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Review Article **Open Access**

Microbiomes and immune checkpoint mechanisms in cancer progression: A structured narrative review

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Abstract:

The role of the microbiome in modulating immune responses has garnered significant attention in cancer research, particularly in the context of immune checkpoint mechanisms. The microbiome influences the tumor micro-environment (TME) and the efficacy of immune checkpoint inhibitors (ICIs) by shaping immune cell function and responses. Immune checkpoints, such as PD-1, PD-L1, LAG-3, and CTLA-4, are critical in regulating immune tolerance and preventing excessive immune activation. However, in cancer, these checkpoints often suppress anti-tumor immunity, contributing to tumor immune evasion and therapeutic resistance. Emerging evidence suggests that the microbiome plays a pivotal role in regulating these checkpoints, enhancing or inhibiting the effectiveness of ICIs. Studies have shown that a balanced microbiome can promote a more favorable immune response, while dysbiosis may lead to increased inflammation and tumor progression. Additionally, the combination of microbiome modulation through probiotics, prebiotics, or dietary interventions, with immune checkpoint blockade has shown potential in overcoming resistance mechanisms and improving treatment outcomes. This comprehensive review explores the complex relationship between the microbiome and immune checkpoint mechanisms, highlighting how microbiome composition impacts immune checkpoint expression and function in cancer. It also discusses the therapeutic potential of microbiome-based interventions in conjunction with ICI therapies, emphasizing the need for further research to fully understand these interactions. Integrating microbiome science with cancer immunotherapy holds the promise of more effective, personalized treatment strategies that could improve patient outcomes and survival rates across various cancer types.

Keywords: Microbiome, immune checkpoint inhibitors, cancer progression, tumor microenvironment.

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Microbiomes et mécanismes des points de contrôle immunitaires dans la progression du cancer: une revue narrative structurée

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Résumé:

Le rôle du microbiome dans la modulation des réponses immunitaires a suscité une attention particulière dans la recherche sur le cancer, notamment dans le contexte des mécanismes des points de contrôle immunitaires. Le microbiome influence le microenvironnement tumoral (TME) et l'efficacité des inhibiteurs des points de contrôle immunitaires (ICI) en modulant la fonction et les réponses des cellules immunitaires. Les points de contrôle immunitaires, tels que PD-1, PD-L1, LAG-3 et CTLA-4, sont essentiels à la régulation de la tolérance immunitaire et à la prévention d'une activation immunitaire excessive. Cependant, dans le cancer, ces points de contrôle inhibent souvent l'immunité antitumorale, contribuant ainsi à l'évasion immunitaire tumorale et à la résistance thérapeutique. De nouvelles données suggèrent que le microbiome joue un rôle essentiel dans la régulation de ces points de contrôle, renforçant ou inhibant l'efficacité des ICI. Des études ont montré qu'un microbiome équilibré peut favoriser une réponse immunitaire plus favorable, tandis qu'une dysbiose peut entraîner une inflammation accrue et une progression tumorale. De plus, l'association de la modulation du microbiome par des probiotiques, des prébiotiques ou des interventions alimentaires, avec le blocage des points de contrôle immunitaire, a montré un potentiel pour surmonter les mécanismes de résistance et améliorer les résultats

thérapeutiques. Cette revue exhaustive explore la relation complexe entre le microbiome et les mécanismes des points de contrôle immunitaires, soulignant l'impact de la composition du microbiome sur l'expression et la fonction des points de contrôle immunitaires dans le cancer. Elle aborde également le potentiel thérapeutique des interventions basées sur le microbiome en association avec les thérapies par ICI, soulignant la nécessité de poursuivre les recherches pour comprendre pleinement ces interactions. L'intégration de la science du microbiome à l'immunothérapie anticancéreuse est prometteuse pour des stratégies thérapeutiques plus efficaces et personnalisées, susceptibles d'améliorer les résultats des patients et les taux de survie pour différents types de cancer.

Mots clés: Microbiome, inhibiteurs de points de contrôle immunitaires, progression du cancer, micro-environnement tumoral.

Introduction:

The concept of microbial communities and their influence on human health dates back to the 19th and 20th centuries. However, the word microbiome was first used by Joshua Lederberg in the early 2000s to describe a vast, complex community of commensal, symbiotic, and microorganisms that comprised of bacteria, viruses, fungi, archaea, and protozoa found in various ecosystems including living and non-living environments. Using their genetic materials, they interact with the essential component of life, influencing human health, environmental stability, and disease processes (1).

Microbiomes significantly influence the immune checkpoint pathways, thereby modulating immune regulation, disease progression, and responses to immunotherapy (2). The immune system itself is a complex natural defense network of cells, tissues, and organs whose mechanical synergy is useful in identifying and neutralizing infectious pathogens and harmful substances, including cancerous cells (3). Program cell death one (PCD-1), cytotoxic T-lymphocyte associated protein-4 (CTLA-4), lymphocyte activation gene-3 (LAG-3), and T-cell immunoglobulin and mucin domain containing-3 (TIM-3) are examples of immune-checkpoint pathways that function as on-and-off switches**,** inhibiting and switching off excessive immune activation and, in another way, switching on the activity of T-cells by promoting strong and sustainable activation against infections and development of cancer (4).

The inhibition of excessive immune responses is a preventive measure against autoimmunity, hypersensitivities, and graft rejection. While activation of T-cells is to eradicate infectious disease agents and cancer cells, dysregulation of immune checkpoint pathways whether through overactivation (allowing immune evasion in cancer) or suppression (leading to autoimmunity) plays a critical role in the development and progression of various chronic and life-threatening diseases (5). Factors contributing to immune checkpoint failure include; checkpoint ligand upregulation, where tumor cell overexpresses PD-L1 and inhibits T-cell activity. In another way, tumor cell can activate TIM-3, LAG-3, and V-domain Ig suppressor of T cell activation (VISTA), causing immune suppression and evasion. Furthermore, loss of major histocompatibility class I (MHC I) expression by tumor cell due to defective antigen presentation can prevent T-cell recognition and dampens immune activation. Mutation in immune-related genes can also lead to immune activation.

Microbiome dysbiosis is the hallmark of immune alteration. Certain microorganisms and their metabolites have the ability to inhibit or activate the immune checkpoint (6). Bacteria such as *Akkermansia muciniphila, Bifidobacterium,* and *Faecalibacterium* are implicated in T-cell activation and anti-tumor promotion. They also produce short-chain fatty acid (SCFAs) like butyrate and propionate that regulate effector T-cells balancing and immune tolerance activation. Some bacteria are also specifically involved in immune suppression of myeloid-derived suppression cells (MDSCs) and tumor-associated macrophages (TAMs) mounting immune surveillance response against cancer cells (7).

The exact molecular signaling pathway used by microbes to enhance anti-cancer immune checkpoint remains unclear. Microbial metabolites that influence T-cell activation and immune checkpoint specific impact on tumor microenvironments are understudied. Most research focuses on the gut microbiome, but the role of lung, skin and tumor microbiome in modulating immune checkpoint is underexplored and requires further research. Host factors such as sex, diet, genetic and pre-existing immune conditions influences microbiome-driven immune checkpoint and a standardized model to study immune interaction in different patient population is a major concern in clinical practice. This review therefore seeks to address the anti-tumoral roles of microbiomes and immune checkpoint, with the aim of providing the innovative prospects of immune checkpoint for anti-cancer immunity.

Methodology:

This review adopts a structured narrative review/evidence-based approach aimed at synthesizing current insights into the intricate relationship between microbiomes, immune checkpoint mechanisms, and cancer progression. An extensive literature search was cond-

ucted using peer-reviewed journal articles, expert reports, book chapters, and conference proceedings published over the last 30 (1995-2025) years.

The selection of literature was guided by keyword combinations including 'microbiome', 'immune checkpoint', 'cancer progression', 'immunotherapy', 'tumour microenvironment', and 'immune regulation'. Both theoretical discussions and experimental studies were reviewed, focusing on the molecular interactions, immune regulation pathways, and therapeutic implications of microbiome-immune system dynamics in cancer development. Information obtained was systematically evaluated and arranged to address the main research questions of the study (Fig 1).

For systematic approach to our main topic of review, we sectioned the article in four major parts addressing the following sections. Section 1 on human microbiome and cancer development outlines the composition, diversity, and functional roles of microbiomes, emphasizing their influence on cancer initiation and progression. Section 2 on immune checkpoint mechanisms and cancer progression highlights the molecular pathways of immune checkpoints, exploring their tumour inhibitory and tumour-promoting roles in cancer immunity. Section 3 on challenges of microbiome and immune checkpoint inhibitions in anti-tumoral efficacy discusses the factors limiting the effectiveness of microbiome manipulation and immune checkpoint blockade in cancer therapy, including inter-individual variation and tumour microenvironment complexity. Section 4 on innovative immune modulation checkpoints for future anti-cancer strategies presents emerging therapeutic concepts aimed at improving cancer treatment by synergistically targeting both the microbiome and immune checkpoint pathways.

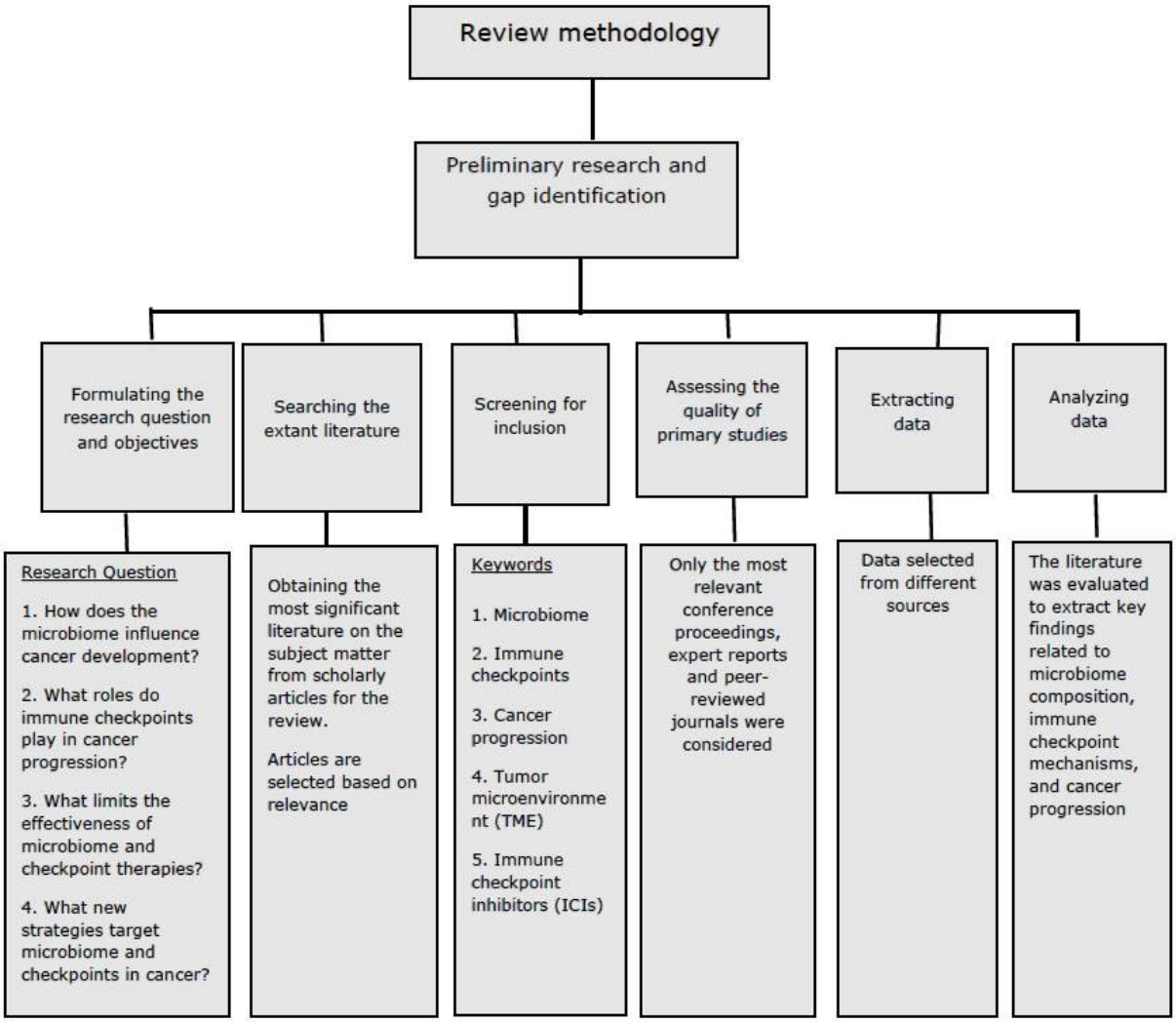


Fig 1: Schematic layout of the research review method

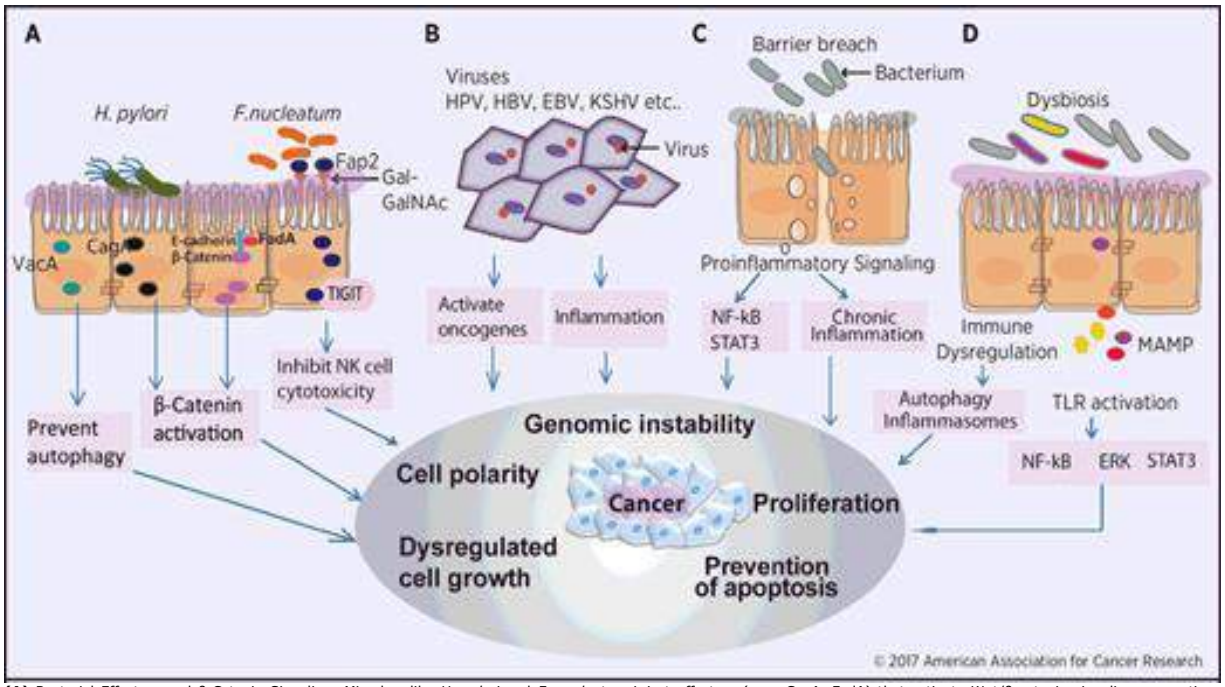
Human microbiome and cancer:

According to Rajagopala et al., (7), the human microbiome, especially the gut microbiota, influences cancer development by modulating immune responses, inflammation, and DNA repair. Specific microbial communities can either promote or inhibit cancer progression depending on their composition. Their findings revealed that pathogens promote cancer development through well-described genetic mechanisms.

There are 10 specific biological agents that have been designated by the International Agency for Cancer Research (IACR) as carcinogenic to humans. One of them, *Helicobacter pylori*, infect the stomach mucosa of half of the world population and cause chronic gastric inflammation, that can lead to gastric cancer (8) (Fig 2). The IACR noted that there are viruses that are known to cause various malignancies, some of which are sufficiently ubiquitous in the community to be considered part of the human virome. Recognized associations include human papillomaviruses (HPV) causing cervical carcinoma, hepatitis B (HBV) and C viruses (HCV) being the causative agents of hepatocellular carcinomas and human T-cell leukemia virus-1 (HTLV) being involved in T-cell leukaemia.

The composition of the gut microbiota can affect cancer onset and progression. Dysbiosis (imbalances in the microbiota) has been linked to the development of various cancers, such as colorectal cancer, breast cancer, and liver cancer. Certain microbial species, such as *Fusobacterium nucleatum*, have been associated with promoting cancer development by influencing inflammation and immune responses in the tumor microenvironment. The microbiome plays a significant role in modulating the effectiveness of cancer therapies, particularly immunotherapy, chemotherapy, and targeted therapies.

A diverse and balanced microbiome is crucial for enhancing the immune system's ability to respond to treatments like anti–PD-1 immunotherapy, as it can stimulate the immune system and reduce resistance to treatment. Some bacterial species, such as *Bifidobacterium* and *Faecalibacterium*, have been shown to enhance the effectiveness of immunotherapy by influencing immune cells like T-cells (9). The study of Gao et al., (10) emphasized the potential of microbiome modulation (through probiotics, antibiotics, diet, or fecal microbiota transplantation) to enhance cancer treatment outcomes. This can be particularly important in overcoming treatment resistance and improving overall survival rates of cancer patients.



(A) Bacterial Effectors and β-Catenin Signaling: Microbes like *H. pylori* and *F. nucleatum* inject effectors (e.g., CagA, FadA) that activate Wnt/β-catenin signaling, promoting tumorigenesis. *H. pylori* VacA protein inhibits autophagy. *F. nucleatum* also evades immune response by interacting with T cell immunoglobulin (TIG) and immunoreceptor tyrosine-based inhibitory motif (ITIM) (TIGIT) via its Fap2 protein. **(B)** Oncogenic Viruses: Viruses such as human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), human T-lymphotropic virus (HTLV), Epstein-Barr Virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV) encode oncoproteins that disrupt DNA repair, alter epigenetics, and cause genomic instability, a hallmark of cancer. **(C)** Inflammation and Genomic Instability: Barrier failure triggers chronic inflammation and DNA damage, contributing to carcinogenesis. **(D)** Dysbiosis and Immune Dysregulation: Microbial imbalance leads to bacterial translocation and immune dysregulation. Microbial associated molecular patterns (MAMPs) activate Toll-like receptors (TLRs) (e. g., TLR4), triggering nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and signal transducer and activator of transcription 3 (STAT3) pathways and promoting cancer. Inflammasome activation and autophagy also play roles.

Fig 2: Mechanisms by which microbes promote carcinogenesis [Source: Sun et al., (8)]

Immune checkpoint mechanisms and cancer progression

Immune cells express chemicals called immune system checkpoints, which alter the degree of activity of the immune system. To stop excessive or unchecked immune activity, these checkpoints serve as essential regulators of the host body immunological response. During the last few years, a variety of control molecules of the immune system have been identified, including programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1), cytotoxic T lymphocyte-associated protein 4 (CTLA-4), lymphocyte activating gene 3 (LAG-3), T cell immunoglobulin and mucin domain-containing gene 3 (TIM-3) among others.

Immune checkpoint inhibitors (ICIs) increase immune activation against cancer cells by blocking negative immune regulation. The review by Rui et al., (11) underlined that the importance of the immune system as an active part of carcinogenetic process and its clinical significance provides the fundamental scientific basis for therapeutic and immunotherapeutic cancer remedies leveraging on the inhibitory checkpoint blockade with friendly characteristics.

Inhibitory mechanisms of PD-1 and cancer development:

Programmed death-1 (PD-1) is a transmembrane receptor belonging to the CD28 family and is characterized by two functional domains, an extracellular Ig V-like domain that binds to its ligands, Programmed death-ligand 1 (PD-L1, also known as B7-H1) and Programmed death-ligand 2 (PD-L2, also known as B7-DC), and an intracellular domain containing immunoreceptor tyrosine-based inhibitory motifs (ITIM and ITSM) responsible for mediating inhibitory signaling (12). Under physiological conditions, PD-1 plays a crucial role in suppressing excessive immune activation and inflammation, thereby preventing immune-mediated tissue damage and maintaining immune homeostasis, especially during infections. However, tumor cells can exploit this pathway to evade immune surveillance, leading to immune escape and cancer progression (13).

During inflammatory responses, PD-1 binds to its ligands PD-L1 and PD-L2, which are typically expressed on antigen-presenting cells (APCs) such as dendritic cells and macrophages, as well as on epithelial and endothelial cells. This interaction inhibits T-cell receptor signaling and activation, resulting in immunosuppression. The Src homology region 2-containing phosphatase-2 (SHP-2) is recruited by PD-1 interaction and aids in the dephosphorylation of important T-cell signaling molecules such as CD28 (needed for co-stimulation), zeta-chain-associated protein kinase 70 (ZAP-70, essential for signal transduction), and the phosphoinositide 3-kinase (PI3K)-AKT mammalian target of rapamycin (mTOR) pathway, which is vital for T-cell metabolism and proliferation. This inhibitory cascade leads to reduced interleukin-2 (IL-2) production, impaired release of pro-inflammatory cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and IL-2, ultimately dampening T-cell immune activity. The suppersion of T-cell function not only weakens the immune response but also creates a microenvironment favorable for tumor survival and progression (14).

Immune mechanism of CTLA-4 and cancer development:

The immune checkpoint inhibitory role of CTLA-4 is crucial in downregulating early T-cell activation, which is essential for the prevention of autoimmune reactions and maintaining immune homeostasis (16). Tumor cells can evade this immune mechanism, impeding natural T-cell immune surveillance and promoting cancer development (17). The CTLA-4 (CD152) is a transmembrane receptor found on activated $CD4^+$ and $CD8^+$ T cells. Its binding on regulatory T cells enhances their immunosuppressive functions (18). In situations where the CTLA-4 receptor has to perform activation and inhibitory roles synergistically, CTLA-4 competes with CD28, for binding to B7-1 (CD80) and B7-2 (CD86) ligands on antigen-presenting cells (APCs) (19). Although CTLA-4 has 10 to 20 times higher binding affinity for the B7 molecules than CD28, it is important to note that the binding of CD28 to B7 ligands activates T cells, leading to cytokine production (IL-2, IFN-γ), enhancing immune responses against infections and tumor cells (20).

When CTLA-4 binds to B7 ligands, T-cell activation is suppressed because B7 is no longer available to stimulate CD28 (17). Intracellularly, there is recruitment of SHP-2 and protein phosphatase 2A (PP2A). SHP-2 dephosphorylates ZAP-70 and PI3K, inhibiting TCR signaling, while PP2A dephosphorylates the AKT-mTOR pathway, reducing T-cell metabolism and survival (18). CTLA-4 signaling inhibits IL-2 transcription, decreases T-cell proliferation and activation, and prevents progression to the G1 phase of the cell cycle (21). The overall immune signalling cascade in cancer development and progression centers on T-cell anergy (dysfunction), exhaustion, and apoptosis, contributing to the increased prevalence of cancer (21).

Stimulatory checkpoint mechanism and cancer development:

The anti-tumoral role of stimulatory checkpoint mechanisms in cancer develop-

ment relies on the complete activation of T cells and the modulation of regulatory T cells (Tregs). These checkpoints also influence interactions within the tumor microenvironment, promote synergy with inhibitory checkpoints, enhance long-term immune memory, and support the activation of dendritic cells, natural killer (NK) cells, and cytotoxic T lymphocytes (CTLs) (22). Stimulatory checkpoints are primarily responsible for providing co-stimulatory signals, such as the interaction between CD28 on T cells and B7 (CD80/CD86) on antigen-presenting cells (APCs). This interaction lowers the activation threshold of T cells and strengthens a durable immune response against tumor antigens (19).

Furthermore, co-stimulatory signaling involving OX40, CD137 (4-1BB), and inducible T-cell co-stimulator (ICOS) promotes T-cell proliferation, cytokine production, and survival, which are essential for establishing long-lasting anti-tumor immunity (23). It is also important to note that stimulatory checkpoints can exert dual immune effects on Tregs in cancer. For instance, under certain conditions, the engagement of OX40, ICOS, and glucocorticoid-induced tumor necrosis factor receptor (GITR) can enhance Treg function, promoting immune suppression and facilitating tumor immune evasion, which may increase the risk of cancer progression (24). However, the interaction of OX40 and CD137 immune signaling has been shown to strengthen T-cell differentiation, thereby preventing tumor relapse after initial clearance and ensuring sustained immune surveillance (25).

Challenges of microbiome and immune checkpoint in anti-tumoral efficacy:

The influence of inter-individual differences on the anti-tumoral efficacy of the microbiome and immune checkpoints is substantial, particularly with respect to variability of major histocompatibility complex (MHC), also known as human leukocyte antigen (HLA) in human. The MHC or HLA is a group of genes located on chromosome 6 that encode proteins responsible for distinguishing self from non-self, making them essential mediators of immune recognition in contexts such as organ transplantation, autoimmune diseases, and cancer immunotherapy. Studies have shown that individuals with high HLA heterozygosity tend to exhibit improved responses to programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) inhibitors in cancer treatment. Conversely, the diversity of HLA alleles shapes antigen presentation and immune responses to tumors, directly influencing the success of immunotherapeutic strategies (24).

Additionally, germline mutations contribute to variability in the anti-tumoral efficacy of both the microbiome and immune checkpoints. Pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and the MHC/HLA are germline-encoded and play central roles in immune cells recognizing pathogen-associated molecular patterns (PAMPs) and initiating immune clearance. Mutations in TLR genes can influence the composition of the gut microbiome and impair immune surveillance mechanisms, thereby affecting the response to immune checkpoint blockade therapy. Furthermore, single nucleotide polymorphisms (SNPs) in genes encoding CTLA-4, PD-1, FoxP3, and interleukin-10 have been shown to modulate immune checkpoint signaling and microbiome composition, further influencing the effectiveness of cancer immunotherapy (26).

Dietary influence on microbiome and immune checkpoint:

High fiber, Mediterranean diet improves microbiome community such as *Bifidobacterium, Faecalibacterium prausnitizii* and *Akkermansia muciniphila,* which in turns promote better ICI efficacy, reduce inflammation and promote anti-tumor immunity. However, easy accessibility and availability of excessive processed foods also known as ultra-processed food (UPFs) containing artificial additives, preservatives and unhealthy fats are often low in fiber, high in sugar, and sodium. This group of foods contributes to metabolic and cardiovascular diseases. Wei et al., (27) reported 95% cardiovascular related deaths attributed to low fiber diets. They also reported that the benefit of high fiber intake was statistically significant ($p<0.001$) among study groups, highlighting the link between dietary fiber consumption and a lower risk of metabolic syndrome (28).

Environmental factors and their effects on microbiome and immune checkpoint:

Geographical settlement, urban or rural, developing or under developing exposes and individual to diverse microbiome, alter immune system priming, different types of toxins and pollutants. All these can contribute to chronic inflammatory shift in microbiome composition, increase the presence of pro-inflammatory species, impairing ICI efficacy (29-31).

Microbiome dysbiosis in cancer development:

Johnson et al., (29) reported that microbiome dysbiosis is associated with disease duration and increased inflammatory gene expression. Research has also indicated that this microbiome imbalance can lead to deregulation of bodily functions, contributing to cardiovascular disease condition, cancer and respiratory diseases. The microbiome im-

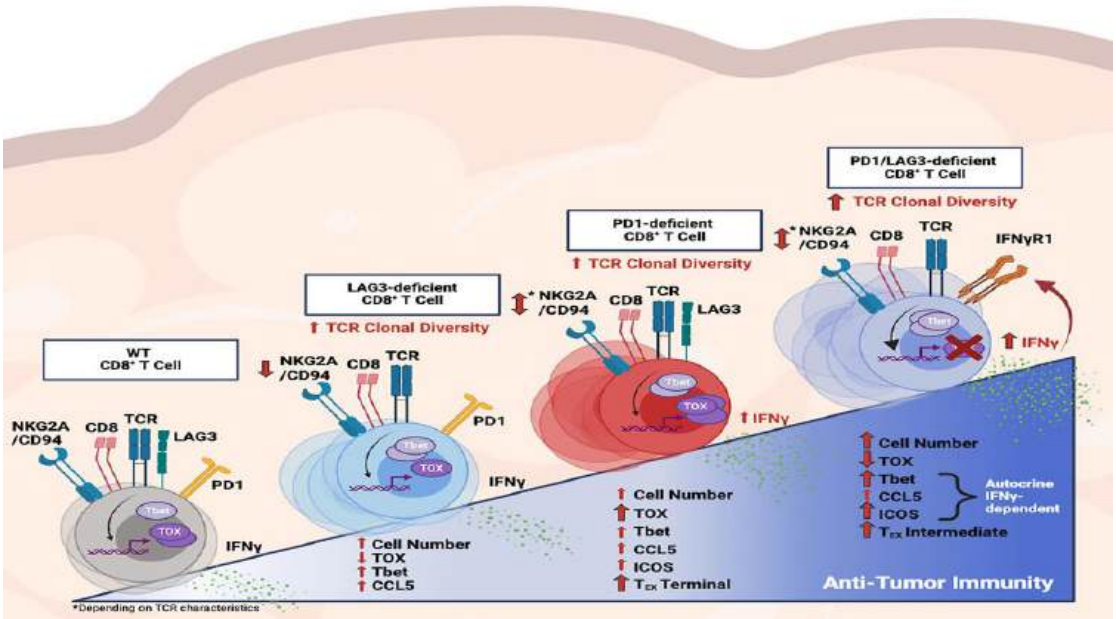
balance (dysbiosis) increases the release of pro-inflammatory (IL-6, TNF-α) cytokines leading to chronic inflammation, the hallmark of tumor progression. In dysbiosis, there is loss of beneficial microbiome which lead to promotion of myeloid-derived suppressor cell (MDSCs) and regulatory T cells (Tregs) which suppresses anti-tumor immunity. Alteration in drug metabolism leads to reduction in chemotherapy efficacy and allow tumor cell to evade immune destruction.

Innovative immune modulation checkpoints for future anticancer therapy:

Emerging research is expanding beyond traditional immune checkpoint inhibitors (ICIs) of PD-1/PD-L1, LAG-3, and CTLA-4 to identify next-generation immune modulation checkpoints. These innovations aim to enhance anti-tumor immunity and overcome the resistance mechanisms observed in cancer therapies. T cells that are worn out or malfunctioning express the immunoglobulin superfamily member lymphocyte activation gene-3 (LAG-3). LAG-3 is structurally and functionally similar to CD4, with multiple binding partners. Its expression negatively regulates T cell proliferation and differentiation, particularly inhibiting $CD8^+$ T cells that express higher levels of LAG-3 than $CD4^+$ T cells. Although they can express themselves separately, LAG-3 and PD-1 frequently coexist in the tumor microenvironment. LAG-3 contributes to T cell fatigue by suppressing anti-tumor T cell activity, just like PD-1 does.

Bispecific dual blockade of PD-1 and LAG-3 in the tumor microenvironment, where they are co-expressed, offers an opportunity to utilize immune checkpoint blockade while minimizing relapse and systemic toxicity (32). Research by Andrews et al., (24) demonstrated that the synergistic blockade and deletion of PD-1 and LAG-3 lead to distinct transcriptional and functional profiles in $CD8^+$ T cells, unleashing the full potential of the anti-tumor immune response. This finding offers valuable insight into potential therapeutic strategies for cancer immunotherapy (Fig. 3).

In particular, bispecific blockade of PD-1 and LAG-3 enhances T cell anti-tumor activity. In wild-type models, $CD8^+$ T cells with functional PD-1 and LAG-3 showed a higher degree of clonal diversity. Deleting LAG-3 in $CD8^+$ T cells induces clonal diversity by removing inhibitory signals, allowing more T cell variants to participate in tumor antigen recognition (32). Furthermore, in PD-1-deficient $CD8^+$ T cells, there is an upregulation of IFN-γ, accompanied by the production of other cytokines. Blocking PD-1 restores the maximum immune activity of T cells, leading to the production of IFN-γ, which in turn enhances cytotoxicity, promotes tumor cell killing, remodels the tumor microenvironment (TME), stimulates tumor immunogenicity, increases tumor antigen presentation, and promotes type I immune polarization (32).



The increasing improvement of anti-tumor immunity in several $CD8^+$ T cell populations, such as wild-type (WT), LAG3-deficient, PD1-deficient, and PD1/LAG3 double-deficient cells, is shown in this figure. When comparing modified T cells to WT, the image demonstrates increased expression of exhaustion markers (TOX, Tbet, ICOS, and CCL5), T cell clonal diversity, and IFN-γ production. Notably, $CD8^+$ T cells with PD1/LAG3 double-deficit have the highest levels of IFN-γ production and anti-tumor potential, most likely due to an IFN-γ autocrine feedback loop. This demonstrates how PD1 and LAG3 work together as inhibitors and how dual inhibition of them may be used therapeutically in cancer immunotherapy.

Fig. 3: PD1 and LAG3 play distinct functions in CD8 T cell-mediated anti-tumor immunity
[Source: Andrews et al., (24)]

Mice lacking both PD-1 and LAG-3 exhibit prolonged anti-tumor immunity due to enhanced T-cell receptor (TCR) clonal diversity, elevated autocrine IFN-γ, and an anti-tumor impact in the TME. The clinical relevance of dual PD-1 and LAG-3 blockade has led to the development of approved monoclonal antibody drugs such as Relatlimab (FDA-approved combination with Nivolumab), which shows potential for use in melanoma, lung cancer, colorectal cancer, and other solid tumors. Additionally, investigational drugs such as MBG453 and TSR-022 are in clinical trials for advanced solid tumors and haematological cancers (23,33,34).

Conclusion:

The interaction between the microbiome and immune checkpoint mechanisms plays a significant role in cancer progression and treatment outcomes. The microbiome can modulate immune responses, influencing the effectiveness of immune checkpoint inhibitors (ICIs) by enhancing anti-tumor immunity or contributing to immune evasion. Targeting immune checkpoints such as PD-1, PD-L1, LAG-3, and CTLA-4, in combination with microbiome modulation, shows promising potential for improving efficacy of cancer therapy.

However, further research is needed to better understand the complex interactions and optimize microbiome-based strategies for personalized cancer treatments. Ultimately, integrating microbiome research with immune checkpoint therapy offers a promising approach to improving patient outcomes in cancer treatment.

Contributions of authors:

EMO conceptualized the review topic, performed the initial literature search, and drafted the first version of the manuscript; ANN contributed to organizing the structure of the review and assisted in literature synthesis and referencing; CG critically reviewed the manuscript, ensuring clarity, accuracy, and intellectual depth and EGM supervised the entire process, provided strategic direction and final approval of the manuscript for publication.

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Original Article **Open Access**

Female genital schistosomiasis in Matta health area, Western Cameroon Region: Prevalence, risk factors and biomarkers profile

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Abstract:

Background: Schistosomiasis is a disease affecting particularly people living in low-income countries. Egg sequestration in the female genitalia, leading to female genital schistosomiasis (FGS), is associated with the release of reactive oxygen species (ROS) that can lead to secondary infertility, which remains underdiagnosed. This study was conducted to determine the prevalence and risk factors associated with FGS and urogenital schistosomiasis (UGS) and to assess some biochemical markers associated with these conditions.
Methodology: This was a descriptive cross-sectional survey of 330 women aged 15 to 50 years, conducted in selected fishing communities in the Matta health area of Western Cameroon. Information on socio-demographic characteristics and risk factors were collected from each participant using a structured questionnaire. UGS and FGS were diagnosed from analysis of urine and vaginal secretion respectively by microscopy and PCR amplification of extracted DNA of *Schistosoma haematobium* and *Schistosoma mansoni*. Biochemical analyses of markers of oxidative stress [levels of malondialdehyde (MDA), nitric oxide (NO) and reduced glutathione (GSH), and activities of superoxide dismutase (SOD) and catalase (CAT)] and cytokine profiling (TNF-α, INF-γ, IL-1β, IL-6 and IL-10) were determined in serum samples and vaginal secretions. Univariate and multivariate logistic regression analyses were used to determine associations of FGS, UGS, and potential risk factors.
Results: The prevalence of UGS and FGS was 45.7% and 60.0% respectively. Detection of schistosome DNA by PCR revealed a prevalence of 100.0% and 94.5% for *S. haematobium* and *S. mansoni* infection respectively. Nevertheless, among those infected, just 23.5% shed eggs in urine. Marital status was a risk factor of infection. Analysis of the oxidative stress markers revealed significantly higher levels of NO and MDA in serum and vaginal secretions of infected persons with highest level in women with FGS. Lower levels of GSH, CAT and SOD activity in serum and vaginal secretion in women with UGS and FGS was observed particularly in women with FGS. High IL-10 level was observed in both urine and vaginal secretions.
Conclusion: These results indicate increased oxidative stress in UGS and FGS particularly in the cervicovaginal tract. However, high levels of IL-10 might prevent immunopathology and limit severity of the disease, from a shift to T_{h2} immune response.

