ISSN 1595-689X

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY

JAN 2024; VOLUME 25; NUMBER 1; PAGE 01-119

Official Publication of the African Society for Clinical Microbiology

ISSN 1595-689X

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY (AJCEM)

https://www.ajol.info/index.php/ajcem and https://www.afrjcem.org

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ISSN 1595-689X

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY (AJCEM)

https://www.ajol.info/index.php/ajcem and https://www.afrjcem.org

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AJCEM/2294. https://www.ajol.info/index.php/ajcem

Review Article **Open Access**

Ribonucleic acid extraction: A mini-review of standard methods

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Abstract:

Different techniques have been proposed for RNA extraction, many of which have found extensive use in biological research. The introduction of these methods has greatly improved molecular diagnostics, drug discovery, and numerous other research and clinical endeavors. In this review, the working principles of the most commonly used RNA extraction methods for research and clinical applications are discussed. Current automation efforts and the quest for more efficient and cost-effective methods are highlighted.

Keywords: RNA, extraction, RNases, mini-review

Received Sept 1, 2023; Revised Oct 8, 2023; Accepted Oct 9, 2023

Extraction de l'acide ribonucléique: une mini-revue des méthodes standards

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Résumé:

Différentes techniques ont été proposées pour l'extraction de l'ARN, dont beaucoup ont été largement utilisées dans la recherche biologique. L'introduction de ces méthodes a considérablement amélioré le diagnostic moléculaire, la découverte de médicaments et de nombreux autres efforts de recherche et cliniques. Dans cette revue, les principes de fonctionnement des méthodes d'extraction d'ARN les plus couramment utilisées pour la recherche et les applications cliniques sont discutés. Les efforts d'automatisation actuels et la recherche de méthodes plus efficaces et plus rentables sont mis en évidence.

Mots clés: ARN, extraction, RNases, mini-revue

Introduction:

Initially, RNA was simply viewed as a short-lived genetic intermediate between DNA and proteins (1). Messenger RNAs (mRNAs) indeed perform this function. However, other types of RNA were, soon after, characterized as components of the cellular protein synthesis machinery. Ribosomal RNAs (rRNAs) are structural and functional components of ribosomes, while transfer RNAs (tRNAs) are adapters for amino acid delivery to ribosomes. Thomas Cech's group (2) was the first to observe RNA catalysis in the protein-independent splicing of Tetrahymena 26S rRNA. Numerous ribozymes, notably group I and II introns and ribonuclease P, have since been identified.

Many other functional classes of noncoding RNAs (ncRNAs) have been discovered. Small noncoding RNAs (sncRNAs) such as microRNAs (miRNAs), small interfering RNAs (si RNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), Piwi-interacting RNAs (piRNAs), tRNA-derived fragments (tRFs), tRNA halves (tiRNAs), and small rDNA-derived RNAs (srRNAs), perform a host of regulatory functions, including RNA silencing, DNA methylation, histone modification, posttranscriptional RNA modification, and posttranscriptional silencing of repeat-derived transcripts (3,4). Long noncoding RNAs (lncRNAs) are distinguished from sncRNAs by their length (> 200 nucleotides) and function in chromatin remodeling, transcriptional regulation, and RNA mo-

dification and degradation (5).

There has been extensive research into RNA biogenesis, structure, function, interactions, posttranscriptional modification, localization, trafficking, turnover, pathogenesis, and prophylaxes, with many consequential discoveries. Most of the methods in molecular biology that enable these investigations require an initial extraction of RNA from tissues, cells, and other biological materials. RNA extraction typically involves obtaining a crude mixture of RNA and other biomolecules from biological samples, removing unwanted macromolecules and chemical contaminants, and concentrating so as to obtain high-quality RNA in concentrations suitable for various downstream applications.

When trying to capture the state of the transcriptome at the time of sample collection, the integrity of RNA molecules is of utmost importance. The success of gene expression analyses, including RT-qPCR and RNA-Seq, is greatly affected by RNA quality (6,7). Other RNA-centric assays are similarly affected (8, 9). Many methods have been proposed for assessing RNA yield, purity, and integrity (10-13). Given the importance of RNA quality, researchers usually adopt RNA quality thresholds for different sample types and workflows on the basis of previous performance studies and optimizations.

RNA extraction is complicated by the molecule's short half-life and susceptibility to ribonuclease (RNase) degradation. Unlike DNA, RNA nucleosides contain a ribose sugar with the typical 2'-hydroxyl group, making RNA more susceptible to spontaneous hydrolysis. RNases are a considerable problem in RNA extraction and manipulation. *In vivo*, RNases contribute to RNA biogenesis and homeostasis and serve as the body's first line of defense against non-self RNA, including RNA viruses (14). However, once released from cells and protection of accessory proteins, RNA is exposed to RNases simultaneously released from the sample. Additionally, due to their high stability, RNases accumulate in the environment and are readily introduced into *in vitro* preparations, contributing to RNA degradation.

There are multiple proven strategies for minimizing RNA degradation. Lowering the temperature slows down self-hydrolysis and RNase activity, reducing RNA degradation. Stable low temperatures are maintained by flash-freezing biological samples in liquid nitrogen immediately after harvesting, prechilling extraction buffers, conducting extraction and downstream manipulations on ice, and using refrigerated centrifuges where applicable. RNA in biological samples stored at -80°C remains stable for extended periods (15,16).

Additionally, following extraction, RNA samples are aliquoted before freezing at -80°C to avoid freeze-thawing. As an alternative to flash-freezing, biological samples are homogenized in chaotropic-based cell lysis solutions or permeated with RNA stabilization solutions before storage. RNA degradation is further prevented by: maintaining an RNase-free workspace using RNase-free filter pipette tips, tubes and reagents; using RNase-decontaminated equipment; and wearing appropriate personal protective equipment (PPE) to avoid introducing RNases from skin and saliva.

Methods of RNA extraction:

Phenol-chloroform extraction

Ingle and Burns (17) reported the effectiveness of phenol:chloroform:isoamyl alcohol (PCI; 25:24:1; v/v) extraction of total nucleic acids from aqueous lysed biological samples. When an emulsion is formed by mixing an aqueous biological lysate with phenol and chloroform (both organic solvents) and allowed to settle, proteins contained in the lysate are permanently denatured and preferentially displaced from the aqueous lysate into the organic solvents, leaving polar nucleic acids in the aqueous phase. When centrifuged, the heavier organic phase containing proteins separates to the bottom of the tube, displacing the aqueous phase containing nucleic acids to the top, where it can easily be aspirated for subsequent alcohol precipitation.

Chomczynski and Sacchi's (18) modified method that uses an acid-guanidinium thiocyanate-phenol-chloroform mixture, has become widely used for isolating total RNA from biological samples of different sources (19-21). The increased acidity causes DNA to separate into the lower organic phase and interphase, leaving RNA in the aqueous phase. The DNA can be separately recovered from the organic phase by precipitation with ethanol or isopropanol. Additionally, guanidinium isothiocyanate, a chaotropic salt, lyses cells and inactivates RNases, removing the need for prior lysis. The method provides a pure preparation of undegraded total RNA in high yield and can be completed within 4 hours.

Great care is required when aspirating the aqueous phase, as disturbing the interphase or organic phase will result in organic and DNA contamination. In addition to careful aspiration, different strategies have been proposed to reduce contamination. For example, phase lock gels eliminate interphase protein and DNA contamination, ensuring faster results with improved aqueous phase recoveries (22). More commonly, methods employ additional chloroform extractions of the aqueous phase and additional ethanol washes of the alcohol precipitate (23,24).

Column-based extraction:

Chaotropic agents disrupt the hydrogen bonding network between water molecules, interfering with the noncovalent forces required for macromolecular structure and supramolecular assembly. Through this means, chaotropes permanently denature proteins, dissolve lipids, and lyse cells. Conversely, chaotropic agents destroy the native hydration shells that maintain the solubility of nucleic acids, making them less soluble in water. Eliminating hydration shells permits positively charged ions to form salt bridges between the negatively charged phosphate backbone of nucleic acids and negatively charged hydroxyl groups on the surface of solid matrices such as silica and cellulose.

Vogelstein and Gillespie (25) first reported the near-quantitative binding of DNA to flint and borosilicate glass particles and silica powder under chaotropic salt concentrations and efficient DNA recovery in any convenient buffer. Silica binding and recovery of nucleic acids have since been incorporated into many centrifugation column-based workflows as one of the most common methods for nucleic acid extraction. Plant-derived cellulose binding materials have also been described for nucleic acid extraction (26,27). Solid-phase RNA extraction is especially appealing because it avoids the dangerous organic solvents used in liquid-liquid methods.

Spin-column workflows typically start with a lysis step in which suitable detergents, lytic enzymes and chaotropes lyse biological samples. Next, using centrifugation or vacuum suction, the lysate is passed through the binding material under chaotropic conditions. The bound nucleic acids are washed to remove unwanted biomolecules and chemical contaminants. For pure RNA, a single DNase treatment can be performed between multiple washes. Additionally, pH, ionic strength, and alcohol-to-sample ratios are adjusted to retain or exclude different types and sizes of nucleic acids (28-30). Finally, purified RNA is eluted from the binding material using low-ionic buffers.

Magnetic beads extraction:

Professor John Ugelstad of the Norwegian Institute of Technology revolutionized the separation of biological materials when he succeeded in producing monosized monodispersed magnetizable microspheres under laboratory conditions (31-34). Magnetic beads, usually magnetite (Fe_3O_4) particles coated according to the application, exhibit super paramagnetism, which means that they are strongly reversibly magnetized in the presence of an external magnetic field. This allows magnetic beads to be separated in suspension, along with any molecules that are bound to their coating. Since the magnetism is completely reversible, once the magnetic field is removed, magnetic beads can be easily resuspended in binding, wash, or elusion buffers.

A wide range of surface coatings are available for magnetic beads to suit different applications. Negatively charged carboxyl and silica coatings, both of which reversibly bind nucleic acids, are widely used for nonspecific nucleic acid purification (35-37). However, silica-coated magnetic beads are favored when sample amounts are low. By varying pH, ionic strength, alcohol concentration, and other buffer conditions, magnetic beads can be made to preferentially retain or exclude different types and sizes of nucleic acids. To obtain pure RNA, a single DNase treatment is often performed between washes. Along with their use in sample lysis and RNA binding, detergents, chaotropes, and RNase inhibitors prevent RNA degradation during extraction.

Oligo(dT) coating covalently hybridizes with the poly(A) tail present in most eukaryotic mRNA, enabling mRNA isolation from biological samples (38,39). RNA containing known sequences are also enriched or depleted from RNA pools in this way (40,41). Alternatively, streptavidin- and other avidin-coated beads form a very strong, highly specific, reversible bond with biotin, enabling the isolation of biotin-labeled targets, including RNA and RNA-DNA hybrids (40,42,43).

Conclusion:

Since the first organic isolation of total nucleic acid by Ingle and Burns (17) and the subsequent total RNA extraction by Chomczynski and Sacchi (18), many techniques for RNA purification have been developed. From column-based extraction of total RNA, small RNA, and RNA clean-up to bead-based methods, RNA extraction has played a fundamental role in various scientific and medical applications. Most biological endeavors, including gene expression analyses, transcriptomics, drug development, functional genomics, molecular diagnostics, cancer research, developmental biology, environmental monitoring, and forensics, are predicated on our ability to obtain good-quality RNA from different sources.

Not much can be done to remedy degraded RNA. However, low-yield and contaminated samples can be concentrated and cleaned up with the same methods used for extracting RNA from crude samples. Furthermore, automated RNA extraction systems have been developed due to modern advancements in engineering. Automation is appealing because it can increase throughput, reproducibility, quality, safety, and labor savings. Solid-phase

reversible immobilization (SPRI) bead-based RNA extraction is ideal for automation due to its yield, reproducibility, and ease of manipulation with magnetic fields. However, numerous organic and column-based liquid handling platforms for RNA extraction are also available. Existing technologies are constantly being improved, but additional discoveries may be required to further lower costs, improve throughput, enable single-cell extraction, and much more.

Contributions of author:

The sole author, OIA, approves the manuscript and is accountable for its content.

Source of funding:

Author received no external funding.

Conflict of interest:

The author declares that the review was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jan 2024; Vol.25 No.1
AJCEM/2306. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Falciparum malaria is associated with risk markers of type 2 diabetes mellitus in individuals with or without COVID-19 exposure

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Abstract:

Background: Scientific information on the impact of malaria on the risk of developing type 2 diabetes mellitus (T2DM) after recovery from the coronavirus disease 2019 (COVID-19) is limited in the Ghanaian context. The purpose of this study was to examine the association between selected risk markers of T2DM in *falciparum* malaria patients post-COVID-19 or not at a tertiary hospital in Ghana.
Methodology: This was a descriptive cross-sectional comparative study of 38-recovered COVID-19 adult participants with malaria and 40 unexposed COVID-19 adults with malaria at the Tamale Teaching Hospital, Ghana. Demographic, anthropometric and levels of glucose, insulin, C-reactive protein and lipid profiles were measured in the two groups of participants under fasting conditions. Parasitaemia was assessed microscopically but insulin resistance and beta-cell function were assessed by the homeostatic model.
Results: The COVID-19 exposed participants were older (p=0.035) with lower parasitaemia (p=0.025) but higher mean levels of insulin, insulin resistance, and beta-cell function compared with their unexposed counterparts (p<0.05). Parasitaemia correlated positively with a number of the measured indices of diabetogenic risk markers in the COVID-19 exposed group only, and predicted (Adjusted R^2=0.751; p=0.031) by beta-cell function, C-reactive protein and triglycerides with the model explaining about 75% of the observed variation. Parasitaemia could only be predicted (Adjusted R^2=0.245; p=0.002) by C-reactive protein with the model explaining just about a quarter of the observed variation in the COVID-19 unexposed group. Insulin resistance and sub-optimal beta-cell function were detected in both groups of participants.
Conclusion: *Falciparum* malaria is associated with risk markers for development of T2DM irrespective of COVID-19 exposure. Insulin resistance, inflammation and sub-optimal beta-cell secretory function may drive the risk. The observed diabetogenic risk is higher in the recovered COVID-19 participants.

Keywords: insulin resistance, *falciparum* malaria, type 2 diabetes mellitus, inflammation, COVID-19

Received Oct 19, 2023; Revised Dec 18, 2023; Accepted Dec 20, 2023

Le paludisme à *falciparum* est associé à des marqueurs de risque de diabète sucré de type 2 chez les individus avec ou sans exposition au COVID-19

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Résumé:

Contexte: Les informations scientifiques sur l'impact du paludisme sur le risque de développer un diabète sucré de type 2 (DT2) après la guérison de la maladie à coronavirus 2019 (COVID-19) sont limitées dans le contexte ghanéen. Le but de cette étude était d'examiner l'association entre certains marqueurs de risque de DT2 chez les patients atteints de paludisme à falciparum après le COVID-19 ou non dans un hôpital tertiaire du Ghana.
Méthodologie: Il s'agissait d'une étude comparative transversale descriptive portant sur 38 participants adultes atteints de COVID-19 guéris et atteints de paludisme et 40 adultes COVID-19 non exposés atteints de paludisme à l'hôpital universitaire de Tamale, au Ghana. Les niveaux démographiques, anthropométriques et de glucose, d'insuline, de protéine c-réactive et le profil lipidique ont été mesurés dans les deux groupes de participants à jeun. La parasitaémie a été évaluée au microscope, mais la résistance à l'insuline et la fonction des cellules bêta ont été évaluées par le modèle homéostatique.
Résultats: Les participants exposés au COVID-19 étaient plus âgés (p=0,035) avec une parasitémie plus faible (p=0,025) mais des niveaux moyens d'insuline, de résistance à l'insuline et de fonction des cellules bêta plus élevés que leurs homologues non exposés (p<0,05). La parasitaémie était corrélée positivement avec un certain nombre d'indices mesurés de marqueurs de risque diabétogène dans le groupe exposé au COVID-19 uniquement et prédite (R^2 ajusté=0,751; p=0,031) par la fonction des cellules bêta, la protéine C-réactive et les triglycérides avec le modèle expliquant environ 75% de la variation observée. La parasitaémie ne pouvait être prédite (R^2 ajusté=0,245; p=0,002) que par la protéine C-réactive, le modèle expliquant à peine environ un quart de la variation observée dans le groupe non exposé au COVID-19. Une résistance à l'insuline et une fonction sous-optimale des cellules bêta ont été détectées dans les deux groupes de participants.
Conclusion: Le paludisme à *falciparum* est associé à des marqueurs de risque de développement du DT2, quelle que soit l'exposition au COVID-19. La résistance à l'insuline, l'inflammation et la fonction sécrétoire sous-optimale des cellules bêta peuvent entraîner ce risque. Le risque diabétogène observé est plus élevé chez les participants guéris du COVID-19.

Mots-clés: Résistance à l'insuline, paludisme à *falciparum*, diabète sucré de type 2, inflammation, COVID-19

Introduction:

The coronavirus disease 2019 (COVID-19) which has caused a serious disruption in the global health system appears to be under control in a number of countries, especially, those in the sub-Saharan African region. In spite of the seeming success in curtailing the spread and eradication of COVID-19, notably, in sub-Saharan African countries, its potential long-term impact is yet to be unraveled. Developing countries in general continue to grapple with the coexistence of communicable and non-communicable disease conditions.

Generally, non-communicable diseases are chronic in nature whilst communicable ones, though acute, can have chronic sequalae depending on the specific agent of interest. Several evidence abound through comprehensive reviews, to demonstrate the susceptibility of diabetes patients to several infectious agents including parasites, viruses and bacteria (1-4). The role of such infectious agents in the pathogenesis of diabetes has been acknowledged (1,5).

Type 2 diabetes mellitus (T2DM) is a chronic metabolic non-communicable disease associated with dysregulation of those aspects of carbohydrate and lipid metabolism mediated by insulin. The condition is strongly promoted by obesity, westernized diet and sedentary lifestyle, which together, affect negatively, the biological role of insulin. Insulin resistance and beta-cell dysfunction have been implicated in the development of T2DM. Insulin resistance connotes diminished responsiveness of cells to insulin action.

Insulin is supposed to facilitate glucose uptake into cells for breakdown for energy generation or synthesis of glycogen and other macromolecules for storage. When cells develop resistance to these actions of insulin, glucose accumulation in blood is favoured. Insulin resistance can be caused by obesity, infection and several other factors that cause inflammation. Indeed, T2DM is known to develop through chronic inflammatory mechanisms suggesting that any inflammation-induced condition could have a probable link to T2DM.

COVID-19 is considered generally as an acute condition of inflammatory nature although its chronic potential as well as the severity of the disease cannot be overlooked

(6,7). Irrespective of the nature and severity of infection, inflammation is critical for driving the pathogenesis especially in cases of reinfection (8). Malaria is a disease caused predominantly by *Plasmodium falciparum* in our setting and remains the number one cause of morbidity in Ghana with multiple episodes over the life course of an individual. In severe cases, the disease has high potential for multiple organ damage just like COVID-19. Although the disease is in most cases mild to moderate in severity, and is generally treatable, its prevalence is higher than COVID-19 in our setting. In spite of this, COVID-19 is given a higher priority, due probably to its easy transmissibility and associated high global mortality in the adult population.

Several studies have considered malaria-COVID-19 co-infection in terms of prevalence, clinical profile and disease outcomes where coinfected individuals seem to have an unfavorable prognosis compared to those with only COVID-19 infection (9-11). Other studies have reported associations between diabetes mellitus and infections (1-5). COVID-19 for instance, is thought to have a bidirectional relationship with diabetes whereby the infection promotes the development of diabetes in non-diabetic individuals and worsens glycaemic control in patients with diabetes (12).

However, not much has been done to evaluate the probable effect of malaria on risk markers for T2DM development in individuals who have recovered from COVID-19 compared with their counterparts without COVID-19 exposure. This information is important for appropriate preventive measures to address the probable long-term impact of COVID-19 in malaria-endemic regions of the globe as far as diabetes development is concerned. Therefore, the current study was designed to examine the effect of malaria on selected markers of T2DM risk in COVD-19 exposed individuals compared with their non-exposed counterparts at a tertiary facility where malaria treatment is uncommon.

Materials and method:

Study site:

The study was conducted at Tamale, in Ghana's Northern Region. The laboratory evaluation was done at the Public Health Reference Laboratory (PHRL) of the Tamale Teaching Hospital (TTH). TTH is a tertiary health facility serving as the major referral hospital for the Upper East, Upper West, Oti, North-East and Savanna regions and parts of the Bono East and Brong-Ahafo regions.

In Ghana, malaria is commonly treated at the primary and secondary health facilities but not the tertiary health facilities. The PHRL of the TTH is accredited by the International Organization for Standardization (ISO: 9001) and currently serves as the national reference laboratory for bacterial meningitis in Ghana. PHRL is currently the major testing site for COVID-19 for the five Northern Regions of Ghana.

The Northern Regions have a total area of 26,524 square kilometres and a population of 2,320,939, with Tamale metropolis having a population of 374,744 and a total area of 454 square kilometres, according to the 2021 Population and Housing Census (13). Agriculture accounts for more than 85% of all economic activities in the region. Savanna and grassland are the main types of vegetation, with baobab trees scattered throughout. The area has a generally dry climate with just one rainy season that lasts from May to October. The range of yearly rainfall observed is between 750 mm and 1050 mm. High temperatures occur around the end of the dry season, which begins in November and run through to March or April. The Northern Region is bounded on the north by the North-East Region, on the east by the international boundary separating Ghana and Togo, on the south by the Oti Region, and on the west by the Savannah Region. The capital of the Northern Region is Tamale.

Study design and participants:

The study was a descriptive cross-sectional comparative design conducted among malaria patients within the Tamale metropolis who had recovered from confirmed COVID-19 in comparison with their counterpart without any history of COVID-19. The COVID-19 exposed participants had recovered completely from the disease at least six months prior infection with the *Plasmodium* parasite. On the other hand, the comparative group had malaria but no history of COVID-19.

Data from the PHRL suggest a total of 9,658 suspected cases of COVID-19 in the Tamale metropolis but only 561 tested positive in 2021 resulting in a positivity rate of 5.8%. With a metropolitan population of 374,744 according to the 2021 Population and Housing Census of the Ghana Statistical Service (13), the prevalence of COVID-19 will be 0.15%. Although malaria is endemic in the region, patients with malaria are rarely managed at the TTH facility because of its tertiary care role in the health system.

In this study, only 78 individuals could be confirmed for malaria after inviting 280 individuals to participate in the study (Fig 1) through a simple random process over a 6-month period (February – July, 2023). Being a preliminary study, the flat

'rule of thumb' principle was employed, where 35 participants per study group was anticipated.

Sample and data collection:

Designed questionnaires were used for the collection of socio-demographic information and clinical data of participants. Specific information included age, history of COVID-19 exposure and other health conditions necessary for the assessment of the inclusion and exclusion criteria.

Ten milliliters of venous blood were obtained from each participant through a routine venipuncture technique under fasting conditions. The blood was aliquoted appropriately into serum separator tubes (4 ml) and fluoride tubes (4 mls) for the respective preparation of serum and plasma for measurement of indices. The remaining 2 ml whole blood was then used for the preparation of thick and thin films for malaria microscopy.

Malaria parasite, blood pressure and anthropometric measurements:

Malaria parasitaemia was determined microscopically using a standard thick blood film under oil immersion procedure by an experienced microscopist. Thin blood film procedure was used for species identification. COVID-19 exposure was determined by real-time polymerase chain reaction and serological tests protocols approved at the time by the Ghana Health Service in line with acceptable World Health Organisation protocol for COVID-19 diagnosis and surveillance.

Blood pressure was measured by an experienced nurse with a standard mercury sphygmomanometer on the right arm of participants in sitting position after resting for at least five minutes. The average of three measurements at 5 minutes apart per measurement was recorded as the blood pressure for the participant. Meanwhile, weight was measured to the nearest 0.1 kg with height to the nearest 0.1 cm. Body mass index was computed as the ratio of weight in kilogramme to the square of the height in metre (kg/m^2). Weight and height were measured in light clothing without footwear.

Waist circumference was measured in centimetres with an inflexible tape measure at the midpoint between the lower margin of the last rib and the top of the iliac crest (14). In terms of hip circumference, it was measured around the widest portion of the buttocks. Waist-to-hip ratio was then computed by dividing waist circumference by the hip circumference. Waist-to-height ratio was computed by dividing the waist by the height.

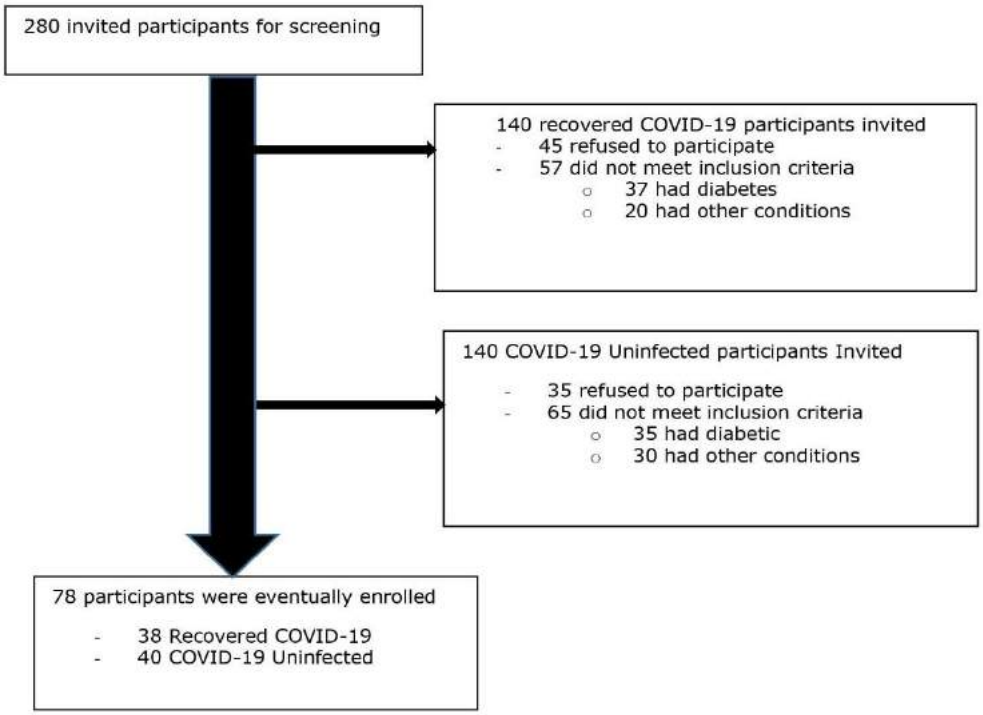

Fig 1: Flow chart for recruitment of participants

Estimation of fasting laboratory indices:

All measurements were done after samples and reagents were brought to room temperature. Blood glucose was measured by the glucose-oxidase method using a standard procedure with the Mindray BS240 automated chemistry analyser (Mindray Diagnostics, Nanshan Shenzhen, China). Additionally, lipid profile was measured by a standard method with the same autoanalyser.

Serum insulin level was determined by a commercial immunoturbidimetric test kit (Kamiya Biomedicals Company, K-ASSAY Seattle, USA). The assay is based on the principle that immune complexes formed in solution between sample insulin and human insulin-specific antibody coated on latex particles, scatter light proportional to insulin concentration in samples. In this specific case, reagents and samples were brought to room temperature. Exactly 16 µL of serum was aspirated into 175 µL of reagent 1 (buffer reagent) and 65 µL of reagent 2 (Latex suspension) in a multi-point end point platform for measurement at 578 nm main and 800 nm sub reactions in the programmed autoanalyser. The concentration of insulin in sample was determined from a calibration curve prepared by plotting absorbance against standard concentration of insulin in accordance with instructions from the kits manufacturer. Insulin resistance was then calculated by the homeostatic model assessment formulae developed by Matthews et al., (15) for insulin resistance, HOMA-IR=(glucose×insulin)/22.5 and beta-cell function, HOMA-B=20 × fasting insulin (µIU/ml)/fasting glucose (mmol/ml) − 3.5 (15).

Ethical approval:

The study was approved by the Tamale Teaching Hospital Ethical Review Committee (TTHERC/24/02/23/01). All protocols followed were in accordance with the ethical requirements of the Ghana Health Service and the World Medical Association declaration of Helsinki (16). A written informed consent was obtained from each study participant.

Data analysis:

The Statistical Package for the Social Sciences (SPSS Inc., Chicago, USA) version 17.0 software was used for the data analysis. Data were log transformed for improved normality of distribution and presented as mean ± standard deviation. Mean levels of indices between COVID-19 exposed and non-exposed groups were compared with independent sample *t*-test. Bivariate correlation was used to examine linear relationship among measured indices in each study group followed by multiple stepwise linear regression analyses to identify predictors of indices of interest. In all analyses, $p<0.05$ was applied for the determination of statistical significance.

Results:

The study involved a total of 78 malaria patients made up of 38 COVID-19 exposed and 40 COVID-19 unexposed individuals. The COVID-19 exposed malaria patients were confirmed to have recovered completely from COVID-19 by the approved protocol at least six months before the commencement of the study while the unexposed group had no history of COVID-19 infection. Both groups had no history of diabetes or other health condi tions known to influence any of the measured indices. The COVID-19 exposed participants were older ($p=0.035$) with lower parasitaemia ($p=0.025$), but higher mean levels of HOMAIR ($p=0.049$), total cholesterol ($p=0.042$) and triglycerides ($p=0.001$) compared with their unexposed counterparts (Table 1).

Pearson bivariate correlational test revealed that parasitaemia correlated positively ($p<0.05$) with insulin, insulin resistance, beta-cell function, fasting blood glucose, C-reactive protein, age, triglyceride and blood pressure in malaria patients with prior exposure to COVID-19. However, in participants without prior COVID-19 exposure, parasitaemia correlated positively ($r=0.525$; $p=0.001$) with C-reactive protein only (Table 2).

Low-density lipoprotein cholesterol (LDLc) correlated ($p<0.05$) positively with insulin resistance, beta-cell function and fasting blood glucose in the COVID-19 unexposed participants only with no such observations in the group with prior exposure to COVID-19. Generally, the pattern of correlation was positive in both groups but the strength of the correlation differed between the two groups and the specific parameters that correlated also differed between the two groups for a number of the measured indices (Table 2).

Table 1: Comparison of mean levels of indices between COVID-19 exposed and COVID-19 unexposed patients with *falciparum* malaria in Tamale Teaching Hospital, Ghana

Parameter	COVID-19 exposed (n = 38)	COVID-19 unexposed (n = 40)	*t*	*p* value
Age (years)	35.23 ± 1.42	29.28 ± 1.40	2.156	0.035*
Parasites (μL^{-1})	112.00 ± 2.00	168.00 ± 2.00	-2.304	0.025*
Insulin (mIU/L)	26.17 ± 2.02	19.51 ± 1.74	1.868	0.066
HOMAIR	5.39 ± 2.40	3.70 ± 1.86	2.006	0.049*
HOMAB (%)	108.86 ± 1.78	91.20 ± 1.70	1.277	0.206
FBG (mmol/L)	4.63 ± 1.24	4.27 ± 1.13	1.967	0.054
TCHOL (mmol/L)	3.18 ± 1.42	2.57 ± 1.57	2.077	0.042*
LDL (mmol/L)	2.78 ± 0.81	3.40 ± 0.96	1.013	0.315
HDL (mmol/L)	1.48 ± 1.48	1.35 ± 1.49	0.96	0.341
CRP	1.39 ± 2.70	0.95 ± 1.70	1.996	0.05
Triglyceride (mmol/L)	1.40 ± 1.45	0.96 ± 1.54	3.645	0.001*
Body mass index (kg/m^2)	24.30 ± 1.08	23.83 ± 1.19	0.544	0.588
Waist-to-hip ratio	0.90 ± 1.02	0.88 ± 1.06	1.621	0.11
Waist-to-height ratio	0.45 ± 1.02	0.44 ± 1.19	0.612	0.543
Waist circumference (cm)	74.79 ± 1.13	71.34 ± 1.24	1.031	0.307
Systolic BP (mmHg)	126.14 ± 1.08	126.76 ± 1.12	-0.196	0.845
Diastolic BP (mmHg)	78.77 ± 1.12	80.34 ± 1.15	-0.602	0.549

* = statistically significant at $p<0.05$; LDL=low-density lipoprotein cholesterol; HDL=high-density lipoprotein cholesterol; BP = blood pressure; CRP = C-reactive protein; TCHOL = total cholesterol; FBG = fasting blood glucose: HOMAIR = homeostatic model of insulin resistance; HOMAB = homeostatic mode of beta cell function

In subsequent stepwise multiple linear regression analyses controlling for appropriate confounders, parasitaemia was predicted (Adjusted R^2=0.751; p=0.031) jointly by the levels of beta-cell function, C-reactive protein and triglycerides, with the model explaining about 75% of the observed variation in participants with prior exposure to COVID-19 (Table 3). However, in the control COVID-19 unexposed group, parasitaemia could only be predicted (Adjusted R^2= 0.245; p=0.002) by C-reactive protein with the model explaining just about a quarter of the observed variation of parasitaemia in that study group (Table 4).

Interestingly, in both study groups, variation in insulin level could be fully explained by fasting glucose and insulin resistance levels (Adjusted R^2 = 1, p <0.001 for both groups, Tables 3 & 4). Similarly, levels of fasting blood glucose, insulin resistance and beta cell function could generally be predicted to perfection or almost perfection by the same set of independent variables in both study groups (Adjusted R^2 range = 0.911-1.0, p <0.001, Tables 3 & 4). Total cholesterol level was however, predicted by age (Adjusted R^2 = 0.439, p <0.001, Table 3) only in the COVID-19 exposed group with the model being able to explain just about 40% of the observed variation. Systolic and diastolic blood pressures partially predicted each other to a similar extent in both groups but the final models included different parameters in each of the study groups.

Discussion:

Diabetes mellitus is postulated to exert a bidirectional relationship with COVID-19. This implies that DM patients have poorer COVID-19 outcome on the one hand and the risk of developing DM is increased by COVID-19 at the other end. This bidirectional relationship poses a challenge to elucidation of the role of other infectious agents in the development of DM in individuals who have recovered from COVID-19. However, in malaria-endemic regions of the globe, malaria remains a major threat to health before, during and after the COVID-19 pandemic (17). This comparative cross-sectional observational study sought to examine the probable role of malaria in the evolution of T2DM post COVID-19 by comparing levels of selected markers of insulin resistance, beta-cell function,

Table 2: Bivariate correlations of measured parameters in COVID-19 exposed and COVID-19 unexposed participants

Parameter	COVID-19 exposed participants		Parameter	COVID-19 unexposed participants	
	Correlation coefficient	***p* value**		**Correlation coefficient**	***p* value**
Parasitaemia & Insulin	0.81	<0.001	Parasitaemia & C- reactive protein	0.525	0.001
Parasitaemia & Insulin resistance	0.781	<0.001	Insulin & Age	0.503	002
Parasitaemia & beta cell function	0.817	<0.001	Insulin & fasting blood glucose	0.489	003
Parasitaemia fasting glucose & blood	0.543	0.002	Insulin & Diastolic blood pressure	0.476	0.003
Parasitaemia & Age	0.437	0.003	Insulin resistance & Age	0.498	0.002
Parasitaemia & Triglyceride	0.436	0.021	Insulin resistance & Fasting blood glucose	0.628	<0.001
Parasitaemia & Systolic blood pressure	0.433	0.021	Insulin resistance & LDL	0.49	0.002
Parasitaemia & Diastolic blood pressure	0.41	0.03	Insulin resistance & Diastolic blood pressure	0.488	0.003
Parasitaemia & C- reactive protein	0.772	<0.001	Beta cell function & Diastolic blood pressure	0.424	0.01
Insulin & Age	0.594	0.001	Beta cell function & LDL	0.431	0.009
Insulin & fasting blood glucose	0.771	<0.001	Beta cell function & Age	0.516	0.001
Insulin & Systolic blood pressure	0.42	0.026	Fasting blood glucose & LDL	0.368	0.027
Insulin & Diastolic blood pressure	0.475	0.011	Fasting blood glucose & Diastolic blood pressure	0.331	0.048
Insulin & C-reactive protein	0.705	<0.001			
Insulin resistance & Age	0.615	<0.001			
Insulin resistance & Fasting blood glucose	0.86	<0.001	Age & LDL	0.42	0.011
Insulin resistance & Systolic blood pressure	0.452	0.016	Age & Systolic blood pressure	0.363	0.029
Insulin resistance & Diastolic blood pressure	0.5	0.007	Age & Diastolic blood pressure	0.593	<0.001
Insulin resistance & C-reactive protein	0.698	<0.001	Total cholesterol & C-reactive protein	0.376	0.024
Beta cell function & Age	0.532	0.004	LDL & Diastolic blood pressure	0.472	0.009
Beta cell function & Fasting blood glucose	0.592	0.001			
Beta cell function & Diastolic blood pressure	0.411	0.03			
Beta cell function & C-reactive protein	0.681	<0.001			
Age & Fasting blood glucose	0.575	0.001			
Age & C-reactive protein	0.375	0.049			
Systolic blood pressure & C-reactive protein	0.452	0.016			

inflammation and lipid metabolism in malaria patients with prior exposure to COVID-19 to their counterparts without COVID-19 exposure in the Tamale metropolis of Ghana.

Our results show that, the COVID-19 exposed individuals were older with higher levels of insulin resistance, total cholesterol and triglycerides but lower level of parasitaemia than their counterparts without prior exposure to COVID-19. Although COVID-19 virtually affects all ages, available data points to a higher proportion of the affected in the adult age group (18,19). This is to be expected when viewed from the perspective of immunosenescense with the effectiveness of immunological responses to various challenges decreasing with age (20). Therefore, the higher age of COVID-19 exposed participants compared with their unexposed counterparts in the current study is consistent with findings of previous reports (18,19). It suggests that the younger COVID-19 unexposed group could mount a more effective immunological response needed for COVID-19 prevention.

Table 3: Multiple linear regression analyses for predictors of measured indices in COVID-19 exposed participants

Parameter	Predictor(s)	Unstandardized coefficients Beta (std error)	Standardized coefficients Beta	Adjusted R^2	*p*-value
Parasitaemia	Constant	1.618 (0.086)			
	HOMAB	0.606 (0.189)	0.45	0.621	<0.001
	CRP	0.309 (0.105)	0.4	0.703	0.013
	TG	0.494 (0.214)	0.249	0.751	0.031
Insulin	Constant	3.114 (0.000)			
	HOMAIR	1.000 (0.000)	1.251	0.974	<0.001
	FBG	-1.000 (0.000)	-0.307	1.000	<0.001
HOMAIR	Constant	-3.114 (0.000)			
	Insulin	1.000 (0.000)	0.799	0.974	<0.001
	FBG	1.000 (0.000)	0.245	1.000	<0.001
HOMAB	Constant	2.915 (0.006)			
	Insulin	1.033 (0.002)	1.27	0.934	<0.001
	FBG	-1.041 (0.006)	-0.393	1.000	<0.001
FBG	Constant	3.114 (0.000)			
	HOMAIR	1.000 (0.000)	4.078	0.727	<0.001
	Insulin	-1.000 (0.000)	-3.259	1.000	<0.001
TCHOL	Constant	-1.332 (0.534)			
	Age	0.757 (0.149)	0.725	0.439	<0.001
Systolic BP	Constant	2.937 (0.367)			
	DBP	0.464 (0.081)	0.673	0.551	<0.001
	WHR	1.188 (0.302)	0.366	0.67	0.005
Diastolic BP	Constant	-1.392 (0.853)			
	SBP	1.165 (0.175)	0.805	0.55	<0.001
	TCHOL	0.11 (0.038)	0.346	0.657	0.009

HOMAIR = homeostatic model of insulin resistance; HOMAB = homeostatic mode of beta cell function; FBG = fasting blood glucose; DBP = diastolic blood pressure; SBP = systolic blood pressure; TCHOL = total cholesterol; TG = triglyceride; CRP = C-reactive protein.

Table 4: Multiple linear regression analyses for predictors of measured indices in the COVID-19 unexposed participants

Dependent variable	Independent variable(s)	Unstandardized coefficients beta (std error)	Standardized coefficients beta	Adjusted R^2	*p* value
Parasitaemia	Constant	5.171 (0.099)			
	CRP	0.633 (0.185)	0.518	0.245	0.002
Insulin	Constant	3.114 (0.000)			
	HOMAIR	1.000 (0.000)	1.123	0.97	<0.001
	FBG	-1.000 (0.000)	-0.219	1.000	<0.001
HOMAIR	Constant	-3.114 (0.000)			
	Insulin	1.000 (0.000)	0.891	0.97	<0.001
	FBG	1.000 (0.000)	0.195	1.000	<0.001
HOMAB	Constant	3.085 (0.331)			
	Insulin	1.007 (0.057)	1.052	0.866	<0.001
	FBG	-1.075 (0.259)	-0.246	0.911	<0.001
FBS	Constant	3.114 (0.000)			
	HOMAIR	1.000 (0.000)	5.118	0.373	<0.001
	Insulin	1.000 (0.000)	-4.559	1.000	<0.001
Systolic BP	Constant	1.774 (0.349)			
	DBP	0.763 (0.086)	0.947	0.588	<0.001
	TCHOL	-0.071 (0.027)	-0.271	0.671	0.005
	HOMAB	-0.047 (0.023)	-0.218	0.702	0.048
Diastolic BP	Constant	-0.238 (0.503)			
	SBP	0.713 (0.127)	0.575	0.588	<0.001
	WC	0.467 (0.09)	0.703	0.745	<0.001
	BMI	-0.259 (0.108)	-0.331	0.779	0.023

HOMAIR = homeostatic model of insulin resistance; HOMAB = homeostatic mode of beta cell function; FBS = fasting blood sugar; DSB = diastolic blood pressure; SBP = systolic blood pressure; TCHOL = total cholesterol; BMI = body mass index; WC = waist circumference; CRP = C-reactive protein.

However, both groups of participants succumbed to the malarial disease, implying that, no group could mount an adequate immunological response to prevent the establishment of the disease in relation to malaria.

Indeed, parasitaemia correlated positively with almost every evaluated risk index of T2DM development in the COVID-19 exposed group as far as this study is concerned but in the COVID-19 unexposed group, only C-reactive protein correlated positively with parasitaemia. This observation points to the probable critical role of malaria in promoting risk of T2DM in individuals who have recovered from COVID-19 compared with their counterparts who did not get infected with the COVID-19 disease. This finding is further buttressed by the results of the stepwise multiple linear regression analyses that revealed C-reactive protein, triglycerides and beta-cell function as predictors for parasitaemia in the COVID-19 exposed group with the final model accounting for about 75% of the observed variation in parasitaemia. This is in sharp contrast to the linear regression analysis results of the COVID-19 unexposed group that showed only C-reactive protein as a predictor of parasitaemia with the model explaining just 24.5% of the observed variation in parasite levels. The higher number of predictors of parasitaemia coupled with the superior nature of the model in accounting for the variation in parasitaemia for the COVID-19 exposed group suggests that the risk of developing T2DM in this group is higher than in their unexposed counterparts.

Additionally, this observation points to a probable heightened future burden of T2DM in malaria-endemic regions of the globe because of the COVID-19 pandemic. This is because multiple bouts of malaria are highly possible in one's lifetime (21), especially for individuals residing in malaria-endemic regions of the globe (22). Therefore, with multiple episodes of malaria, the secretory function of the beta-cell can be unduly heightened, beyond sustainable level and result in eventual exhaustion and the consequent decline in insulin levels to favour hyperglycaemia as observed in diabetes (23,24).

Although direct evidence of beta-cell failure through exhaustion is non-existent in the current study, a critical examination of the HOMAB values points to some hypersecretory activity in the COVID-19 exposed group but a relatively reduced beta-cell secretory function for the COVID-19 unexposed group in spite of the statistically comparable HOMAB values of the two study groups. The relatively reduced secretory function of beta cells in the COVID-19 unexpo sed participants may suggest reduced beta-cell mass due probably to inflammation-induced apoptosis (25). This increases the risk of those participants to the development of T2DM in the future when metabolic demand on the beta cells increases through obesity, multiple bouts of malaria and other environmental factors. Thus, in both groups of participants, the risk of T2DM development in future through beta-cell failure will increase if metabolic load and bouts of malaria increase (23,24).

Indeed, in both groups of participants, insulin resistance was clearly established during malaria in support of earlier findings except that the degree of insulin resistance observed for COVID-19 exposed group was significantly higher than their COVID-19 unexposed counterparts (5,26). This observation of a clear establishment of insulin resistance in the current study further points in the direction of increased risk for future development of T2DM. A study on Sprague-Dawley rats to examine the nature of *Plasmodium berghei*-induced insulin resistance demonstrated that effective clearance of the infectious agent resulted in a reduced insulin resistance (27). However, in that study, a second episode of infection, at a relatively lower parasitaemia, induced insulin resistance comparable to the level of the first episode (27).

In humans, several episodes of malaria are possible in a lifetime, implying that at every episode of malaria, comparable level of insulin resistance could be established at a rather relatively reduced parasite level than the previous episode to a point that even at asymptomatic level of infection, insulin resistance could be present. This is a challenge because such individuals may remain undetected for treatment due to their milder symptoms and therefore continue to maintain their insulin resistant and low-grade inflammatory state for adequate duration to pave way for possible development of T2DM under favourable conditions such as increased metabolic demand caused by nutrients overload (23, 24).

Dyslipidaemias of various components of the lipid profile have been associated with T2DM development or its complications in a number of studies (28-30). The components of lipid profile include total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol and triglycerides, and abnormal levels of any of these components poses a risk to cardiovascular health (28-30). In the current study, dyslipidaemia was not detected in the participants, probably due to the relatively younger age compared to previous studies (28-30), coupled with the relatively small sample size. However, the total cholesterol and triglycerides components of the lipid profile in this study differed between the two groups; with the COVID-19 exposed group demonstrating significantly higher

levels compared to their unexposed counterparts although the levels fell within the normal range. The normal levels of the components of lipid profile could easily be considered as indicative of low risk to the development of T2DM. However, a recent large sample prospective longitudinal study in the Netherlands (31), has demonstrated that even in healthy individuals without metabolic syndrome, raised triglycerides levels within the normal range increased the risk of development of T2DM. Considering that, the participants in the current study appear younger, the observed normal lipid profile could still represent a critical risk to T2DM development in the future in the context of the findings by Szili-Torok et al., (31) because of the likelihood of increase in the triglyceride component of the lipid profile as one ages. Therefore, the older COVID-19 exposed group with higher triglycerides level appear more vulnerable to developing T2DM than their counterparts without prior exposure to COVID-19 in future if triglyceride levels rise within the normal range with time in line with the postulation of Szili-Torok et al (31). Above all, the association of total cholesterol with blood pressure in the study participants further supports this view of increased risk of lipid-driven future development of T2DM in the current study.

Our study is not without limitations. Firstly, being a cross-sectional study, causality could not be established. Secondly, the sample size appears relatively small making it difficult for undue generalization of findings. However, the current sample size is enough for statistical analysis and extrapolation of findings to populations that are very similar in characteristics to those of the current study under similar disease conditions, for a proof-of-concept study of this nature. Thus, our evidence of malaria association with increased diabetogenic risk in clinically exposed COVID-19 participants compared with their unexposed counterparts, is a worthy baseline information with implications for the evolution of T2DM in malaria-endemic regions of the globe.

Conclusion:

Falciparum malaria is associated with increased risk for development of T2DM irrespective of COVID-19 exposure through the association of inflammation with insulin resistance and beta-cell secretory function.

Acknowledgements:

The authors are very grateful to the study participants and staff of the Tamale Teaching Hospital whose support made this work possible.

Contributions of authors:

SA, LDK, FP, KD, ASB, PN, and JS contributed to conception, design and the conduct of the study; RA and EWW were responsible for sample selection, data collection and laboratory analysis; SA, LDK, FP, KD, ASB, PN, JS, EAB, RA and EWW were involved in data analysis and drafting of manuscript; SA, LDK, FP, KD, ASB, EAB, PN, and JS revised the manuscript for important intellectual contents. All authors read and approved the final manuscript.

Source of funding:

Authors received no external funding for the study

Conflict of interest:

No conflict of interest is declared

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Adejo et al. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (1): 17 - 27 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jan 2024; Vol.25 No.1
AJCEM/2303. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Prevalence of high-risk human papillomavirus genotypes among apparently healthy women with normal and abnormal cervical cytology in Kaduna State, Nigeria

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Abstract:

Background: About 99.7% of cervical dysplasia and cancer cases are caused by persistent genital high-risk human papillomavirus (hrHPV) infection. Most HPV infections are subclinical and self-limiting but may persists in about 5 to 10% of infected women, resulting in pre-cancerous lesions that can progress to invasive cancer years later. This study is aimed at detecting hrHPV among apparently healthy women of reproductive age in Kaduna State, thus providing more information for effective control of HPV and cervical cancer in Nigeria.
Methodology: Cervical smears were taken from 515 randomly selected apparently healthy women across selected secondary and tertiary facilities from 3 Local Government Areas (LGAs) in each Senatorial Zone of Kaduna State, Nigeria. Liquid-based cytology (LBC) technique was used to collect cervical smears and prepare smears for cytology study, while the remaining samples were stored at -80ºC for molecular studies. HPV DNA were extracted from the samples and amplified by convectional PCR using specific hrHPV (HPV 16,18,31 and 45) primer sets and a broad spectrum MY09/11 and GP5+/6+ primers for a wider range of HPV genotypes. Data were analysed using the Statistical Package for Social Sciences (SPSS) version 23.0 and relationship between prevalence of hrHPV and socio-demographic factors such as age and marital status were determined using Chi-square or Fisher Exact test with $p<0.05$ considered statistically significant.
Results: The prevalence of total HPV and hrHPV infections in the study population was 11.8% (61/515) and 9.3% (48/515) respectively. A total of 100 HPV genotypes were detected by PCR in the 61 positive smears, with 66 hrHPV types from 48 women, and 34 other HPV types from 13 women. The frequency of hrHPV genotypes detected was HPV 31 (5.8%, n=30), HPV 45 (4.1%, n=21), HPV 16 (1.7%, n=9), and HPV 18 (1.2%, n=6), with other HPV genotypes (6.6%, n=34). The frequency of cervical dysplasia was 6.4% (33/515), which was significantly associated with all HPV genotypes except HPV 16. Single HPV infection was seen in 31 (51.8%) women while multiple infections were seen in 30 (49.2%), with double infection in 21 (34.4%) and triple infections in 9 (14.7%).
Conclusion: The prevalence of hrHPV infection was high among women in Kaduna State, Nigeria. DNA-based screening for hrHPV genotypes and production of new vaccine that will protect against the predominant hrHPV genotypes are thus recommended for the prevention of cervical cancer in Nigeria, Africa and beyond.

