	4.1×10^3	*Aspergillus* spp*., Penicillium* spp. and *Rhizopus* spp.

Discussion:

The comparative assessment of the microbial load of grilled food from roadside vendors in three busiest locations in Calabar metropolis, Cross River State was carried out in order to compare the safety of this highly demanded roasted ripe or unripe plantain with grilled mackerel fish commonly referred to as "bole" to ascertain which vendor complies with the appropriate hygienic and food safety practices. The microflora of the roasted ripe and unripe plantain is an assessment index of the quality of the grilled food, the hygienic conditions of the food handler, environment and the cooking utensils employed in the preparation of the food (17).

From the present study, the total bacterial counts ranged from 8.0×10^3 to 4.0×10^6 CFU/g, and the total fungal load ranged from 3.6×10^3 to 2.8×10^4 CFU/g. These values, mostly the bacterial loads, were above the safe limit stated by the International Commission on Microbiological Specifications for grilled food, with $0\text{-}10^3$ as safe, $10^4\text{-}10^5$ as bearable and $>10^6$ as unacceptable (18). The high microbial load of the grilled food from roadside vendors implicates their consumption as a risk factor of food borne illnesses, as most of the isolated pathogens have been

linked to the illness.

However, the total bacterial counts recorded in the present study were lower than those reported by Henry, et al., (4), Brooks (19) and Ekpenyong et al., (1) for ready-to-eat foods, fruits salads and soups respectively, sold in the streets of Calabar metropolis. The variation in the total bacterial counts observed between the present study and previous findings may be attributed to handling and processing methods employed in the preparation of the various foods, as the present study employed roasting, where the pathogens must have been subjected to a very high temperature, compared to the cooking process used in the previous studies. This therefore underscore the recent reports of studies (20-23) that independently revealed that heating/roasting reduced the level of harmful compounds in food, thereby promoting the therapeutic potentials of processed foods as they have been implicated in the fight against cardio-metabolic disorders (24).

The present study revealed variation in the total bacterial counts among the three samples location, with Marian samples having the highest bacterial load. The high microbial load from the Marian location suggests unhygienic conditions and preparation, use of unclean water and utensils, exposure of the grilled food to air, as well as unhygienic behavior of the food vendors. Marian route can be referred to as unclean location due to high industrial activities, and the heavy presence of street children (urchins), locally called "scolombo", who are known for poor hygienic practices, going days without having a bath or change of clothes, sleeping in the street at nights, defecating and urinating in open places, thereby contaminating the location with faecal matters. Unfortunately, grilled food vendors usually prepare and sell these foods in this vicinity with complete exposure to air and dust, resulting in poor food handling safety practices, leading to high microbial loads in the area.

Our analysis further revealed *Streptococcus* spp, *S. aureus*, *E. coli* and *B. subtilis* as the bacterial pathogens present in the grilled food, with *E. coli* having the highest frequency of occurrence. The isolation of *E. coli* could be linked to processing contamination, faecal contamination and poor sanitary practice of food handlers. The fecal contamination may also be attributed to the high existence of water activity (fluid) in the fish, following contamination of the rivers/ponds where the mackerel fish were obtained. The results of the present study agree with previous findings of Lambu et al., (25) and Brooks (19), who separately reported the presence of *E. coli* in foods sold in the metropolis of Kano and Calabar, respectively. *Staphylococcus aureus* and *Streptococcus* spp were also among the most frequent pathogens present, and this finding is in line with the report of Ire and Imuh (26), who reported the presence of these isolates in ready-to-eat food in Port-Harcourt, Nigeria. The presence of these pathogens in food may be linked to the untidiness and dusty vending locations, hand contact during processing and vending, drops of saliva from both the vendor and customer's mouths, contact with skin and superficial wounds, as well as cross contamination after roasting. While, some of the isolates are beneficial, their continuous and high concentration when ingested have been implicated in food poisoning, as well as foodborne diseases (27). Hence, their presence in roadside grilled foods is a subject of concern, thereby posing a serious health risk to ignorant consumers.