Keywords: Schistosomiasis; female genital; urogenital; inflammation; oxidative stress

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Schistosomiase génitale féminine dans la zone de santé de Matta, région de l'Ouest du Cameroun: Prévalence, facteurs de risque et profil des biomarqueurs

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Résumé:

Contexte: La schistosomiase est une maladie qui touche particulièrement les populations des pays à faible revenu. La séquestration d'ovules dans les organes génitaux féminins, responsable de la schistosomiase génitale

féminine (SGF), est associée à la libération d'espèces réactives de l'oxygène (ERO) pouvant entraîner une infertilité secondaire, encore sous-diagnostiquée. Cette étude visait à déterminer la prévalence et les facteurs de risque associés à la SGF et à la schistosomiase urogénitale (SUG), ainsi qu'à évaluer certains marqueurs biochimiques associés à ces affections.
Méthodologie: Il s'agissait d'une enquête transversale descriptive auprès de 330 femmes âgées de 15 à 50 ans, menée dans des communautés de pêcheurs sélectionnées dans la zone de santé de Matta, dans l'ouest du Cameroun. Des informations sur les caractéristiques sociodémographiques et les facteurs de risque ont été recueillies auprès de chaque participante à l'aide d'un questionnaire structuré. L'UGS et la FGS ont été diagnostiquées à partir de l'analyse des urines et des sécrétions vaginales respectivement par microscopie et amplification par PCR de l'ADN extrait de *Schistosoma haematobium* et *Schistosoma mansoni*. Français Des analyses biochimiques des marqueurs du stress oxydatif [taux de malondialdéhyde (MDA), d'oxyde nitrique (NO) et de glutathion réduit (GSH), et activités de superoxyde dismutase (SOD) et de catalase (CAT)] et le profilage des cytokines (TNF-α, INF-γ, IL-1β, IL-6 et IL-10) ont été déterminés dans des échantillons de sérum et des sécrétions vaginales. Des analyses de régression logistique univariée et multivariée ont été utilisées pour déterminer les associations entre le FGS, l'UGS et les facteurs de risque potentiels.
Résultats: La prévalence de l'UGS et du FGS était respectivement de 45,7% et 60,0%. La détection de l'ADN de schistosome par PCR a révélé une prévalence de 100,0% et 94,5% pour l'infection à *S. haematobium* et *S. mansoni* respectivement. Néanmoins, parmi les personnes infectées, seulement 23,5% ont excrété des œufs dans l'urine. L'état matrimonial était un facteur de risque d'infection. L'analyse des marqueurs du stress oxydatif a révélé des taux significativement plus élevés de NO et de MDA dans le sérum et les sécrétions vaginales des personnes infectées, les taux les plus élevés étant observés chez les femmes atteintes de SGF. Des taux plus faibles d'activité du GSH, de la CAT et de la SOD dans le sérum et les sécrétions vaginales ont été observés chez les femmes atteintes de SGF et de SGF, en particulier chez celles atteintes de SGF. Un taux élevé d'IL-10 a été observé dans les urines et les sécrétions vaginales.
Conclusion: Ces résultats indiquent une augmentation du stress oxydatif dans les SGF et les SGF, en particulier au niveau du tractus cervico-vaginal. Cependant, des taux élevés d'IL-10 pourraient prévenir l'immunopathologie et limiter la gravité de la maladie, notamment en favorisant une réponse immunitaire T_{h2}.

Mots-clés: Schistosomiase; génital féminin; urogénital; inflammation; stress oxydatif

Introduction:

Schistosomiasis, also called Bilharzia, is a chronic and debilitating parasitic disease caused by trematode blood flukes of the *Schistosoma* genus. It belongs to the phylum Platyhelminthes and affects a number of vertebrates. Schistosomiasis is a neglected tropical disease that infects over 251.4 million people yearly with about 800 million people at risk of infection (1). This disease is present in over 78 different countries around the globe, precisely low-income countries in Africa, South America and Asia. The disease is ranked second place below malaria in the list of WHO important parasitic diseases due to its impact on public health, causing over 200 thousand deaths annually (1). The species of schistosomes that infect humans reside at specific sites in the human system. *S. mansoni* and *S. japonicum* resides in the intestines of the human host, causing intestinal schistosomiasis whereas *S. haematobium* on the contrary resides in the urinary bladder and urethra of their human definitive host and causes urogenital schistosomiasis (UGS) (2).

Investigations have shown that schistosomiasis is highly endemic in Cameroon, but its distribution is uneven and varies greatly with geographical location across the national territory (3,4). The most affected regions are the Southwest, Adamawa, North, Far North, West and Littoral regions (4). Hotspots of transmission have been observed in several localities around lakes and dams such as Barombi Kotto in the Southwest region and Malantouen in the West region, where water contacts are intense and lead to high reinfection patterns (5). Prevalence of the disease rapidly returns near to the initial level within 6–12 months post-treatment in these localities and the prevalence of the disease within the country ranges from 1.7 to 55.5% (3-6) with rural communities being the most affected. In areas where it is endemic, schistosomiasis can persist for decades without treatment (7). The accumulation of adult *Schistosoma* worm eggs within the host's tissues results in granuloma development, fibrosis, and portal hypertension, and is linked to the chronic morbidity of the disease (8). This is largely due to repeated exposure to freshwater bodies contaminated with cercariae and the longevity of adult worms.

The clinical manifestations of schistosomiasis are associated with the host immune response to the parasite eggs as they circulate within the hosts system (9). The aim of the immune response to eradicate the parasite but it ends up being unsuccessful resulting into an inter-relationship that keeps both parasite and host alive (10). When these schistosome eggs lodged into specific sites in the human system, they elicit an immune response characterized by granuloma formation in the organs and tissues (10). The granuloma formation, which involves the action of white blood cells, results in the release of high levels of reactive oxygen species (ROS) that expose the system to severe oxidative stress. Eggs produced mainly by *S. haematobium* in the urinary system can lodge in the genitals resulting to granulomatous lesions in the genital tract. This causes a form of the disease called genital schistoso-

miasis.

Female genital schistosomiasis (FGS) has a more prominent consequence compared to that of the male, and the WHO estimates its prevalence in about 56 million women around the world (11). FGS in most cases remains undiagnosed due to low index of suspicion among physicians and presents symptoms such as bloody vaginal discharge, bleeding after intercourse or spotting, genital itching or burning sensation (11,12). FGS can result in further complications such as infertility, abortion or ectopic pregnancy, tumors and genital ulcers (12).

Although the presence of the parasite itself in the host is somehow harmless, it results to dysuria and haematuria when infected with *S. haematobium* and occasionally dysentery when infected with intestinal schistosomes (10). The clinical manifestation of schistosomiasis progresses in three phases beginning with a dermatitis reaction following cercariae penetration through the skin that causes an allergic response and later on proceeds to a symptomatic acute phase (Katayama syndrome) characterized by fever, arthralgia and vasculitic skin eruption, eosinophilia and high serum IgM before progressing into a chronic infection (13). During established acute and late chronic stages of the disease, infected persons develop organ-specific morbidity, caused by the accumulation of parasite eggs and granuloma formation around schistosome eggs. The site of inflammation correlates with the infecting *Schistosoma* species as the adult worms of different species localize and lay eggs in specific preferred sites (14).

Adult worms of *S. mansoni* and *S. japonicum* live and lay eggs in the mesenteric veins, some of which get trapped in the intestinal tissues inducing mucosal granulomatous inflammation with micro ulcerations, superficial bleeding and pseudopolyposis during chronic inflammation (15). Some of the eggs released by *S. japonicum* or *S. mansoni* eggs from the mesenteric veins are carried through venous circulation into the small portal branches of the liver through the portal vein, where some get trapped in the pre-sinusoidal periportal tissues inducing the formation of granulomas around the eggs resulting in liver inflammation. In high-intensity, established active infection results in enlargement of the spleen and liver, especially the left liver lobe characteristic of hepatosplenic schistosomiasis (13). In some individuals with prolonged or recurrent infection, egg-induced granulomatous responses may result in severe periportal fibrosis, known as Symmer's pipe stem fibrosis, characterised by deposition of collagen around the portal vein, blockage of the smaller portal branches and severe, often irreversible, pathology (16).

Adult *S. haematobium* worms on the contrary live in the pelvic venous plexus, resulting in UGS symptoms and pathological changes closely associated with the passage of eggs through the urinary bladder wall and egg deposition in bladder tissue and genital organs (13). The passage of egg clusters into the lumen of the bladder is often accompanied by breakage of the epithelial surface of the bladder, ulceration and bleeding which results in haematuria, characteristic of UGS (17). Intense or prolonged egg-induced tissue inflammation can result in bladder wall thickening and development of polypoid protrusions (2) around the ostium, blocking the passage of urine resulting in hydronephrosis which in some cases result in a non-functioning kidney (18). Investigations have shown that UGS is significantly associated with squamous cell carcinoma (SCC) of the urinary bladder, and *S. haematobium* has been classified by the International Agency for Research on Cancer (IARC) as a carcinogen, as infection may result in bladder cancer after many years of exposure, infection and urinary tract inflammation (19).

During established active infection, *S. haematobium and S. mansoni* may migrate from their primary sites through the intercommnections between the different pelvic venous plexuses to the uterovaginal venous plexus where they release eggs that migrate to the genital tract (20). In the genital tract, the eggs cannot be released to the environment, hence, are trapped and their antigens evoke an inflammatory response, resulting in FGS (9). FGS is considered a risk factor for the transmission of sexually transmitted diseases (STDs) such as HIV and HPV because the disease leads to open sores, inflammation and exposure to blood, providing easy access for any virus to enter the body (21). Depending on where eggs are released, the clinical pathology develops in vulva and vagina, cervix, uterus, fallopian tubes, ovaries and all genital organs may be affected simultaneously. Women suffering from FGS may experience infertility, irregular menstruation, pregnancy complications, and problems of sexual intercourse like bleeding (9).

The immune response profile of the disease is attributed to a supervening T_{h1} pro-inflammatory immune reaction and high levels of tumor necrosis factor (TNF-α) and interferon gamma (IFN-γ), in concert with a building up of a T_{h2} modulatory profile during the chronic phase of the infection (9). This switch takes place gradually over a long period and coincides with egg over-deposition during which inflammatory cells are gradually being replaced by fibroblasts and granulomas calcified as a result of humoral mediators of the host and parasite in the presence of deposited eggs. The most serious manifestations of the disease such as fibrotic lesions, glomerulo-

nephritis, amyloidosis and malignancy can take place over several weeks or years after infection (10).

Schistosomes depend on human host for its life processes by feeding on red blood cells during which hemoglobin is catabolized. Hb is broken down by schistosomes to produce dipeptides or free amino acids that are ingested by the parasite and broken down by proteolytic enzymes, producing waste products that are regurgitated into the bloodstream of the host as toxic heme and cellular debris (22). Accumulation of these substances within the bloodstream and organs of the host, particularly the liver induces oxidative stress and also inhibits mechanisms set in place to defend against ROS (9).

Toxic heme, also known as free iron (II)-heme, has earned this name because of its capacity to oxidize para- site biomolecules and reduce molecular oxy- gen to produce superoxide anion (O_2), which is crucial for the generation of reactive oxygen species (ROS), which have a direct impact of lipid peroxidation (22). Reactions between lipids, intracellular H_2O_2, and the active heme exacerbate this. The free iron (II)-heme (Fe^{2+}) is converted to non-functional ferric heme (Fe^{3+}) and O_2 that in turn generates H_2O_2 via the Fenton (iron-catalyzed Haber-Weiss) reaction. H_2O_2 produced has the potential to react with Fe^{3+} in the bloodstream to produce the ferryl heme (Fe^{4+}) intermediate.

Extremely hazardous, these products and their intermediates are known to oxidize lipids, amino acids, and nucleic acids (22). They also create toxic heme, which in turn causes the loss of heme and the release of free iron. There is therefore the need to have more data about the complications of schistosomiasis in women in particular, the impact on the immune system and the development of oxidative stress among infected women

Materials and method:

Study site and design:

This study was a descriptive cross-sectional study carried out in selected fishing communities in the Matta health area (5°57'N 11°13'E) which is one of the 18 health areas of the Malantouen Health District in the West Region of Cameroon. These communities included Matta Barrage, Matta Village, Mabonkor Bore, Mabonkor Village and Makossa. These communities were chosen based on their presence within the Matta health area, higher population size, proximity to the lake, prevalence of *S. haematobium* infection and the socioeconomic activities (fishing and farming) carried out among the people as described by Masong et al., (23).

The Matta health area is made up of one Government Integrated Health Centre and two private health centers (4). According to the socio-economic statistics of Cameroon in 2015, the Matta health area has an estimated population of 5000 people with women representing 51% or 2,550 of the population and age group 15–64 years representing 54.6% or 1,400 of the female population (28% of the total population).

Ethical considerations:

The study was approved by the Institutional Review Board of the Faculty of Health Science of the University of Buea (IRB-FHS) (Ref N° 2022/1790-04UB/SG/IRB/FHS). An administrative authorization was gotten from the Regional Delegation of Public Health for the West Region of Cameroon (Enregistrement S/N° 163 /2022).

Written and verbal informed consent was provided by each participant to take part. Participants under the age of eighteen gave their approval after receiving permission to participate from their parents, spouses, or guardians. Furthermore, as required by cultural rules, husbands of married women or partners had to give their verbal consent before they were approached.

Study participants and method of selection:

The participants for this study were females aged 15-50 years residing in the Matta Health area. These participants were selected from 5 rural fishing communities surrounding the Mape Dam, a known transmission hotspot for *S. haematobium*, with most participants living within 200 meters of the dam and relying on it for household water and income-generating activities such as fishing.

Participants for the study were selected by simple random sampling technique. After selection, participants (or their guardians for minors) were informed about the study objectives, procedures, and potential risks. Only those who gave consent were included in the study.

Sample size determination:

The sample size was determined using the formula described by Charan and Biswas (24), predicated on achieving a 95% precision rate with a 5% error margin, as follows; $n = \frac{Z^2 p(1-p)}{d^2}$, where Z=standard normal variate (at 5% type 1 error = 1.96), p = expected proportion of 0.59 in population based on a previous study by Masong et al., (23), and d = absolute error or precision = 0.05. Thus, $n = \frac{1.96^2 \times 0.59\ (1-0.59)}{0.05^2}$, which gave the calculated sample size of 372.

Sample and data collection:

Urine, stool, vaginal swab and blood samples were collected from girls 15 years and above and women within the target communities and codes assigned to each participant.

The sample collection exercise was supervised by trained medical professionals working within the Matta health area.

On the day of enrollment, each participant was given 2 sterile, wide mouthed, screw capped plastic bottles carrying their identification information. About 250 ml urine were collected from each participant and a small scoop of faeces. The samples were stored in cooling boxes containing cooler ice packs to prevent the eggs from hatching during transportation to the laboratory of the Integrated Health Center of Matta Barrage. Questionnaires were administered to participants during sample collection to obtain information on frequency and history of fresh water contact and related symptoms of FGS.

Sample processing:

The urine samples collected were used for microscopic examination to detect eggs of *S*. *haematobium* excretion, from which we inferred the possibility of UGS in correlation with microhematuria from dipstick tests. A single dose (40 mg/kg) of Praziquantel was offered and administered to those who had microhematuria from the dipstick tests (12).

Laboratory identification of *Schistosoma* by microscopy:

The urine samples were tested for microhematuria and proteinuria using urine reagent strips (Siemens Multistix 10). In the laboratory, the urine samples were processed using the membrane filtration technique and examined microscopically for the presence of eggs of *S*. *haematobium* using Binocular compound light microscope. Terminal-spined eggs, characteristics of *S*. *haematobium* were identified and counted manually. Fecal samples were processed and examined microscopically for the presence of *S. mansoni* infection by identifying lateral-spined eggs characteristic of *S. mansoni*.

Molecular detection of *Schistosoma* species:

Polymerase chain reaction was used to amplify schistosomes DNA extracted from dried blood spot and vaginal secretions to detect schistosomiasis and FGS respectively.

DNA extraction:

DNA was extracted from dried blood spotted on Whatman paper number 2 using the Chelex method as described by Ategeka (25) with minor modifications. Each coded dried blood spot was cut and transferred into their corresponding 1.5µl Eppendorf tube using sterile equipment and 1ml of 0.5% saponin was added into the tubes and incubated overnight at 4℃. The brown solution was discarded and replaced with 1ml of 1x PBS and the tubes incubated at 4℃ for 30 minutes. A stock 20% Chelex resin (50µl) of and 150 µl of autoclaved distilled water were both transferred into newly labelled tubes and heated at 99℃ for 30 minutes on a heat block. The tubes containing PBS were centrifuged at 4000 rpm for 2 minutes and each filter paper was carefully transferred into their corresponding hot Chelex tubes. The tubes were vortexed for 30 seconds and placed on a heat block at 99℃ for 10 minutes. The tubes were then centrifuged at 4000 rpm for 2 minutes and 100µl supernatant were transferred into freshly labelled tubes. The supernatant contained the DNA template and was stored at -20°C for PCR analysis.

DNA was extracted from vaginal secretion using the Qiagen kit following the manufacturer's protocol. Lysis buffer AL, 100µl, 50µl proteinase K and 100µl lysis buffer ATL were pipetted respectively into 1.5ml Eppendorf tubes containing 100 µl vaginal secretion. The tubes were vortexed for 15 seconds then, incubated for 10 minutes followed by a brief centrifugation to remove the drops from the inner lid. Ethanol (100 µl, 95%) was added to the samples and vortexed for 15 seconds. Following a brief centrifugation of the tubes, the mixtures was carefully transferred to respective QIAamp spin columns placed in a 2ml collection tubes and centrifuged at 8000 rpm for 1 minute. The QIAamp spin column was removed and placed in a new 2ml collection tube and 500µl washing buffer (AW1) carefully pipetted in the QIAamp spin column and centrifuged at 14000 rpm for 1 minute.

The QIAamp spin column was removed for the second time and placed in a new 2 ml collection tube and 700µl washing buffer (AW2) was carefully pipetted in the QIAamp spin column and centrifuged at 14000 rpm for 3 minutes. The QIAamp spin column was removed for the third time, placed in a new 2 ml collection tube and was centrifuged at 14000 rpm for 1 minute. Finally, the QIAamp spin column was placed in a clean 1.5 ml micro centrifuge tube, 100 µL of distilled water were added followed by incubation at room temperature for 1 minute and centrifugation at 8000 rpm for 1 minute and the solution collected stored at -20℃ until used.

DNA amplification and detection:

The DNA of *S. haematobium* and *S. mansoni* in the DNA extract was detected by amplification of tandemly arranged 121bp long units of *Dra*1 repeats gene of *S. haematobium* and *Sm*1-7 repeats gene of *S. mansoni* (26) in a PCR machine (Bio-Rad T100™ Thermal Cycler, Berkeley California, USA). These repeats genes constitute approximately 15% of *S. haematobium* genome and 12% of *S. mansoni* genome respectively.

Amplification was carried out in a total volume of 10 µL including 5 µL of One Taq Hot Start 2x Mastermix (New England Biolabs),

Table 1: Primer sequences for *Schistosoma* species identification by polymerase chain reaction

Schistosome species	Primer sequence	Expected band size	Reference
Schistosoma haematobium	SHF: 5' -GATCTCACCTATCAGACGAAAC- 3'	97 bp	Lodh et al., (27)
	SHR: 5' -TCACAACGATACGACCAAC- 3'		
Schistosoma mansoni	SMF: 5'-GATCTGAATCCGACCAACCG- 3'	110 bp	
	SMR: 5'-ATATTAACGCCCACGCTCTC- 3'		

Table 2: Polymerase chain reaction conditions for *Schistosoma* species identification

Schistosome species	Initial denaturation	Denaturation	Annealing	Extension	Number of cycles	Final extension
Schistosoma haematobium	95℃ for 10 minutes	95℃ for 30 seconds	53℃ for 90 seconds	72℃ for 1 minute	33	60℃ for 5 minutes
Schistosoma mansoni	95℃ for 10 minutes	95℃ for 30 seconds	60℃ for 90 seconds	72℃ for 30 seconds	35	72℃ for 5 minutes

Lodh et al., (27)

0.5 µL of both forward and reverse primers (primer sequence listed in Table 1), 1 µL of the DNA template and 3 µL DNase free water, with reaction conditions as set in Table 2 (27). The amplified fragments together with a 100bp molecular weight marker were separated in a 2% agarose gel and observed using a gel documentation system (Molecular Imager® Gel DocTM XR+ System with Image LabTM Software, Bio-Rad, Berkeley California, USA).

Biochemical assay:

Using a 5 ml syringe, 4 ml of venous blood was obtained from each participant and poured into sterile plain blood tubes carrying participants' identification numbers and centrifuged to obtain serum. The serum was transferred to clean sterile Eppendorf tubes and then transported in cooling boxes containing cooler ice parks from Matta to the University of Buea Molecular and Cell Biology Laboratory (MCBL) for storage at -20℃ (28). Biochemical analysis of markers of oxidative stress (malondialdehyde (MDA), nitric oxide (NO) and reduced glutathione (GSH) levels, superoxide dismutase (SOD) and catalase activities) and cytokine profiling (TNF-α, INF-γ, IL-1β, IL-6 and IL-10) was then carried out using vaginal secretion and serum samples.

Analysis of oxidative stress markers:

Assay of malondialdehyde level:

Malondialdehyde (MDA) level, a measure of lipid peroxidation based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA); forming an MDA-TBA2 adduct that absorbs strongly at 532 nm was determined as described by Hamburger et al., (26). The reagents, 1.5 mL acetic acid (20% at pH 3.5), and 1.5 ml thiobarbituric acid (0.67%) were added to 0.1 mL of sample, and then heated at 90°C for 60 minutes. The mixture was cooled, centrifuged at 3000 rpm for 10 min, the supernatant collected and absorbance measured at 532 nm using a spectrophotometer (Tecan, Infinite M200). The concentration of MDA was quantified by the extinction coefficient (ε) of 1.56×10^5 M/cm using the formula; C=A/ℰl, where A is the absorbance at 532 nm, ε is the extinction coefficient, C is the concentration of MDA and l is the length of the cuvette. The concentration of MDA was expressed in nmol/ml of sample.

Assay of reduced glutathione level:

Reduced glutathione (GSH) was assayed by Ellman method as described by Fotio et al., (29). The method is based on a thiol-exchange reaction between GSH and Ellman's reagent (5 mg of Disthio-bis 2-nitrobenzoic acid (DTNB) in 250 ml of phosphate buffer) forming one equivalent of 5-thio-2-nitrobenzoic acid (TNB). The produced TNB is detected by spectrophotometry in the visible region at 405 nm.

The sample (20µl) was added to 3 ml of Ellman reagent. The mixture was kept at room temperature for 1 hour and the absorbance read using a spectrophotometer (Tecan, Infinite M200) at 405nm. The concentration of GSH was quantified with the extinction coefficient (ε) of 14,150 $M^{-1}cm^{-1}$ using the formula; C=A/ℰl, where, A is the absorbance, l is the path length in centimeters, C is the concentration in moles/liter (M) and ε is the extinction coefficient. The obtained concentration was converted to µg of protein per ml of sample.

Measurements of nitric oxide:

The method for nitric oxide levels was based on the Griess reaction as described by Hamburger et al., (26). It is based on the reaction between nitrite and Griess reagent (sulfanilamide and N-naphthyl ethylene diamine) in acidic medium leading to the formation of a pink/magenta azo product that

absorbs light at 540nm (30). 100 µl of sample were measured and mixed with an equal volume of Griess reagent and the absorbance determined spectrometrically at 540nm. Absorbance readings obtained were compared with a standard curve plotted with known concentrations of nitrite and the results obtained expressed in µg/ml of sample.

Measurement of superoxide dismutase (SOD) activity:

Superoxide dismutase activity was determined according to the method of Misra and Fridovich (31); a method which is based on the superoxide dismutase inhibition of epinephrine autoxidation to adrenochrome at pH 10.2. Presence of superoxide dismutase prevents the conversion of epinephrine to adrenochrome which can absorb light at 480 nm. Sample (134 µl) was mixed with 1666 µL of carbonate buffer (0.05M, pH 10.2) followed by 200µl of epinephrine (0.06 mg/ml). Absorbance was recorded at 20 and 80 seconds. A blank was prepared by adding 1800 µl of the carbonate buffer to 200 µl of epinephrine and absorbance recorded at 20 and 80 seconds. The change in absorbance (ΔA) within one minute (absorbance at 20 S – absorbance at 80 S) was calculated for the blank and for each sample.

The activity of SOD was determined as percentage inhibition of epinephrine autoxidation as; % inhibition = 100 – [(Δ A sample x 100)/Δ A blank]. One unit of SOD was defined as the enzyme amount causing 10% inhibition in the epinephrine oxidation rate (U), and results were expressed in units of SOD per ml of mixture.

Measurement of catalase activity:

The catalase activity was measured following the spectrometric method by Fotio et al., (32) which is based on the fact that hydrogen peroxide (H_2O_2) is broken down into oxygen (O_2) and water (H_2O) by the enzyme catalase (CAT). Catalase in the sample is allowed to split H_2O_2 and the remaining hydrogen peroxide is measured spectrometrically at 340 nm. A volume of 250µl of sample was added to 250µl of phosphate buffer and 1ml of H_2O_2 (30 mM) added to the mixture and the absorbance read at 340 nm at 30, 60, and 90 S consecutively after the addition of the hydrogen peroxide in the medium. The catalase activity was expressed as units of catalase per ml of sample.

Cytokine profiling:

The values of inflammatory cytokines (TNF-α, INF-γ, IL-1β, IL-6 and IL-10) in both serum and vaginal secretion samples were determined using Quantikine ELISA kit (R&D Systems, USA) following the manufacturer's instructions. This assay employs the quantitative sandwich enzyme immunoassay technique. The concentration of cytokines was determined and recorded in pg/ml by comparing the absorbances with those of a standard curve for the respective recombinant cytokine as described by Misra and Fridovich (31). The intensity of the colour measured is proportional to the amount of the specific cytokine bound in the initial step.

Monoclonal antibodies specific for human TNF-α, INF-γ, IL-1β, IL-6 and IL-10 were pre-coated onto the wells of separate 96 well microplate and 100 µl of sample pipetted into the wells. The plates were incubated for 2 hours at room temperature such that any cytokine (TNF-α, INF-γ, IL-1β, IL-6 and IL-10 respectively) present should be captured by the immobilized antibody. Washing buffer (400 µl) was used to remove any unbound substances, followed by the addition of 100 µl of enzyme-linked polyclonal antibody specific for the human cytokine present in the wells, respectively and incubated at room temperature for 2 hours. Following a wash to remove any unbound antibody-enzyme reagent, 100 µl of substrate solutions were added to the wells of each plate and incubated for 30 minutes at room temperature away from light. The stop solution (50µl) was then added. This yielded to an enzyme reaction that turned the blue products to yellow. The optical densities were determined within 30 minutes, using a microplate reader (Thermo Scientific; MultiskanTM FC Microplate Photometer) set at 450 nm.

Data analysis:

Data obtained were entered into Microsoft Excel 2019 and checked, with tabulations, graphs and statistical analyses conducted using IBM Statistical Package for the Social Sciences (IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp) and GraphPad Prism version 9.

Chi-square and Fisher's Exact tests were used to check for associations between UGS, FGS, site, risk factors and morbidity. Univariate and multivariate logistic regression analyses were conducted for *S. haematobium* and *S. mansoni* associations with independent variables. Age group, village site, marital status and economic activity were included as core variables in regression models, and a stepwise regression approach was used to arrive at the most parsimonious adjusted model, applying a 5% level of statistical significance.

Results:

Socio-demographic characteristics of the study participants:

A total of 350 females (girls and women) from five (5) mainland communities in the Matta health area were considered from which 165 participants were enrolled into the

study (Table 3). The age range of the enrolled participants is 15 to 50 years old (median age: 23 years), 60.6% were married and 39.4% were single women. Majority of the participants use the lake (95.8%, n=158) for domestic activities, and most of them use it on daily bases (49.7%, n=79) due to lack of access to improved water source (72.7%).

Prevalence of urogenital schistosomiasis (UGS) by microscopy:

After urine analysis for *S. haematobium* egg excretion, and microhematuria test, the prevalence of UGS (with or without eggs in urine) was 45.7% (n=75) from all selected sites.

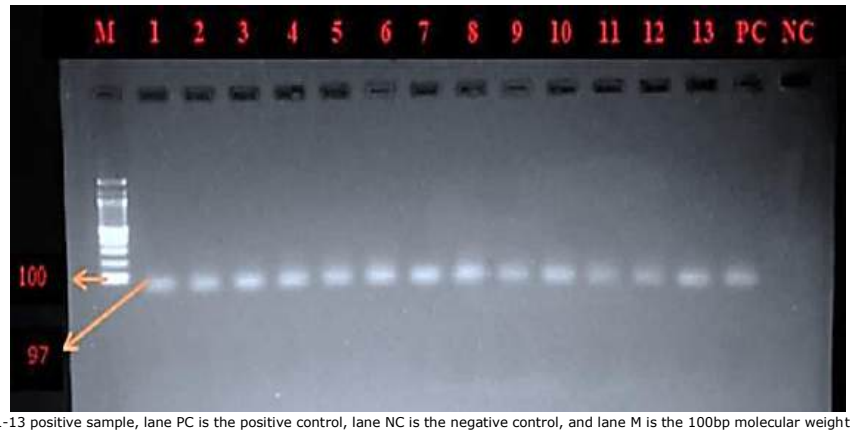
Prevalence of schistosomiasis by molecular detection method (PCR):

PCR detection of *S. haematobium* and *S. mansoni* DNA extracted from both dried blood and vaginal secretion revealed a band of 97 bp and 110 bp for positive samples respectively (Figs 2 and 3). All participants blood samples (100.0%, n=165) tested positive for *S. haematobium* by PCR, with 94.5% (n=158) co-infected with *S. mansoni* (schistosomiasis).

Most of the participants (86.0%, n= 142) provided vaginal secretion samples but no *S. mansoni* was detected in the vaginal secretion while *S. haematobium* was detected in 59.9% (n=85) of vaginal secretion by PCR (FGS).

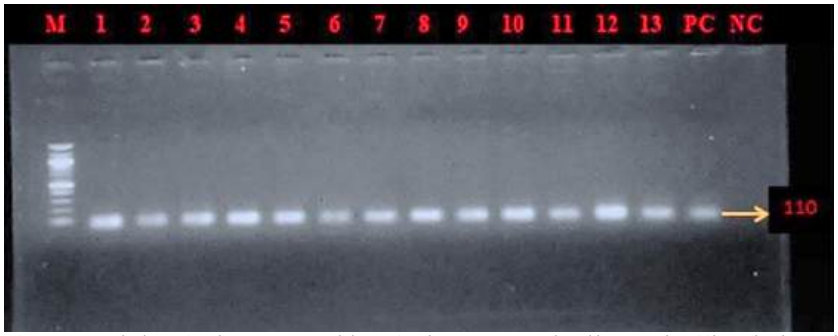
Table 3: Socio-demographic characteristics of the study participants in the Matta Health Area, Western Region of Cameroon

Variables	Category	Frequency (%)
Area of residence	Matta barrage	74 (44.8)
	Matta village	32 (19.4)
	Mambonkor bord	28 (17.0)
	Mambonkor village	14 (8.5)
	Makossa	17 (10.3)
Age group (years)	15-24	88 (53.3)
	25-34	42 (25.5)
	≥35	35 (21.2)
Marital status	Single	65 (39.4)
	Married	100 (60.6)
Level of education	Primary	112 (67.9)
	Secondary	53 (32.1)
Occupation	Housewife	35 (21.2)
	Farmer	58 (35.2)
	Fishing	12 (7.3)
	Pupil/students	53 (32.1)
	Civil servants	7 (4.2)
Religion	Christianity	107 (64.8)
	Islam	58 (35.2)



Lane1-13 positive sample, lane PC is the positive control, lane NC is the negative control, and lane M is the 100bp molecular weight marker

Fig 2: Amplicons gel visualization of representative *Schistosoma haematobium* in dried blood and vaginal secretion



Lane1-13 positive sample, lane PC is the positive control, lane NC is the negative control, and lane M is the 100bp molecular weight marker

Fig 3: Amplicons gel visualization of representative *Schistosoma mansoni* in serum

Self-reported clinical symptoms of female genital and urogenital schistosomiasis associated with *S. haematobium* egg excretion:

Self-reported symptoms and descriptions from the questionnaire survey included lower abdominal pain, foul-smelling vaginal discharges, inconsistent/painful menstruation, genital itch or burning sensation, difficulty in conceiving, frequent miscarriage and pelvic pain or pain during intercourse. The frequency of these self-reported clinical symptoms of FGS by the participants include; vaginal itches (20%, n=33), burning sensation (12.1%, n=20), bleeding after sex (1.2%, n=2), stress incontinence (12.7%, n=21), hypermenorrhoea (21.8%, n=36), difficulty in conceiving (12.1%, n=20), frequent miscarriage (7.3%, n=12), foul-smelling vaginal discharge (42.4%, n=70), irregular/painful menstruation (37.8%, n=61) and abdominal pain (32.7%, n=54) (Table 4).

Relating the participants reported FGS symptoms with prevalence of egg excretion, there was significant association between egg shedding and abdominal pain (p=0.003), vaginal itches (p=0.002) and burning sensation (p=0.03). Other reported symptoms such as smelly vaginal discharge, stress incontinence, pain during/after sex, burning sensation, irregular menstruation, difficulty in conceiving, frequent miscarriage and hypermenorrhea that may be associated with FGS were not significantly related (p>0.05) with egg excretion (Table 4).

Table 4: Summary of self-reported FGS symptoms and their relationship with *Schistosoma haematobium* egg excretion

Reported FGS symptoms	Category	Number (%) egg excretion	*p* value
Abdominal pain	Yes (n=54) No (n=111)	20 (37.0) 18 (16.2)	0.003*
Vaginal itches	Yes (n=33) No (n=132)	14 (43.4) 24 (18.2)	0.002*
Burning sensation	Yes (n=20) No (n=145)	10 (50.0) 28 (19.3)	0.003*
Bleeding after sex	Yes (n=2) No (n=163)	0 38 (23.3)	0.431
Stress incontinence	Yes (n=21) No (n=144)	4 (19.1) 34 (23.6)	0.609
Smelly vaginal discharge	Yes (n=70) No (n=95)	11 (15.7) 27 (28.4)	0.689
Hypermenorrhoea	Yes (n=36) No (n=129)	10 (27.8) 28 (21.7)	0.420
Irregular menstruation	Yes (n=61) No (n=104)	11 (18.0) 27 (26.0)	0.274
Difficulty in conceiving	Yes (n=20) No (n=145)	3 (15.0) 35 (24.1)	0.340
Frequent miscarriage	Yes (n=12) No (n=153)	4 (33.3) 73 (47.7)	0.564

* = statistically significant; FGS = female genital schistosomiasis

Table 5: Association of self-reported UGS symptoms and *Schistosoma haematobium* egg excretion

UGS reported symptom	Category	Number (%) of egg excretion	*p* value
Painful urination	Yes (n=51)	25 (49.0)	0.01*
	No (n=114)	13 (11.4)	
Blood in urine	Yes (n=41)	12 (29.3)	0.260
	No (n=124)	26 (21.0)	
Frequent urination	Yes (n=50)	11 (22.0)	0.770
	No (n=115)	17 (14.8)	

* = statistically significant; UGS = urogenital schistosomiasis

The frequency of UGS symptoms reported by participants include blood in urine (24.8%, n=41), painful urination (30.9%, n=51) and frequent urination (30.3%, n=50). Associating symptoms reported by participants and *S. haematobium* egg shed in urine showed that among the UGS clinical manifestation reported, only painful urination was significantly associated (p=0.01) with egg shedding as shown in Table 5. Blood in urine and frequent urination had p>0.05, therefore not significantly associated with egg excretion

Association of UGS and FGS with sociodemographic factors of participants:

Differences in prevalence rate of UGS and FGS were considered along several variables such as area of residence, educational level, age, marital status, religion, occupation, infertility and sub-fertility. The relationship between UGS and FGS with the sociodemographic factors is summarized in Table 6. Factors such as the area of residence, religion, marital status and age group shows no significant relationship with the prevalence of UGS. However, it was observed that level of education and occupation had significant relationship with UGS (p<0.05) with the disease being more prevalent among participants with only primary education (43.8%), housewives (51.4%) and fishing (83.3%) compared to other groups (Table 6).