Keywords: High-risk human papillomavirus; genotypes; cytology; cervical cancer

Received Oct 07, 2023; Revised Nov 22, 2023; Accepted Dec 07, 2023

Prévalence des génotypes du virus du papillome humain à haut risque chez les femmes apparemment en bonne santé présentant une cytologie cervicale normale et anormale dans l'État de Kaduna, au Nigeria

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Résumé:

Contexte: Environ 99,7% des cas de dysplasie cervicale et de cancer sont causés par une infection génitale persistante au papillomavirus humain à haut risque (hrHPV). La plupart des infections au VPH sont subcliniques

et spontanément résolutives, mais peuvent persister chez environ 5 à 10% des femmes infectées, entraînant des lésions précancéreuses pouvant évoluer vers un cancer invasif des années plus tard. L'étude vise à détecter le hrHPV chez les femmes apparemment en bonne santé et en âge de procréer dans l'État de Kaduna, fournissant ainsi plus d'informations pour un contrôle efficace du VPH et du cancer du col de l'utérus au Nigeria.
Méthodologie: Des frottis cervicaux ont été effectués sur 515 femmes apparemment en bonne santé sélectionnées au hasard dans des établissements secondaires et tertiaires sélectionnés de 3 zones de gouvernement local (LGA) dans chaque zone sénatoriale de l'État de Kaduna, au Nigeria. La technique de cytologie en milieu liquide (LBC) a été utilisée pour collecter des frottis cervicaux et préparer des frottis pour une étude cytologique, tandis que les échantillons restants ont été conservés à -80°C pour des études moléculaires. L'ADN du VPH a été extrait des échantillons et amplifié par PCR convectionnelle en utilisant des ensembles d'amorces spécifiques du hrHPV (HPV 16, 18, 31 et 45) et des amorces MY09/11 et GP5+/6+ à large spectre pour une gamme plus large de génotypes du VPH. Les données ont été analysées à l'aide du progiciel statistique pour les sciences sociales (SPSS) version 23.0 et la relation entre la prévalence du hrHPV et les facteurs sociodémographiques tels que l'âge et l'état civil ont été déterminées à l'aide du test du chi carré ou de Fisher Exact avec $p<0,05$ considéré comme statistiquement significatif.
Résultats: La prévalence des infections totales par le VPH et le hrHPV dans la population étudiée était respectivement de 11,8% (61/515) et 9,3% (48/515). Au total, 100 génotypes HPV ont été détectés par PCR dans les 61 frottis positifs, avec 66 types hrHPV provenant de 48 femmes et 34 autres types HPV provenant de 13 femmes. La fréquence des génotypes hrHPV détectés était HPV 31 (5,8%, n=30), HPV 45 (4,1%, n=21), HPV 16 (1,7%, n=9) et HPV 18 (1,2%, n=6), avec d'autres génotypes de VPH (6,6%, n=34). La fréquence de la dysplasie cervicale était de 6,4% (33/515), ce qui était significativement associé à tous les génotypes de VPH, à l'exception du VPH 16. Une infection unique au VPH a été observée chez 31 (51,8%) femmes, tandis que des infections multiples ont été observées 30 (49,2%), avec double infection chez 21 (34,4%) et triple infection chez 9 (14,7%).
Conclusion: La prévalence de l'infection par le hrHPV était élevée chez les femmes de l'État de Kaduna, au Nigeria. Le dépistage basé sur l'ADN des génotypes hrHPV et la production d'un nouveau vaccin qui protégera contre les génotypes hrHPV prédominants sont donc recommandés pour la prévention du cancer du col de l'utérus au Nigeria, en Afrique et au-delà.

Mots-clés: Papillomavirus humain à haut risque; génotypes; cytologie; cancer du col de l'utérus

Introduction:

It is estimated that viral infections contribute to 15–20% of all human cancers (1). Viruses are obligate parasites which encode proteins that reprogram host cell metabolism and the immune system. Infection by oncogenic viruses can promote different stages of carcinogenesis. Among many types of human papillomaviruses (HPV), around 15 are linked to cancer. Human papillomaviruses are members of the Papovaviridae and consist of almost 8000 bp long circular DNA molecules that are wrapped into a protein shell which is composed of two molecules, L1 and L2 (2).

Cervical dysplasia is caused by the high-risk human papillomavirus (hrHPV) and can develop into cancer at any age. However, follow up and treatment can help to prevent cancer will depend on age of the woman. Human papillomavirus is a common virus that is spread through sexual contact. Persistent infection by certain genotypes of hrHPV plays a crucial role in creating tumors. Human papillomaviruses are a diverse group of viruses that can infect numerous epithelial sites and cause a variety of epithelial lesions, including common warts, verrucous, laryngeal papillomas, and genital condylomata, depending on the HPV types (3).

Human papillomaviruses are found to be present in 99.7% of cervical cancer speci mens (4). Most sexually active and unvaccinated men and women get the virus at some point in their life (5). There are over 100 types of HPV which causes of genital warts and may resolve without treatment in immuno-competent individuals but may persist and spread widely in patients with decreased cell-mediated immunity. The different types that infect the female genital tract have been divided into hrHPV, which includes types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68 and low-risk HPV (lrHPV) which are types 6, 11, 40, 42, 54, and 57.

The lrHPVs are associated with benign exophytic genital warts (condylomata acuminata) and are rarely associated with high-grade squamous intraepithelial lesions (HSILs) or invasive squamous cancers. Conversely, HPV 16, the most prevalent virus infecting the cervix, are associated with the entire spectrum of cervical intraepithelial neoplasia (CIN) lesions as well as invasive squamous carcinomas. Recent studies have demonstrated that hrHPV types account for almost 90% of all cervical cancers (6). The study of HPVs has been driven not by these widespread inapparent infections, but by the severity to which some hrHPV-associated diseases can progress. Most significant of these is cervical cancer, which can result from persistent hrHPV infection (3).

Describing the association between multiple HPV infections and cervical disease is important in generating hypotheses regarding its pathogenesis (7). Identifying the individual hrHPV types in each grade of cervical neoplasia is important for the development of HPV vaccines and screening strategies. Provision of appropriate hrHPV vaccines is necessary to protect against many of the HPV strains that

can cause genital warts and cancer. The cervical disease is strongly influenced by cultural and religious practices that govern sexual behavior and transmission of HPV. The sub-Saharan Africa has the highest estimated rates of cervical cancer, and in Guinea, Malawi, and Zambia, the age-standardized incidence rate is over 50 per 100,000 population (8).

In Nigeria, cervical cancer ranks as the second most frequent cancer among women and the second most frequent cancer death among women between the ages of 15 and 44 years (9). The seroprevalence of hrHPV has been reported to be 66.7% and Magaji et al., (10) noted that cancer of the cervix is the most common malignancy among women in Kaduna State, Nigeria. Almost all of cervical cancer deaths could be avoided by early detection and diagnosis of cervical dysplasia. An effective intervention could be made available to women with pre-cancerous lesions caused by persistent HPV after cervical screening. The objective of this study is to determine the prevalence of hrHPV among apparently healthy women of reproductive age in Kaduna State, in order to provide more information for effective control of HPV and cervical cancer in Nigeria.

Materials and method:

Study setting:

This study was conducted in 3 selected health facilities from each senatorial district in 9 randomly selected Local Government Areas (LGAs) of Kaduna State, Nigeria. The health facilities included the Ahmadu Bello University Teaching Hospital Zaria in Kaduna North Senatorial district, Barau Dikko Teaching Hospital (BDTH) in Kaduna Central Senatorial district and the General hospitals located in the selected LGAs in the State.

Study design:

The study is a hospital based cross-sectional comparative study which was conducted among apparently healthy women of reproductive age (15-65 years), irrespective of ethnicity, educational status and place of residence who were attending the Reproductive Health and Family Planning clinics in the selected facilities.

Sample size and sampling method:

The minimum sample size was determined using the Fisher formula (11), $n=Z^2p(1-p)/d^2$, where 'Z' is the standard normal variate (=1.96), 'p' is the local prevalence of 48.1% from a previous study (12), and 'd' is the degree of precision (=0.05). After adjusting for 10% attrition, the sample size of 422 was obtained, although a total of 515 consenting women were eventually enrolled for the study.

Participants were consecutively recruited at the clinics of each selected facility until the sample size was obtained. Exclusion criteria were women older than 65 years and those with previous operative or therapeutic history of related to gynaecologic diseases.

Ethical consideration:

Ethical approvals for the study were obtained from the ethics committees of Ahmadu Bello University Teaching Hospital Zaria (ABUTH), Barau Dikko Teaching Hospital (BD TH) and Kaduna State Ministry of Health. Informed consent was obtained from each participant and the procedure for obtaining samples was explained to each participant before samples for investigations were collected.

Data collection:

A predesigned structured questionnaire was interviewer-administered to collect socio-demographic information such as age, education, occupation, type of family, geographic location, annual income, and possible risk factors from each participant.

Sample collection and transportation:

Liquid-based cytology (LBC) technique was used to collect cervical smears by the attending physician assisted by a reproductive health nurse following standard procedures. This was carried out by using a cytology brush to take the smears. The brush was rotated over the whole surface of the cervix, making sure that the squamo-columnar junction was well and truly scrapped. The smeared brush was detached into a special vial containing the preservative liquid and labeled appropriately.

The specimens were transported to the Pathology laboratory of the Ahmadu Bello University Teaching Hospital Zaria where an aliquot of the smear was vortexed to obtain homogeneous mixture and strained to remove other elements such as mucor. The mixture was then centrifuged into layers based on density gradient. With the aid of a special polycarbonate filter, a thin layer of cells was placed on a clean grease free glass slide and deposit was processed using standard procedures. The remaining sample was stored at -80°C for molecular studies at the Institute of Human Virology Regional Laboratory, Jos, Nigeria.

Cytology technique:

A total of 5ml of vortexed cervical sample was centrifuged at 3000 revolution per minute (rpm) for 2 mins. The supernatant was decanted and 2ml of clearing fluid was added and vortexed again after which it was centrifuged at 3000 rpm for 2 mins, and the supernatant discarded. Two drops of polymer base were added to the sediment and thoroughly mixed to make the LBC smear on a clean grease-free glass slide.

The slides were washed with distilled water, stained in a solution of Haris haemat-

oxylin for 5 mins and washed in distilled water. Scott tap-water was used to blue the stained slide before washing in 95% ethanol following which Orange-G-6 was applied to stain the smear for 90 seconds and washed with 95% ethanol again. The smear was stained with Eosin Azure 50 for another 90 seconds and washed again in 95% ethanol. The preparation was finally dehydrated in absolute alcohol, cleared with xylene and mounted on Distrene Polystrene Xylene (DPX) for microscopic examination by a cytologist using the Bethesda system of reporting cytology slides.

DNA extraction:

Extraction of the DNA was performed using Quick-DNA™ viral kit (Zymo Research) following the manufacturer's instructions. Briefly, in a 1.5 ml Eppendorf tube, 800 µl of viral DNA buffer containing beta-mercaptoethanol to a final dilution of 0.5% (v/v), was added to 200 µl of each sample followed by brief vortexing and incubation for 10 mins at room temperature. Subsequently, the mixture was transferred to Zymo-Spin™ immuno chromatographic (IC) column in a collection tube and centrifuged at 10, 000 x g for 1 min.

The samples were washed with 300 µl DNA wash buffer containing 100% ethanol and the columns dried by centrifugation at 10,000 x g for 1 min. The flow through with collection tubes were discarded. The Zymo-Spin™ IC columns were transferred into new 1.5 ml Eppendorf microcentrifuge tubes and the DNA was eluted by addition of 10 µl DNA elution buffer directly onto the column matrix, incubated at room temperature for 1min and centrifuged at 10, 000 x g for 1 min to elute the DNA. The processed samples were then stored at -20°C until analysis.

Polymerase chain reaction set up:

Each sample was amplified by PCR as adapted from the work of Shikova et al., (13) using the following primer sets; consensus primers MY09/MY11 and GP5+/GP6+, and the type-specific primers for HPV16, 18, 31 and 45 as shown in Table 1. PCR assays were carried out in a final volume of 50 µl containing 25 µl One Taq Quick-Load 2X Master Mix (New England Biolabs, Inc.), 2 µl of each primer, 13 µl of water and 10 µl of genomic DNA sample. Amplifications were performed with the following cycling outline; initial denaturation at 94°C for 5 mins, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 40°C for 2 mins, and extension at 72°C for 1 min, followed by a final extension of 10 mins at 72°C.

Gel electrophoresis of PCR amplicons:

The PCR products were run on a 2% agarose gel stained with ethidium bromide. The amplicons were loaded into wells created in the agarose gel with a gel comb. The first well was loaded with 100 bp size ladder (Promega) which served as the standard DNA marker. Electrophoresis was carried out at 90V for 30 mins and the gel was visualized under UV light in Bio-Rad Gel Doc™ Universal Hood II Imaging System Lab.

Statistical analysis:

Data were analysed using the Statistical Package for the Social Sciences (SPSS) version 23.0, and presented in frequency distribution tables, and bar and pie charts. The relationship between prevalence of hrHPV and socio-demographic factors such as age and marital status were determined using Chi-square or Fisher Exact test with $p<0.05$ considered statistically significant.

Table 1: Sequence of primers used in the study

Primer name	Primer sequence	No of bp
MY09	CGT CCA CAA GAG GGA TAC TGA TC	23
MY11	GCA CCA GGG ATC ATA ACT AAT GG	23
GP5	TTTGTTACTGTGGTAGATACTAC	23
GP6	GAAAAATAAACTGTAAATCATATTC	25
16L1F	TGC TAG TGC TTA TGC AGC AA	20
16L1R	ATT TAC TGC AAC ATT GGT AC	20
16E6/F	TTG CTT TTC GGG ATT TAT GC	20
16E6/R	AGA TCA GTT GTC TCT GGT TGC A	22
18F	AAG GAT GCT GCA CCG GCT GA	20
18R	CAC GCA CAC GCT TGG CAG GT	20
31F	ATG GTG ATG TAC ACA ACA CC	20
31R	GTA GTT GCA GGA CAA CTG AC	20
45F	ACC AGA TTT GTG CAC AGA AT	20
45R	TTT TTT CCA GTG TCT CTC CA	20

bp=base pair

Results:

Cervical smear samples of 515 apparently healthy women were examined for the presence of HPV and hrHPV DNA (16, 18, 31, and 45). Human papillomavirus DNA was detected in smears of 61 women, of which 48 were positive for hrHPV (and 13 were positive for other HPVs), giving HPV and hrHPV prevalence rates of 11.8% and 9.3% respectively among the study participants.

Figures 1, 2 and 3 are of the gel electrophoresis pictures of representative DNA amplicons from the samples. A total of 100 HPV DNA types were detected by PCR in the 61 positive smears, with 66 hrHPV DNA types from 48 women, and 34 other HPV DNA types from 13 women. The frequency of hrHPV genotypes detected was HPV 16 (n=9), HPV 18 (n=6), HPV 31 (n=30) and HPV 45 (n=21), with other HPV genotypes (n=34) (Fig 4).

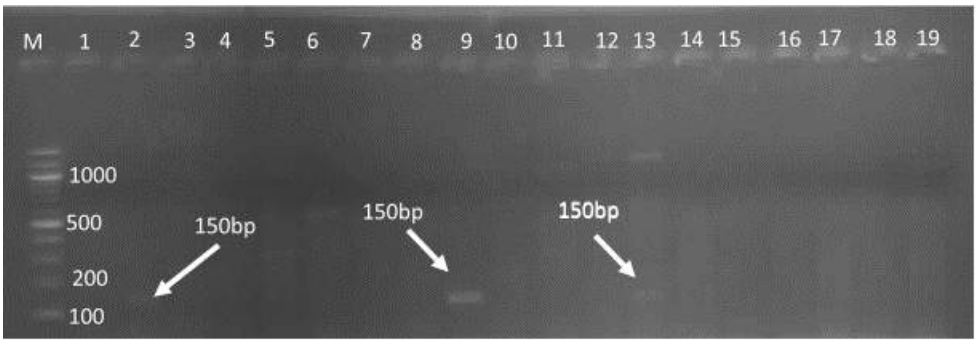

Fig 1: Gel electrophoresis picture of HPV DNA amplicons (Lanes 2, 9, and 13) with consensus primer GP5+/GP6+ (150 bp)

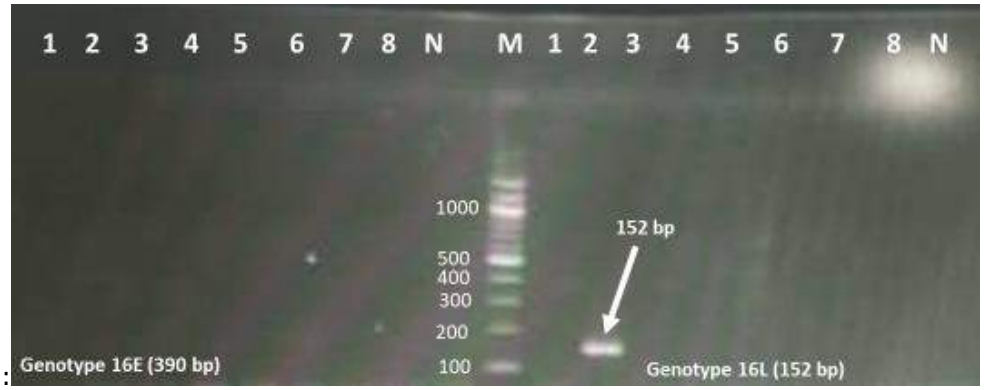

Fig 2: Gel electrophoresis picture of PCR amplicon of HPV 16 in lane 2 (152 bp)

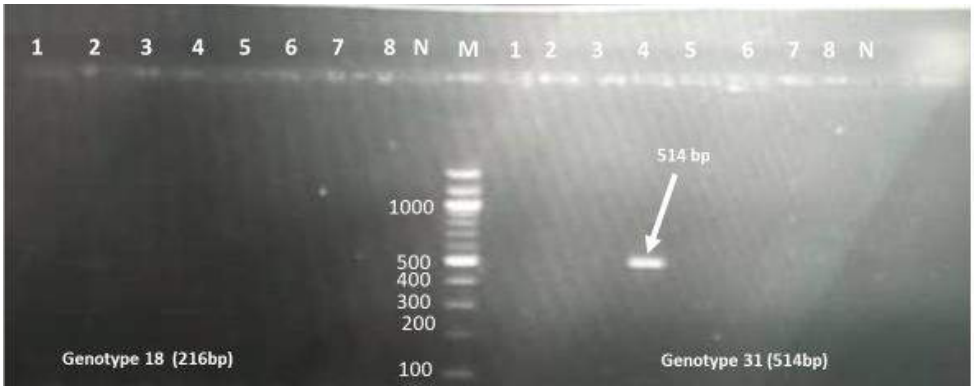

Fig 3: Gel electrophoresis of PCR amplicon of HPV 31 in lane 4 (514 bp)

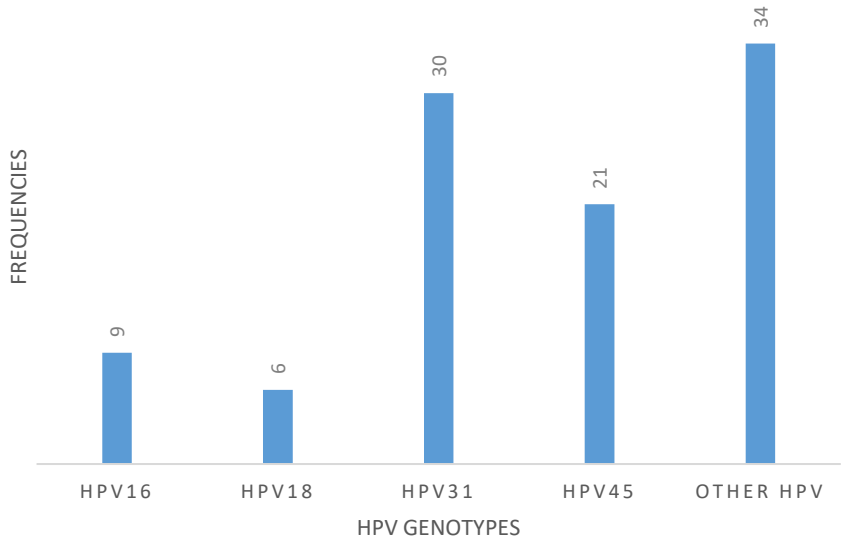

Fig 4: Distribution of HPV genotypes among women of reproductive age in Kaduna State, Nigeria

Table 2: Distribution of human papillomavirus genotypes and cervical dysplasia in association with socio-demographic characteristics of apparently healthy women in Kaduna State, Nigeria

Socio-demographic Variable	Number of women examined	No of women with HPV infection (%)	X^2	*p* value	No of women with cervical dysplasia (%)	X^2	*p* value
Age group (years)							
16-25	31	2 (6.5)	9.09	0.0609	0	19.069	0.0008*
26-35	119	16 (13.4)			7 (5.9)		
36-45	173	29 (16.8)			5 (2.9)		
46-55	116	8 (6.9)			17 (14.7)		
56-65	76	6 (7.9)			4 (5.3)		
Total	515	61 (11.8)			33 (6.4)		
Educational status							
None	66	4 (6.1)	2.585	0.694	5 (7.6)	0.3919	0.9831
Primary	74	10 (13.5)			4 (5.4)		
Secondary	144	19 (13.2)			10 (6.9)		
Tertiary	199	24 (12.1)			12 (6.0)		
Qur'anic	32	4 (12.5)			2 (6.3)		
Total	515	61 (11.8)			33 (6.4)		
Marital status							
Single	34	3 (8.8)	1.173	0.7596	3 (8.8)	3.914	0.2710
Married	422	53 (12.6)			23 (5.5)		
Divorced	33	3 (9.1)			4 (12.1)		
Widowed	26	2 (7.7)			3 (11.5)		
Total	515	61 (11.8)			33 (6.4)		
Ethnicity							
Hausa	224	25 (11.2)	5.942	0.1145	12 (5.4)	1.575	0.6651
Yoruba	38	1 (2.6)			4 (10.5)		
Igbo	65	12 (18.5)			4 (6.2)		
Others	188	23 (12.2)			13 (6.9)		
Total	515	61 (11.8)			33 (6.4)		

*Statistically significant at $p<0.05$

The socio-demographic characteristics of the participants as presented in Table 2 shows that HPV infection was highest among women in the age group 36-45 years with 16.7% (29/173), and lowest among women in age group 16-25 years (6.4%, 2/31), although there was no significant difference in HPV prevalence among the different age group of participants (x^2=9.09, p=0.0609). However, the prevalence of cervical dysplasia was highest among women in the age group 46-55 years (14.7%, 17/116), which was statistically significant (x^2=19.069, p=0.0008).

Women with primary and secondary education have higher frequency of HPV infection with 13.5% and 13.2% respectively (x^2= 2.585, p=0.694) while cervical dysplasia was highest in women with no education (7.6%)

and women with secondary level education (6.9%) (x^2=0.392, p=0.9831), although the prevalence differences in HPV infection and cervical dysplasia were not statistically significant with respect to level of education.

The prevalence of HPV infection was highest among married women (12.6%, p= 0.7596) while cervical dysplasia was highest among divorced (12.1%) and widowed women (11.5%), but the difference was also not statistically significant (p=0.2710). Women of the Igbo tribe had the highest frequency of HPV infection (18.5%, p=0.1145) while women of Yoruba tribe had the highest frequency of cervical dysplasia (10.5%, p=0.6651) but the difference was not statistically significant.

In all, hrHPV 31 was the most prevalent high-risk HPV genotype occurring in 30 (49.2%) of the 61 women with HPV infection. The distribution of single and multiple HPV infections in Table 3 showed that 31 (51.8%) women had single and 30 (49.2%) women had multiple infections [21 (34.4%) double and 9 (14.7%) triple HPV infections] (Fig 5). Single HPV infection was highest among women with HPV 45 (n=6) and lowest among those with HPV 18 (n=3).

Table 3: Distribution of single and multiple HPV genotypes among apparently healthy women in Kaduna State, Nigeria

Type of infection/HPV genotype	No of participants (%)
Single infection	
HPV 16	4 (6.5)
HPV 18	3 (4.9)
HPV 31	5 (8.2)
HPV 45	6 (9.8)
Other HPV	13 (21.3)
	31 (50.8)
Double infection	
HPV 16, Other HPV	1 (1.6)
HPV 18, Other HPV	1 (1.6)
HPV 31, Other HPV	11 (19.6)
HPV 16, HPV 45	1 (1.6)
HPV 31, HPV 45	7 (11.5)
	21 (34.4)
Triple infection	
HPV 16, HPV 31, HPV 45	1 (1.6)
HPV 16, HPV 45, Other HPV	2 (3.3)
HPV 18, HPV 31, Other HPV	2 (3.3)
HPV 31, HPV 45, Other HPV	4 (6.5)
	9 (14.8)
Total	**61 (100.0)**

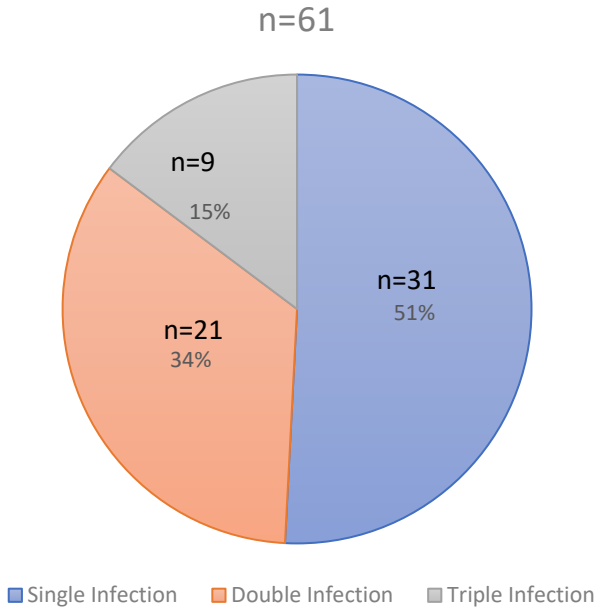

Fig 5: Distribution of single and multiple HPV infections among women in Kaduna State, Nigeria

Table 4 displays the distribution of HPV genotypes in the 33 women with cervical dysplasia, which when compared with women without cervical dysplasia, showed significant association of cervical dysplasia with all the HPV genotypes detected except HPV 16. The frequency of cervical dysplasia was highest among women with other HPV infections with 30.3% (10/33, OR=8.297, 95% CI=3.552-19.383, $p<0.0001$) followed by women with HPV 31 infections with 21.2% (7/33, OR= 5.373, 95% CI=2.111-13.672, $p=0.0016$), HPV 45 with 12.1% (4/33, OR=3.773, 95% CI=1.192-11.942, $p=0.0385$) and HPV 18 with 9.1% (3/33, OR=15.967, 95% CI=3.089-82.531, $p=0.0042$). The frequency of cervical dysplasia was lowest in women with HPV 16 infections (6.1%, 2/33), and when compared with the frequency of HPV 16 infections in women without cervical dysplasia (1.5%, 7/482), was not statistically significant (OR=4.378, 95% CI=0.8722-21.975, $p=0.1081$).

Table 5 shows the distribution of hr-HPV genotypes among women with cervical epithelial cell abnormalities (CEA) which indicates that HPV 16 and HPV 18 were implicated in 2 cases of low-grade squamous intra epithelial lesion (LSIL). High-grade squamous intra-epithelial lesion (HSIL) was detected in 3 women infected with HPV 31 and 4 women with unidentified (other) HPV genotypes. Multiple HPV genotypes were also detected among 4 women who had HSIL and LSIL.

Table 4: Frequency distribution of HPV genotypes and association with cervical dysplasia among the women participants

HPV types	No of HPV genotypes detected in all women (%) (n=515)	No of HPV detected in cervical dysplasia-positive women (%) (n=33)	No of HPV detected in cervical dysplasia-negative women (%) (n=482)	χ^2	OR (95% CI)	*p* value
HPV16	9 (1.7)	2 (6.1)	7 (1.5)	1.608	4.378 (0.8722-21.975)	0.1081
HPV18	6 (1.2)	3 (9.1)	3 (0.6)	12.584	15.967 (3.089-82.531)	0.0042*
HPV31	30 (5.8)	7 (21.2)	23 (4.8)	12.368	5.373 (2.111-13.672)	0.0016*
HPV45	21 (4.1)	4 (12.1)	17 (3.5)	3.842	3.773 (1.192-11.942)	0.0385*
Other HPV	34 (6.6)	10 (30.3)	24 (4.9)	28.146	8.297 (3.552-19.383)	<0.0001*
Total	100 (19.4)	26 (78.8)	74 (19.4)	75.427	20.479 (8.573-48.918)	<0.0001*

OR=Odd Ratio; CI=Confidence Interval; *= Statistically Significant at $p<0.05$

Table 5: Distribution of HPV genotypes in samples of women with cervical epithelial cell abnormalities in Kaduna State, Nigeria

No of HPV genotypes	No of HPV detected in sample with cervical dysplasia			No of HPV detected in sample with cervical non-dysplasia lesions		
	LSIL	HSIL	ASCUS	Anthropic changes	Inflammation	Cervicitis
HPV16 (n=9)	2	0	0	1	1	0
HPV18 (n=6)	2	1	0	0	0	0
HPV31 (n=30)	3	3	0	0	1	1
HPV45 (n=21)	4	1	0	0	1	1
Other HPV (n=34)	4	4	2	2	1	1
Total (n=100)	**15**	**9**	**2**	**3**	**4**	**3**
Multiple HPV (n=30)	4	4	1	1	2	1
Single HPV (n=31)	4	0	1	2	1	0
Total (n=61)	**8**	**4**	**2**	**3**	**3**	**1**

LSIL=Low-grade Squamous Intra-epithelial Lesion; HSIL= High-grade Squamous Intra-epithelial Lesion; ASCUS=Atypical Squamous Cells of Undermined Significance

Discussion:

Despite the high burden of cervical cancer morbidity and mortality in Nigeria, there is no reliable national prevalence of HPV genotypes in women. This study determined the prevalence of some hrHPV among the study population in Kaduna State, Nigeria and found that many women who were apparently healthy were actually infected with hrHPV. The prevalence of cervical HPV in this study is 11.8 % while the prevalence of hrHPV is 9.3%.

The HPV prevalence in our study is low compared to rates reported by similar studies across Nigeria. In Kano, northwest Nigeria, a high HPV prevalence of 76.0% was reported by Auwal et al., (14). Manga et al., (12) reported cervical HPV prevalence among women of 48.1% in a study carried out in Gombe, northeast Nigeria. In southwest Nigeria, cervical HPV was detected in 26.3% of sexually active women above 15 years in Ibadan and 14.7% among 1282 women in Irun (15). In Okene, northcentral Nigeria, a prevalence of 21.6% among 231 women was reported, and in Abuja, the Federal Capital of Nigeria, the prevalence was 37% among 275 women studied (16). The variation in the HPV prevalence in our study from those of others could be due to the differences in methodology and nature of samples used. Most studies determined HPV seroprevalence using ELISA but our study used molecular detection of HPV DNA on cervical samples using liquid-based technique. There is high probability of false positive detection of HPV antigens or antibodies in serological studies without detection of viral DNA in the cervical scrapings as done in our study.

Age is a significant socio-demographic factor because the chances of a woman developing cervical dysplasia increases with increasing age. According to Mosuro et al., (17), the mean age for developing dysplasia and carcinoma-in-situ ranged from 34.7 to 38.6 years, and 39.6 to 43.5 years, respectively. In our study, the age group 46-55 years had the highest frequency of cervical dysplasia (14.7%, 17/116) that was statistically significant (p= 0.0008), which agrees with the reports of Oguntayo and Samaila (18) who observed a peak age specific prevalence rate of cervical intraepithelial neoplasia (CIN) in their study. The prevalence of HPV infection however was highest among the age group 36-45 years in our study (16.2%, 28/173), although this was not statistically significant (p=0.0609). This finding differs from those of Kolawole et al., (19) and Akarolo-Anthony et al., (15) in similar HPV studies in Lokoja and Abuja, Nigeria respectively, where they reported high HPV infection rates among younger women less than 30 years of age, with decrease in HPV infection rate with age. This is probably because younger women including teenagers are now more sexually active with higher number of partners compared to the older women.

Reports from different parts of Nigeria indicate that there is paucity of data on the prevalence of HPV infections. Several authors have raised the possibility of certain HPV types being more common in sub-Saharan African women than elsewhere. Our study showed that HPV 31 and HPV 45 were more commonly detected compared to HPV 16 and HPV 18 in Kaduna State. This agrees with the finding of Nejo et al., (20) who reported that HPV 31 followed by HPV 35, were the most predominant high-risk circulating HPV in Ibadan, south west Nigeria. HPV 35, for instance, was slightly more common than HPV 16 in Mozambique both in women with normal cytology and in those with high-grade squamous intra-epithelial lesion (HSIL) or worse (21,22). Our finding is however contradicted by that of Ezebialu et al., (16) who reported that HPV 16 and HPV 18 rates were higher than other hrHPV genotypes in their study among women in Awka, southeast Nigeria. Manga et al., (14) also reported that HPV 16 and 18 were the most prevalent HPV genotypes in northern Nigeria. Geographical and socio-cultural diversities in the different regions of Nigeria may be account for variations in hrHPV prevalence rates in these studies.

Emeribe et al., (23) noted that data on HPV genotypes, geographical distribution and risk factors among women of child bearing age are important to determine the best vaccines needful for the protection against cervical cancer. In our study, 49.2% (30/61) of the women infected with HPV had more than one HPV genotypes. This observation has been a common occurrence in most HPV genotype studies across Nigeria. A systematic review of 16 studies reported that HPV 31 poses a similar or higher risk for CIN3+ disease compared to HPV 18, above the 4% American Society for Colposcopy and Cervical Pathology (ASCCP) immediate risk of CIN3+ threshold for referral to colposcopy (24).

The prevalence of multiple HPV infections in this population is high as 30% (9/30) of women who had multiple hrHPV infection were reportedly positive for cervical dysplasia. This finding agrees with that of Schmitt et al., (7) who reported that multiple HPV were found in 75.9% of HPV positive samples. The distribution of hrHPV genotypes among women with cervical dysplasia in this study showed that hrHPV 31 and hrHPV45 may play significant roles in the development of cervical cancer in Kaduna State, Nigeria. A similar study in Maiduguri, northeast Nigeria, reported that both single and multiple high-risk HPV infections were observed among slides prepared from women with cervical cancer (25). Further

observations were reported in studies from two African countries, Malawi and Ghana, with multiple HPV infections that accounted for 54.0% and 52.2% respectively (26,27).

Conclusion:

The prevalence of hrHPV genotypes of 9.3% in this study is high among women in Kaduna State, Nigeria. Our study showed that HPV 31 and HPV 45 were predominant genotypes detected both as single and multiple infections. This justifies the need to review the currently available HPV vaccines with the view to developing new types that will be potent against other predominant oncogenic hrHPV types such as HPV 31 and 45 in Nigeria and Africa.

DNA-based screening for hrHPV genotypes and vaccination of young girls are recommended for early detection of hrHPV and preventive management of cervical dysplasia, which can result in significant reduction in cervical cancer in Nigeria and beyond. Our study contributes to the understanding of HPV epidemiology and allows for hrHPV genotype screening programs to better assess the cancer-developing risks associated with individual hrHPV infections.

Contributions of authors:

ADS was involved is study conceptualization, project administration, investigations, methodology, resources and writing of the original draft; MA was involved in supervision, validation of data, review and editing of the manuscript; EE was involved in supervision, methodology, visualization, investigation, review and editing of manuscript; AOO was involved in supervision, investigation, cytology validation and review of manuscript; and FOO was involved in molecular analysis, methodology and data validation. All authors approved the manuscript for submission.

Source of funding:

Authors received no external funding.

Conflict of interest:

Authors declared no conflict of interest

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Ogban et al. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (1): 28 - 37 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jan 2024; Vol.25 No.1
AJCEM/2304. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Knowledge and awareness of hepatitis B amongst students of Pamo University of Medical Sciences (PUMS), Port Harcourt, Rivers State, Nigeria

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Abstract:

Background: Globally, 296 million people were infected by hepatitis B in 2019, with 1.1 million deaths. Africa is one of the endemic regions. Good knowledge and awareness of hepatitis B remain pivotal to the biosafety of medical students. This study sought to determine the levels of knowledge and awareness of hepatitis B among students of Pamo University of Medical Sciences (PUMS), Port Harcourt, Nigeria, and the predicting factors associated with this knowledge and awareness. The is with the aim of providing recommendations for improving and sustaining biosafety levels for medical and other health-related students of the University.
Methodology: The study was a descriptive cross-sectional design conducted amongst 528 randomly selected medical students of PUMS, Port Harcourt, Nigeria. Structured questionnaires were interviewer-administered to collect socio-demographic information and participants' responses to questions on knowledge and awareness of hepatitis B. Data were analysed using SPSS version 26.0 and relationships of socio-demographic characteristics and predictive factors with knowledge and awareness of hepatitis B were tested using binary logistic regression analysis with p value for statistical significance set at <0.05.
Results: A total of 528 students participated in the study, 202 (38.3%) males and 326 (61.7%) females. Most participants (296, 56.1%) were between 15-19 years of age with mean age of 19 ±2.43 years. The mean (±SD) of participants responses with good knowledge of hepatitis B was 249±121.5 while for good awareness, it was 181±88.3. The percentage average for good knowledge and good awareness was 47.2% and 34.2% respectively, with positive correlation between knowledge and awareness of hepatitis B (r=0.720, $p<0.0001$). Age was significantly associated with participants percentage average knowledge (OR=0.77, 95% CI 0.70-0.84, $p<0.0001$) and awareness of hepatitis B (OR=0.84, 95%CI 0.78-0.90, $p=0.004$). No other factor was significantly associated with knowledge and awareness of hepatitis B except Ijaw tribe (OR=0.4, 95%CI 0.24-0.66, $p=0.034$) and attendance of Federal Government College (OR=0.4, 95% CI 0.24-0.68, $p=0.046$).
Conclusion: The percentage average good knowledge of 47.2% and awareness of 34.2% for hepatitis B in this study are low, although most participants in the study were between the ages of 15-19 years and in their first and second year of study. This gives room for improvement in knowledge and awareness of hepatitis B with progression in age and year of training. Good knowledge and awareness of hepatitis B are central to the biosafety of medical students. It is recommended that the National Universities Commission (NUC) and the Medical and Dental Council of Nigeria (MDCN) review the current medical school curriculum to increase the teaching of medical and health-related students that will impact more on knowledge and awareness of infectious diseases and infection prevention and control.

Keywords: Knowledge, awareness, hepatitis B, Pamo University, Nigeria.

Received Oct 07, 2023; Revised Dec 14, 2023; Accepted Dec 16, 2023

Connaissance et sensibilisation à l'hépatite B parmi les étudiants de l'Université des sciences médicales de Pamo (PUMS), Port Harcourt, État de Rivers, Nigéria

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Résumé:

Contexte: Dans le monde, 296 millions de personnes ont été infectées par l'hépatite B en 2019, avec 1,1 million de décès. L'Afrique fait partie des régions endémiques. Une bonne connaissance et sensibilisation à l'hépatite B reste essentielle à la biosécurité des étudiants en médecine. Cette étude visait à déterminer les niveaux de connaissance et de sensibilisation à l'hépatite B parmi les étudiants de l'Université des sciences médicales de Pamo (PUMS), à Port Harcourt, au Nigeria, ainsi que les facteurs prédictifs associés à ces connaissances et sensibilisation. L'objectif est de fournir des recommandations pour améliorer et maintenir les niveaux de biosécurité pour les étudiants en médecine et dans d'autres domaines liés à la santé de l'Université.
Méthodologie: L'étude était une conception transversale descriptive menée auprès de 528 étudiants en médecine sélectionnés au hasard au PUMS. Port Harcourt, Nigéria. Des questionnaires structurés ont été administrés par l'intervieweur pour recueillir des informations sociodémographiques et les réponses des participants aux questions sur la connaissance et la sensibilisation à l'hépatite B. Les données ont été analysées à l'aide de SPSS version 26.0 et les relations entre les caractéristiques sociodémographiques et les facteurs prédictifs avec la connaissance et la sensibilisation à l'hépatite B. ont été testés à l'aide d'une analyse de régression logistique binaire avec une valeur de *p* pour la signification statistique fixée à <0,05.
Résultats: Au total, 528 étudiants ont participé à l'étude, 202 (38,3%) hommes et 326 (61,7%) femmes. La plupart des participants (296, 56,1%) étaient âgés de 15 à 19 ans, avec un âge moyen de 19±2,43 ans. La moyenne (± ET) des réponses des participants ayant une bonne connaissance de l'hépatite B était de 249±121,5 tandis que pour une bonne connaissance, elle était de 181±88,3. Le pourcentage moyen de bonne connaissance et de bonne sensibilisation était respectivement de 47,2% et 34,2%, avec une corrélation positive entre la connaissance et la sensibilisation à l'hépatite B (r=0,720; *p*<0,0001). L'âge était significativement associé au pourcentage de connaissances moyennes des participants (OR=0,77; IC à 95% 0,70-0,84; *p*<0,0001) et à la connaissance de l'hépatite B (OR=0,84; IC à 95% 0,78-0,90; *p*=0,004). Aucun autre facteur n'était associé de manière significative à la connaissance et à la sensibilisation à l'hépatite B, à l'exception de la tribu Ijaw (OR=0,4; IC à 95% 0,24-0,66; *p*=0,034) et de la fréquentation du Collège du Gouvernement Fédéral (OR=0,4; IC à 95% 0,24-0,68; *p*=0,046).
Conclusion: Le pourcentage moyen de bonnes connaissances de 47,2% et de sensibilisation de 34,2 % à l'hépatite B dans cette étude est faible, bien que la plupart des participants à l'étude étaient âgés de 15 à 19 ans et en première et deuxième années d'études. Cela laisse place à une amélioration des connaissances et de la sensibilisation à l'hépatite B avec une progression en âge et en année de formation. Une bonne connaissance et sensibilisation à l'hépatite B est essentielle à la biosécurité des étudiants en médecine. Il est recommandé que la Commission Nationale des Universités (NUC) et le Conseil Médical et Dentaire du Nigéria (MDCN) réexaminent le programme actuel des facultés de médecine afin d'accroître l'enseignement aux étudiants en médecine et dans les domaines de la santé, ce qui aura davantage d'impact sur la connaissance et la sensibilisation aux maladies infectieuses. et la prévention et le contrôle des infections.

Mots-clés: Connaissance, sensibilisation, hépatite B, Université de Pamo, Nigéria

Introduction:

Hepatitis B, an inflammatory disease of the liver, is a leading cause of liver cancer and cirrhosis, affecting 296 million people globally (1), and accounting for 1.1 million global deaths in 2019 (2). An estimated 3.6% of the global population is affected by chronic hepatitis B (3,4). There is disproportionate distribution of hepatitis B amongst regions of the world. The prevalence is highest in the Western Pacific, accounting for 116 million infections, followed by the African region, accounting for 81 million, and the Eastern Mediterranean region and Southeast Asia, each accounting for 60 million and 18million infections respectively. Europe and the Americas account for 14 and 5 million respectively (5).

In 2016, the World Health Assembly endorsed the goal of eliminating viral hepatitis as a public health threat by 2030, including the elimination of mother-to-child transmission (EMTCT) of hepatitis B virus (HBV), documented by demonstration of ≥90% coverage and three doses of HepB (HepB3) vaccine, and ≤0.1% hepatitis B surface antigen (HBsAg) seroprevalence among children ≤5 years of age (6).

Hepatitis B is transmitted via inoculation with contaminated sharps or hospital devices, transfusion with unscreened blood, mother-to-child vertical transmission or through breast feeding, sexual route amongst others. Vulnerable groups include health workers, medical and nursing students, sexually active individuals, people who engage in same sex intercourse, intravenous drug abusers, renal dialysis patients, sickle cell patients, inmates and staff of correctional centres (7).

Nigeria is rated as one of the countries hyper-endemic for hepatitis B infections with prevalence greater than 8% (8). About 1 in 10 Nigerians living with hepatitis B are not aware of their status and therefore not captured in global public health statistics due to interplay of unawareness, resource constrains and lack of political will to address headlong this national plight (9-11). A study done in 2021 (12) reported hepatitis B prevalence rate of 5.1% in Port Harcourt city with male to female distribution of 7.9% and 3.4% respectively, with two peak age groups; 24-29 years and

30-34 years of 8.29% and 9.2% respectively. Nigeria ranks as one of the countries with the highest percentage prevalence of hepatitis B related malignancies in West Africa with an age-standardised incidence estimate of 2.6 to < 5.1 cases per 100,000 person-years (13, 14).

Hepatocellular carcinoma (HCC) is one of such malignant neoplasm associated with hepatitis B. HCC has limited treatment options which are yet to be popular in resource poor settings and in Nigeria where cost of treatment is out-of-pocket, these treatment options, even where available, are not affordable to the vulnerable population (8). Furthermore, there is lack of advance diagnostic facilities such as immunoassays, nucleic acid amplification tests and the absence of focused public enlightenment programs with emphasis on enhancing knowledge and awareness of debilitating diseases such as HCC. These missing gabs constitute impedance on the path to achieving prevention, control or possible elimination of hepatitis infections in Nigeria as declared by WHO.

It is on this premise that this study sought to evaluate the knowledge and awareness of hepatitis B amongst the students of Pamo University of Medical Sciences (PUMS), Port Harcourt, Nigeria, a newly established medical university, yet having 500-level stream of Medicine and Surgery students as the topmost undergraduate class in the Faculty of Clinical Sciences of the University. The objectives of this study are to determine the knowledge and awareness levels of hepatitis B amongst students of the University and the predicting factors influencing this knowledge and awareness. The outcome of this study is intended to provide empirical evidence of the need or otherwise for early inclusion of infectious diseases prevention and control programs in the curricular of training for medical schools in Nigeria.

Materials and method:

Study design, setting and period:

This study was a descriptive cross-sectional design conducted amongst the students of Pamo University of Medical Sciences, Port Harcourt, Rivers State, Nigeria, from February 17 to March 25, 2022. Pamo University of Medical Sciences was established in 2017 as the first private medical university in Nigeria with an initial student population of 123. The current student population is under 1000, all residential within the campus.

The university has a serene environ ment for learning and steady power and water supply. The university has a sick bay to take care of the health of staff and students. Those with serious health challenges are referred to multi-specialties Pamo hospital, located along Aba Road, Port Harcourt. Presently, the university has 4 faculties and 2 academic units. The faculties are the Faculty of Clinical Sciences, Faculty of Basic Clinical Sciences, Faculty of Basic Medical Sciences and Faculty of Allied Health Sciences, while the academic units are Basic Science, and General & Entrepreneurial units. There are total of 29 academics departments in the University.

Ethical approval:

Approval for the study was given by the office of the Vice Chancellor, after a thorough review of ethical issues involved in the study.

Determination of sample size:

The sample size was determined using the Fisher formula (15); $N=Z^2pq/d^2$, where N=sample size, Z=1.96 (i. e. 95% confidence interval), d=0.05 (margin of error), p=0.051, which is the prevalence of hepatitis B in Port-Harcourt from a previous study (12), and q=1-p (0.949). This gave a calculated minimum sample size of 206, which was increased to 528, allowing the recruitment of all the participants who gave informed consent.

Inclusion/Exclusion criteria:

To be included in the study, one was mandatorily a student of Pamo University of Medical Sciences, irrespective of the discipline. Staff of the University were excluded.

Data collection:

Structured questionnaires were used to collect socio-demographic information from the 528 participants and their responses to probing questions to establish their levels of knowledge and awareness of hepatitis B. The questionnaire contains 3 sections, with the first section for collecting sociodemographic information of the participants. The second and third sections, each with 15 questions, respectively assessed knowledge and awareness of hepatitis B amongst the participants.

The questionnaires were interviewer-administered. The consent form was first explained to each participant before data collection with assurance of the safety of the exercise, secrecy of data submitted, freedom to opt out of the study without any consequence, assurance of no cost on their part and that the interview would take about 10 minutes. Each participant gave signed informed consent before administration of questionnaire.

To ensure that only eligible students of a particular class were enlisted, two laboratory scientists and an administrative staff were used to administer the questionnaires to all eligible students while seated to commence a semester examination and given 10 minutes to complete and turn them in before commencement of the examination. In the questionnaire, correct responses under 'yes' were indications for "good knowledge or awareness",

wrong responses under 'no' were indications for "poor knowledge or awareness'' and "I don't know" responses were indications for "no knowledge or awareness" of hepatitis B.

The frequency of participants responses under each category of responses were summed up, divided by 15, and multiplied by 100 to derive the percentage average responses of participants with "good knowledge or awareness", "poor knowledge or awareness" and "no knowledge or awareness" of hepatitis B respectively (16). The mean and standard deviation (SD) of participants' responses for each category were also calculated. The percentage response values in the three categories of knowledge or awareness of hepatitis B were deemed high or low depending on whether the value was above or below 50%.

Statistical analysis of data:

Data were analysed with the Statistical Package for the Social Sciences (SPSS) version 26.0. Continuous and categorical variables were summarised and reported as mean ±SD and percentage values respectively. The percentage knowledge and awareness scores were dichotomised into poor and good knowledge/awareness.

Binary logistic regression model was used to test for association between outcome variables (e. g. poor knowledge score vs good knowledge score) and the independent variables (predictors), with calculation of their odd ratios (ORs) and 95% confidence intervals (CIs). Statistical significance was set at *p* less than 0.05

Results:

Socio-demographic data of the participants:

A total of 528 students of Pamo University of Medical Sciences, Port Harcourt, Nigeria, participated in this study by completing and turning in their questionnaires. Table 1 shows the distribution of the socio-demographic characteristics of the participants. Majority of the study participants (296, 56.1%) were between 15-19 years of age whereas the least number of participants (2, 0.4%) were in ≥ 30 years age group. The mean age of the participants was 19±2.43 years. Female participants (376, 71.2%) dominated the study.