In addition, *Bacillus subtilis* was also isolated, predominately from Marian and Highway samples. The presence of *Bacillus* spp in the grilled foods could be attributed to the fact that it is regarded as ubiquitous lipase-producing bacteria related to pathogenicity, with high presence in water, soil, and the environment. The spores producing nature of the *Bacillus* spp, makes them extremely resilient to the high temperature of roasting, heating and ultraviolet radiation (4). Their presence in ready-to-eat soup in Calabar metropolis has been recently reported by Ekpenyong et al., (1). However, the presence of *E. coli*, *S. aureus, Streptococcus* spp and *B. subtilis* in roadside grilled foods indicates a possible health threat as these bacterial are disease-causing microbes, which have been linked to progress of foodborne infections (11).

In addition to the bacteria isolates, five different fungal pathogens (*Penicillium digitatum, Aspergillus flavus, Rhizopus stolonifer*, *Saccharomyces cerevisiae* and *Mucor* spp) were isolated from the samples and roasted unripe plantain with grilled mackerel fish from Marian route recorded the highest total fungi counts, with *P. digitatum, A. flavus* and *R. stolonifer* being the most prevalent. Our findings also revealed that the total fungal count was higher in the ripe plantain samples, compared to the unripe plantain samples. The reason for the pathogen variation may be attributed to the sugar content in the samples, as the ripe plantain tends to contain more sugar from the carbohydrate hydrolysis during the ripening process, which may in turn favor microbial growth. The results of our findings substantiate the reports of Ahmad et al., (10) and Oranusi and Braide (11), who reported the presence of these fungal pathogens in foods sold in the metropolis of Katsina State and Onitsha-Owerri southeast, Nigeria respectively. The presence of these fungi species in grilled foods is worrying, as most of these isolates have been

implicated in various diseases condition, ranging from food poison to cancer. For instance, mycotoxins of *Aspergillus* spp are known to be carcinogenic and have also been linked to various forms of human and animal infections, including respiratory, local and superficial fungal infections (17,28). The spore-producing nature of mold (*Aspergillus and Mucor spp*) makes them heat resistant to the high temperature of roasting, heating and ultraviolet radiation, thereby favoring their growth in grilled foods (29,30).

From the results of this study, the presence of the isolated pathogens is a public concern, as they have been implicated in food borne diseases, among other devastating illnesses. The most disturbing situation is the high loads of the isolated pathogens, mostly above the safe limit as reported by the ICMSF (18) and the high patronage these vendors receive on daily basis by the ever-growing populace of Calabar metropolis. Consequently, this condition is detrimental not only to the consumers of the roadside grilled foods, but also to the fragile Nigerian healthcare system, which may not be capable of combating any potential foodborne disease outbreaks as a result of continuous consumption of the roadside grilled foods, which has become a culture in the city of Calabar, among other cities in Nigeria.

There is therefore a need for public health guidelines and enactment of dietary hygiene practices regarding the preparation, handling and vending of roadside grilled foods. Also, roadside grilled food vendors should be properly educated, monitored and possibly licensed by the food regulatory agencies before serving the public, in order to safeguard the lives of the teaming consumers of grilled foods and that of the generation yet unborn.

Conclusion:

The results of this study showed that roadside grilled ripe or unripe plantain with mackerel fish sold at Marian, Etagbor and Murtala Mohammed high way in Calabar metropolis were contaminated with high bacterial and fungal count of $4.0x10^6$ CFU/g and $2.8x10^4$ CFU/g respectively, with ripe samples sold along Marian routes recording the highest microbial loads. Poor food handling and unhealthy sanitary practices by the vendors are among the possible causes of pathogens contamination of these roadside grilled foods. Unfortunately, these pathogens have been implicated in foodborne diseases and other devastating health conditions. Hence, public health regulation and enactment of dietary hygiene practices regarding the preparation, handling and vending of roadside grilled foods are among the measures capable of reducing the microbial contamination of grilled foods.

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Contributions of authors:

MAA and ANI conceptualized and designed the study; MAA, ANI, EDE, EOO and JEM were involved in acquisition, analysis, and interpretation of data; and MAA, ANI, EDE, OOA, JEM and EOO were involved in manuscript drafting, revision, and provision of resource materials. All authors read and approved the submitted for publication.

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Conflict of interests:

Authors declare no conflict of interest

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