No significant association was observed when relating FGS with sociodemographic factors such as age (x^2=0.758, p=0.158), marital status (x^2=3.715, p=0.156), area of residence (x^2=13.685, p=0.188), level of education (x^2=4.269, p=0.640), occupation (x^2 = 1.211, p=0.997), and religion (x^2=0.113, p= 0.945). However, the prevalence of UGS was significantly higher in participants with primary level education (43.8%, n=49, p=0.038) and fishing (83.3%, n=12, p=0.000) as occupation.

There was no significant association between intensity of egg excretion and FGS. Further analysis revealed that the prevalence of FGS only was 45.8% and prevalence of FGS with egg excretion in urine was 14.1%. Females between the ages of 15–24 and 25–34 years had the highest prevalence of FGS with egg excretions, while the prevalence of FGS increased with age (Fig 1).

Table 6: Association of UGS and FGS with socio-demographic characteristics of the study participants

Variable	Category	No (%) with UGS	Chi square & *p* values	No (%) with FGS	Chi square & *p* values
Area of residence	Matta Barrage (n=74)	31 (41.9)	x^2 = 1.453	32 (43.2)	x^2 = 13.685
	Matta Village (n=32)	12 (37.5)	p = 0.918	18 (56.3)	p = 0.188
	Mambonkor Bord (n=14)	5 (35.7)		7 (50.0)	
	Mambonkor Village (n=28)	11 (39.3)		17 (60.7)	
	Makossa (n=17)	5 (29.4)		11 (64.7)	
Age group (years)	15 – 24 (n=88)	34 (38.6)	x^2 = 19.563	43 (48.9)	x^2 = 0.758
	25 – 34 (n=42)	14 (33.3)	p = 0.927	23 (54.8)	p = 0.158
	≥ 35 years (n=35)	16 (45.7)		19 (54.3)	
Marital status	Married (n=100)	39 (39)	x^2 = 0.039	52 (52.0)	x^2 = 3.715
	Single (n=65)	25 (38.5)	p = 0.843	33 (50.8)	p = 0.156
Level of education	Primary (n=112)	49 (43.8)	x^2 = 8.446	53 (47.3)	x^2 = 4.269
	Secondary (n=53)	15 (28.3)	p = 0.038*	32 (60.4)	p = 0.640
Occupation	Housewife (n=35)	18 (51.4)	x^2 = 21.042	18 (51.4)	x^2 = 1.211
	Farmer (n=58)	15 (25.9)	p = 0.000*	31 (53.4)	p = 0.997
	Fishing (n=12)	10 (83.3)		5 (41.7)	
	Pupil/students (n=53)	21 (39.6)		27 (50.9)	
	Civil servant (n=7)	0		4 (57.1)	
Religion	Christianity (n=107)	43 (40.2)	x^2 = 0.115	56 (52.3)	x^2 = 0.113
	Islam (n=58)	21 (36.2)	p = 0.734	29 (50.0)	p = 0.945

* = statistically significant; FGS = female genital schistosomiasis; UGS = urogenital schistosomiasis

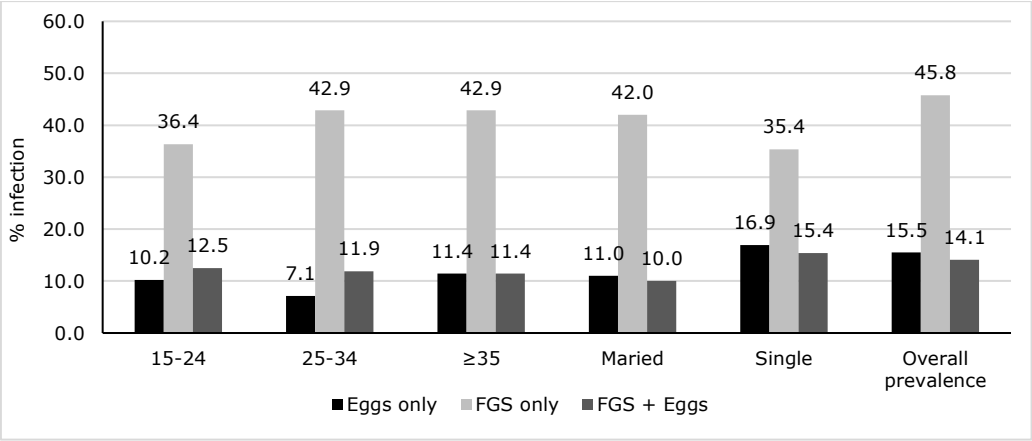


Fig 1: Distribution of participants with female genital schistosomiasis (FGS) across age groups and marital status

Table 7: Association between urogenital schistosomiasis (UGS) and water contact behavior

Variable	Category	No (%) with UGS	Chi square and *p* values
Lake usage	Yes (n=158) No (n=7)	64 (40.5) 0	x^2 = 4.880 *p* = 0.027*
Lake usage frequency	Daily (n=79) Weekly (n=53) Monthly (n=33)	40 (50.6) 18 (34) 6 (18.2)	x^2 = 9.583 *p* = 0.008*
Bathing	Yes (n=70) No (n=95)	37 (52.9) 27 (28.4)	x^2 =8.571 *p* = 0.003*
Laundry	Yes (n=139) No (n=26)	61 (43.9) 3 (11.5)	x^2 = 8.897 *p* = 0.003*
Crossing	Yes (n=100) No (n=65)	43 (43) 21 (32.3)	x^2 = 1.924 *p* = 0.165
Swampy farming	Yes (n=113) No (n=52)	47 (41.6) 17 (32.7)	x^2 = 0.829 *p* = 0.363
Fishing	Yes (n=24) No (n=141)	13 (54.2) 51 (36.2)	x^2 = 2.361 *p* = 0.124
Playing in the lake	Yes (n=43) No (n=122)	17 (39.5) 47 (38.5)	x^2 = 0.049 *p* = 0.824
Access to improved water source	Yes (n=120) No (n=45)	47 (39.2) 17 (37.8)	x^2 = 0.047 *p* = 0.828

* = statistically significant

Association between UGS and water contact behavioral pattern of the population:

The association between UGS and water contact behavior is represented in Table 7. It was observed that lake usage, frequency of lake usage and domestic lake activities had significant relationships with UGS ($p<0.05$), with the disease being more common in those who use the lake compared to those who do not. Other factors such as access to improved water source, crossing, swampy farming, fishing and recreational lake activities (playing) show no significant relationship with UGS with $p>0.05$.

Markers of oxidative stress:

Table 8 shows the effects of UGS and FGS on the levels of oxidative stress markers in serum and vaginal secretion. GSH levels observed was significantly lower ($p<0.05$) in the serum of participants with UGS and FGS compared to the uninfected. There was no significant ($p=0.085$) difference in serum GSH levels of participants with UGS compared to those with FGS. The level of GSH in vaginal secretions was significantly lower ($p<0.05$) in participants with UGS and FGS compared to the uninfected (mean=7.85±0.464). There was a significant difference in the levels of GSH between participants with FGS and UGS ($p=0.043$).

Catalase activity was observed to be significantly higher in serum of uninfected participants (mean=2.13±0.41) compared to participants with UGS (mean=0.99±0.31) ($p=0.019$). Catalase activity was also significantly higher in serum of uninfected participants (mean=2.21±0.32) compared to participants with FGS (mean=0.88±0.25) ($p=0.011$). However, there was no significant differ-

Table 8: Mean values of oxidative stress markers in women with different *Schistosoma* infection status

		Serum				Vaginal secretion			
Parameter	**Pairs**	**Mean ± SD (µg/mL)**	**SE of mean**	***t***	***p* value**	**Mean ± SD (µg/mL)**	**SE of mean**	***t***	***p* value**
GSH	Uninfected	11.85±0.784	0.105	3.375	0.041	7.65 ± 0.682	0.110	3.715	0.037
	UGS	9.05 ± 0.713	0.153			5.19 ± 0.701	0.113		
	Uninfected	11.77 ± 0.612	0.113	5.681	0.037	7.85 ± 0.464	0.106	6.616	0.031
	FGS	8.21 ± 0.442	0.120			3.45 ± 0.492	0.112		
	UGS	9.12 ± 0.575	0.144	0.633	0.085	5.00 ± 0.637	0.146	2.456	0.043
	FGS	8.21 ± 0.442	0.120			3.45 ± 0.492	0.112		
CAT	Uninfected	2.13 ± 0.41	0.020	3.175	0.019	1.52 ± 0.28	0.015	3.575	0.003
	UGS	0.99 ± 0.31	0.010			0.89 ± 0.23	0.009		
	Uninfected	2.21 ± 0.32	0.019	3.899	0.011	1.49 ± 0.27	0.021	5.856	0.003
	FGS	0.88 ± 0.25	0.008			0.42 ± 0.11	0.008		
	UGS	0.94 ± 0.33	0.009	0.726	0.058	0.91 ± 0.21	0.009	3.456	0.003
	FGS	0.88 ± 0.25	0.008			0.42 ± 0.11	0.008		
NO	Uninfected	39.03 ± 4.73	0.767	4.736	0.001	37.34 ± 5.30	0.679	3.136	0.001
	UGS	70.6 ± 7.77	1.261			67.1 ± 8.93	0.991		
	Uninfected	38.2 ± 5.23	1.169	5.123	0.001	36.1 ± 5.23	1.041	4.326	0.001
	FGS	74.63 ±3.38	0.757			78.37 ± 5.82	0.821		
	UGS	72.34 ± 4.01	0.897	0.377	0.103	66.2 ± 4.91	0.897	.775	0.031
	FGS	74.63 ± 3.38	0.757			78.37 ± 5.82	0.821		
SOD	Uninfected	3.97 ± 0.41	0.038	2.736	0.011	3.02 ± 0.38	0.031	6.236	0.001
	UGS	3.09 ± 0.36	0.031			2.08 ± 0.33	0.019		
	Uninfected	3.83 ± 0.43	0.036	3.236	0.032	3.83 ± 0.43	0.036	12.316	0.032
	FGS	2.88 ± 0.45	0.027			1.52 ± 0.30	0.017		
	UGS	3.17 ± 0.36	0.029	0.377	0.091	2.10 ± 0.31	0.022	1.177	0.04
	FGS	2.88 ± 0.45	0.027			1.52 ± 0.30	0.014		
MDA	Uninfected	0.599 ± 0.111	0.031	2.136	0.001	0.634 ± 0.114	0.031	2.736	0.001
	UGS	1.143 ±0.265	0.019			1.253 ± 0.210	0.013		
	Uninfected	0.587 ± 0.102	0.036	2.636	0.032	0.587 ± 0.102	0.056	4.236	0.032
	FGS	1.168 ± 0.247	0.017			1.625 ± 0.231	0.017		
	UGS	1.101 ± 0.210	0.022	0.375	0.04	1.201 ± 0.190	0.011	1.377	0.041
	FGS	1.168 ± 0.247	0.014			1.625 ± 0.231	0.014		

ence in serum catalase activity in participants with UGS and FGS (p=0.058). The catalase activity in vaginal secretion was significantly lower in participants with FGS and UGS compared to the uninfected (p<0.05). It was also observed that the difference in catalase activity in the secretions of participants with UGS and FGS was statistically significant (p= 0.003).

The level of nitric oxide in serum of the uninfected participants (mean=39.03±4.73) was observed to be significantly lower (p= 0.001) than that of participants with UGS and FGS (mean=70.6±7.77) as shown in Table 8. However, there was no significant difference in the serum NO levels between participants with UGS and FGS (p=0.103). The level of NO in vaginal secretions of participants with UGS and FGS were significantly higher than that of the uninfected (p<0.05). NO levels were also significantly higher in vaginal secretions of participants with FGS than in those with UGS (p=0.031).

The SOD activity in serum of the uninfected participants was observed to be significantly higher than those of participants with UGS and FGS (p<0.05) as shown in Table 8. No significant difference in serum SOD activity was observed between participants with UGS and FGS (p=0.091). However, there was a significant difference in SOD activities of vaginal samples of participants with UGS and FGS compared to the SOD activity levels in the uninfected participants as shown in Table 8.

SOD activity was also significantly lower in those with FGS compared to those with UGS.

Results from pairwise comparison of MDA levels in participants with different infection status showed significantly lower levels of serum MDA in uninfected participants compared to those of participants with UGS and FGS. MDA levels in participants with UGS showed no significant difference to those with FGS ($p< 0.05$). There were significantly higher MDA levels in vaginal secretions of participants with UGS and FGS compared to the uninfected. There was also a significantly higher MDA level in vaginal secretion of participants with FGS compared to secretion of participants with UGS.

Levels of inflammatory cytokines in serum and vaginal secretions:

The mean concentration of levels of inflammatory cytokines in both serum and vaginal secretion were determined and FGS effect on the cytokine levels is shown in Table 9. Results obtained shows that there was no significant correlation between FGS infected TNF-α, INF-γ, IL-1β, IL-6 and IL-10 levels in serum and vaginal secretion. There was no significant difference ($p>0.05$) in the cytokine profiles in serum and vaginal secretion between participants with FGS and non-infected participants.

Discussion:

The molecular identification of *S. haematobium* and *S. mansoni* infection gave a prevalence of 100% and 94.5% respectively. This reveals the co-endemicity of the area with both schistosome species. However, the prevalence of *S. haematobium* infection in this study is higher than the 63.8% UGS prevalence reported by Masong et al., (23). This higher prevalence may be due to the use of a more sensitive diagnostic method that was able to detect schistosomes in all its stages of development in blood. Nevertheless, among those infected, just 23.5% shed eggs in urine which is relatively lower compared to that reported by Njunda et al., (4) in Matta secondary and primary schools (41.1%) and that reported by Masong et al., (23) in some fishing communities in the Matta health area (63. 8%).

The prevalence of UGS (haematuria + egg shed) in this study was observed to be 45.7%, which is similar to that of a study carried out by Njunda et al., (4) across Matta Barrage community alone. The prevalence observed was relatively similar to that reported in other schistosomiasis foci in the South West region; 40.27% in Munyenge and 41.3% in Marumba (33). However, the prevalence of UGS observed in this study is higher compared to the overall prevalence of schistosomiasis reported in the West Region by Tchuem-Tchuente et al., (34) and in other areas of Cameroon including 37% in Tiko (33), 1.7% in Kékem (6), and 22.9% in Maroua (35). The higher prevalence of UGS reported in our study might be due to the difference in geographical settings, while Tiko, Kekem and Maroua are semi-urban/urban areas with improved water sources which limits the frequency of human water contacts (35), Matta health area is a rural area with natural and artificial ponds, and the presence of a lake on which their daily activities rely. Because Matta health area is an endemic area that has been targeted regularly for control of schistosomiasis and soil transmitted helminths through mass drug administrations, this may have accounted for the lower prevalence of urine egg excretion reported in the current study. Independent of the lower egg excretion prevalence, the higher prevalence with results from PCR revealed that either the infection was still at its early stage or some eggs were trapped in body tissues.

The overall estimated prevalence of FGS

Table 9: Association of inflammatory cytokines and FGS status

		Serum			Vaginal secretion		
Parameter	**Pairs**	**Mean ± SD (pg/ml)**	***t***	***p* value**	**Mean ± SD (pg/ml)**	***t***	***p* value**
TNF-α	Uninfected	13.75 ± 1.45	0.06	0.995	20.31 ± 1.64	-0.538	0.592
	FGS	13.71 ± 2.05			21.80 ± 2.32		
INF-γ	Uninfected	15.58 ± 2.22	0.195	0.845	22.51 ± 2.32	-0.198	0.843
	FGS	14.85 ± 3.10			23.25 ± 2.99		
IL-1β	Uninfected	13.92 ± 0.24	-0.99	0.922	13.52±0.24	-0.593	0.554
	FGS	13.98 ± 0.69			13.85±0.57		
IL-6	Uninfected	134.95 ± 21.36	0.546	0.586	151.54±21.86	0.435	0.664
	FGS	116.20 ± 26.27			136.94±24.81		
IL-10	Uninfected	277.99 ± 40.77	0.910	0.365	347.81± 40.61	-0.06	0.995
	FGS	220.05 ± 45.83			348.20±49.1		

(59.9%) in this study is similar to Masong et al., (23), who reported a prevalence of 58%. This similarity could be as a result of the sensitivity of the diagnostic techniques and the signs and symptoms on which diagnosis was based. Our study explored PCR identification of schistosome DNA and signs such as vaginal itches, burning sensation and abdominal pain for diagnosis whereas vaginal lesions by colposcopy, egg load in urine and other similar criteria, which were used in the study by Masong et al., (23). FGS prevalence was solely due to *S. haematobium* infection and no association with *S. mansoni* was observed. FGS appeared to be higher in married women than in unmarried women and was also higher in females aged ≥ 35 years compared to other age groups. This is similar to Masong et al., (23) who reported a higher FGS prevalence in those aged ≥29 years and those married. Unlike Masong et al., (23) which reported that single females did not show any "FGS + eggs", our current study reported a higher prevalence of "FGS + eggs" in singles (15.4%) than that in married women (10%).

Painful urination was identified as the most common symptom associated with *S. haematobium* infection which is not very different from the findings of a similar study by Saotoing et al., (35) on urinary schistosomiasis in the Northern regions of Cameroon and Njunda et al., (4) in the Matta Barrage community. Frequent contact with the lake was identified as the associated factor for infection with the *Schistosoma* parasite. Many inhabitants in Cameroon especially in rural areas have limited access to portable water for domestic use leaving them with the option of using natural water bodies such as lakes, rivers, ponds and dams which may be infested with the parasite (34). As the lake was the main source of water for fishing, irrigation and domestic use, it is more likely that the people became infected as they come in contact with the infested water.

The study participants appeared to have good knowledge on schistosomiasis with most of the information coming from community health workers. This result is different from that reported by Njunda et al., (4) and Kamga et al., (36) which reported lack of knowledge of the disease in Cameroon. This improvement in knowledge can be attributed to the mass drug administration of Praziquantel under the National Program for the Control of Schistosomiasis and Soil Transmitted Helminths which is being conducted yearly and usually involves sensitization of the population (37). This mass drug administration has made it possible for some rural areas to receive education concerning schistosomiasis and its clinical manifestations.

Given that there is need for the human host to maintain a critical balance between antioxidant defence mechanisms and the generation of reactive oxygen species and other free radicals, the measurement of the antioxidant capacity, level of oxidative damage and level of reactive oxygen species were carried out. The measurement of the antioxidant capacity was done using the levels of reduced glutathione, superoxide dismutase and catalase, the level of oxidative destruction was assessed using malondialdehyde, and the level of reactive oxygen species was carried out by measuring nitric oxide. Catalase is responsible for the conversion of hydrogen peroxide to water and oxygen and it was observed that the levels of catalase activity was significantly lower in serum and vaginal secretions of women with UGS and FGS compared to the uninfected. This result is in line with that reported by Aloho-Bekong et al., (38) which evaluated the level of oxidative stress is serum of school children with schistosomiasis and other helminthiasis.

The level of catalase activity was also seen to be significantly lower in vaginal secretions of women with FGS compared to those with UGS. This significant reduction of catalase activity can be attributed to the effect of the *Schistosoma* parasite on the liver. The liver is the major organ involved in the production of catalase and schistosomiasis is known to induce liver fibrosis (39), which can account for the decreased catalase level during infection. The significant reduction of catalase activity in vaginal secretion of women with FGS compared to those with UGS can also be attributed to the fact that the parasite feeds on red blood cell which is one of the cells with the most amount of catalase (9). Also, since accumulation of schistosome egg in host tissue causes inflammation and granuloma characterized by reactive oxygen species production, it can be said that the reactive oxygen species produced in FGS has resulted to the modification of the catalase enzyme and its synthesis. This is because reactive oxygen species has been shown to have several dysfunctional effects on various cellular components that are involved in the transcription and translation of enzymes including catalase (15).The reduced activity of catalase will result to severe oxidetive stress due to the accumulation of hydrogen peroxide within host tissues.

Superoxide dismutase (SOD) acts as the first line of defence against oxidative stress by converting superoxide to hydrogen peroxide and the hydrogen peroxide is further converted to water and oxygen by catalase (40). The level of SOD activity was observed to be significantly lower in serum and vaginal secretion of women with UGS and FGS compared to the uninfected. This result is similar to that reported by Aloho-Bekong et al., (38) which evaluated the level of oxidative stress is serum of school children with schistosomiasis

and other helminthiasis. The results obtained in this study can be attributed to the fact that hydrogen peroxide is released by phagocytic cells as a means to fight against helminthic infections resulting to high levels of hydrogen peroxide which will exert a negative feedback mechanism on the activity of SOD (41). Also, schistosomiasis has been shown to interfere with the activity of antioxidant enzymes thr-ough a mechanism that affects the expres-sion of the enzymes (42).

Nitric oxide (NO) produced by the enzyme nitric oxide synthase during inflam-mation act as a signalling molecule and a vasodilator for rapid blood flow to the inflamed organ (43). The level of NO was observed to be significantly higher in serum and vaginal secretion of women with UGS and FGS com-pared to the uninfected, which is similar to the findings of a study conducted by Nkam et al., (44) on the implication of serum NO and IgE in urinary schistosomiasis in Bamendjing, West region of Cameroon. The higher level of NO can be attributed to the ability of schisto-somal eggs to induce inflammation in various tissues and organs and investigations have shown that during schistosomiasis induced inflammation there is increased nitric oxide synthase expression in macrophages which are not normally expressed in resting cells (22). This nitric oxide acts as a precursor of other reactive species like nitroxyl anion, nitrosonium cation and higher oxides of nitrogen, S-nitrosothiols and dinitrosyl iron complexes. These substances have the ability to induce oxidative damage to other biomole-cules (43).

Malondialdehyde (MDA) is known to be a characteristic product of lipid peroxidation induced by reactive oxygen species during oxidative stress conditions (38). The level of MDA in serum and vaginal secretion of UGS and FGS infected persons was observed to be higher than that of the uninfected. This result is in line with that reported by Aloho-Bekong et al., (38) which investigated on the level of oxidative stress in serum of school children with schistosomiasis and other helminthiasis in Makenene, Centre Region of Cameroon. The results are also similar to the findings of Facundo et al., (45) which studied the levels of lipids peroxidation in patients with schistoso-miasis. These results can be attributed to the increased release of reactive oxygen species in schistosomiasis principally by phagocytic macrophages as a means to fight infection (22). There was also a significantly higher level of MDA in vaginal secretion of women with FGS compared to those with UGS indi-cating severe oxidative stress induced in the urogenital tract due to the presence of parasite eggs.

Reduced glutathione (GSH) plays a very important role in removing hydrogen per-oxide produced to prevent oxidative stress. The enzyme glutathione peroxidase catalyses the reduction of GSH by hydrogen peroxide to oxidised glutathione (GSSG) which is reversed by the enzyme glutathione reductase (46). The level of GSH in both serum and vaginal secretion was observed to be higher in the uninfected compared to those with UGS and FGS. This reduction in GSH in infected persons can be attributed to the increased cytotoxicity associated with hydrogen peroxide release which has an inactivation effect on the enzyme glutathione reductase responsible for GSH turnover from GSSG (47). The measurement of these biomarkers of oxidative stress was done in both serum and vaginal secretion due to the fact that serum levels do not give a clear indication of the level of tissue specific damage (46). This was observed to be so given that though there was no significant difference in levels of the various biomarkers in serum of women with UGS compared to those with FGS, there exist a significant difference in these biomarkers in vaginal secretions of the women with UGS when compared to those with FGS.

In this study, the mean TNF-α, INF-γ, IL-1β, IL-6 and IL-10 levels between women with FGS and uninfected for both serum and vaginal secretion was not statistically different from each other. The presence of other helminths in the study site might have infected the participants together with schistosomes and this might be responsible for these find-ings. No correlation was observed between TNF-α, INF-γ, IL-1β, IL-6 and IL-10 levels in serum and vaginal secretion of women with FGS. This agrees with the study by Njaanake et al., (48) which reported no correlation between levels of urinary and serum IL6, IFN-γ, TNF-α and IL-10 among children. These findings suggest that there may be differences between systemic and local mucosal cytokine secretion during infections in the genital tract. T_{h1} immune cytokines (TNF-α, INF-γ, IL-1β and IL-6) favour the production of proinflam-matory responses to schistosomes infection and activate immune cells while T_{h2} immune cytokines (IL-10) tend to produce anti-inflam-matory response.

IL-10 plays a crucial role in immuno-regulation and inflammation. It represents an important element in the down-regulation of the T_{h1} response by preventing excessive inflammation in the early stages of the infec-tion limiting tissue damage. However, this may also allow the schistosome to persist in its host, as the immune response is not strong to eliminate it, leading to chronic disease. This study shows a significantly higher IL-10 cyto-kine level compared to that of T_{h1} cytokines levels. The review done by Masamba et al., (22) indicate that a shift towards a more anti-inflammatory immune response of schistoso-miasis infection may contribute to the severity

of the disease. TNF-α, INF-γ and IL-1β are proinflammatory cytokines that are typically elevated during the acute phase of the disease (22). They play a role in activating the immune cells, promoting inflammation and recruiting immune cells to the site of infection. As the infection progresses, there is a shift toward a more anti-inflammatory immune response leading to a decrease in these cytokines level as seen in the current study.

IL-6, is a pleotropic cytokine that has both anti-inflammatory and proinflammatory response (49). It is produced in response to the infection and tissue damage. In this study, its level was significantly higher compared to other proinflammatory cytokines evaluated. This might be due to the fact that it also helps to mobilise immune cells to the site of infection thereby facilitating the pathogenesis of the disease (50).

Conclusion:

The present study reports a *S. haematobium* prevalence of 23.5% by microscopic and morphological identification. Molecular identification gave prevalence of 100.0% and 94.5% in bloodspot for *S. haematobium* and *S. mansoni* respectively, and the prevalence of FGS in the Matta Health area was 60%. Lake usage, marital status and symptoms such as abdominal pain, painful urination, vaginal itches and burning sensation were significantly associated with UGS in the Matta Health area. Marital status was identified as a key risk factor among participants, with single women at higher risk of contracting schistosomiasis compared to married women.

Higher levels of malondialdehyde and nitric oxide, and lower levels of reduced glutathione, catalase and superoxide peroxidase activities in UGS and FSG indicate oxidative stress in women living in the Matta Health area. High levels of IL-10 in serum and vaginal secretion compared to proinflammatory cytokines (IL-1β, IL-6, INF-γ and TNF-α) might prevent immunopathology and also limit the severity of the disease, with a shift to T_{h2} immune response.

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Contributions of authors:

MSDN conceived and designed the study; SIU, MDE, FIA, and CDN collected samples and carried out the major part of study; MSDN, SIU, CDN, and PAN analyzed the data and wrote the manuscript; MSDN, MBT, and AFL made provision of reagents and proofread the manuscript. All authors reviewed and approved the manuscript submitted for publication.

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Original Article **Open Access**

Prevalence of urinary schistosomiasis among school-aged children in Ipogun community, Ondo State, Nigeria

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Abstract:

Background: Urinary schistosomiasis remains a public health challenge among school-aged children in Nigeria. The disease is caused by the blood fluke parasite, *Schistosoma haematobium* and harbored by freshwater snail intermediate hosts. Contact with infected surface water bodies made children vulnerable. This study investigated the prevalence of urinary schistosomiasis among school-aged children in Ipogun community, Ondo State, Nigeria.
Methodology: A total of 182 school-aged children (5-12 years of age) were randomly selected from the community for the study. Urine samples were collected from each participant on three consecutive days for macroscopic and microscopic examinations of the sediment to detect *S. haematobium* ova and determine the mean egg counts for positive cases. Information on children activities, knowledge of schistosomiasis and its treatment, socioeconomic status, demographics, risk factors and clinical manifestations, were obtained through structured questionnaires. Data were analysed using the Statistical Package for Social Sciences. Associations between variables were determined using ANOVA and Chi square test. Significant level was determined at $p \leq 0.05$.
Results: The prevalence of urinary schistosomiasis among the children was 32.4%, with prevalence of 23.1% for haematuria. The prevalence of urinary schistosomiasis was higher in males (34.5%) than females (28.6%) but the difference was not statistically significant ($p=0.42$). Participants in the 8-10 and 11-12-year age groups had the highest prevalence of urinary schistosomiasis (35.3% each, $p=0.36$) and those in the age group 11-12 years had the highest haematuria prevalence (35.3%, $p=0.18$) but the difference in prevalence was not statistically significant. Difference in the mean *S. haematobium* egg count across the age groups was also not statistically significant ($p=0.85$).
Conclusion: Since relatively high prevalence of urinary schistosomiasis was observed in the study area, there is need for urgent attention to control the infection in the study area.

Keywords: urinary schistosomiasis, egg count; prevalence, school-aged children, Ondo State, Nigeria

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Prévalence de la schistosomiase urinaire chez les enfants d'âge scolaire de la communauté d'Ipogun, État d'Ondo, Nigéria

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Résumé:

Contexte: La schistosomiase urinaire demeure un problème de santé publique chez les enfants d'âge scolaire au Nigéria. Français La maladie est causée par le parasite de la douve du sang, *Schistosoma haematobium*, et hébergée par des hôtes intermédiaires d'escargots d'eau douce. Le contact avec des plans d'eau de surface infectés a rendu les enfants vulnérables. Cette étude a examiné la prévalence de la schistosomiase urinaire chez les enfants d'âge scolaire de la communauté d'Ipogun, dans l'État d'Ondo, au Nigéria.
Méthodologie: Un total de 182 enfants d'âge scolaire (5 à 12 ans) ont été sélectionnés au hasard dans la communauté pour l'étude. Des échantillons d'urine ont été prélevés sur chaque participant pendant trois jours consécutifs pour des examens macroscopiques et microscopiques du sédiment afin de détecter les œufs de *S. haematobium* et de déterminer le nombre moyen d'œufs pour les cas positifs. Des informations sur les activités des enfants, les connaissances sur la schistosomiase et son traitement, le statut socio-économique, les données

démographiques, les facteurs de risque et les manifestations cliniques ont été obtenues au moyen de questionnaires structurés. Les données ont été analysées à l'aide du progiciel statistique pour les sciences sociales. Les associations entre les variables ont été déterminées à l'aide d'une ANOVA et du test du Chi carré. Le niveau de signification a été déterminé à $p \leq 0,05$.
Résultats: La prévalence de la schistosomiase urinaire chez les enfants était de 32,4%, avec une prévalence de 23,1% pour l'hématurie. La prévalence de la schistosomiase urinaire était plus élevée chez les hommes (34,5%) que chez les femmes (28,6%), mais la différence n'était pas statistiquement significative (p=0,42). Les participants des groupes d'âge 8-10 et 11-12 ans avaient la prévalence la plus élevée de schistosomiase urinaire (35,3% chacun, p=0,36) et ceux du groupe d'âge 11-12 ans avaient la prévalence la plus élevée d'hématurie (35,3%, p=0,18), mais la différence de prévalence n'était pas statistiquement significative. La différence dans le nombre moyen d'œufs de *S. haematobium* entre les groupes d'âge n'était pas non plus statistiquement significative (p=0,85).
Conclusion: Étant donné la prévalence relativement élevée de la schistosomiase urinaire observée dans la zone d'étude, il est urgent de lutter contre l'infection.

Mots-clés: schistosomiase urinaire, numération des œufs ; prévalence, enfants d'âge scolaire, État d'Ondo, Nigéria

Introduction:

Urinary schistosomiasis is a water borne parasitic disease affecting over 240 million people of all ages globally (1,2). The disease is second to malaria in parasitic burden (2). It is also known as snail fever or bilharzia, named after its discoverer, Theodor Bilharz (3,4). Urinary schistosomiasis is caused by the blood fluke trematode, *Schistosoma haematobium*, and man serves as the definitive host (5). Adult schistosome worms lay eggs in the blood vessels supplying the bladder of man (6) and these eggs are released into the water bodies through urination. Under appropriate conditions the eggs hatch and release miracidia which swim to locate freshwater snail intermediate hosts. The miracidia undergo series of developmental changes inside the freshwater snail intermediate hosts until a final stage called cercariae emerged (5). Infection takes place when cercariae of the schistosome parasite penetrates human skin and then migrate through the circulatory system to develop into adult worms (7).

Urinary schistosomiasis has an important impact on human health, leading to reduced productivity and reproductive issues, and causing severe medical conditions (2). It is prevalent in many parts of the world including Nigeria (8,9), posing a significant challenge to public health with social and economic consequences (10). The disease disproportionately affects school-aged children due to their frequent water contact during domestic and recreational activities which lead to severe morbidity such as haematuria, bladder dysfunction, and chronic kidney damage (11-13).

Nigeria is one of the countries bearing highest burdens of urinary schistosomiasis globally and endemic in many rural and peri-urban areas especially where there is limited access to clean water and sanitation facilities (14,15,16). The transmission is still active in Nigeria and poses a serious health challenge. Although Mass Drug Administration (MDA) has been used to reduce morbidity, but this has not been adequate to abate transmission in many areas in the country (1). The country in its National Schistosomiasis Elimination Progr amme (NSCHEP) with the support of international partners has been implementing preventive chemotherapy with the use of praziquantel for fifteen years which has yielded reduction in morbidity but yet transmission in some communities is still noticeable. Urinary schistosomiasis related issues such as haematuria are sometimes linked to school-aged group and some communities regard the condition as normal and sign of maturity, due to lack of knowledge about the disease (13,17).

The disease is known to reduce cognitive and academic abilities of children and also results in poor physical development. The World Health Organization (WHO) set morbidity reduction of <5% prevalence target in children aged 5 to 14 years by 2020 for endemic countries, yet Nigeria is still far from achieving this target despite efforts to mitigate the transmission (1). Five years beyond, the WHO target calls for determination of the infection status in most endemic areas in Nigeria. This study focused on urinary schistosomiasis among school-aged children in Ipogun community of Ondo state, Nigeria with the objective of determining the prevalence of urinary schistosomiasis among school-aged children in the community. Although various researches on schistosomiasis had been carried out in this community (18-20) which had invoked control interventions, schistosomiasis appeared to persist in this area. Thus, monitoring the successes of such interventions becomes necessary, hence, the need for this study.

Materials and method:

Study area:

The study was conducted in Ipogun community (7°19'N; 5°05'E), a town in Ifedore Local Government Area (LGA) of Ondo State, Nigeria. The "Aponmu" river, which flows through the community serves as major source of water for agricultural and domestic purposes. The river had been reported to harbour freshwater snail species including *Bulinus* species (19), which are good intermediate hosts of *S. haematobium* that cause urinary schistosomi-

asis.

Children usually visit the river for recreational activities while women visit the river to fetch water for domestic and household usage, and most men who are primarily farmers use the water for farming activities. Other sources of water in the community include "Asala" and "Odo-lona-oko" rivers which are also visited by the community members. Ipogun community was purposely chosen for this study based on the earlier findings of urinary schistosomiasis in the area (18).

Ethical consideration:

Ethical approval (OSHREC08/09/22/472) to conduct this study was obtained from the State Health Research Ethics Committee, Ministry of Health, Akure, Ondo State, Nigeria. Approval to conduct the study was also granted by the community leaders, school heads and parents of the children who participated in the study.

Study design and participants:

This was a descriptive cross-sectional study of school-aged children (5-12 years old) carried out between January and March 2024, upon obtaining the consent of their parents.

Sample size calculation and sampling:

The sample size of 182 children was determined using the formula of Taro Yamane for a finite population (21). The children were randomly selected from six primary schools in the local government area that gave approval for the children to participate in the study. Only children enrolled in the selected schools and those whose parents consented to participate were included in the study. Children who had not been in the community for a year or more, as well as those whose parents or guardians declined consent were excluded from the study.