A total of 226 (42.8%) participants belonged to a collection of minor tribes described in this study as 'other tribes', followed by participants of Ikwerre tribe (20.4%, n= 108) and Ijaw tribe (16.8%, n=80). Hausa tribe with just 2 (0.4%) participants was the least recruited into the study. Majority of the participants' (80.3%, n=424)) attended private colleges. Medicine and Surgery students (63.6%, n=332) were in the majority followed by nursing students (18.6%, n=98). Students of the departments of biochemistry (1.1%, n=6) and physiology (1.1%, n=6) were the least frequently recruited participants to the study. Participants in their first year of study (26.7%, n=140) were in the majority, followed by third year students (22.7%, n=119) and second year students (22.1%, n=116).

Participant responses to questions on knowledge of hepatitis B

Table 2 shows the distribution of participants based on their responses to questions on knowledge of hepatitis B. Majority of the participants (73.9%, n=387) responded that hepatitis B was infectious and 70.8% (n=374) also responded that hepatitis B was a public health hazard to medical and health students. Two hundred and sixteen participants (41.1%) responded that hepatitis B was transmitted through ingestion of contaminated food and drinks and 60.2% (n=305) responded that hepatitis B was transmitted through hand shake with an infected person. Three hundred and eleven (59.1%) participants did not know whether or not hepatitis B was primarily a disease of the heart and 48.6% (n=255) participants did not know whether or not the disease could be transmitted vertically from mother to foetus in-utero.

The mean (± SD) of participants with good knowledge responses is 249±121.5, poor knowledge responses 81±115.3 and no knowledge responses 198±126.0. The percentage average of participants with good knowledge of hepatitis B was 47.2%, poor knowledge 15.3% and no knowledge 37.5%.

Table 1: Socio-demographic characteristics of medical students at Pamo University of Medical Sciences, Port Harcourt, Nigeria

Socio-demographic variables	Number of respondents (%)
Age in years (Mean ± SD)	19 ±2.43
Age group (years)	
15-19	296 (56.1)
20-24	207 (40.7)
25-29	14 (2.6)
≥30	2 (0.4)
Total	528 (100.0)
Gender	
Male	152 (28.8)
Female	376 (71.2)
Total	528 (100.0)
Tribe	
Ibo	50 (9.4)
Yoruba	6 (1.1)
Hausa	2 (0.4)
Ibibio	11 (2.1)
Ijaw	89 (16.8)
Ogoni	45 (8.5)
Ikwerre	108 (20.4)
Others	226 (42.8)
Total	528 (100.0)
Type of secondary school	
State Public College	41 (7.8)
Federal Government College	63 (11.9)
Private College	424 (80.3)
Total	528 (100.0)
Department	
Anatomy	11 (2.1)
Biochemistry	6 (1.1)
Physiology	6 (1.1)
Medical Laboratory Sciences	39 (7.4)
Medicine and Surgery	332 (62.9)
Nursing	98 (18.6)
Pharmacology	26 (4.9)
Radiology	10 (1.9)
Total	528 (100.0)
Year of study	
1	149 (28.2)
2	116 (22.0)
3	119 (22.5)
4	77 (14.6)
5	67 (12.7)
6	0
Total	528 (100.0)
Monthly allowance (`000 Naira)	
≤99.99	216 (40.9)
10-19.999	157 (29.3)
20-39.999	101 (19.1)
≥40	54 (10.2)
Total	528 (100.0)

Participant responses to questions on awareness of hepatitis B:

Table 3 shows that 60.9% (n=310) of the participants responded that hepatitis B was a risk factor for liver cancer, and 48.3% (n=245) responded that jaundice was a common symptom of hepatitis B. Two hundred and seventy-six participants (54.0%) responded that hepatitis B could be prevented by exercise and 56.3% (n=289) responded that hepatitis B cannot be diagnosed by blood serology. Two hundred and ninety-four (57.8%) participants did not know whether or not Government has free anti-hepatitis B vaccination program for newborns and 66.3% (n=339) did not know whether or not a complete set of hepatitis B vaccination required 3 doses of the vaccine.

The mean (±SD) of participants with good awareness responses was 181±88.3, poor awareness responses 106±86 and no awareness responses 234±49.3. The percentage average of participants with good awareness of hepatitis B is 34.2%, poor awareness 21.5% and no awareness 44.3%.

Table 2.: Distribution of participants responses to questions on knowledge of hepatitis B

Questions	**Number of respondents**		
	Yes (%)	**No (%)**	**Don't know (%)**
Have you heard about hepatitis B infection?	489 (92.6)	19 (3.6)	20 (3.8)
Is hepatitis B an infectious disease?	387 (73.3)	28 (5.3)	113 (21.4)
Is hepatitis B a hazard to medical and health students?	374 (70.8)	24 (4.6)	130 (24.6)
Is hepatitis B infection caused by a virus?	324 (61.3)	16 (3.0)	188 (35.7)
Does hepatitis B infection primarily affect the liver?	189 (35.8)	134 (25.4)	205 (38.8)
Hepatitis B infection does not primarily affect the heart?	110 (20.9)	105 (20.0)	311 (59.1)
Hepatitis B infection does not primarily affect the kidneys?	146 (27.9)	86 (16.4)	292 (55.7)
Is hepatitis B infection is transmitted through food and drinks?	72 (13.6)	219 (41.5)	237 (44.9)
Is hepatitis B infection transmitted through blood transfusion?	348 (65.9)	13 (2.5)	167 (31.6)
Is hepatitis B infection transmitted through tattoos?	183 (34.7)	90 (17.0)	255 (48.3)
Is hepatitis B infection transmitted through sex?	296 (56.1)	44 (8.3)	188 (35.6)
Is hepatitis B infection transmitted via contaminated sharps injury?	304 (57.6)	22 (4.1)	202 (38.3)
Is hepatitis B infection transmitted from mother to baby in-utero?	258 (48.9)	15 (2.8)	255 (48.3)
Is hepatitis B infection transmitted via contaminated hair clippers?	196 (37.1)	94 (17.8)	238 (45.1)
Hepatitis B infection is not transmitted through hand-shake?	58 (11.0)	305 (57.8)	165 (31.2)
Mean ± SD of participants' responses	249±121.5	81±115.3	198±126
Percentage average responses on knowledge of hepatitis B	47.2%	15.3%	37.5%

SD=Standard deviation

Table 3: Distribution of participants responses to questions on awareness of hepatitis B

Questions	**Number of respondents**		
	Yes (%)	**No (%)**	**Don't Know (%)**
Can Hepatitis B be prevented by vaccination?	357 (67.6)	18 (3.4)	153 (29.0)
Is Hepatitis B not preventable with exercise?	42 (8.0)	276 (52.3)	210 (39.8)
Is Hepatitis B not preventable by dietary measures?	116 (22.0)	170 (32.2)	242 (45.8)
Can Hepatitis B be prevented by good hand hygiene?	225 (42.6)	96 (18.2)	207 (39.2)
Can hepatitis B be prevented by wearing PPE?	192 (36.4)	139 (26.3)	197 (37.3)
Can Hepatitis B be diagnosed by serology?	23 (4.4)	304 (57.6)	201 (38.1)
Is there antiviral therapy for hepatitis B?	243 (46.0)	39 (7.4)	246 (46.6)
Is Hepatitis B a risk factor for liver cancer	310 (58.7)	25 (4.7)	193 (36.6)
Is hepatitis B vaccination to newborns free in Nigeria?	186 (35.2)	48 (9.1)	294 (55.7)
Does hepatitis B vaccination require 3 doses of the vaccines?	156 (29.5)	33 (6.3)	339 (64.2)
Is Jaundice one of the commonest symptoms of Hepatitis B?	266 (50.4)	19 (3.6)	243 (46.0)
Does Government provide free hepatitis B vaccination to adults in Nigeria?	186 (35.2)	50 (9.5)	292 (55.3)
Do you know the hepatitis B infection status of your siblings?	165 (31.3)	173 (32.8)	190 (36.0)
Do you know your hepatitis B status?	220 (41.7)	101 (19.1)	207 (39.2)
Have you completed your hepatitis B vaccination?	124 (23.5)	197 (37.3)	207 (39.2)
Mean ± SD of participants responses	181±88.03	106±86.0	234±49.3
Percentage average responses on awareness of hepatitis B	34.2%	21.5%	44.3%

SD=Standard deviation

Relationship between participants knowledge and awareness of hepatitis B:

There was a statistically significant positive correlation between knowledge and awareness of hepatitis B among the study participants (r=0.720, N=516, $p<0.0001$).

Predictors of knowledge of hepatitis B among participants using binary logistic regression analysis:

Table 4 shows the relationship between participants knowledge of hepatitis B and predicting/associated factors using binary logistic regression analysis model. The percentage average knowledge was set as the dependent variable while predictors included the following factors; tribe, school, departments, year of study, and age group, which was entered as the covariate, with calculation of their corresponding odds ratio (ORs) and respective 95% confidence intervals (95% CIs).

None of the explanatory variables was significant effective predictor except the age of the participants (OR=0.77, 95% CI 0.70-0.84, $p<0.0001$), which indicates that with a unit decrease in age of the participants by a factor of 0.77, there is a corresponding decrease in participants knowledge of hepatitis B.

Predictors of awareness of hepatitis B among participants using binary logistic regression analysis:

Binary logistic regression analysis was also used to determine the association of participants awareness of hepatitis against some potential predictors/factors. The predictor variables included the participants age (entered as a covariate) and others such as participants' tribe, school, departments, and year of study, were entered as factors (Table 5).

Table 4: Binary logistic regression analysis of predictors of knowledge of hepatitis B among the study participants

Predictors	Statistics of percentage average knowledge of hepatitis B			
	Odds Ratio	95% CI		*p* value
		Lower	Upper	
Mean age	0.77	0.70	0.84	<0.0001*
Tribe				
Ibo	1.03	0.67	1.60	0.929
Yoruba	0.29	0.11	0.76	0.134
Hausa	0.0	0.0	.	0.999
Ibibio	.0.78	0.35	1.73	0.718
Ijaw	1.01	0.70	1.44	0.988
Ogoni	2.33	1.31	4.15	0.091
Ikwerre	0.97	0.70	1.34	0.909
Others+				
Secondary College				
State Public College	0.66	0.44	1.00	0.25
Federal Government College	0.90	0.61	1.33	0.7
Private College+				
Department				
Anatomy	1.13	0.31	4.04	0.916
Biochemistry	4.04E+8	0.00	.-	0.999
Physiology	1.25	0.26	5.91	0.869
Medical Laboratory Sciences	0.83	0.30	2.29	0.835
Medicine and Surgery	0.90	0.36	2.27	0.898
Nursing	0.49	0.19	1.27	0.387
Pharmacology	6.25	1.42	27.58	0.158
Radiology+				
Year of Study				
Year 1	1.36	0.37	4.94	0.787
Year 2	2.7	0.72	10.20	0.390
Year 3	1.39	0.38	5.09	0.772
Year 4	0.25	0.07	0.92	0.219
Year 5	0.12	0.03	0.43	0.058
Year 6+				

Significant at $p<0.05$, + = Reference variable

There was statistically significant association of percentage average awareness of hepatitis B with age of participants (OR=0.84, 95% CI 0.78 - 0.90, p=0.004), Ijaw tribe (OR=0.4, 95% CI 0.24 - 0.66, p=0.034), and attendance of Federal Government College (OR=0.4, 95% CI 0.24 - 0.68, p=0.046). This indicated that the odds of being aware of hepatitis B is decreased by a factor of 0.4 if the participant is an Ijaw compared to being from another tribe. Also, the odds of being aware of hepatitis B is decreased by a factor of 0.4 if the participant attended Federal Government College compared to those who attended a private college.

Discussion:

Students between the ages of 15-19 years (56.1%, 296) dominated this study and they were mostly females (71.2%, 376). This finding is similar to the report of a related study on hepatitis among students in northwest Nigeria which reported students in the age group 15-25 years (67.8%) who were mostly males (61.0%) as dominating the study (17). Students of medical and health sciences, irrespective of disciplines are mandatorily expected to interact at one time or the other with human body fluids and other potentially infectious materials in the course of their training. The training curriculum should be such that will create enabling environment for good knowledge and awareness build-up on infectious threats including hepatitis B which they might be exposed to in the course of career acquisition and practice. There are reports that hepatitis B is transmitted 2 to 10 times higher amongst medical professionals (18,19).

In this study, the percentages average good knowledge and awareness of hepatitis B among the participants were 47.2% and 34.2% respectively. These findings are similar to that of a related study in Malaysia (20), which reported that 36.9% and 38.8% of the participants had good knowledge and awareness of hepatitis B respectively. A similar study in Senegal (21) reported that only 27% of the study population had good knowledge of hepatitis B whereas 14.5% and 38.8% had poor knowledge and no knowledge of hepatitis B respectively. Although these findings call for concern, they are however better than the finding of a study in northwest Nigeria where 64.4% of the participants had poor knowledge of hepatitis B (17).

Also, 92.6% (n=489) of the study par-

Table 5: Binary logistic regression of predictors of awareness of hepatitis B among the study participants

Predictors	Statistics of percentage average awareness of hepatitis B			
	Odds Ratio	95% CI		P value
		Lower	Upper	
Age	0.84	0.78	0.90	0.004*
Tribe				
Ibo	0.99	0.47	2.14	0.929
Yoruba	1.02E+8	0.0		0.99
Hausa	102E+8	0.0	.	0.999
Ibibio	0.64	0.18	2.23	0.718
Ijaw	0.4	0.24	0.66	0.034*
Ogoni	0.89	0.42	1.91	0.863
Ikwerre	0.97	1.05	4.72	0.218
Others+				
Secondary College				
State Public College	0.53	0.28	1.02	0.265
Federal Government College	0.40	0.24	0.68	0.046*
Private College+				
Departments				
Anatomy	1.8E+8	0.00		0.999
Biochemistry	1.8E+8	0.00		0.999
Physiology	1.8E+8	0.00		0.999
Medical Laboratory Sciences	0.61	0.16	2.28	0.667
Medicine and Surgery	1.83	0.53	6.34	0.578
Nursing	1.17	0.32	4.22	0890
Pharmacology	1.8E+8	0.00		0.158
Radiology+				
Year of Study				
Year 1	0.0	0.0		0.999
Year 2	0.0	0.0		0.999
Year 3	0.0	0.0		0.999
Year 4	0.0	0.0		0.999
Year 5	0.0	0.0		0.999
Year 6+				

Significant at $p<0.05$, + = Reference

ticipants in our study responded that they have heard about hepatitis B and 73.0% (n= 307) correctly responded that hepatitis B was infectious, but 38.8% (n=205) did not know whether or not hepatitis B was a primary disease of the liver, only 58.0% (n=304) knew that hepatitis B could be transmitted through injury from contaminated sharps, 4.5% (n= 23) of participants were aware that serology was a screening test for hepatitis B, while 48.3% (n=245) knew jaundice as a common symptom of the disease. Although these findings are not impressive, they appeared to be encouraging when compared with the findings of a study in Sri Lanka (22) where out of 64 first year students, only 1 (1.6%) student was aware of all risk factors of hepatitis B including piercings, tattoos, transfusion of blood, and dental care visits, and 40.6% (n=52) of the students were unaware that contaminated blood, contaminated needles, unprotected sex with an infected person, and birth to an infected mother were all modes of hepatitis B transmission (p=0.000).

Most of the participants (56.1%, n= 196) in our study were between the ages of 15-19 years and were mostly in their first and second year of study. This raised the hope of improvement in their percentage knowledge of hepatitis B with progressing age, more so when backed by enabling curriculum of training. Our study supports this because we showed statistically significant association between percentage average knowledge of hepatitis B and age of participants (OR=0.77, 95% CI 0.70-0.84, p<0.0001), which implies that with a unit decrease in age of participant by a factor of 0.77, there is a corresponding decrease in knowledge of participant about hepatitis B. Hence, increased age will conversely be associated with increased knowledge of hepatitis B.

Our study also showed significant statistical associations of participants age (OR= 0.84, 95% CI 0.78-0.90; p=0.004), Ijaw tribe (OR=0.4, 95% CI: 0.24-0.66, p=0.034), and attendance of Federal Government College (OR=0.4, 95% CI 0.24-0.68, p=0.046). This implies that the odds of being aware of hepatitis B is decreased by a factor of 0.4 if participant was an Ijaw compared to being from other tribes. Also, the odds of being aware of hepatitis B is decreased by a factor of 0.4 if participant attended Federal Government College as against attending a Private College.

Similar to the statistically significant association between participants knowledge of hepatitis B and age, for every unit decrease in

the age of participant by a factor of 0.84, there would be a corresponding decrease in participants awareness about hepatitis B. This also raised the hope that this study population, dominated by youthful participants between the ages of 15-19 years of age and still in their junior classes of training, would likely gain remarkable awareness of hepatitis B before entering the clinical segment of their training where they would start interacting with infectious threats, including hepatitis B.

The lower odds of awareness of hepatitis B among Ijaw tribe (OR=0.4) and attendance of Federal Government College (OR= 0.4) cannot be readily explained. The Federal Government Colleges, also known as Unity Schools, were established and funded directly by the Federal Government of Nigeria, which provides them with serene environment for learning characterized by quality teachers, boarding facilities, laboratories, clinics, and well-articulated training curriculum that provides balanced knowledge for the students. It is therefore expected that the level of awareness of hepatitis B should be higher in these Colleges, hence the difficulty for us in explaining the reasons for the lower odds. There is however the possibility that the high standards of these schools at the time they were established may have been negatively affected by the socio-economic challenges of the country leading to lowering of training standards including those of knowledge and awareness of infectious diseases such as hepatitis B. One limitation of our study is the cross-sectional design which cannot establish cause-effect relationship between education/awareness levels and the predictors.

Conclusion:

The percentage average good knowledge of 47.2% and awareness of 34.2% for hepatitis B in our study are low. However, most of participants in the study were between the ages of 15-19 years and mostly in their first and second year of study. There is thus the hope of improvement in knowledge and awareness of hepatitis B with progression in age and year of training. Good knowledge and awareness of hepatitis B remained one of the pivotal strategies for the realization of the planned global elimination of the disease as a public health challenge by the World Health Organization on or before 2030.

The National Universities Commission (NUC) in conjunction with the Medical and Dental Council of Nigeria (MDCN) should review the current academic curricula of medical schools to promote amongst others, good knowledge and awareness of hepatitis B and other infectious diseases that the students would be exposed to in the course of their clinical skill acquisitions.

Introduction of a yearly campus seminar on infection prevention and control as part of the orientation programme for fresh students aimed at promoting knowledge and awareness of not only hepatitis B but infectious diseases generally is recommended. This will in the long term, institutionalise good knowledge and awareness of infectious diseases among medical students, well enough to safely guide them in the course of their training.

Contributions of authors:

The study was conceptualized by GIO and AAI. All authors were involved in the literature searches. USN and EUE wrote the manuscript. EUE and EL edited the final copy of the manuscript which was read and approved by all the authors.

Source of funding:

Authors received no external funding.

Conflict of interest:

Authors declared no conflict of interest

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Gbegbe et al. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (1): 38 - 47 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jan 2024; Vol.25 No.1
AJCEM/2300. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Antibiotic resistance profiles of uropathogenic bacterial isolates in Haut-Sassandra Region, Côte d'Ivoire from January 2019 to December 2022

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Abstract:

Background: The escalating issue of bacterial resistance is a profound universal peril. This looming crisis has evolved from a mere forecast to a tangible reality globally. Urinary tract infections (UTIs) significantly influence antibiotic prescriptions in primary care, thus crucially impacting the selective pressure and the emergence of antibiotic-resistant bacteria. A profound comprehension of the microorganisms involved in UTIs and their resistance patterns is crucial, particularly in Daloa city, Côte d'Ivoire. This research aims to review the antibiotic resistance profiles of uropathogens isolated from patients in the Regional Hospital Center (CHR) of Daloa, Côte d'Ivoire from January 2019 to December 2022.
Methodology: This was a descriptive cross-sectional study of 1,513 patients whose voided urine samples were received at the Bacteriology-Virology Laboratory of CHR for cyto-bacteriological examination and aerobic culture using standard microbiological protocols over a period of 4 years. Bacterial isolates were routinely identified by colony morphology, Gram staining reaction and conventional biochemical tests. The antibiotic susceptibility of the bacterial isolates was determined by the agar diffusion method and interpreted following the Antibiogram Committee of the French Society of Microbiology (CASFM) guidelines.
Results: Of the 1,513 patient urine samples examined, 246 (16.3%) were positive for microbial organisms, 216 (14.3%) were positive for significant bacterial isolates, 9 (0.6%) were positive for fungi, and 21 (1.4%) were positive for ova of *Schistosoma haematobium*. Among the samples with significant bacteriuria, 91.2% were due to Gram-negative bacilli, 5.9% to Gram-positive cocci, and 2.9% to Gram-negative cocci. *Escherichia coli* was the most predominant bacterial pathogen, accounting for 73.2% of the isolates. Antibiotic susceptibility testing showed high *in vitro* resistance of the bacterial isolates to tested antibiotics, with Enterobacteriaceae exhibiting resistance rate between 56.0% for nalidixic acid (NAL) and 67.0% for amoxicillin/clavulanic acid (AMC). *Pseudomonas aeruginosa* isolates exhibited 50.0% resistance rate to ceftazidime (CAZ), ciprofloxacin (CIP), and ticarcillin (TIC) while *Staphylococcus* isolates demonstrated 100.0% resistance rate to ofloxacin (OFX), clindamycin (CMN), erythromycin, trimethoprim/sulfamethoxazole (SXT), and fusidic acid (FA). The extended-spectrum beta-lactamase (ESBL)-producing isolates were identified in 15.1% of the Enterobacteriaceae.
Conclusion: The high prevalence of antibiotic resistant bacterial isolates from significant bacteriuria in our study highlights the pressing need for the formulation and implementation of strategies to address this potential public health menace. The findings of our study may be useful for healthcare authorities to plan strategic interventions that will assist in optimizing the management of bacteriuria and UTI in the city of Daloa.

Keywords: Urinary tract infection, bacterial resistance, antibiotic, ESBL, Enterobacteriaceae

Received Oct 03, 2023; Revised Dec 13, 2023; Accepted Dec 17, 2023

Profils de résistance aux antibiotiques des isolats bactériens uropathogènes dans la région du Haut-Sassandra, Côte d'Ivoire de janvier 2019 à décembre 2022

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Résumé:

Contexte: Le problème croissant de la résistance bactérienne constitue un grave péril universel. Cette crise imminente est passée d'une simple prévision à une réalité tangible à l'échelle mondiale. Les infections des voies urinaires (IVU) influencent considérablement les prescriptions d'antibiotiques en soins primaires, ayant ainsi un impact crucial sur la pression sélective et l'émergence de bactéries résistantes aux antibiotiques. Une compréhension approfondie des micro-organismes impliqués dans les infections urinaires et de leurs modèles de résistance est cruciale, en particulier dans la ville de Daloa, en Côte d'Ivoire. Cette recherche vise à examiner les profils de résistance aux antibiotiques des uropathogènes isolés chez les patients du Centre Hospitalier Régional (CHR) de Daloa, Côte d'Ivoire de janvier 2019 à décembre 2022.
Méthodologie: Il s'agit d'une étude transversale descriptive portant sur 1513 patients dont les échantillons d'urine vidés ont été reçus au Laboratoire de Bactériologie-Virologie du CHR pour examen cyto-bactériologique et culture aérobie selon des protocoles microbiologiques standards sur une période de 4 ans. Les isolats bactériens ont été systématiquement identifiés par la morphologie des colonies, la réaction de coloration de Gram et les tests biochimiques conventionnels. La sensibilité aux antibiotiques des isolats bactériens a été déterminée par la méthode de diffusion sur gélose et interprétée selon les directives du Comité Antibiogramme de la Société Française de Microbiologie (CASFM).
Résultats: Sur les 1513 échantillons d'urine de patients examinés, 246 (16,3%) étaient positifs pour les organismes microbiens, 216 (14,3%) étaient positifs pour des isolats bactériens significatifs, 9 (0,6%) étaient positifs pour des champignons et 21 (1,4%) étaient positifs pour ovules de *Schistosoma haematobium*. Parmi les échantillons présentant une bactériurie significative, 91,2% étaient dus à des bacilles à Gram négatif, 5,9% à des coques à Gram positif et 2,9% à des coques à Gram négatif. *Escherichia coli* était le pathogène bactérien le plus prédominant, représentant 73,2% des isolats. Les tests de sensibilité aux antibiotiques ont montré une résistance in vitro élevée des isolats bactériens aux antibiotiques testés, les Enterobacteriaceae présentant un taux de résistance compris entre 56,0% pour l'acide nalidixique (NAL) et 67,0% pour l'amoxicilline/acide clavulanique (AMC). Les isolats de *Pseudomonas aeruginosa* présentaient un taux de résistance de 50,0% à la ceftazidime (CAZ), à la ciprofloxacine (CIP) et à la ticarcilline (TIC), tandis que les isolats de *Staphylococcus* présentaient un taux de résistance de 100,0% à l'ofloxacine (OFX), la clindamycine (CMN), l'érythromycine, le triméthoprime/ sulfaméthoxazole (SXT) et l'acide fusidique (FA). Les isolats producteurs de bêta-lactamases à spectre étendu (BLSE) ont été identifiés chez 15,1% des Enterobacteriaceae.
Conclusion: La forte prévalence d'isolats bactériens résistants aux antibiotiques provenant d'une bactériurie importante dans notre étude souligne le besoin urgent de formuler et de mettre en œuvre des stratégies pour faire face à cette menace potentielle pour la santé publique. Les résultats de notre étude pourraient être utiles aux autorités sanitaires pour planifier des interventions stratégiques qui contribueront à optimiser la gestion de la bactériurie et des infections urinaires dans la ville de Daloa.

Mots-clés: Infection des voies urinaires, résistance bactérienne, antibiotique, BLSE, Enterobacteriaceae

Introduction:

The incidence of urinary tract infections (UTIs) caused by multidrug-resistant pathogens is escalating at an alarming rate globally (1,2), as these infections lead to extensive antibiotic prescriptions in primary care, contributing significantly to the selection pressure of bacterial resistance to antibiotics (3). The rising rates of antibiotic resistance have resulted in substantial morbidity, mortality, and increased healthcare costs (4). Economically, bacterial resistance leads to extension of hospital stays, necessitating more intensive care and costlier medications (5). Moreover, in low-income countries, the lack of knowledge about proper use, non-adherence to prescription protocols, and the abusive use of antibiotics often lead to increased phenomena of bacterial resistance to these molecules (6).

Microbial resistance to antibiotics is one of the top ten global threats to public health. According to the World Health Organization (WHO), sub-Saharan Africa is the most affected by this problem, where in 2019, 1.27 million deaths were directly attributable to bacterial resistance (7). Given this situation, it is imperative to deepen the understanding of the epidemiology and antibiotic resistance of uropathogens to ensure rational and regular surveillance of their sensitivity to antibiotics to enhance clinical outcomes. Therefore, our aim is to analyze the antibiotic resistance profiles of uropathogens from patients at the Regional Hospital Center (CHR) of Daloa, Côte d'Ivoire over a four-year period (2019 to 2022).

Materials and method:

Study setting and design:

This research was a descriptive cross-sectional study conducted in the Bacteriology-Virology Laboratory of the Regional Hospital Center (CHR) of Daloa (Côte d'Ivoire), over a period of 4 years (January 2019 to December 2022).

Urine collection and microbiological analysis:

From 2019 to 2022, a total of 1,513

urine samples were received and routinely processed at the Bacteriology-Virology Laboratory of CHR in Daloa, Côte d'Ivoire. Upon reception, the initial analysis included a macroscopic examination to identify characteristics such as appearance (clear, cloudy, purulent, haematuria, presence of sediment). This was followed by microscopic examination, which entailed a wet mount to detect cells (leukocytes, erythrocytes, epithelial cells), bacteria, ova of parasites (e. g. nematodes), yeasts, and nitrites, along with direct Gram stain.

Urine samples exhibiting significant leukocyturia were cultured using the quantitative calibrated loop method. For this, 10 µl (0.01 ml) of the sample was spread onto agar media and incubated at 37°C for 18 to 24 hrs. Nutrient agar or Uriselect medium were used for the general enumeration of urinary pathogens. The isolation of different species was facilitated by selective media such as Cetrimide agar for *Pseudomonas*, Chapman medium for *Staphylococcus*, Sabouraud agar with chloramphenicol for fungal isolation, eosin methylene blue (EMB) agar for Enterobacteriaceae, and Hektoen agar for *Salmonella*.

After culture incubation, quantitative bacterial colony counting was conducted. The interpretation of urine cultures adhered to the criteria defined by Kass in 1956, which includes a homogeneous culture (monomicrobial infection), significant leukocyturia ($\geq 10^4$ leukocytes/ml of urine), and significant bacteriuria ($\geq 10^5$ bacteria/ml of urine). Bacterial counts of 10^3 CFU/ml or 10^4 CFU/ml was also indicative of infections, particularly in samples from non-catheterized paraplegic men, women with cystitis, cases of high diuresis under antibiotic treatment or when slow-growing bacteria are present.

Following bacterial enumeration, Gram stain was performed on the colonies. Conventional biochemical such as oxidase and catalase were conducted on colonies from the ordinary agar. Enterobacteriaceae were characterized using the reduced Le Minor tray, *Staphylococcus* isolates were identified by DNase, mannitol fermentation, and motility tests, *Enterococcus* by haemolysis pattern, and *Pseudomonas* by oxidase and motility tests.

Antibiotic sensitivity testing:

From a 24-hour culture of the test isolates, a bacterial suspension was prepared in 2 ml of 0.85% NaCl solution to achieve the turbidity equivalent to 0.5 McFarland standard. The inoculum density was adjusted using a densitometer. Subsequently, Mueller Hinton (MH) agar was inoculated, followed by the placement of antibiotic-impregnated disks (as listed in Table 1). After incubation at 37°C in aerobiosis for 18 to 24 hrs, readings were taken, and results were interpreted according to the Antibiogram Committee of the French Society of Microbiology (CASFM) guidelines.

Phenotypic confirmation of extended-spectrum β-lactamase (ESBL) production:

Phenotypic confirmation of isolates producing extended-spectrum β-lactamases (ESBLs) was carried out using the double disc synergy test of amoxicillin-clavulanic acid with either of cefotaxime, ceftriaxone, or aztreonam arranged on Müeller-Hinton agar that has been pre-inoculated with the test isolates. The characteristic "champagne cork" or "funnel" zones appearing between discs of amoxicillin-clavulanic acid and the cephalosporin phenotypically confirms an isolate to be ESBL producer (8).

Data analysis:

Data were analysed using descriptive statistics on the R software (version R64 × 4.3.1), with *p* values of less than 0.05 considered statistically significant.

Results:

Prevalence of pathogens isolated in urine at CHR of Daloa:

In total, 1,513 urinary samples were received at the Bacteriology-Virology Laboratory of the CHR of Daloa from 2019 to 2022. Among these, microbial pathogens were recovered from 246 samples, giving an overall prevalence of positive urine cultures (PUCs) of 16.3%, while significant bacteria were isolated from 216 (14.3%) samples, fungi (*Candida* sp.) from 9 (0.6%) samples, and oval of nematodes (*Schistosoma haematobium*) from 21 (1.4%) samples (Fig 1).

Table 1: List of antibiotics tested on the bacterial isolates

Antibiotic class	Antibiotic name	Abbreviation	Content (µg)
Aminoglycosides	Amikacin	AKN	30
	Gentamicin	GMN	10
Beta-lactams	Cefuroxime	CXM	30
	Amoxicillin	AMX	25
	Amoxicillin/clavulanic acid	AMC	20/10
	Ticarcillin	TIC	75
	Cefepime	FEP	30
	Cefotaxime	CTX	30
	Cefoxitin	FOX	30
	Ceftriaxone	CRO	30
	Ceftazidime	CAZ	10
	Aztreonam	ATM	30
Carbapenem	Imipenem	IPM	10
Fluoroquinolones	Ciprofloxacin	CIP	5
	Nalidixic acid	NAL	30
	Ofloxacin	OFX	5
Macrolide-Lincosamide	Erythromycin	ERY	15
	Clindamycin	CMN	2
Sulphonamides	Trimethoprim/sulfamethoxazole	SXT	1.25/23.75
Others	Fosfomycin	FOS	200
	Fusidic acid	FA	10
	Rifampicin	RA	5

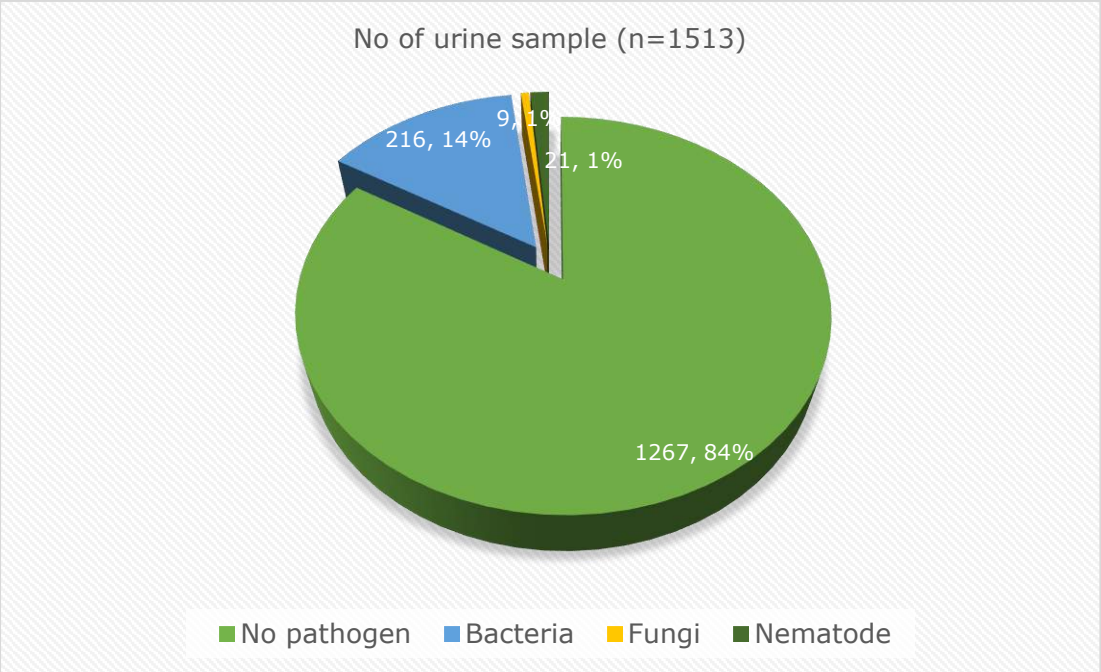

Fig 1: Prevalence of microbial pathogens from urinary tract infections in Daloa (2019 to 2022)

Distribution of bacteria species isolated from urine by Gram stain and biochemical characteristics:

Based on Gram staining reaction, significant bacteriuria was caused by Gram-negative bacilli (91.2%), Gram-positive cocci (5. 9%) and Gram-negative cocci (2.9%) (Fig 2).

Fig 3 depicts the frequency of bacterial isolates, which shows that *Escherichia coli* was the most frequently isolated with 73.2%, followed by unknown Enterobacteriaceae (4.5%), *Klebsiella* sp. (4.5%), *Klebsiella pneumoniae* (4.0%) and other bacterial isolates (13.8%).

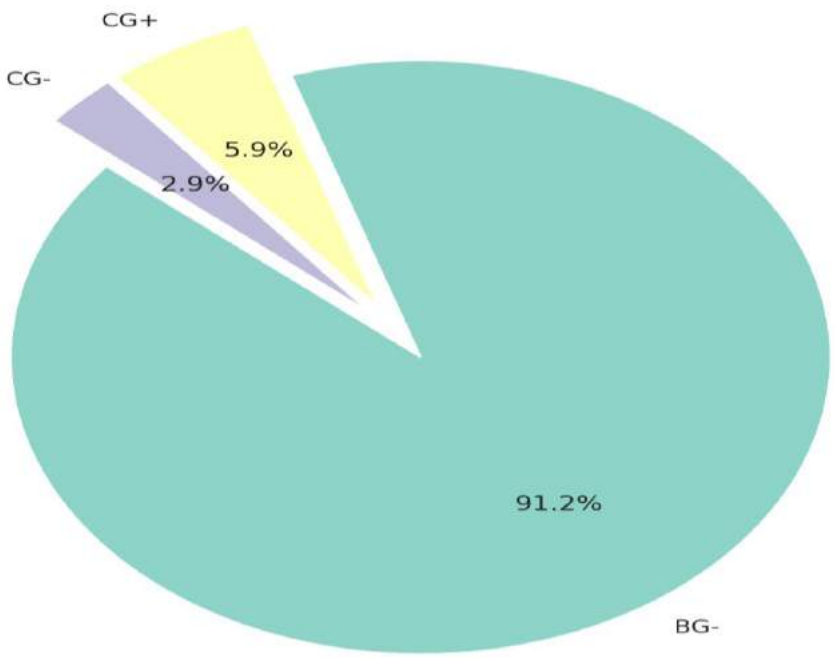

Fig 2: Frequency distribution of bacteria isolates based on Gram staining reaction

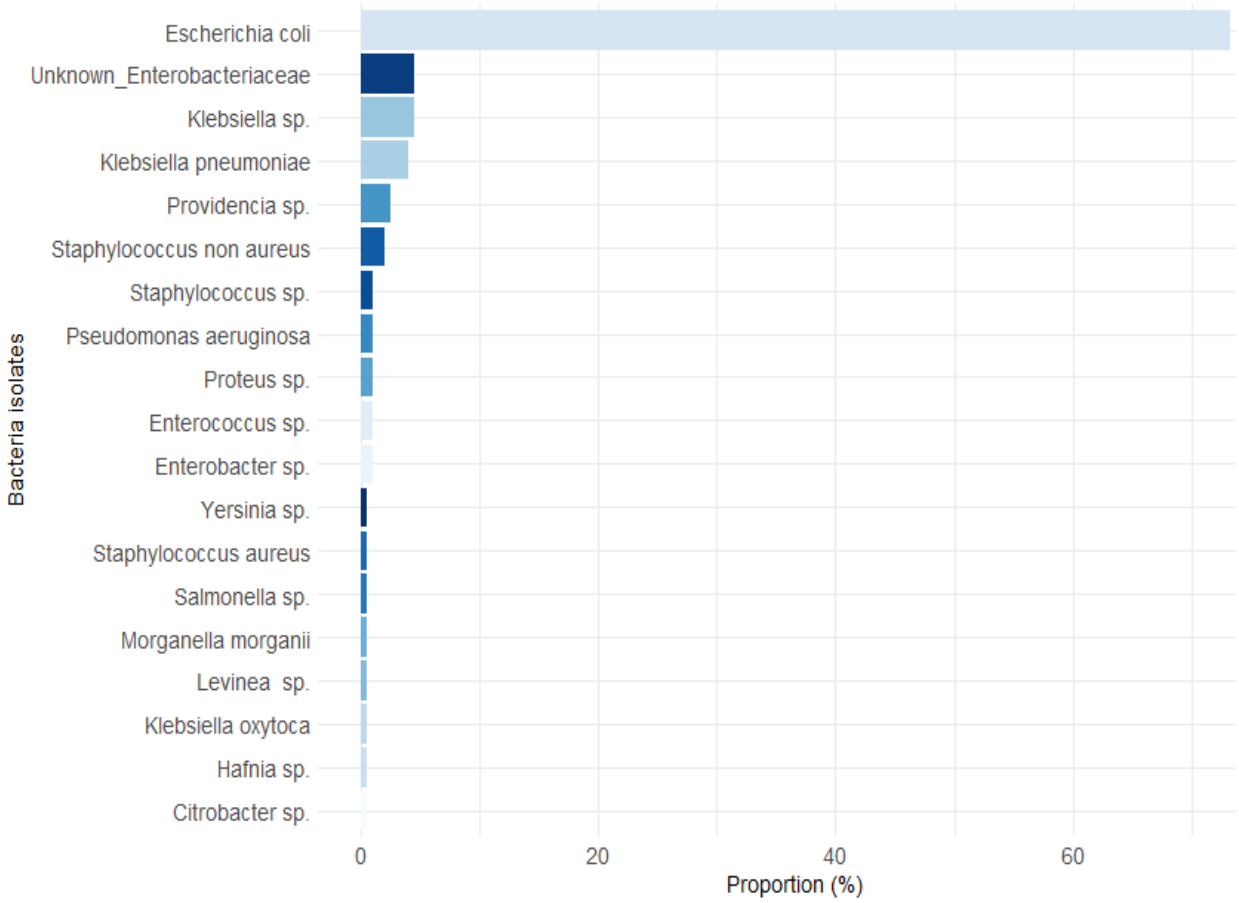

Fig 3: Frequency distribution of bacterial isolates of urinary tract infections.

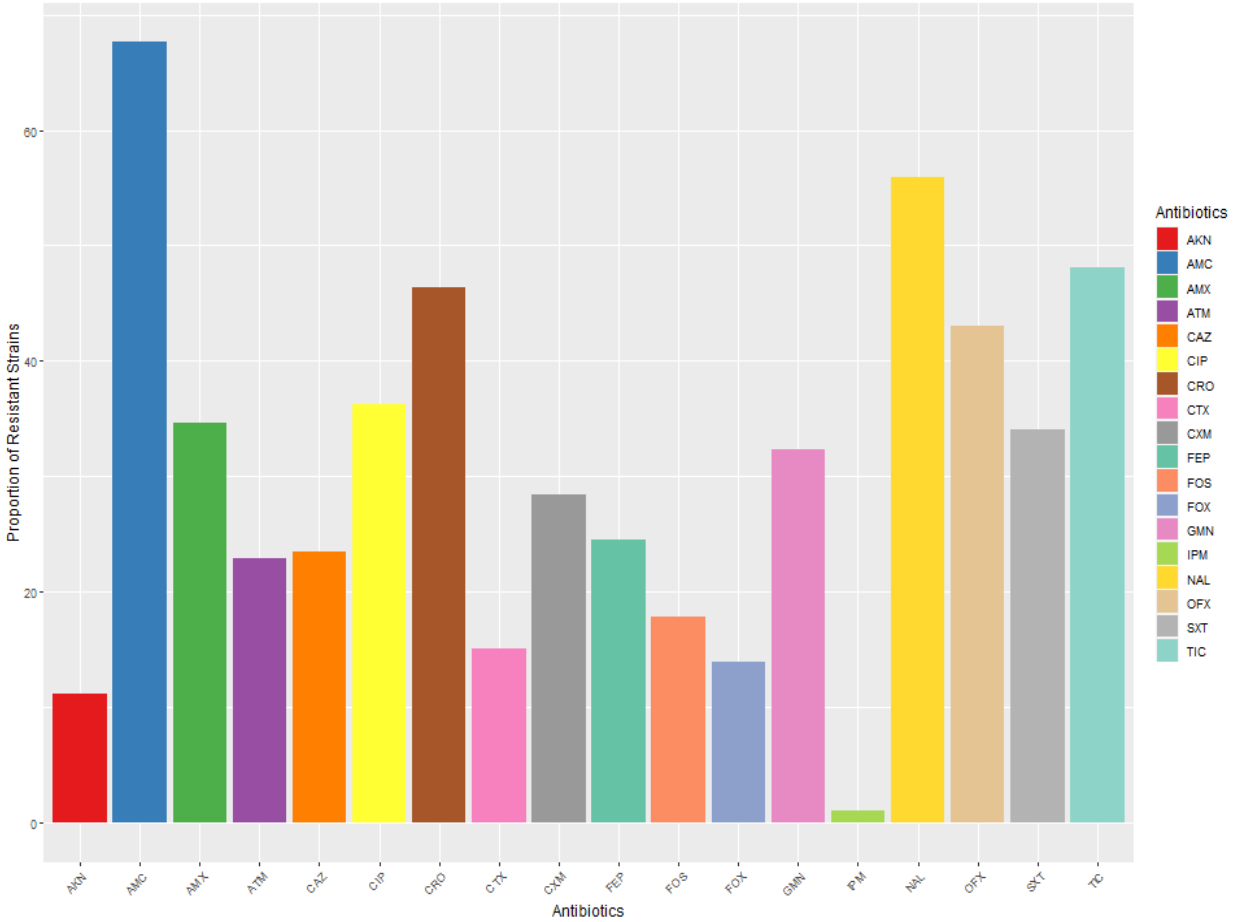

Fig 4: Resistance of Enterobacteriaceae to standard antibiotics.

Antibiotic susceptibility results of Enterobacteriaceae:

Fig 4 showed that the Enterobacteriaceae (n=179) demonstrated *in vitro* resistance to most of the antibiotics tested. However, carbapenem (represented by imipenem) showed high *in vitro* inhibitory activity (with 95% sensitivity and only 5% resistance rate) against the isolates over the 4-year period. The highest resistance rates were recorded for amoxicillin/clavulanic acid (AMC) and nalidixic acid (NAL) with resistance rates of 67.0% and 56.0% respectively.

Resistance of Enterobacteriaceae isolates of urinary tract infections to standard antibiotics:

The results of antimicrobial susceptibility showed high resistance rates to all tested antibiotics. Specifically, *Enterobacter* species were 100.0% resistant to fosfomycin (FOS), amoxicillin/clavulanic acid (AMC), trimethoprim/sulfamethoxazole (SXT), and cefoxitin (FOX). However, they were 100.0% sensitive to ciprofloxacin (CIP), cefepime (FEP), gentamicin (GMN), imipenem (IPM), and cefotaxime (CTX). For *E. coli* isolates, resistance was pronounced to ticarcillin (TIC) with a rate of 91.0%, amoxicillin (AMX) 85.0%, trimethoprim/sulfamethoxazole (SXT) 84.0%, and nalidixic acid (NAL) 82%. However, they were considerably sensitive to IPM (99.0%).

For *Klebsiella pneumoniae*, resistance rate to AMX and TIC was 100.0%, while to GMN and FOS, the rates were 75% and 67% respectively. Conversely, resistance rate to IMP, aztreonam (ATM), and CTX was 0%. The resistance rate of *Klebsiella* sp., was 100.0% to CXM, TIC, SXT, AMX, and ceftazidime (CAZ) but were 100.0% sensitive to IMP, amikacin (AKN) and cefuroxime (CTX).

For *Proteus* sp., 50.0% of the isolates were resistant to NAL, TIC and SXT. For *Providencia* sp., 100.0% were resistant to CIP, TIC, SXT and AMX. For unknown Enterobacteriaceae, 100.0% were resistant to AMX and CTX, and 80.0% to OFX. In contrast, they were 100% sensitive to IMP, GMN, and AKN.

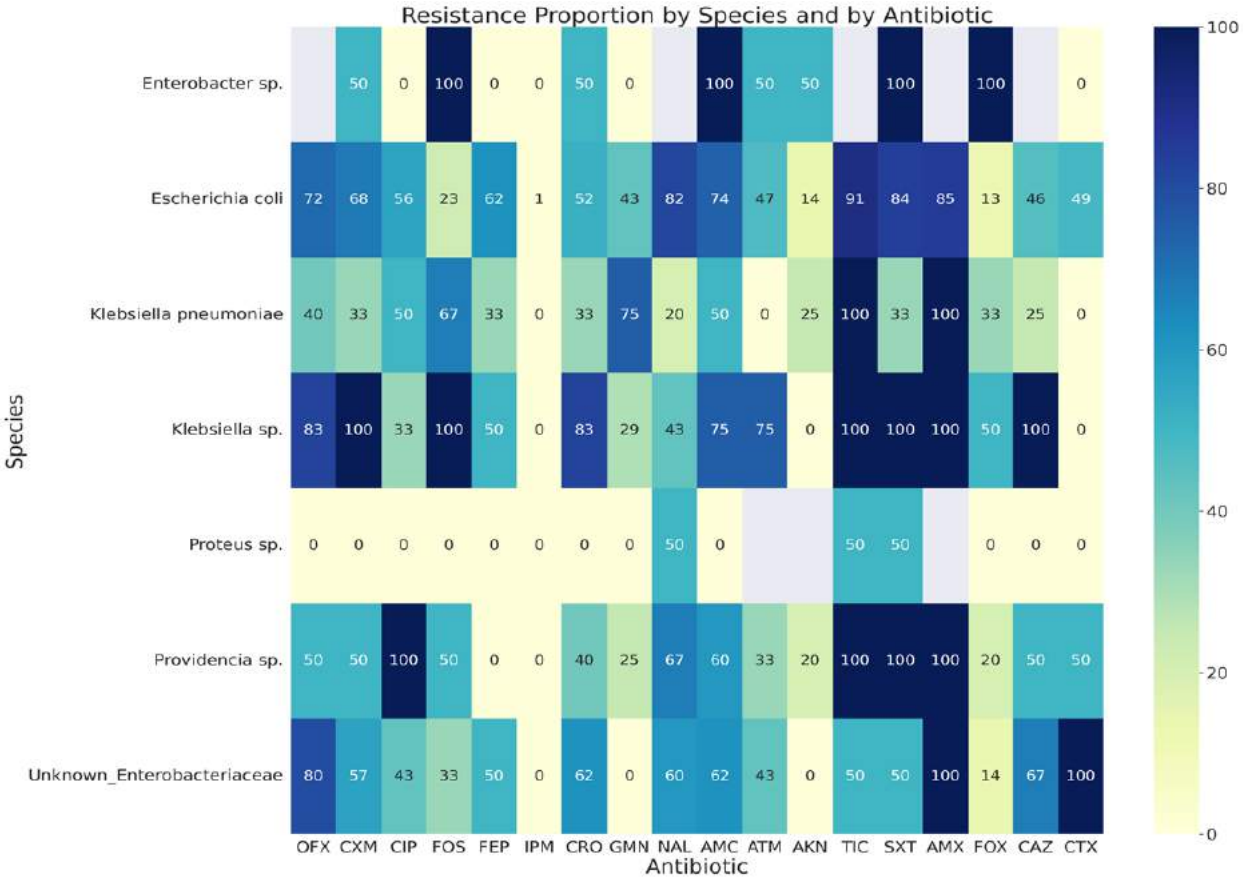

Fig 5: Resistance of Enterobacteriaceae isolates from urinary infections to common antibiotics.

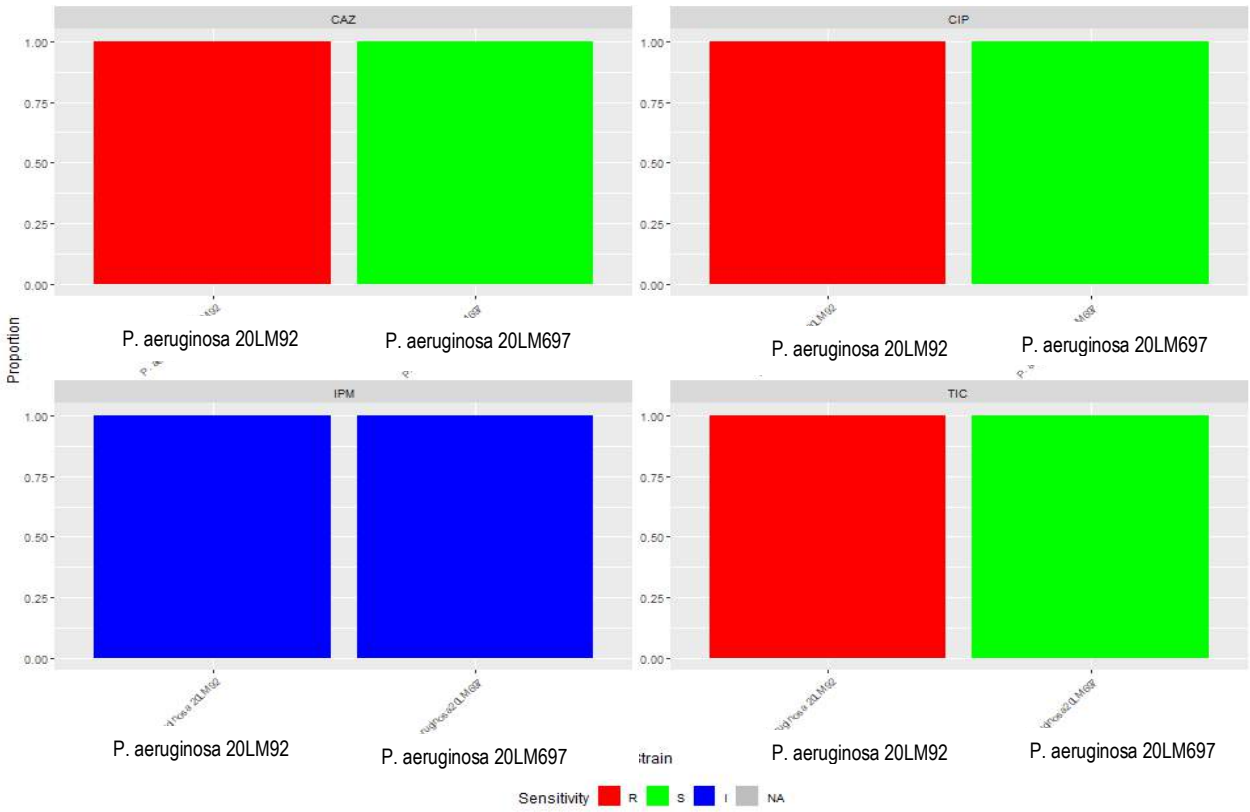

Fig 6: Resistance of *Pseudomonas aeruginosa* isolates to standard antibiotics.

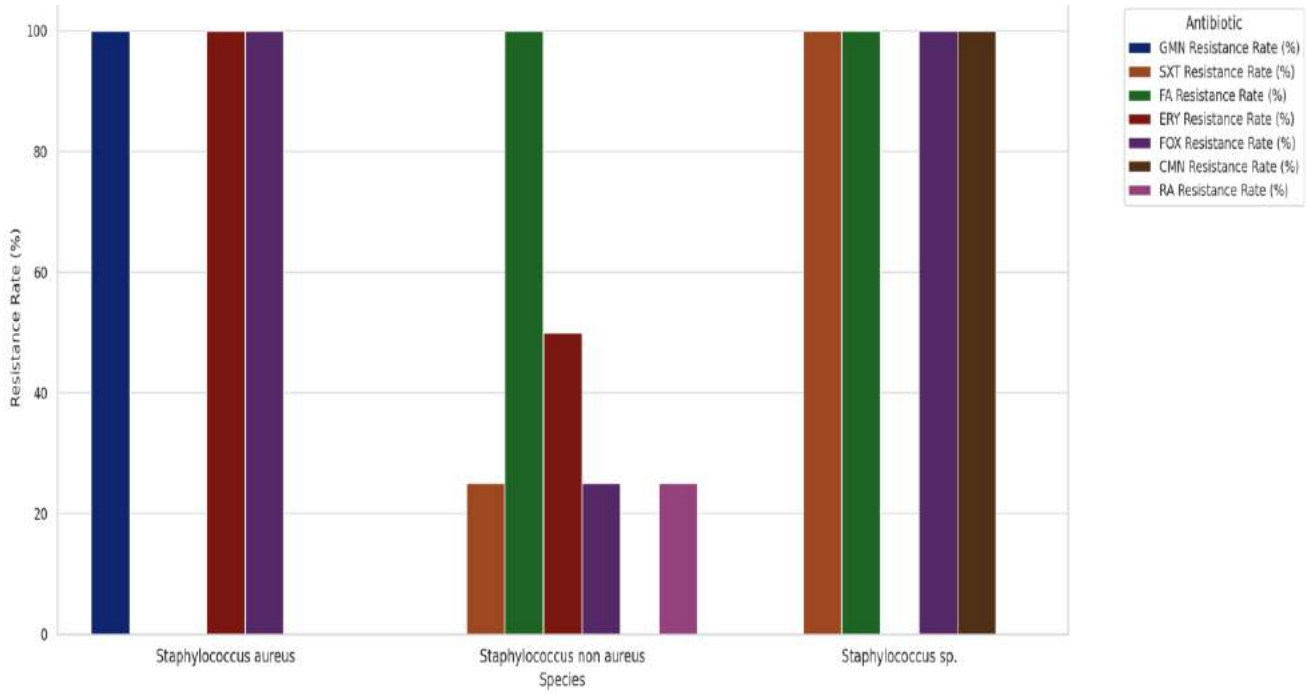

Fig 7: Resistance of *Staphylococcus* isolates to standard antibiotics.

Resistance of *Pseudomonas aeruginosa* isolates to standard antibiotics:

Fig 6 shows the resistance patterns of *Pseudomonas aeruginosa* isolates (n=2) to standard antibiotics, with 50.0% of the isolates resistant to ceftazidime (CAZ), ciprofloxacin (CIP), and ticarcillin (TIC) and 100.0% of the isolates with intermediate resistance to imipenem (IPM).

Resistance of *Staphylococcus* isolates to standard antibiotics:

The result of the antibiotic sensitivity test of the *Staphylococcus* isolates is presented in Fig 7, which showed that 100.0% of the isolates were resistant to erythromycin (ERY), clindamycin (CMN), ofloxacin (OFX), and trimethoprim/sulfamethoxazole (SXT). Also, the non-aureus *Staphylococcus* isolates were resistant to fusidic acid (FA) and ERY with 100.0% and 66.7% rates respectively. For *Staphylococcus aureus* isolates, they were 100.0% resistant to fusidic acid (FA), gentamicin (GMN), cefoxitin (FOX) and erythromycin (ERY).

Prevalence of observed resistance phenotypes resulting from antibiotic resistance in isolated bacterial isolates:

Over the 4-year period, the antibiotic sensitivity and resistance tests performed at the Bacteriology-Virology Laboratory at CHR of Daloa identified a single resistance phenotype, extended-spectrum beta-lactamase (ESBL)-producing strains among the Enterobacteriaceae isolates. This phenotype was identified in 27 of the 179 Enterobacteriaceae isolates, representing a rate of 15.1%. *Escherichia coli* accounted for 81.5% (22/27) of the ESBL-producing isolates, 14.8% (4/27) were unidentified Enterobacteriaceae, and 3.7% (1/27) were *Klebsiella* sp.

Discussion:

Urinary tract infection is a condition characterized by the inflammation of the urinary pathways resulting from abnormal colonization of detrimental microorganisms (9). Discerning the microbial aetiology and resistance patterns of these microorganisms to conventional antibiotics is pivotal to guide clinical decisions more accurately. This study, spanning four years (2019 to 2022), showed that UTIs in Daloa are predominantly bacterial in nature, with significant bacteriuria accounting for 14.3% (216/1513) of the UTI cases, followed by infections due to *Schistosoma haematobium* (1.4%,21/1513) and candiduria (0.6%,9/1513).

The predominance of bacteriuria in UTI as observed in our study has been reported by several authors (10,11). This is attributed to several factors including age, frequent sexual activity, inadequate or excessive hygiene, menopause, and homosexuality (12). The results indicated a predominant incidence of Gram-negative bacilli accounting for 91.2% of bacteriuria, with *E. coli* being the most prevalent species with 73.2%. The high prevalence of *E. coli* is corroborated by other studies (13), and could be attributed to specific factors such as presence of bacterial adhesins in *E. coli* which facilitate its binding to the urinary epithelium (14,15].

The results of antibiotic susceptibility test for the Enterobacteriaceae in particular

revealed significant resistance to all tested antibiotics, with the highest resistance to amoxicillin/clavulanic acid (67,0%), followed by nalidixic acid (56.0%), ticarcillin (48.0%), and ceftriaxone (47.0%). *Escherichia coli* showed pronounced resistance to ticarcillin (91.0%), amoxicillin (85.0%) and trimethoprim/sulfamethoxazole (84.0%). *Enterobacter* sp. isolates also exhibited profound resistance (100%) to several antibiotics including fosfomycin, amoxicillin/clavulanic acid, trimethoprim/sulfamethoxazole and cefoxitin. The non-Enterobacteriaceae isolates such as *Pseudomonas aeruginosa* also exhibited 50.0% resistance to ceftazidime, ciprofloxacin and ticarcillin, and *Staphylococcus* sp. showed 100.0% resistance rate to ofloxacin, clindamycin, erythromycin and trimethoprim/sulfamethoxazole, while the non-aureus *Staphylococcus* isolates showed 100.0% resistance to fusidic acid and 66.7% to erythromycin. These high resistance rates could be attributed to the selective pressure from extensive and often inappropriate use of broad-spectrum antibiotics in both hospital and community settings (16).

Our study primarily identified ESBL resistance phenotype among the Enterobacteriaceae, with a prevalence of 15.1%. *Escherichia coli* alone accounted for 81.5% of the ESBL-producing Enterobacteriaceae, while other unidentified Enterobacteriaceae isolates accounted for 14.8% and *Klebsiella* sp. accounted for 3.7%, findings that are in agreement with reports of other studies (17,18,19). Our study provides crucial insights into the understanding of the microbial aetiology of UTIs and their antibiotic resistance patterns to available antibiotics in Daloa, which can assist healthcare authorities in devising effective strategies for the management of significant bacteriuria and UTIs in the region.

Conclusion:

The prevalence of significant bacteriuria in our study over a 4-year period in Daloa, Cote D'Ivoire, is 14.3%, while candiduria and urinary schistosomiasis constituted 0.6% and 1.4% respectively. Bacteriuria was predominantly caused by Gram-negative bacilli (91.2%), with *E. coli* being the most prevalent isolate (73.2%). The ESBL phenotype was detected in 15.1% of the Enterobacteriaceae, with *E. coli* comprising 81.5%, *Klebsiella* sp. 3.7%, and unknown Enterobacteriaceae 14.8%. The *in vitro* resistance rates of the isolates to standard antibiotics were high.

The findings of our study may be useful for healthcare authorities to plan interventions that can assist in optimizing the management of bacteriuria and UTI in the city of Daloa.

Contributions of authors:

GDA and ADM conceived the study idea; GDA, NNP and MS were involved in the study design; GDA, NNP, MS, GN and ADM were involved in analysis and interpretation of data; GDA and NNP produced the manuscript draft; and GN and ADM critically reviewed the manuscript for intellectual content. All authors read and approved the final manuscript submitted.

Source of funding:

No funding was received for the study.

Conflict of interest:

Authors declare no conflict of interest.

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Bassey et al. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (1): 48 - 59 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jan 2024; Vol.25 No.1
AJCEM/2309. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Prevalence of symptomatic significant bacteriuria and associated risk factors among patients attending major hospitals in Calabar, Nigeria

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Abstract:

Background: Urinary tract infections (UTIs) are among the most encountered bacterial infections of humans and affect both male and female of all age groups, resulting in high mortality, without proper management. This study aimed to assess the prevalence, aetiological agents, and factors associated with symptomatic significant bacteriuria/UTI among patients attending selected hospitals in Calabar metropolis, Nigeria.
Methodology: This was a cross-sectional study of 240 patients with suspected UTI, from whom mid-stream voided urine samples were collected for culture on Cystine Lactose Electrolyte Deficient (CLED) agar. Uropathogens growth on the culture media were characterized using conventional microbiological and biochemical tests, and confirmed with API® 20E and 20NE (BioMérieux) identification system. Data on socio-demographic, clinical symptoms and potential risk factors were obtained using structured questionnaire. Pearson Chi-square was employed to determine association between categorical variables with $p<0.05$ considered statistically significant.
Results: Of all the urine samples collected from the 240 patients, 13 samples were contaminated during collection, leaving 227 samples for analysis. Sixty-five (28.6%) of the 227 patients had symptomatic significant bacteriuria. Previous history of UTI (OR=2.863, 95% CI=1.582-5.180, p=0.008), contraceptive use (OR=3.469, 95% CI=1.446-8.320, p=0.012), pregnancy (OR=9.94, 95% CI=3.867-25.571, $p<0.0001$) and history of urinary catheterization (OR=4.417, 95% CI=1.024-19.053, p=0.045) were significantly associated with prevalence of symptomatic significant bacteriuria/UTI. *Klebsiella pneumoniae* (23.1%) was the most predominant isolate, followed by coagulase-negative staphylococci (CoNS) (16.9%) and *Escherichia coli* (12.3%).
Conclusion: The prevalence of symptomatic significant bacteriuria among patients attending selected hospitals in Calabar, Nigeria, was 28.6% (65/227), with *K. pneumoniae* and CoNS being the major aetiologic agents. Our study shows that previous history of UTI, pregnancy, history of urinary catheterization, contraceptive use, dysuria and occupation were significantly associated with symptomatic significant bacteriuria/UTI ($p<0.05$). Routine screening for UTI is recommended for pregnant women, patients with dysuria, previous episodes of UTI, and catheterized patients.

Keywords: Bacteriuria, prevalence, significant, symptomatic, urinary

Received Oct 31, 2023; Revised Dec 18, 2023; Accepted Dec 19, 2023

Prévalence de la bactériurie symptomatique significative et des facteurs de risque associés chez les patients fréquentant les principaux hôpitaux de Calabar, Nigeria

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Résumé:

Contexte: Les infections des voies urinaires (IVU) font partie des infections bactériennes les plus rencontrées chez l'homme et touchent à la fois les hommes et les femmes de tous les groupes d'âge, entraînant une mortalité élevée, sans prise en charge appropriée. Cette étude visait à évaluer la prévalence, les agents étiologiques et les facteurs associés à une bactériurie/IVU symptomatique significative chez les patients fréquentant des hôpitaux sélectionnés dans la métropole de Calabar, au Nigeria.
Méthodologie: Il s'agissait d'une étude transversale portant sur 240 patients suspectés d'infection urinaire, chez lesquels des échantillons d'urine mictionnés à mi-jet ont été collectés pour être cultivés sur une gélose cystine lactose déficiente en électrolytes (CLED). La croissance des uropathogènes sur les milieux de culture a été caractérisée à l'aide de tests microbiologiques et biochimiques conventionnels, et confirmée par le système d'identification API® 20E et 20NE (BioMérieux). Les données sur les symptômes sociodémographiques, cliniques et les facteurs de risque potentiels ont été obtenues à l'aide d'un questionnaire structuré. Le chi carré de Pearson a été utilisé pour déterminer l'association entre les variables catégorielles avec $p<0,05$ considéré comme statistiquement significatif.
Résultats: Sur tous les échantillons d'urine prélevés sur les 240 patients, 13 échantillons ont été contaminés lors du prélèvement, laissant 227 échantillons pour analyse. Soixante-cinq (28,6%) des 227 patients présentaient une bactériurie symptomatique significative. Antécédents d'infection urinaire (OR=2,863, IC à 95%=1,582-5,180, p=0,008), utilisation de contraceptifs (OR=3,469, IC à 95%=1,446-8,320, p=0,012), grossesse (OR =9,94, 95% IC=3,867-25,571, $p<0,0001$) et les antécédents de cathétérisme urinaire (OR=4,417, IC à 95% =1,024-19,053, p=0,045) étaient significativement associés à la prévalence des bactériurie/IVU symptomatique significative. *Klebsiella pneumoniae* (23,1%) était l'isolat le plus prédominant, suivi des staphylocoques à coagulase négative (CoNS) (16,9%) et d'*Escherichia coli* (12,3%).
Conclusion: La prévalence de la bactériurie symptomatique significative parmi les patients fréquentant certains hôpitaux de Calabar, au Nigéria, était de 28,6% (65/227), *K. pneumoniae* et CoNS étant les principaux agents étiologiques. Notre étude montre que les antécédents d'infection urinaire, de grossesse, de cathétérisme urinaire, d'utilisation de contraceptifs, de dysurie et d'occupation professionnelle étaient significativement associés à une bactériurie/IVU symptomatique significative ($p<0,05$). Le dépistage systématique des infections urinaires est recommandé pour les femmes enceintes, les patients souffrant de dysurie, d'épisodes antérieurs d'infection urinaire et les patients cathétérisés.

Mots clés: Bactériurie, prévalence, significative, symptomatique, urinaire

Introduction:

Urinary tract infection (UTI) is any infection that occurs along the length of the urinary tract. It is characterized by the presence of bacteria in a supposedly sterile urinary tract, resulting in an increased bacterial load (often greater than 10^5/ml) in voided urine sample (1). Urinary tract infections are widespread and affect a large proportion of the human population. Approximately, 150 million people are affected by UTI every year worldwide, with an estimated cost of $5 billion each year in the United States (2).

Although other microbial groups cause UTI, the predominant organisms responsible for UTI are members of the Enterobacteriaceae, with *Escherichia coli* accounting for over 80% of cases (3,4). Among the Gram-positive bacteria, *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) are mostly implicated in community acquired UTIs. Fungi and viruses rarely cause UTI, however yeast, especially *Candida albicans*, are occasionally recovered from catheterized and/or immunocompromised patients (1). Other bacterial isolates implicated include *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterobacter cloacae*, and *Enterococcus faecalis* (2,3).

The risk factors for UTI vary with geographical locations and country. Gender, personal hygiene, prostate problems, compromised immunity, diabetes mellitus, use of spermicidal contraception and urinary catheterization are some of the risk factors for UTI (4). Most cases of UTI are seen in women due to their short urethra, proximity of the rectum to the urethra, change in vaginal pH due to depletion of the commensal bacteria, hormonal imbalance occasioned by menstrual flow and/ or pregnancy (5).

Antibiotic resistance among uropathogens is a consistent problem making clinical management of the disease challenging (6) and our previous study showed that this influence the prevalence of UTIs across different geographical areas. Several studies in different sub-Saharan Africa locations show variations in incidence of UTIs as well as the causative organisms (7-10). Mwang'onde and Mchami (4) reported a median prevalence rate of 32.1% in sub-Saharan Africa and the commonest associated causative organism was *E. coli.* Therefore, this current study was conducted to determine the prevalence, aetiological agents and factors associated with bacterial UTIs among patients attending selected hospitals in Calabar metropolis, Cross River State, Nigeria.

Materials and method:

Study setting:

This study was conducted in Calabar, the capital of Cross River State, located in Southern Nigeria. Calabar is administratively divided into Calabar Municipal and Calabar

South Local Government Areas. It is located between latitudes 8°11'21" and 8°27'00" East of the Meridian and between latitudes 04° 45'30" and 05°08'30" North of the Equator. Calabar metropolis has two major rivers: The Great Kwa River and Calabar River.

The city has a total land area of 406 square kilometers (11). According to the 2006 Nigeria Census Report, Calabar Metropolis had a total population of 375,196 (12). However, the rate of urbanization has greatly impacted on the population of the city (11). This population is primarily served by three hospitals; the University of Calabar Teaching Hospital (UCTH), the Nigeria Naval Reference Hospital (NNRH) and General Hospital Calabar (GHC).

Study design:

A descriptive cross-sectional study was conducted between September and December 2021 to determine the prevalence and associated risk factors of bacterial UTIs among patients attending major hospitals in Calabar, Cross River State, Nigeria.

Study participants, sample size and method of sampling:

Both inpatients and outpatients (>5 years) with clinical diagnosis of UTIs were randomly recruited after informed consent was obtained. Patients who had taken antibiotics in the last seven days prior to the day of sample collection, those below 5 years of age, and those who willingly withdrew their consent were excluded from the study. The sample size of 240 was calculated using the Fisher formula with 95% confidence level, 5% precision and 19% prevalence of UTI (7). A total of 240 patients with clinical diagnosis of UTI were enrolled into the study.

Data collection:

Structured questionnaire was used to obtain information on socio-demographic characteristics such as age, gender, educational status, occupation, and marital status. Female patients ≥12 years of age were screened for pregnancy.

Ethical consideration:

Ethical approval for the study was obtained from the Cross River State Health Research Ethics Committee (CRS-HREC) with REC No: CRSMOH/RP/2021/183. Data were collected after an informed, voluntary and oral consent had been secured from each study participant. Confidentiality of subjects' information was ensured. Positive results were reported to attending physician for appropriate treatment and management of the patients.

Urine collection and analysis:

Clean catch mid-stream voided urine specimens were collected into sterile universal containers with each bottle labelled with the patient's identity number and the assigned hospital code. Samples were subsequently transported to the laboratory for microbiological analysis.

Macroscopic and microscopic examinations of urine:

Prior to microscopic examination, each specimen was checked macroscopically for color and transparency and the results recorded. Each sample (5 mL) of urine was centrifuged for 10 minutes at 3000 rpm. The supernatant was examined with 10 x objective lens and then 40 x with the condenser iris closed. Samples with leukocytes ≥ 10 per high power field (40 x objective) were considered to be pyuric (14).

Culture and phenotypic identifications:

Uropathogens were isolated using the semi-quantitative culture technique previously described by Kass (13). Each urine sample was inoculated on Cystine Lactose Electrolyte Deficient (CLED) agar plates using a calibrated loop (0.002 ml) and then incubated aerobically at 37°C for 18-24 hours. Colony forming units (CFUs) were counted to determine significant bacteriuria. Urine samples with colonies ≥ 10^5 CFU/ml were considered significant for bacteriuria.

Distinctive colonies from significant cultures were sub-cultured onto fresh nutrient agar plates. The isolated uropathogens were identified using colonial morphology, growth characteristics, Gram stain reaction, conventional biochemical tests (14), and confirmed with commercial biochemical test kits; Analytical Profile Index (API) 20E and API 20NE (BioMérieux).

Screening for pregnancy:

Pregnancy was screened using a qualitative human chorionic gonadotropin (hCG) urine test (10). In this test, LabACON® (Hangzhou Biotest Biotec Co., Ltd) pregnancy rapid test strip was used to detect hCG hormones in patient's urine samples. The test strip was dipped into a bottle of freshly collected urine and the result read within 3 minutes. For positive result, two lines color bands were observed. For negative result, only one color band was observed.

Statistical analysis:

Data derived from questionnaire and microbiological survey were analyzed using Statistical Package for Social Sciences (SPSS) version 20.0. Descriptive statistics such as frequencies, percentages, means and standard deviations, were derived. Pearson Chi-square was used to determine associations between categorical variables. P value of < 0.05 was considered statistically significant.

Results:

Socio-demographic data of participants:

Two hundred and forty participants who met the inclusion criteria were enrolled in the study. Of this, 98 were enrolled from the NNRH, 78 from GHC and 62 from UCTH. Thirteen of the 240 urine samples were contaminated due to inappropriate samples collection measures, and these were excluded from data analysis.

More females (59.5%, 135/227) participated in the study compared to males (40.5%, 92/227). The age of the study participants ranged from 5 to 75 years with the mean age of 34.1±0.8 years (Table 1). About 39.6% (90/227) were in the age group 30-45 years and 49.3% (112/227) were single. Most participants (56.4%, 128/227) had tertiary educational qualification while 44.9% (102/227) of the respondents were students (Table 1).

Table 1: Frequency distribution of socio-demographic characteristics, clinical symptoms and probable risk factors for urinary tract infection among the study participants

Characteristics	Total n (%)	NNRH n (%)	UCTH n (%)	GHC n (%)
Age group (years)				
5-18	15 (6.6)	9 (9.6)	0	6 (8.5)
19-29	82 (36.1)	31 (33.0)	17 (27.4)	34 (47.9)
30-45	90 (39.6)	41 (43.6)	28 (45.2)	21 (29.6)
Above 45	40 (17.6)	13 (13.8)	17 (27.4)	10 (14.1)
Gender				
Male	92 (40.5)	37 (39.4)	29 (46.8)	26 (36.6)
Female	135 (59.5)	57 (60.6)	33 (53.2)	45 (63.4)
Marital status				
Single	112 (49.3)	43 (45.7)	24 (38.7)	45 (63.4)
Married	110 (48.5)	47 (50.0)	37 (59.7)	26 (36.6)
Divorced	3 (1.3)	3 (3.2)	0 (0.0)	0 (0.0)
Widow	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Widower	2 (0.9)	1 (1.1)	1 (1.6)	0 (0.0)
Educational qualification				
No formal education	9 (4.0)	2 (2.1)	5 (8.1)	2 (2.8)
Primary	25 (11.0)	10 (10.6)	8 (12.9)	7 (9.9)
Secondary	65 (28.6)	33 (35.1)	15 (24.2)	17 (23.9)
Tertiary	128 (56.4)	49 (52.1)	34 (54.8)	45 (63.4)
Occupation				
Farming	16 (7.0)	3 (3.2)	9 (14.5)	4 (5.6)
Trading	46 (20.3)	20 (21.3)	15 (24.2)	11 (15.5)
Student	102 (44.9)	38 (40.4)	22 (35.5)	42 (59.2)
Civil servant	56 (24.7)	33 (35.1)	9 (14.5)	14 (19.7)
Artisan	7 (3.1)	0 (0.0)	7 (11.3)	0 (0.0)
Clinical symptoms				
Urine urgency	12 (5.3)	4 (4.3)	6 (9.7)	2 (2.8)
Dysuria	46 (20.3)	17 (18.1)	16 (25.8)	13 (18.3)
Fever	54 (23.8)	25 (26.6)	8 (12.9)	21 (29.6)
Flank or supra-pubic pain	50 (22.0)	23 (24.5)	9 (14.5)	18 (25.4)
Frequency of urination	59 (26.0)	16 (17.0)	25 (40.3)	19 (26.8)
Probable risk factors				
Pregnancy status	22 (16.3)	9 (15.8)	5 (15.2)	8 (17.8)
Use of drug without prescription	63 (15.9)	24 (25.5)	15 (24.2)	24 (33.8)
Previous history of UTI	85 (37.4)	44 (46.8)	18 (29.0)	23 (32.4)
History of catheterization	8 (3.8)	2 (2.1)	5 (8.1)	1 (1.4)
Family history of UTI	121 (53.3)	48 (51.1)	33 (53.2)	42 (59.2)
Diabetes	3 (1.3)	0 (0.0)	2 (3.2)	1 (1.4)
Use of contraceptive	27 (20.0)	7 (12.3)	6 (18.2)	14 (31.1)

NNRH = Nigeria Navy Reference Hospital Calabar; UCTH = University of Calabar Teaching Hospital; GHC = General Hospital Calabar; n = number of participants; % = percentage

Table 2: Association of socio-demographic characteristics of the study participants with symptomatic significant bacteriuria/urinary tract infections

Sociodemographic characteristics	**No of participants with significant bacteriuria/urinary tract infection**				***p* value**
	Total (N=227) n (%)	**NNRH (N=94) n (%)**	**UCTH (N=62) n (%)**	**GHC (N=71) n (%)**	
	65 (28.6)	31 (33.0)	15 (24.2)	19 (26.8)	0.4519
Age group (years)					
5-18 (n=15)	4 (26.6)	4 (44.4)	0	0	
19-29 (n=82)	25 (30.5)	9 (29.0)	4 (23.5)	12 (35.3)	
30-45 (n=90)	24 (26.7)	13 (31.7)	6 (21.4)	5 (23.8)	
Above 45 (n=40)	12 (30.0)	5 (38.5)	5 (29.4)	2 (20.0)	
x^2	.3733	.9605	.3732	3.782	
p value	.9457	.8108	.8298	.2860	
Gender					
Male (n=92)	26 (28.3)	11 (29.7)	11 (37.9)	4 (15.4)	
Female (n=135)	39 (28.9)	20 (35.1)	4 (12.1)	15 (33.3)	
x^2	.01056	.09941	4.284	1.870	
OR (95% CI)	.97 (.54-1.74)	.78 (.32-1.91)	4.43 (1.22-16.05)	.36 (0.11-1.25)	
p value	.9182	.7275	0.0384*	0.1714	
Marital status					
Single (n=112)	27 (24.1)	13 (30.2)	3 (12.5)	11 (24.4)	
Married (n=110)	37 (33.6)	17 (36.2)	12 (32.4)	8 (30.8)	
Divorced (n=3)	1 (33.3)	1 (33.3)	0	0	
Widow (n=0)	0	0	0	0	
Widower (n=2)	0	0	0	0	
x^2	3.305	.8555	3.478	7.153	
p value	.3469	.8361	.1757	0.0280*	
Educational status					
No formal education (n=9)	1 (11.1)	0	1 (20.0)	0	
Primary (n=25)	7 (28.0)	3 (30.0)	3 (37.5)	1 (14.3)	
Secondary (n=65)	22 (33.8)	12 (36.4)	6 (40.0)	4 (23.5)	
Tertiary (n=128)	35 (27.3)	16 (32.7)	5 (14.7)	14 (31.1)	
x^2	2.326	1.198	4.532	1.812	
p value	0.5076	.7536	.2094	.6124	
Occupation					
Farming (n=16)	1 (6.3)	0	1 (11.1)	0	
Trading (n=46)	11 (23.9)	7 (35.0)	2 (13.3)	2 (18.2)	
Student (n=102)	25 (24.5)	11 (28.9)	3 (13.6)	11 (26.2)	
Civil servant (n=56)	23 (41.1)	13 (39.4)	4 (44.4)	6 (42.9)	
Artisan (n=7)	5 (71.4)	0	5 (71.4)	0	
x^2	15.786	2.407	13.670	3.732	
p value	0.0033*	,4923	0.0084*	0.2919	

NNRH = Nigeria Navy Reference Hospital Calabar; UCTH = University of Calabar Teaching Hospital; GHC = General Hospital Calabar; *Statistically significant at $p<.05$; N = Total number of participants with bacteriuria; n = no of participants with significant bacteriuria/urinary tract infection

Clinical symptoms and risk factors of UTI:

Clinical symptoms and risk factors for UTIs observed among the participants are depicted in Table 1. The frequency of clinical symptoms among the study participants were 26.0% (59/227), 23.8% (54/227), 22.0% (50 /227), 20.3% (46/227) and 5.3% (12/ 227) for frequent urination, fever, flank or supra-pubic pains, dysuria and urine urgency, respectively. Of all considered risk factors, 53.3%

(121/227) of the participants had family relatives with history of UTI, 37.4 % (85/227) had recurrent history of UTI, 15.9% (63/227) used drugs without prescription, 3.5% (8/227) had history of urinary catheter, while 1.3% (3 of 227) were diabetic. Out of the 135 female participants, 20.0% (27/135) used contraceptives and 16.3% (22/135) were pregnant (Table 1).

Prevalence of UTI among participants:

Sixty-five out of the 227 urine samples had significant bacterial growth, giving an overall UTI prevalence of 28.6% (Table 2). In NNRH, 31 of the 94 urine samples had significant bacterial growth, giving a UTI prevalence of 33.0%; in UCTH 15 (24.2%) of 62 samples were positive for UTI, and 19 of 71 (26.8%) samples from patients in GHC, had significant bacteriuria (Table 2).

Associated socio-demographic characteristics with UTI:

The prevalence of UTI was slightly higher in females (28.9%, 39/135) than in males (28.3%, 26/92) which was not statistically significant (p=0.918). The prevalence of UTI was also slightly higher among patients in the age group 19-29 years (30.5%) and age >45 years (30.0%) but the difference was not statistically significant (p=0.946). The prevalence of UTI in relation to marital status showed that the prevalence was slightly higher (33.6%, 37/110) among married participants but this was not significantly different from the other marital status (p=0.347).

The prevalence of UTI was highest among patients with secondary educational qualification (33.8%, 22/65) but the difference was also not statistically significant (p= 0.508). With respect to occupational status, artisans had the highest prevalence of UTI (71.4%, 5/7) followed by civil servants (41.1%, 23/56), and these prevalence rates were significantly higher than the prevalence in other occupational groups (p=0.003) (Table 2).

Associated clinical symptoms & risk factors with UTI:

The results revealed that, of all assessed clinical symptoms, only dysuria (OR= 2,065, 95% CI=1.051-4.059, p=0.044) was significant associated with UTI. Pearson Chi-square showed that urine urgency (OR=1.845, 95% CI=0.564-6.039, p=0.3315), fever (OR= 0.5637, 95% CI=0.270-1.177, p=0.1672), flank or supra-pubic pain (OR=0.7388, 95% CI=0.358-1.524, p=0.4808) and frequency of urination (OR=1.260, 95% CI=0.134-1.187, p=0.5055) were not significantly associated with UTI.

Measurement of association between potential risk factors and occurrence of UTI showed that previous history of UTI (OR= 2.863, 95% CI=1.582-5.180, p=0.008), pregnancy (OR=9.94, 95% CI=3.867-25.571, p<0.0001), history of urinary catheterization (OR=4.417, 95% CI=1.024-19.053, p=0.045) and contraceptive use (OR=3.469, 95% CI=1.446-8.320, p=0.012) were significantly associated with occurrence of UTI. However, use of drug without prescription (OR=0.996, 95% CI=0.5234-1.894, p=1.000), family history of UTI (OR=1.227, 95% CI=0.687-2.191, p=0.5568) and diabetes mellitus (OR=5.111, 95% CI=0.455-57.368, p=0.1979) were not significantly associated with the occurrence UTI (Table 3).

Prevalence of uropathogens from the study:

Ten different bacterial species were isolated from the samples of 65 patients with significant bacteriuria/ urinary tract infection. Gram-negative uropathogens constituted 80% (52/65) while 20% (13/65) were Gram-positive cocci. The most predominant bacteria were *Klebsiella pneumoniae* (23.1%), followed by coagulase-negative staphylococci (CoNS) (16.9%), *Escherichia coli* (12.3%), *Enterobacter cloacae* (10.8%), *Citrobacter freundii* (9.2%), *Proteus mirabilis* (7.7%), *Serratia marcescens* (7.7%), *Pseudomonas aeruginosa* (3.1%), *Cronobacter* sp (3.1%), *Enterococcus* sp (3.1%), *Citrobacter koseri* (1.5%) and *Pseudomonas luteola* (1.5%) (Table 4).

Discussion:

In this study, 65 of 227 voided urine samples yielded significant bacterial growths, giving an overall prevalence of 28.6% for symptomatic significant bacteriuria/urinary tract infection among the study participants. This is similar to 31.3% reported from among symptomatic UTI patients in Enugu, Nigeria (16). Outside the shore of Nigeria, Odoki et al., (10) reported 32.2% in Bushenyi District, Uganda, and 29.0% was reported in Ismaila City, Egypt, which are comparable to the present study.

The prevalence of UTI in the present study was slightly higher than the rates previously reported in the same study area (7, 17). Other researchers have also reported UTI prevalence rates higher or slightly lower when compared to the current study (18-20). The observed difference in prevalence of UTI could be attributed to differences in personal hygiene status, study population, socio-economic status, immune status of the study participants and geographical variation. Interestingly, the highest prevalence (33.0%) of UTI was recorded among patients who visited the Nigeria Naval Reference Hospital (NNRH) Calabar when compared to other study sites, although the prevalence difference between the study sites was not statistically significant (p= 0.4519). The higher prevalence of UTI in these patients could be attributed to the status of NNRH as reference hospital where advanced

Table 3: Association of clinical symptoms and potential risk factors with symptomatic significant bacteriuria/urinary tract infection among the study participants

Characteristics		**No of participants with significant bacteriuria**				**OR (95% CI)**	***p* value**
		NNRH (N = 94) n (%)	**UCTH (N = 62) n (%)**	**GHC (N = 71) n (%)**	**Total (N=227)**		
Clinical symptoms							
Urine urgency	Yes	3 (9.7)	0	2 (10.5)	5 (7.7)	1.85 (0.56-6.04)	.3315
	No	28 (90.3)	15 (100.0)	17 (89.5)	60 (92.3)		
Dysuria	Yes	7 (22.6)	5 (33.3)	7 (36.8)	19 (29.2)	2.07 (1.05-4.06)	**.**044*
	No	24 (77.4)	10 (66.7)	12 (63.2)	46 (70.8)		
Fever	Yes	4 (12.9)	1 (6.7)	6 (31.6)	11 (17.0)	0.56 (0.27-1.18)	.1672
	No	27 (87.1)	14 (93.3)	13 (68.4)	54 (83.1)		
Flank or supra-pubic pain	Yes	7 (22.6)	2 (13.3)	3 (15.8)	12 (18.5)	0.74 (0.36-1.52)	.4808
	No	24 (77.4)	13 (86.7)	16 (84.2)	53 (81.5)		
Frequency of urination	Yes	4 (12.9)	9 (60.0)	7 (36.8)	19 (29.2)	1.26 (0.13-1.19)	.5055
	No	27 (87.1)	6 (40.0)	12 (63.2)	46 (70.8)		
Risk factors							
Pregnancy status	Yes	5 (25.0)	3 (75.0)	5 (33.3)	13 (33.3)	9.94 (3.87-25.57)	<.0001*****
	No	15 (75.0)	1 (25.0)	10 (66.7)	26 (66.7)		
Use of drug without prescription	Yes	7 (22.6)	5 (33.3)	6 (31.6)	18 (28.6)	0.99 (0.52-1.89)	1.000
	No	24 (77.4)	10 (66.7)	13 (68.4)	47 (71.4)		
Previous history of UTI	Yes	17 (54.8)	11 (73.3)	8 (42.1)	36 (55.4)	2.86 (1.58-5.18)	.0008*
	No	14 (45.2)	4 (26.7)	11 (57.9)	29 (44.6)		
History of catheterization	Yes	1 (3.2)	3 (20.0)	1 (5.3)	5 (7.7)	4.42 (1.02-19.05)	.0450*
	No	30 (96.8)	12 (80.0)	18 (94.7)	60 (92.3)		
Family history of UTI	Yes	16 (51.6)	9 (60.0)	14 (73.7)	37 (57.0)	1.23 (0.69-2.19)	.5568
	No	15 (48.4)	6 (40.0)	5 (26.3)	28 (43.1)		
Diabetes	Yes	0	2 (13.3)	0	2 (3.1)	5.11 (0.46-57.37)	.1979
	No	31 (100.0)	13 (86.7)	19 (100.0)	63 (97.0)		
Use of contraceptive	Yes	2 (10.0)	2 (50.0)	6 (40.0)	10 (25.6)	3.47 (1.45-8.32)	.012*
	No	18 (90.0)	2 (50.0)	9 (60.0)	29 (74.4)		

NNRH = Nigeria Navy Reference Hospital Calabar; UCTH = University of Calabar Teaching Hospital; GHC = General Hospital Calabar; OR=Odds ratio; CI=Confidence interval; *Statistically significant at $P < .05$; N = Total number of participants with bacteriuria; n = no of participants with significant bacteriuria/urinary tract infection

Table 4: Prevalence of uropathogens isolated from patients by gender, study site, and age group

Identified bacteria/ participant characteristics	**No of bacteria pathogen isolated from significant urine culture**									
	Gender (%)		**Study location (%)**			**Age group (years) (%)**				**Total (%)**
	Males	**Females**	**NNRH**	**UCTH**	**GHC**	**5-18**	**19-29**	**30-45**	**>45**	
Gram negative bacteria										
Serratia marcescens	1 (3.8)	4 (10.3)	2 (6.5)	1 (6.7)	2 (10.5)	0	3 (12.0)	2 (8.3)	0	5 (7.7)
Escherichia coli	4 (15.4)	4 (10.3)	3 (9.7)	2 (13.3)	3 (15.7)	0	4 (16.0)	3 (12.5)	1 (8.3)	8 (12.3)
Cronobacter species	0	2 (5.1)	1 (3.2)	0	1 (5.3)	0	0	2 (8.3)	0	2 (3.1)
Klebsiella pneumoniae	7 (26.9)	8 (20.5)	8 (25.8)	5 (33.3)	2 (10.5)	1 (25.0)	5 (20.0)	7 (29.2)	2 (16.7)	15 (23.1)
Enterobacter cloacae	1 (3.8)	6 (15.4)	4 (12.9)	1 (6.7)	2 (10.5)	2 (50.0)	2 (8.0)	2 (8.3)	1 (8.3)	7 (10.8)
Citrobacter freundii	1 (3.8)	5 (12.8)	2 (6.5)	1 (6.7)	3 (15.7)	1 (25.0)	3 (12.0)	1 (4.2)	1 (8.3)	6 (9.2)
Pseudomonas luteola	1 (3.8)	0	1 (3.2)	0	0	0	1 (4.0)	0	0	1 (1.5)
Proteus mirabilis	3 (11.5)	2 (5.1)	3 (9.7)	0	2 (10.5)	0	3 (12.0)	0	2 (16.7)	5 (7.7)
Citrobacter koseri	1 (3.8)	0	0	0	1 (5.3)	0	1 (4.0)	0	0	1 (1.5)
Pseudomonas aeruginosa	1 (3.8)	1 (2.6)	1 (3.2)	1 (6.7)	0	0	0	1 (4.2)	1 (8.3)	2 (3.1)
Gram positive bacteria										
Enterococcus species	1 (3.8)	1 (2.6)	1 (3.2)	0	1 (5.3)	0	1 (4.0)	1 (4.2)	0	2 (3.1)
Staphylococcus species	5 (19.2)	6 (15.4)	5 (16.1)	4 (26.7)	2 (10.5)	0	2 (8.0)	5 (20.8)	4 (33.3)	11 (16.9)
Total	26 (40.0)	39 (60.0)	31 (47.8)	15 (23.1)	19 (29.2)	4 (6.2)	25 (38.5)	24 (36.9)	12 (18.5)	65 (100.0)

NNRH = Nigeria Navy Reference Hospital Calabar; UCTH = University of Calabar Teaching Hospital; GHC = General Hospital Calabar

level of healthcare services and diagnoses are conducted. This observation is in consonance with the study of Karikari et al., (9) in Ghana, who reported high incidence of UTI in a referral hospital.

The age group 19-29 years had the highest prevalence of UTI (30.5%, 25/82) among the study participants closely followed by age group >45 years (30.0%, 12/40). This age group also had the highest UTI prevalence in GHC (35.3%, 12/34) but the age group 5-18 years had the highest prevalence in NNRH (44.4%, 4/9) while age group >45 years had the highest prevalence in UCTH (29.4%, 5/17). Nevertheless, this prevalence difference in the entire study participants (and with respect to study sites) was not statistically significant (p=0.946). This finding is in consonance with Ndako et al., (19) in southwest Nigeria, Abdul et al., (21) in Maiduguri, northeast Nigeria, and Mokube et al., (8) in Cameroon. In contrast, Obirikorang et al., (22) reported highest prevalence of UTI in age group 30-34 years in Kumasi, Ghana, and Marami et al., (23) in age group 35-44 years in Ethiopia. This variation in prevalence of UTI with respect to age group might be due to the target population under study, previous untreated history, family history, underlying medical condition, sexual activity, and educational level, among other factors as previously reported (5,24).

In this study, the prevalence of UTI was slightly higher in females (28.9%, 39/135) than in males (28.3%, 26/92), although this was not statistically significant (p=0.918). This finding is consistence with previous studies supporting that women are more vulnerable to contracting UTI due to their short urethra and its proximity to the anal opening (25,26). Other factors which may contribute to high incidence of UTI in women are physiological changes in women which deplete the vaginal flora, unauthorized administration of contraceptives, pregnancy, and family history (4). However, in UCTH, the UTI prevalence of 37.9% (11/29) in the male participants was significantly higher than 12.1% (4/33) in the female participants (p=0.0384). This is contrary to what has been previously established, and the cause of this reversal is not apparent in this center.

The prevalence of UTI was higher among the married (33.6%, 37/110) compared to single participants (24.1%, 27/112). Although this difference was not statistically significant among the entire study participants (p=0.347), it was statistically significant for participants in GHC, where the prevalence was 30.8% (8/26) in married compared to 24.4% (11/45) in singles (p=0.0280). This is in agreement with the finding of Ezugwu et al., (27) but contradicts Wanja et al., (28) in Kenya, who reported highest prevalence of UTIs among widowed (57.1%). The highest prevalence of UTI among married participants in this study may be attributed to increase in parity or a higher number of pregnancies (27).

Participants with secondary educational qualification had the highest prevalence of UTI (33.8%, 22/65), followed by those with primary (28.0%, 7/25) and tertiary education (27.3%, 35/128). Although the difference in the prevalence was not statistically significant (p=0.508), in GHC, participants who attended tertiary education had the highest prevalence of UTI (31.1%, 14/45). This variation may be attributed to the proximity of University of Calabar to this site. Multiple sexual partners and unfaithfulness to one sex partner have been found as indicators of UTI among university students (29). Studies have shown that people with little or no formal education had higher prevalence of UTIs (30,31), which agrees with the present findings but contradicts those of Ndako et al., (19) and Mokube et al., (8). On the basis of occupation, the UTI prevalence was highest among artisan (71.4%, 5/7) followed by civil servants (44.4%, 4/9) and lowest among students (13.6%, 3/22), trader (13.3%, 2/15) and farmers (11.1%, 1/9). The observed difference was statistically significant (p=0.003), which agrees with findings of previous studies (32,33).

Of all the clinical symptoms, only dysuria was significantly associated with UTI (OR =2,065 95% CI=1.051-4.059, p=0.044). This contradicted the study of Seifu and Gebissa (34) who reported that fever, urgency, frequency and supra-pubic pain were significantly associated with UTI, but agrees with Al-Kashif (35) who reported statistical association of dysuria with UTI. In line with earlier studies (5,6,35,36), pregnancy, previous history of UTI, history of catheterization and contraceptive use were significantly associated with the occurrence of UTI in our current study. This may be attributed to factors such as contamination of catheter during insertion, and physiological changes of pregnancy and oral contraceptive pills on the urinary system in the females. Contrary to Odoki et al., (10) and Ahmed (37), the present study showed that family history of UTI and diabetes mellitus were not significantly associated with UTI (p> 0.05).

The severity of UTI is greatly influenced by the types of organisms involved. In this study, Gram-negative uropathogens constituted 80% while Gram-positive constituted 20%. This supports the assertion that Gram-negative bacteria constitute 80-90% of uropathogens (38). The uropathogens recovered in this study had previously been known to cause UTI (6,7,17,26). The highest prevalence of *K. pneumoniae* in our study is an indication that

this organism is achieving more prominence as causative agents of UTI. This finding is inconsistent with that of Seifu and Gebissa (34) in Ethiopia who reported *E. coli* as the most common uropathogens from UTI patients. In other reports, Many et al., (39) in Democratic Republic of Congo (DRC) reported *P. mirabilis* (41.2%), Labi et al., (24) in Ghana reported *Enterococcus* sp. (26.7%) and Musonda et al., (40) in Zambia reported *S. aureus* (32%) as the most predominant organism. The variation in the type of bacteria uropathogens in this study and others reported might be attributed to physiological state of patients, techniques of sample collection, sample size used, environmental or personal hygi ene levels.

Our study reported the presence of *Cronobacter* sp. in the urine samples. *Cronobacter* sp. has been reported as emerging pathogens from infant food (41). UTI caused by *Cronobacter* sp. in the study area is rare. However, this study agrees with Hayashi et al., (42) who reported the occurrence of *Cronobacter sakazakii* in a 69-year-old man presenting with UTI in Shimane University Hospital, Shimane, Japan. The possible route of infection with *Cronobacter* sp could be via oral ingestion from external sources and retrograde transmission through the urinary tract (42).

Conclusion:

In our study, the overall prevalence of symptomatic significant bacteriuria/UTI among patients attending hospitals in Calabar, Nigeria was 28.6%. Of all considered risk factors, previous history of UTI, pregnancy, contraceptives and history of urinary catheterization were significantly associated with prevalence of UTI. Similarly, there was significant association of dysuria symptom with UTI and artisans had significantly higher prevalence of UTI compared to other occupational groups.

Klebsiella pneumoniae was the most predominant uropathogens, followed by coagulase-negative staphylococci and others such as *E. coli*, *E. cloacae*, *C. freundii*, *P. mirabilis*, *S. marcescens*, *P. aeruginosa*, *Cronobacter* sp, *Enterococcus* sp, *C. koseri*, and *P. luteola*. Routine screening for UTI is recommended for pregnant women, patients with dysuria, previous episodes of UTI, and catheterized patients. Appropriate antimicrobial drugs should be promptly administered for positive cases.

Acknowledgements:

The authors appreciate the participants, management and staff of the University of Calabar Teaching Hospital (UCTH), Nigeria Navy Reference Hospital Calabar (NNRH), and General Hospital Calabar (GHC), for their contributions towards the success of this research.

Contributions of authors:

EEB conceptualized and designed the study, collected, analyzed, interpreted the data, drafted and critically reviewed the manuscript; AAAA assisted in fund acquisition and critically reviewed the manuscript; EEI, MM and SSA critically reviewed the manuscript. All authors read and approved the manuscript.

Source of funding:

This study was funded by the Tertiary Educational Trust Fund (TETFund) under the Institution Based Research (IBR) Grant. The funder played no role in the study design, data collection and analysis, and manuscript preparation.

Conflict of interests:

Authors declare no conflict of interest.