Sample and data collection:

Clean-catch specimen of terminal urine were collected from selected children between the period 12:00 noon and 2:00 p.m. Individual participant/parent/guardian received a sterile, screw-capped universal bottle labelled with a unique identification number (22). The urine samples were collected on three consecutive days from each study participant. The samples were immediately stored in a cool box with ice packs before transportation to the Biological Sciences Laboratory, Joseph Ayo Babalola University, Ikeji-Arakeji, in black polythene bags to prevent the hatching of eggs from light penetration (22,23).

Structured questionnaires were also administered to collect information on socio-economic status, demographics, risk factors, and clinical manifestations from each participant/parent/guardian (22).

Parasitological examination of urine:

Macroscopic examination of the urine samples in each universal bottle was done to determine the colour for presence of blood and for clarity (22). The urine sample was first properly mixed to re-suspend urine deposits, following which 10 ml was transferred into a centrifuge tube using a sterile disposable syringe, and centrifuged for 5 minutes at 1500 rpm. Following the decantation of the supernatant, the tube was gently tapped to re-suspend the urine deposit. A drop of the re-suspended deposit was transferred to a clean grease-free glass slide with a Pasteur pipette and covered with a cover slide. The preparation was then examined under a compound light microscope (Olympus model CX31RBS FA) for the presence of *S. haematobium* eggs with 40x objective lens and an iris diaphragm suitably closed to obtain good contrast (22).

The entire sample on the glass slide was examined and the eggs with characteristic terminal spine were counted. The egg count was determined as; total eggs counts/total volume of the urine (×100). The mean (±standard deviation) egg count for each participant was determined from the egg counts of three consecutive samples.

Statistical analysis:

Data were analysed using the Statistical Package for Social Sciences (SPSS) version 21.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was used to determine differences in the mean ova count and Chi square test was used to determine association of infection prevalence with demographic and other factors, among the study participants. Significant level was determined at $p \leq 0.05$.

Results:

Socio-demographic characteristics and risk factors in the study participants:

A total of 182 children participated in the study (Table 1). Majority of the participants (n=119, 65.4%) were males while 63 (34.6%) were females. Of the 182 participants, 15 (8.2%) usually fetch water from rivers for domestic use while 167 (91.8%) usually fetch water for the same purpose from wells. Meanwhile, 123 (67.6%) of the participants claimed not to have contact with surface water either by entering to fetch water or by swimming. However, 59 (32.4 %) responded that they used to have contact with surface water.

Majority of the participants (n=174, 95.6%) claimed to have good knowledge of schistosomiasis while only 8 (4.4%) did not have any knowledge of the disease. However, 63 (34.6%) of the participants had received

treatment against schistosomiasis at one time or the other (Table 1). The occupation of the parents of the participants is also presented in Table 1. Those who engaged in farming were 33.0%, while 12.6% of them were fishermen. Others were civil servants and traders.

Prevalence and clinical features of urinary schistosomiasis among the study participants:

The prevalence of urinary schistosomiasis among the participants in the study area is presented in Table 2. The overall prevalence among the participants was 32.4% (59/182)**.** The prevalence of the infection was higher in males (34.5%, 41/119) than females (28.6 %, 18/63), but the difference was not statistically significant (χ^2**=**0.65, *p*=0.42).

The appearance of the urine of the study participants is shown in Table 3. Majority of the of the study participants (40.7%) had light yellow and cloudy urine, 27.5% had pale yellow and highly turbid urine, 12.1% had transparent and colorless urine, 11.0% had yellow and slightly turbid urine and 8.8 % had amber and moderately cloudy urine. Among the participants with light yellow and cloudy urine (n=74), 25.7% were infected, for pale yellow and highly turbid urine (n=50), 42.0% were infected, for yellow and slightly turbid urine (n=20), 50.0% were infected, for transparent and colorless urine (n=22), 22.7% were infected and for amber and moderately cloudy urine (n=16), 25.0% were infected. The prevalence of urinary schistosomiasis with respect to the different appearance of the urine was statistically significant (*p*=0.01) (Table 3).

Table 1: Demographic characteristics of the study participants in Ipogun community, Ondo State, Nigeria *

Variable	Categories	Frequency	Percentage (%)	95% CI
Sex	Male	119	65.4	60.21 – 69.91
	Female	63	34.6	30.09 – 49.79
Source of water for use	River	15	8.2	6.34 – 10.67
	Well	167	91.8	86.66 – 92.64
Water contact	No	123	67.6	61.50 – 70.74
	Yes	59	32.4	25.92 – 35.49
Informed about schistosomiasis	Yes	174	95.6	88.66 – 97.64
	No	8	4.4	2.44 – 6.51
Treated for schistosomiasis	No	119	65.4	61.50 – 70.74
	Yes	63	34.6	29.26 – 38.50
Occupation of parents	Civil servant	48	26.37	7.57 – 14.65
	Crop farming	60	33.00	25.92 – 35.49
	Trading	51	28.02	7.23 – 13.86
	Fishing	23	12.61	4.47 – 26.43

CI = Confidence interval.

Table 2: Prevalence of urinary schistosomiasis in relation to sex of study participants in Ipogun community, Nigeria

Sex	Number examined	Number infected (%)	χ^2	OR (95% CI)	*p* value
Male	119	41 (34.5)	0.65	1.314 (0.68-2.56)	0.42
Female	63	18 (28.6)			
Total	182	59 (32.4)			

OR=Odd ratio; CI=Confidence interval

Table 3: Prevalence of urinary schistosomiasis in relation to urine appearance of study participants in Ipogun community

Appearance of urine	Number examined (%)	Number infected	Prevalence of infection (%)
Light yellow and cloudy	74 (40.7)	19	25.7
Pale yellow and highly turbid	50 (27.5)	21	42.0
Yellow and slightly turbid	20 (11.0)	10	50.0
Colorless and Transparent	22 (12.1)	5	22.7
Amber and moderately cloudy	16 (8.8)	4	25.0
Total	**182 (100)**	**59**	**32.4**

% is calculated down the group, χ^2 = 7.78, *P* = 0.01.

Table 4: Prevalence of urinary schistosomiasis, haematuria and mean egg counts with respect to age groups of the study participants in Ipogun community, Ondo State, Nigeria

Age group (years)	Number examined	Number infected (%)	Number with haematuria (%)	Mean egg count/10 ml of urine
5 – 7	29	5 (17.2)	3 (10.3)	5 ± 2.4
8 – 10	119	42 (35.3)	27 (22.7)	8 ± 4.0
11-12	34	12 (35.3)	12 (35.3)	6 ± 3.0
Total	**182**	**59 (32.4)**	**42 (23.1)**	**5 ± 2.5**
		χ^2=2.05	χ^2=3.48	F=0.52
		p=0.36	p=0.18	p=0.85

Analysis was made at a significance level of 5%

The prevalence of urinary schistosomiasis in relation to age groups of the participants is presented in Table 4. The highest prevalence of 35.3% each was recorded among the participants in the age groups 8-10 and 11-12 years while the least prevalence of infection (17.2%) was recorded in the age group 5-7 years. However, the difference in prevalence was not statistically significant (χ^2 =2.05, p=0.36).

Similarly, participants in the age group 11-12 years had highest prevalence of haematuria (35.3%) followed by participants in the age group 8-10 years (22.7%) while the least (10.3%) was observed in group 5-7 years, but no statistically significant difference (χ^2=3.48, p=0.18) existed in the prevalence of haematuria among the different age groups (Table 4). Likewise, the there was no statistically significant difference in the mean *S. haematobium* egg count among the different age groups (F=0. 524, p=0.85).

Discussion:

The high prevalence of urinary schistosomiasis of 32.4% reported in this study confirms the continued endemicity of schistosomiasis in Ipogun community of Ondo State, Nigeria. This is in consonance with the findings of Kone et al., (24) and Balogun et al., (25) who reported schistosomiasis as still a common disease among Nigerian school-age children. The relatively high prevalence of urinary schistosomiasis in this study may be due to low mass treatment with praziquantel, the drug of choice for Mass Drug Administration (MDA) programme, in the study area.

Findings in this study showed that although the prevalence of urinary schistosomiasis was higher in males (34.5%) than females (28.6%), there was no significant disparity of infection based on gender (p= 0.42) whereas, past studies established significant differences in infection based on sex (26,27). The reason why this study did not show disparity of infection in relationship with gender might not be unconnected with the wide difference in the number of the males who participated in this study compared with the female participants. The number of male participants recruited was far higher than the female participants which was due to the low consent received from the parents of female children to participate in the study. Other studies however, attributed gender disparity of urinary schistosomiasis with swimming, fishing and herding of livestock, in which male children are more involved than the females (28).

Children within the age group 8-10 and 11-12 years had the highest prevalence of infection, which is in line with the report of Oniya et al., (18). In their study, children in these age groups were mostly affected by urinary schistosomiasis which they linked to frequent water contact and weaker immune defense. Therefore, providing health education that involve transmission and treatment of schistosomiasis at the elementary schools where majority of children at this age group could be easily reached is required to curb further increase in the prevalence of infection in the study area. Such education must be properly delivered to provide accurate and adequate knowledge. Also, there is need for community and government intervention to control the infection in the study area and possibly extended to other communities in Ondo State, Nigeria.

The behavioral practice of the study participants involving bathing or swimming in water bodies agrees with the findings in other African countries such as Malawi (29), Egypt (30) and Tanzania (31), which means, children naturally love to visit rivers and streams for recreations. Therefore, establishing public swimming pools that are well managed for recreational purposes is necessary to prevent children contact with the infected surface water bodies. In addition, potable water needs to be provided for community use to minimize the rate of parasitic infections and diseases, more especially, in the communities where urinary

schistosomiasis is endemic.

The color or appearance of urine may not necessarily be used as a diagnostic clinical symptom for people harboring schistosome parasites. This is because the participants who had the same urine appearance in this study consisted of individuals who both harbored urinary schistosome parasites and those without infection. However, the proportion of participants with urine that appeared yellow and highly turbid (42.0%) and those with pale yellow and turbid urine (50.0%) had significantly higher prevalence of urinary schistosomiasis than others (p=0.01), which may indicate that turbid urine is associated with urinary schistosomiasis. Urine colour is usually caused by factors such as urinary tract infection, kidney stones, insufficient water intake, or types of diet (32). Although, none of the urine of the participants was red or cola-cola/ brown colored, this does not rule out the presence of infection with *S. haematobium*.

Conclusion:

Findings in this study confirm continuing existence of schistosomiasis in the study area which calls for comprehensive interventions. Such interventions should include targeted health education that focuses on transmission of the disease and mass treatment of the people in the study area. Further researches are also recommended, especially to survey the dynamics and distribution of freshwater snail intermediate hosts of schistosomes in the study area.

Contributions of authors:

OOA and SYS were involved in the study conceptualization, design and data acquisition; OSS and OFO were involved in analysis and interpretation of data; OOA, SYS and OSS were in drafting of the manuscript; OOA and OFO were involved in critical revision of the manuscript for important intellectual content; SYS and OSS were involved in the statistical analysis; and OOA and OFO supervised the study. All authors approved the manuscript submitted for publication.

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Original Article **Open Access**

Factors associated with parasitic contamination of fresh fruits retailed at major markets in Ibadan, Nigeria: A public health concern

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Abstract:

Background: Globally, cases of protozoan and helminths infections in humans have been linked to the consumption of unwashed fresh fruits and this has been prevalent especially in developing countries. The ingestion of parasite-contaminated fruits can lead to intestinal parasitosis that can result in childhood diarrhea, malnutrition and growth retardation. Hence, the aim of the study was to determine the prevalence of parasites contaminating fresh fruits sold in major markets in Ibadan, Nigeria, the sources and factors associated with contamination.
Methodology: This was a descriptive cross-sectional study of contamination rates of fresh fruits sold by vendors in 3 selected major markets from 3 local government areas in Ibadan, Nigeria. A total of 108 fresh fruits of 7 different types (orange, watermelon, mango, apple, banana, pineapple and carrot) were purchased from the vendors using proportional allocation method for the 3 markets, over a period of 4 weeks. The fruits were at each purchase, transported immediately to the parasitology laboratory of the University College Hospital Ibadan for analysis. Wet film microscopy of concentrated fruit samples through sedimentation technique was used to detect helminths eggs, cysts and larvae, and Kinyoun acid-fast stain was used to detect protozoan cysts and oocysts. Information on possible sources of fruit contamination and associated factors were collected from the fruit vendors and from environmental surveys, using a designed collection form. Data analysis was performed on SPSS version 28.0. Fisher's exact test was used to determine association between potential risk factors and parasitic contamination rates, and p-value ≤ 0.05 was considered statistically significant.
Results: Out of the 108 fruits examined, a total of 12 samples were positive for parasites (11.1%), with 2 (16.7%) of the 12 samples contaminated with multiple parasite species. *Strongyloides stercoralis* (n=6, 42.9%) was the most commonly identified parasite, followed by *Cryptosporidium* spp (n=5, 35.7%), *Trichomonas hominis* (n=2, 14.3%) and *Ascaris lumbricoides* (n=1, 7.2%). There was significant association (p<0.05) between parasitic contamination rates and factors such as method of fruit display, vendors' defecation practices, and presence of dirt around the vendors' premises. Although, parasitic contamination rates were higher with factors such as poor drainage systems, proximity of sellers to waste collection points, presence of animal droppings, and infrequently washed display containers, the differences were not statistically significant (p>0.05).
Conclusion: This high level of contamination of fresh fruits with intestinal parasites poses a health risk to the consumers, thus thorough washing of fruits before ingestion is highly recommended. Food safety should encompass the prevention of parasitic contamination at all stages from planting to ingestion.

Keywords: Intestinal Parasites, Protozoa, Helminths, WASH, Food safety, food-borne illnesses

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Facteurs associés à la contamination parasitaire des fruits frais vendus au détail sur les principaux marchés d'Ibadan, au Nigéria: un problème de santé publique

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Résumé:

Contexte: À l'échelle mondiale, des cas d'infections à protozoaires et à helminthes chez l'homme ont été associés à la consommation de fruits frais non lavés, et cette prévalence est particulièrement élevée dans les pays en développement. Français L'ingestion de fruits contaminés par des parasites peut entraîner une parasitose intestinale susceptible de provoquer des diarrhées infantiles, de la malnutrition et un retard de croissance. Par conséquent, l'objectif de l'étude était de déterminer la prévalence des parasites contaminant les fruits frais vendus sur les principaux marchés d'Ibadan, au Nigéria, ainsi que les sources et les facteurs associés à la contamination.
Méthodologie: Il s'agissait d'une étude transversale descriptive des taux de contamination des fruits frais vendus par des vendeurs sur 3 grands marchés sélectionnés dans 3 zones de gouvernement local d'Ibadan, au Nigéria. Un total de 108 fruits frais de 7 types différents (orange, pastèque, mangue, pomme, banane, ananas et carotte) ont été achetés auprès des vendeurs en utilisant la méthode d'allocation proportionnelle pour les 3 marchés, sur une période de 4 semaines. Les fruits ont été, à chaque achat, transportés immédiatement au laboratoire de parasitologie de l'Hôpital universitaire d'Ibadan pour analyse. Français La microscopie à film humide d'échantillons de fruits concentrés par technique de sédimentation a été utilisée pour détecter les œufs, les kystes et les larves d'helminthes, et la coloration acido-résistante de Kinyoun a été utilisée pour détecter les kystes et les oocystes de protozoaires. Des informations sur les sources possibles de contamination des fruits et les facteurs associés ont été recueillies auprès des vendeurs de fruits et à partir d'enquêtes environnementales, à l'aide d'un formulaire de collecte conçu. L'analyse des données a été réalisée sur SPSS version 28.0. Le test exact de Fisher a été utilisé pour déterminer l'association entre les facteurs de risque potentiels et les taux de contamination parasitaire, et la valeur $p \leq 0,05$ a été considérée comme statistiquement significative.
Résultats: Sur les 108 fruits examinés, un total de 12 échantillons étaient positifs pour les parasites (11,1%), avec 2 (16,7%) des 12 échantillons contaminés par plusieurs espèces de parasites. *Strongyloides stercoralis* (n=6, 42,9%) était le parasite le plus fréquemment identifié, suivi de *Cryptosporidium* spp (n=5, 35,7%), *Trichomonas hominis* (n=2, 14,3%) et *Ascaris lumbricoides* (n=1, 7,2%). Une association significative ($p<0,05$) a été observée entre les taux de contamination parasitaire et des facteurs tels que le mode de présentation des fruits, les pratiques de défécation des vendeurs et la présence de saleté autour des locaux. Bien que les taux de contamination parasitaire soient plus élevés en raison de facteurs tels que la mauvaise qualité des systèmes de drainage, la proximité des vendeurs avec les points de collecte des déchets, la présence d'excréments d'animaux et le lavage peu fréquent des contenants de présentation, les différences n'étaient pas statistiquement significatives ($p>0,05$).
Conclusion: Ce niveau élevé de contamination des fruits frais par des parasites intestinaux présente un risque pour la santé des consommateurs. Il est donc fortement recommandé de laver soigneusement les fruits avant leur ingestion. La sécurité alimentaire doit inclure la prévention de la contamination parasitaire à toutes les étapes, de la plantation à la consommation.

Mots-clés: Parasites intestinaux, Protozoaires, Helminthes, WASH, Sécurité alimentaire, maladies d'origine alimentaire

Introduction:

Fruits are one of the major sources of vitamins and minerals, high in fiber and help in promoting good health (1). A diet rich in fruits can lower blood pressure, reduce the risk of heart disease and stroke, lower the risk of eye and digestive problems, prevent some types of cancer and have a positive effect on blood sugar (2,3). In spite of these benefits, fruits could also constitute risk to human health when consumed unclean (4). A major risk from consumption of such unclean fruits is parasitic infections which are acquired through consumption of food or water contaminated with feces containing the infective stages of the parasite. Improperly cooked meat, vegetables and fresh fruits are the main sources of such contaminated food items (5).

Intestinal parasites feed off nutrients from their host, causing significant damage to human health including malnutrition, intestinal obstruction, amoebic liver abscess, skin rash, liver damage, malabsorption and are major contributors to childhood diarrhea and growth retardation (6,7). The consumption of fresh fruits plays a major role in the transmission of parasitic food borne diseases. Routes of contamination of fresh fruits vary as they can be contaminated throughout the process from planting to consumption. During the planting process, application of organic wastes to agricultural land as fertilizers, fecal matter contaminating irrigation water, poor personal hygiene, poor sanitation, and direct contamination by birds, wild animals and livestock can result in contamination (8).

In many developing countries the practice of irrigation of soil with untreated wastewater and use of manure and night soil as fertilizers in the cultivation of fruits often lead to parasitic infections when these fruits are eaten unwashed or uncooked (6). This practice which is growing prominence in Nigeria because of the high cost of mineral fertilizers and poor socio-economic conditions that is further worsened by poor environmental hygiene (9).

Inadequate hygiene practices during harvesting, packaging, transportation, and storage of fruits also contribute to the contamination of fruits. Globalization of food supply post-harvest has led to introduction of pathogens into new geographical areas through importation (10). Post harvesting contamination of fruits may occur when fresh fruits are rinsed, washed and sprinkled with contaminated and untreated water posing hazardous effects

on humans (11). The rate of contamination and species of parasites is affected by various factors including weather conditions such as temperature, rainfall, soil type, sociocultural status, season of sample collection and fruits examined (10).

In recent times, there has been a global increase in the cases of food borne illnesses and outbreaks linked to consuming fresh fruits (12). Previous studies have shown that many fresh fruits purchased from markets in different regions from developing countries were contaminated with helminths eggs and larvae including *Ascaris lumbricoides, Trichuris trichiura, Ancylostoma duodenale,* and *Necator americanus* (hookworms), as well as protozoan cysts such as *Giardia intestinalis, Entamoeba histolytica, and Cryptosporidium* spp (5,6,13-15).

In Ibadan, Nigeria, a city highly endemic for Soil Transmitted Helminths (STHs), contamination is particularly concerning due to inadequate sanitation and open defecation practices, which exacerbate the risk of soil-transmitted helminth infections (16). However, there has been little information available about the burden of parasitic contamination in recent times. Furthermore, previous studies did not investigate factors determining such contaminations. It is crucial to identify contaminating parasites as well as their sources with the goal of providing suitable, effective, and feasible intervention methods for monitoring and controlling them before these food items reach the final consumers. Hence, this study evaluated the current prevalence of parasites on fresh fruits sold in major markets in Ibadan, Oyo state, Nigeria and the likely sources of contamination.

Materials and method:

Study design and setting:

This was a descriptive cross-sectional study of fresh fruits retailed by vendors in three selected markets in Ibadan, the capital of Oyo state, south-western Nigeria and a major center of commerce in the state. Bodija, Oje, and Ayeye markets in three local government areas of Ibadan were purposively selected for this study to reflect the major sources of fruit in the city. These are major markets where farmers bring in their fruit harvests to be sold.

Bodija is situated in Ibadan North local government is the largest food market in Ibadan with a large fruit section, Oje market is situated in Ibadan North-East local government and is the most popular fruit market, while Ayeye Market situated in Ibadan North-West local government is also a popular fruit market. Sample collection and analysis was conducted within a 4-week duration from April to May 2023.

Ethical considerations:

This research was ethically approved by the local institutional review board with code number UI/EC/23/0186. Informed consent was obtained from all participating fruit vendors.

Study population, sample size and sampling:

The study population comprised of fresh fruits sold in the selected markets by fruit vendors. The fruits include orange (*Citrus sinensis*), watermelon (*Citrullus lanatus*), mango (*Mangifera indica*), apple (*Malus domestica*), banana (*Musa acuminate*), pineapple (*Ananas comosus*), and carrot (*Daucus carota*). The sample size was estimated using the formular for cross-sectional studies and the prevalence determined from a previous study in Nigeria (17). A minimum sample size of 98 fresh fruits was obtained and a 10% non-response rate was included to obtain adjusted sample size of 108.

Approximately 17 to 18 fruit vendors were randomly sampled weekly at the selected markets, with proportionate allocation of number of fruit samples for the markets (1.5: 1.5: 1 for Oje: Bodija: Ayeye) based on the estimated size of each market. Sampling was done over a period of 4 weeks to obtain the sample size of 108.

Data and sample collection:

Structured questionnaire were designned and used to collect information on the socio-demographic characteristics of the fruit vendors such as age, sex, and level of education. Information such as market place, source of fruits, means of transportation, method of fruit display, method of storage, and the fingernail status of the vendors were collected. Observations of the market environment for the presence of waste bins, dumping site, wastes and toilet facilities were documented.

Each set of fruit sampled was packaged into sterile polythene bags, labelled with a unique laboratory identification, name of market, date of purchase, and transported to the parasitology laboratory for analysis.

Macroscopic/microscopic examination of samples:

Each fruit sample was examined macroscopically for presence of segments or adult forms of helminths. The fruit samples were then concentrated by the sedimentation technique before microscopic examination for the presence of ova or cyst of helminths using saline and iodine mounts, and for oocysts using modified Ziehl Neelsen (ZN) staining.

Concentration of parasites using the sedimentation method:

Each fruit sample was thoroughly washed in 100ml of physiological saline (0.85% NaCl) for 2 minutes. The suspension was strained using a sterile sieve to remove undesirable materials. The filtrate was left to stand for about 12 hours. The supernatant was discarded and the sediment was transferred to a 15ml centrifuge tube. For concentrating the parasite, the tube was centrifuged at 3000 rpm for 5 minutes and the supernatant was carefully decanted without shaking. The sediment was agitated by gently shaking the tube to redistribute the parasites (18). The resultant suspension was used for wet smear and modified ZN acid-fast staining.

Wet smear preparation for microscopy:

A drop each of the suspension was placed on both ends of a clean grease-free microscopic slide, one for saline and the other for iodine mounts (a drop of Lugol's iodine was added to one of the suspensions). A thin smear was made, and the suspension drops were carefully covered with cover slip each and observed under light microscope using 10x and 40x objective for the presence of parasites.

Modified Ziehl Neelsen staining procedure:

Fixed slides of fruit suspension were stained with cold carbol fuchsin for 15 mins, rinsed, decolorized with 1% hydrochloric acid alcohol and counterstained with 0.5% malachite green. These were allowed to air dry and examined microscopically under immersion oil for oocysts of parasites (19).

Data analysis:

Data analysis was performed on SPSS version 28.0. The data were summarized and presented using descriptive statistics. Fisher Exact test was used to compare the rate of parasitic contaminations among different fruit types sold in different markets and to determine the association between the risk factors and parasitic contamination. A p-value ≤ 0.05 was considered statistically significant.

Results:

A total of 108 samples of fruits were purchased, consisting of 42 (38.9%) in Oje market, 40 (37.0%) in Bodija market and 26 (24.1%) in Ayeye market based on proportional ratio of 1.5: 1.5: 1. (Table 1). A total of 12 (11.1%) of the 108 fruit samples were positive for parasites during the study. The distribution of parasitic contamination according to the different markets showed highest contamination rate in Ayeye (19.2%, 5/26) market, followed by Oje (14.3 %, 6/42) market and lowest in Bodija (2.5%, 1/40) market. However, statistical analysis did not indicate a significant association between contamination rates and the type of markets (p=0.057) (Table 1).

Table 1: Prevalence of parasitic contamination of fresh fruits in major markets in Ibadan, Nigeria

Markets	No of fruits examined	No of fruits contaminated (%)	Fisher	*p* value
Bodija	40	1 (2.5)	5.569	0.057
Oje	42	6 (14.3)		
Ayeye	26	5 (19.2)		
Total	**108**	**12 (11.1)**		

Table 2: Frequency of parasites contaminating fresh fruits in major markets in Ibadan, Nigeria

Parasite	Frequency	Percentage (%)
Ascaris lumbricoides	1	7.1
Trichomonas hominis	2	14.3
Cryptosporidium spp.	5	35.7
Strongyloides stercoralis	6	42.9
Total	**14**	**100.0**

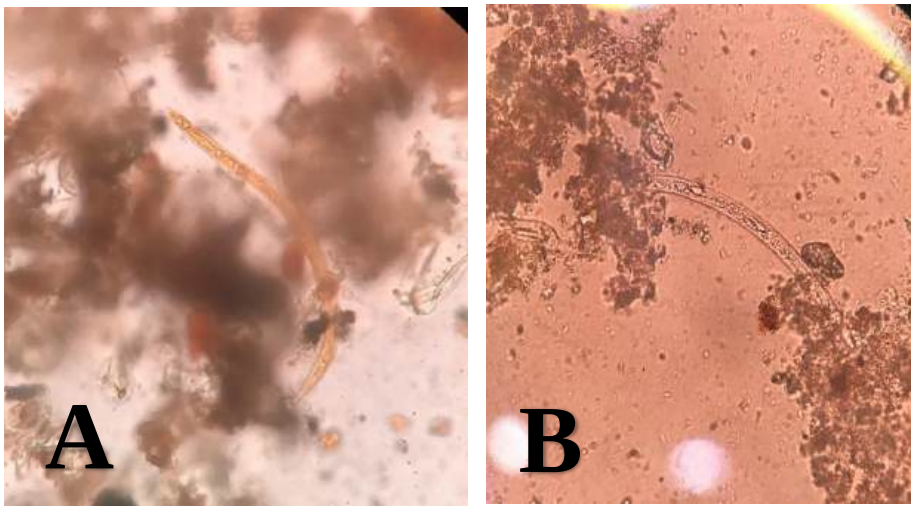
Table 3: Parasite contamination rates for different fresh fruit types in major markets in Ibadan, Nigeria

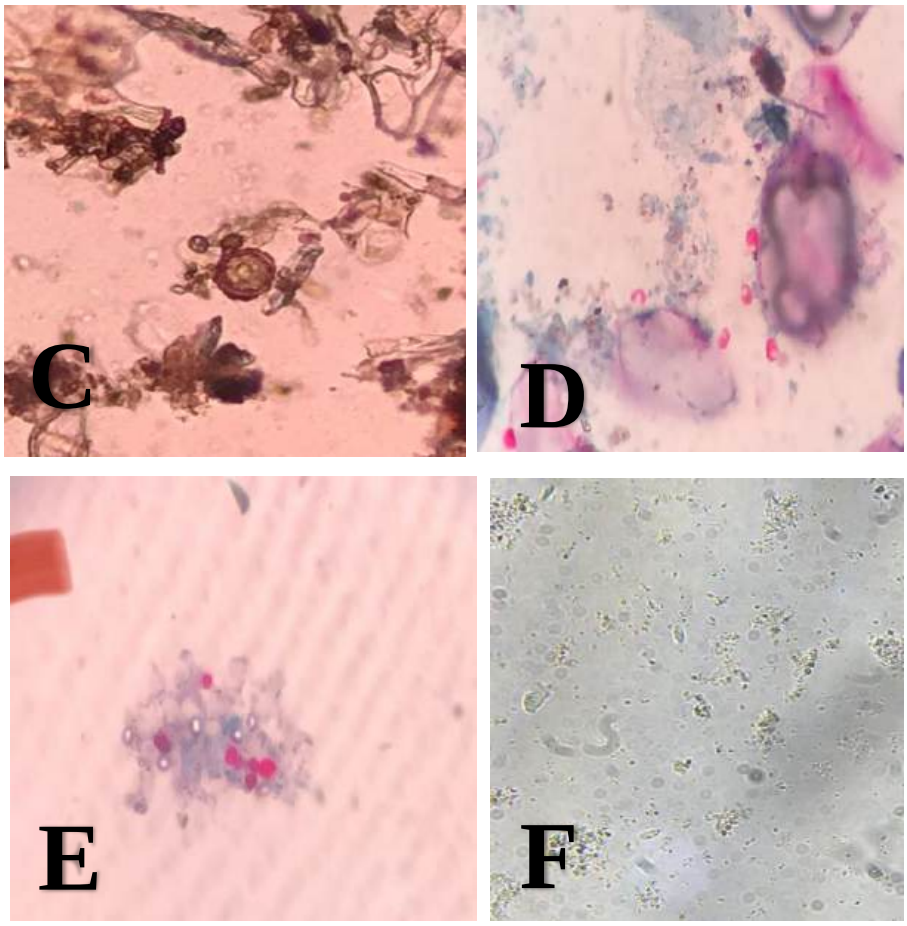
Fruits/markets	Bodija		Oje		Ayeye		Total examined	Total contaminated
	Examined	Contaminated	Examined	Contaminated	Examined	Contaminated		
Apple	3	0	2	0	3	0	8	0
Banana	2	0	6	0	5	0	13	0
Carrot	4	1	3	0	3	1	10	2 (20.0)
Mango	8	0	6	0	6	1	20	1 (5.0)
Orange	7	0	14	2	2	0	23	2 (8.7)
Pineapple	8	0	6	3	4	2	18	5 (27.8)
Watermelon	8	0	5	1	3	1	16	2 (12.5)
Total	**40**	**1 (2.5)**	**42**	**6 (14.3)**	**26**	**5 (19.2)**	**108**	**12 (11.1)**

Fisher Exact test 7.574, p=0.189

Parasites identified include *Ascaris lumbricoides, Cryptosporidium* spp*, Strongyloides stercoralis,* and *Trichomonas hominis* (Fig 1). The most frequent parasitic contaminant of the fruits is *S. stercoralis* (n=6, 42.9%), followed by *Cryptosporidium* spp (n=5, 35.7%), *T. hominis* (n=2, 14.3%), and *A. lumbricoides* (n=1, 7.1%) (Table 2).

Parasitic contamination rate was highest for pineapple fruits (27.8%, 5/18), followed by carrots (20.0%, 2/10), watermelon (12.5%, 2/16), orange (8.7%, 2/23), and mango, with the least (5.0%, 1/20) (Table 3). However, statistical analysis did not indicate a significant association between parasitic contamination rates and type of fruits (p=0.189) (Table 3). Contamination with more than one parasite specie was observed in some of the fruits examined, with 2 (16.7%) of the 12 contaminated samples showing contamination with two different parasites species.





A – Larva of *Strongyloides stercoralis* in iodine wet mount; B - Larva of *Strongyloides stercoralis* in normal saline wet mount; C – Ova of *Ascaris lumbricoides;* D – Oocyst of *Cryptosporidium* spp in modified Ziehl Neelsen stain; E – Oocyst of *Cryptosporidium* spp in modified Ziehl Neelsen stain; F – Trophozoite of *Trichomonas hominis* in normal saline wet mount

Fig 1: Microscopic pictures of parasitic types contaminating fresh fruits at major markets in Ibadan, Nigeria

With respect to potential vendor factors associated with parasitic contamination, most of the vendors (67.0%) reported defecation during market hours, however only 42.9% regularly washed hands after bowel movement (Table 4). Furthermore, an assessment of the nails of the vendors revealed that majority of the vendors (64.3%) had short but dirty finger nails. The analysis indicated a significant association between the vendors defecation practice and parasitic contamination rate (p=0.01).

Although the highest contamination rate was seen with fruits transported by public buses or taxi (12% vs 0% for other means), there was no statistically significant association between the means of transportation and parasitic contamination rate (p=1.000). However, there was a significant difference between contamination rates of fruits displayed on the floor (20.0%) compared to those displayed by other means (p=0.026). Fruits sold by vendors who do not regularly wash their fruits before displaying, had three times more contamination rate (13.1%) compared to those who regularly wash hands before displaying fruits (4.2%) (p=0.22). Also, fruits sold by vendors who kept their left-over fruits by covering outside with clothes were the most contaminated (Table 4).

The assessment of the overall general condition of the vendors' environment, whether clean or dirty is presented in Table 5. The highest contamination rate was seen in vendors with dirty environment (13.3% vs 4%). However, statistical analysis did not indicate any significant association between the general condition of the environment and parasitic contamination rate (p=0.288). All the fruit samples with parasitic contamination were from vendors who were not aware of a market refuse dump site.

There was a statistically significant association between the presence of dirt around the vendors and parasitic contamination rate (p=0.032). The fruits sold by vendors with dirt around them were 5 times more likely to be contaminated when compared to those sold by vendors with no dirt around them. Notably vendors with waste bin within one meter from their selling point had twice parasitic contamination rate compared to those with waste bins farther away that one meter (17.9% vs 8.8%).

Table 4: Post-harvest factors associated with the parasitic contamination of fresh fruits sold in major markets in Ibadan, Nigeria

Post-harvest factors		**Contamination**		**Fisher**	**P value**
		No (%)	**Yes (%)**		
Source of fruits	Directly from farm	52 (89.7)	6 (10.3)	0.074	1.000
	Middlemen	44 (88.0)	6 (12.0)		
Mean of transportation	Personal vehicle	2 (100.0)	0	0.499	1.000
	Bus or Taxi	88 (88.0)	12 (12.0)		
	Truck	6 (100.0)	0		
Packaging during transportation	Arranged on the floor of vehicle	34 (85.0)	6 (15.0)	1.750	0.610
	Basket	26 (86.7)	4 (13.3)		
	Carton	5 (100.0)	0		
	Sacks	31 (93.9)	2 (6.1)		
Means of display of fruits	On the floor	36 (80.0)	9 (20.0)	6.112	0.026*
	Bucket	1 (100.0)	0		
	Cart	6 (100.0)	0		
	In a tray	9 (100.0)	0		
	On a table	43 (93.5)	3 (6.5)		
Frequency of washing of the display materials	Daily	17 (100.0)	0	4.851	0.144
	Weekly	23 (95.8)	1 (4.2)		
	Monthly	6 (5.6)	1 (14.2)		
	Never	50 (83.3)	10 (16.7)		
Washing of fruits with water before displaying	No	73 (86.9)	11 (13.1)	1.590	0.219
	Yes	23 (95.8)	1 (4.2)		
Source of water for washing fruits	Not applicable	73 (86.9)	11 (13.1)	2.905	0.366
	Pipe – borne	13 (100.0)	0		
	Well	10 (90.9)	1 (9.1)		

* = statistically significant

Table 5: Environmental factors associated with the parasitic contamination of fruits sold in major markets in Ibadan, Nigeria

Environmental factors		**Contamination**		**Fisher**	***p* value**
		No (%)	**Yes (%)**		
Presence of drainage system	No	52 (92.9)	4 (7.1)	1.854	0.226
	Yes	44 (74.6)	8 (15.4)		
Condition of drainage system	Good	2 (100.0)	0	3.515	0.325
	Fair	17 (89.5)	2 (10.5)		
	Poor	24 (80.0)	6 (20.0)		
Presence of waste bin 1 m from selling point	No	73 (91.3)	7 (8.8)	1.742	0.292
	Yes	23 (82.1)	5 (17.9)		
Presence of dumping site	No	92 (88.5)	12 (11.5)	0.519	1.000
	Yes	4 (100.0)	0		
Presence of dirt around the seller.	No	49 (96.1)	2 (3.9)	5.057	0.032*
	Yes	47 (82.5)	10 (17.5)		
Presence of insects, animals or pest in the surroundings	No	49 (90.7)	5 (9.3)	0.375	0.761
	Yes	47 (87.0)	7 (13.0)		
Presence of animal excreta around selling point	No	92 (89.3)	11 (10.7)	0.419	0.452
	Yes	4 (75.0)	1 (25.0)		
General condition of environment	Clean	24 (96.0)	1 (4.0)	1.666	0.288
	Dirty	72 (86.7)	11 (13.3)		
Condition of storage area.	Clean	18 (100.0)	0	2.700	0.211
	Dirty	78 (86.7)	12 (13.3)		
Condition of storage and display materials	Clean	14 (100.0)	0	2.011	0.358
	Dirty	82 (87.2)	12 (12.8)		

Presence of toilet close to selling point, presence of water source(s) close to selling point and presence of hand wash area close to selling point were all constant (No), thus excluded from the table; * = statistically significant

Discussion:

In accordance with the prevailing circumstances seen in many developing nations, Nigeria exhibits a widespread distribution of intestinal parasites, and this is largely attributed to the favorable climatic conditions and unsanitary environments that facilitate the contamination of water, food items, and soil by fecal matter (20,21). This study assessed the level of parasitic contamination, the prevalence of different intestinal parasites and the possible source of contamination of fruits sold in major fruit markets in Ibadan, Nigeria. The overall parasitic contamination rate was 11.1% which agrees with similar findings from Bauchi, northern Nigeria with reported parasitic contamination rate of 14.3% (22). However, the rate from this study is lower than the rates from other parts of Nigeria such as Onitsha markets in Anambra State, and in Katsina State, with prevalence of 43.8% and 41.7% respectively. Outside Nigeria, similar higher rates of 39.0%, and 42.6% respectively were reported from Egypt and Southwest Ethiopia (6,10,23,24).