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Abayomi et al. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (1): 60 – 67 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jan 2024; Vol.25 No.1
AJCEM/2291. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Faecal carriage of extended spectrum β-lactamase producing Enterobacterales (ESBL-PE) in children under five years of age at a tertiary hospital in southwest Nigeria

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Abstract:

Background: The main reservoir of Enterobacterales is the human gut, which has been reported as a source of hospital acquired infection. Enterobacterales carrying the extended spectrum β-lactamase (ESBL) genes have emerged over the years as significant multidrug resistant (MDR) pathogens, that have hindered effective therapy of infections caused by them, and limited treatment to a small number of drugs such as carbapenems, leading to selection pressure and emergent resistance to carbapenems. The objective of this study was to determine the faecal carriage of ESBL-producing Enterobacterales (ESPL-PE) among children under 5 years of age at the Ladoke Akintola University of Technology Teaching Hospital, Ogbomoso, Nigeria.
Methodology: A total of 144 children under 5 years of age were consecutively recruited over a period of 5 months from the paediatrics outpatient clinic, children emergency, paediatrics ward, and neonatal unit of the hospital. Rectal swabs were collected from selected children and transported to the medical microbiology laboratory of the hospital for inoculation on MacConkey agar plates and aerobic incubation at 37°C for 24 hours. All positive growth on the culture plates were identified by colony morphology, Gram stain reaction and conventional biochemical tests scheme. Antimicrobial susceptibility test was performed by the disc diffusion method against selected antibiotics, and ESBL production was confirmed by the double disc synergy test (DDST). Association of risk factors with ESBL-PE faecal carriage was determined using Chi-square or Fisher Exact test, with statistical significance set at $p < 0.05$.
Results: The prevalence of ESBL-PE faecal carriage was 37.5% (54/144), with 34.7% (50/144) for *Escherichia coli* and 2.1% (3/144) for *Klebsiella pneumoniae.* The overall resistance rate of both ESBL and non-ESBL producing isolates were to ampicillin (100.0%), amoxicillin-clavulanic acid (96.2%), ceftazidime (94.3%) and ciprofloxacin (90.6%), while resistance to carbapenems was low at 22.2%. Significant risk factors associated with ESBL-PE faecal carriage were age group 24-59 months (p=0.0187), prior intake of antibiotics (p=0.014), and intake of antibiotics without prescription (p=0.0159), while gender (p=0.8877), mother's education level (p=0.3831) and previous hospital visit (p=0.8669) were not significantly associated with faecal ESBL carriage.
Conclusion: The relatively high faecal carriage rate of ESBL-PE in children <5 years of age in our study highlights the risk for antimicrobial resistance transmission within the hospital and community.

Keywords: Antimicrobial resistance; Faecal carriage; ESBL; Enterobacterales; Children

Received Aug 18, 2023; Revised Oct 10, 2023; Accepted Oct 12, 2023

Transport fécal d'entérobactéries productrices de β-lactamases à spectre étendu (ESBL-PE) chez des enfants de moins de cinq ans dans un hôpital tertiaire du sud-ouest du Nigéria

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Résumé:

Contexte: Le principal réservoir d'Enterobacterales est l'intestin humain, qui a été signalé comme source d'infections nosocomiales. Les Entérobactéries porteuses des gènes des β-lactamases à spectre étendu (BLSE) sont apparues au fil des années comme d'importants agents pathogènes multirésistants (MDR), qui ont entravé le traitement efficace des infections provoquées par elles et ont limité le traitement à un petit nombre de médicaments tels que les carbapénèmes, conduisant à une pression de sélection et à une résistance émergente aux carbapénèmes. L'objectif de cette étude était de déterminer le portage fécal d'entérobactéries productrices de BLSE (ESPL-PE) chez les enfants de moins de 5 ans à l'hôpital universitaire de technologie Ladoke Akintola, à Ogbomoso, au Nigeria.
Méthodologie: Au total, 144 enfants de moins de 5 ans ont été recrutés consécutivement sur une période de 5 mois dans la clinique externe de pédiatrie, les urgences pédiatriques, le service de pédiatrie et l'unité néonatale de l'hôpital. Des écouvillons rectaux ont été prélevés sur des enfants sélectionnés et transportés au laboratoire de microbiologie médicale de l'hôpital pour inoculation sur plaques de gélose MacConkey et incubation aérobie à 37°C pendant 24 heures. Toutes les croissances positives sur les plaques de culture ont été identifiées par la morphologie des colonies, la réaction de coloration de Gram et le schéma de tests biochimiques conventionnels. Le test de sensibilité aux antimicrobiens a été réalisé par la méthode de diffusion sur disque contre des antibiotiques sélectionnés, et la production de BLSE a été confirmée par le test de synergie à double disque (DDST). L'association des facteurs de risque avec le portage fécal des BLSE-PE a été déterminée à l'aide du test du Chi carré ou de Fisher Exact, avec une signification statistique fixée à $p<0,05$.
Résultats: La prévalence du portage fécal des BLSE-PE était de 37,5% (54/144), dont 34,7% (50/144) pour *Escherichia coli* et 2,1% (3/144) pour *Klebsiella pneumoniae*. Le taux de résistance global des isolats produisant et non des BLSE était à l'ampicilline (100,0%), à l'amoxicilline-acide clavulanique (96,2%), à la ceftazidime (94,3%) et à la ciprofloxacine (90,6%), tandis que la résistance aux carbapénèmes était faible à 22,2%. Les facteurs de risque significatifs associés au portage fécal de BLSE-PE étaient le groupe d'âge de 24 à 59 mois (p=0,0187), la prise antérieure d'antibiotiques (p=0,014) et la prise d'antibiotiques sans ordonnance (p= 0,0159), tandis que le sexe (p=0,8877), le niveau d'éducation de la mère (p=0,3831) et la visite antérieure à l'hôpital (p=0,8669) n'étaient pas significativement associés au portage fécal de BLSE.
Conclusion: Le taux de portage fécal relativement élevé d'EP-BLSE chez les enfants de moins de 5 ans dans notre étude met en évidence le risque de transmission de la résistance aux antimicrobiens au sein de l'hôpital et de la communauté.

Mots-clés: Résistance aux antimicrobiens; Transport fécal; BLSE; Enterobacterales; Enfants

Introduction:

Bowel carriage has been identified as the main reservoir of Enterobacteriaceae and they have been reported to cause hospital acquired infections (HAIs) which are defined as infections not present and without evidence of incubation at the time of admission to a healthcare setting, and associated with prolonged hospital stay (1). The order Enterobacterales carrying the extended-spectrum β-lactamase (ESBL) genes have emerged over the years as significant human pathogens. Such strains are resistant to multiple antimicrobial agents, and can be challenging to treat, as therapeutic options for them are few (2).

Resistance to β-lactam antibiotics in Enterobacterales is primarily due to β-lactamases-mediated antibiotic hydrolysis. Alteration in the expression of efflux pumps and/or porins also play important roles in bacteria resistance to antibiotics, as they limit interaction of the drug with its intracellular target and consequently its deleterious effect on the cell (3).

Extended-spectrum β-lactamases are enzymes which can hydrolyze virtually all penicillins and cephalosporins, including extended-spectrum cephalosporins such as cefotaxime or ceftazidime (4). Many ESBL producing bacteria are multi-resistant to non-β-lactam antibiotics such as the fluoroquinolones, aminoglycosides, tetracyclines, trimethoprim, and sulfonamides (5,6). Consequently, effective antibiotic therapy for treating these infections is limited to a small number of drugs such as carbapenems and thus increasing the chance of resistance to carbapenems among the Enterobacterales (7).

Epidemiologically, Gram-negative bacteria are frequent cause of infections in both adults and paediatric population globally, especially of the urinary tract, and as a group are second to staphylococcus as a cause of bloodstream infections (8). They have also been reported to be the most common cause of serious bacterial infections in the paediatric age groups (8). The global emergence and spread of the extended spectrum β-lactamase producing Enterobacterales (ESBL-PE) have threatened the ability to treat infection

caused by them. Studies in many countries have reported increasing emergence of ESBL-PE (9,10) in recent years, providing information necessary to understand the mechanism of and the threats posed by ESBL-PE and other multi-resistant bacteria (3,11).

Over the years in Europe, there have been increasing reports of invasive infections caused by *Klebsiella pneumoniae* and *Escherichia coli* resistant to the third-generation cephalosporins, and this is believed to be due to the dissemination of ESBL-producing bacteria in both the hospitals and communities. An increase in carbapenemase producing clinical bacterial isolates has also been reported and this raises grave concerns on the future of antimicrobial therapy (10). For example, the prevalence of faecal carriage of ESBL-PE in children under 5 years of age in a study by Tola et al., (12) was 17.1%. In the SMART (Study for Monitoring Antimicrobial Resistance Trends) study of urinary tract isolates conducted between 2009 and 2010 in Europe, the prevalence rates of ESBL producing *E. coli* and *K. pneumoniae* were 17.6% and 38.9% respectively (13). In North America, the respective prevalence rates were 8.5% and 8.8% (14). In Asia, the respective ESBL prevalence rates were 5.0% and 0%, while New Zealand, they were 67.0% and 61.0% respectively (15).

In the study performed at a Dutch hospital in Switzerland, the prevalence of ESBL-PE carriage was 4.5% (25/559) from the faecal swabs analyzed (16). In Nigeria, a study done by Jewoola et al., (1) reported prevalence of rectal carriage of ESBL-PE to be 25.3%, with the common strains isolated being *K. pneumoniae* (44.2%), *E. coli* (34.2%) and *Enterobacter* species (12.8%). The common risk factors identified for acquisition of ESBL-PE in children includes nutritional status such as the consumption of poorly cooked meat and chicken, dairy products (milk, yoghourt and cheese) and poor personal hygiene (12,17).

Due to paucity of data on faecal carriage of ESBL-PE in developing countries, this study aimed to determine the faecal carriage rate of ESBL-PE in children under 5 years of age at the Ladoke Akintola University of Technology (LAUTECH) Teaching Hospital Ogbomoso, southwest Nigeria.

Materials and method:

Study area, design and ethical approval

This was a descriptive cross-sectional study conducted on a total of 144 children recruited from the outpatient clinic, children emergency, paediatrics wards and neonatal unit of the Paediatrics and Child Health Department of the LAUTECH Teaching Hospital, Ogbomoso. Approval of the Research and Ethics Committee (REC) of the hospital was obtained before the commencement of the study. Informed consent of the parent/guardian was also obtained.

Study population and participants:

All children aged 0 to 5 years including those placed on observation for < 48 hrs in the hospital either for medical or surgical treatment were included in the study. Children who have been on admission for > 48 hrs prior to commencement of the study as well as children whose parents/guardian did not give consent were excluded from the study.

Sample size and participants selection:

The sample size of 144 patients was calculated using the Fisher formula, with an estimated ESBL-PE prevalence rate of 17.1%, determined in a previous study (12), and adjusting for 10% attrition. Children who satisfied the inclusion criteria were recruited consecutively over a period of 5 months until the sample size of 144 was attained.

Data and sample collection:

Structured questionnaires were designed and interview-administered to collect relevant children's demographic and clinical data including age, gender, educational level of child and mother/guardian, family size, underlying medical conditions/co-morbidities, intake of anti-microbial drugs prior to study, and hospitalization history within previous 12 months. Information on potential risk factors such as dietary habits (intake of yoghourt, milk, cheese, meat, chicken), source of drinking water, toilet facility, and personal hygiene habits of parents/guardians when caring for children (i. e. after child's urination, defaecation or diaper change, parents wash intimate parts of the child with water only or with soap and water, or use dry tissue or wipes), were also collected.

Rectal swab samples were collected using sterile swabs from all the selected children. The samples were kept in re-sealable polyethylene bag and transferred to the Medical Microbiology Research Laboratory of the hospital for immediate processing and microbiological analysis.

Culture isolation and identification of bacterial isolates:

The samples were inoculated on MacConkey agar plate and incubated aerobically at 37°C for 24 hrs. All positive cultures were characterized by colonial morphology, Gram stain reaction and conventional biochemical test scheme including indole, citrate, and urease tests (18). Quality control strains (*E. coli*

ATCC25922 and *K. pneumoniae* ATCC70063) were included in each run as negative and positive controls respectively.

Antimicrobial susceptibility testing:

Antimicrobial susceptibility testing (AST) was performed by the Kirby-Bauer disc diffusion technique on Mueller-Hinton (MH) agar plates against the following antibiotics; amikacin 30µg, ampicillin 30µg, amoxicillin-clavulanic acid (co-amoxiclav) 30µg, piperacillin-tazobactam, ceftazidime 30µg, cefotaxime 30µg, ciprofloxacin 5µg, gentamicin 10µg, cefepime 30µg, and meropenem 10µg. The diameter of inhibition zone was interpreted as susceptible, intermediate or resistant according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (19).

Screening identification of ESBL-PE:

Enterobacterales isolate was presumptively identified to be ESBL producer on AST plate when the zone diameter of inhibition was ≤22 mm to ceftazidime disc or ≤27 mm to cefotaxime disc or to both (19).

Phenotypic confirmation of ESBL-PE by double disc synergy test:

Presumptive ESBL-PE isolates were confirmed by the double disc synergy test (DDST) on MH agar plate, with cefotaxime (30µg) and ceftazidime (30µg) discs placed 25-30 mm apart on either side of amoxicillin-clavulanic acid (20/10µg) disc. ESBL positive (*K. pneumoniae* ATCC 700603) and ESBL negative (*E. coli* ATCC 25922) control strains were similarly tested.

A difference in inhibition zone diameter of ≥5 mm produced on the amoxicillin-clavulanic acid disc side compared to the zone diameters produced by either of the cephalosporin discs alone, confirmed phenotypic ESBL production by the isolate (20).

Data analysis:

Data were analyzed using SPSS for windows version 21.0. Categorical data were presented as proportions and percentages while quantitative data were presented as mean, median, standard deviation, and range as appropriate. Statistical association of a risk factor and faecal ESBL carriage was determined using Chi-square or Fisher Exact test with statistical significance put at $p<0.05$.

Results:

Demographic characteristics of the children:

A total of 144 children aged <5 years (from whom faecal swab specimens were collected) were studied. There were 77 (53.5%) males and 67 (46.5%) females, with 40 (28%) in age group 1-23 months (infants) and 104 (72.0%) in age group 24-59 months (children). Most of the children (73, 50.6%) had family members of one to five in number. Majority (70.1%) of the mothers fall between age-range 25-34 years. Majority (108, 75%) of the mothers of the children had tertiary education level. The source of drinking water in the majority (85, 59.0%) of the family is tap water while the toilet type used by majority of the children's family (122, 85.0%) is private latrines (Table 1)

Clinical characteristics of participants:

Most children had received antibiotics 3 weeks prior to the study (122, 84.7%), and most had been given antibiotics by their mothers without medical prescription (118, 82.0%). Most of the mothers (131, 91.0%) claimed to clean anogenital region of their children after defecation with water while the remaining (13, 9.0%) use tissue paper. A total of 83 (58.0%) children had history of previous hospital visits, 40 (28.0%) had history of previous hospital admission, and 12 (8.0%) had history of previous surgery.

Prevalence of ESBL-PE faecal carriage:

Enterobacterales (*E. coli* and *K. pneumoniae*) were isolated from faecal samples of all 144 children, with 137 (95.1%) *E. coli* and 7 (4.9%) *K. pneumoniae*. The overall prevalence of ESBL-PE faecal carriage among the children is 37.5% (53/144), with prevalence of ESBL-producing *E. coli* faecal carriage of 34.7% (50/144) and ESBL-producing *K. pneumoniae* faecal carriage rate of 2.1% (3/144).

Of the 53 ESBL-PE, 50 (94.3%) are *E. coli* while 3 (5.7%) are *K. pneumoniae* isolates. However, 36.5% (50/137) of the *E. coli* isolates were ESBL-producing compared to 42.9% (3/7) of *K. pneumoniae* isolates (OR = 0.7663; 95%CI = 0.1647-3.564; p=0.7083).

Antimicrobial susceptibility test result:

The AST result of the ESBL-producing and non-ESBL producing *E. coli* and *K. pneumoniae* isolates is presented in Table 3. The overall resistance rate of both ESBL and non-ESBL-producing isolates were to ampicillin (100.0%), amoxicillin-clavulanic acid (96.2%), ceftazidime (94.3%) and ciprofloxacin (90.6%). Resistance rate was higher to cephalosporins and fluoroquinolones among ESBL-producing *E. coli* while it was higher to aminoglycoside, cephalosporins and amoxicillin-clavulanic acid in ESBL-producing *K. pneumoniae* compared to the non-ESBL producers that showed lower resistance rates. Resistance of both ESBL and non-ESBL producing *E. coli* and *K. pneumoniae* isolates to carbapenems was low at the rate of 22.2%.

Risk factors for ESBL-PE faecal carriage

Risk factors analysis (Table 3) showed that children aged 24-59 months (p=0.019), those with prior intake of antibiotics within 1

and 2 weeks (p=0.014), and those who have taken antibiotics without prescription (p=0.02) had significantly higher faecal carriage rate. However, gender of children (p=0.8877), educational level of the mother (p=0.3831) and previous hospital visit by children (p=0.8669) were not significantly associated with faecal carriage of ESBL-PE.

Table 1: Socio-demographic characteristics of children under five years of age recruited for the study at the Ladoke Akintola University of Technology Teaching Hospital, Ogbomoso, Nigeria

Characteristics		**Frequency**	**Percent (%)**
Age of children (in months)	1 -23	40	28.0
	24-59	104	72.0
Gender of children	Male	77	53.5
	Female	67	46.5
Age group of mothers (years)	15-24	11	7.6
	25-34	101	70.1
	35-44	28	19.4
	45-54	4	2.8
Family size	1-3	73	50.6
	3-5	55	38.2
	6 and above	16	11.1
Educational level of mother	None	5	3.4
	Primary	2	1.4
	Secondary	29	20.0
	Tertiary	108	75.0
Educational status of child	None	75	52.1
	Creche	36	25.0
	Nursery	33	22.9
Source of drinking water	Bore hole	85	59.0
	Boiled water	16	11.1
	Bottled water	34	23.6
	Well water	9	6.3
Toilet facility for family use	Private	122	84.7
	Communal	12	8.3
	Bush faecal disposal	10	6.9

Table 2: Antimicrobial resistance pattern of faecal ESBL and non-ESBL producing Enterobacterales isolated from children under five years of age at Ladoke Akintola University of Technology Teaching Hospital, Ogbomoso, Nigeria

Antibiotics	**No of resistant ESBL-PE (%)**			**No of resistant non-ESBL-PE (%)**			**Total no of resistant Enterobacterales (%)**
	***E. coli* (n=50)**	***K. pneumo* (n=3)**	**Sub-total (n=53)**	***E. coli* (n=87)**	***K. pneumo* (n=4)**	**Sub-total (n=91)**	**(n = 144)**
Ampicillin	50 (100.0)	3 (100,0)	53 (100.0)	87 (100.0)	4 (100.0)	91 (100.0)	144 (100.0)
Ciprofloxacin	46 (92.0)	2 (66.7)	48 (90.6)	70 (80.5)	4 (100.0)	74 (81.3)	122 (84.7)
Amikacin	7 (14.0)	3 (100,0)	10 (18.9)	29 (33.3)	1 (25.0)	30 (33.0)	40 (27.8)
Meropenem	11 (22.0)	2 (66.7)	13 (24.5)	18 (20.7)	1 (25.0)	19 (20.9)	32 (22.2)
Gentamicin	35 (70.0)	3 (100.0)	38 (71.7)	27 (31.0)	1 (25.0)	28 (30.8)	66 (45.8)
Cefotaxime	46 (92.0)	2 (66.7)	48 (90.6)	22 (25.3)	1 (25.0)	23 (25.3)	71 (49.3)
Piperacillin-tazobactam	17 (34.0)	0	17 (32.1)	23 (26.4)	3 (75.0)	26 (28.6)	43 (29.9)
Cefepime	45 (90.0)	3 (100.0)	48 (90.6)	31 (35.6)	3 (75.0)	34 (37.4)	82 (56.9)
Amoxicillin-clavulanate	48 (96.0)	3 (100.0)	51 (96.2)	78 (89.6)	3 (75.0)	81 (89.0)	132 (91.7)
Ceftazidime	49 (98.0)	1 (33.3)	50 (94.3)	63 (72.4)	4 (100.0)	74 (81.3)	117 (81.3)

n = no of isolates; ESBL-PE = Extended spectrum β-lactamase producing Enterobacterales

Table 3: Demographic characteristics and risk factors for ESBL faecal carriage among under 5 years old children in Ladoke Akintola University of Technology Teaching Hospital, Ogbomoso, Nigeria

Characteristics		**ESBL**			x^2	**OR (95% CI)**	***p* value**
		Positive (%)	**Negative (%)**	**Total (n=144)**			
Age group (months)	1-23	27 (33.7)	53 (66.3)	80	5.532	0.4221 (0.22-0.83)	0.0187*
	24-59	35 (55.0)	29 (45.0)	64			
Gender	Male	33 (43.0)	44 (57.0)	77	0.01994	1.111 (0.57-2.16)	0.8877
	Female	27 (40.3)	40 (59.7)	67			
Prior intake of antibiotics	1 week	9 (69.2)	4 (30.8)	13	8.531	NA	0.014*
	2 weeks	7 (87.5)	1 (12.5)	8			
	≥ 3 weeks	28 (41.2)	40 (58.8)	68			
Intake of antibiotics without prescription	Yes	18 (69.2)	8 (30.7)	26	5.817	3.491 (1.34-9.07)	0.0159*
	No	29 (39.2)	45 (60.8)	74			
Hospital visit	Yes	14 (45.1)	17 (54.8)	31	0.02809	1.071 (0.48-2.38)	0.8669
	No	50 (39.1)	65 (61.9)	105			
Educational level of mother	None	3 (60.0)	2 (40.0)	5	3.056	NA	0.3831
	Primary	0	2 (100.0)	2			
	Secondary	16 (59.3)	11 (40.7)	27			
	Tertiary	34 (49.3)	35 (50.7)	69			

*=statistically significant; x^2=Chi square; OR=Odds ratio; CI=Confidence interval; ESBL=Extended spectrum beta-lactamase; NA=Not applicable

Discussion:

In this study, the overall prevalence of ESBL producing *E. coli* and *K. pneumoniae* fecal carriage among children under 5 years of age was 37.5%. This prevalence is lower when compared with the reports of some previous studies such as that reported by Desta et al., (21) with a prevalence of 52.0% faecal ESBL carriage. It is also lower than the prevalence of 55.4% reported by Sageerabanoo et al., (22). In contrast, the prevalence in our study is comparable with the study carried out by Isendahl et al., (23) who reported a prevalence of 32.6% of ESBL carriers and by Tellevik et al., (24) who reported a prevalence of 34.3% in their study. On the other hand, the prevalence of ESBL reported in our study is higher when compared to the study carried out by Tola et al., (11) who reported a rate of 17.1% in Nigeria. Another study carried out by Jewoola et al., (1) reported the prevalence of rectal carriage of ESBL-PE to be 25.3%. The differences in prevalence rates in these studies might be due to differences in the study participants, study settings, and geographical locations.

In this study, the most common ESBL producing strains isolated from the children was *E. coli* (34.7%), similar to the studies from Turkey and Korea that reported *E. coli* as the most common ESBL-producing isolates (25,26), as well as a study from India where *E. coli* was the most common ESBL producer (77.0%), followed by *Klebsiella* spp (16.4%) (22). In contrast, the study by Jewoola et al., (1) reported the most common ESBL-PE to be *K. pneumoniae* (44.2%), followed by *E. coli* (34.2%) and *Enterobacter* spp (12.8%). While *E. coli* was the most common ESBL-PE in this study, the rate of ESBL-production by *K. pneumoniae* was slightly higher (42.9%, 3/7) than *E. coli* isolates (36.5%, 50/137), although this difference in rate was not statistically significant (OR=0.7663; 95%CI=0.165-3.564; *p*=0.7083), probably due to the rather small number of *K. pneumoniae* isolates

In our study, ESBL producing *E. coli* and *K. pneumoniae* isolates showed high resistance rate to ampicillin (100.0%), ceftazidime (98.0%), amoxicillin-clavulanate (96%), cefotaxime (92.0%), and cefepime (90.0%). This is a similar pattern to another study in Nigeria which reported ESBL-producing isolates exhibiting high resistance to cefotaxime (98.6%), ceftazidime (95.1%), ciprofloxacin (72.7%) and cefepime (58.7%) (1). A study in Ethiopia similarly reported ESBL-producing isolates with high resistance rates to ampicillin (94.9%), cefepime (74.4%), and ceftazidime (71.8%) (11). However, up to 22.0% of the ESBL producing *E. coli* and *K. pneumoniae* isolates in our study were resistant to carbapenem, the currently most active drug

against them. This rate is higher than the 2% resistant rate of ESBL producing isolates to carbapenem, reported in the study by Desta et al., (21).

Significant risk factors associated with faecal ESBL carriage in this study were aged 24-59 months, intake of antibiotics 1 or 2 weeks preceding the study and intake of antibiotics without medical prescription while gender of children, level of mother's education and previous hospital visit by children were not significantly associated with faecal ESBL carriage.

Conclusion:

The findings of our study showed that faecal carriage rate of ESBL-PE (*E. coli* and *K. pneumoniae*) was high at 37.5% among children under 5 years of age in LAUTECH Teaching Hospital, Ogbomoso, southwest Nigeria. The ESBL producing isolates also exhibited high rate of multi-drug resistance (MDR) to ampicillin, amoxicillin-clavulanic acid, ceftazidime, cefotaxime, and cefepime.

Routine screening of Gram-negative bacterial isolates for phenotypic ESBL production is crucial for surveillance of antimicrobial resistance (AMR) in the hospital. Surveillance information is important for the hospital antimicrobial stewardship (AMS) programme, particularly in guiding appropriate antibiotic selection and the development of antibiotic guideline for initial empirical therapy of suspected infections caused by ESBL-producing pathogens.

Acknowledgements:

The authors appreciate the support of the nurses and doctors in the Department of Paediatrics during the recruitment of eligible participants from whom rectal swab samples were collected for the study

Contributions of authors:

ASA conceived and designed the study, and wrote the initial manuscript draft; OOT coordinated fieldwork during sample collection; LOA reviewed the literature; OKI was involved in data collection and sample processing; AOA was involved in data collection and editing of the manuscript; and OMO was involved in coordination of the fieldwork and review of the manuscript. All authors read and approved the final manuscript.

Source of funding:

Authors receive no external funding

Conflict of interest:

Authors declare no conflict of interest

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Umo et al. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (1): 68 – 74 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jan 2024; Vol.25 No.1
AJCEM/2265. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Evaluation of serum concentration of essential trace elements during therapy among tuberculosis patients in Uyo, Nigeria

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Abstract:

Background: Nutritional status is one of the most important determinants of immune response to infection. The objective of this study was to assess the serum concentrations of selected trace elements in selected patients on anti-tuberculosis (TB) therapy in Uyo, Akwa Ibom State, Nigeria
Methodology: This was a prospective observational study of selected TB patients attending the TB treatment centers of selected hospitals in Uyo, Akwa Ibom State, Nigeria, for assessment of the serum concentrations of some essential trace elements during anti-TB therapy. First, participants with suspected pulmonary TB were consecutively selected and sputum samples were collected from each of them into wide mouth containers for GeneXpert TB analysis. Then, 5 millilitres of venous blood were collected from participants who tested positive for *Mycobacterium tuberculosis* (MTB) on GeneXpert test into plain specimen containers at the time of diagnosis, and at the 2nd, 4th and 6th month of anti-TB therapy. Blood samples were also collected from randomly selected apparently healthy individuals as controls. The samples were centrifuged at 3,000 rpm for 5 minutes, and serum concentrations of copper (Cu), zinc (Zn), iron (Fe), selenium (Se) and chromium (Cr) were measured using flame atomic absorption spectrometry.
Results: A total of 155 participants with suspected TB were selected for the study, 83 (53.5%) were females while 72 (46.5%) were males. Majority of the participants were in age group 31-50 years. Thirteen (8.4%) participants were positive for MTB on GeneXpert analysis and placed on standard anti-TB therapy, while 1 participant defaulted. The mean serum concentrations of all the trace elements measured for the 12 positive participants at the different stages of anti-TB therapy was statistically significant ($p<0.05$). The mean serum concentrations of Zn, Fe and Se were significantly increased at the 4th and 6th month of therapy compared to the concentration at diagnosis and at 2nd month of treatment. However, the mean serum concentrations of Cu and Cr significantly decreased at the 6th month of treatment compared to their concentrations at initial diagnosis.
Conclusion: Assessment of the serum concentrations of Zn, Fe, Cu, Se and Cr could serve as indicator of nutritional status and oxidative stress, as well as serve as treatment indices to assess patients on anti-TB therapy.

Keywords: tuberculosis; trace elements; serum concentration; therapeutic response; Uyo; Nigeria

Received Apr 26, 2023; Revised Jul 3, 2023; Accepted Jul 4, 2023

Évaluation de la concentration sérique des oligo-éléments essentiels au cours du traitement chez les patients tuberculeux à Uyo, Nigeria

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Résumé:

Contexte: L'état nutritionnel est l'un des déterminants les plus importants de la réponse immunitaire à l'infection. L'objectif de cette étude était d'évaluer les concentrations sériques d'oligo-éléments sélectionnés chez des patients sélectionnés sous traitement antituberculeux (TB) à Uyo, dans l'État d'Akwa Ibom, au Nigeria.
Méthodologie: Il s'agissait d'une étude observationnelle prospective de patients tuberculeux sélectionnés fréquentant les centres de traitement de la tuberculose d'hôpitaux sélectionnés à Uyo, dans l'État d'Akwa Ibom, au Nigeria, pour l'évaluation des concentrations sériques de certains oligo-éléments essentiels pendant le traitement antituberculeux. Tout d'abord, les participants suspects de tuberculose pulmonaire ont été sélectionnés consécutivement et des échantillons d'expectorations ont été prélevés sur chacun d'eux dans des récipients à large ouverture pour l'analyse GeneXpert TB. Ensuite, 5 millilitres de sang veineux ont été prélevés sur des participants testés positifs pour *Mycobacterium tuberculosis* (MTB) sur le test GeneXpert dans des récipients d'échantillons simples au moment du diagnostic et aux 2e, 4e et 6e mois de traitement antituberculeux. Des échantillons de sang ont également été prélevés sur des individus apparemment en bonne santé choisis au hasard comme témoins. Les échantillons ont été centrifugés à 3 000 tr/min pendant 5 minutes et les concentrations sériques de cuivre (Cu), de zinc (Zn), de fer (Fe), de sélénium (Se) et de chrome (Cr) ont été mesurées par spectrométrie d'absorption atomique à flamme.
Résultats: Un total de 155 participants suspects de tuberculose ont été sélectionnés pour l'étude, 83 (53,5%) étaient des femmes et 72 (46,5%) étaient des hommes. La majorité des participants appartenaient à la tranche d'âge des 31 à 50 ans. Treize (8,4%) participants étaient positifs pour MTB lors de l'analyse GeneXpert et placés sous traitement antituberculeux standard, tandis qu'un participant a abandonné. Les concentrations sériques moyennes de tous les oligo-éléments mesurées pour les 12 participants positifs aux différentes étapes de la thérapie antituberculeuse étaient statistiquement significatives ($p<0,05$). Les concentrations sériques moyennes de Zn, Fe et Se ont été significativement augmentées au 4ème et 6ème mois de traitement par rapport à la concentration au moment du diagnostic et au 2ème mois de traitement. Cependant, les concentrations sériques moyennes de Cu et Cr ont significativement diminué au 6ème mois de traitement par rapport à leurs concentrations lors du diagnostic initial.
Conclusion: L'évaluation des concentrations sériques de Zn, Fe, Cu, Se et Cr pourrait servir d'indicateur de l'état nutritionnel et du stress oxydatif, ainsi que servir d'indices de traitement pour évaluer les patients sous traitement antituberculeux.

Mots clés: tuberculose; oligo-éléments; concentration sérique; réponse thérapeutique; Uyo; Nigeria

Introduction:

Tuberculosis (TB) is a chronic granulomatous infectious disease that mostly affects the lungs but can affect other parts of the body, caused by members of the *Mycobacterium tuberculosis* complex (1). It remains the top infectious killer in the world claiming close to 4,000 lives a day (2). Common symptoms of active TB are cough with sputum and blood, chest pains, weakness, weight loss, fever and night sweats. Nutritional status is one of the most important determinants of immune response to resist TB, and undernutrition has been reported to be a risk factor for the progression of latent TB to active TB disease. Tuberculosis may cause malnutrition through increased metabolic demands and decreased nutrient intake (3). Food and nutri tional care are essential for a successful TB prevention and health promotion.

Trace elements are elements that occur in nature or in perturbed environment in small amount, and that when present in sufficient bioavailable concentration are toxic to living organism (4). The essential ones are known to play a variety of important roles, including acting as structural components of vitamins (e. g. cobalt), co-factors in metalloenzymes, glutathione peroxidase (e. g. selenium), catalytic components of numerous enzymes (e. g. zinc and copper), and as structural components of some proteins that are significant in immune reactions (5).

Deficiencies in various essential trace elements have been associated with decreased immunity against TB, moreover, trace elements are believed to have an impact on clinical outcome and are thus related to disease control (3). The World Health Organization (WHO) guideline on nutritional care and support for TB patient states that their nutritional status should be assessed at diagnosis and throughout treatment, and they should receive appropriate counseling based on their nutritional status (6). Various direct and indirect assays such as measurement of enzymatic activity or nutrient metabolites are used to assess the quantity of nutrients in specimens such as blood, tissue and urine (5). The objective of this study is therefore to determine the serum concentration of selected trace elements in newly diagnosed TB cases attending some selected hospitals in Uyo, Akwa Ibom State, Nigeria.

Materials and method:

Study setting and design:

This study was a prospective observational study of persons with clinical suspicion of pulmonary tuberculosis (TB) attending the TB treatment centers of selected hospitals in Uyo, Akwa Ibom State, Nigeria from November 2021 to July 2022. The selected hospitals were randomly selected and included Saint Luke's Hospital Anua, and University of Uyo Teaching Hospital.

Study population, sample size & participants selection:

A total of 155 with clinical suspicion of pulmonary TB were consecutively selected as participants for the study. The sample size of 155 was calculated based on TB prevalence of 10.3% from a previous study carried out in Enugu, Nigeria by Kenneth et al., (7).

Patients with clinical diagnosis of pulmonary TB and co-infected with HIV, and patients who were yet to commence TB treatment after diagnosis were included in the study, while patients with another diagnosed pulmonary morbidity, who tested negative for TB and persons below 18 years of age were excluded.

Of the 155 suspected TB persons, 13 were positive for TB by GeneXpert MTB, one of whom defaulted, leaving only 12 confirmed TB patients for the trace elements determination at diagnosis, 2nd, 4th and 6th month of TB treatment. Ten randomly selected apparently healthy individuals aged 18 years and above, formed the control group.

Ethical consideration:

Ethical approval was obtained from the Akwa-Ibom State Ethical Review Committee of the Ministry of Health. Written informed consent was obtained from patients, stating that they understood and agreed to participate in the research.

Collection of blood sample:

Five millilitres of venous blood were collected from the 12 selected participants by venipuncture into plain blood container at the time of diagnosis, and at follow up in the 2nd, 4th and 6th month (completion) of TB treatment regimen. The blood samples were centrifuged at 3,000 rpm for 5 minutes at room temperature to separate the sera. Serum samples were stored at -20^{0}C until ready for trace element analysis. Approximately, 5 mls of blood were also collected from each of the 10 control participants once.

Measurement of serum concentration of trace elements:

Serum concentrations of copper, zinc, iron, selenium and chromium were measured using Varian AA240 Atomic Absorption Spectrophotometer at the Michael Okpara University of Agriculture Research Institute laboratory. Reference ranges of each of the selected trace elements are copper 1.6-2.4 µmol/L, zinc 0.66-1.10 µmol/L, iron 10.09-16.82 µmol/L, selenium 110-165 µg/L, and chromium 0.05 to 0.16 µg/L

Statistical analysis of data:

The data were analysed using IBM SPSS Version 20.0. Continuous variable was expressed as mean and standard deviation (mean ± SD). The mean difference between more than two groups was analysed by One-way ANOVA. The categorical variables results were expressed in frequency and percentages. Comparison of proportion of distribution was analysed by Chi-square. P-value less than 0.05 was considered statistically significant difference/association between group(s).

Results:

The demographic distribution of the study participants is shown in Table 1. Of the total 155 participants included in the study, 83 (53.5%) were females, while 72 (46.5%) were males. Forty-one (20%) were 20 years or below, 54 (34.8%) were aged 31-30 years, 42 (27.1%) were 31-40 years, 19 (12.3%) were 41-50 years, 5 (3.2%) were 51-60 years and 4 (2.6%) were 60 years or above. The mean age of the participants is 25.83±17.21 years.

Table 1: Demographic distribution of the study participants

Characteristics	Frequency	Percentage
Gender		
Female	83	53.5
Male	72	46.5
Age group (years)		
≤ 20	31	20.0
21-30	54	34.8
31-40	42	27.1
41-50	19	12.3
51-60	5	3.2
>60	4	2.6
Total	**155**	**100**
Mean age (years)	25.83±17.21	

The mean serum concentrations of trace elements for the 12 confirmed pulmonary TB participants at the various stages of TB treatment compared with the controls (n=10) are shown in Table 2. The difference in the mean serum concentrations of all the trace elements between the various stages of treatment was statistically significant (p<0.05) by ANOVA test.

However, the mean serum concentration of copper at the time of initial diagnosis (Cu0, 1.03±0.27) and at the 2nd month of treatment (Cu1, 0.99±0.25) was not significantly different, but was significantly higher than the mean serum concentration at the 4th (Cu2, 0.25±0.10) and 6th month (Cu3, 0.33± 0.14), and in the control subjects (0.63± 0.13). From the ANOVA post-hoc analysis, the mean serum concentrations of copper at the 4th and 6th month were significantly lower than the mean serum concentration at initial diagnosis and in the controls (p<0.001).

Similarly for zinc, the mean serum concentration at diagnosis (Zn0, 0.59±0.26) and at the 2nd month (Zn1, 0.53±0.19) was not significantly different from each other and from the controls (0.72±0.11), but was signi-

ficantly lower than the mean serum concentrations at the 4th (Zn2, 1.28±0.27) and 6th month (Zn3, 1.29±0.19) of the treatment (p<0.001). For iron, the mean serum concentration at diagnosis (Fe0, 0.37±0.29) and at the 2nd month (Fe1, 0.34±0.1) was not significantly different, but was significantly lower than the controls (0.94±0.18), and at the 4th (Fe2, 0.87±0.33) and 6th month (Fe3, 0.94± 0.18) of the treatment regimen (p<0.001).

The mean serum concentration of selenium at the initial diagnosis (Se0, 20.42± 6.61) and at the 2nd month (Se1, 20.17± 6.58) was not significantly different, but significantly lower than the controls (38.60± 6.43), and at 4th (Se2, 33.42±7.81) and 6th month (Se3, 34.17±10.36) of the treatment regimen (p<0.001). The mean serum concen trations of chromium were high in the control subjects (5.60±5.03) but not significantly different from the mean serum concentrations of the TB patients at initial diagnosis (Cr0, 8.0±5.69) and at 2nd (Cr1, 3.75±2.6) and 4th month (Cr2, 3.92±3.12), however, it was significantly higher than at the 6th month (Cr3, 2.17±1.53) of the treatment regimen (p=0.02).

Discussion:

Micronutrients play a crucial role in the pathophysiology of tuberculosis (8). Its deficiency suppresses immune functions by affecting the innate T-cell-mediated immune response and adaptive antibody response, and this leads to dysregulation of the balanced host response and also increases the susceptibility to infections, with increased morbidity and mortality (9). Undernutrition has been reported to be a risk factor for the progression of latent TB infection (LTBI) to active TB disease (ATBD), and its presence at the initial diagnosis of active TB has been reported to be a predictor of increased risk of death and TB relapse (10).

In this study, the TB prevalence was 8.4%, which is lower compared to the rate reported in Uyo by Ibokette et al., (11) with TB prevalence of 12.4%. It is also lower than 13.7% reported by Itah and Udofia (12) in another study in Uyo on epidemiology and endemicity of pulmonary TB in South-Eastern Nigeria. A study by Ulasi et al., (13) in Enugu, Nigeria reported prevalence of 6.8%, which is lower than the rate in our current study. In Lagos a higher prevalence of 23.4% was reported by Adejumo et al., (14).

Trace elements levels have been reported to be associated with diseases (15). The present study shows that mean serum concentration of Zn at diagnosis (Zn0) was significantly low, but with progression of therapy at the 4th (Zn2) and 6th month (Zn3), there was significant increase in Zn concentration compared to control group. The same findings were reported in a study by Pourfallah et al., (16) in Iran and Festus et al., (17) in Edo, Nigeria. Decreased level of Zn could be due to pre-existing malnutrition and increased usage of Zn by the tuberculosis bacteria itself. Thus, these parameters directly reflect the pathophysiological state of the disease process. Zinc insufficiencies among MDR-TB patients have a negative impact on the immune system. In effect, zinc deficiency is responsible for an alteration in the macrophage function and reduction in production of tumor necrosis factor (TNF-α) and interferon-γ (INF-γ). These low zinc concentrations could also be responsible for decrease in proliferation and differentiation of T and B lymphocytes (18), which are at the forefront of immune system defenses and protection against active TB (9). In addition, it was reported that zinc and vitamin A supplementation in adult patients with active TB could allow elimination of *Mycobacterium,* thus leading to quick cure of these patients (19).

This study also shows that mean serum iron concentration in patients with TB was significantly lower at initial diagnosis (Fe0) and at second month of therapy (Fe1) compared to control, but serum Fe increased significantly in the 4th and 6th month of therapy. These findings are consistent with the work of Pourfallah et al., in Iran (16). Lower serum concentration was also reported among TB patients by Lawn et al., (20) in Northern Nigeria (20). Iron is a micronutrient that is important for both the host and MTB metabolism. Pathogenic mycobacteria compete with the host for iron, either by directly depleting intracellular iron from the host cytoplasm or by synthesizing siderophores and micromolecules, including transferrin, ferritin or lactoferrin, which have high affinity to capture extracellular ferric ion (21). Anaemia, as a result of inflammation caused by MTB infection, is predominantly caused by an iron delivery problem, where erythrocyte iron is poorly used and dietary iron is enriched in intestinal enterocytes (22).

Table 2: Comparison of the mean serum concentrations of different trace elements at various stages of TB treatment for the twelve participants with confirmed pulmonary TB and the controls

Trace element	Time of measurement	Serum concentration (µmol/L) (Mean ± SD)	F value (ANOVA)	*p* value
Cu 0	At diagnosis	1.03±0.27[a]	39.52	<0.001
Cu 1	2 months	0.99±0.25[a]		
Cu 2	4 months	0.25±0.10[b]		
Cu 3	6 months	0.33±0.14[b]		
Cu control (n=10)		0.63±0.13[c]		
Zn 0	At diagnosis	0.59±0.26[a]	32.43	<0.001
Zn 1	2 months	0.53±0.19[a]		
Zn 2	4 months	1.28±0.27[b]		
Zn 3	6 months	1.29±0.19[b]		
Zn control (n=10)		0.72±0.11[a]		
Fe 0	At diagnosis	0.37±0.09[a]	25.29	<0.001
Fe 1	2 months	0.34±0.1[a]		
Fe 2	4 months	0.87±0.33[b]		
Fe 3	6 months	0.94±0.18[b]		
Fe control (n=10)		0.94±0.18[b]		
Se 0	At diagnosis	20.42±6.61[a]	11.27	<0.001
Se 1	2 months	20.17±6.58[a]		
Se 2	4 months	33.42±7.81[b]		
Se 3	6 months	34.17±10.36[b]		
Se control (n=10)		38.60±6.43[b]		
Cr 0	At diagnosis	8.0±5.69[a]	4.14	0.02
Cr 1	2 months	3.75±2.6[a,b]		
Cr 2	4 months	3.92±3.12[b]		
Cr 3	6 months	2.17±1.53[b]		
Cr control (n=10)		5.60±5.03[a,b]		

a, b, c values with same superscript are not significantly different at pairwise comparison; SD = Standard deviation ANOVA = Analysis of Variance

Our study also shows significant decrease in mean serum selenium concentration in patients with TB at diagnosis (Se0) compared to control participants. This decrease was found to cut across the second month (Se1) of therapy. However, there was significant increase in selenium concentration at the 4th (Se2) and 6th month (Se3) of therapy. This is because in inflammation, selenoenzymes are translocated as a result of increased vascular permeability, and selenium passes into the tissues. Therefore, serum selenium levels may not represent the actual level of selenium in the body. These findings are consistent with the report by Moraes et al., (23) in Brazil and Kassu et al., (24) in Ethiopia. Selenium deficiency induces a systematic redox imbalance and inflammation in the blood and causes pathological changes in the liver (25). Reduced selenium status in turn can pathologically increase inflammation cascade, thus contributing to disease manifestation, forming a vicious circle (26).

In this study, we found that mean serum concentration of chromium at TB diagnosis (Cr0) was high but not significantly different from the control. At month 2 (Cr1) and month 4 (Cr2) of TB therapy, there was also no significant difference in concentration of chromium. However, at the final stage (month 6, Cr3) of therapy, there was significant decrease in the chromium level compared to the level at diagnosis, but no significant difference compared to the control. No reported work on serum concentration of chromium in TB patients was found in the literature to support these findings. However, Festus et al., (17) reported a decrease in chromium concentration in TB patients (17). Nevertheless, a study by Akkas et al., (27) on serum trace elements and heavy metal levels in patients with sepsis reported an increase in the concentration of chromium in patients with sepsis.

Also reported in this study is the fact that the serum concentration of copper was significantly higher than that of the control group at diagnosis (Cu0) and at the second month of therapy (Cu1). At month 4 (Cu2) and month 6 (Cu3) of TB treatment, there was significant decrease in serum copper concentration compared to that at diagnosis and the control subjects. The findings on Cu level in this study can be compared with study done by Reza et al., (28), which reported a rise in serum Cu level in TB patients than in normal subjects. Copper is used as a bactericidal agent within macrophages, accumulating in phagolysosomes during infection (29), and both systemic and localized copper concentrations in mammals are increased during infection (30). However, Edem et al., (31) showed decreased serum copper level in PTB patients due to utilization by both macrophages and *Mycobacterium* (31). Another study reported that mycobacteria require copper for survival (32).

Conclusion:

The analysis of levels of the elements Fe, Cu, Zn, Se and Cr may help in the under-

standing the contributions of these elements to immune function during development of TB disease. The enhancement or reduction in the level of these elements may be linked with typical symptoms of TB. There is need for periodical nutrition supplementation during TB treatment.

Serum trace elements analysis should be included as one of the diagnostic procedures for TB since malnutrition and TB are both problems of considerable magnitude in most of the underdeveloped regions of the world. It is important to consider how these two problems tend to interact with each other. Awareness programs should be created in high-risk areas on need for good feeding habits and general nutrition.

Contribution of authors:

UAN was involved in conceptualization of the study, supervision, and writing of the manuscript draft; JNF was involved in methodology, investigation, and project administration; ASA was involved in formal analysis, reviewing and editing of the manuscript. All authors read and approved the manuscript submitted for publication.

Source of funding:

Authors received no external funding.

Conflict of interest:

No conflict of interest is declared.

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Isichei-Ukah et al. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (1): 75 - 85 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jan 2024; Vol.25 No.1
AJCEM/2290. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

A survey of antibiotic resistance and virulence factors in *Enterococcus* species isolated from poultry farms in Benin City, Nigeria

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Abstract:

Background: Enterococci are commensal bacteria resident in the gastrointestinal tract of humans and animals. However, their increasing resistance to clinically important antimicrobial agents remain a global threat. The objective of this study is to determine the prevalence, antimicrobial resistance profile and virulence factors of *Enterococcus* isolated from selected poultry farms in Benin City, Nigeria.
Methodology: Sixty samples (20 feed, 20 water and 20 faecal samples) were randomly collected from five selected poultry farms in different commercial farming areas between August and September 2020. The samples were first enriched in Tryptone Soy Broth (TSB) and then cultured on Bile Aesculin Azide (BAA) agar aerobically at 37°C for 18-24 hours. Black colonies on BAA agar were presumptively identified as *Enterococcus* and confirmed by conventional biochemical tests and Analytical Profile Index (API) rapid ID 32 STREP. The antibiotic susceptibility of the isolates was determined by the Kirby-Bauer disc diffusion method. The virulence factors and biofilm formation were evaluated using standard bacteriological and microtitre plate methods.
Results: In total, *Enterococcus*-positive samples were 32/60 (53.3%) with a total of 45 *Enterococcus* isolates. The speciation of the *Enterococcus* isolates based on API rapid ID 32 STREP were *Enterococcus faecium* 15/45 (33.3%), *Enterococcus faecalis* 12/45 (26.7%), *Enterococcus durans* 8/45 (17.8%), *Enterococcus casseliflavus* 5/45 (11.1%) and *Enterococcus hirae* 5/45 (11.1%). The isolates showed the highest antibiotic resistance to ampicillin (100.0%), fosfomycin (95.6%) and penicillin G (88.9%) and the least resistance to ciprofloxacin (22.2%) and chloramphenicol (28.9%). The virulence factors of *Enterococcus* species observed were gelatinase, β-hemolytic and hyaluronidase activity, biofilm, and S-layer formation. The degree of biofilm formation by the *Enterococcus* species was strong biofilm formation (19/45, 42.2%), moderate biofilm formation (10/45, 22.2%), weak biofilm formation (11/45, 24.4%) and no biofilm formation (5/45, 11.1%).
Conclusion: Findings from this study emphasized on the potential health implications associated with antimicrobial resistance and phenotypic virulence factors of *Enterococcus* in poultry products.