The difference in rates between our study and others might be due to the variations in geographical location, climatic and environmental conditions, poor post-harvest handling method and socioeconomic status. Other procedural factors including variation in sample size and techniques used for identification of parasites may also account for the observed differences (6). For example, a previous study in Ibadan, Oyo state, Nigeria compared the extent of parasitic contamination using two conventional techniques, sedimentation and floatation techniques for isolation of parasites. It was observed that the sedimentation method had a much higher recovery rate of 86.9% while the floatation method had just 13.1% (25). The overall contamination rate in the present study though lower than the previously cited studies, is however higher than the findings from a study in Kogi, Nigeria, which reported a low prevalence of 6.8% (17). The factors mentioned above could also have contributed to the lower reported prevalence. For example, unlike this present study, the study done in Kogi did not include Modified Ziehl-Neelsen staining technique for detection of protozoan oocysts.

In this study, pineapple was the most frequently contaminated fruit (27.8%) while mango was the least contaminated (5%). This result is similar to the findings from studies carried out much earlier in by Alli in 2011 and by Okunlola in 2021 where pineapple was also reported as the most frequently contaminated with 62.5 % and 46.7% contamination respectively (25,26). This may be due to the rough surface of pineapple fruit which makes the adhesion of parasitic eggs, larvae or cyst to the surface easier either on the farm or during handling (27). Carrot, watermelon and orange also recorded a high level of contamination rate with 20.0%, 12.5% and 8.7% contamination rates respectively. The high contamination rate recorded in carrots may be because carrots grow in the ground, leading to higher risk of contamination particularly if night soil is used as fertilizer (28). We did not identify any contamination in bananas and apples, in agreement with a study done in South India which reported no contamination for banana and 1 (6.6%) contamination for apple (29). This might be attributed to the smooth surface of these fruits which impede the adherence of parasitic eggs, larvae, or cysts to their surfaces (29).

Although there was no significant association between parasitic contamination and market types, the rate of contamination in Oje and Ayeye markets were over 5 times higher than that of Bodija market (OR=7.526, 95% CI =1.0-60.7, p=0.0303). This finding could be attributed to differences in drainage, personal hygiene, source of water and other environmental factors in these markets (25). The isolated parasites are mainly of faecal origin implying the fecal contamination of the samples from humans or animals (22). This contamination may arise from the use of manure for cultivation and sewage water for irrigation of these fruits as studies have shown the soil and rivers in Nigeria and other developing countries to be contaminated with fecal parasites (25,30-32). The level of sanitation in the market premises apparently plays a major role in the occurrence of parasitic contamination. In this present study, there was a significant association between the presence of dirt around the vendors, waste disposal practices, where the vendors defecate and the rate of parasitic contamination. Although majority of the vendors used the public toilets in the markets to defecate, their poor maintenance and hygiene could contribute to the contamination of fruits. There is also a high probability of the vendors in these markets were handling the fruits with faecally contaminated hands.

Additionally, the means of fruit transportation from farms to markets may also contribute to contamination. Most of the vendors used public vehicles, with their fruits arranged on the floor inside the vehicle, to convey fruits to the markets. They also moved these fruits in vehicles used for transporting other items thus encouraging cross contamination. Although, it was not statistically significant, in this study, fruits sold by vendors that use public transport were more prone to contamination compared to those who use personal vehicles which had no contamination.

The means of display of fruits could also play a major role in the variation in para-

sitic contamination among the markets. Most vendors display fruits on the floor and by the roadside, exposed to dust, flies and other insects making them prone to contamination. Flies such as housefly could easily carry parasite eggs and cyst from the dirty market premises, the refuse, uncovered drainages, and surroundings and transfer them mechanically to the displayed fruits (24). We observed that fruits displayed on the floor had a higher contamination rate of 20.0% when compared to those displayed on the table which was about one third of the floor rate. The detection of intestinal parasites on fruits sold in these selected markets has a significant health implication, particularly given the consumption of unwashed fruits potentially leading to infections and diseases. Our results emphasize the importance of adequate water, sanitation and hygiene (WASH) practices in reducing the spread of infectious agents (33).

Strongyloides stercoralis, *Cryptosporidium* spp*, T. hominis,* and *A. lumbricoides,* were the four types of parasites we identified from the fruit samples collected from these markets. However, these findings from our study differ from those from other parts of Nigeria and such variation in the parasites identified may be due to the geographical differences based on location of the study. For instance, a study in River State identified *Entamoeba coli, Entamoeba histolytica, Giardia lamblia, Balantidium coli, A. lumbricoides, Ancylostoma* and *Fasciola* spp (14). Larvae of *S. stercoralis* (42.9%) was the most frequently detected parasite in our study. This might be due to the free-living state of the parasite making it abundant in the environment, hence easily contaminating fruits. In contrast to this study, a previous study in Ibadan, Oyo state, Nigeria over a decade earlier reported a lower prevalence of *S*. *stercoralis* in fruits (25). In agreement with our findings, a study in Benue State, Nigeria reported *S*. *stercoralis* as the second most common parasite identified in fruits with a similar observation reported by some researchers in South India (29,34).

It has been reported that flies can act as vectors for various pathogenic parasites including *Cryptosporidium* spp (35). Oocyst of *Cryptosporidium* spp (35.7%) was the second most detected parasite in the present study. A study conducted in markets of Northwest Mexico, reported *Cryptosporidium* spp as the most prevalent parasitic pathogen (11.7%) (4). The high contamination of fruits in this study with *Cryptosporidium* spp also agrees with a study done in Ghana which reported a high prevalence of 11.1% for *Cryptosporidium* spp (36). Another study in Southern Ethiopia also reported a high prevalence of *Cryptosp oridium* spp (4.7%). The third most prevalent parasite detected in this present study was *T. hominis* (14.3%). This finding is similar to that of a study in Jos, Nigeria which reported *T. hominis* as the parasite with the highest contamination rate of 24.9% (9). It is remarkable that among the four parasites identified in this study, *T. hominis,* a flagellate that does not have a cystic form to withstand adverse environmental conditions recorded a high prevalence. This contamination is thus likely to be recent and might be from the water used in washing the fruits (9).

Ova of *A. lumbricoides* were the least detected parasites (7.1%). Although this finding is similar to a study done in Southern Thailand, which reported a low prevalence of 2.6%, it is in contrast to reports from other parts of Nigeria (37). A study in Owerri, Imo State, Nigeria, reported *A. lumbricoides* as the most prevalent parasite detected in fruit with rate of 37% (38). Another study in rural villages of Ebonyi State, Nigeria, also reported *A. lumbricoides* (54.5%) to be the most prevalent parasite detected in fruits (27). Other studies done outside Nigeria including Southwest Ethiopia, have also reported high prevalence of *A. lumbricoides* (10). The variation in the prevalence could be attributed to several factors such as difference in geographic locations, local hygiene and sanitation practices, samples sizes, methods of detection and even seasonal variation. For example, in the study done in Ebonyi State, Nigeria, the fruits were analyzed with both sedimentation and floatation techniques and this could have contributed to a higher prevalence (27). Contamination with multiple species was observed in some of the fruits in this study. This might indicate the possibility of a high-level contamination of fruits, which may result in multiple parasitic infections in humans. Additionally, such findings suggest the persistence of intestinal parasitic infections within the region (17).

Although not reported, other microbial organisms apart from parasites were identified during the study, including bacteria, fungi and rotifers. This is similar to a study done in Osogbo, southwest Nigeria which identified six bacteria and one fungal isolate (39). Rotifers also identified in this present study are microscopic, multicellular aquatic invertebrate organisms. Because of their size, shape and habitat, they can be confused with protozoans but have little significance to humans. They have some economic significance but do not cause diseases in humans (40). Our study is limited by the relatively small sample size which affected the statistical conclusions we could have made from the data collected. A study with larger sample size is thus needed.

Conclusion:

Our study showed that parasitic contamination rate in fruits sold in markets in Iba-

dan, Oyo State, Nigeria, is considerably high, indicating the potential risk of contracting helminths and protozoan infection through ingestion of unwashed or uncooked fruits bought from these markets. Although, the prevalence rate has reduced when compared to previous studies done in the same region, it still poses a serious public health challenge.

The findings of this present study also revealed poor socio-economic conditions, poor sanitation practices, and inadequate public health enlightenment. It is essential to educate market vendors on proper fruit washing and maintaining good hand hygiene while selling to ensure food safety. Public health departments should promote awareness campaigns focusing on proper fruit handling and emphasizing food sanitation and personal hygiene. Governments should reinforce environmental policies and sanitation laws and prioritize the provision and maintenance of modern amenities like toilets, running water, and effective drainage systems within market areas to ensure good sanitary infrastructure.

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Contributions of authors:

DHO designed the study; MOB and OYM conducted the literature search; OYM and DHO conducted the experimental work; OYM and MOB analyzed the data and wrote the manuscript. MOB revised the manuscript, and all authors approved the final manuscript.

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Conflicts of interest:

Authors declare no conflict of interest.

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Original Article **Open Access**

Antifungal susceptibility patterns of *Candida* species colonizing the oral cavity of cancer patients attending two tertiary hospitals in southwest Nigeria

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Abstract:

Background: *Candida* species inhabit humans as commensals but become opportunistic pathogens in immunocompromised individuals. This yeast has developed resistance to anti-fungal medications and infection caused by it is associated with increased morbidity and mortality. Information on the yeast colonization profile and the antifungal sensitivity pattern in the Nigerian population is sparse. This study determined the prevalence and antifungal susceptibility patterns of oral *Candida* species among cancer patients attending two tertiary care hospitals in Osun State, southwest Nigeria.
Methodology: This was a case-control study of 165 cancer patients attending the Obafemi Awolowo University Teaching Hospitals Complex (OAUTHC) Ile-Ife and Osun State University Teaching Hospital (OSUTH) Osogbo, and 165 age and sex matched apparently healthy controls recruited from Ife and Modakeke communities in Osun State, southwest Nigeria. Socio-demographic and clinical information were collected from each participant to determine potential risk factors for *Candida* colonization and resistance. Oral rinse samples were collected from each cancer patient and the healthy control. *Candida* species were isolated on Hicrome chromogenic agar and identified by characteristic yeast-like colonies and Gram-staining reaction. Antifungal susceptibility of the *Candida* species against six selected antifungal agents was determined by the disc diffusion method. Data were analyzed using Chi-square and Fisher Exact tests, with $p<0.05$ considered as statistical significance.
Results: *Candida* species were significantly more prevalent in oral cavity of the cancer patients (38.8%, n=64) than the controls (26.7%, n=44) ($p=0.0258$)**.** The prevalence was highest among elderly cancer patients above 70 years of age (58.3%, n=7), higher in females (42.3%, n=44) than males (32.8%, n=20) and higher among cancer patients on anti-cancer therapy (40.7%, n=50) than those who had not received therapy (33.3%, n=13). *Candida* species identified include *Candida albicans* (55.1%, n=70)*, Candida krusei/Pichia kudriavzevii* (27.6%, n=35)*, Candida parapsilosis* (11.8%, n=13), *Candida tropicalis* (3.1%, n=4), *Candida/Diutina rugosa* (1.6%, n=2) and *Candida/Nakaseomyces glabrata* (0.8%, n=1). The highest and lowest susceptibilities of the *Candida* species were to nystatin (95.2%, n=118) and fluconazole (42.7%, n=53) respectively. The frequency of multi-drug resistance (MDR) was significantly higher ($p<0.01$) among non-*albicans Candida* species (43.6%, n=24) than *C. albicans* (31.9%, n=22).
Conclusion: It was generally observed that *Candida* species were more prevalent in cancer patients than apparently healthy persons. The observed high resistance rates to commonly used azoles underscore the concerns about current empirical treatment options. Nystatin was the most effective drug against *Candida* species in the study population. Regular surveillance of anti-fungal susceptibility and anti-fungal stewardship aew urgently needed to mitigate resistance and improve patients outcomes.

Keywords: Oral colonization; *Candida*; anti-fungal; resistance; immunocompromised; cancer

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Profils de sensibilité aux antifongiques des espèces de *Candida* colonisant la cavité buccale de patients atteints de cancer, hospitalisés dans deux hôpitaux tertiaires du sud-ouest du Nigéria

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Résumé:

Contexte: Les espèces de *Candida* vivent chez l'homme commensales, mais deviennent des pathogènes opportunistes chez les personnes immunodéprimées. Cette levure a développé une résistance aux antifongiques et les infections qu'elle provoque sont associées à une augmentation de la morbidité et de la mortalité. Les informations sur le profil de colonisation des levures et le profil de sensibilité aux antifongiques au sein de la population nigériane sont rares. Cette étude a déterminé la prévalence et les profils de sensibilité aux antifongiques des espèces de *Candida* buccales chez des patients atteints de cancer fréquentant deux hôpitaux de soins tertiaires de l'État d'Osun, au sud-ouest du Nigéria.
Méthodologie: Il s'agissait d'une étude cas-témoins portant sur 165 patients atteints de cancer fréquentant le Complexe hospitalier universitaire Obafemi Awolowo (OAUTHC) d'Ile-Ife et l'Hôpital universitaire d'État d'Osun (OSUTH) d'Osogbo, et 165 témoins apparemment sains, appariés selon l'âge et le sexe, recrutés dans les communautés d'Ife et de Modakeke, dans l'État d'Osun, au sud-ouest du Nigéria. Des informations socio-démographiques et cliniques ont été recueillies auprès de chaque participant afin de déterminer les facteurs de risque potentiels de colonisation et de résistance à *Candida*. Des échantillons de bain de bouche ont été prélevés chez chaque patient atteint de cancer et chez le témoin sain. Les espèces de Candida ont été isolées sur gélose chromogène Hicrome et identifiées par des colonies caractéristiques de type levure et une réaction de coloration de Gram. La sensibilité antifongique des espèces de *Candida* à six agents antifongiques sélectionnés a été déterminée par la méthode de diffusion sur disque. Les données ont été analysées à l'aide des tests du Chi carré et de Fisher Exact, avec une valeur de $p<0,05$ considérée comme statistiquement significative.
Résultats: Les espèces de *Candida* étaient significativement plus prévalentes dans la cavité buccale des patients atteints de cancer (38,8%, n=64) que chez les témoins (26,7%, n=44) ($p=0,0258$). La prévalence était la plus élevée chez les patients atteints de cancer âgés de plus de 70 ans (58,3%, n=7), plus élevée chez les femmes (42,3%, n=44) que chez les hommes (32,8%, n=20) et plus élevée chez les patients atteints de cancer sous traitement anticancéreux (40,7%, n=50) que chez ceux n'en ayant pas reçu (33,3%, n=13). Français Les espèces de *Candida* identifiées comprennent *Candida albicans* (55,1%, n=70), *Candida krusei*/*Pichia kudriavzevii* (27,6%, n=35), *Candida parapsilosis* (11,8%, n=13), *Candida tropicalis* (3,1%, n=4), *Candida*/*Diutina rugosa* (1,6%, n=2) et *Candida*/*Nakaseomyces glabrata* (0,8%, n=1). Les sensibilités les plus élevées et les plus faibles des espèces de *Candida* étaient respectivement à la nystatine (95,2%, n=118) et au fluconazole (42,7%, n=53). La fréquence de la multirésistance aux médicaments (MDR) était significativement plus élevée ($p=0,01$) parmi les espèces de *Candida* non *albicans* (43,6%, n=24) que chez *C. albicans* (31,9%, n=22).
Conclusion: Il a été généralement observé que les espèces de *Candida* étaient plus répandues chez les patients atteints de cancer que chez les personnes apparemment en bonne santé. Les taux élevés de résistance observés aux azolés couramment utilisés soulignent les inquiétudes quant aux options thérapeutiques empiriques actuelles. La nystatine s'est avérée le médicament le plus efficace contre les espèces de *Candida* dans la population étudiée. Une surveillance régulière de la sensibilité aux antifongiques et une gestion responsable des antifongiques sont nécessaires de toute urgence pour atténuer la résistance et améliorer l'état de santé des patients.

Mots-clés: Colonisation buccale; *Candida*; antifongique; résistance; immunodépression; cancer

Introduction:

Candida albicans belong to the kingdom fungi, phylum Ascomycota, class Saccharomycetes, order Saccharomycetales and the family Saccharomycetaceae (1). This yeast that was in the past considered harmless is now known to be primarily responsible for

morbidity and mortality from fungal infections in immunocompromised and debilitated individuals, including cancer patients (2). It is a paradox that medical advancement over past few decades has led to a significant escalation in the incidence of life-threatening invasive fungal infections. However, the extent of the problem is under-reported due to challenges faced in diagnosing fungal infection. It is worrying to know that the mortality estimates due to fungal diseases could be five times higher than what is reported (3). Oladele and Denning (4) estimated that more than 11.8% of Nigerian population yearly suffer from serious fungal infection which is associated with substantial mortality. Among human fungal diseases, candidiasis caused by *Candida* species is the commonest and it is increasingly being reported in recent years (1,4).

However, a decline in the host immunity contributes to the development of candidiasis since *Candida* species exist as commensals in the host's skin as well as the gastrointestinal and genitourinary systems (5,6). Thus, they act as opportunistic pathogens which contribute to high morbidity and mortality from fungal infections among the immunocompromised (7). Factors that compromise the immune system includes low birth weight, cancer, diabetes, HIV/AIDS, burns, chemotherapy and organ transplantation. As a result of the immunocompromised state and the effect of chemotherapy in cancer patients, they are prone to fungal infections, majorly candidiasis, caused by *Candida* species (8).

Globally, after bacterial infection caused by *Clostridioides difficile, Neisseria gonorrhoeae* and Enterobacteriaceae, candidiasis now ranks as third to fourth most occurring nosocomial infections (9). Asymptomatic oral colonization often progresses to oropharyngeal candidiasis, characterized clinically by white pseudomembranous plaques (oral thrush) or erythematous ulcerations. In vulnerable patients, *Candida* species can cause systemic infections including candidaemia and disseminated candidiasis affecting multiple organs such as the heart valves, eyes, central nervous system, spleen, liver and bones. These invasive forms are associated with high morbidity and mortality, especially in immunocompromised hosts (10,11).

Fewer classes of antifungal drug compared with antibacterial drugs are available for the treatment of fungal infections, thus, limiting therapeutic options. A major factor that limits the scope of antifungal drug discovery and development is the fact that evolutionarily, when compared with other pathogens, fungi are the closest to man (12). Several classes of antifungal drugs which have been developed to treat infections caused by *Candida* species include the nucleoside analogs, allylamines (e.g. terbinafine), the azoles (e.g. fluconazole, itraconazole and voriconazole), echinocandins (e.g. caspofungin) and polyenes (amphotericin B and nystatin). However, *Candida* species have developed resistance to practically most of these drugs.

Almost all surveys on *Candida* colonization and infections in cancer patients come from the USA, Europe, and other developed countries (13,14). To date, there is lack of published report on oral *Candida* colonization and antifungal susceptibilities among cancer patients in Nigeria. Therefore, this study was aimed at determining the prevalence, species distribution and antifungal susceptibility of *Candida* species in cancer patients receiving treatment at two southwest Nigerian tertiary hospitals, in comparison to matched apparently healthy controls from the same geographical area.

Materials and method:

Study area and design:

This was a case-control study involving a total of 165 cancer patients receiving treatment at two tertiary hospitals in Osun State, Nigeria; Obafemi Awolowo University Teaching Hospital (OAUTHC) Ile-Ife and Osun State University Teaching Hospital (OSUTH), Osogbo, and 165 apparently healthy individuals, matched for sex and age, recruited from Ife and Modakeke communities in Osun State, Nigeria, as controls.

Processing of samples was carried out at the Department of Medical Microbiology and Parasitology, Faculty of Basic Medical Sciences, College of Health Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria as well as the Department of Drugs Research and Production Unit, Faculty of Pharmacy at the same institution.

Ethical clearance:

Ethical clearance for this study was obtained from the Ethics and Research Committee of OAUTHC with national number; NHREC/27/02/2009a and international number approval number: IRB/IEC/0004553. Additional ethical clearance was obtained from the Ethics and Research Committee of OSUTH with protocol number: UTH/EC/2021/09/537.

Informed consent and data Collection:

Both written and verbal informed consents were obtained from all participants prior to enrolment. Structured questionnaires were administered to all the participants by the researchers to collect socio-demographic and clinical data. The information obtained from cancer patients include hospital number, age, sex, marital status, educational background, occupation, types of cancer, type of cancer therapy, duration of treatment, mouth

symptoms and the hospital ward.

Similar socio-demographic data excluding those that had to do with hospitalization were also obtained from the controls. All data were analysed anonymously throughout the study and confidentially kept. Occupation of participants were grouped based on the modified International Standard Classification of Occupation (ISCO-08) by the International Labour Organization.

Sample collection:

Samples were collected by using concentrated oral rinse technique in which 10ml of sterile distilled water was given in Universal bottles to cancer patients and the healthy controls recruited in this study to rinse their mouths (15). All the samples collection from cancer patients was carried out between June and November 2021 while collection from healthy controls was done between December 2021 and January 2023.

Isolation of *Candida* species from oral rinse:

Hicrome agar (Himedia, India) was prepared according to the manufacturer's specification. Oral rinse sample was cultured on Hicrome agar plates and incubated at 37°C for 48 hours after which the culture plates were observed for growth. Isolates with yeast-like colonies were selected for further studies. Gram-stained cells were observed under a light microscope.

Identification of *Candida* species

Candida species were identified on Hi-chrome agar, a chromogenic agar, based on their colonial colours. A dark green yeast colony which appeared different from *C. albicans* was molecularly identified. Each of the different coloured colonies was selected, sub-cultured on Sabouraud Dextrose Agar (SDA) and incubated at 37^{o}C for 18-24 hours for purity. The pure isolates were stored on SDA slants in cryovials and refrigerated at 4^{o}C for further studies.

Antifungal susceptibility testing

Antifungal susceptibility of the *Candida* isolates was determined against six antifungal agents (Liofilchem, Italy) which included fluconazole (100µg), itraconazole (50µg), voriconazole (1µg), amphotericin B (10µg), caspofungin (5µg) and nystatin (100IU) by the disc diffusion method in line with the Clinical and Laboratory Standards Institute (CLSI) document M44A2 (16). Zones of inhibition were measured and interpreted as susceptible (S), susceptible-dose dependent (SDD) or intermediate, and resistant (R) according to the CLSI guideline.

Data analysis:

Data were analysed using the R Core Team (2022) and the Jamovi project (2023) analysis software (17,18). The data analysis method used was Pearson's Chi-squared test while $p \leq 0.05$ was taken to be statistically significant.

Results:

Demographic characteristics of study participants:

The age of the cancer patients ranged from 7-87 years with a mean of 46.58±17.35 years while that of the controls ranged from 7 -95 years with a mean of 46.51±17.72 years (Table 1). Among the cancer patients, the most frequently occurring cancer type was leukaemia (41.2%, n=68), followed by breast cancer (30.9%, n=51). All the cancer types were grouped into two classes, solid tumour (50.9%, n=84) and haematological malignancies (49.1%, n=81) (Table 2).

Prevalence of *Candida* species in cancer patients:

The prevalence of *Candida* species in the oral cavity of the cancer patients (38.8%, n=64) was significantly higher (p=0.0258) than the healthy controls (26.7%, n=44) (Table 3). The association of demographic characteristics with prevalence of *Candida* species in cancer patients is shown in Table 4. The prevalence of *Candida* species was higher (though not statistically significant) in cancer patients who had started cancer therapy (40.7%, n=50) than those who were yet to start therapy (33.3%, n=13) (p=0.414).

Candida species isolated from cancer patients include *Candida albicans, Candida krusei/Pichia kudriavzevii, Candida parapsilosis*, *Candida tropicalis*, *Candida rugosa* and *Candida/Nakaseomyces glabrata* (Table 5). Similar species except *C. rugosa* and *C/N. glabrata* were isolated from the controls. Most of the Hicrome agar plates yielded single *Candida* species (84.3%, n=91) while a few had multiple *Candida* species (15.7%, n=17) (Table 6).

Table 1: Socio-demographic characteristics of case and control participants

Characteristics	Cases (%) (n=165)	Controls (%) (n=165)	Total (%) (n=330)	*p* value
Age group (years)				
≤10	8 (4.8)	8 (4.8)	16 (4.8)	1.000
11-20	7 (4.2)	7 (4.2)	14 (4.2)	
21-30	12 (7.3)	12 (7.3)	24 (7.3)	
31-40	25 (15.2)	25 (15.2)	50 (15.2)	
41-50	45 (27.3)	45 (27.3)	90 (27.3)	
51-60	35 (21.2)	35 (21.2)	70 (21.2)	
61-70	21 (12.7)	21 (12.7)	42 (12.7)	
>70	12 (7.3)	12 (7.3)	24 (7.3)	
Gender				
Female	104 (63.0)	104 (63.0)	208 (63.0)	1.000
Male	61 (37.0)	61 (37.0)	122 (37.0)	
Marital status				
Divorced/Separated	1 (0.6)	3 (1.8)	4 (1.2)	0.197
Married	116 (70.3)	121 (74.2)	237 (72.3)	
Single	29 (17.6)	30 (18.4)	59 (18.0)	
Widowed	19 (11.5)	9 (5.5)	28 (8.5)	
Level of Education				
No formal education	7 (4.3)	7 (4.3)	14 (4.3)	0.113
Primary	23 (14.0)	21 (12.8)	44 (13.4)	
Secondary	53 (32.3)	35 (21.3)	88 (26.8)	
Tertiary	81 (49.4)	101 (61.6)	182 (55.5)	
Occupation				
Agriculture	5 (3.0)	9 (5.8)	14 (4.4)	0.047
Clerical	2 (1.2)	1 (0.6)	3 (0.9)	
Crafts	8 (4.8)	4 (2.6)	12 (3.8)	
Machine	5 (3.0)	6 (3.9)	11 (3.4)	
Unemployed	11 (6.7)	4 (2.6)	15 (4.7)	
Professional	41 (24.8)	63 (40.6)	104 (32.5)	
Retiree	16 (9.7)	9 (5.8)	25 (7.8)	
Service and Trade	59 (35.8)	41 (26.5)	100 (31.2)	
Student	18 (10.9)	18 (11.6)	36 (11.2)	

Table 2: Frequency of cancer types among the cancer patients

Cancer	Frequency (%)
Cancer types	
Cervical	16.0 (9.7)
Breast	51.0 (30.9)
GIST	4.0 (2.4)
Leukaemia	68.0 (41.2)
Liver	3.0 (1.8)
Lung	2.0 (1.2)
Lymphoma	7.0 (4.2)
Ovarian	3.0 (1.8)
Pancreatic	2.0 (1.2)
Others	9.0 (5.5)
Cancer category	
Haematological	81 (49.1)
Solid malignancies	84 (50.9)
Total	**165 (100)**

The 9 cancer types classified as 'others' include colorectal tumour, neuroblastoma, right colonic tumour, head and neck cancer, kidney cancer, Wilm's tumour, prostate cancer, multiple myeloma and febrile neutropenia; GIST=Gastrointestinal stroma (GIST); leukaemia, lymphoma, multiple myeloma and febrile neutropenia were classified as haematological malignancies while the rest were classified as solid tumours

Table 3: Prevalence of oral *Candida* colonization in the case and control participants

Parameter	Case		Control	
	No (%)	*p* value	No (%)	*p* value
Females	44 (42.3)	0.226	33 (31.7)	0.055
Males	20 (32.8)		11 (18,0)	
Total	**64 (38.8)**		**44 (26.7)**	**0.0258***

* = statistically significant

Table 4: Prevalence of *Candida* species in relation to demographic characteristics of cancer patients

Characteristics	Colonized (%)	Not colonized (%)	Total	*p* value
Age group (years)				
≤ 10	1 (12.5)	7 (87.5)	8	0.217
11-20	1 (14.3)	6 (85.7)	7	
21-30	4 (33.3)	8 (66.7)	12	
31-40	13 (52.0)	12 (48)	25	
41-50	17 (37.8)	28 (62.2)	45	
51-60	11 (31.4)	24 (68.6)	35	
61-70	10 (47.6)	11 (52.4)	21	
>70	7 (58.3)	5 (41.7)	12	
Sex				
Male	20 (32.8)	41 (67.2)	61	0.226
Female	44 (42.3)	60 (57.7)	104	
Marital status				
Married	49 (42.2)	67 (57.8)	116	0.038*
Single	5 (17.2)	24 (82.8)	29	
Widowed	10 (52.6)	9 (47.4)	19	
Separated/divorced	0	1 (100)	1	
Level of education				
No formal education	3 (42.9)	4(57.1)	7	0.454
Primary	11 (47.8)	12(52.2)	23	
Secondary	16 (30.2)	37(69.8)	53	
Tertiary	33 (40.7)	48(59.3)	81	
Occupation				
Agriculture	2 (40.0)	3(60.0)	5	0.078
Clerical service	2 (100.0)	0	2	
Crafts	1 (12.5)	7 (87.5)	8	
Machine	1 (20.0)	4 (80.0)	5	
Unemployed	6 (54.5)	5 (45.5)	11	
Professional	14 (34.1)	27 (65.9)	41	
Retired	6 (37.5)	10 (62.5)	16	
Service and Trade	29 (49.2)	30 (50.8)	59	
Student	3 (16.7)	15 (83.3)	18	
Total	**64 (38.8)**	**101 (61.2)**	**165**	

* = statistically significant

Table 5: Frequency of *Candida* species isolated from cases and controls

***Candida* isolates**	**Case (%)**	**Control (%)**	**Total (%)**
Candida albicans	42 (55.3)	28 (54.9)	70 (55.1)
Candida krusei/Pichia kudriavzevii	26 (34.2)	9 (17.6)	35 (27.6)
Candida parapsilosis	3 (3.9)	12 (23.5)	13 (11.8)
Candida tropicalis	2 (2.6)	2 (3.9)	4 (3.1)
Candida/Nakaseomyces glabrata	1 (1.3)	0	1 (0.8)
Candida rugosa	2 (2.6)	0	2 (1.6)
Total	**76 (59.8)**	**51 (40.2)**	**127 (100.0)**

Table 6: Distribution of *Candida* species isolated from case and control participants with respect to colonization status

***Candida* species**	**Cases (%) (n=165)**	**Controls (%) (n=165)**	**Total (%) (n=330)**
Single agent			
C. albicans	33 (20.0)	20 (12.1)	53 (16.1)
C. krusei/P. kudriavzevii	20 (12.1)	7 (4.2)	27 (8.2)
C. parapsilosis	1 (0.6)	8 (4.8)	9 (2.7)
C. tropicalis	0	1 (0.6)	1 (0.3)
C. rugosa	1 (0.6)	0	1 (0.3)
Subtotal	55 (33.3)	36 (21.8)	91 (27.6)
Multiple (≥2) agents			
C. albicans + C. parapsilosis	1 (0.6)	3 (1.8)	4 (1.2)
C. albicans + C. tropicalis	1 (0.6)	1 (0.6)	2 (0.6)
C. albicans + C. krusei/P. kudriavzevii	6 (3.6)	4 (2.4)	10 (3.0)
C. albicans + C. parapsilosis + C. tropicalis + C/N. glabrata + C. rugosa	1 (0.6)	0	1 (0.3)
Subtotal	9 (5.5)	8 (4.8)	17 (5.1)
Total	**64 (38.8)**	**44 (26.7)**	**108 (32.7)**

Table 7: Antifungal Susceptibility patterns of the *Candida* isolates

Antifungal agent/ Interpretation	***C. albicans* (n=69)**	**non *albicans Candida* (n=55)**						**Total (n=124)**	***p* value**
		C. krusei/P. kudriavzevii	***C. parapsilosis***	***C. tropicalis***	***C/N. glabrata***	***C. rugosa***	**Total**		
Voriconazole									
S	22 (31.9)	27 (75.0)	10 (83.3)	-	-	1 (50.0)	38 (69.1)	60 (48.4)	<0.001*
I	0	3 (8.3)	-	-	-	-	3 (5.5)	3 (2.4)	
R	47 (68.1)	6 (17.6)	2 (16.7)	4 (100)	1 (100)	1 (50.0)	14 (25.5)	61 (49.2)	
Fluconazole									
S	18 (26.1)	26 (72.2)	8 (66.7)	0	0	0	35 (63.6)	53 (42.7)	<0.001*
I	4 (5.8)	3 (8.3)	2 (16.7)	0	0	0	5 (9.1)	9 (7.3)	
R	47 (68.1)	7 (19.4)	2 (16.7)	4 (100)	1 (100)	4 (100)	15 (27.3)	62 (50.0)	
Itraconazole									
S	17 (24.6)	28 (77.8)	10 (83.3)	0	0	1 (50)	39 (70.9)	56 (45.2)	<0.001*
I	1 (1.4)	0	0	0	0	0	0	1 (0.8)	
R	51 (73.9)	8 (22.2)	2 (16.7)	4 (100)	1 (100)	1 (50)	16 (29.1)	67 (54.0)	
Amphotericin B									
S	43 (62.3)	12 (33.3)	3 (25)	1 (25)	0	0	16 (29.1)	59 (47.6)	<0.011*
R	26 (37.7)	24 (66.7)	9 (75)	3 (75)	1 (100)	2 (100)	39 (70.9)	65 (52.4)	
Caspofungin									
S	58 (84.1)	22 (61.1)	8 (66.7)	4 (100)	1 (100)	1 (50)	36 (65.5)	94 (75.8)	0.085
R	11 (15.9)	14 (38.9)	4 (33.3)	0	0	1 (50)	19 (34.5)	30 (24.2)	
Nystatin									
S	66 (95.7)	34 (94.4)	11 (91.7)	4(100)	1 (100)	2 (100)	52 (94.5)	118 (95.2)	0.980
I	3 (4.3)	2 (5.6)	1 (8.3)	0	0	0	3 (5.5)	6 (4.8)	
Multi-drug resistant									
Yes	22 (31.9)	14 (37.8)	5 (41.7)	3 (75.0)	1 (100)	2 (100)	24 (43.6)	53 (42.7)	0.01*
No	47 (68.1)	23 (62.2)	7 (58.3)	1 (25.0)	-	-	31 (56.4)	71 (57.2)	

S=Sensitive, I = Intermediate (Susceptible dose-dependent), R = Resistant; *p* values represent differences in susceptibility between *Candida. albicans* and non-*albicans Candida* species; * = statistically significant at $p<0.05$ *

Antifungal susceptibility pattern of isolated *Candida* species:

Candida albicans were more resistant than non-*albicans Candida* species to the azoles. However, the reverse was observed for other antifungal agents. The frequency of multi-drug resistance (MDR) was significantly higher (p=0.01) in non-*albicans Candida* species (43.6%, n=24) than *C. albicans* (31.9%, n=22) (Table 7).