Keywords: Antibiotic resistance; *Enterococcus*; Poultry; Virulence factors; Benin City

Received Aug 13, 2023; Revised Oct 11, 2023; Accepted Oct 13, 2023

Une enquête sur la résistance aux antibiotiques et les facteurs de virulence chez les espèces d'*Enterococcus* isolées dans des élevages de volailles à Benin City, Nigeria

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Résumé:

Contexte: Les entérocoques sont des bactéries commensales résidant dans le tractus gastro-intestinal des humains et des animaux. Cependant, leur résistance croissante aux agents antimicrobiens cliniquement importants reste une menace mondiale. L'objectif de cette étude est de déterminer la prévalence, le profil de résistance aux antimicrobiens et les facteurs de virulence d'*Enterococcus* isolés dans des élevages de volailles sélectionnés à Benin City, au Nigeria.
Méthodologie: Soixante échantillons (20 échantillons d'aliments, 20 d'eau et 20 échantillons de matières fécales) ont été collectés de manière aléatoire dans cinq élevages de volailles sélectionnés dans différentes zones d'élevage commercial entre août et septembre 2020. Les échantillons ont d'abord été enrichis dans du bouillon tryptone soja (TSB), puis cultivés sur Gélose bile-esculine-azide (BAA) en aérobiose à 37 °C pendant 18 à 24 heures. Les colonies noires sur gélose BAA ont été présumées identifiées comme étant Enterococcus et confirmées par des tests biochimiques conventionnels et par l'indice de profil analytique (API) ID rapide 32 STREP. La sensibilité aux antibiotiques des isolats a été déterminée par la méthode de diffusion sur disque de Kirby-Bauer. Les facteurs de virulence et la formation de biofilm ont été évalués à l'aide de méthodes bactériologiques et de plaques de microtitrage standard.
Résultats: Au total, les échantillons positifs pour *Enterococcus* étaient de 32/60 (53,3 %) avec un total de 45 isolats d'*Enterococcus*. La spéciation des isolats d'*Enterococcus* basée sur l'API rapid ID 32 STREP était *Enterococcus faecium* 15/45 (33,3%), *Enterococcus faecalis* 12/45 (26,7%), *Enterococcus durans* 8/45 (17,8%), *Enterococcus casseliflavus* 5/45 (11,1%) et *Enterococcus hirae* 5/45 (11,1%). Les isolats présentaient la résistance aux antibiotiques la plus élevée à l'ampicilline (100,0%), à la fosfomycine (95,6%) et à la pénicilline G (88,9%) et la moindre résistance à la ciprofloxacine (22,2%) et au chloramphénicol (28,9%). Les facteurs de virulence des espèces d'*Enterococcus* observés étaient la gélatinase, l'activité β-hémolytique et hyaluronidase, le biofilm et la formation de couche S. Le degré de formation de biofilm chez l'espèce *Enterococcus* était une forte formation de biofilm (19/45, 42,2%), une formation modérée de biofilm (10/45, 22,2%), une faible formation de biofilm (11/45, 24,4%) et aucune formation de biofilm (5/45, 11,1%).
Conclusion: Les résultats de cette étude mettent l'accent sur les implications potentielles sur la santé associées à la résistance aux antimicrobiens et aux facteurs de virulence phénotypique d'Enterococcus dans les produits de volaille.

Mots-clés: Résistance aux antibiotiques; Entérocoque; La volaille; Facteurs de virulence; Bénin Ville

Introduction:

Enterococcus is an autochthonous microbiota of the gastrointestinal and skin flora tract of birds, humans and diverse animal species (1). Enterococci are Gram-positive, non-spore-forming, catalase-negative and facultative anaerobic bacteria. In domestic animals, especially in the poultry industry, enterococcal probiotics are beneficial in infection control, improving the immune system and growth promotion (2). Globally, the poultry industry is one of the fastest and largest growing agro-based protein production industries. The intense desire to meet up with the high demand for poultry products usually involve the usage of enterococcal probiotic supplements (3). However, despite their intrinsic potentials in the food industry, they are not generally recognized as safe (GRAS), and their presence could also be attributed to faecal contamination (4).

The activities of *Enterococcus,* like other opportunist pathogens, can also trigger an infection in animals and humans when it invades other mucosal and skin surfaces, especially in cases of reduced host immunity (5). The foremost species responsible for enterococcal-related infections in humans are *E. faecalis* and *E. faecium,* and they are usually associated with urinary tract infections, liver infections, endocarditis and septicemia (1). The ability of these microorganisms to cause can be attributed to several virulence factors (6). However, their resistance to various antibiotics notably enhances the pathogenic strength expressed by these virulence factors (7). This makes the absence of transferable antibiotic resistance an essential criterion for selecting enterococci as probiotic food supplements (8,9).

Enterococci of food origin have not been explicitly determined as immediate causes of clinical infections (10). Still, the presence of antibiotic-resistant enterococci has been reported in retail poultry meats (11). This tends to be a potential risk of transmitting antimicrobial resistance genes to humans when consumed (10). Antimicrobial resistance in enterococci also enhances their ability to withstand a variety of host defenses including innate immune system (6). Although the strains of enterococci linked with clinical infections may vary from animal-related strains, antibiotic-resistant strains that are genetically related have been linked to both animals and human colonization (12, 13).

The exchange of vancomycin resistance between animals and humans has also been noticed *in vitro* and *in vivo* (14). The surveillance of antimicrobial resistance (AMR) in poultry production and the use of specific therapeutic agents are, therefore, imperative

concerning public food safety and environmental health concerns (15). The objective of this study is to determine the antibiotic resistance profile and phenotypic virulence properties associated with *Enterococcus* species isolated from poultry farms in Benin City, Edo State, Nigeria.

Materials and method:

Description of study area:

The samples were collected from five poultry farms in Benin City, Edo State, Nigeria. The five poultry farms were selected by simple random sampling from the different commercial farming areas within Benin City; Ekenwan road (Farm A), Sapele road (Farm B), Aruogba (Farm C), New Benin (Farm D) and Ugbowo (Farm E).

Sample collection:

Sixty samples were randomly collected from each poultry farm (12 random samples from each farm) between July and September 2020. The samples include 20 feed samples, 20 water samples and 20 faecal samples. Sterile containers were used to collect the water, feeds and fecal samples from the various farms and transported immediately to the Applied Microbial Processes and Environmental Health Research Group (AMPEHREG) laboratory, University of Benin, for analysis within 4 hours of sample collection.

Ethical consideration:

The samples were collected as recommended in "Institutional Animal Care and Use Committee" guidelines on ethics concerning the usage of animals and animal products for research purposes according to Suckow and Lamberti (16).

Enrichment and isolation:

Enrichment and isolation were carried out according to the method previously described by Sanlibaba et al., (17). Ten grams of the samples (feed and faecal) and 10 ml of water samples were introduced into 90 ml sterile distilled water. An aliquot of 1 ml from each stock solution was aseptically pipetted into 9 ml tryptone soy broth (TSB, Merck, Darmstadt, Germany). The TSB was incubated at 37°C for 18–24 hours. Subsequently, a loopful of bacterial culture in the TSB was streaked on bile aesculin azide (BAA) agar (TM Media, Rajasthan, India). The culture plates were incubated for 18-24 hours at 37°C.

Black colonies on BAA agar were considered to be presumptive *Enterococcus* isolates. The colonies were sub-cultured on fresh BAA agar and incubated for another 18-24 h at 37°C. Presumptive *Enterococcus* colonies that were recovered were purified on nutrient agar for 18-24 hours at 37°C. Purified isolates were stored on nutrient agar (Lab M, Lancashire, United Kingdom) slants until needed for further analysis.

Characterization and identification of *Enterococcus*:

Morphological characteristics and biochemical tests were determined using purified isolates as previously described (17,18). The purified isolates on Nutrient agar were characterized using Gram reaction with potassium hydroxide (3% KOH), oxidase test, catalase test, temperature tolerance range assay (10°C, 45°C), sodium chloride (NaCl) tolerance assay and Pyrrolidonyl-beta-naphthylamide (PYR) test. Following the manufacturer's instruction, the isolates were subsequently confirmed using Analytical Profile Index (API) rapid 32 STREP strips (BioMerieux, France).

Antimicrobial susceptibility screening:

Enterococcus isolates were screened for antibiotic resistance using the Kirby-Bauer disc diffusion method. Suspension of the test isolates with of 0.5 McFarland's approximated turbidity was pipetted and aseptically spread on Mueller-Hinton agar plates (Lab M, Lancashire, United Kingdom). The antibiotics discs (Mast Diagnostics, Merseyside, United Kingdom) were aseptically placed on the Mueller-Hinton agar culture plates. The antibiotics tested include penicillin G (10 units), ampicillin (10µg), rifampin (5µg), erythromycin (15µg), vancomycin (30µg), ciprofloxacin (5µg), chloramphenicol (30µg), fosfomycin (200µg) and nitrofurantoin (300 µg).

The culture plates were incubated at 37°C for 18-24 hours. The diameter of inhibition zones was measured and interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines (19,20).

Multiple antibiotic resistance index:

Multiple antibiotic resistance index (MARI) was determined according to the formula of Chitanand et al., (21) as simplified by Ogofure and Igbinosa (22); MARI = y/nx, where 'y' is the number of resistant isolates, 'n' is the number of isolates, and 'x' is the number of antibiotics tested. MAR index higher than 0.2 indicates that the organisms originate from high-risk sources of contamination and are, therefore of public health significance.

Determination of phenotypic virulence:

The colonies were cultured on TSA (Merck, Darmstadt, Germany) and re-suspended in 20 ml TSB. The turbidity of the suspension was adjusted to 10^6 cells/ml using McFarland guidelines for virulence determination. Gelatinase production was determined on gelatin medium. The β-haemolytic activity

was determined on sheep blood agar plate. Hyaluronidase activity was evaluated by spot inoculation using brain heart infusion broth supplemented with 1.0 g of agar-agar. The presence of surface-layer (S-layer) was assessed by streaking cultures on TSA plates, augmented with 0.1 mg/ml Coomassie brilliant blue R 250 (Merck, Darmstadt, Germany). All experiments were performed in triplicates and assessed in accordance with the method previously described (23).

Biofilm characterization:

The biofilm formation potential of the *Enterococcus* isolates was assessed quantitatively using the microtitre plate method. Suspension of overnight cultured *Enterococcus* (20µl) were re-standardized to 0.5 McFarland turbidity, inoculated into 96-wells microtitre plates containing 200 µl of nutrient broth and incubated at 37°C for 18-24 hours. Constituents of respective wells were removed, plates were rinsed with sterile phosphate buffered saline (PBS) and air-dried. The plates were then stained with 1% crystal violet (200 µl) for 30 mins. Respective wells were rinsed with de-ionized water to remove the crystal violet and then dried at 28±2°C. Crystal violet dye that bound to adherent cells was solubilized using 150 µl of absolute ethanol.

The optical density (OD) of the plates was determined at a wavelength 570 nm with a micro-plate reader (Synergy MxBiotekR, USA). The OD of each triplicate result, negative and positive controls was calculated. Isolates were classified as strong (ODi>0.12), moderate (ODi=0.1<0.12), weak (ODc<ODi <0.1) and non-biofilm producer (ODi<ODc), accordingly as previously described (24,25).

Statistical analysis:

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 21.0 and Microsoft Excel 2013. Mean values were expressed using descriptive statistics.

Results:

A total of 60 samples which comprised of 20 feeds, 20 water and 20 faecal samples obtained from 5 different poultry farms in Benin City, Nigeria, was assessed in this study. In overall, the total positive samples for *Enterococcus* isolates were 32/60 (53.3%). The frequency of *Enterococcus* isolation from the different samples is shown in Table 1 with 60.0% (12/20) from feeds, 75.0% (15/20) from water and 25.0% (5/20) from faeces

Table 1: Frequency of *Enterococcus* isolation from the different samples

Sample types	No of samples	No of *Enterococcus* positive samples (%)
Feed	20	12 (60.0)
Water	20	15 (75.0)
Faeces	20	5 (25.0)
Total	60	32 (53.3)

Fig 1 shows the frequency of *Enterococcus* isolation from the samples in each poultry farm, with 66.7% (8/12) in Farm A, 50.0% (6/12) in Farm B, 41.7% (5/12) in Farm C, 66.7% (8/12) in Farm D and 41.7% (5/12) in Farm E. Table 2 shows the phenotypic characterization and speciation of the 45 *Enterococcus* isolates based on API rapid ID 32 STREP. The frequency of *Enterococcus faecium* is 33.3% (15/45), *Enterococcus faecalis* 26.7% (12/45), *Enterococcus durans* 17.8% (8/45), *Enterococcus casseliflavus* 11.1% (5/45) and *Enterococcus hirae* 11.1% (5/45).

The antibiotic resistant profile of *Enterococcus* species is shown in Table 3, with resistance to penicillin G (88.9%, 40/45), rifampin (75.6%, 34/45), erythromycin (77.8%, 35/45), vancomycin (68.9%, 31/45), ciprofloxacin (22.2%, 10/45), chloramphenicol (28.9%, 13/45), ampicillin (100%, 45/45), fosfomycin (95.6%, 43/45) and nitrofurantoin (86.7%, 39/45).

The multiple antibiotic resistance index (MARI) of *Enterococcus* species is shown in Table 4. It was observed that a total of 38/45 (84.4%) isolates demonstrated resistance to at least five antibiotics. In comparison, all the isolates (45/45, 100.0%) demonstrated resis-

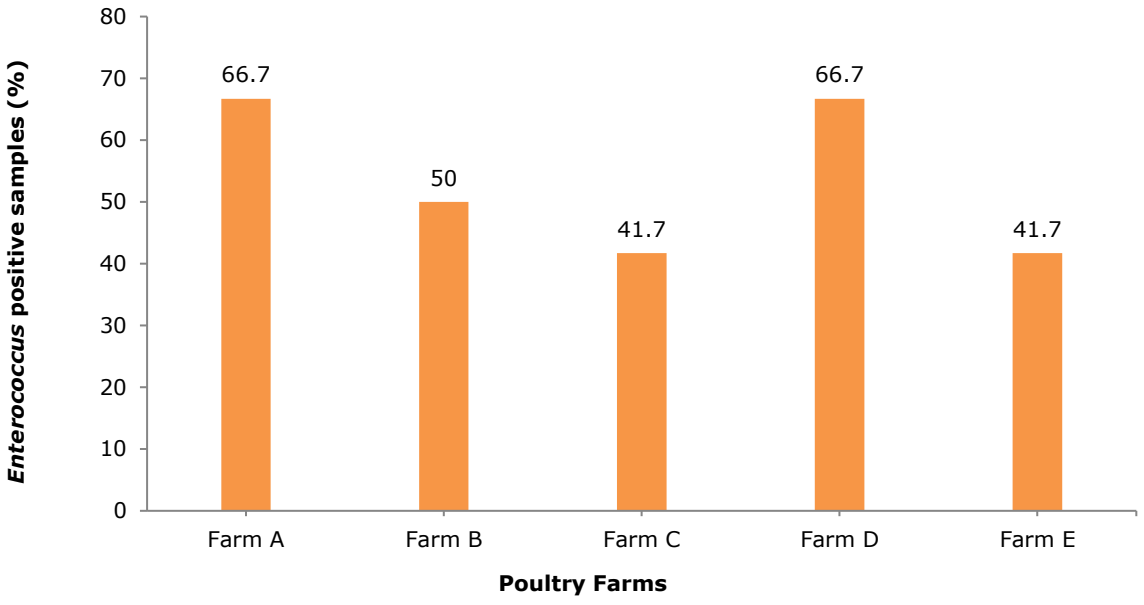

Fig 1: Frequency of occurrence of *Enterococcus* positive samples in each poultry farms

Table 2: Phenotypic characterization of the isolated *Enterococcus* species by conventional biochemical tests and API ID STREP

Group of isolates	Conventional biochemical tests						API ID 32 STREP	Number of isolates (%)
	Gram reaction (3% KOH)	Temperature of growth		Growth in 6.5% NaCl	PYR Test	Genus Identification		
		10 °C	45 °C					
Group A	+	+	+	+	+	*Enterococcus* spp.	*E. faecalis*	12 (26.7)
Group B	+	+	+	+	+	*Enterococcus* spp.	*E. faecium*	15 (33.3)
Group C	+	+	+	+	+	*Enterococcus* spp.	*E. durans*	8 (17.8)
Group D	+	+	+	+	+	*Enterococcus* spp.	*E. casseliflavus*	5 (11.1)
Group E	+	+	+	+	+	*Enterococcus* spp.	*E. hirae*	5 (11.1)
Total								**45 (100.0)**

KOH: Potassium hydroxide; PYR: Pyrrolidonyl-β- naphthylamide; NaCl: Sodium chloride
Group A: AF, BD, 2AF, CF, DD, 2AW, 2EW2, 4DW2, 2BF, 4BF2, DF, 3BW1
Group B: DW, 2EW1, 4AW2, 4DW1, AW, AD, CW, 4EW1, BF, CD, 2DF, 3DF1, ED, 2DW, 3DF2
Group C: 3BW2, 3DW1, 3CW1, 4AF2, 4CW1, 4AW1, 4CW2, 4EW2
Group D: 3DW2, 4BF1, EF, 3AF1, 3CW2
Group E: 4AF1, EW, 3AF2, 4EW1, BW

tance to at least three antibiotics used in this study. The MARI of the *Enterococcus* species ranged from 0.3–0.9, with 38/45 (84.4%) isolates having a MARI of ≥ 0.5. A total of 2 of 45 (4.4%) of the isolates were resistant to four antibiotics with a MARI of 0.4 while 5/45 (11.1%) were resistant to three antibiotics with MARI of 0.3. All the isolates in the study (45/45, 100%) had MARI of ≥ 0.3.

The virulence factors of *Enterococcus* species are shown in Fig 2. The virulence factors observed in *Enterococcus faecalis* include gelatinase activity in 10/12 (83.3%), β-haemolytic activity in 12/12 (100.0%), hyaluronidase activity in 11/12 (91.7%) and S-layer formation in 12/12 (100,0%).

The virulence factors observed in *Enterococcus faecium* include gelatinase activity in 11/15 (73.3%), β-haemolytic activity in 10 of 15 (66.7%), hyaluronidase activity in 13 of 15 (86.7%) and S-layer formation in 15 of 15 (100.0%) isolates.

The virulence factors observed in *Enterococcus durans* include gelatinase activity in 3/8 (37.5%), β-haemolytic activity in 4/8 (50%), hyaluronidase activity in 4/8 (50%) and S-layer formation in 7/8 (87.5%) isolates.

The virulence factors observed in *Enterococcus hirae* include gelatinase activity in 1/5 (20.0%), β-haemolytic activity in 2/5 (40.0%), hyaluronidase activity in 2/5 (40%) and S-layer formation in 4/5 (80.0%).

Table 3: Antibiotic susceptibility profile of *Enterococcus* species isolated from poultry in selected farms in Benin City, Nigeria

Antibiotics	Antibiotic susceptibility profile (%)															Resistant strains (%)
	***E. faecalis* (n=12)**			***E. faecium* (n=15)**			***E. durans* (n=8)**			***E. casseliflavus* (n=5)**			***E. hirae* (n=5)**			
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	
Penicillins																
PEN (10 units)	12 (100)	0	0	11 (73.3)	0	4 (26.7)	8 (100)	0	0	4 (80)	0	1 (20)	5 (100)	0	0	40 (88.9)
AMP (10µg)	12 (100)	0	0	15 (100)	0	0	8 (100)	0	0	5 (100)	0	0	5 (100)	0	0	45 (100)
Ansamycins																
RIF (5µg)	9 (75)	0	3 (25)	12 (80)	0	3 (20)	5 (62.5)	0	3 (37.5)	3 (60)	0	2 (40)	5 (100)	0	0	34 (75.6)
Macrolides																
ERY (15µg)	10 (83.3)	1 (8.3)	1 (8.3)	14 (93.3)	0	1 (6.7)	5 (62.5)	3 (37.5)	0	2 (40)	2 (40)	1 (20)	4 (80)	1 (20)	0	35 (77.8)
Glycopeptides																
VAN (30µg)	9 (75)	2 (16.7)	1 (8.3)	10 (66.7)	2 (13.3)	3 (20)	6 (75)	2 (25)	0	3 (60)	1 (20)	1 (20)	3 (60)	2 (40)	0	31 (68.9)
Fluoroquinolones																
CIP (5µg)	1 (8.3)	4 (33.3)	7 (58.3)	5 (33.3)	2 (13.3)	8 (53.3)	2 (25)	2 (25)	4 (50)	1 (20)	1 (20)	3 (60)	1 (20)	1 (20)	3 (60)	10 (22.2)
Phenicols																
CHL (30µg)	5 (41.7)	3 (25.0)	4 (33.3)	4 (26.7)	8 (53.3)	3 (20)	1 (12.5)	6 (75)	1 (12.5)	1 (20)	3 (60)	1 (20)	2 (40)	2 (40)	1 (20)	13 (28.9)
Fosfomycins																
FOS (200µg)	12 (100)	0	0	15 (100)	0	0	7 (87.5)	1 (12.5)	0	4 (80)	0	1 (20)	5 (100)	0	0	43 (95.6)
Nitrofurans																
NIT (300µg)	9 (75)	2 (16.7)	1 (8.3)	13 (86.7)	2 (13.3)	0	8 (100)	0	0	5 (100)	0	0	4 (80)	1(20)	0	39 (86.7)

PEN: Penicillin G (10 units); RIF: Rifampin (5µg); ERY: Erythromycin (15µg); VAN: Vancomycin (30µg); CIP: Ciprofloxacin (5 µg); CHL: Chloramphenicol (30µg); AMP: Ampicillin (10µg); FOS: Fosfomycin (200µg) and NIT: Nitrofurantoin (300 µg). Values in parenthesis represent percentage (%).

Table 4: Resistance phenotypes and multiple antibiotic resistance index of *Enterococcus* species

Isolates Code	Number of antibiotics	Resistance phenotype	MARI
AW, CF, 4DW2	8	PEN^R - RIF^R - ERY^R - VAN^R - CHL^R - AMP^R - FOS^R - NIT^R	0.9
BW	8	PEN^R - RIF^R - VAN^R - CIP^R - CHL^R - AMP^R - FOS^R - NIT^R	0.9
CW, ED, 3AF2, 4BF2	8	PEN^R - RIF^R - ERY^R - VAN^R - CHL^R - AMP^R - FOS^R - NIT^R	0.9
DF, EF, 3DF1, 4EW2, 4EW3	8	PEN^R - RIF^R - ERY^R - VAN^R - CIP^R - AMP^R - FOS^R - NIT^R	0.9
AF	7	PEN^R - RIF^R - ERY^R - VAN^R - CHL^R - AMP^R - FOS^R	0.8
AD, BF, BD, DD, EW, 3BW2, 3DF2, 3DW1	7	PEN^R - RIF^R - ERY^R - VAN^R - AMP^R - FOS^R - NIT^R	0.8
DW	7	PEN^R - RIF^R - VAN^R - CIP^R - AMP^R - FOS^R - NIT^R	0.8
3BW1	7	PEN^R - ERY^R - VAN^R - CHL^R - AMP^R - FOS^R - NIT^R	0.8
CD	7	PEN^R - ERY^R - VAN^R - CIP^R - AMP^R - FOS^R - NIT^R	0.8
4AW2	7	RIF^R - ERY^R - CIP^R - CHL^R - AMP^R - FOS^R - NIT^R	0.8
4CW2	7	PEN^R - ERY^R - VAN^R - CHL^R - AMP^R - FOS^R - NIT^R	0.8
2BF, 4AF2, 4EW1	6	PEN^R - RIF^R - ERY^R - AMP^R - FOS^R - NIT^R	0.7
2EW2, 4AW1, 4BF1	6	PEN^R - RIF^R - VAN^R - AMP^R - FOS^R - NIT^R	0.7
3AF1	6	RIF^R - ERY^R - VAN^R - AMP^R - FOS^R - NIT^R	0.7
3DW2	5	PEN^R - CHL^R - AMP^R - FOS^R - NIT^R	0.6
4AF1	5	PEN^R - RIF^R - ERY^R - AMP^R - FOS^R	0.6
4CW1	5	PEN^R - VAN^R - AMP^R - FOS^R - NIT^R	0.6
4DW1	5	RIF^R - ERY^R - AMP^R - FOS^R - NIT^R	0.6
2DF	4	ERY^R - AMP^R - FOS^R - NIT^R	0.4
3CW1	4	PEN^R - CIP^R - AMP^R - NIT^R	0.4
2AF, 2DW	3	PEN^R - AMP^R - FOS^R	0.3
2AW	3	ERY^R - AMP^R - FOS^R	0.3
2EW1	3	RIF^R - AMP^R - FOS^R	0.3
3CW2	3	PEN^R - AMP^R - NIT^R	0.3

PEN: Penicillin G (10 units); RIF: Rifampin (5µg); ERY: Erythromycin (15µg); VAN: Vancomycin (30µg); CIP: Ciprofloxacin (5 µg); CHL: Chloramphenicol (30µg); AMP: Ampicillin (10µg); FOS: Fosfomycin (200µg) and NIT: Nitrofurantoin (300 µg); MARI: Multiple antibiotic resistance index

The virulence factors observed in *Enterococcus casseliflavus* include gelatinase activity in 3/5 (60.0%), β-haemolytic activity in 2/5 (40.0%), hyaluronidase activity in 3/5 (60%) and S-layer formation in 5/5 (100%). In total, the virulence factors formation of *Enterococcus* species observed were gelatinase activity in 28/45 (62.2%), β-hemolytic activity in 30/45 (66.7%), hyaluronidase activity in 33/45 (73.3%) and S-layer formation in 43/45 (95.6%) isolates.

The frequency distribution of biofilm forming *Enterococcus* species is shown in Fig 3. Biofilm formation ability observed in *E. faecalis* includes strong biofilm formation in 5/12 (41.7%), moderate biofilm formation in 3/12 (25.0%), weak biofilm formation in 4 of 12 (33.3%) and no biofilm formation in nil isolate.

Biofilm formation capacity observed in *E. faecium* includes strong biofilm formation in 6/15 (40.0%), moderate biofilm formation in 4/15 (26.7%), weak biofilm formation in 3/15 (20.0%) and no biofilm formation in 2/15 (20.0%) isolates.

Biofilm formation capacity in *E. durans* includes strong biofilm formation in 3/8 (37.5%), moderate biofilm formation in 1/8 (12.5%), weak biofilm formation in 2/8 (25.0%) and no biofilm formation in 2/8 (25.0%) isolates.

Biofilm formation capacity observed in *E. hirae* includes strong biofilm formation in 2/5 (40.0%), moderate biofilm formation in 1/5 (20.0%), weak biofilm formation in 1/5 (20.0%) and no biofilm formation in 1/5 (20.0%).

Biofilm formation capacity observed in *E. casseliflavus* includes strong biofilm formation in 3/5 (60.0%), moderate biofilm formation in 1/5 (20.0%), weak biofilm formation in 1/5 (20.0%) and no biofilm formation in nil isolate.

In total, the frequency of biofilm formation observed in *Enterococcus* species was 42.2% (19/45) for strong biofilm formation, 22.2% (10/45) for moderate biofilm formation, 24.4% (11/45) for weak biofilm formation 11.1% (5/45) for no biofilm formation.

Discussion:

Enterococci are widely known for their probiotic potential in birds including poultry. However, the presence of antibiotic-resistant strains of enterococci remains a global health concern as it tends to influence animal pathology. In this study, *Enterococcus* species were detected in the feeds, water and faecal samples of poultry birds. Previous studies have equally reported the detection of enterococci in feeds, water and faecal samples poultry (26,27).

The detection of enterococci in faecal samples could be attributed to the fact that enterococci are gastrointestinal flora of animals including poultry (4). Furthermore, Lata et al., (28) reported that enterococci in water could indicate fecal contamination. The water samples investigated in this study could have been exposed to enterococcal contamination through unclean water trough or enterococci dissemination through air. This affirmed pre-

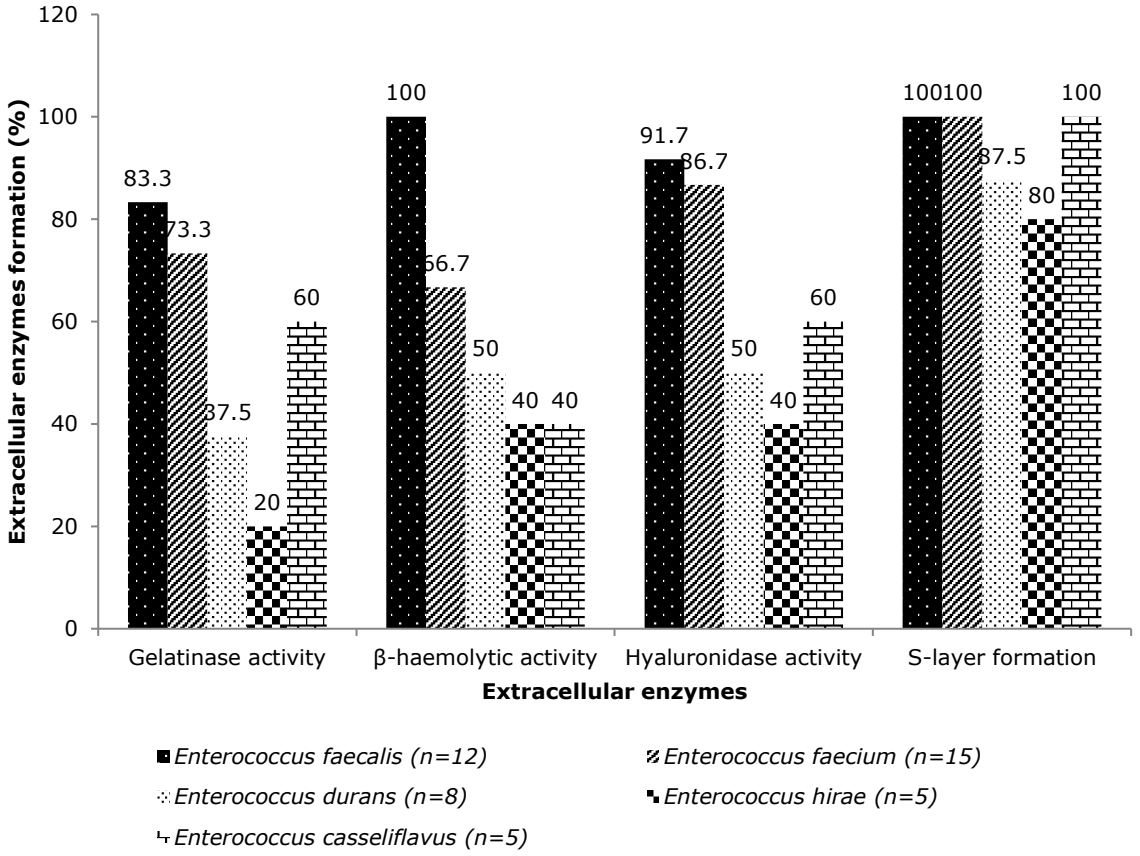

Fig 2: Distribution of phenotypic virulence factors of the *Enterococcus* isolates

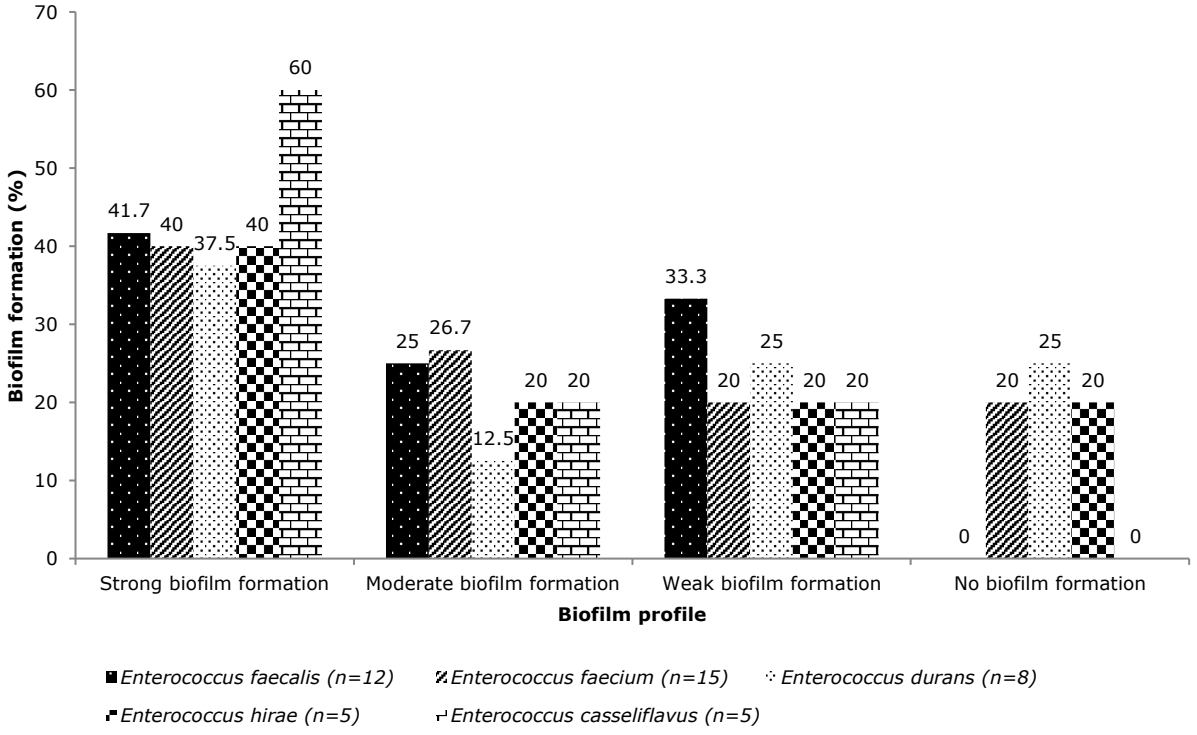

Fig 3: Biofilm formation distribution of *Enterococcus* species

vious studies which reported that microorganisms associated with faecal discharge, including enterococci, can be disseminated through air (29). The presence of enterococci in the feeds could be attributed to their usage as probiotic supplements and contaminations arising from faecal matters. Several studies have reported using enterococci strains as probiotic supplements in animal feeds for growth promotion and disease control (30,31).

The most prevalent *Enterococcus* spp isolated in this study were *E. faecium* (33.3%) and *E. faecalis* (26.7%), followed by *E. durans* (17.8%), *E. casseliflavus* (11.1%) and *E. hirae* (11.1%). This agrees with previous studies that reported the detection of *E. faecium*, *E. faecalis, E. durans*, *E. casseliflavus* and *E. hirae* in poultry and its environment in which *E. faecium* and *E. faecalis* are the most prevalent species (26,29). Although enterococci may be involved in the pathology of birds, enterococci from food animals have not been exclusively implicated as pathogenic in human because investigations have attributed resulting infections in human to nosocomial and community-associated strains (10). Nevertheless, enterococci isolated from poultry and several other food chains can still adversely affect human and animal health as they could influence the acquisition and dissemination of antibiotic resistance (32).

In this study, it was observed that enterococci demonstrated high resistance to ampicillin (100%), fosfomycin (95.6%), penicillin G (88.9%) and nitrofurantoin (86.7%) while the least resistance was demonstrated to chloramphenicol (22.2%) and ciprofloxacin (28.9%). In agreement with this study, it has been previously envisaged that enterococci show significantly high resistance to β-lactam antibiotics and lower towards quinolones (6). Contrary to this study, enterococci isolates investigated in the study by Bertelloni et al., (27) reported a lower resistance to chloramphenicol (19.1%) compared to the 22.2% observed in our study. However, the resistance rate of enterococci in the study to nitrofurantoin (48.7%), ampicillin (29.6%), rifampicin (22.6%) and vancomycin (10.0%) were lower than the rate reported in our study. In agreement with our study, previous studies reported significant resistance of enterococci to erythromycin, penicillin and ampicillin (33,34).

The different antimicrobial resistance rates of *Enterococcus* in these studies could be due to variations in geographical locations and intensity of antibiotics usage in different settings (35). Unrestrained use of antimicrobial agents is acknowledged as the most essential factor contributing to the development of resistant microorganisms which could spread to humans via the food chain.

The multiple antibiotic resistance index (MARI) of *Enterococcus* species in this study, showed that 84.4% of the isolates were resistant to at least five antibiotics, while all the isolates (100.0%) were resistant to at least three antibiotics. The MARI of the *Enterococcus* species ranged from 0.3 - 0.9 in which all the isolates demonstrated MARI of ≥ 0.3. The MARI is a good risk assessment tool, and MARI > 0.2 indicates that isolates are from high-health risk sources where frequency of antibiotic use is high (36). The MARI in all the enterococci isolates in our study was greater than the 0.2 threshold value, further intensifying the possibility of antibiotic resistance dissemination.

The virulence factors investigated in this study showed that 62.2% of the enterococci isolates demonstrated gelatinase activity, 66.7% β-haemolytic activity, 73.3% hyaluronidase activity and 95.6% showed S-layer formation. The virulence factors detected in this study have been implicated in previous investigation on enterococci isolates from animal products meant for human consumption and its environment (37). In addition, the degree of biofilm formation in the enterococci isolates showed that 42.2% were strong biofilm forming, 22.2% moderate biofilm forming, 24.4% weak biofilm forming while only 11.1% were non-biofilm forming enterococci isolates.

The linkage of enterococci from food origin with virulence production, which is an effector molecule that enhances pathogenicity further increases their clinical significance as opportunistic pathogens. This agrees with previous studies which emphasized that the demonstration of biofilm and other virulence factors in enterococci of non-clinical origin increases their chances of causing infections (37,38). This makes it essential for enterococci originating from food sources to be monitored regarding potential antibiotic resistance (39). This is to strategize on how to minimize their potential threat to animal and human health. In view of this, proper monitoring and surveillance of virulence traits and antimicrobial resistance exchange among animal, human or indirectly through environmental interface could help reduce the possible health risks associated with using enterococci as probiotics in poultry.

Conclusion:

Our study shows that the poultry environment is a potential reservoir of virulent enterococci with antibiotic-resistant capabilities.

The linkage of the isolated enterococci with extracellular virulence properties, biofilm potential and resistance to multiple antibiotics signal that enterococci of non-clinical origin remain possible route of disseminating antimicrobial resistance and virulence traits to human microbiota. Therefore, it remains fundamental to emphasize proper hygiene practices and antibiotic use in poultry farms. Furthermore, the use of probiotic supplements in poultry feeds should also be strictly monitored.

Contribution of authors:

IEO conceptualized the study and designed the laboratory methods; IBO, AO and NCN were involved in material preparation, data collection and analysis; IBO, AO and NCN prepared the initial manuscript draft; IBO, AO and IEO revised the manuscript. All authors read and approved the final manuscript.

Source of funding:

No fund was received for the study

Conflict of interest:

Authors declare no conflict of interest

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Otaigbe et al. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (1): 86 - 94 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jan 2024; Vol.25 No.1
AJCEM/2298. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Pattern of inappropriate antibiotic use among patients in the medical wards of a tertiary hospital in southwest Nigeria

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Abstract:

Background: The inappropriate use of antibiotics results in the emergence of antimicrobial resistance and adverse clinical and economic outcomes in hospital in-patients. A lack of institutional and national antibiotic guidelines promotes inappropriate antibiotic use. The objectives of this study are to evaluate the appropriateness of antibiotic prescribing, and the quality of antibiotic use in medical wards of the Lagos University Teaching Hospital, Lagos, Nigeria.
Methodology: This was a descriptive cross-sectional study of patients admitted and placed on antibiotics in the medical wards of Lagos University Teaching Hospital between July 2013 and August 2014. The appropriateness of antibiotic therapy was determined by compliance with the guidelines of the Infectious Diseases Society of America (IDSA).
Results: A total of 350 hospitalized patients on antibiotic therapy during the period of the study were reviewed, including 197 (56.3%) males and 153 females (43.7%). The mean age of the patients was 48.7±17.6 years and a total of 539 initial antibiotics were empirically prescribed. Antibiotic therapy was considered inappropriate in 290 (82.9%) patients, of which 131 (37.4%) patients had no evidence of infection. Pneumonia (23.1%) was the most common indication for antibiotic use, out of which 59.3% had inappropriate antibiotic therapy. Overall, the most frequently prescribed initial empirical antibiotic classes were imidazole derivatives (32.4%) and cephalosporins (22.0%), while the most frequently prescribed inappropriate antibiotic classes were carbapenems (100.0%) and quinolones (89.3%).
Conclusion: The study revealed a high rate of inappropriate antibiotic therapy. There is an imperative need to establish antimicrobial stewardship programmes to curb the inappropriate use of antibiotics in the hospital.

Keywords: Antibiotic use; Prescribing practice; Empirical; Antimicrobial resistance; Antimicrobial stewardship

Received Sept 25, 2023; Revised Oct 9, 2023; Accepted Oct 10, 2023

Modèle d'utilisation inappropriée d'antibiotiques chez les patients des services médicaux d'un hôpital tertiaire du sud-ouest du Nigeria

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Résumé:

Contexte: L'utilisation inappropriée d'antibiotiques entraîne l'émergence d'une résistance aux antimicrobiens et

des résultats cliniques et économiques défavorables chez les patients hospitalisés. L'absence de directives institutionnelles et nationales sur les antibiotiques favorise une utilisation inappropriée des antibiotiques. Les objectifs de cette étude sont d'évaluer la pertinence de la prescription d'antibiotiques et la qualité de l'utilisation des antibiotiques dans les services médicaux de l'hôpital universitaire de Lagos, Lagos, Nigeria.
Méthodologie: Il s'agissait d'une étude transversale descriptive portant sur des patients admis et placés sous antibiotiques dans les services médicaux de l'hôpital universitaire de Lagos entre juillet 2013 et août 2014. La pertinence de l'antibiothérapie a été déterminée par le respect des directives de l'Infectious Diseases Society. d'Amérique (IDSA).
Résultats: Au total, 350 patients hospitalisés sous antibiothérapie au cours de la période de l'étude ont été examinés, dont 197 (56,3%) hommes et 153 femmes (43,7%). L'âge moyen des patients était de 48,7±17,6 ans et un total de 539 antibiotiques initiaux ont été prescrits de manière empirique. L'antibiothérapie a été jugée inappropriée chez 290 (82,9%) patients, dont 131 (37,4%) patients ne présentaient aucun signe d'infection. La pneumonie (23,1%) était l'indication la plus courante d'utilisation d'antibiotiques, dont 59,3% avaient un traitement antibiotique inapproprié. Dans l'ensemble, les classes d'antibiotiques empiriques initiales les plus fréquemment prescrites étaient les dérivés de l'imidazole (32,4%) et les céphalosporines (22,0%), tandis que les classes d'antibiotiques inappropriés les plus fréquemment prescrites étaient les carbapénèmes (100,0%) et les quinolones (89,3%).
Conclusion: L'étude a révélé un taux élevé d'antibiothérapie inappropriée. Il est impératif d'établir des programmes de gestion des antimicrobiens pour lutter contre l'utilisation inappropriée des antibiotiques à l'hôpital.

Mots clés: Utilisation d'antibiotiques; Pratique de prescription; Empirique; Résistance aux antimicrobiens; Gestion des antimicrobiens

Introduction:

The introduction and use of antimicrobial agents in modern medicine contributed to a reduction in the morbidity and mortality of humans (1). However, inappropriate use of antibiotics has become a global concern (1,2), which results in adverse clinical and economic outcomes in patients (3). The adverse clinical outcomes include increased length of hospital stay, morbidity, and mortality (3), increased consumption of antibiotics by hospital in-patients (4), and emergence of drug resistance (5). The adverse economic outcomes include increased costs of healthcare borne by patients (3).

The problem of inappropriate antibiotic therapy and its adverse clinical and economic outcomes is exacerbated by a paucity of new and effective antimicrobials due to diminishing research and development efforts in antibiotic discovery (6). This scenario infers the possibility of a post-antibiotic era, in which simple infections would no longer be treatable due to a lack of effective antibiotics (5).The impact of a post-antibiotic era would be particularly severe in Africa and other resource constrained parts of the world due to an already existing vicious circle of therapeutic failures from multidrug resistant organisms, weak regulatory systems, inadequate diagnostic facilities, poorly funded healthcare delivery systems, and a paucity of antibiotic stewardship programs (7). It is therefore absolutely necessary to safeguard currently available and effective antibiotics (8).

There is however, a paucity of data in Africa regarding the appropriateness of antibiotic prescribing (9). Sadly, this knowledge gap frustrates effective policy making to curtail inappropriate antibiotic use and antimicrobial resistance in healthcare facilities in Africa (9). The aim of this study was therefore to evaluate the appropriateness of initial antibiotic therapy and the quality of antibiotic use in Lagos University Teaching Hospital, Nigeria.

Materials and method:

Study location and design:

This study was a cross sectional study conducted between July 2013 and August 2014 on patients admitted and placed on antibiotics in the medical wards of the Lagos University Teaching Hospital, southwest Nigeria. The hospital has 761 beds while its medical wards have 152 beds. Each ward admits about 30 patients monthly.

Study participants:

The study participants were adult patients over 18 years old, who were hospitalized in any of the medical wards for a minimum, of 24 hours and who were on antibiotics for treatment and not for prophylaxis.

Data collection:

Data were collected from the patients' case notes, observation charts, prescription sheets, microbiology and pathology, and radio-diagnostic results. Data collected included: patient's bio-data; diagnosis; site of infection; indication for antibiotic therapy; length of hospital stay in days; whether clinical samples were obtained for culture before antibiotic treatment; information related to the micro-organisms isolated from culture; and details of antibiotics prescribed including name, dose, duration, route and frequency of administration while on admission.

With regards to initial antibiotic use, all antibiotics were documented using the Anatomic Therapeutic Chemical (ATC) classification (10) and by the WHO Access, Watch and Reserve (AWaRe) classification of antibiotics

(11). To ensure confidentiality all data collected were de-identified and given study identification numbers.

Determining appropriateness of initial antibiotic therapy:

The antibiotic prescribing guidelines of the Infectious Diseases Society of America (IDSA) (12) was used to determine the appropriateness of initial antibiotics. In this study, inappropriate initial antibiotic therapy was categorized as; (i) inappropriate indication (no indication) where there was no evidence of infection and antibiotics were not needed; (ii) inappropriate choice where wrong choice of antibiotic based on the indication was used, and (iii) inappropriate dosing where there was wrong dose or dosage interval or route or duration. Antibiotic therapy was assessed to be inappropriate when any of the above criteria was fulfilled.

Ethical approval:

Ethical approval was obtained from the Health Research and Ethics Committee (HREC) of the hospital.

Data analysis:

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 20.0. Categorical variables were summarized using percentages for inappropriate and appropriate initial antibiotic therapy based on demographics, clinical features, site of infection and patterns of antibiotic use

Results:

Three hundred and fifty in-patients on antibiotics for treatment in the medical wards of the hospital were recruited into the study between July 2013 and August 2014. There were 197 (56. 3%) males and 153 (43.7%) females in the study, with mean age of 48.7 ± 17.6 years (Table 1). A total of 539 antibiotic prescriptions were given for 350 patients over the period of study. One hundred and forty-four in-patients (41.1%) were prescribed one initial empirical antibiotic while 206 (58.9%) patients were prescribed a combination of initial empirical antibiotics (Table 1).

Table 1: Demographic characteristics and antibiotic prescribing pattern of the inpatients in Lagos University Teaching Hospital, Lagos, Nigeria (2013-2014)

Characteristics	Frequency	Percentage (%)
Gender		
Male	197	56.3
Female	153	43.7
Mean age (years)	48.7 ± 17.6	
Initial Antibiotic therapy		
Single antibiotic use	144	41.1
Combination therapy	206	58.9
Route of antibiotic administration		
Parenteral/Intravenous	322	92.0
Oral	28	8.0
Indication for antibiotic therapy		
Healthcare associated infection	4	1.1
Community acquired infection	215	61.4
Unknown or no identified infection	131	37.4
Appropriateness of antibiotic therapy		
Appropriate	60	17.1
Inappropriate	290	82.9
Inappropriate indication	131	37.4
Inappropriate choice of antibiotic	142	40.6
Inappropriate dosing/duration	17*	4.9*
Wrong dose	10*	2.9*
Wrong duration of administration	16*	4.6*

*=One prescription can include more than one inappropriate dosing.

Table 2: Indication for antibiotic prescribing and inappropriateness of antibiotic therapy among inpatients in Lagos University Teaching Hospital, Lagos, Nigeria (2013-2014)

Indication for antibiotic prescribing	No of patients receiving antibiotics (%)	No of inappropriate use (%)
Pneumonia	81 (23.1)	48 (59.3)
Sepsis	67 (19.1)	56 (83.6)
Skin and soft tissue infection	32 (9.1)	25 (78.1)
Urinary tract infection	17 (4.9)	15 (88.2)
Intra-abdominal infection	11 (3.2)	10 (90.9)
Central Nervous System Infections	11 (3.2)	5 (45.5)
No evidence of infection	131 (37.4)	131 (10.0)
Total	**350 (100.0)**	**290 (82.9)**

Three hundred and twenty-two (92%) in-patients were prescribed parenteral antibiotics while 28 (8.0%) were prescribed oral antibiotics. Most of the patients (61.4%) were treated with antibiotics for community acquired infection. A total of 219 (62.6%) patients had evidence of infections and the most common indications for antibiotic therapy were pneumonia, accounting for 23.1% of patients in the study, followed by sepsis (19.1%) and skin and soft tissue infections (9.1%) (Table 2). Antibiotic therapy was appropriate in 60 patients (17.1%) while in 290 (82.9%) patients, antibiotic therapy was inappropriate (Table 1).

Specifically, in 131 (37.4%) patients, there was no indication for antibiotic therapy because there was no evidence of infection and therefore antibiotic therapy was unnecessary. In 142 (40.6%) patients, antibiotic choice was inappropriate and in 17 (4.9%) patients, there was inappropriate antibiotic dosing based on the dose and/or duration of treatment. The highest level of inappropriate antibiotic use (90.9%) was seen in patients with presumptive intra-abdominal infection while the lowest level of inappropriate antibiotic use was seen in patients with central nervous system infections (45.5 %) (Table 2).

Of the 131 inpatients with no evidence of infection, 36 (27.5%) had an admission diagnosis of cardiac failure, 24 (18.3%) had cerebrovascular disease, and 22 (16.8 %) had chronic kidney disease (Fig 1). Out of the 142 patients with inappropriate choice of antibiotics, only 39 (27.5%) were treated with a single antibiotic while the rest were treated with multiple antibiotics. A total of 539 initial empirical antibiotics were prescribed. Overall, the most frequently prescribed initial empirical antibiotics were metronidazole (32.5%), amoxicillin-clavulanate (18.9%), levofloxacin (18.7%) and ceftriaxone (18.7%). These four antibiotics accounted for 479 (88.9%) prescriptions (Table 3).

Out of the total antibiotics prescribed, 449 (83.3%) antibiotic prescriptions were assessed as inappropriate antibiotic use. The most frequently prescribed inappropriate antibiotic classes were carbapenems (100%), quinolones (89.3%), imidazole derivatives (86.9 %) and third generation cephalosporins (86.4%).