Discussion:

Candida species was significantly more prevalent in the oral cavity of cancer patients (38.8%) compared to the healthy controls (26.7%), which is comparable to that reported by Bakki et al., (19). It is opined that this increase in oral *Candida* colonization might have resulted from their states of immune deficiency. In the cancer patients, the highest prevalence was observed among the elderly of age 71 years and above (58.3%) and this agrees with previous findings (20-22). This should be expected as old age is also a factor that contributes to weakened immunity and elderly people may be poor in oral hygiene. Good oral hygiene should be encouraged in this set of people so as to prevent susceptibility to *Candida* infection. Regular surveillance for colonisation by *Candida* species should also be carried out on this group of people. Higher prevalence of *Candida* species was also observed in females (42.3%) than in males (32.8%) among the cancer patients, which agrees with the findings of Marak and Dhanashree (23), who reported a higher incidence of clinical *Candida* species in females (54%) than in males (45%). Good hygiene, especially regular practice of hand washing must be imbibed by women as most women carry *Candida* species in their genital areas.

Based on marital status, the highest prevalence of *Candida* species seen among the widowed (52.6%) is well understandable since most of the widows naturally fall into the age group of the elderly (22). In this study, the level of education had no significant impact on rate of *Candida* species colonization, which agrees with previous findings (24). The highest prevalence seen among the clerical staff (100.0%), followed by those in service and trade (48.0%) may be due to horizontal transfer of the yeasts through constant human interactions. In the study, leukaemia (41.2%) was the most frequent cancer type followed by breast cancer (30.9%). This agrees with the findings of a previous research (25). Oral colonization by single *Candida* species of 33.3% (n=55) and multiple species of 5.5% (n=9) among the cancer patients in the study agrees with the report of Aslani et al., (26). *C. albicans* was the most commonly isolated *Candida* species from both the cancer patients (55.3%) and the controls (54.9%). This is similar to a previous study by Lone et al., (21) who reported that *C. albicans* (58.0%) was the predominant species isolated from cancer patients.

Candida isolates showed least susceptibility to fluconazole (42.7%), which agrees with the findings of Soliman et al., (27) who reported that the sensitivity to fluconazole among *Candida* species isolated from clinical samples was 41.6%. Fifty percent of the *Candida* isolates in our study were resistant to fluconazole, similar to the study of Waikhom et al., (24) who reported that 48.1% of *Candida* species isolates were resistant to fluconazole. Higher resistance rate to fluconazole was observed among *C. albicans* (68.1%) compared to the non *albicans Candida* species (25.5%). The only *C./N. glabrata* isolate (100.0%) in this study was resistant to fluconazole, which agrees with previous findings (28). This is most likely due to the fact that this yeast species possesses intrinsic resistance to fluconazole (29). All *C. tropicalis* (100%) isolates were resistance to azoles, which agrees with the reports of Jayachandran et al., (30). *Candida parapsilosis* was highly sensitive (85.7%) to itraconazole, which agrees with the study of Lyon et al., (31) who reported 90% of *C. parapsilosis* isolated from denture wearers to be susceptible to itraconazole. Most *C. krusei/P. kudriavzevii* in this study were susceptible to azoles, which contrast previous report that the yeast is intrinsically resistant to azoles (32). This is because some strains of *C. krusei/P. kudriavzevii* might have their genes mutated.

Resistance rate to itraconazole in all the *Candida* species was 54.0%. This agrees study of Tsega and Mekonnen (33) who reported 57.3% of *Candida* species isolated from pregnant women were resistant to itraconazole. In this study, resistance rate to voriconazole, a third-generation azole, was 48.8%. This disagrees with the findings of Bitew and Abebaw (34), who observed that all isolated *Candida* species isolated from some Ethiopian women with vulvovaginal candidiasis were 100% susceptible to voriconazole. Moreover, significantly higher (p<0.001) resistance rates of 68.1%, 68.1% and 73.9% were observed among *C. albicans* than non-albicans *Candida* species of 25.5%, 27.3% and 29.1% to voriconazole, fluconazole and itraconazole respectively. This contrasts the study of Jayachandran (30) who reported that non-*albicans Candida* species demonstrated higher resistance to fluconazole and itraconazole

than *C. albicans.* The increase in resistance of *C. albicans* to the azoles is probably due to over exposure to this class of antifungal agent.

Worthy of note is the increasing resistance to fluconazole shown in our study when compared with previous studies (20,26, 35-37). This situation might have arisen from frequent administration of fluconazole as first line antifungal drug in the treatment of candidiasis (38). The reduced *in vitro* activities of the azole antifungal agents in this study agree with the findings of Mba and Nweze (39) who reported that *Candida* species demonstrated multi-azole resistance. It was observed that a *Candida* species which was resistant to particular azole was most often resistant to the rest of the azole drugs, which agrees with Owotade et al., (40) who reported that *Candida* species that are resistant to a particular azole are likely to be resistant to other azole drugs. Therefore, other antifungal agents apart from azole should be administered to a patient who does not respond to an azole (40).

Due to the emergence of resistance to azole among *Candida* species, the echinocandins such as caspofungin are now the first-line therapeutic choice in the treatment of invasive candidiasis in some countries (41). Echinocandins generally demonstrate greater activity against *Candida* species than the azoles (42,43). Although, these class of drugs are not available in Nigeria, only 75.8% (n=94) of all the *Candida* isolates in our study were susceptible to caspofungin, which is similar to the finding of a previous study (44), although contrast the report of another study (45). Resistance to caspofungin in *Candida* species is known to be rare. Notwithstanding, certain *Candida* species such as *C. tropicalis* have been reported to demonstrate resistance to this drug. In our study, 25.0% (n=19) of all the *Candida* species were resistant to caspofungin, which agrees with the report of a study by Aslani et al., (26), while 15.9% (n=11) of the *C. albicans* isolates were resistant to caspofungin in agreement with the study of Mahmoudabadi et al., (44) who reported 13.04% (n=3) clinical isolates of *C. albicans* to be resistant to caspofungin. All the *C. tropicalis* were susceptible to caspofungin in our study, which is contrary to the findings of Pasquale (46) who reported the first incidence of caspofungin resistance in *C. tropicalis.* About 33.3% (n= 4) of *C. parapsilosis* were resistant to caspofungin, which is similar to the findings of Cuenca-Estrella et al., (47) who reported *C. parapsilosis* isolates from China and Malaysia to be resistant to caspofungin. Resistance to caspofungin despite the fact that there was no prior exposure might be as result of intrinsic or cross resistance to drugs in *Candida* species.

The susceptibility results for polyene class of antifungals (amphotericin B and nystatin) against the *Candida* isolates showed that only 47.6% were susceptible to amphotericin B, similar to the findings of Soliman et al., (27) who reported that 50.0% of *Candida* species isolated from clinical samples were susceptible to amphotericin B. However, our finding result disagrees with several previous reports where all tested *Candida* isolates (100%) were susceptible to amphotericin B (23,48). Drug misuse and overuse might be the cause for the increased resistance. Among the antifungal agents tested, nystatin was the most effective while the azoles (fluconazole, itraconazole and voriconazole) were the least effective. This is in line with the findings of Ambe et al., (49) who reported that *Candida* species demonstrated highest sensitivity to nystatin and highest resistance to the azoles (ketoconazole and fluconazole). However, earlier reports showed that all *Candida* species isolated were susceptible to nystatin (50,51). Worthy of note is the occurrence of intermediate susceptibility to nystatin in our study, which also raises a concern about emerging resistance to nystatin, that appears to be the only remaining potent antifungal drug of choice available for treating *Candida* infections. Misidentification and misappropriation of drugs often lead to decreased susceptibility to antimicrobial agents.

In this study, multi-drug resistance was most frequently observed in *C. rugosa* (100.0%, n=2) and *C./N. glabrata* (100%, n=1), followed by *C. tropicalis* (75.0%, n=3) and the least was among *C. albicans* (31.9%, n=22). Prolonged use of different antifungal agents in various clinics has led to an increase in multidrug resistant *Candida* species such as *C./N. glabrata*. Multidrug resistance is a major global healthcare challenge which is an impediment to successful treatment of infected patients (52). Antimicrobial stewardship, regular surveillance for *Candida* colonization, precise identification, sensitivity testing and appropriate treatment must be regularly practised in the management of immune suppressed individuals so as to prevent systemic candidiasis which can result in increased debility and mortality among this set of individuals.

Conclusion:

In this study, it was generally observed that *Candida* species were more prevalent in the oral cavities of cancer patients than healthy persons. Cancer therapies may contribute to increased oral colonization by *Candida* species in cancer patients. Nystatin

remains the drug of choice in the treatment of *Candida* infection.

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Contribution of authors:

OEA, JSG, OFO, OFJ, OBW and OO designed the project; OEA carried out the research; OEA, JSG, OFO, OFJ, OBW, OO, and GPT reviewed the manuscript, and JSG and OFJ supervised the research. All authors approved the final manuscript submitted for publication.

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Authors declare no conflict of interest

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Original Article **Open Access**

Detection of influenza virus by real-time polymerase chain reaction (RT-PCR) on archived COVID-19 respiratory specimens in Ibadan, Nigeria

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Abstract:

Background: Every year, influenza virus affects millions of people, causing morbidity and mortality among adults and children prompting many to seek outpatient care. Influenza is largely undetected among infected individuals in lower-middle-income settings. Influenza co-infection with other respiratory viral infections is associated with a severe clinical presentation and a worse clinical outcome. The current study aimed to detect influenza A and B viruses from respiratory samples of patients with suspected CoVID-19, in order to establish possible co-infection, and its implications.
Methodology: Archived respiratory samples from a total of 230 patients with suspected COVID-19 (73 confirmed COVID-19 and 157 without COVID-19) at the Biorepository Clinical Virology Laboratory (BCVL), College of Medicine, University of Ibadan, were included in the study. RNA extraction was carried out on the samples, and then amplified by real-time PCR (RT-PCR) for specific genes of influenza virus. Data obtained were sorted out and analyzed. Descriptive statistics such as percentages were used to summarize variables while inferential statistics such as the Chi-square test was used to compare categorical variables. SPSS version 21.0 was used for statistical computation, R version 4.1.1 was used for graphical illustration.
Results: Of the 73 SARS-COV-2 positive specimens, no influenza virus gene was detected by RT-PCR. However, of the 157 SARS-COV-2 negative specimens, 2 (1.3%) were positive for influenza A virus while 3 (1.9%) for influenza B virus. Co-infection of SARS-COV-2 and influenza virus was not detected.
Conclusion: In this study, the frequency of influenza virus detection in patients with suspected COVID-19 was low for both influenza A and B viruses, but no co-infection of the virus with SARS-CoV-2 was observed. The study emphasizes the need for continuous surveillance for respiratory viral pathogens among individuals presenting with respiratory symptoms in the study setting.

Keywords: Influenza viruses, SARS-CoV-2, COVID-19, Real-Time PCR, Archived samples

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Détection du virus de la grippe par réaction en chaîne par polymérase en temps réel (RT-PCR) sur des échantillons respiratoires COVID-19 archivés à Ibadan, Nigéria

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Résumé:

Contexte: Chaque année, le virus de la grippe touche des millions de personnes, entraînant morbidité et mortalité chez les adultes et les enfants, ce qui incite nombre d'entre elles à consulter en ambulatoire. La grippe passe largement inaperçue chez les personnes infectées dans les milieux à revenu intermédiaire de la tranche inférieure.

Français La co-infection grippale avec d'autres infections virales respiratoires est associée à une présentation clinique sévère et à un pronostic clinique plus défavorable. L'étude actuelle visait à détecter les virus grippaux A et B à partir d'échantillons respiratoires de patients suspectés de COVID-19, afin d'établir une éventuelle co-infection et ses implications.
Méthodologie: Des échantillons respiratoires archivés provenant d'un total de 230 patients suspectés de COVID-19 (73 COVID-19 confirmés et 157 sans COVID-19) au Laboratoire de Virologie Clinique de Biodépositaire (BCVL), Collège de Médecine, Université d'Ibadan, ont été inclus dans l'étude. L'extraction d'ARN a été réalisée sur les échantillons, puis amplifiée par PCR en temps réel (RT-PCR) pour des gènes spécifiques du virus de la grippe. Les données obtenues ont été triées et analysées. Des statistiques descriptives telles que les pourcentages ont été utilisées pour résumer les variables tandis que des statistiques inférentielles telles que le test du Chi carré ont été utilisées pour comparer les variables catégorielles. SPSS version 21.0 a été utilisé pour le calcul statistique, R version 4.1.1 a été utilisé pour l'illustration graphique.
Résultats: Sur les 73 échantillons positifs au SARS-CoV-2, aucun gène du virus de la grippe n'a été détecté par RT-PCR. Cependant, sur les 157 échantillons négatifs au SARS-CoV-2, 2 (1,3%) étaient positifs au virus de la grippe A et 3 (1,9%) au virus de la grippe B. Aucune co-infection par le SARS-CoV-2 et le virus de la grippe n'a été détectée.
Conclusion: Dans cette étude, la fréquence de détection du virus de la grippe chez les patients suspectés d'être atteints de la COVID-19 était faible, tant pour les virus de la grippe A que pour les virus de la grippe B, mais aucune co-infection du virus par le SARS-CoV-2 n'a été observée. L'étude souligne la nécessité d'une surveillance continue des agents pathogènes viraux respiratoires chez les personnes présentant des symptômes respiratoires dans le cadre de l'étude.

Mots-clés: Virus de la grippe, SARS-CoV-2, COVID-19, PCR en temps réel, Échantillons archivés

Introduction:

Members of the *Orthomyxoviridae* family are responsible for the acute viral illness known as influenza. There are three primary types of influenza viruses; A, B, and C. Among these, influenza A virus is particularly significant due to its association with major pandemics, including four global outbreaks in the 20th century. The most catastrophic of these was the "Spanish flu" pandemic of 1918–1919, which claimed approximately 20 million lives worldwide (1,2).

Both influenza A and B viruses are principal contributors to seasonal influenza epidemics (3, 4). Influenza A virus infects a wide range of hosts including humans, birds, pigs, and horses, whereas influenza B and C viruses are predominantly human pathogens. However, influenza type C viruses have occasionally been isolated from pigs and dogs (3). Subtype classification of Influenza A viruses is based on their surface glycoproteins; haemagglutinin (HA) and neuraminidase (NA). The subtypes are denoted using the letters "H" and "N," such as H1N1 and H3N2, which have been the most common circulating strains in humans since the late 1970s (4).

According to the World Health Organization (WHO), influenza affects around 1 billion individuals annually, with 3–5 million cases progressing to severe illness and approximately 290,000 to 650,000 influenza-related respiratory deaths each year (5). Influenza affects all age groups, but accurate incidence estimates are hindered by the fact that many individuals do not seek medical attention or receive formal diagnoses. A meta-analysis incorporating statistical extrapolation from United States hospitalization data estimates the annual incidence at around 8% (range 3–11%) (6). Additionally, several other respiratory viruses, including parainfluenza, adenoviruses, enteroviruses, and paramyxoviruses, can mimic influenza-like illnesses, complicating clinical diagnosis (7).

Historically, viral culture has served as the 'gold standard' for influenza diagnosis. However, this method is time-consuming, technically demanding, and has limited sensitivity, especially during widespread outbreaks. Modern diagnostic approaches include non-culture-based techniques such as immunofluorescence, enzyme immunoassays, and molecular assays. Among these, real-time reverse transcription polymerase chain reaction (RT-PCR), including one-step and multiplex RT-PCR formats, provides superior sensitivity and specificity, along with rapid turnaround time (8). Real-time RT-PCR has thus become the diagnostic method of choice, especially during outbreaks and pandemics.

During the COVID-19 pandemic, reports of co-infection with influenza A virus (IAV) and SARS-CoV-2 varied significantly across different regions and studies (9). This highlights the need for continued surveillance and accurate diagnostic tools to differentiate between respiratory pathogens with overlapping clinical presentations.

Materials and method:

Study site and sample:

This laboratory-based study was conducted at the Biorepository and Clinical Virology Laboratory (BCVL), College of Medicine, University of Ibadan. The laboratory is accredited by the Nigerian Centre for Disease Control (NCDC) and enrolled for external quality assurance with the WHO-accredited organization. To date, the BCVL has tested and archived over 40,000 nasopharyngeal and oropharyngeal swab samples from suspected COVID-19 cases.

The laboratory maintains a collabora-

tive quality control network with Northwestern University and the African Centre of Excellence for Genomics of Infectious Diseases (ACEGID). Samples are stored at –80°C. Archived nasopharyngeal specimens confirmed as SARS-CoV-2 positive and negative were retrieved for this study.

Viral RNA Extraction and RT-PCR:

RNA extraction was performed in a Class II biosafety cabinet using the Da An Gene extraction kit (Da An Gene Co., Ltd., Sun Yat-Sen University) following the manufacturer's instructions. RT-PCR amplification was carried out using the detection kit for influenza A, influenza B, and respiratory syncytial virus (RSV) RNA from Guangdong Huayin Medicine Science Co. (Catalog No. HY06128). The RT-PCR protocol involved reverse transcription at 50°C for 10 minutes, pre-denaturation at 95°C for 2 minutes, followed by 45 amplification cycles with 5 seconds at 95°C for denaturation and 35 seconds at 95°C for annealing and extension.

Data analysis:

Descriptive statistics (percentages) were used to summarize variables. Inferential statistics, including Chi-square tests, were used to compare categorical variables. Statistical computations were performed using SPSS version 21, while graphical illustrations were created using R version 4.1.1.

Results:

The demographic distribution of the COVID-19 patients whose archived samples were retrieved, and the sample collection locations is summarized in Tables 1 and 2. A total of 230 patient samples were analyzed, consisting of 73 samples from patients with COVID-19 and 157 from individuals without COVID-19.

Among patients with COVID-19, 50 (68.5%) were males and 23 (31.5%) were females. Most of the samples (n=167, 72.6%) were collected from patients in the community site at Agodi, Ibadan (Health is Wealth Clinic), while 1 (0.4%) and 62 (27.0%) were collected from hospital-based sources in BCVL and University College Hospital, respectively.

Table 1: Age and gender distribution of the study participants

Characteristics	No of patients with COVID-19 (n=73)	No of patients without COVID-19 (n=157)	Total (n=230)
Age group (years)			
≤ 19 years	3 (4.1)	15 (9.6)	18 (7.8)
20-39 years	44 (60.3)	72 (45.9)	116 (50.4)
40-59 years	10 (13.7)	50 (31.8)	60 (26.1)
≥60 years	16 (21.9)	20 (12.7)	36 (15.7)
Sex			
Male	50 (68.5)	70 (44.6)	120 (52.2)
Female	23 (31.5)	87 (55.4)	110 (47.8)

Table 2: Distribution of respiratory samples by collection sites

COVID-19 sample collection areas	Frequency (%)
Hospital	
Biorepository & Clinical Virology Laboratory, College of Medicine, University of Ibadan	1 (0.4)
University College Hospital	62 (27.0)
Subtotal	63 (27.4)
Community	
Health is Wealth, Agodi-Ibadan	167 (72.6)
Total	230 (100.0)

Regarding patient status, 7 (4.5%) of the patients without COVID-19 were in-patients and 150 (95.5%) were out-patients, while among patients with COVID-19, 6 (8.2%) were in-patients and 67 (91.8%) were out-patients (Fig 1). No significant difference was observed between the groups with respect to inpatient or outpatient location (p=0.356). In terms of clinical symptoms, all the patients with COVID-19, presented with fever. Cough and headache were also common, occurring in 48 (65.8%) and 41 (56.2%) of patients, respectively. Similarly, all the patients without COVID-19 experienced fever (Table 3). Tiredness and cough were reported in 103 (65.6%) and 96 (61.1%) of patients without COVID-19 respectively.

Importantly, no co-infection of SARS-CoV-2 with influenza A or B virus was detected. Of the 157 SARS-CoV-2 negative patients, 2 (1.3%) tested positive for influenza A virus (Ct values 23.37 and 34.57), and 3 (1.9%) tested positive for influenza B virus (Ct values 36.1, 36.03, and 17.79). All influenza virus-positive patients had fever; some also experienced cough, runny nose, and headache (Table 3).

Table 3: Comparative symptom distribution between patients with confirmed COVID-19 and those without COVID-19

Clinical symptoms	**COVID-19 status**	
	Positive (%)	**Negative (%)**
Fever	73 (100.0)	157 (100.0)
Sore throat	26 (35.6)	43 (27.4)
Running nose	23 (31.5)	51 (32.5)
Cough	48 (65.8)	96 (61.1)
Shortness of breath	6 (8.2)	25 (15.9)
Headache	41 (56.2)	87 (55.4)
Vomiting	3 (4.1)	16 (10.2)
Nausea	12 (16.4)	18 (11.5)
Diarrhea	6 (8.2)	14 (8.9)
Tiredness	35 (47.9)	103 (65.6)
Chest pain	6 (8.2)	40 (25.5)
Red eyes	5 (6.8)	19 (12.1)
Loss of taste	14 (19.2)	44 (28.0)
Loss of smell	9 (12.3)	30 (19.1)

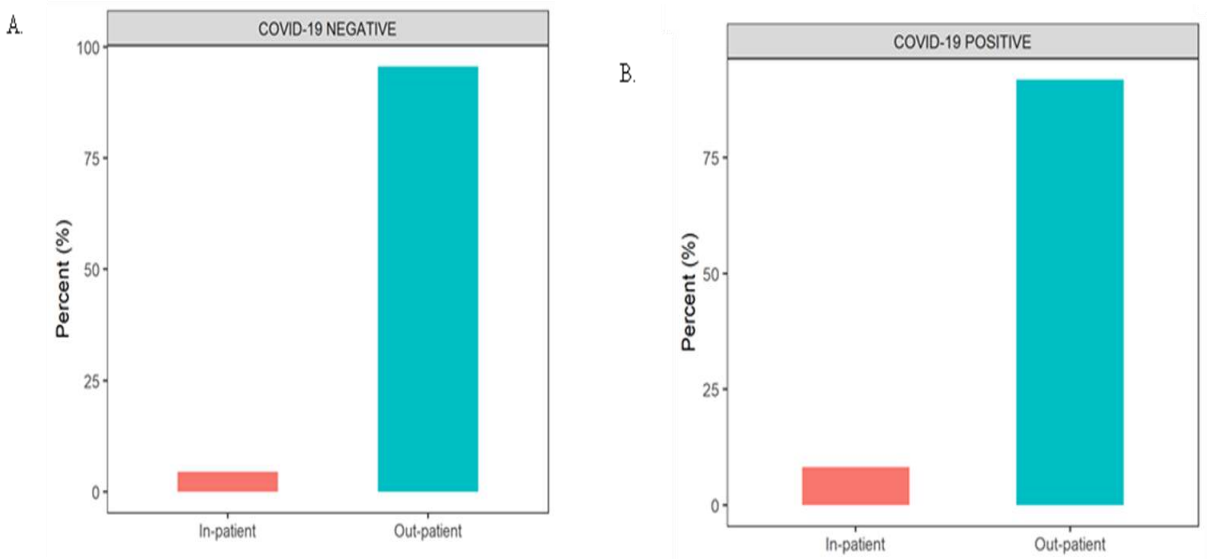

Fig 1: Frequency (%) of patients with and without COVID-19 with respect to in-patient and out-patient locations

Discussion:

This study employed a highly sensitive and specific real-time RT-PCR assay to detect influenza A and B viruses in archived SARS-CoV-2 respiratory specimens. Our findings demonstrated the absence of influenza virus in all SARS-CoV-2 positive samples (n=73). Among the 157 SARS-CoV-2 negative samples, 2 (1.3%) tested positive for influenza A virus, and 3 (1.9%) tested positive for influenza B virus. These findings are consistent with prior surveillance reports in Nigeria and neighboring countries.

A nationwide surveillance program in Nigeria initiated in 2008 and published in 2012 reported a 7.7% influenza virus positivity rate, predominantly influenza A virus (9). In Ghana, monthly influenza virus positivity in co-infection surveillance programs was reported as low as 0.3%, although higher rates were observed in specific age groups, especially in children aged 0–10 years, where the positivity rate was 20% (10).

Reports of co-infection rate of influenza virus with SARS-CoV-2 vary by region. Lansbury et al., (11) estimated a 3.0% co-infection rate with other respiratory viruses among hospitalized COVID-19 patients, with influenza A virus being a frequent co-pathogen. Alotaibi et al., (12) in 2023 reported concurrent detection of influenza A and B viruses in SARS-CoV-2 patients, while a study in Burkina Faso also documented such co-infections (13). Wu et al., (14) in 2020 noted that influenza A can complicate SARS-CoV-2 diagnosis. Case reports suggest that dual infections with SARS-CoV-2 and other viruses, including influenza, may exacerbate disease severity and increase mortality (15). Therefore, accurate and timely identification of such co-infections is vital for improving clinical outcomes.

While our RT-PCR assays for influenza virus were not conducted immediately following COVID-19 diagnosis, prior research confirms that delayed processing (even up to 105 hours) does not compromise RNA integrity for influenza detection. The absence of influenza among SARS-CoV-2 positive patients and the low prevalence among SARS-CoV-2 negative patients may be influenced by sample size, study design, environmental or seasonal factors, and population characteristics. Possible contributing factors include lower viral shedding in older individuals, mucosal dryness, and the use of supplemental oxygen, as well as delayed sample collection beyond the early phase of illness when viral load peaks. Additionally, specimen site variability may influence detection sensitivity, though this remains underexplored.

Moreover, distinguishing COVID-19 from other viral respiratory illnesses during flu season is complicated by overlapping symptoms such as fever, cough, and shortness of breath. Both diseases can present with similar blood abnormalities (e.g., leukopenia and lymphopenia) and radiographic findings (e.g., ground-glass opacities), further complicating diagnosis. Hence, concurrent detection of both SARS-CoV-2 and influenza virus reinforces diagnostic challenges, particularly when SARS-CoV-2 is not detected but other viral infections are present.

Influenza and COVID-19 share overlapping transmission patterns, clinical symptoms, and seasonal peaks. Co-infections are especially concerning in high-risk populations, where they can worsen clinical presentation. Experimental studies suggest that prior influenza infection enhances SARS-CoV-2 entry and replication, potentially worsening disease in susceptible individuals (10,16). Recent studies from China observed frequent SARS-CoV-2/influenza co-infections during the early COVID-19 outbreak in Wuhan, China, associating them with poorer clinical outcomes (17). These findings, along with sporadic African data, motivated our investigation. Our results underscore the need for comprehensive respiratory pathogen surveillance to distinguish between co-circulating viruses. This is crucial for effective patient management, particularly during flu seasons and pandemic surges, where disease severity may be amplified by co-infection (18).

Influenza remains a highly contagious viral respiratory illness with a history of significant morbidity and mortality during epidemics and pandemics. Differentiating it from COVID-19 is essential, as the two are caused by distinct viruses and may require divergent management strategies.

Conclusion:

In this study, influenza virus was not detected in archived samples of patients with confirmed COVID-19. However, influenza virus was detected in samples of patients without COVID-19, albeit at a very low prevalence. This finding may suggest that various clinical mitigation measures such as adherence to patient care protocols, improved clinical conditions, and compliance with hospital policies might have influenced the reduced detection of influenza.

Concurrent infections involving multiple respiratory viruses are commonly observed in human populations and are associated with an increased risk of hospitalization, ICU admission, and mortality. In our study, influenza type B virus was more frequently detected than type A. These findings reinforce the importance of employing advanced molecular methods, beyond RT-PCR alone, for the detection of influenza viruses. It is also important to

note that the genetic variability of influenza viruses may compromise assay sensitivity and increase the risk of false-negative results. Therefore, improved molecular diagnostics and continued surveillance are essential to accurately assess the burden and dynamics of influenza virus infections, particularly during pandemics such as COVID-19.

Contributions of authors:

OJO was involved in the study conceptualization, design, methodological activities, and initial draft production; FA was involved in the study conceptualization, design, methodological activities, initial draft production and supervision, and OOA was involved in the critical review of the initial draft. All authors were involved in findings interpretation, critical review and approval of the final draft.

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No funding was received for the study.

Conflict of interest:

Authors declare no conflict of interest.

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Original Article **Open Access**

Antimicrobial resistance patterns of enteric bacteria in HIV sero-positive patients: A regional hospital-based cross-sectional survey in Kumasi metropolis, Ghana

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Abstract:

Background: Enteric bacterial infections are at least ten times more common in HIV individuals compared to the general population. This results in frequent consumption of antibiotics either for prophylactic or treatment purposes, resulting in a rise in emergence of multidrug-resistant (MDR) bacteria. The paucity of research on antibiotic resistance in enteric bacteria among HIV individuals in secondary and primary healthcare settings in Ghana necessitated this study, which investigated the antibiotic susceptibility and resistance patterns of enteric bacterial isolates from HIV-positive individuals at three healthcare facilities in Kumasi metropolis, Ghana.
Methodology: This was a descriptive cross-sectional survey of 57 HIV-seropositive individuals on anti-retroviral therapy at three selected district hospitals in Kumasi metropolis, for resistance pattern of enteric bacteria. Faecal samples from HIV-positive individuals were collected for microbiological analysis, using differential and selective agar media and conventional biochemical assays for isolation, preliminary characterisation and presumptive identification of bacterial isolates. Confirmatory identification of the isolates was performed using MALDI-TOF MS. Antimicrobial susceptibility testing by the Kirby-Bauer disk diffusion method was used to evaluate the susceptibility of the isolates to a panel of 10 antimicrobial agents. Data were analysed with WHONET software, GraphPad Prism, Microsoft Excel and SPSS. Association of demographic data and other patients' parameters was determined using Chi-square tests with 95% confidence interval and $p<0.05$ was considered significant.
Results: Thirty enteric bacterial isolates were confirmed from the 57 HIV-seropositive individuals, comprising predominantly *Escherichia coli* (80.0%), *Klebsiella pneumoniae* (10.0%), *Enterococcus faecalis* (6.7%), and *Enterobacter cloacae* (3.3%). Antimicrobial susceptibility testing showed resistance rates ranging from 3.3% to 100.0%, with majority of isolates susceptible to ciprofloxacin and meropenem. Sixty per cent of the isolates were MDR and *E. coli* and *K. pneumoniae* isolates were highly resistant to amoxicillin-clavulanic acid and ampicillin.
Conclusion: *E. coli* was the most frequent enteric bacterial isolate carried by HIV-infected individuals in this study. Over 60% of the enteric bacteria were multidrug-resistant. Meropenem and ciprofloxacin were the antibiotics with high *invitro* inhibitory activity against the isolates. The use of these antibiotics for therapy should however be guarded to avoid rapid resistance development by bacterial pathogens to these agents.

Keywords: HIV seropositive. Enterobacteria, *E. coli,* meropenem, ciprofloxacin

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Profils de résistance aux antimicrobiens des bactéries entériques chez les patients séropositifs pour le VIH: une enquête transversale régionale menée en milieu hospitalier dans la métropole de Kumasi, au Ghana

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Résumé:

Contexte: Les infections bactériennes entériques sont au moins dix fois plus fréquentes chez les personnes infectées par le VIH que dans la population générale. Cela entraîne une consommation fréquente d'antibiotiques, que ce soit à titre prophylactique ou thérapeutique, et une augmentation de l'émergence de bactéries multirésistantes (MR). Le manque de recherches sur la résistance aux antibiotiques des bactéries entériques chez les personnes infectées par le VIH dans les établissements de soins de santé primaires et secondaires au Ghana a rendu nécessaire la réalisation de cette étude. Cette étude a examiné la sensibilité aux antibiotiques et les profils de résistance d'isolats de bactéries entériques provenant de personnes infectées par le VIH dans trois établissements de santé de la métropole de Kumasi, au Ghana.
Méthodologie: Il s'agissait d'une enquête transversale descriptive menée auprès de 57 personnes séropositives sous traitement antirétroviral dans trois hôpitaux de district sélectionnés de la métropole de Kumasi, afin d'évaluer le profil de résistance des bactéries entériques. Des échantillons de selles de personnes séropositives ont été prélevés pour analyse microbiologique, à l'aide de milieux gélosés différentiels et sélectifs, ainsi que de dosages biochimiques conventionnels pour l'isolement, la caractérisation préliminaire et l'identification présomptive des isolats bactériens. L'identification confirmatoire des isolats a été réalisée par spectrométrie de masse MALDI-TOF. Des tests de sensibilité aux antimicrobiens par diffusion sur disque de Kirby-Bauer ont été utilisés pour évaluer la sensibilité des isolats à un panel de 10 agents antimicrobiens. Les données ont été analysées avec les logiciels WHONET, GraphPad Prism, Microsoft Excel et SPSS. L'association des données démographiques et des autres paramètres des patients a été déterminée par des tests du Chi carré avec un intervalle de confiance à 95% et une valeur de $p<0,05$ a été considérée comme significative.
Résultats: Trente isolats de bactéries entériques ont été confirmés chez 57 personnes séropositives pour le VIH, comprenant principalement *Escherichia coli* (80,0%), *Klebsiella pneumoniae* (10,0%), *Enterococcus faecalis* (6,7%) et *Enterobacter cloacae* (3,3%). Les tests de sensibilité aux antimicrobiens ont montré des taux de résistance allant de 3,3% à 100,0%, la majorité des isolats étant sensibles à la ciprofloxacine et au méropénème. Soixante pour cent des isolats étaient multirésistants, et les isolats d'*E. coli* et de *K. pneumoniae* étaient hautement résistants à l'amoxicilline-acide clavulanique et à l'ampicilline.
Conclusion: E. coli était l'isolat de bactérie entérique le plus fréquemment présent chez les personnes infectées par le VIH dans cette étude. Plus de 60 % des bactéries entériques étaient multirésistantes. Le méropénème et la ciprofloxacine étaient les antibiotiques ayant une forte activité inhibitrice in vitro contre les isolats. L'utilisation thérapeutique de ces antibiotiques doit toutefois être prudente afin d'éviter le développement rapide d'une résistance des bactéries pathogènes à ces agents.

Mots-clés: Séropositivité au VIH, entérobactéries, *E. coli*, méropénème, ciprofloxacine

Introduction:

HIV/AIDS remains a disease that weakens the host defences against many infections as it affects the immune system even in the face of improved therapy (1). The World Health Organisation (WHO) indicates that over 39 million (33.1–45.7) people were living with HIV at the end of 2022 (2). The Ghana national HIV and AIDS strategic plan (2021-2025) estimates 346,120 (91.71% adults and 8.29% children) people living with HIV (PLHIV), with an adult national HIV prevalence of 1.68%. In Ghana, the Ashanti region is estimated to have an HIV prevalence of 1.8% (3).

Due to the immunocompromised nature of PLHIV, they are at risk of frequent antibiotic consumption, either for treatment or prophylaxis of infections (4). Overuse and misuse of these antibiotics can result in the development of different resistance mechanisms by enteric bacteria (5,6). Unfortunately, HIV-infected individuals may harbour resistant bacterial strains because of their frequent exposure to antibiotic medication and prophylaxis (7).

The current management of bacterial infections could be difficult owing to multidrug-resistant (MDR) bacteria and limited treatment options (8). Some enteric bacterial pathogens, such as *Salmonella* spp., *Klebsiella* spp., *Campylobacter* spp., *Shigella* spp., *Clostridioides difficile* and different strains of *Escherichia coli* have been identified as causative agents of diarrhoea in HIV-infected patients with the potential to induce severe diarrhoeal disease when they are multidrug-resistant (9).

Enteric bacterial infections are at least ten times more common in HIV patients than in the general population (10). Enteric bacterial pathogens with high antibiotic resistance have been reported among PLHIV (11), however such reports are few and unavailable especially in low-and-middle income countries, both globally and in sub-Saharan Africa (10).

Surveillance aids in the identification of factors that contribute to the spread of antimicrobial resistance (AMR) in specific areas, allowing for the implementation of control measures, however, most investigations on the extent of antibiotic resistance in enteric bacteria from immunocompromised individuals are carried out in tertiary-level health institutions, without recourse to the primary and secondary levels, which are typically the initial place of visit for the treatment of most enteric infections.