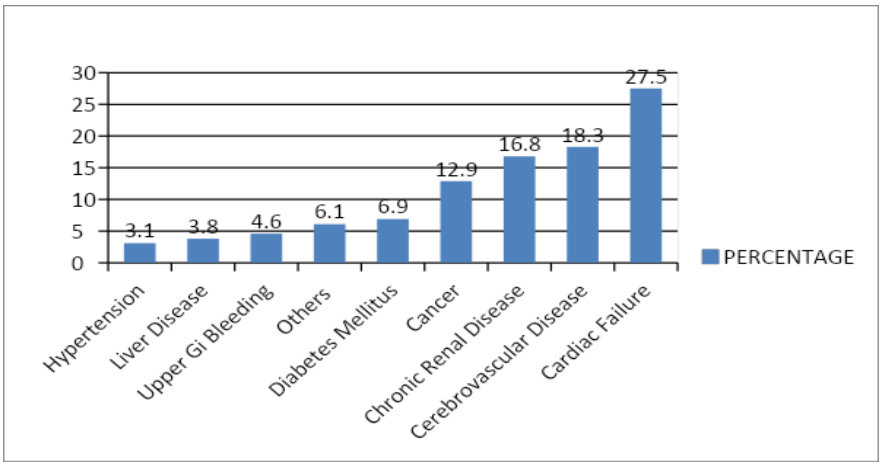

Fig 1: Admission diagnosis for 131 inpatients on antibiotic therapy with no evidence of infection in Lagos University Teaching Hospital, Lagos, Nigeria (2013-2014)

Table 3: Frequency and Anatomical Therapeutic Chemical (ATC) Codes of antibiotics prescribed to inpatients in Lagos University Teaching Hospital, Lagos, Nigeria (2013-2014)

Antibiotics prescribed	ATC Code	Frequency	Percentage (%)
Penicillins	J01C	**106**	**19.7**
Penicillin G	J01CE09	1	0.2
Amoxicillin	J01CA04	1	0.2
Flucloxacillin	J01CF05	1	0.2
Penicillin with B lactamase inhibitor	J01CR	103	19.1
Amoxycillin-clavulanate	J01CR02	102	18.9
Piperacillin-tazobactam	J01CR05	1	0.2
Cephalosporins		**119**	**22.0**
2nd Generation	J01DC	9	1.6
Cefuroxime	J01DC02	9	1.6
3rd Generation	J01DD	110	20.3
Ceftriaxone	J01DD04	101	18.7
Cefixime	J01DD08	2	0.4
Ceftazidime	J01DD02	7	1.2
Carbapenems	**J01DH**	**8**	**1.4**
Meropenem	J01DH02	8	1.4
Quinolones	**J01MA**	**113**	**21.0**
Ciprofloxacin	J01MA02	12	2.3
Levofloxacin	J01MA12	101	18.7
Macrolides	**J01FA**	**12**	**2.2**
Clarithromycin	J01FA09	3	0.6
Azithromycin	J01FA10	9	1.6
Imidazole derivatives	**J01XD**	**175**	**32.5**
Metronidazole	J01XD01	175	32.5
Other antibiotics		**6**	**1.2**
Clindamycin	J01FF01	1	0.2
Trimethoprim-sulphamethoxazole	J01EE01	1	0.2
Gentamicin	J01GB03	1	0.2
Vancomycin	J01XA01	3	0.6
Total		**539**	**100**

Of the total 539 antibiotics prescribed, 35.1% were due to inappropriate indication, 43.4% were inappropriately chosen, and 4.8% were inappropriately dosed. Inappropriate indication of antibiotics was highest for the cephalosporins (38.7%) (Table 4). The carbapenems were inappropriately used, mainly because of inappropriate choice (62.5%).

Most of the inappropriate use of the 2nd generation cephalosporins (66.7%) was due to inappropriate choice. The macrolides (58.3%) were the antibiotics with the least inappropriate use, and most of the macrolides were inappropriately used mainly because of inappropriate choice (41.7%).

A total of 256 of 539 (47.5%) antibiotics prescribed were in the "Watch" group of the WHO AWaRe category of antimicrobials, with 221 of 449 (49.2%) inappropriately prescribed, while there was no antibiotic prescribed (appropriately or inappropriately) from the "Reserve" group (Fig 2).

Table 4: Frequency distribution of types of inappropriate antibiotic usage among inpatients of Lagos University Teaching Hospital, Lagos, Nigeria (2013-2014)

Antibiotics prescribed	**Inappropriate antibiotic usage**			
	Inappropriate indication (%)	**Inappropriate choice (%)**	**Inappropriate dosing (%)**	**Total inappropriate use (%)**
Penicillins (n=106)	36 (33.9)	37 (34.9)	1(0.9)	74 (69.8)
Penicillin + beta lactamase inhibitor (n=103)	35 (34.0)	36 (35.0)	1 (1.0)	72 (70.0)
Other Penicillins (n=3)	1 (33.3)	1 (33.3)	0	2 (66.7)
Cephalosporins (n=119)	46 (38.7)	54 (45.4)	2 (1.7)	102 (85.7)
2nd Generation (n=9)	1 (11.1)	6 (66.7)	0	7 (77.8)
3rd Generation (n=110)	45 (41.0)	48 (43.6)	2 (1.8)	95 (86.4)
Carbapenems (n=8)	0	5 (62.5)	3 (37.5)	8 (100.0)
Quinolones (n=113)	42 (37.2)	48 (42.5)	11 (9.7)	101 (89.3)
Macrolides (n=12)	2 (16.7)	5 (41.7)	0	7 (58.3)
Imidazole derivatives (=175)	63 (36.0)	83 (47.4)	6 (3.4)	152 (86.9)
Other antibiotics (n=6)	0	2 (33.3)	3 (50.0)	5 (83.3)
Total (n=539)	**189 (35.1)**	**234 (43.4)**	**26 (4.8)**	**449 (83.3)**

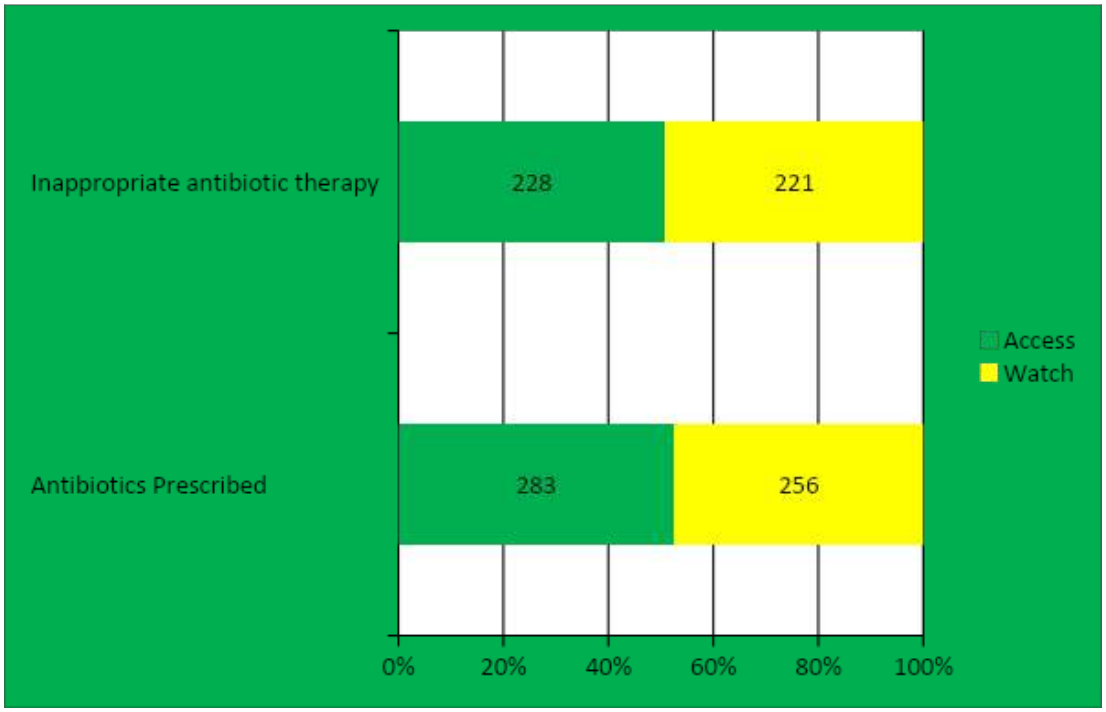

Fig 2: Frequency of inappropriate antibiotic therapy among inpatients of Lagos University Teaching Hospital, Lagos, Nigeria (2013-2014) using the WHO Access, Watch and Reserve (AWaRe) classification

Discussion:

The inappropriate use of antibiotics is a major driver of antibiotic resistance. Regrettably, this study showed that about 4 out of every 5 (82.9%) in patients; treated with antibiotics were placed on inappropriate antibiotic therapy. These findings are higher than those reported in previous studies on inappropriate antibiotic use in hospital in-patients, for example, 50.0% in Nigeria (13), 75.7% in Ethiopia (14), 72.2% in Iran (15), 54.9% in South Africa (16), 38.0% in Namibia (16), 37.0% in Switzerland (17), and 14.0% in Ghana (18). This very high rate of inappropriate antibiotic therapy might lead to increased length of stay in the hospital, cost of hospital stays, morbidity and mortality and also puts the hospital at risk of the emergence of antimicrobial resistance. It is needful to actually develop local

antibiotic guidelines for the hospital's department of medicine and ascertain the compliance rates to show appropriateness of antibiotic prescribing.

Clearly, inappropriate antibiotic prescribing is a complex process which is driven by several factors including lack of antibiotic prescribing guidelines, absence of antimicrobial stewardship programmes and inadequate knowledge of appropriate antibiotic prescribing (19,20). In addition, a major factor driving inappropriate antibiotic prescribing, is an effort by the physicians to use antibiotics to prevent infections i. e. prophylaxis (21). This implies that physicians prescribe antibiotics as a prophylactic measure, even when unnecessary (21). Therefore, it is likely that the antibiotic prescriptions for non-communicable diseases, which occurred in this study, may have been for prophylaxis. However, the IDSA guidelines do not recommend prophylaxis for non-communicable conditions such as hypertension and cerebrovascular disease (12).

In addition, it is important to understand the factors which drive inappropriate prescribing particularly in scenarios where an infection is clearly absent (20). If these factors are not clearly understood, efforts that seek to stimulate behavioural change such as antimicrobial stewardship programmes and antibiotic prescribing guidelines may fail (20,22). Such factors that drive inappropriate antibiotic prescribing will however serve as future areas for research. Data from such research will definitely guide the hospital's antimicrobial stewardship programme in designing effective interventions to curb inappropriate antibiotic use in the hospital.

Furthermore, simple biomarkers of infection such as white blood cell counts, serum procalcitonin (PCT) or C-reactive protein (CRP) are useful to screen for infection in patients admitted to the hospital. For example, a study done in Nigeria showed that 96.2% of patients with culture proven sepsis had high levels of serum PCT (23). The inclusion of such biomarkers in patient management can obviate the inapp- ropriate use of antibiotics (23,24). In addition, the management of patients on antibiotics should involve adequate utilization of the clinical microbiology laboratory in order to ensure guided antibiotic therapy and optimized appropriate antibiotic use (25).

It is noteworthy that most of the patients who had inappropriate choice of antibiotics were placed on multiple antibiotics when the guidelines recommended a single antibiotic. This is similar to other studies done in Nigeria which showed high usage of multiple antibiotic therapy (26). The use of, multiple antibiotic therapy, especially those with similar antibiotic spectrum, results in redundant therapy which could lead to increased costs, increased risk of adverse effects and antagonism and the development of resistance (27). In this regard, it would be important, to educate physicians about the indications for combination therapy.

The rate of inappropriate antibiotic use was lowest among inpatients receiving antibiotics for central nervous system (CNS) infections. This may be due to the fact CNS infections such as meningitis are life threatening conditions and considered medical emergencies; which may have made the physicians pay more attention to the treatment. Overall, the most frequently prescribed initial empiric antibiotics were metronidazole (32.5%), amoxicillin-clavulanate (18.9%), levofloxacin (18.7%) and ceftriaxone (18.7%).

Most of the point prevalence surveys of antimicrobial consumption in Nigeria have shown ceftriaxone and metronidazole as the most commonly prescribed antibiotics in tertiary healthcare facilities like ours (13,26). Similarly, a study done in Uganda showed that the most frequently prescribed antibiotics for in-patients were ceftriaxone (66%), metronidazole (41%), co-trimoxazole (27%), ciprofloxacin (19.0%) and amoxicillin (10.0%) (28). Furthermore, another study done in Pakistan showed that the five most commonly prescribed antibiotics in hospitalized patients were ceftriaxone (21.0%), amikacin (15.2%), cefoperazone plus sulbactam (11.4%), ciprofloxacin (6.4%), metronidazole (5.9%), amoxicillin-clavulanate (5.6%) and clarithromycin (2. 4%) (29).

In this study, the antibiotics most com monly prescribed inappropriately were carbapenems, quinolones, metronidazole and third generation cephalosporins. Nearly half of the total antibiotics prescribed were in the "Watch" group of the WHO AWaRe category of antimicrobials and none in the "Reserve" group. These figures are clearly a deviation from the WHO recommendations (30). According to the WHO, about 60% of antibiotic consumption should be from the Access group (30). Clearly there is a need to curb the inappropriate use of antibiotics in the hospital in order to delay the emergence of antibiotic resistance.

Going by the guidelines, the carbapenems were an inappropriate choice all the time they were used in our study. Carbapenems are the last line of defense against multidrug resistant (MDR) Gram-negative bacterial infections and should be used based on culture results or when there are risk factors for MDR infections (31). Also, narrow spectrum antibiotics such as aztreonam and temocillin, with proven efficacy against MDR infections should be included in antibiotic guidelines (32). The use of such narrow spectrum antibiotics can reduce the inappropriate use of broad-spectrum antibiotics such as the carbapenems (32). Furthermore, in this study, the macrolides had the lowest rate of inappropriate use. This may

be because they were majorly used to treat community acquired pneumonia, which is in accordance with the IDSA guidelines. These findings differ from a study done in Pakistan where 3rd generation cephalosporins (54.2%) were the most frequently prescribed inappropriate antibiotics (29), and in Eritrea where imipenem (100%), amikacin (100%) and piperacillin-tazobactam (100%) were described as the most inappropriately prescribed antibiotics (33).

From the foregoing it is absolutely clear that inappropriate antibiotic therapy is a challenge that must be urgently curbed at the hospital where this study was conducted. Fortunately, antibiotic stewardship programmes can curb the inappropriate use of antibiotics in hospitals (34). However, adequate funding of antibiotic stewardship programmes and the robust support of the hospital's management are necessary to ensure successful programme outcomes (34). Furthermore, efforts such as hospital guidelines and frequent education of physicians on proper antibiotic prescribing are also useful interventions (34).

Conclusion:

In conclusion, a high rate of inappropriate antibiotic therapy (82.9%) was found in this study. The inappropriate antibiotic therapy was due to inappropriate indication where antibiotic therapy was unnecessary, wrong choice of antibiotics according to IDSA guidelines and inappropriate dose and or duration where the choice of antibiotic was correct. The most frequently prescribed antibiotics were metronidazole, amoxicillin-clavulanate, levofloxacin and ceftriaxone. The carbapenems were the antibiotic most prescribed inappropriately. The presence of an antimicrobial stewardship programme in the hospital will curb the problem of inappropriate antibiotic use.

Contributions of authors:

IIO drafted the manuscript and was responsible for data acquisition and analysis. POO and OOO critically reviewed the manuscript for important intellectual content. All authors approved the manuscript for publication.

Source of funding:

Authors received no external funding

Conflicts of interest:

Authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

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Igunma & Lofor. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (1): 95 - 102 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jan 2024; Vol.25 No.1
AJCEM/2310. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Discordant rate between empirical antibiotics administered and antimicrobial susceptibility in infections caused by *Pseudomonas aeruginosa* in a tertiary hospital in Nigeria

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Abstract:

Background Early initiation of appropriate antibiotics is key to the effective management of severe bacterial infections. The initiation of targeted antibiotic therapy is possible only when the causative organism is isolated. As a result, antibiotics are usually administered on an empirical basis guided by the clinical presentation, local antibiotic guidelines and other relevant histories. Generally, empirical antibiotics differ for both community- and hospital-acquired infections (HAIs), as a result of which common HAI pathogens such as *Pseudomonas aeruginosa* should be deliberately targeted, because most routine antibiotics are ineffective against them.
Methodology: This was a retrospective cross-sectional study involving the review of the clinical consults sent to clinical microbiologists at the University of Benin Teaching Hospital (UBTH) between January and December 2022. The consults were analyzed for the initial diagnosis, reasons for the invitation and empirical antibiotics administered. Other relevant informations were obtained from the laboratory records. Susceptibility profiles of *P. aeruginosa* isolates were compared with the empirical antibiotics administered. Discordant empirical antibiotic therapy was defined as the administration of antibiotic regimen with no anti-pseudomonal activity.
Results: Of the 256 consults received over the period of study, *P. aeruginosa* was isolated from 57 (22.3%) patients as pathogens. Out of this, 24.6% (n=14) received at least one anti-pseudomonas antibiotic, which puts the total discordant rate at 75.4%. Metronidazole (22.7%) and ceftriaxone-sulbactam (Tandak) (21.5%) were the most commonly prescribed empirical antibiotics. The most common reason for consultation was a diagnosis of sepsis at 40.2% followed by pan-resistant isolates at 34.8%
Conclusion: Although the commonly prescribed antibiotics in our setting are broad spectrum, they lack coverage for *P. aeruginosa* which is one of the most common pathogens implicated in HAIs.

Keywords: *Pseudomonas aeruginosa,* discordant antibiotics, empiric antibiotics

Received Nov 6, 2023; Revised Dec 18, 2023; Accepted Dec 19, 2023

Taux de discordance entre les antibiotiques empiriques administrés et la sensibilité aux antimicrobiens dans les infections causées par *Pseudomonas aeruginosa* dans un hôpital tertiaire au Nigeria

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Résumé:

Contexte: L'instauration précoce d'un traitement antibiotique approprié est essentielle à la prise en charge efficace des infections bactériennes graves. L'instauration d'une antibiothérapie ciblée n'est possible que lorsque l'organisme causal est isolé. En conséquence, les antibiotiques sont généralement administrés sur une base empirique, guidée par la présentation clinique, les directives locales en matière d'antibiotiques et d'autres antécédents pertinents. En général, les antibiotiques empiriques diffèrent à la fois pour les infections nosocomiales et celles nosocomiales (IAS), de sorte que les agents pathogènes courants des IAS, tels que *Pseudomonas aeruginosa*, doivent être délibérément ciblés, car la plupart des antibiotiques courants sont

inefficaces contre eux.
Méthodologie: Il s'agit d'une étude rétrospective transversale portant sur la revue des consultations cliniques adressées aux microbiologistes cliniciens de l'Hôpital Universitaire du Bénin (UBTH) entre janvier et décembre 2022. Les consultations ont été analysées pour le diagnostic initial, les raisons de l'invitation et antibiotiques empiriques administrés. D'autres informations pertinentes ont été obtenues à partir des dossiers de laboratoire. Les profils de sensibilité des isolats de *P. aeruginosa* ont été comparés à ceux des antibiotiques empiriques administrés. Une antibiothérapie empirique discordante a été définie comme l'administration d'un régime antibiotique sans activité anti-pseudomonas.
Résultats: Parmi les 256 consultations reçues au cours de la période d'étude, *P. aeruginosa* a été isolé comme pathogène chez 57 (22,3%) patients. Sur ce total, 24,6% (n=14) ont reçu au moins un antibiotique anti-pseudomonas, ce qui porte le taux discordant total à 75,4%. Le métronidazole (22,7%) et la ceftriaxone-sulbactam (Tandak) (21,5%) étaient les antibiotiques empiriques les plus couramment prescrits. Le motif de consultation le plus fréquent était un diagnostic de sepsis à 40,2% suivi d'isolats pan-résistants à 34,8%.
Conclusion: Bien que les antibiotiques couramment prescrits dans notre contexte soient à large spectre, ils manquent de couverture pour P. aeruginosa qui est l'un des plus courants agents pathogènes courants impliqués dans les IAS.

Mots clés: *Pseudomonas aeruginosa*, antibiotiques discordants, antibiotiques empiriques

Introduction:

Early initiation of appropriate antibiotics is key to the effective management of bacterial infections. However, initiation of targeted antibiotic therapy is possible only when the causative organism is isolated and subsequently, antimicrobial susceptibility testing performed (1). As a result, antibiotics are often given on an empirical basis guided by the clinical presentation, other relevant clinical histories and local antibiotics guidelines, as complete culture results usually take 24 to 48 hours (2). It has been shown that delay and or administration of inadequate antibiotics for infections in the critically ill is often associated with adverse outcomes including higher morbidity and mortality as well as extended length of hospital stay (1,3).

Empirical therapy is simply defined as the initial antibiotic regimen selected in the absence of definitive microbiological pathogen identification and susceptibility testing. On the hand, targeted or definitive therapy is the antibiotic regimen selected after pathogen identification and susceptibility testing is completed (4). The common approach to prescribing on empirical basis include; the use of broad-spectrum antimicrobial agents as initial therapy, which could be achieved by mono-therapy or a combination of antimicrobial agents, with the aim of covering multiple possible organisms commonly associated with the specific clinical syndrome including Gram-positive, Gram-negative, anaerobes as well as atypical bacteria (3).

Worthy of note is that empirical choices differ for both community and hospital-acquired infections. For example, adults with community-acquired pneumonia should be started on either a respiratory fluoroquinolone such as levofloxacin or combination of a beta-lactam (amoxicillin/amoxicillin-clavulanic acid) and a macrolide (5). However, in hospital-acquired pneumonia, antibiotics regimen for empirical coverage should include antipseudomonals such as piperacillin-tazobactam, cefepime, levofloxacin, imipenem or meropenem (6).

In the case of hospital-acquired infections (HAI)s, which are frequently associated with the presence of invasive devices and procedures that compromises the normal barriers to infection such as intravascular catheter–associated bacteraemia, ventilator-associated pneumonia, and catheter-associated urinary tract infections (CAUTI), the offending pathogens are frequently multi-drug-resistant organisms, which is true for both Gram-positive (e. g. MRSA) and Gram-negative (e. g. *Pseudomonas aeruginosa*) bacteria (4). The sources of these resistance phenotypes have been linked to the hospital environment which often serves as a reservoir for these pathogens, primarily due to selection pressure from frequent antimicrobial use in the hospital (4).

Therefore, to select empirical antimicrobial therapy for HAIs, the following should be considered; (i) the site of infection and the most likely organisms colonizing that site, for example, central-line associated blood stream infection frequently results from skin flora such as staphylococci inoculated into the bloodstream by the process of catheterization, (ii) prior knowledge of bacteria known to colonize a given patient e .g. screening by the use of nasal swab and faecal screening for the carriage of carbapenem-resistant Gram-negative bacteria pathogens, which is currently been conducted routinely by many hospitals before admitting patients to the intensive care unit or before highly invasive surgeries, and (iii) the local bacterial resistance profile or antibiograms (7).

Initial antibiotic therapy in critically ill does not only need to be timely, but should be appropriate. Appropriateness can be defined as antimicrobial coverage that provides adequate *in vitro* activity against all likely pathogens at the clinical infection site of interest (8). When considering targeted therapy, appropriateness is defined as antimicrobials with *in vitro a*ctivity against the isolated pathogen, or appropriate for the underlying clinical syndrome even if no pathogen was isolated.

Empirical antibiotic therapy is considered discordant if the bloodstream isolate does not display *in vitro* susceptibility to any systemic antibiotic administered on the day of blood culture sampling. Such a scenario includes meningitis and antibiotic regimen that crosses the blood-brain barrier (8).

Common predictors of discordant empirical antibiotic therapy (DEAT) include but are not limited to high prevalence of antibiotic-resistant phenotypes in the setting, infections due to non-glucose fermenting Gram-negative organisms (*Acinetobacter baumannii* and *P. aeruginosa*) and presence of *Enterococcus* spp and infection due to resistant phenotypes (9). Among Gram-negative infections, *P. aeruginosa* is one of the most common Gram-negative bacteria causing HAIs in hospitalized patients (10).

The World Health Organization (WHO) has placed carbapenem-resistant *P. aeruginosa* among critical priority pathogen that desperately requires new treatment options (11). Increasing rates of multidrug-resistant (MDR) *P. aeruginosa* in HAIs and among hospitalized patients is a major public health problem (12). Multidrug-resistant *P. aeruginosa* infections in the hospital setting are associated with poor outcomes including increased resource utilization and costs, morbidity, and mortality (10). In the USA, MDR *P. aeruginosa* accounts for 13–19% of the annual HAI burden. The increasing level of resistance in MDR *P. aeruginosa* is often attributed to patient-to-patient transmission of resistant strains, increasing rate of environmental colonization, as well as newly acquired resistance owing to previous antibiotic exposure (13).

In the management of severe systemic infections, multidrug-resistant *P. aeruginosa* must be part of the consideration when selecting empirical treatment to ensure timely and appropriate initial therapy. Instead of universal broad-spectrum antibiotics, specific antibiotic regimens should be determined using a more scientific approach. Such an approach should include acquiring site-specific diagnosis such as previous blood culture information to predict probable causative organisms based on epidemiological and host risk factors including recent infection exposures, evidence of colonization, indwelling devices, comorbidity, recent infections, recent antibiotic exposure in the preceding 3 months and host immunologic status (4).

Initiation of antibiotics such as ceftriaxone and amoxicillin-clavulanic acid with no *P. aeruginosa* coverage often leads to inappropriate initial therapy that adversely impacts health outcomes (14). This study aimed to determine the rate of inappropriate empirical therapy in patients with *P. aeruginosa* infections at the University of Benin Teaching Hospital, Nigeria

Materials and method:

Study setting, period and design:

The study was conducted at the University of Benin Teaching Hospital, Edo State, Nigeria between January and December 2022. This was a retrospective cross-sectional study involving the review of the clinical consults sent to consultant clinical microbiologists at the University of Benin Teaching Hospital for a period of one year.

Study procedure and data collection:

The clinical consults were analyzed for the following parameters; initial diagnosis, reasons for the invitation, and empirical antibiotics administered. Isolates and the *in vitro* susceptibility profile of the initial samples sent for microbiology culture before or immediately after initiating antibiotics were also included in the analysis. Samples that yielded growth of *P. aeruginosa* were included for further evaluations to determine the extent the *in-vitro* susceptibility profiles differ (discordant empirical antibiotic therapy) or aligned with the initial empiric antibiotics prescribed. In cases where multiple samples from one patient grew *P. aeruginosa*, only one isolate was included.

Clinical samples included in the study were blood culture, urine, body fluid aspirates and wound swabs. Empirical antibiotic therapy was considered discordant (DEAT) if *P. aeruginosa* isolate did not display *in vitro* susceptibility to any systemic antibiotic administered shortly before or immediately after sample collection. Pan-resistant isolate was defined as resistance to all antibiotics tested (15).

Results:

Of the 256 consults received during the study period, all but two patients were on antibiotics before review, giving antibiotic prevalence rate of 99.2%. *Pseudomonas aeruginosa* was isolated from 57 (22.3%) patients as pathogens. Of this, only 14 (24.6%) received at least one anti-pseudomonas antibiotics which puts the total discordant rate at 75.4% (Fig 1).

The ICU had the highest number of *P. aeruginosa* infected patients closely followed by the surgical ward at 18 (31.6%) and 15 (26.3%) respectively. The percentage of DEAT was 66.7% each for ICU and Paediatrics wards (Table 1). Metronidazole (22.7%) and ceftriaxone-sulbactam (Tandak) (21.5%) were the most commonly prescribed empirical antibiotics (Fig 3) while the most common reasons for consultation were the diagnosis of sepsis, followed by pan-resistant isolates respectively at 40.2% and 34.8% (Fig 4). The distribution of *P. aeruginosa* by specimen sources shows that urine had the highest yield of the studied pathogens followed by wound swabs at 43.9% and 29.8% respectively (Fig 4)

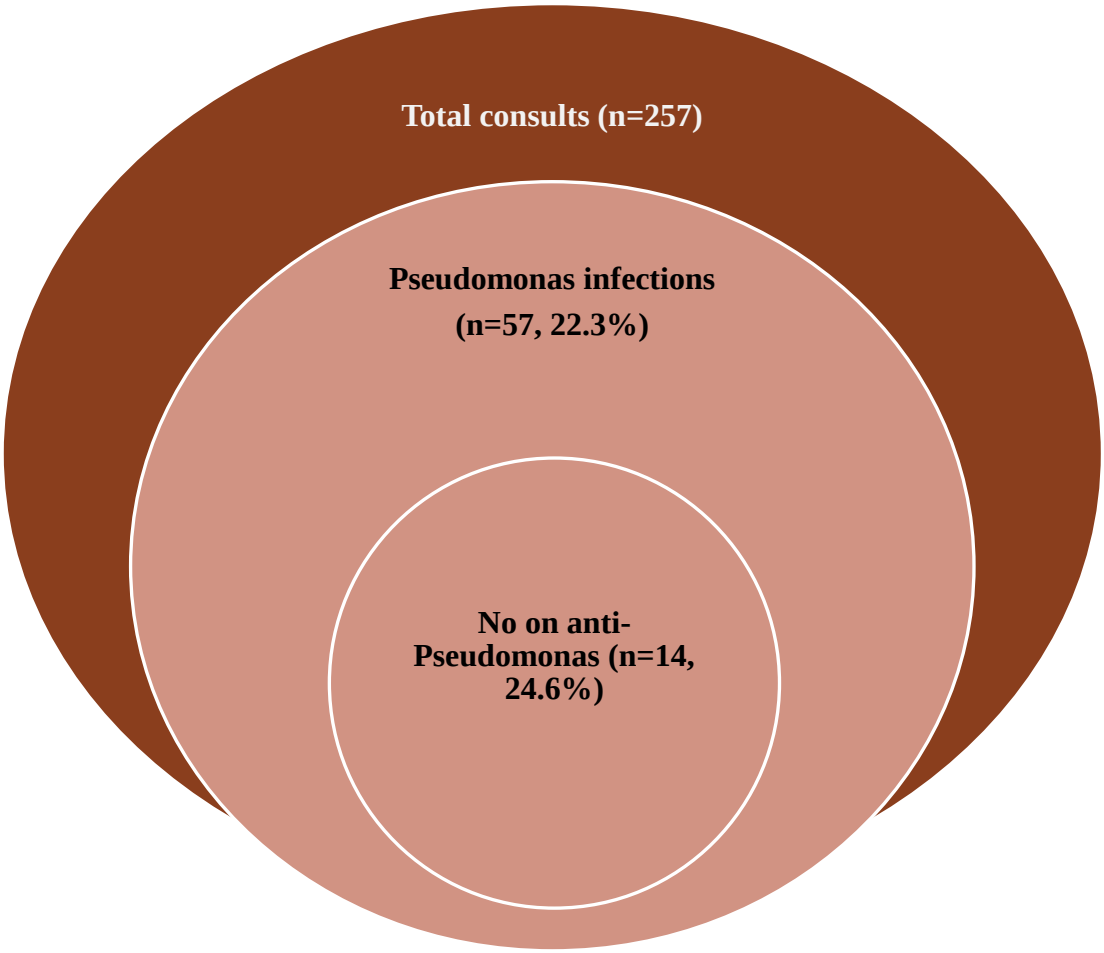

Fig 1 Frequency of *Pseudomonas aeruginosa* isolate and anti-pseudomonas prescribed

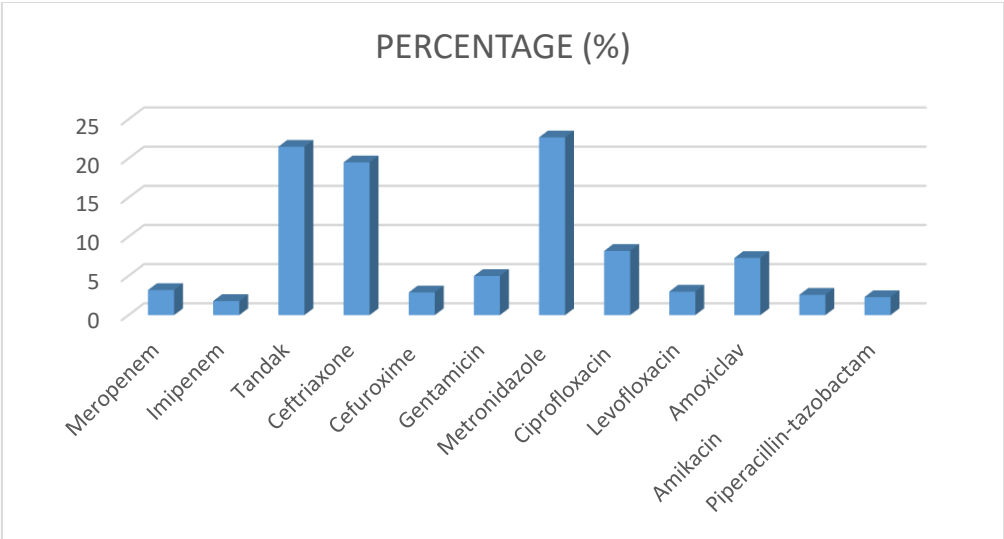

Fig 2: Patterns of empirical antibiotics prescribed before or after sample collection

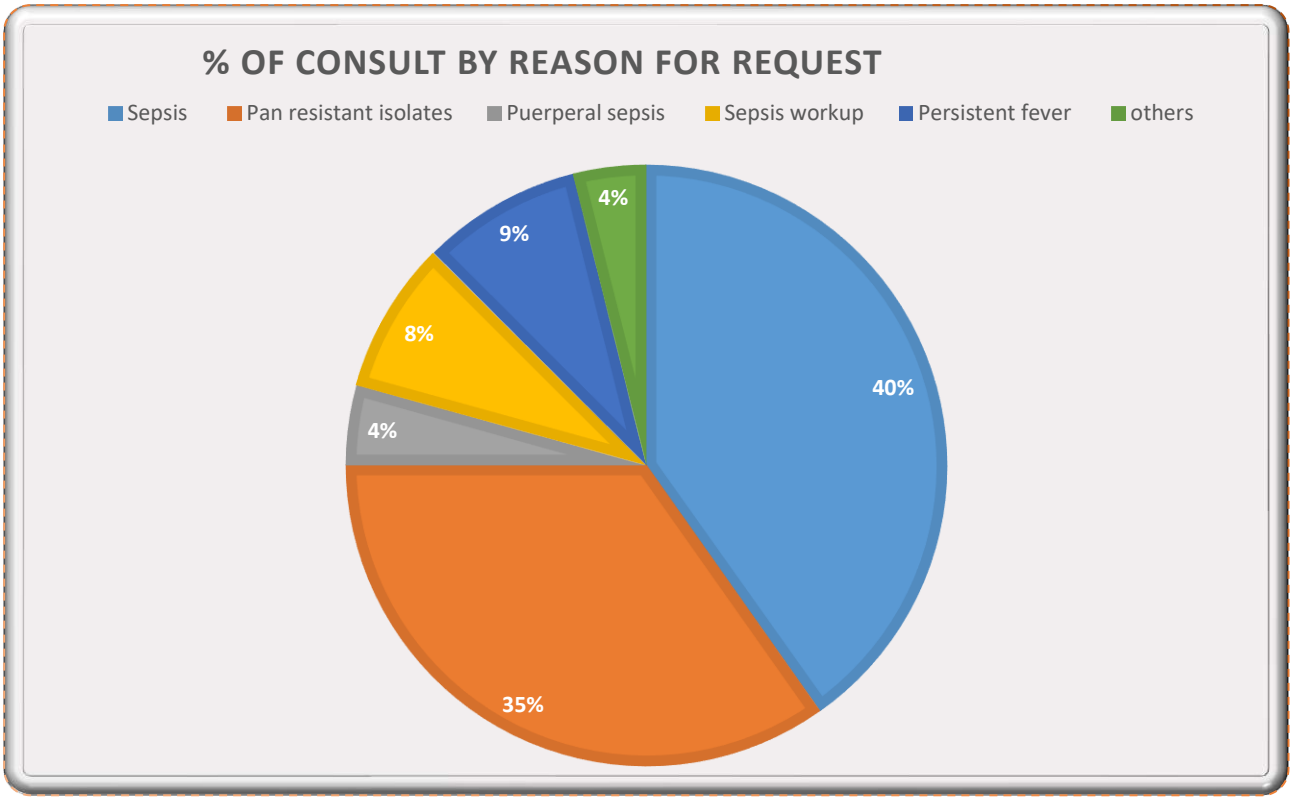

Fig 3: Frequency (%) of consults by reason for request

Table 1: Distribution of *Pseudomonas aeruginosa* infection by ward and discordant empirical antibiotic therapy

Ward/units	**No of patients with *Pseudomonas aeruginosa* infection (%)**	**No on anti-*Pseudomonas aeruginosa* coverage (%)**	**No of DEAT (%)**	***x*²**	***p* value**
ICU	18 (31.6)	6 (33.3)	12 (66.7)	1.806	0.7714
Surgical ward	15 (26.3)	3 (20.0)	12 (80.0)		
O&G	7 (12.3)	1 (14.3)	6 (85.7)		
Medicine	11 (19.3)	2 (18.2)	9 (81.8)		
Paediatrics ward	6 (10.5)	2 (33.3)	4 (66.7)		
Total	**57**	**14 (24.6)**	**43 (75.4)**		

DEAT = Discordant empirical antibiotic therapy; ICU=Intensive Care Unit

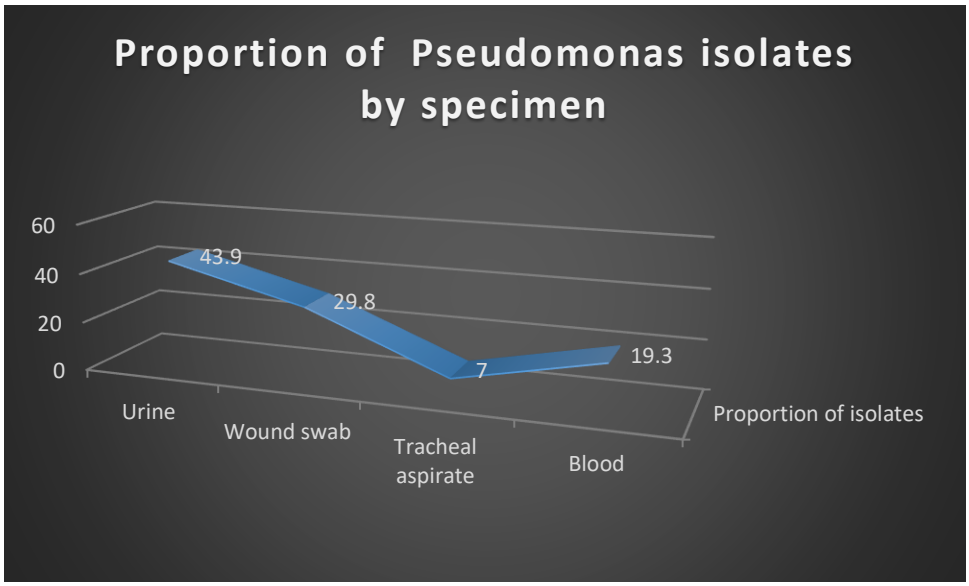

Fig 4: Frequency of *Pseudomonas aeruginosa* isolates by specimen types

Discussion:

Timely administration of effective antibiotic therapy is associated with improved outcomes in patients with severe infections such as sepsis and bloodstream infections (9). However, the uncompromisable lag between sampling of cultures and the availability of *in vitro* susceptibility report make empirical antibiotic selection challenging especially when infection involves pathogen such as *P. aeruginosa* which besides being intrinsically resistant to a number of common antibiotics, are known to possess various mechanism of resistance to different classes of antibiotics (4,9). The key predictors of discordant empirical antimicrobial therapy which include antibiotic-resistant phenotype such as *P. aeruginosa* and high antimicrobial resistance prevalence setting (16) are common features seen in this study. As demonstrated by various studies, there is high degree antibiotics resistance from isolates obtained from clinical samples in Nigerian's hospitals (17).

In this study, the discordant empirical antimicrobial therapy rate was 75.4%. This is much higher than findings from other studies (16) probably because our study was done in a setting with high prevalence of resistance to antibiotics and the focus was on a notable resistant pathogen. Also, the studies with low DEAT rate besides the low resistant prevalence setting were focused on only blood stream infections (9,16). Empirical antibiotics reported as the most administered in the study were ceftriaxone-sulbactam (Tandak) and metronidazole. Although this combination is broad spectrum sufficient to cover most Gram-positive, Gram-negative and anaerobic organisms, it does not have sufficient coverage for *P. aeruginosa (*18). This pattern of prescription was similar to a report by Abubakar et al., (19) and other studies (20,21) which reported metronidazole and ceftriaxone as the most administered antibiotics among patients admitted to tertiary healthcare. The need for a broad-spectrum antibiotic therapy may have informed the general choice of ceftriaxone and metronidazole as among the common empirical antibiotics across most studies, even if it lacks potency for most pathogens with resistant encoding genes.

Sepsis closely followed by pan-resistant isolates were the common reasons for referrals to the clinical microbiologists, which accounted for 40.2% and 34.8% respectively. This is likely due to the understanding that the management of sepsis involves multidisciplinary approach and also clinical microbiologists largely play the roles of infectious diseases physician in our settings (22). In the analysis of the distribution of *P. aeruginosa* by ward/ unit, it somewhat showed direct correlations with HAI, with ICU having the highest recovery of *P. aeruginosa* at the rate of 31.6%. This could be explained by the fact that ICU admission is a strong determinant of HAI, and *P. aeruginosa* is a prominent pathogen in this category of infection (23).

Pseudomonas aeruginosa is a notable pathogen for serious infection especially in the immunocompromised hosts including those with severe burn, surgical site infection and those with indwelling devices such as urethral catheter (24). In this study, the highest number of *P. aeruginosa* were isolated from urine (42.9%) followed by wound swabs (29.8%). A similar observation was reported in another study with urine also the highest source for this pathogen (24,25). The high

yield of *P. aeruginosa* from the urine samples in our study may be due the fact that most of the patients were in patient who have stayed more 48 hours on admission, and many of whom would have been on urethral catheter or other devices which usually encourage bio-film formation by this organism (26).

Our study has some limitations. First, the study findings were based only on sample of patients whose consults were sent to the clinical microbiologists, which might not necessarily be the true picture, and secondly, some of the cases reviewed had no sample collected for culture before clinical microbiologist's review. These were excluded and could have further reduced the number of *P. aeruginosa* in the study

Conclusion:

In general, the routine antibiotics prescribed on the empirical basis in our setting are broad spectrum, however, they lack coverage for the common multi-drug resistant pathogens associated with HAIs such as *P. aeruginosa.* As a result, the unintended use of these antibiotic regimens often results in inappropriate initial therapy that adversely impacts health outcomes.

Contributions of authors:

IJA conceptualized and designed the study, conducted the analysis and wrote the first draft. LPVO reviewed the manuscript. The authors read and approved the final manuscript

Source of funding:

No funding was received for the study.

Conflict of interest:

No conflict of interest is declared.

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Ikinyom et al. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (1): 103 - 111 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jan 2024; Vol.25 No.1
AJCEM/2302. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Antimicrobial activity of selected nutraceutical plants used in Northern Uganda

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Abstract:

Background: Nutraceutical plants (NP) play a vital role as supportive treatment with antiretroviral drugs (ARVs). However, there is limited scientific evidence on the efficacy of NP to justify their extensive use. This study aimed to evaluate the antibacterial activity of three nutraceutical plants which are commonly used as antimicrobials.
Methodology: Leaves of *Cajanus cajan* L. Millsp. and *Eucalyptus globulus* Labill., and stem bark of *Mangifera indica* L. were collected from Northern Uganda. The three samples of each NP were extracted with acetone and the minimum inhibitory concentration (MIC) values of the extracts against *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were determined using the serial broth microdilution technique [1]. The mean MIC values of the extracts against each bacterial species were recorded.
Results: The three NP extracts were active against all the four bacteria species with MIC ranging from 0.08 to 2.5 mg/ml. The extract of *Cajanus cajan* was very active against *Klebsiella pneumoniae* with the lowest recorded MIC value of 0.08 mg/ml. The extract of *Mangifera indica* bark was very active against *Pseudomonas aeruginosa* with the lowest MIC of 0.08 mg/ml.
Conclusion: The results of the present study support the traditional use of the nutraceutical plants as antimicrobials.

Keywords: Antimicrobial, bacteria, nutraceutical plants, MIC, serial microdilution

Received Oct 04, 2023; Revised Dec 14, 2023; Accepted Dec 17, 2023

Activité antimicrobienne de certaines plantes nutraceutiques utilisées dans le nord de l'Ouganda

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Résumé:

Contexte: Les plantes nutraceutiques (NP) jouent un rôle essentiel en tant que traitement de soutien avec des médicaments antirétroviraux (ARV). Cependant, il existe peu de preuves scientifiques sur l'efficacité des NP pour justifier leur utilisation intensive. Cette étude visait à évaluer l'activité antibactérienne de trois plantes nutraceutiques couramment utilisées comme antimicrobiens.

Méthodologie: Feuilles de *Cajanus cajan* L. Millsp. et *Eucalyptus globulus* Labill., et l'écorce de tige de *Mangifera indica* L. ont été collectées dans le nord de l'Ouganda. Les trois échantillons de chaque NP ont été extraits avec de l'acétone et les valeurs de concentration minimale inhibitrice (CMI) des extraits contre *Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa* et *Klebsiella pneumoniae* ont été déterminées à l'aide de la technique de microdilution en bouillon en série. Les valeurs moyennes de la CMI des extraits contre chaque espèce bactérienne ont été enregistrées.
Résultats: Les trois extraits de NP étaient actifs contre les quatre espèces bactériennes avec une CMI allant de 0,08 à 2,5 mg/ml. L'extrait de *Cajanus cajan* s'est montré très actif contre *Klebsiella pneumoniae* avec la valeur de CMI la plus basse enregistrée de 0,08 mg/ml. L'extrait d'écorce de *Mangifera indica* s'est montré très actif contre *Pseudomonas aeruginosa* avec la CMI la plus basse de 0,08 mg/ml.
Conclusion: Les résultats de la présente étude soutiennent l'utilisation traditionnelle des plantes nutraceutiques comme antimicrobiens.

Mots clés: Antimicrobien, bactéries, plantes nutraceutiques, CMI, microdilution en série

Introduction:

Antimicrobial resistance (AMR) to antibiotics has been reported as a major therapeutic concern (1). Based on current trends, AMR is projected to kill 10 million people worldwide each year by 2050 and will cost the global economy US $100 trillion gross domestic product (GDP) loss between 2015 and 2050 (1). AMR occurs when microbes such as bacteria, viruses, fungi and parasites transform over time and no longer respond to any drug (2) and this may be as a result of spontaneous or induced genetic mutations (1).

A weak immune system in persons with HIV infection results in susceptibility to opportunistic infections caused by opportunistic pathogens such as *Candida albicans* causing genital infections; *Staphylococcus aureus* causing eye and wound infections, pneumonia, and septicaemia; *Mycobacterium tuberculosis* causing tuberculosis; *Streptococcus pneumonia*e causing pneumonia and meningitis; *Klebsiella pneumoniae* causing pneumonia, urinary tract infection and septicaemia (1-3).

There is no cure or effective vaccines for HIV, although people living with HIV/AIDS (PLWH) can enjoy healthy, long and productive lives by taking antiretroviral drugs (ARVs) that can effectively control the virus, manage opportunistic infections and help prevent transmission (4). PLWH exposed to subtherapeutic ARVs, are however at increased risk of developing ARV drug resistance (5). There is increased risk of AMR in PLWH across a range of bacterial pathogens and multi-drug classes (6). The use of ARVs in PLWH in Uganda is limited by toxic side effects, poor adherence to treatment, limited access and antimicrobial resistance to ARVs has been reported (7-9).

The World Health Organization (10) observed that AMR was more prevalent in cases of bacterial infections such as respiratory tract infection, diarrhoea, meningitis, syphilis, gonorrhoea and tuberculosis. PLWH had higher odds for colonization and infection with AMR *S. aureus, S. pneumoniae, E. coli* and *K. pneumoniae* (6).

One practical way to circumvent antibiotic resistance is to develop and use new antibiotics (1). There is an urgent need to discover new antimicrobial compounds or extracts to address the problem of increasing microbial resistance against current antibiotics (11). Among the potential sources of new agents are nutraceutical plants because they contain many bioactive compounds, have low toxicity and there is a long tradition of using nutraceutical plants in Uganda folk medicine (9,12). Currently drug discovery focus has moved onto plants due to beneficial attributes of plants (13). Plant based antimicrobials have immense potential to combat bacterial, fungal, protozoal and viral diseases without known side effects (13).

Nutraceutical plants play a vital role as supportive treatment with ARVs in the management of opportunistic infections associated with HIV/AIDS especially among the rural poor (9,12,13). Nutraceutical plants possess nutritional and pharmaceutical properties or a combination of both (9). Living organisms can grow, maintain themselves and reproduce by assimilation of nutritious plants that contain vitamins A, C, K, fibre, riboflavin and minerals, which are essential requirements for the health of HIV positive patients (14).

There are approximately 5,000 species of higher plants in Uganda, of which 70 are endemic (15). There are more than 200 species of non-cultivated edible plants and 75 species of edible fruit trees in Uganda, while forestland covers approximately 3.3 million hectares (15). Ethnobotanical research in Uganda has identified more than 300 trees, shrubs and herbs growing wild associated with medicinal value (15). Traditional knowledge of plants with medicinal value is passed on from one generation to another (15).

Eucalyptus globulus Labill belongs to family of Myrtaceae, an evergreen broadleaf tall tree with a straight trunk, is cultivated in Uganda and worldwide because of adaptability and fast growth rate (16). Phytochemical analysis of leaf extract of *E. globulus* proved the presence of tannins, saponins, terpenoids, glycosides, alkaloids, phenolic compounds, cardiac glycosides, terpenes, reducing sugars, carbohydrates, and flavonoids (17).

Mangifera indica L. also known as Mango,

belonging to family Anacardiaceae, is a large evergreen tree growing to a height of 10-15m. The green leaves are linear-oblong and release an aromatic strong odour when crushed (16). The tree bark is thick, grey to brown in color and with age exfoliates in the form of flakes. *M. indica* inflorescence occurs in panicles consisting of tiny whitish-red oy yellowish –green flowers. The mango fruit is a drupe (16). Selles et al., (18) reported that *M. indica* stem bark extracts contain ketones, phenols, terpenoids, steroids, nitrogen compounds, and one sulphur compound. The phytochemical constituents of *M. indica* stem bark and leaves was reported to contain alkaloids, flavonoids, saponins, tannins, phenols, and vitamins (19).