Efforts to reduce enteric pathogen transmission cannot be overemphasised, however, the rate of resistance of diverse infectious agents

varies by geographical area and country (4). This study south to investigate the antibiotic resistance situation in enteric bacteria from HIV-infected patients from secondary-level health institutions in the Ashanti region of Ghana.

Materials and method:

Study setting and design:

This was a descriptive cross-sectional study conducted at three hospitals; Suntreso Hospital, Manhyia Hospital and Kumasi South Hospital, in Kumasi metropolis, Ghana. The three district hospitals serve as primary health centres for over 70% of the inhabitants in the Kumasi metropolis.

Ethical approval:

The study was approved by the Research and Development Unit of the Komfo Anokye Teaching Hospital (RD/CR20/095). Consent from the various hospitals was granted, and verbal and written consent was obtained from the study participants.

Study population, participants and sample size:

The study included HIV/AIDS patients who were visiting the hospitals for routine check and fill-up of their Highly Active Anti-retroviral Therapy (HAART) medications. To be eligible for inclusion in the study, participants should not have been on or received any specific antibiotic therapy in the previous two weeks prior to the study. Diabetic patients, pregnant women, smokers and patients on oestrogen were also exempted from the study.

The sample size was determined using the formulae (12); $(Z_{1-\alpha/2})^2 \times p\ (1-p)/d^2$, where $Z_{1-\alpha/2}$ is the standard normal variate at 5% (p<0.05 =1.96), p is prevalence of PLHIV (2% or 0.02) in the Ashanti region (13), and d is margin of error of 5% (0.05). This gave a calculated sample size of 30 HIV/AIDS patients, but 57 patients were recruited into the study.

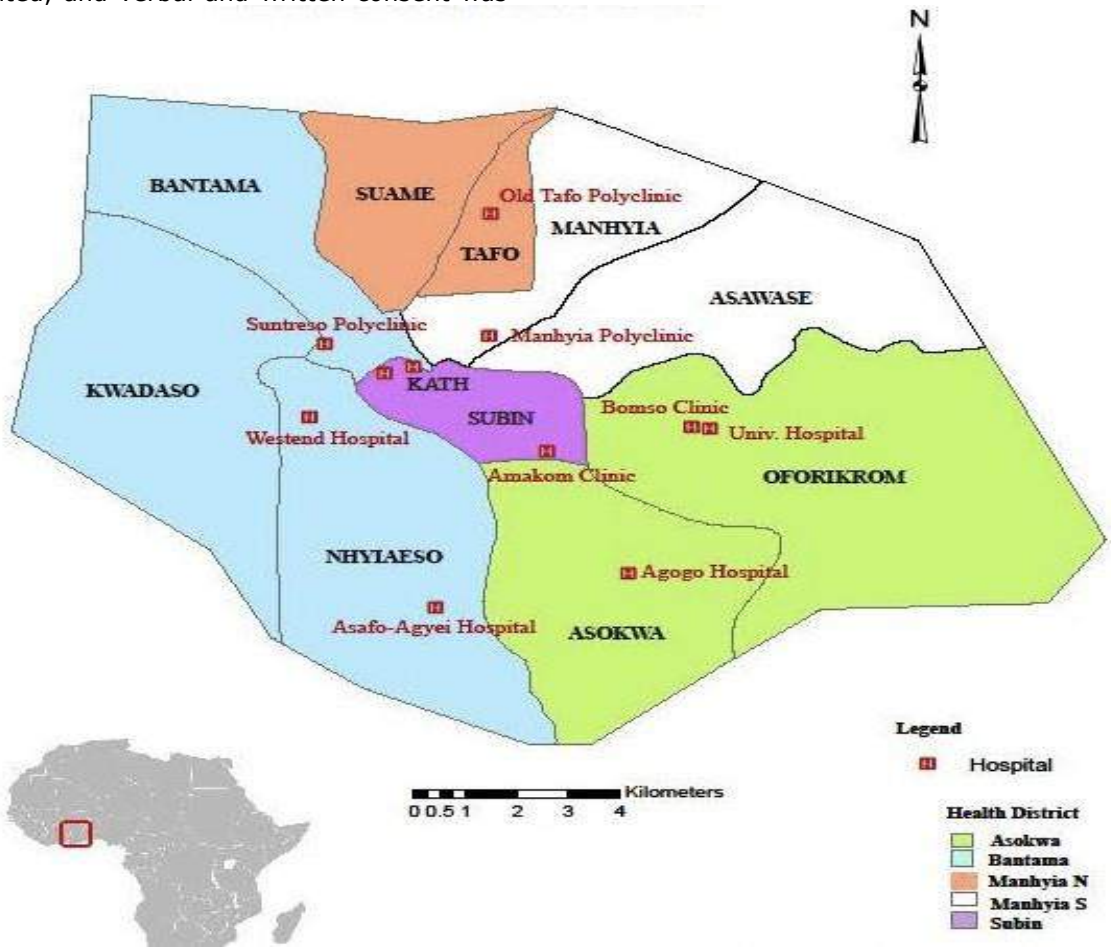

Fig 1: Demographical representation of the Kumasi metropolis health district and health facilities (http://mci.ei.columbia.edu/millennium-cities/kumasi-ghana/kumasi-maps-and-population-data/)

Data collection:

Participants were given a consent form to sign, after the research had been explained and they agreeing to submit their stool samples for testing and giving consent to access personal data from their hospital records. The personal data accessed included their name, age, sex, contact details, medication history (duration of HAART treatment) and current medication.

Sample collection, bacterial isolation and preliminary identification:

Stool samples were collected from participants using sterile disinfectant-free containers. The specimens were sent to the labo ratory, emulsified with sterile water and cultured on Xylose Lysine Deoxycholate agar (XLD agar), MacConkey agar and Mannitol Salt Agar plates, and incubated at 37°C for 24 h. The isolated organisms were preliminarily identified based on colonial morphology, Gram stain, and conventional biochemical tests, and confirmed by Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (14).

Confirmation of isolates using MALDI-TOF:

MALDI-TOF of ribosomal and housekeeping proteins was used to confirm the isolates. Preliminarily identified bacteria isolates were cultured on tryptone soya agar, a colony was picked from a cultured plate and spotted (forming a thin film layer of the colony) on a MALDI-TOF MS target plate. The cells were then treated with formic acid (Surechem, Needham, UK) on the target plate on the MALDI Biotyper (Bruker Daltonic GmbH). Generated spectra were compared with current libraries (MALDI Flex Control software system Server Version: 4.1.31 and 60 Hz Nitrogen Laser (337 nm wavelength) and log scores generated (Scores of the log were classified as: > 2.0 = High confidence identification, 1.7–2.0 = Low confidence and < 1.7.0 = Unreliable).

Antimicrobial susceptibility testing:

Antibiotic susceptibility tests on the confirmed isolates were done by the Kirby–Bauer method. The antibiotics tested included amikacin (AMK 30µg), amoxicillin/clavulanic acid (AMC 20/10µg), ampicillin (AMP 10µg), aztreonam (ATM 30µg), cefotaxime (CTX 30µg), ceftriaxone (CRO 30µg), cefuroxime (CXM 30µg), chloramphenicol (CHL 30µg), ciprofloxacin (CIP 5µg), gentamicin (CN 30 µg), meropenem (MEM 10µg), and tetracycline (TEN 30µg).

Colonies of the bacterial isolates were suspended in 3ml nutrient broth and incubated for 24 hours. The turbidity of the suspension of the isolate was standardised by adjusting its turbidity to match 0.5 McFarland standard (Hardy Diagnostics, Santa Maria, USA), which is equivalent to approximately 1.5×10^8 colony forming units (CFU) per millilitre (15). A sterile cotton swab was dipped into the standardised bacteria suspension and swabbed evenly across the entire surface of Mueller Hinton (MH) agar plate to obtain a semiconfluent growth. The plate was incubated aerobically at 37°C for 24 hours.

After incubation, clear zones around the antibiotic discs (zones of inhibition) were measured; taking the average of the horizontal diameter, vertical diameter and diagonal diameter of the zones and interpreted as sensitive, intermediate or resistant based on the breakpoint criteria of the Clinical and Laboratory Standards Institute (16). Isolates showing resistance to three or more categories of antibiotics were considered as multidrug-resistant bacteria (17).

Analysis of data:

All data obtained were stored on a secured hard drive at the Department of Pharmaceutics, KNUST. WHONET software, GraphPad Prism from GraphPad Software Inc., USA, version 8.0 and Microsoft Excel from Microsoft Office Professional Plus 2016, USA, were used to analyse the data obtained. WHONET software was used to determine the sensitivity profile of isolates using zone breakpoints from 2024 CLSI. A statistical association of demographic data and other parameters was performed using the Statistical Package for the Social Sciences (IBM SPSS Statistics V. 30) with Chi-square tests and 95% confidence interval, and $p<0.05$ considered significant.

Results:

Sources of samples and prevalence of enteric bacteria carriage among the participants:

Stool samples were obtained from 57 HIV-seropositive patients in the 3 hospitals (Suntreso Hospital, Kumasi South Hospital and Manhyia Hospital) within the Kumasi metropolis of Ghana. Forty (40) (70.2%) were from Suntreso Hospital, 11 (19.3%) from Manhyia Hospital and 6 (10.5%) from Kumasi South Hospital (Table 1).

Table 1: Sources of samples and prevalence of enteric bacteria carriage among HIV-seropositive patients

Facility	HIV- seropositive samples	Samples positive for enteric bacteria	x^2	*p* value
Suntreso	40 (70.2)	15 (37.5)	12.832	0.0016*
Manhyia	11 (19.3)	9 (81.8)		
Kumasi	6 (10.5)	6 (100.0)		
Total	57 (100.0)	30 (52.6)		

x^2 = Chi square; * = statistically significant

Table 2: Sociodemographic characteristics and medication history of the HIV-seropositive participants

Parameters	Number	Percentage (%)
Sex		
Female	48	84.2
Male	9	15.8
Age group (years)		
0-20	1	1.8
20-40	23	40.4
40-60	25	43.9
> 60	8	14.0
Duration of therapy (months)		
0-24	20	35.1
24-48	13	22.8
48-72	10	17.5
72-96	6	10.5
96-120	4	7.0
120-144	3	5.3
144-168	1	1.8
Medication history		
3TC/TDF/DTG	40	70.2
3TC/TDF/EFV	14	24.6
3TC/ZDV/EFV	1	1.8
ABC/3TC/LPV/r	1	1.6
FTC/TDF/EFV	1	1.6
Total	**57**	**100.0**

3TC – Lamivudine, TDF – Tenofovir, DTG – Dolutegravir, EFV – Efavirenz, ZDV – Zidovudine, LPV/r – Lopinavir/Ritonavir, FTC – Emtricitabine

Sociodemographic characteristics and medication history of the participants

Forty-eight (84.2%) of the samples were obtained from females while 9 (15.8%) samples were from males (Table 2). One (1.75%) sample was obtained from a patient aged 0-20 years, 23 (40.4 %) between 20-40 years, 25 (43.9 %) between 40-60 years and 8 (14.0%) above 60 years. A total of 20 participants (35.1%) have been on HAARTs for 0 to 24 months, with just one person having been on HAARTs for about 156 months (13 years). The most common HAART combination used was Lamivudine/Tenofovir/Dolutegravir (used by 70.2 % of the participants).

Frequency distribution of confirmed enteric bacterial isolates:

Thirty (30) bacterial isolates were identified and confirmed from the samples based on the culture media, biochemical reactions and MALDI-TOF (Table 3), giving enteric bacteria carriage rate of 52.6% in the study population. Fifteen (37.5%) isolates were obtained from Suntreso Hospital (SH), 9 (81.8%) from Manhyia Hospital (MH), and 6 (100.0%) from Kumasi South Hospital (KH) (x^2=12.832, p=0.0016) (Table 1).

Twenty-four (80.0%) of the isolates were *Escherichia coli*, 3 (10.0%) *Klebsiella pneumoniae*, 2 (6.7%) *Enterococcus faecalis*, and 1 (3.3%) *Enterobacter cloacae* isolate (Table 4). From the 15 isolates obtained from SH, 13 (86.7%) were *E. coli*, and 1 (6.7%) each were *E. faecalis* and *K. pneumoniae.* From the 9 isolates obtained from MH, 6 (66.7%) were *E. coli*, 2 (18.2%) were *K. pneumoniae* and 1 (9.1%) was *E. cloacae*. From the 6 isolates obtained from KH, 5 (83.3%) were *E. coli*, and 1 (16.7%) was *E. faecalis* (Table 4).

Table 3: MALDI-TOF confirmatory identification lists of the microbial isolates

Sample Number	Organism (First match)	Score value	Organism (second match)	Score value
1.	*Escherichia coli*	2.41	*Escherichia coli*	2.26
2.	*Escherichia coli*	2.28	*Escherichia coli*	2.26
3.	*Enterococcus faecalis*	2.21	*Enterococcus faecalis*	2.21
4.	*Escherichia coli*	1.75	*Escherichia coli*	1.84
5.	*Escherichia coli*	2.40	*Escherichia coli*	2.37
6.	*Enterococcus faecalis*	2.22	*Enterococcus faecalis*	2.19
7.	*Escherichia coli*	2.17	*Escherichia coli*	2.16
8.	*Escherichia coli*	2.42	*Escherichia coli*	2.33
9.	*Escherichia coli*	2.36	*Escherichia coli*	2.35
10.	*Escherichia coli*	2.40	*Escherichia coli*	2.35
11.	*Escherichia coli*	2.35	*Escherichia coli*	2.25
12.	*Escherichia coli*	2.41	*Escherichia coli*	2.37
13.	*Klebsiella pneumoniae*	2.33	*Klebsiella pneumoniae*	2.33
14.	*Escherichia coli*	2.07	*Escherichia coli*	2.07
15.	*Escherichia coli*	1.74	*Escherichia coli*	1.73
16.	*Escherichia coli*	2.01	*Escherichia coli*	1.81
17.	*Klebsiella pneumoniae*	2.21	*Klebsiella pneumoniae*	2.17
18.	*Escherichia coli*	2.21	*Escherichia coli*	2.13
19.	*Escherichia coli*	2.00	*Escherichia coli*	1.86
20.	*Escherichia coli*	2.15	*Escherichia coli*	1.94
21.	*Escherichia coli*	2.12	*Escherichia coli*	2.08
22.	*Escherichia coli*	2.33	*Escherichia coli*	2.30
23.	*Escherichia coli*	2.17	*Escherichia coli*	2.16
24.	*Escherichia coli*	2.32	*Escherichia coli*	2.29
25.	*Escherichia coli*	2.28	*Escherichia coli*	2.22
26.	*Escherichia coli*	2.20	*Escherichia coli*	2.20
27.	*Escherichia coli*	2.29	*Escherichia coli*	2.21
28.	*Escherichia coli*	1.89	*Escherichia coli*	1.80
29.	*Klebsiella pneumoniae*	2.25	*Klebsiella pneumoniae*	2.07
30.	*Enterobacter cloacae*	2.20	*Enterobacter cloacae*	2.10

Table 4: Frequency of enteric bacterial isolates with respect to hospital facility

Bacterial isolate	Number of bacterial isolate (%)			Total (%_
	SH	MH	KH	
Escherichia coli	13 (86.7)	6 (66.7)	5 (83.3)	24 (80.0)
Klebsiella pneumoniae	1 (6.7)	2 (18.2)	0	3 (10.0)
Enterococcus faecalis	1 (6.7)	0	1 (16.7)	2 (6.7)
Enterococcus cloacae	0	1 (9.1)	0	1 (3.4)
Total	**15**	**9**	**6**	**30 (100.0)**

SH: Suntreso Hospital; MH: Manhyia Hospital; KH: Kumasi South Hospital.

Antibiotic resistance profile of isolates:

Ten enteric bacterial isolates (33.3%) were resistant to cefotaxime, 15 (50.0%) to ceftriaxone and 5 (16.7%) to cefuroxime. Two isolates (6.7%) each were resistant to gentamicin and amikacin. Also, 29 (96.7%) and 30 (100.0%) isolates were resistant to ampicillin and amoxicillin-clavulanic acid, respectively. One isolate (3.3%) each was resistant to meropenem and ciprofloxacin. Twelve isolates (40.0%) were resistant to aztreonam, 19 (63.3%) to tetracycline and 3 (10.0%) to chloramphenicol (Fig 2).

Multidrug-resistant isolates:

Thirteen (54.2%) of the 24 *E. coli* isolates were MDR, 7 of these 13 (53.8%) were resistant to 4 antibiotics, 2 (15.4%) to 5 antibiotics, and 4 (30.8%) to 6 antibiotics (Table 6). Eight MDR *E. coli* isolates were from SH, 3 from MH, and 2 from KH (Fig 3). Two *K. pneumoniae* isolates were MDR and were resistant to 4 antibiotics, and all from MH. Two *E. faecalis* isolates were MDR, with 1 isolate from SH resistant to 10 antibiotics, and 1 isolate from KH resistant to 7 antibiotics (Fig 3, Table 6)

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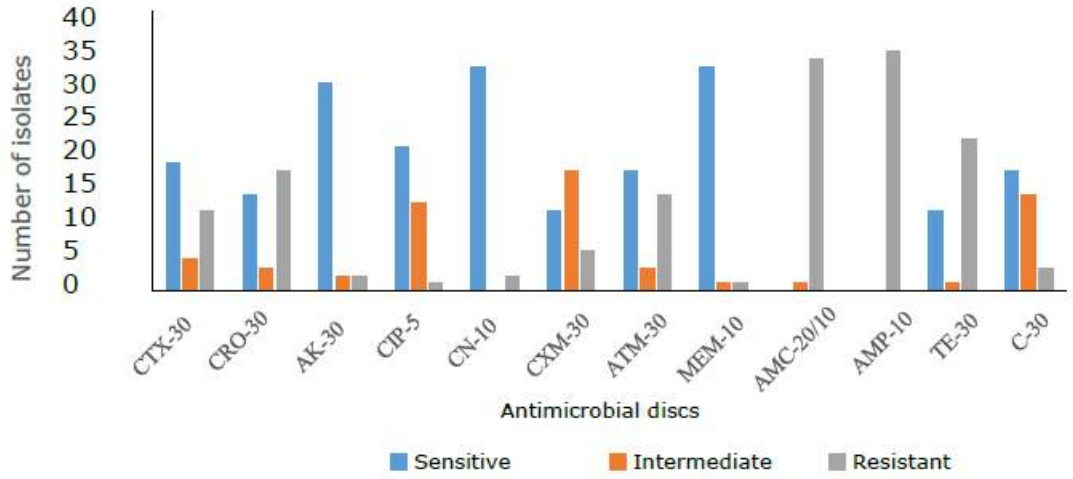

AK-30: Amikacin 30 µg; AMC-20/10: Amoxicillin 20 µg + clavulanic acid 10 µg; AMP-10: Ampicillin 10 µg; ATM-30: Aztreonam 30 µg; CTX-30: Cefotaxime 30 µg; CRO-30: Ceftriaxone 30 µg; CXM-30: Cefuroxime 30 µg; C-30: Chloramphenicol 30 µg; CIP-5: Ciprofloxacin 5 µg; CN-30: Gentamicin 30 µg; MEM-10: Meropenem 10 µg; TE-30: Tetracycline 30 µg

Fig 2: Antibiotic susceptibility and resistance of total bacterial isolates from all samples

Table 5: Antimicrobial resistance of individual bacterial species

Antimicrobial agent	***E. coli* (n=24)**	***K. pneumoniae* (n=3)**	***E. faecalis* (n= 2)**	***E. cloacae* (n= 1)**
CTX-30	6 (25.0)	2 (66.7)	2 (100.0)	0
CRO-30	13 (54.2)	0	2 (100.0)	0
AK-30	0	1 (33.3)	1 (50.0)	0
CIP-5	1 (4.2)	1 (33.3)	0	0
CN-10	0	0	1 (50.0)	0
CXM-30	3 (12.5)	0	2 (100.0)	0
ATM-30	8 (33.3)	1 (33.3)	2 (100.0)	1 (100.0)
MEM-10	0	0	1 (50.0)	0
AMC-20/10	24 (100.0)	3 (100.0)	1 (50.0)	1 (100.0)
AMP-10	24 (100.0)	3 (100.0)	2 (100.0)	1 (100.0)
TE-30	16 (66.7)	0	2 (100.0)	1 (100.0)
C-30	2 (8.3%)	0	1 (50.0)	0

AK-30: Amikacin 30 µg; AMC-20/10: Amoxicillin 20 µg + clavulanic acid 10 µg; AMP-10: Ampicillin 10 µg; ATM-30: Aztreonam 30 µg; CTX-30: Cefotaxime 30 µg; CRO-30: Ceftriaxone 30 µg; CXM-30: Cefuroxime 30 µg; C-30: Chloramphenicol 30 µg; CIP-5: Ciprofloxacin 5 µg; CN-30: Gentamicin 30 µg; MEM-10: Meropenem 10 µg; TE-30: Tetracycline 30 µg

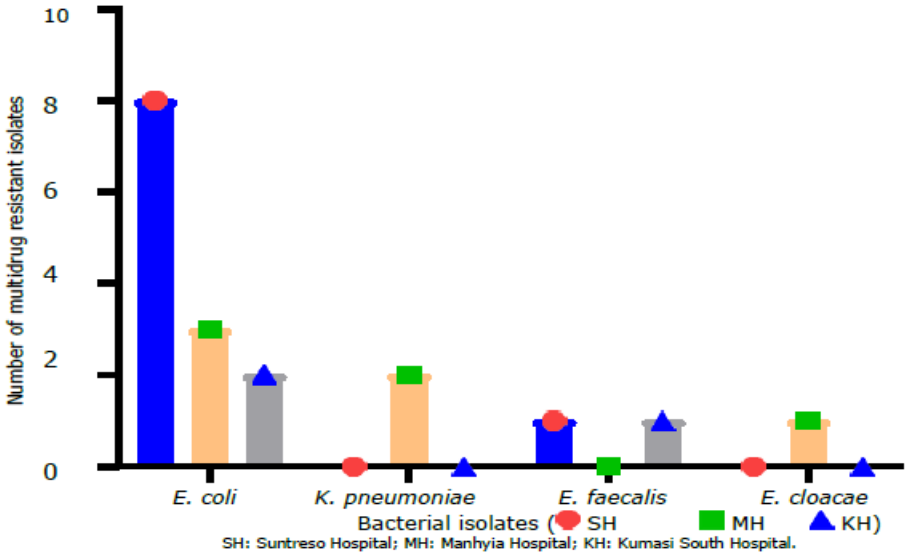

Fig 3: Sources of multidrug-resistant bacterial isolates

Table 6: Multidrug-resistance pattern of enteric bacterial isolates from stool samples of HIV-sero-positive patients from selected hospitals in Kumasi, Ghana

Bacterial isolates and MDR profiles	**Frequency (%)**
***Escherichia coli* (n=24)**	
CRO, AMP, AMC, TE	3 (23.0)
ATM, AMP, AMC, TE	2 (15.4)
AMP, AMC, TE, C	1 (7.7)
CTX, ATM, AMP, AMC	1 (7.7)
CTX, CRO, ATM, AMP, AMC	1 (7.7)
CRO, AMP, AMC, TE, C	1 (7.7)
CTX, CRO, ATM, AMC, AMP, TE	1 (7.7)
CTX, CRO, CXM, ATM, AMC, AMP	3 (23.0)
Total	13 (54.2)
***Klebsiella pneumoniae* (n=3)**	
CTX, ATM, AMP, AMC	1 (50.0)
CRO, CIP, AMP, AMC, TE	1 (50.0)
Total	2 (67.7)
***Enterococcus faecalis* (n=2)**	
CTX, CRO, CXM, ATM, AMC, AMP, TE	1 (50%)
CTX, CRO, AK, CN, CXM, ATM, MEM, AMP, TE, C	1 (50%)
Total	2 (100.0)

AK-30: Amikacin 30 µg; AMC-20/10: Amoxicillin 20 µg + clavulanic acid 10 µg; AMP-10: Ampicillin 10 µg; ATM-30: Aztreonam 30 µg; CTX-30: Cefotaxime 30 µg; CRO-30: Ceftriaxone 30 µg; CXM-30: Cefuroxime 30 µg; C-30: Chloramphenicol 30 µg; CIP-5: Ciprofloxacin 5 µg; CN-30: Gentamicin 30 µg; MEM-10: Meropenem 10 µg; TE-30: Tetracycline 30 µg; SH: Suntreso Hospital; MH: Manhyia Hospital; KH: Kumasi South Hospital

Statistical analysis of sociodemographic data and resistance patterns:

The analysis shows that there was significant association between isolate resistance to amikacin, cefuroxime and ciprofloxacin, with respect to age, current medication and duration of therapy ($p<0.05$) (Tables 7).

All isolates were susceptible to meropenem and tetracycline and resistant to ampicillin, therefore, no statistical association could be performed between the sociodemographic data and these antibiotics.

Table 7: Association of patient demographics with antimicrobial resistance and current medications

Demographics	***p*-values of statistical association of patients' parameters against antimicrobial resistance**							**Current medication vrs Demographics**
	ATM	AK	CN	CRO	CXM	C	CIP	
Age	0.456	0.001	0.231	0.072	0.001	0.001	0.009	0.001
Current medication	0.017	0.002	0.001	0.197	0.001	0.113	0.008	-
Duration of therapy	0.001	0.028	0.001	0.023	0.001	0.001	0.041	0.001
Facility	0.701	0.005	0.915	0.001	0.001	0.185	0.033	0.038
Sex	0.003	0.251	0.002	0.017	0.001	0.003	0.550	0.057

ATM-30: Aztreonam 30 µg; AK-30: Amikacin 30 µg; CN 30: Gentamicin 30 µg; CRO-30: Ceftriaxone 30 µg; CXM-30: Cefuroxime 30 µg; C-30: Chloramphenicol 30 µg; CIP-5: Ciprofloxacin 5µg: A *p*-value < 0.05 indicates a significant association between parameters (at a 95% confidence interval and 0.05 margin of error)

Discussion:

Antibiotic resistance has emerged as a significant public health concern globally, including in Ghana (18). The prevalence of infectious diseases remains high in lower-middle-income countries like Ghana, primarily due to constrained health systems and widespread poverty, limiting access to effective antibiotics (19,20). Specifically, HIV patients are vulnerable to enteric bacterial infections and bacterial pneumonia due to their compromised immune systems (10). Frequent bacterial infections lead to increased antibiotic usage, causing resistance development through repeated microbial exposure to these antibiotics. This complicates treatment, leading to prolonged hospitalization, increased mortality, and substantial financial burdens on families and societies (10,21). This study investigated antibiotic resistance patterns of enteric bacteria in HIV-infected patients in secondary healthcare centres in a region in Ghana.

The study predominantly had female participants (84%), which aligns with global and national trends indicating a higher HIV prevalence among women (2,22). Majority of participants were recruited from Suntreso hospital, located in the Kwadaso district, which reported the third-highest HIV adult prevalence rate (6.40%) in Ghana in 2019 (23). Microbiological analysis showed *E. coli* as the predominant isolate, accounting for 80.0% of the confirmed isolates from stool samples, consistent with previous research reporting increased prevalence in rectal carriage of *E. coli* among HIV-infected individuals (24,25). This supports the initiation of empiric antibiotic therapy targeting *E. coli* or Gram-negative enterobacteria in diarrhoea illnesses in HIV patients. *Klebsiella pneumoniae*, a common intestinal commensal (26), were confirmed in 10.0% of the isolated bacteria, mirroring the findings of Najjuka (24). The remaining 10.0% consisted of *E. faecalis* (6.7%) and *E. cloacae* (3.3%), the latter being a member of the normal gastrointestinal flora and commonly associated with infections in immunocompromised or hospitalised patients (27).

The susceptibility test showed 100.0% resistance among *E. coli* isolates to amoxicillin/clavulanic acid and ampicillin, suggesting the production of β-lactamase enzymes that destroys these antibiotics (28). A similar study in South Africa, reported 50.0% of Enterobacteriaceae isolates produced extended-spectrum beta-lactamases (ESBL), with 90.0% of *E. coli* isolates exhibiting resistance to penicillins (29). Furthermore, Cotton et al., (30) reported 50.0% resistance rate to ceftriaxone, a third-generation cephalosporin, similar to the finding of this current study of 54.2% resistance rate to the third-generation cephalosporins.

However, *E. coli* in our study showed low resistance to aminoglycosides, carbapenems, chloramphenicol, and ciprofloxacin, mirroring the findings of Najjuka (25). Nevertheless, multi-drug resistance, defined as acquired resistance to at least one agent in three or more antimicrobial categories (30), was seen in 54% of the *E. coli* isolates in our study. In a study by Stanley (31), a similar trend was reported where about 57% of *E. coli* isolates were MDR. Other studies also reported a high resistance to amoxicillin/clavulanic acid, ampicillin, and 3rd and 4th generation cephalosporins in combination with tetracycline and cotrimoxazole (32,33). The resistance to cotrimoxazole may be a result of the wide use of the drug for prophylaxis in HIV care (34).

Klebsiella pneumoniae isolates showed resistance to both amoxicillin/clavulanic acid and ampicillin and were highly susceptible to ceftriaxone, cefuroxime, gentamicin, meropenem, tetracycline, chloramphenicol and ciprofloxacin. *K. pneumoniae* has been shown to have high susceptibility to aminoglycosides (35,36). The *Klebsiella* genus has inherent resistance to ampicillin due to its carriage of chromosomally located beta-lactamase gene SHV-1 (31).

The two *E. faecalis* isolates, which were both MDR, showed resistance to all the selected antibiotics except ciprofloxacin. Enterococci species are known to demonstrate intrinsic resistance to cephalosporins, amino-

glycosides, tetracycline and chloramphenicol. They are also known to have acquired genetic determinants that confer resistance to many classes of antimicrobial agents such as cephalosporins, aminoglycosides, tetracycline and chloramphenicol (37,38). *Enterobacter cloacae* isolate was resistant to aztreonam, amoxicillin-clavulanic acid, ampicillin and tetracycline. *E. cloacae* are generally more susceptible to fluoroquinolones, chloramphenicol, aminoglycosides, tetracyclines and carbapenems. On the other hand, most of them are intrinsically resistant to β-lactams owing to the production of constitutive AmpC β-lactamase (39). Resistance of *Enterobacter* to 3rd generation cephalosporins and aztreonam result from mutations in AmpD, which causes the constitutive hyperproduction of AmpC (40).

There was statically significant association ($p<0.01$) of resistance to cefuroxime, gentamicin and ciprofloxacin with the duration of therapy and the current medications of participants. This can be attributed to the fact that these medications are readily available, inexpensive drugs used to empirically treat common infections caused by a wide variety of organisms (41). The current medication of the participants was not significantly associated ($p>0.05$) with isolation of MDR pathogens. One of the potential causes of the observed high level of antibiotic resistance could be the misuse of antibiotics, both in the community and hospital settings (19).

Clinically, there are reports indicating the inappropriate prescribing practices as a cause of increased drug resistance. The wrongful use and prescription of antibiotics without microbial culture and sensitivity testing, and the unavailability of functional microbiology laboratories in most low-income countries may contribute significantly to this high prevalence in resistance (31). Another practice that leads to antibiotic resistance is patient non-compliance in completing the course of antibiotics prescribed for them (42,43), as well as the unregulated dispensing and purchase of antibiotics (mostly suboptimal doses), usually from over-the-counter facilities (31,44). The findings of our study showed high resistance to the most commonly available and affordable antibiotics but increased susceptibility to the more expensive reserve antibiotics. This may lead to increase in the cost of treatment of enteric bacterial infections in HIV patients, with increased use of effective but more expensive second line antibiotics.

A significant strength of our study was the use of MALDI-TOF and standard AST protocol, that ensured accurate identification and susceptibility of bacterial isolates. However, the study is limited by the small sample size due to the low prevalence of HIV (2%) in the region of study. This restricts the generalizability of our findings. The study was carried out in a few secondary hospitals, which might not be an accurate representation of other facilities.

Conclusion:

The enteric bacterial isolates recovered from the study population showed a predominance of *E. coli* and *K. pneumoniae*. Antimicrobial susceptibility testing showed that *E. coli* exhibited high resistance to β-lactams (amoxicillin/clavulanic acid and ampicillin) but were susceptible to ciprofloxacin and meropenem. A substantial proportion (60.0%) of the enteric isolates exhibited multidrug resistance (MDR), primarily characterised by concurrent resistance to β-lactams and cephalosporin classes, in combination with resistance to other antimicrobial agents.

These findings underscore the imperative for stringent antibiotic stewardship policies and rigorous monitoring of antimicrobial usage among HIV-positive individuals. Also, healthcare providers should emphasise patient education and counselling to combat self-medication with antibiotics and mitigate the emergence of MDR pathogens.

Contributions of authors:

DOM carried out the study. VEB and CA designed and supervised the study. DOM and YDB analysed data and drafted the original manuscript. VEB, YDB and CA edited the final manuscript. All the authors read and approved the final manuscript.

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Original Article **Open Access**

Prevalence and antimicrobial resistance patterns of bacterial isolates of pyogenic infections in a tertiary care centre in Southern India

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Abstract:

Background: Pyogenic infections, characterized by pus formation due to acute inflammation, remain a common clinical problem, particularly in hospital settings. Antimicrobial resistance (AMR) among causative bacterial pathogens poses a significant threat to treatment efficacy. The objective of this study is to assess the prevalence and antimicrobial resistance patterns of bacterial isolates from pyogenic infections in a tertiary care hospital in Southern India.
Methodology: An observational laboratory-based study was conducted from January to April 2025 in the Microbiology Department of Apollo Institute of Medical Sciences and Research, Hyderabad, India. A total of 138 pus, aspirates and wound swab samples were processed using standard aerobic culture techniques. Bacterial identification and antimicrobial susceptibility testing were performed using the VITEK 2 Compact system following the CLSI 2025 guidelines.
Results: Out of 138 specimens, 79 (57.3%) were culture-positive for clinically significant bacterial isolates. Gram-negative isolates predominated (73.4%, n=58), with *Klebsiella pneumoniae* (22.9%) as the most prevalent pathogen, followed by *Staphylococcus aureus* (20.3%) and *Pseudomonas aeruginosa* (15.2%). Notably, 50.0% of *K. pneumoniae* and 16.7% of *P. aeruginosa* isolates were resistant to carbapenems. Multidrug resistance (MDR) was observed in 16.5% of the isolates. Among *S. aureus* isolates, 75.0% were methicillin-resistant (MRSA) but all the Gram-positive isolates were susceptible to vancomycin and linezolid.
Conclusion: This study underscores the predominance of *K. pneumoniae*, *S. aureus*, and *P. aeruginosa* in pyogenic infections and highlights alarming levels of antimicrobial resistance, including carbapenem resistance and MRSA. The findings emphasize the urgent need for robust antimicrobial stewardship, continuous surveillance, and adherence to infection control measures to manage AMR effectively.

Keywords: Pyogenic infections; Antimicrobial resistance; Multidrug-resistant bacteria; VITEK 2; Southern India

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Prévalence et profils de résistance aux antimicrobiens d'isolats bactériens d'infections pyogènes dans un centre de soins tertiaires du sud de l'Inde

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Résumé:

Contexte: Les infections pyogènes, caractérisées par la formation de pus due à une inflammation aiguë, restent un problème clinique fréquent, notamment en milieu hospitalier. La résistance aux antimicrobiens (RAM) des bactéries pathogènes responsables constitue une menace importante pour l'efficacité des traitements. L'objectif de cette étude est d'évaluer la prévalence et les profils de résistance aux antimicrobiens d'isolats bactériens d'infections pyogènes dans un hôpital de soins tertiaires du sud de l'Inde.
Méthodologie: Une étude observationnelle en laboratoire a été menée de janvier à avril 2025 au département de microbiologie de l'Institut Apollo des sciences médicales et de la recherche, à Hyderabad, en Inde. Français Un total de 138 échantillons de pus, d'aspirats et d'écouvillons de plaies ont été traités à l'aide de techniques de culture aérobie standard. L'identification bactérienne et les tests de sensibilité aux antimicrobiens ont été réalisés

à l'aide du système VITEK 2 Compact conformément aux directives CLSI 2025.
Résultats: Sur 138 échantillons, 79 (57,3%) étaient positifs à la culture pour des isolats bactériens cliniquement significatifs. Les isolats Gram négatifs prédominaient (73,4%, n=58), *Klebsiella pneumoniae* (22,9%) étant l'agent pathogène le plus répandu, suivi de *Staphylococcus aureus* (20,3%) et de *Pseudomonas aeruginosa* (15,2%). Notamment, 50,0% des isolats de *K. pneumoniae* et 16,7% des isolats de *P. aeruginosa* étaient résistants aux carbapénèmes. Une multirésistance aux médicaments (MDR) a été observée chez 16,5% des isolats. Parmi les isolats de *S. aureus*, 75.0% étaient résistants à la méthicilline (SARM), mais tous les isolats à Gram positif étaient sensibles à la vancomycine et au linézolide.
Conclusion: Cette étude souligne la prédominance de *K. pneumoniae*, *S. aureus* et *P. aeruginosa* dans les infections à pyogènes et met en évidence des niveaux alarmants de résistance aux antimicrobiens, notamment aux carbapénèmes et au SARM. Ces résultats soulignent l'urgence d'une gestion rigoureuse des antimicrobiens, d'une surveillance continue et du respect des mesures de contrôle des infections pour gérer efficacement la RAM.