Cajanus cajan L. Millsp (also known as pigeon pea-English, Lapena-Acholi) is an erect branched hairy shrub, about 1-2m high. Green leaves are oblong-lanceolate with three leaflets, flowers are yellow in color and pod is hairy, containing 2-7 seeds (20). *Cajanus cajan* is an important grain-legume food crop and forage crop of rainfed agriculture in semi-arid tropics with high levels of proteins (16,20). Sahu et al., (21) reported that phytochemical analysis of the leaf, stem and seed extracts of *C. cajan* showed presence of saponins, tannins, alkaloids, flavonoids, anthraquinones and reducing sugars. Research found *C. cajan* leaves to be rich in flavonoids, saponins, tannins, reducing sugars and coumarins (20). This study aimed to provide scientific evidence on effectiveness of use of nutraceutical plants to justify their extensive use, even as widespread and empirical use of nutraceutical plants demands accurate and reliable information on efficacy (9).

Studies of *in vitro* screening for antibacterial activity on nutraceutical plants in Europe, Asia and Africa have been conducted on various microbial organisms causing opportunistic diseases and managed with nutraceutical plants. In Pakistan, *Cassia fistula* and *Punica granatum* have been used against fungal opportunistic infections associated with HIV (22). In Thailand, 12 medicinal plants used among HIV patients were evaluated for antibacterial activities (23). Ten Nigerian medicinal plants showed potential antimycobacterial activity following preliminary MIC assay (24). The plants *A Juss Turraea floribunda* and *Warburgia ugandensis* showed antimicrobial activity against *E. coli, P. aeruginosa* and *Salmonella* (25). Fresh extracts of Ugandan medicinal plants, *Zanthroxylum chalybeum* and *Enclea latidens* had antibacterial effects on carcinogenic and periodontopathic bacteria (26). Kuglerova *et al.,* (27) reported antimicrobial activity of Ugandan medicinal plants.

The pharmacological activity of nutraceutical plants can be predicted by the identification of the biologically active compounds in plants called phytochemicals (28). The phytochemicals are derived from various parts of plants such as leaves, flowers, seeds, bark, roots and pulps (29,30). Nutraceutical effects of plants are attributed to the interaction of phytochemicals such as alkaloids, phenols, flavonoids, saponins and tannins (28,31). The World Health Organization (WHO) recommends that quality evaluation further involves naming the major chemical constituent, chemical structure of the selected major constituents and drawing of the chemical structures where appropriate (32). According to Chandra et al., (33), antimicrobial properties of plants are attributed to the presence of active compounds.

Different bioactive compounds with antimicrobial properties have been isolated from various nutraceutical plants. Quinones possess antimicrobial activity against *Pseudomonas aeruginosa* and *Bacillus anthracis* (33, 34). Coumarins isolated from *Angelica lucida* L. is active against the oral pathogens, *Streptococcus mutans* and other viridian streptococci (33). Terpenoids extracted from the bark of *Acacia nilotica* have antimicrobial properties and the flavonoids such as kaemferol, rutin and quercetin have antifungal properties (33). The tannin of sorghum has antimicrobial activity against *S. aureus* and *Salmonella typhimurium* (33). According to Aerts *et al.*, (35), the plant *Raphanus sativum* has peptides which are effective antifungal against *Candida albicans*. Banso (36) and Ragasa et al., (37) observed that terpenoids and essential oils from plants are effective against *S. aureus, P. aeruginosa* and viridian streptococci (34). Alkaloids from plants are effective against *S. aureus, Streptococcus mutans* and *Microsporum canis* (38). This study aimed to evaluate the antibacterial activity of three nutraceutical plants which are commonly used as antimicrobials in Uganda.

Materials and method:

Study setting, sample collection and authentication:

From the ethnobotanical study in Northern Uganda (39), three plants were selected for *in vitro* antimicrobial analysis based on their high frequency of mention in managing opportunistic infections associated with HIV/AIDS. Plants for analysis were collected from Pader district, located in northern Uganda at 2^0 49′ 59.9″ N and $33^0$04′ 60.0 0″ E (15) with the total area of 3.362 square kilometres. Generally, altitude ranges between 1000-1200 metres above sea level. The district experience tropical climate with average annual rainfall of 1507 mm and average temperatures is 23^0C, vegetation is intermediate savannah grassland. Food crops grown are beans, peas, cassava, cotton, groundnuts, and sunflower. Ninety percent (90%) of the economic activity

is subsistence agriculture.

Plant specimens were identified in the field by the principal researcher (a trained Taxonomist/Botanist), based on the African Plant database and Tropical plants of East Africa catalogue (16). Plant names were checked and updated with online website (www.theplantlist.org). The voucher plant specimens were identified, collected and processed according to standard procedures (16,40). Voucher specimens were pressed, dried, mounted, coded and deposited at the Herbarium in the Department of Plant Science, Microbiology and Biotechnology, Makerere University Kampala, Uganda. Further identification of botanical specimens was done by the herbarium curator via comparison with herbarium material stored in the Makerere University Herbarium. Each plant was given an accession number; *M. indica* L. (MHU 41712), *C. cajan* L. Millsp (MHU 51151), and *E. globulus* Labill (MHU 51152).

Ethical approval:

Ethical approval for the study was obtained from Gulu University Research Ethics Committee (Ref. GUREC-062-20) and Uganda National Council for Science and Technology (Ref. HS983ES). Plants and seeds were collected, permission to collect was obtained from Gulu University Research Ethics Committee (Ref. GUREC-062-20) and Uganda National Council for Science and Technology (Ref. HS983ES). All local, national and international guidelines and legislation were adhered to in the conduct of this study.

Preparation of plant extract:

The three plants were evaluated for their *in vitro* antimicrobial activities at the Microbiology Bioscience Laboratory, Gulu University. Stem bark of *M. indica* L. (MHU 417 12), leaves of *C. cajan* L. Millsp (MHU 51151) and leaves of *E. globulus* Labill (MHU 51152) were air dried in shade for 10 days and later dried in oven (Memmert) at 37.2^{0}C for 10 hours. Plant parts were ground to fine powder using a grinder (IKA Merke model M20). Plant powder was weighed on a weighing scale (OHaus Neo-tech SA). Acetone was used as an extractant in the assay because it has been shown to extract compounds of a wide range of polarities and its low toxicity to bioassay systems (41).

Approximately 10g of the plant samples were dissolved in 200 ml 99% acetone (Loba chemie PVT Ltd) in glass beaker, shaken in shaker (Stuart orbital shaker SSL1, Neotech SA) for 16 hours, and then soaked/macerated for 24 hours. The supernatants were then filtered using Whatman no. 1 filter paper. The filtrates were put in round bottom flask and evaporated to dryness using a Rotary evaporator (Hei-VAP Precision, Heidolph), and dried extracts were transferred into pre-weighed falcon tubes and stored in freezer till further analysis.

Test microorganisms and media:

The panel of microbial organisms used were four human pathogenic species commonly causing opportunistic infections associated with HIV/AIDS and included Gram-positive cocci (*Staphylococcus aureus* ATCC 25923 and *Streptococcus pneumoniae* ATCC 49619) and Gram-negative bacilli (*Pseudomonas aeruginosa* ATCC 10231 and *Klebsiella pneumoniae* ATCC 700603) (43). The ATCC strains were purchased from Mulago Referral Hospital Microbiology Laboratory, Kampala Uganda, and handled according to performance standard procedures (42). The strains were maintained at the Gulu University Bioscience Laboratory.

Analysis was carried out in line with the guidelines and standards of Serial Microtitre Dilution Methods using tetrazolium salts, which has been shown to produce best (reproducible) results (42). All the microbial strains were sub-cultured from original culture on nutrient agar (HiMedia), kept in Eppendorf tubes in sucrose and glycerol and stored in deep freezer (Energ, Beko HS530) at -80^{0}C. Prior to antimicrobial assay, all the strains were sub-cultured onto a fresh appropriate Muller-Hinton (MH) agar (BioLab chemicals) plate at 37^{0}C for 24 h.

Quantitative antibacterial activity assay by minimum inhibitory concentration:

Petri dishes and microtitre culture plates were sterilised in autoclave (Sturdy SA-300 VMA). The organisms were sub-cultured onto MH agar (BioLab Chemicals) and incubated aerobically for 24 hours at 37^{0}C in oven (Memmert). The freshly sub-cultured strains were used for the test against leaf, root and bark extracts in a biosafety cabinet. The bacterial turbidity of each species was prepared and standardized.

Inocula were prepared by transferring several single colonies of microbes to 5 ml sterile normal saline solution to produce a suspension. The turbidity of the suspension was adjusted to 1.0 McFarland standard using spectrophotometer (NeoTech SA, Jenway, Genova plus), which is equivalent to 3.0 x 10^8 CFU/ml.

Preparation of crude extracts and antibiotics, and determination of MIC:

The dried plant extract was reconstituted in 99% acetone (Loba chemie PVT Ltd) to make 10 mg/ml stock extract (42). Stock extract was serially diluted 1:1 with sterile distilled water. 1 ml of the final extract concentrations of 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0.08, 0.04 mg/ml was made in culture plates.

100µl concentration of the plant extract were added to the wells of the sterile 96-well microtitre plate. A positive control of a standard antibacterial ciprofloxacin (R_X Farma, Hertford shire, UK) was also serial diluted accordingly. Acetone and water were used as negative control. After the dilution process, 100µl of Mueller-Hinton broth (Accumix chemicals) for bacteria was added to each well, followed by 100µl of test organisms. The microtitre plates were covered and incubated at 37°C for 24 hours.

The minimum inhibitory concentration of the extract was determined following addition of 40µl of 0.2mg/ml 2,3,5-triphenyltetrazolium chloride (TTC) (Loba Chemie) for colorimetric assay, incubated at 37°C and observed for 4 hours. Microbial growths were determined by observing the change of color of TTC in the microplate wells, with pinkish-red formazan when there is growth, and clear solution when there is no growth. The colourless well immediately after a red well was recorded as the MIC, which is the lowest extract concentration showing no color change (clear) indicating complete inhibition of bacterial growth (42). The experiments were carried out in triplicate, and the mean MICs were recorded.

Statistical analysis:

The results were expressed as mean MICs of 3 replicates. Descriptive statistics and the ANOVA single factor statistical tool was used to analyze results of the MICs of plant extracts from the serial microdilutions. Statistical significance was defined at $p< 0.01$ level (42).

Results:

All the nutraceutical plants exhibited low MICs against the bacteria strains. The serial microdilution results by single factor analysis of variance (ANOVA) indicated that there is significant difference in the sensitivity of the tested microorganisms to the various extracts ($p<0.01$). The microbial sensitivity to different extracts represented by mean MICs values ranged from 0.08 to 2.5 mg/ml (Table 1 and Fig 1). *Pseudomonas aeruginos*a and *Klebsiella pneumoniae* (MIC=0.08mg/ml) were the most sensitive, followed by *Streptococcus pneumoniae* (MIC=0.16mg/ml).

Antibacterial activity of *Mangifera indica* stem bark extract:

The bark extract of *M. indica* exhibited strong antimicrobial activity against *S. aureus* and *P. aeruginosa* in this study. Antibacterial tests results indicated that *M. indica* bark extract inhibited growth of *S. aureus* and *P. aeruginosa* at mean MICs of 0.63 mg/ml and 0.08 mg/ml respectively.

Antibacterial activity of *Cajanus cajan* leaf extract:

The leaf extracts of *C. cajan* exhibited growth-inhibitory activity against *S. pneumoniae* and *K. pneumoniae* that causes pneumonia. *Cajanus cajan* leaf extract inhibited the growth of *S. pneumoniae* and *K. pneumoniae* at MICs of 0.16 mg/ml and 0.08 mg/ml respectively.

Antibacterial activity of *Eucalyptus globulus* leaf extract:

Eucalyptus globulus exhibited growth-inhibitory activity against *S. pneumoniae* and *K. pneumoniae* at MICs of 1.25 mg/ml and 2.5 mg/ml respectively.

Discussion:

Our results of the antibacterial activity

Table 1: Minimum inhibitory concentrations of nutraceutical plant extracts against the bacteria strains

Plant species	Bacteria strain	Mean MIC (mg/ml)
Mangifera indica bark extract	*Staphylococcus aureus* ATTC 25923	0.63
	Pseudomonas aeruginosa ATTC 10231	0.08
Cajanus cajan leaf extract	*Streptococcus pneumoniae* ATTC 49619	0.16
	Klebsiella pneumoniae ATTC 700603	0.08
Eucalyptus globulus leaf extract	*Streptococcus pneumoniae* ATTC 49619	1.25
	Klebsiella pneumoniae ATTC 700603	2.5
Ciprofloxacin (standard)	*Staphylococcus aureus* ATTC 25923	0.04
	Pseudomonas aeruginosa ATTC 10231	0.04
	Streptococcus pneumoniae ATTC 49619	0.04
	Klebsiella pneumoniae ATTC 700603	0.04

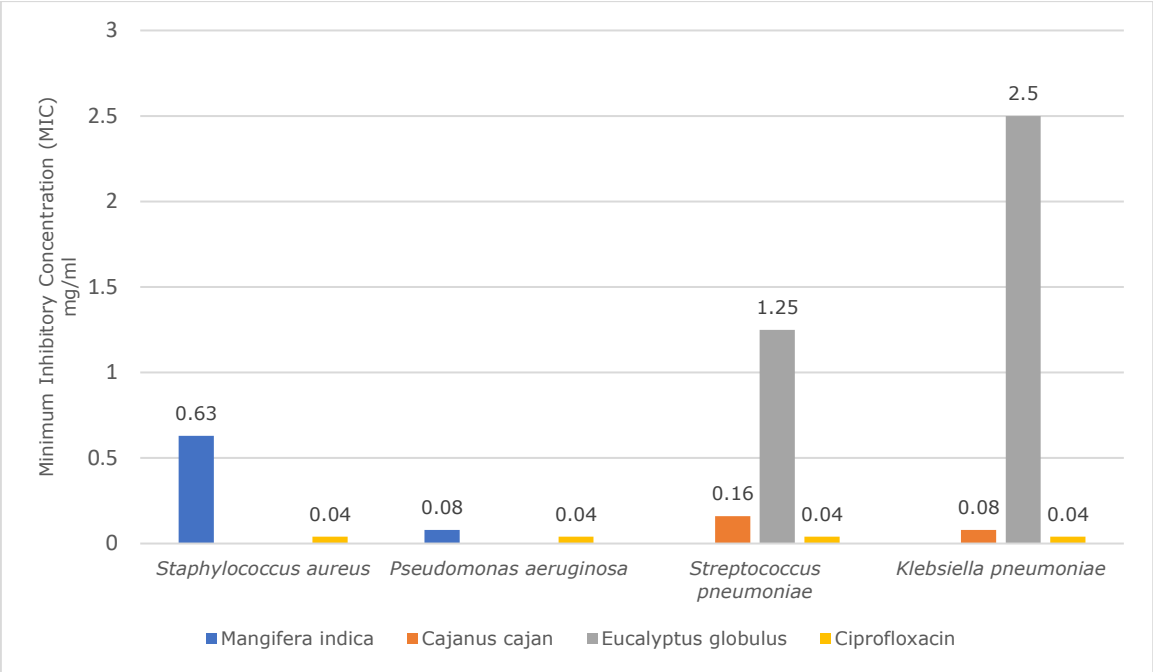

Fig 1: Antibacterial activity (minimum inhibitory concentration) of nutraceutical plant extracts against the four bacterial strains

of *M. indica* stem bark extract agrees with the findings of Bbosa et al., (44), who reported antimicrobial effect of ethanolic extract of *M. indica* against *S. aureus. E. coli* and *P. aeruginosa* with MIC range of 5.48 to 43.75 mg/ml. Mushore and Matuvhunye (45) also reported that stem bark extract of *M. indica* had antimicrobial activity against *S. aureus* using broth dilution MIC method of 0.16-1.25 mg/ml. *Mangifera indica* stem bark extract inhibits the growth of microorganisms with varying degrees of susceptibility depending on the bacterium and extract, with MIC values ranging from 6.25-50 mg/ml (46).

The antimicrobial activity in our study supports the claim by local communities for use of *M. indica* stem bark decoctions for treating opportunistic infections such as diarrhoea and gastrointestinal infections. Aqueous and methanolic extracts of *M. indica* stem bark possess antimicrobial and anti-diarrhoeic properties with methanolic extract MIC being 256 µg/ml for both Gram-positive and Gram-negative bacteria (43). The study of Osei-Djarbeng et al., (47) showed that bark and leaf extracts of *M. indica* has antimicrobial activity. *Pseudomonas aeruginosa* and *S. aureus* showed susceptibility to inhibitory activity of *M. indica* bark extract (48). Sanusi et al., (46) reported that the antimicrobial activity obtained in their study indicated presence of bioactive compounds and support the claim by the local communities for the use of *M. indica* stem bark decoction for treatment of infections such as diarrhoea. *Mangifera indica* bark extract showed significant activity against four clinical strains of *S. typhi, B. subtilis, E. coli* and *K. pneumoniae*, where all solvent extracts at dose range 2-4 mg/ml showed significant antibacterial activity (49). Phytochemical screening of crude stem bark extracts of *M. indica* revealed the presence of tannins, saponins, alkaloids, flavonoids, cardiac glycosides and phytosterols (46). This could be said to be responsible for the efficacy of *M. indica* bark studied in the treatment of different diseases.

Our findings on antibacterial activity of *C. cajan* leaf extract are in agreement with previously published research by Pratima and Mathad (50), who reported that extract of *C. cajan* inhibited growth of both Gram-positive and Gram-negative bacteria (*S. aureus, S. pneumoniae, K. pneumoniae*). *Cajanus cajan* showed antibacterial activities against *Streptococcus mutans* (51). The extracts of *C. cajan* showed potential activity against *S. pneumoniae*, *S. aureus*, and *P. aeruginosa* (52) and is capable of preventing and treating bronchitis, cough, pneumonia and respiratory infections (53). Oke (54) reported *C. cajan* leaves to contain alkaloids, flavonoids, tannins, saponins, terpenes, phlobatannins, anthraquinones and sterols. Mohanty et al., (55) found steroids, phenolic compounds, saponins, glycosides, flavonoids in *C. cajan* and this could be said to be responsible for the efficacy of *C. cajan* leaves studied in the treatment of different diseases.

The results obtained on antibacterial activity of *E. globulus* leaf extract are in agreement with those obtained by Mulyaningsih et al., (56), who reported that *Eucalyptus globulus* exerted promising antibacterial activity against methicilin resistant *S. aureus* (MIC 250 µg/ml), and Cermelli et al., (57) who observed that *S. pneumoniae* was susceptible

to antibacterial activity of *E. globulus*. Bachir and Benali (58) reported that essential oil in leaves of *E. globulus* has antimicrobial activity against Gram-negative and Gram-positive bacteria. Alvarenga et al., (59) also reported that 32 air-bone anti-tuberculosis components were identified in *Eucalyptus citriodora*. Eucalyptus essential oil was effective against *Staphylococcus* and *Streptococcus* (60). Phytochemical analysis of leaf extract of *E. globulus* proved the presence of tannins, saponins, terpenoids, glycosides, alkaloids, phenolic compounds, cardiac glycosides, terpenes, reducing sugars, carbohydrates, flavonoids (18) and this could be said to be responsible for the efficacy of *E. globulus* studied in the treatment of different diseases.

Conclusion:

The results of the present study support the traditional use of the studied nutraceutical plants for the management of opportunistic infections associated with HIV/AIDS. We confirmed the ability of selected plant extracts to inhibit bacterial growth. The antimicrobial activity obtained in this study indicated presence of bioactive compounds and support the claim by the local communities for the use of plant parts decoction for treatment of opportunistic infections such as diarrhoea, tuberculosis, oral candidiasis, pneumonia. Our results provide useful baseline information for the potential use of the studied nutraceutical plants in the fight against ARV drug resistant bacteria and the possibility of developing plant-based drugs to help in long term management of opportunistic infections associated with HIV/AIDS.

A high degree of medical pluralism has been observed among local community, therefore communication and collaboration between biomedical clinicians and traditional medicine practitioners should be encouraged. We recommend that the Ministry of Health in Uganda should coordinate this collaboration. Bakibinga (61) reported that contemporary Intellectual Property Law permits only the patenting of an identified "active principle" from a plant, and not the plant or folk information relating to medicinal properties of a plant, according to Copyright and Intellectual Property Rights (CIPR). Therefore, we recommend that the issues of traditional medicine practitioners should be fully exploited with the help of Gulu University and the Registrar of Patents office in Uganda.

Acknowledgements:

The authors appreciate the funding support from the Regional Universities Forum (RUFORUM) and Gulu University for the facilities and technical support.

Contributions of authors:

NI was involved in study conceptualization, funding acquisition, methodology, investigation, data curation, validation, formal analysis, writing original draft and editing, and visualization; AL, ATM and EN were involved in reviewing and editing of manuscript and supervision of the project. All authors read and approved the final manuscript.

Source of funding:

The Regional Universities Forum (RUFORUM) provided financial support for the conduct of the research. The funding source had no involvement in the study design, collection, analysis and interpretation of data, writing report and decision to submit manuscript for publication.

Conflict of interests:

Authors declare no conflict of interest

Availability of data and materials:

The datasets during and /or analysed during the current study are available from the corresponding author on reasonable request.

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Antwi. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (1): 112 - 119 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Apr 2024; Vol.25, No.1
AJCEM/2227. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Antagonistic activity of secondary metabolites from rhizofunctional bacteria extracts against *Fusarium* species

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Abstract:

Background: *Fusarium* species remain important fugal pathogens that produce several mycotoxins with adverse effects on both plant and animals. This work aimed to identify biocontrol agent from rhizofunctional bacteria and assess its antagonistic activity against *Fusarium* sp. using dual culture technique.
Methodology: Briefly a circular disc of the *Fusarium* sp. was inoculated at the center of Potato Dextrose Agar (PDA) plate and incubated for three days. The bacterial isolates were then inoculated about 2cm from the *Fusarium* hyphal tips and incubated for three days, and zone of inhibition was examined. Isolates that showed antagonistic activities against the fungi were subculture in nutrient broth for three days and the metabolites were extracted using ethyl acetate. The metabolic extracts were tested against the fungi using the agar disc diffusion method.
Results: Of the 20 rhizofunctional bacterial isolates screened for antagonistic activities against *Fusarium* sp., 5 showed active antagonism against the fungi with observed clear zone of inhibition in the dual culture, and microscopic examination of the fungal hyphae showed excessive and diffused hyphal branching with hyphal swelling. Ethyl acetate extracts from nutrient broth cultures did not show any zone of inhibition in dual culture against the *Fusarium* sp. All the 5 bacterial isolates were Gram positive strains but only 2 isolates (2a and 3K) were lipase positive, which may indicate that the mechanisms of antagonism could be due to the production of enzymes that have the ability to hydrolyze the cell wall and membrane lipids of the fungi.
Conclusion: The rhizoplane and rhizosphere of plants could be great sources of biocontrol agents and that bacterial isolates 2a and 3K have the potential to be used as antifungal agents against *Fusarium* sp. Molecular identification of 2a and 3K bacterial isolates to the species level is recommended.

Keywords: antagonistic; secondary metabolites; rhizofunctional; bacteria; *Fusarium*

Received Oct 6, 2022; Revised Dec 15, 2022; Accepted Oct 19, 2023

Activité antagoniste des métabolites secondaires d'extraits de bactéries rhizofonctionnelles contre les espèces de *Fusarium*

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Résumé:

Contexte: Les espèces de Fusarium demeurent d'importants agents pathogènes fongiques qui produisent plusieurs mycotoxines ayant des effets néfastes sur les plantes et les animaux. Ce travail visait à identifier un agent de lutte biologique à partir de bactéries rhizofonctionnelles et à évaluer son activité antagoniste contre *Fusarium* sp. en utilisant la technique de double culture.
Méthodologie: Brièvement un disque circulaire de *Fusarium* sp. a été inoculé au centre d'une plaque de Potato Dextrose Agar (PDA) et incubé pendant trois jours. Les isolats bactériens ont ensuite été inoculés à environ 2 cm des extrémités des hyphes de Fusarium et incubés pendant trois jours, et la zone d'inhibition a été examinée. Les isolats qui ont montré des activités antagonistes contre les champignons ont été sous-cultivés dans un bouillon nutritif pendant trois jours et les métabolites ont été extraits à l'aide d'acétate d'éthyle. Les extraits métaboliques ont été testés contre les champignons en utilisant la méthode de diffusion sur disque d'agar.
Résultats: Sur les 20 isolats bactériens rhizofonctionnels criblés pour les activités antagonistes contre Fusarium sp., 5 ont montré un antagonisme actif contre les champignons avec une zone claire d'inhibition observée dans la double culture, et l'examen microscopique des hyphes fongiques a montré une ramification excessive et diffuse des hyphes avec des hyphes gonflement. Les extraits à l'acétate d'éthyle des cultures en bouillon nutritif n'ont

montré aucune zone d'inhibition en double culture contre Fusarium sp. Tous les 5 isolats bactériens étaient des souches Gram positives mais seulement 2 isolats (2a et 3K) étaient positifs pour la lipase, ce qui peut indiquer que les mécanismes d'antagonisme pourraient être dus à la production d'enzymes qui ont la capacité d'hydrolyser la paroi cellulaire et les lipides membranaires des champignons.
Conclusion: Le rhizoplan et la rhizosphère des plantes pourraient être d'excellentes sources d'agents de lutte biologique et les isolats bactériens 2a et 3K ont le potentiel d'être utilisés comme agents antifongiques contre *Fusarium* sp. L'identification moléculaire des isolats bactériens 2a et 3K au niveau de l'espèce est recommandée.

Mots clés: antagoniste; métabolites secondaires; rhizofonctionnel; bactéries; *Fusarium*

Introduction:

Fusarium is a large genus of hyaline filamentous molds best known as the most important group of mycotoxigenic plant pathogens. This group of fungi are able to produce different toxins such as deoxynivalenol, nivalenol, T2, zearelenone, fusaric and moniliformin with adverse effects on both plants and animal (1). They have also emerged over the past three decades as opportunistic pathogens of immunocompromised hosts. Infections in healthy individuals typically remain localized and include keratitis, especially in association with ocular trauma, onychomycosis of the toenails or fingernails, allergic sinusitis, paronychia, and dermatomycoses. When crops such as wheat, barley, oats, rice and maize are infected with *Fusarium*, it leads to yield loss through low growth rate, reduction of grain size and weakening of the straw.

In the United States of America, *Fusarium* outbreak in the 1990's resulted in losses in the region up to $3 billion (2). The fungus produces a mycotoxin known as deoxynivalenol that poses a significant threat to domestic animals and humans (3). The strain that specifically attacks banana is called *Fusarium oxysporum* f. sp. *cubense*. Apart from field infections, *F. culmorum* is also known to cause storage rot of sugar beet, potatoes and apples (4). Parasitism mediated by degradation of cell wall of pathogenic fungi relies on extracellular lytic enzymes. Several *Bacillus* species produce enzymes that degrade chitin, an insoluble linear polymer of 1,4-N-acetylglucosamine, which is a major component of most fungal cell wall. Among these species, *B. circulans* (5), *B. licheniformis* (6), *B. cereus* (7) and *B. thuringiensis* have been implicated as potential biocontrol agents. These species are reported to secrete chitinases and the role of these chitinolytic enzymes in the biocontrol of fungal pathogens has been elucidated in experiments involving bacterial and fungal antagonists (8, 9).

Several basic mechanisms of the bacterial-induced biocontrol of plant pathogenic fungi have been described, particularly concerning the *Pseudomonas* genus (7); antibiosis, fungistasis, competition for nutriments, modification of the biophysical root environment, active exclusion of pathogenic fungi from the rhizosphere, detoxification of pathogen virulence factors and induction of plant disease resistance. In recent years, it has become apparent that bacteria coordinate their interactions and associations with higher organisms by using intercellular communication systems that rely on small diffusible molecules in a process known as quorum sensing.

Screening microbial secondary metabolites is an established method to identify novel biologically active molecules. Microbial extracts have been and continue to be productive sources of new biologically active molecules for drug discovery. It is well known that production of secondary metabolites by microorganism is influenced by fermentation conditions. In a study of 29 *Nodulisporium* strains, Li et al., (10) reported that synthesis of secondary metabolites was directly influenced by fermentation conditions and that there were differences of up to 400-fold in the concentrations of secondary metabolites between conditions. In addition, rare metabolites were consistently reported in extracts containing larger numbers of secondary metabolites (10). There are a number of previously developed methods which use direct chemical measurement to classify microorganisms but most previous studies focused on characterizing microorganisms by detecting the presence of known secondary metabolites. Frisvad et al., (11) used high-performance liquid chromatography (HP LC) diode array detection and flow injection analysis together with Electrospray Ionization Mass Spectrometry (ES-MS) to detect secondary metabolites characteristic of fungal strains responsible for spoilage of stored cereals. There has also been much success in obtaining biological control of plant pathogens using bacterization techniques. Bacteria used as inoculants are mostly *Pseudomonas fluorescens-putida* types obtained from soils and plant surfaces (12).

Investigations on microbial metabolites is gaining greater momentum in the agrochemical industry as a source for the development of new pesticide products. Several such products have been developed and used as bactericide, fungicide, acaricide or insecticide in agriculture. *Fusarium* species cause a lot of diseases in a lot of plant and crop thereby reducing the yield and quality of crop products. Hence an effective biocontrol approach such as the use of bacteria that are antagonistic to the growth of the fungi could be adopted to prevent or control the devastating effect of the fungi on crops. The main purpose of this

research is to identify rhizo-functional bacteria that possess antagonistic activity against *Fusarium* sp. and to test extracts of secondary metabolites from potent bacterial isolates against *Fusarium* sp.

Materials and method:

Sources of bacterial and *Fusarium* isolates:

Pure culture of *Fusarium* sp. was obtained from the Department of Molecular Biology and Biotechnology, University of Cape Coast, Ghana. Twenty rhizofunctional bacterial previously isolated from the rhizosphere of *Carica papaya* were obtained from the same department.

Sub-culturing of bacteria isolates and storage:

The 20 rhizo-functional bacterial previously isolated from the rhizosphere of *Carica papaya* and stored in 10% glycerol at 40°C were allowed to thaw at room temperature and vortexed for 10 sec. The streak plate method using Potato Dextrose Agar (PDA) medium was used to subculture the bacterial isolates under aseptic conditions. The inoculated plate was incubated at 28°C for 18-24 hours. The purified isolates were then transferred into 1.5ml Eppendorf tubes containing 10% glycerol which were then stored in the refrigerator at –40°C.

Sub-culture of *Fusarium* sp.

A 5-mm diameter sterile cork-borer was used to create several agar discs at the tips of the young hyphae of *Fusarium* growth on PDA medium. A sterile inoculation needle was then used to transfer the agar discs containing the *Fusarium* onto the surface of fresh PDA agar and incubated in the dark at room temperature for 3-7 days.

Determination of antagonistic activity of bacterial isolates against *Fusarium* sp.

A 5-mm mycelial disc from a 7-day old *Fusarium* culture was obtained using a sterile cork-borer was transferred onto the surface of a sterile PDA plate. The culture was then incubated at room temperature for 2 days. A single colony of pure bacterial cultures from the streak plate was picked with sterile inoculation loop and transferred onto the PDA plate containing the 2-day old pure culture of *Fusarium* sp.

Five bacterial isolates were inoculated 2cm away from the growing *Fusarium* sp. and about 2cm apart between them. A control experiment was set up without any bacteria growing concurrently with the *Fusarium* culture. Bacterial isolates able to inhibit the growth of the fungus were considered as having an antagonistic activity and were therefore selected for further screening. Such bacterial isolates were further subjected to a second screening to determine the most potent antagonist using the same approach described. The zone of inhibition between the fungus and the bacteria was measured with a ruler and the bacterial isolate that produced the widest induced zone of inhibition was selected for metabolite extraction in nutrient broth.

Extraction of crude metabolites using ethyl acetate:

Inoculum from the most potent antagonistic bacterial isolate was aseptically transferred into a conical flask containing 100ml of sterile nutrient broth and incubated on an orbital shaker at 120 rpm at 28°C for 7 days. After the incubation period, the culture was centrifuged at 10,000 rpm for 15 minutes to separate the supernatant from the bacterial cells (pellets). The crude metabolites were then extracted from the supernatant by partitioning with equal volume of ethyl acetate. Various concentrations of the crude metabolite extract were then prepared; 0.1%, 1%, 2%, 3%, 5%, and 100% and their antifungal potential was tested against a three-day old *Fusarium* culture using the disc infusion method.

Briefly sterile paper disc was impregnated with 0.5ml of the prepared concentrations of the metabolite extract and placed on an agar plate containing a 3-day old *Fusarium* culture such that each disc was 2cm away from the fungi. The culture was incubated at 28°C for 3 days. Observations were made after every 24-hour period. A negative control was set-up using filter paper disc impregnated with ethyl acetate only.

Biochemical and morphological characteristics of bacterial isolates:

The bacterial isolates exhibiting antagonistic activity were biochemically characterized using amylase, lipase, catalase and hydrogen cyanide production tests and morphologically using Gram stain and motility test.

Microscopic examination of fungi growth:

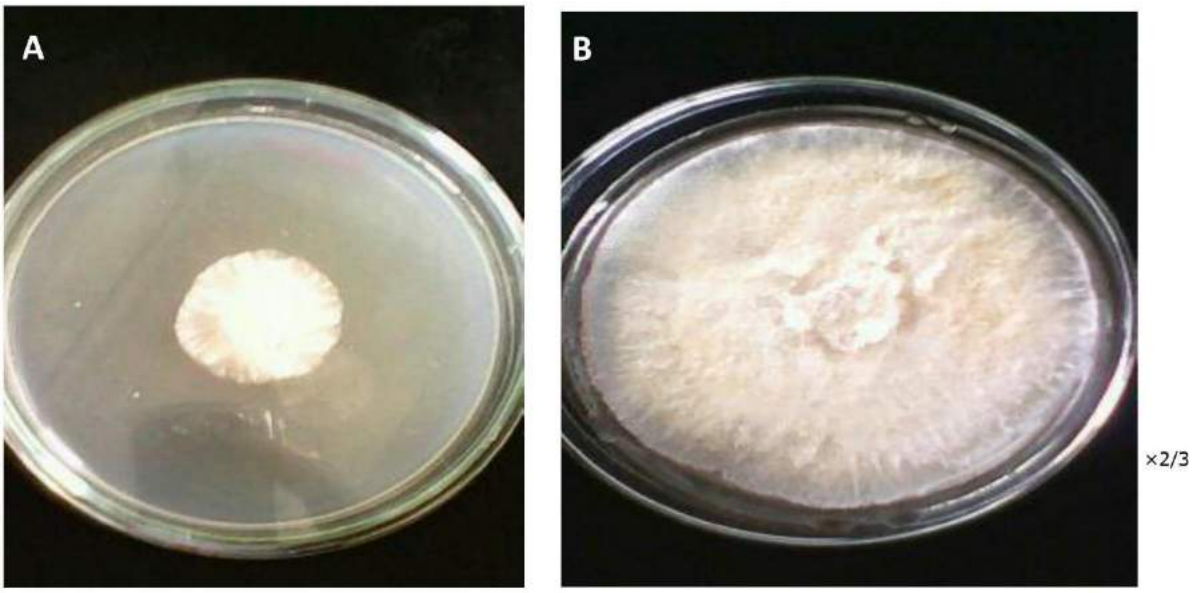
The effect of the antagonist bacterial isolates on hyphal and mycelial growth was examined using a compound light microscope.

Results:

Of the 20 bacterial isolates screened, 5 showed antagonistic activity against *Fusarium* species. The zones of inhibition varied from one bacterial isolate to the other with the bacterial isolate designated HSG2 having the highest inhibition zone (0.92 mm), followed by isolates 3k, 1a, and 2a. The bacterial isolate 4f recorded the least zone of inhibition (0.61mm) (Table 1). Pure cultures of *Fusarium* sp used for antagonistic screening are shown on Plate 1, with photographs of the first and second antagonistic screening on Plate 2.

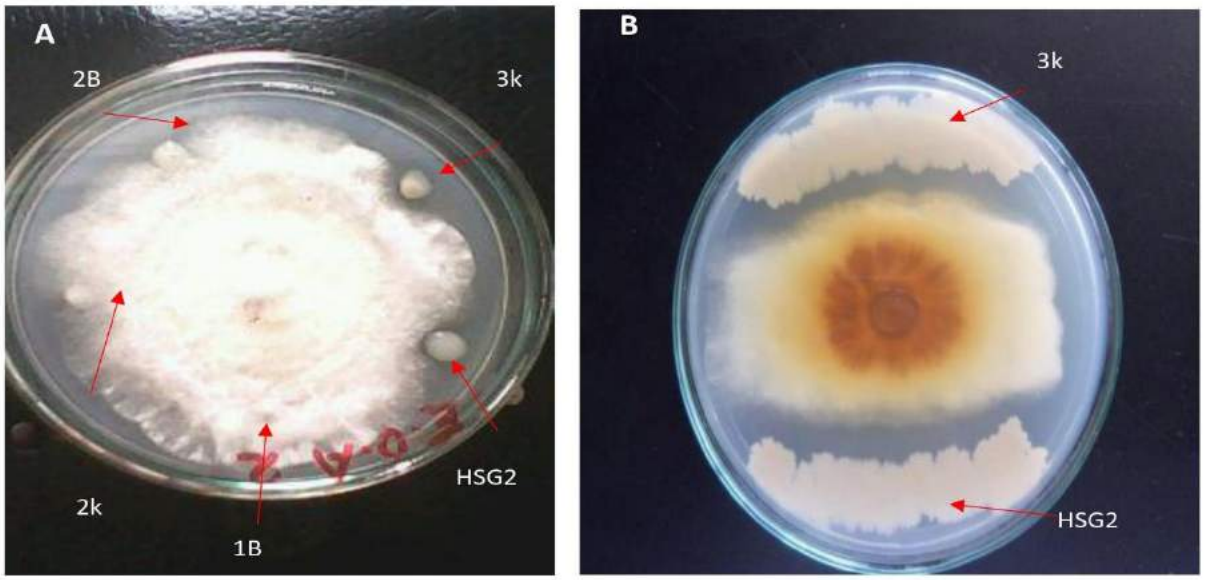
Table 1: Zone of inhibition (mm) induced by five antagonistic bacterial isolates against *Fusarium* species

Bacterial isolate	Zone of inhibition (mm)
2a	0.64
4f	0.61
1a	0.70
3k	0.71
HSG2	0.92



A: 3day old culture; B: 7day old culture

Plate 1: Pure cultures of *Fusarium* species on potato dextrose agar in different incubation periods at 28°C



A: First screening Fusarium sp + bacterial isolates (2b, 3k, HSG2, 1b and 2k); B: Second screening (*Fusarium* sp + bacterial isolates HSG2 and 3k)

Plate 2: Screening for antagonism showing mixed cultures of *Fusarium* species concurrently on potato dextrose agar after 7 days incubation at 28 °C

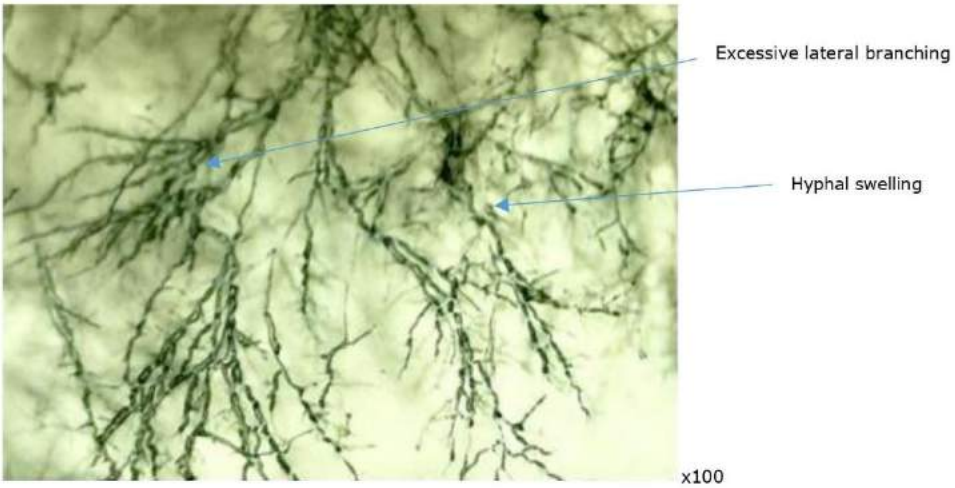
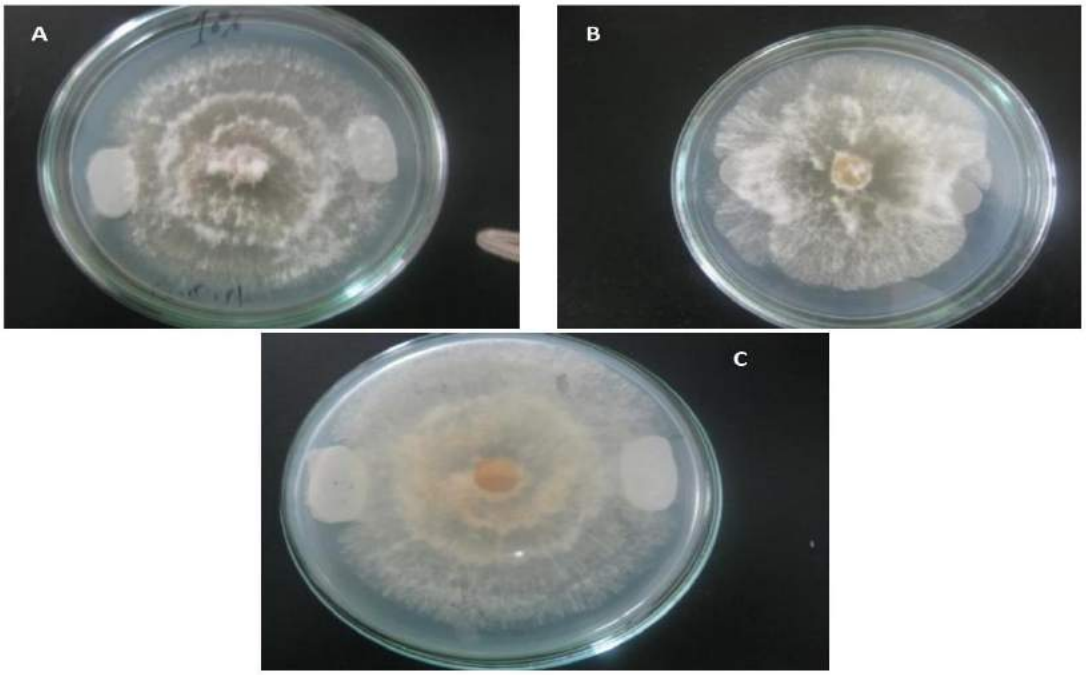


Fig 1: Morphological changes in *Fusarium* hyphae in response to antagonistic bacterial isolates

Microscopic examination of fungi growth: Both the portion of *Fusarium* mycelium close to the zone of inhibition and that far from the zone of inhibition were observed under a light microscope. Morphophysiological changes including excessive lateral branching and hyphal swellings were observed in *Fusarium* hyphae in response to the antagonistic activity of the bacterial isolates (Fig 1).

Antifungal property of crude metabolite extract:

No zone of inhibition was recorded when the filter paper discs impregnated with the various concentrations of ethyl acetate crude metabolite extracts were placed on the Potato Dextrose Agar plate containing the *Fusarium* culture (Plate 3).



(A) 1% crude metabolites extract + *Fusarium* culture; (B) 100% crude metabolites extract + *Fusarium* culture (C) Ethyl acetate + *Fusarium* culture (control)

Plate 3: Results after 7-day old culture of *Fusarium* sp from the action of the filter paper discs impregnated with 1% and 100% crude metabolite extracts

Table 2: Biochemical and morphological characteristics of the five rhizobacterial isolates

Bacterial isolate	Biochemical characteristics				Morphological characteristics	
	Amy	**Lip**	**Cat**	**Hcn**	**Mot**	**Gram stain**
2a	-	+	+	X	+	Gram +ve rod
4f	-	-	+	X	+	Gram +ve rod
1a	-	-	+	X	-	Gram +ve rod
3k	-	+	+	-	+	Gram +ve rod
HSG2	-	-	+	x	+	Gram +ve rod

Amy=Amylase, Lip=Lipase, Cat=Catalase, Hcn=Hydrogen cyanide, +ve=Positive, -=negative, +=positive, x=not performed, Mot=Motility

Biochemical and physiological characteristics of bacterial isolates:

Data obtained from biochemical and physiological tests are presented in Table 2. All the 5 bacterial isolates were amylase negative and 2 of the isolates were lipase positive and while the other 3 were lipase negative. Furthermore, all 5 isolates were catalase positive. The hydrogen cyanide test was performed only on one isolate (3k) and this isolate gave a negative reaction. The Gram staining revealed that all 5 isolates were Gram-positive rods. The motility test however revealed that 4 out of the 5 isolates were motile and only one was non-motile.

Discussion:

This research aimed to identify biofungicidal agents against phytopathogenic *Fusarium* sp. All the five most bacterial antagonists were Gram-positive rod shaped and catalase positive. This is because these isolates produced the enzyme catalase that is able to hydrolyse hydrogen peroxide into water and oxygen leading to the evolution of white bubbles. This helps the bacteria to survive under hydrogen peroxide polluted environment. The lipase positive bacteria, 2a and 3k, produce the enzyme lipase that act on lipid and hydrolyse it into glycerol and fatty acids. This property would enable the bacteria to act directly on the phospholipid bi-layer of the cell membranes or the cell wall of other microorganisms causing disintegration in their cell wall. This therefore enables the bacteria to antagonize the growth of other competitors in its environment. The bacterial isolate 3k profoundly inhibited radial growth of the hyphae and also induced morpho physiological changes including excessive lateral branching, hyphal swellings and cytoplasmic extrusion at the tips of the hyphae as revealed by the light microscope.

According to Asante et al., (13), abnormal nuclear divisions occur at the sub-epical region of the hyphal tip before excessive lateral branching occurs, and according to Semighini and Harris (14), hyphal branching in fungi is due to mitotic cell division. On the other hand, the affected hyphal tip extrusion was most likely to be cytoplasmic substances that became extruded through the cell wall of the hyphae. This phenomenon likely reflects the exclusive targeting of exocystic vesicle laden with components required for cell surface expansion and cell wall deposition to the hyphal tip at the expense of potential branching site (3,14). This may also be due in part to the production of the enzyme lipase by the bacterial isolate 3k, which might have affected the transport of cytoplasmic substances responsible for the growth and extension of the fungal hyphae across the cell wall to the tips leading to the accumulation of cytoplasmic substances resulting in the observed hyphal swellings. This therefore supports the finding that in fungi, branching forms a central development of mycelial colony and also appears to play a major role in fungal interaction with other organisms and that there are two partings of hyphal branching: apical and lateral branching (15).

Schmid et al (3) affirmed that in fungi, branching is dominant at the apical tip and hence turn to suppress the formation of lateral branching. Therefore, the inhibition of apical branching induced by the antagonistic bacteria might have accounted for the observed excessive lateral branching in the *Fusarium* sp. in the dual culture assay. This would reduce the ability of the fungi to colonize the environment, which in turn would reduce its ability to effectively utilize the nutrients in its vicinity. This is because the hyphal tip is involved in the production of enzymes necessary for nutrient break down and assimilation as well as hyphal growth and extension. On the contrary, bioefficacy studies on the various concentrations of the crude metabolites against the *Fusarium* sp. in a dual culture did not produce any antagonistic activity. This might have been due to insufficient dissolution of the metabolites by the extraction solvent, ethyl acetate hence, the optimum inhibitory concentration was not attained.

The bacterial isolate (3k) probably occupies the rhizoplane of the plant and likely plays a role in host defense using other mechanism(s) such as hydrogen cyanide production or ion depletion mechanism either than its secondary metabolites. Among the alternative means of assimilating iron are surface reduction to the more soluble ferrous species, lowe-

ring the pH, utilization of heme, or extraction of protein-complex metal. Thus, bacterial isolate 3k might have produced small molecules called siderophores that are high affinity iron chelators or undergone iron homeostasis to capture and to use different forms of bond iron by using molecules containing iron, such as lactoferrin, ferritin or heme. Heme utilizing bacteria produce the enzyme called heme oxygenase that oxidatively cleaves the heme molecules to form intracellular ferrous iron called biliverdin and carbon mono-oxide resulting in protoporphyrin ring degradation (15). Mathieu et al., (16) again found that Gram-positive bacteria exhibit functional redundancy in iron transporter mechanism, that is, siderophore-mediated iron uptake, heme uptake and /or ferrous iron uptake, (15). Therefore, the test bacterial isolate 3k being Gram-positive may have the ability to use this mechanism to antagonize the growth of the *Fusarium* sp. by utilizing and making the iron unavailable to the fungus.

This alternative possibility for the anta gonistic relationship between the *Fusarium* sp. and the bacteria is supported by the fact that, the role of iron in the virulence mechanism of some pathogenic organisms attacking crops and animals is well established. For example, the siderophore system of *Yersinia enterocolitica* is correlated with the virulence of the organism (16). In addition, the potency of common antibiotics has been elevated by building into the molecules the iron-binding functional groups of siderophores. The objective here is to take advantage of the high affinity, siderophore-mediated iron uptake system of the bacteria. Also in agriculture, the ability of some bacteria to produce chemicals such as pseudobactin or pyoverdine type siderophores has been applied to improved plant growth either through a direct effect on the plant or through control of noxious organisms in the soil. This may also be due in part to the production of the enzyme, lipase by the bacterial isolate 3k which might have affected the transport of cytoplasmic substances responsible for the growth and extension of the fungal hyphae across the cell wall to the tips leading to the accumulation of cytoplasmic substances resulting in the observed hyphal swellings. This therefore supports the finding that in fungi, branching forms a central development of mycelial colony and also appears to play a major role in fungal interaction with other organisms and that there are two partings of hyphal branching; apical and lateral branching (15).

The finding of this study suggests that, the rhizosphere and the rhizoplane is inhabited by bacteria species that could be utilized as biocontrol or bio-fungicidal agents against *Fusarium* sp. This study therefore recommends that the potent bacterial isolate be tested on other pathogenic fungi to determine the spectrum of action. Further biochemical analysis should be conducted to identify the isolate to the species level and elucidate their mechanism of inhibition including iron utilization. In addition, different extraction solvents should be tested for their efficacy to ensure efficient extraction of the metabolites.

Contributions of author:

The author performed the bench work and writing of the manuscript.

Source of funding:

Author received no external funding

Conflict of interest:

No conflict of interest is declared.

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ISSN 1595-689X

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY (AJCEM) ISSN 1595-689X

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ISSN 1595-689X

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