Mots-clés: Infections à pyogènes; Résistance aux antimicrobiens; Bactéries multirésistantes; VITEK 2; Inde du Sud

Introduction:

Pyogenic infections, characterized by the production of pus due to acute inflammation, are among the most common presentations in clinical practice. These infections may arise from wounds, abscesses, surgical sites or secondary bacterial invasions in immunocompromised individuals (1). The etiological agents vary widely depending on the anatomical sites and patient factors but *Staphylococcus aureus, Escherichia coli*, *Klebsiella* spp, *Pseudomonas aeruginosa* and *Proteus* spp are frequently implicated (2).

The growing threat of antimicrobial resistance (AMR) among pyogenic pathogens has emerged as a serious challenge in both community and hospital settings. The irrational and indiscriminate use of antibiotics, coupled with poor infection control practices, has led to the emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR), and even pandrug-resistant (PDR) organisms (3,4). Infections caused by these resistant pathogens are associated with higher morbidity, prolonged hospital stays, increased treatment costs, and poor patient outcomes (5).

In India, recent surveillance reports indicate alarmingly high resistance rates among common pathogens, especially in tertiary care settings (6). Studies have shown that MRSA rates range from 30–50%, while Gram-negative bacilli such as *Klebsiella* spp and *E. coli* exhibit increasing resistance to carbapenems and the third-generation cephalosporins (7,8). The Indian Council of Medical Research (ICMR) and the World Health Organization (WHO) have highlighted AMR as a critical public health concern, especially in the context of limited antibiotic development and inadequate stewardship (9,10).

Despite the growing burden, there is a paucity of region-specific data on the microbiological profile and resistance patterns of pyogenic infections, particularly from tertiary care centres in Southern India. Understanding local resistance trends is crucial to guide empirical therapy and develop region-specific antibiotic policies. Thus, this study was conducted to determine the most prevalent pathogens in pyogenic samples and their resistance pattern.

Materials and method:

Study setting:

This four-month observational study was conducted in the Department of Microbiology of Apollo Institute of Medical Sciences, Hyderabad, India from January 2025 to April 2025. Apollo Medical College and Hospital, Hyderabad is an established hospital in the southern part of India with a 660-bed capacity.

Sample collection and laboratory analysis:

During the study period, 138 samples (pus aspirate and wound swab) were received for aerobic culture in the Microbiology Laboratory, as part of medical care for patients from various outpatient and inpatient departments, and intensive care units. Within two hours of their aseptic collection, the samples were transferred to the laboratory for analysis. The specimens were cultured on MacConkey and Blood agar media and incubated for 48 hours at 37°C. In accordance with conventional laboratory procedure, species identification was carried out using colony features and the BioMérieux Vitek 2 compact identification system.

Antimicrobial susceptibility testing (AST) was also carried out using BioMérieux Vitek 2 compact system. Aminoglycosides, cephalosporins, quinolones, carbapenems, piperacillin/tazobactam, and cotrimoxazole were tested against Gram negative isolates, while penicillin, aminoglycosides, quinolones, erythromycin, clindamycin, and cotrimoxazole were tested against Gram-positive isolates. The 35th edition (2025) of the Clinical and Laboratory Standards Institute (CLSI) guideline M-100 was followed in AST interpretation process.

Statistical analysis:

Microsoft Excel was used to evaluate and analyse the data. The quantitative data were presented in tabular form and figures as numbers and percentages.

Results:

Demographic characteristics of the patients with suspected pyogenic infections:

During the study period from January to April 2025, a total of 138 clinical specimens were collected from patients with suspected pyogenic infections. The study population comprised 86 (62.3%) male and 52 (37.7%) female patients, with age ranging from 1 to 82 years (mean age: 45.5±18.5 years). The most commonly affected age group was the 51-60 years category, accounting for 11.6% of the cases. The frequency distribution of the samples of patients is shown in Fig 1.

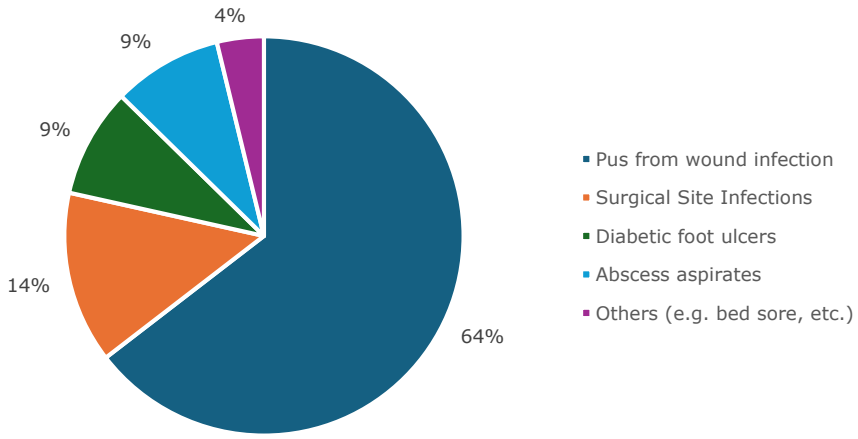


Fig 1: Frequency distribution of clinical samples from patients with pyogenic infections received at the microbiology laboratory of Apollo Institute of Medical Sciences and Research, Hyderabad, India

Table 1: Frequency distribution of bacterial isolates from clinical samples of patients with pyogenic infections at Apollo Institute of Medical Sciences and Research, Hyderabad, India

Bacterial isolate	Number of Isolates	Percentage (%)
Klebsiella pneumoniae	18	22.8
Staphylococcus aureus	16	20.3
Pseudomonas aeruginosa	12	15.2
Escherichia coli	10	12.7
Proteus mirabilis	4	5.1
Enterobacter cloacae	4	5.1
Enterococcus faecalis	3	3.8
Pseudomonas luteola	2	2.5
Morganella morganii	2	2.5
Enterococcus faecium	1	1.3
Burkholderia cepacia	1	1.3
Streptococcus agalactiae	1	1.3
Acinetobacter haemolyticus	1	1.3
Providencia rettgeri	1	1.3
Stenotrophomonas maltophilia	1	1.3
Serratia marcescens	1	1.3
Enterobacter aerogenes	1	1.3

Prevalence and bacterial isolates of pyogenic infections:

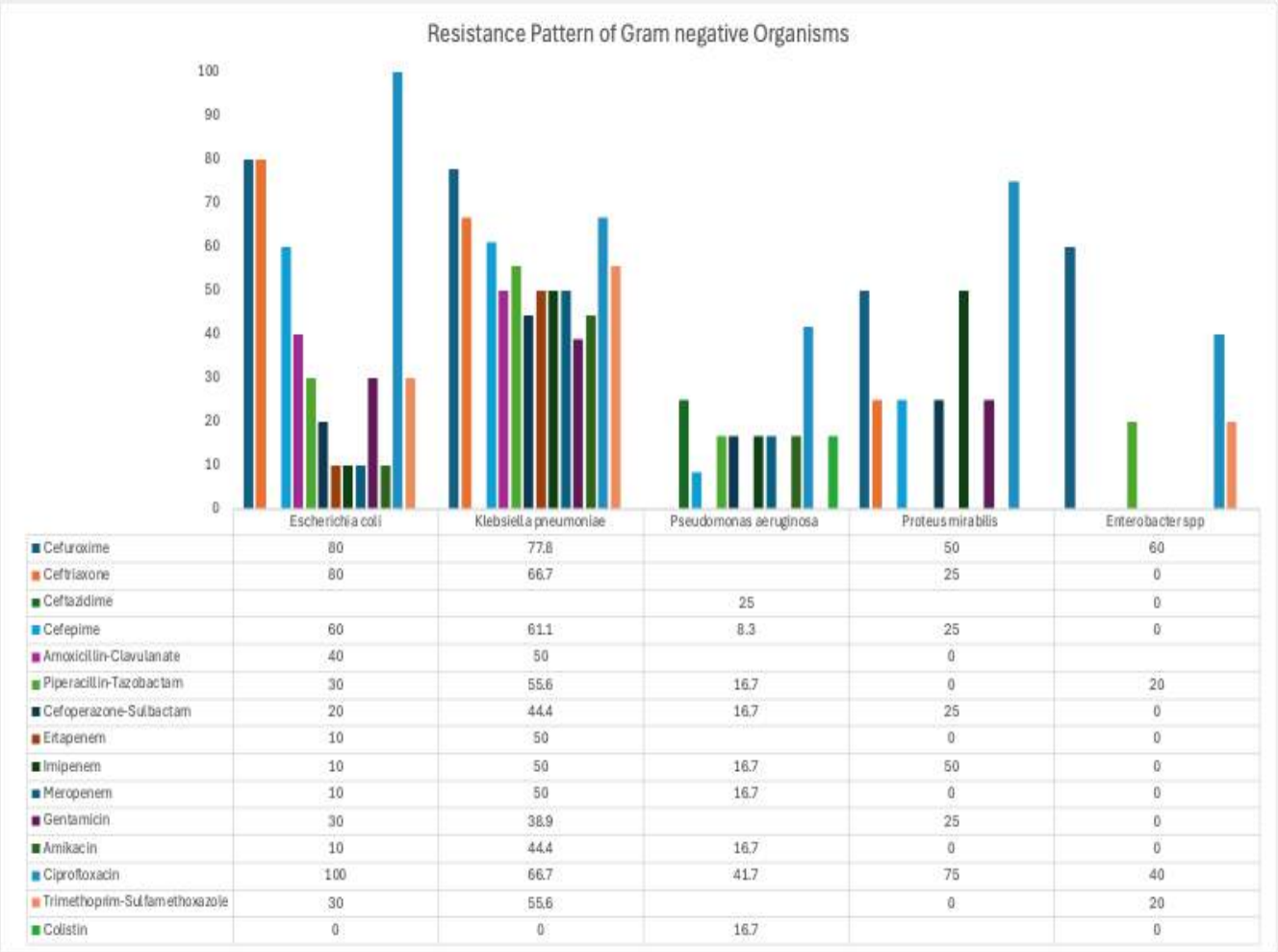
Of the 138 clinical samples from the patients, 79 (57.3%) yielded clinically significant bacterial isolates on culture and were included in the analysis, while the remaining 59 (42.8%) showed either no bacterial growth or grew contaminants and were excluded. Among total culture positives, 58 (73.4%) isolates were Gram negative and 21 (26.6%) isolates were Gram positive.

The most prevalent isolate was *Klebsiella pneumoniae* (n=18, 22.9%), followed by *Staphylococcus aureus* (n=16, 20.3%) and *Pseudomonas aeruginosa* (n=12, 15.2%). The distribution of bacterial isolates is shown in Table 1.

Antimicrobial resistance of bacterial isolates of pyogenic infections:

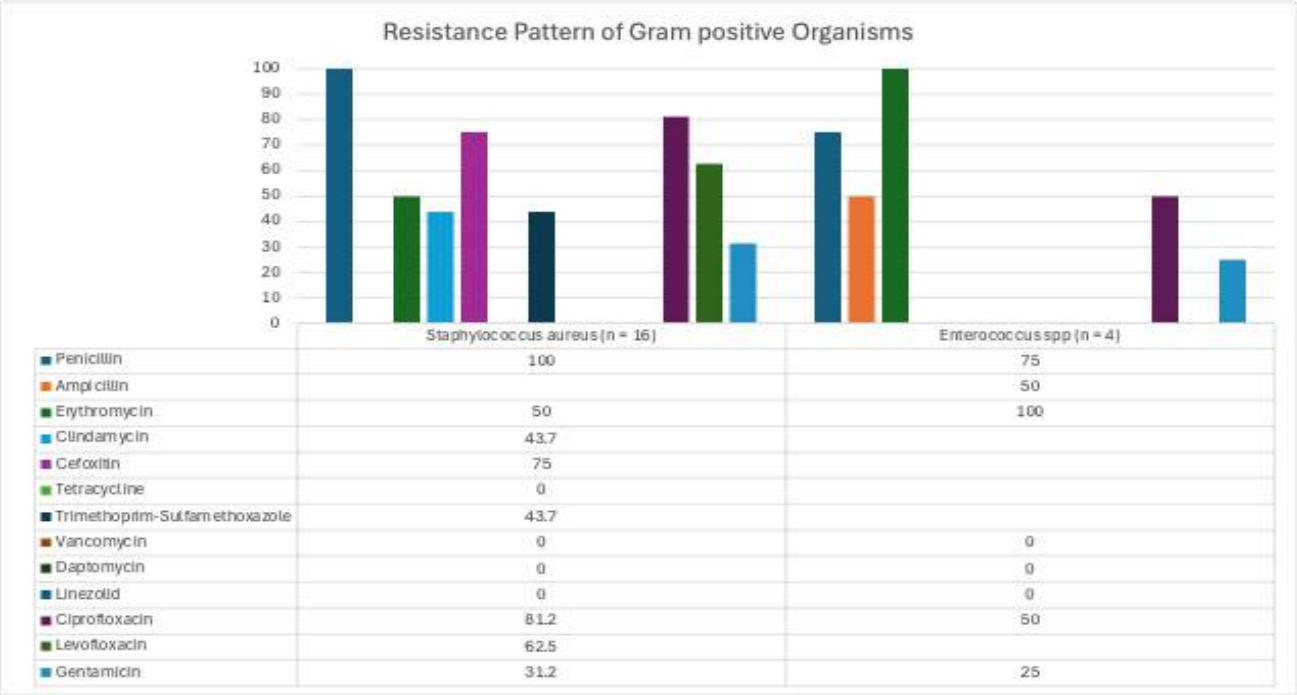
The antimicrobial resistance pattern of the Gram-negative bacteria is detailed in Fig 2. Only 16.7% of *P. aeruginosa* demonstrated resistance to colistin while all the others showed no resistance to colistin, except for the ones with intrinsic resistance. Most of the Gram-negative bacteria exhibited high resistance rates to cephalosporins and quinolones. Carbapenem resistance was observed in 50% of *K. pneumoniae*, 16.7% of *P. aeruginosa* and 10% of *Escherichia coli* isolates. Multidrug resistance (MDR), defined as resistance to at least one antimicrobial agent in three separate antimicrobial groups or classes (11), was observed in 13 Gran-negative bacterial isolates (8 *K. pneumoniae*, 3 *P. aeruginosa* and 2 other isolates).

Among the *S. aureus* isolates, methicillin resistance was observed in 12 isolates (75%). The *S. aureus* isolates showed high resistance rates against penicillin, erythromycin and fluoroquinolones. However, none of the Gram-positive isolates was resistant to vancomycin or linezolid. The resistance pattern of the Gram-positive isolates is shown in Fig 3.



	Escherichia coli	Klebsiella pneumoniae	Pseudomonas aeruginosa	Proteus mirabilis	Enterobacter spp
Cefuroxime	80	77.8		50	60
Ceftriaxone	80	66.7		25	0
Ceftazidime			25		0
Cefepime	60	61.1	8.3	25	0
Amoxicillin-Clavulanate	40	50		0	
Piperacillin-Tazobactam	30	55.6	16.7	0	20
Cefoperazone-Sulbactam	20	44.4	16.7	25	0
Ertapenem	10	50		0	0
Imipenem	10	50	16.7	50	0
Meropenem	10	50	16.7	0	0
Gentamicin	30	38.9		25	0
Amikacin	10	44.4	16.7	0	0
Ciprofloxacin	100	66.7	41.7	75	40
Trimethoprim-Sulfamethoxazole	30	55.6		0	20
Colistin	0	0	16.7		0

Fig 2: Resistance pattern of Gram-negative bacterial isolates from clinical samples of patients with pyogenic infections at Apollo Institute of Medical Sciences and Research, Hyderabad, India



	Staphylococcus aureus (n = 16)	Enterococcus spp (n = 4)
Penicillin	100	75
Ampicillin		50
Erythromycin	50	100
Clindamycin	43.7	
Cefoxitin	75	
Tetracycline	0	
Trimethoprim-Sulfamethoxazole	43.7	
Vancomycin	0	0
Daptomycin	0	0
Linezolid	0	0
Ciprofloxacin	81.2	50
Levofloxacin	62.5	
Gentamicin	31.2	25

Fig 3: Resistance pattern of Gram-positive bacterial isolates from clinical samples of patients with pyogenic infections at Apollo Institute of Medical Sciences and Research, Hyderabad, India

Discussion:

This study provides a snapshot of the bacterial pathogens implicated in pyogenic infections in a tertiary care hospital in Southern India, with *K. pneumoniae* as the predominant isolate, followed by *S. aureus* and *P. aeruginosa*. These findings align with previous studies conducted in similar settings, where *K. pneumoniae* and *P. aeruginosa* were frequently reported as leading causes of nosocomial and community-acquired infections, especially in immunocompromised individuals and patients with prolonged hospital stays (12–14).

The high prevalence of *K. pneumoniae* (22.9%) is concerning, particularly given the observed 50.0% resistance rate to carbapenems. This reinforces growing global concerns about carbapenem-resistant *Klebsiella* spp, which are associated with limited therapeutic options and poor clinical outcomes (15). Similarly, the detection of carbapenem resistance in *P. aeruginosa* (16.7%) and *E. coli* (10.0%) underscores the need for stringent antimicrobial stewardship and robust infection control practices in the hospital.

Multidrug resistance (MDR), observed in 16.5% (13/79) of the isolates, particularly among *K. pneumoniae* and *P. aeruginosa*, further complicates management. This is consistent with data from the Indian Council of Medical Research (ICMR), which reported rising MDR rates among Gram-negative bacterial pathogens in tertiary care centres across India (16). The MDR criteria applied in this study follow the international guidelines established by the CDC and ECDC, which provide a standardized framework for resistance classification in bacteria (11).

Among the Gram-positive isolates, the high prevalence of methicillin-resistant *S. aureus* (MRSA) at 75% is significant. MRSA remains a major cause of healthcare-associated infections and often exhibits resistance to multiple non-beta-lactam antibiotics (17,18), as was evident in the resistance to penicillin, erythromycin, and quinolones in this study. Encouragingly, all Gram-positive isolates remained susceptible to vancomycin and linezolid, preserving these agents as viable therapeutic options, which is in line with global trends (19).

The age distribution indicated that individuals in the 51–60 years age group were most commonly affected by pyogenic infections, likely reflecting a combination of age-related immune decline, comorbidities, and increased healthcare interactions in this demographic. Male predominance was also noted, which may relate to higher exposure risks or differences in health-seeking behaviours, as observed in other regional studies (17,20).

Overall, the findings of this study highlight the evolving bacterial landscape of pyogenic infections and the mounting threat of antimicrobial resistance. Continuous Surveillance, rational antibiotic use, and implementation of infection control policies are imperative to curb the spread of resistant pathogens. Future studies with larger sample sizes and

molecular characterization of resistance genes may provide further insights into the mechanisms and transmission dynamics of AMR in this region.

Conclusion:

This study highlights the predominance of *K. pneumoniae*, *S. aureus*, and *P. aeruginosa* as the leading bacterial pathogens causing pyogenic infections in our tertiary care centre. The high prevalence of MDR, particularly among Gram-negative bacteria, and the significant proportion of methicillin resistance among *S. aureus* isolates underscore the urgent need for enhanced antimicrobial stewardship and routine surveillance programs.

The continued susceptibility of Gram-positive bacteria to vancomycin and linezolid provides some reassurance, but the rising resistance to other antibiotic classes remains a serious concern. Implementing strict infection control measures, promoting rational antibiotic use, and conducting further research, including molecular characterization of resistance genes, are critical steps toward curbing the spread of antimicrobial resistance and improving patient outcomes.

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Contributions of authors:

AB was involved in the conception and design of the study, data collection, laboratory analysis, data interpretation, and drafting of the manuscript. PG provided overall supervision, critical review, and final approval of the manuscript. Both authors read and approved the final version of the manuscript.

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Conflict of interest:

Authors declare no conflict of interest

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Original Article **Open Access**

Genotypic identification and antibiotic susceptibility of coagulase negative staphylococci isolates from urine samples of patients in southwest Nigeria

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Abstract:

Background: Urinary tract infection (UTI) largely affects women and is mostly caused by bacteria, among which coagulase negative staphylococci has received little attention. The objectives of this study are to isolate and genotypically identify coagulase negative staphylococci (CoNS) from urine samples of adult females attending selected healthcare facilities from three States (Oyo, Osun and Ogun) in southwest Nigeria, and to determine their susceptibility to selected antibiotics.
Methodology: A case-control study of 471 adult female participants with urinary symptoms in 205 (case) and no urinary symptoms in 266 (control), was conducted over a period of 10 months. Mid-stream voided urine samples were collected from the participants. The samples were cultured on mannitol salt and blood agar plates, and bacteria growths were presumptively identified for CoNS and other bacterial isolates using routine biochemical tests. The CoNS isolates were confirmed by conventional PCR amplification with *Staphylococcus saprophyticus* specific primers and 16S rRNA sequencing with phylogenetic analysis using MegaX software 18 version 10.0.5. Antibiotic susceptibility testing was performed on genotypically identified CoNS isolates using VITEK 2 system. Chi square and Fisher Exact tests were used to compare the prevalence of bacterial UTIs across the States and between symptomatic and asymptomatic participants at α=0.05.
Results: Of the urine samples of 471 participants examined, 178 (37.8%) had bacterial UTIs on cultures, 66 of 117 (56.4%) were from Oyo State, 98 of 241 (40.7%) from Osun State, and 14 of 113 (14.1%) from Ogun State (x^2=49.113, p<0.0001). A total of 34 (7.2%) CoNS isolates were phenotypically identified, with 29 (14.1%) from 205 symptomatic and 5 (1.9%) from 266 asymptomatic participants (OR=8.601, 95% CI=3.266-22.653, p<0.0001). *Staphylococcus saprophyticus* PCR amplified 19 of the 34 CoNS isolates while 16S rRNA sequencing identified 5 CoNS species among the 19 isolates, with *S. saprophyticus* (n=10, 52.6%) *S. lentus* (n=5, 2.8%), *S. xylosus* (n=2, 1.1%), *S. sciuri* (n=1, 0.6%), and *S. rodentium* (n=1, 0.6%). All the CoNS isolates were resistant to daptomycin, sulfamethoxazole-trimethoprim and oxacillin indicating they were multi-drug resistant (MDR) but were all sensitive to nitrofurantoin.
Conclusion: The high resistance rates to commonly used antibiotics such as the β-lactams, sulfamethoxazole-trimethoprim and daptomycin from our study underscore the need for routine genotypic surveillance and antibiotic stewardship in the management of UTIs. Our findings emphasize the clinical relevance of CoNS in UTIs and the necessity for targeted diagnostics and treatment strategies.

Keywords: *Staphylococcus saprophyticus*, Urinary tract infection, multi-drug resistance

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Identification génotypique et sensibilité aux antibiotiques d'isolats de staphylocoques à coagulase négative issus d'échantillons d'urine de patients du sud-ouest du Nigéria

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Résumé:

Contexte: Les infections urinaires (IU) touchent majoritairement les femmes et sont principalement d'origine bactérienne, parmi lesquelles les staphylocoques à coagulase négative ont reçu peu d'attention. Les objectifs de cette étude sont d'isoler et d'identifier génotypiquement les staphylocoques à coagulase négative (SCN) à partir d'échantillons d'urine de femmes adultes fréquentant des établissements de santé sélectionnés dans trois États (Oyo, Osun et Ogun) du sud-ouest du Nigéria, et de déterminer leur sensibilité à certains antibiotiques.
Méthodologie: Une étude cas-témoins a été menée sur une période de 10 mois auprès de 471 femmes adultes, dont 205 présentaient des symptômes urinaires (cas) et 266 n'en présentaient aucun (témoin). Des échantillons d'urine mictionnelle ont été prélevés chez les participantes. Les échantillons ont été cultivés sur des plaques de gélose au sel de mannitol et au sang, et les croissances bactériennes ont été identifiées de manière présomptive pour le CoNS et d'autres isolats bactériens à l'aide de tests biochimiques de routine. Les isolats de CoNS ont été confirmés par amplification PCR conventionnelle avec des amorces spécifiques de *Staphylococcus saprophyticus* et séquençage de l'ARNr 16S avec analyse phylogénétique à l'aide du logiciel MegaX 18 version 10.0.5. Des tests de sensibilité aux antibiotiques ont été réalisés sur les isolats de CoNS identifiés génotypiquement à l'aide du système VITEK 2. Les tests du chi carré et de Fisher Exact ont été utilisés pour comparer la prévalence des infections urinaires bactériennes à travers les États et entre les participants symptomatiques et asymptomatiques à α=0,05.
Résultats: Sur les échantillons d'urine de 471 participants examinés, 178 (37,8%) présentaient des infections urinaires bactériennes sur les cultures, 66 sur 117 (56,4%) provenaient de l'État d'Oyo, 98 sur 241 (40,7%) de l'État d'Osun et 14 sur 113 (14,1%) de l'État d'Ogun (x^2=49,113, p<0,0001). Au total, 34 (7,2%) isolats de CoNS ont été identifiés phénotypiquement, avec 29 (14,1%) provenant de 205 participants symptomatiques et 5 (1,9%) de 266 participants asymptomatiques (OR=8,601, IC à 95%=3,266-22,653, p<0,0001). Français La PCR de *Staphylococcus saprophyticus* a amplifié 19 des 34 isolats de CoNS tandis que le séquençage de l'ARNr 16S a identifié 5 espèces de CoNS parmi les 19 isolats, avec *S. saprophyticus* (n=10, 52,6%), *S. lentus* (n=5, 2,8%), *S. xylosus* (n=2, 1,1%), *S. sciuri* (n=1, 0,6%) et *S. rodentium* (n=1, 0,6%). Tous les isolats de CoNS étaient résistants à la daptomycine, au sulfaméthoxazole-triméthoprime et à l'oxacilline, indiquant qu'ils étaient multirésistants aux médicaments (MDR), mais étaient tous sensibles à la nitrofurantoïne.
Conclusion: Les taux élevés de résistance aux antibiotiques couramment utilisés, tels que les bêta-lactamines, le sulfaméthoxazole-triméthoprime et la daptomycine, observés dans notre étude, soulignent la nécessité d'une surveillance génotypique systématique et d'une gestion responsable des antibiotiques dans la prise en charge des infections urinaires. Nos résultats soulignent la pertinence clinique de la neurosynthèse des antibiotiques dans les infections urinaires et la nécessité de diagnostics et de stratégies thérapeutiques ciblés.

Mots-clés: *Staphylococcus saprophyticus*, infection urinaire, multirésistance aux médicaments

Introduction:

Urinary tract infection (UTI) is the most common type of bacterial infections usually reported in the healthcare facilities (1). It is an infection of the urinary system which consists of the bladder, ureter, kidney and urethra with significant symptoms which include increase rate of urination, enhanced desire to empty the bladder and painful urination, thereby adding to the hospital bills of the affected individual, most especially if it involves multi-drug-resistant (MDR) isolates (1,2). Urinary tract infection is confirmed by the presence of 10^5 microorganisms or of a single strain of bacterium per milliliter in two consecutive mid-stream samples of urine (3).

Among the bacteria that colonize urinary system, *Staphylococcus saprophyticus* is the second most common bacterium in UTIs, mostly common in females due to menarche, menopause and sexual intercourse (4,5). *Staphylococcus saprophyticus* is a commensal Gram-positive, coagulase negative staphylococcus (CoNS) and therefore has the potential to exist among contaminating bacteria, which often leads to lower UTI in young women as a community-acquired UTI (6). Colonization of the gut and genital areas has been linked to this kind of UTI (7).

Antimicrobial resistance (AMR) is an inevitable outcome of evolutionary adaptation of microbes which has emerged as epidemic crisis in clinical medicine worldwide (8,9). Bacterial resistance to antimicrobial drugs is one of the most serious menaces to global public health (10,11). Human and improper uses of antimicrobial drugs have driven the intense rapid and prevalent outburst of resistance in both pathogenic and commensal organisms (12). The challenge of AMR knows no limitation, drug-resistant microbes of all kinds can move among humans and animals, and from countries to countries (13).

The looming threat of incurable staphylococcal infections most especially coagulase negative staphylococci is at the latest corkscrew in an international public health night-

mare, increasing bacterial resistance to many antibiotics that once cured (14). When *Staphylococcus* species are present and multiply in generally sterile sites such as the urinary tract, they cause harm to the host. However, *Staphylococcus* species are usually harmless on the skin, gastrointestinal tract, and periurethral area.

The dominance of *S. saprophyticus* infections had been in reality since the inception of mankind because the organism has the ability to adhere to urothelial cells and produce urease. Colonization is a very common phenomenon even in an environment thought to be nearly sterile (15). *Staphylococcus saprophyticus* has been accorded little attention as an important causative agent of UTIs. This study was therefore carried out to determine the prevalence of *S. saprophyticus* in UTI and antibiotic susceptibility of the isolates to selected antibiotics.

Materials and method:

Study setting and design:

This was a laboratory-based case-control study carried out in selected healthcare facilities from three States (Oyo, Ogun and Osun) in southwest region of Nigeria. The facilities from Oyo State were University Health Service (UHS) and Oyo State Hospital Management Board (OYSHMB); from Ogun State, Sacred Heart Hospital (SHH) and Federal Medical Centre (FMC) Abeokuta, and from Osun State, Osun State University Teaching Hospital and Osun State Hospital Management Board (OSHMB) Osogbo.

Study participants, sample size and method of sampling:

The sample size of 471 participants (205 with urinary symptoms as case and 266 without urinary symptoms as control) was determined using the appropriate sample size formula. Participants were individuals attending the outpatient departments of each selected hospital in the States and were randomly selected over a period of 12 months, with 117 from Oyo, 113 from Ogun and 241 from Osun State. Participants who have received antibiotics in the 30 days preceding recruitment and those who declined to give consent were excluded.

Ethical approval and informed consent:

Ethical approval was obtained from the Ethical Committees of the three selected hospitals in Oyo, Osun and Ogun States approval numbers AD16/479/634, OSHREC/PRS/569T/173 and HPRS/381/341 respectively. Informed consent was obtained from each participant, and the rights of each participant to remain anonymous and decline study participation were upheld.

Urine sample collection:

Clean catch specimen of voided midstream urine was collected from each participant into a sterile urine sample bottle and transported to the Pharmaceutical Microbiology laboratory, University of Ibadan, within one hour of collection for microbiological analysis.

Urine culture, isolation and identification of coagulase negative staphylococci:

The urine sample of each participant was carefully mixed and about 1µL inoculated by streaking onto mannitol salt and blood agar plates using a sterile wire loop and incubated aerobically at 37^{o}C overnight. The culture plates were observed for growth of staphylococcal-like colonies and identified by Gram staining and biochemical tests including catalase, coagulase, and novobiocin disc tests (17). Urinary tract infection caused by CoNS was confirmed when there was ≥ 10^5 colony-forming units of a single CoNS per milliliter (CFU/ml) of urine.

Antibiotic susceptibility testing of CoNS isolates using VITEK 2 system:

Inoculum of the overnight culture of each CoNS isolate was prepared in 0.85% saline and the turbidity adjusted to 0.5 McFarland turbidity standard using VITEK 2 densitometer. The inoculum was then transferred to a second tube containing 3.0 ml of saline. The bacterial suspension tubes containing susceptibility card cassette were loaded into the Vitek 2 compact processor, having loaded the cassette information into the workstation computer.

The AST cards containing benzyl-penicillin, clindamycin, erythromycin, ciprofloxacin, gentamicin, levofloxacin, daptomycin, nitrofurantoin, oxacillin, rifampin, teicoplanin, tetracycline, tigecycline, trimethoprim-sulfamethoxazole, linezolid and vancomycin, was automatically loaded, sealed, and introduced into VITEK 2 reader-incubator module (incubation temperature, 35°C), where it was subjected to kinetic absorbance spectra every 15 min using a vacuum device.

The results were analyzed using the AST-GPC database, and the final results were calculated automatically. All of the used cards were instantly discarded. The MIC for each antimicrobial agent was determined and used to classify each CoNS isolate as susceptible, intermediate or resistant in line with Clinical Laboratory and Standards Institute (CLSI) guidelines (18).

Extraction of DNA:

Bacterial DNA was extracted using Quick DNA^{TM} Bacterial Miniprep Kit. About 50-100µg of bacterial cells was re-suspended in 200 µl of isotonic buffer (PBS) in a Zymo Research (ZR) Bashing-bead^{TM}. The bacterial

DNA was extracted with Zymo Research (ZR) Bashing-bead™ following manufacturer's instruction.

Polymerase chain reaction (PCR) amplification of *S. saprophyticus* specific gene:

Molecular identification of *S. saprophyticus* isolate was carried out following the method described by Morot-Bizot et al., (19), using *S. saprophyticus* primers; F-5'-TCAAAA AGTTTTCTAAAAAATTTAC-3' (annealing position 169 to 193) and R-5'-ACGGGCGTCCAC AAAATCAATAGG A-3' (annealing position 355 to 379) with the amplicon size of 221bp alongside the positive control. The following PCR conditions were used; 5 min at 95°C, 40 cycles of 30 sec at 95°C, 60 sec at 55°C, 30 sec at 72°C and a final extension of 3 min at 72°C.

Agarose gel electrophoresis of PCR amplicons:

Electrophoresis of amplicon DNA was carried out in 1.5% agarose in 1xTBE buffer on horizontal gel apparatus. Ten microliters of amplicon DNA and 2 µL of loading buffer were mixed well and loaded into the gel containing 10 µL ethidium bromide (1 µg/mL in water) and placed in Tris-borate buffer according to the method of (20)**.** The electrophoresis was conducted for 60-90 min at constant voltage 75v. The gel was examined on transilluminator at wavelength 312 nm. Photography was carried out using a Polaroid Camera SP 34. The positive control was *S. saprophyticus* subsp*. bovis* DSM 18669 and nuclease free water was used as the negative control.

16S rRNA sequencing and phylogenetic analysis of *S. saprophyticus* isolates:

About 50 ng DNA templates of 20 selected CoNS isolates amplified with *S. saprophyticus* primers were further amplified with the V5 region of 16S rRNA which spanned the site of the primer (27F 5'-AGAGTTTGATCCT GGCTCAG-3'; 1492R 5'-GGTTACCTTGTTACGA CTT-3'). The following conditions were used for PCR: 95°C for 10 minutes, then 30 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec, followed by a 10 min extension at 72°C. The PCR products were purified using the Big-Dye Terminator Purification x kit (Applied Biosystems) and sequenced using 1 µL of forward and reverse 16s rRNA primer set (Thermo Fisher Scientific, Waltham, MA) in a 3130 Genetic Analyzer (Applied Bio-systems).

To identify *Staphylococcus* species, the partial sequences of 16S rRNA were compared to sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Sequences were aligned using Mega X software (version 10.0.5), which was also utilized to establish phylogenetic relationships between several *Staphylococcus* species and build phylogenetic trees (21).

Statistical analysis of data:

The prevalence of UTI due to CoNS and other bacterial isolates was determined among the case and control participants and compared across the facilities in the three States using Chi square and Fisher Exact tests. Statistical significance was determined at *p*-value < 0.05.

Results:

Distribution of the participants by States and healthcare facilities:

Of 471 recruited participants, 205 (43.5%) had urinary symptoms (case) while 266 (56.5%) were asymptomatic (control); 117 (24.8%) were from Oyo, 113 (24.0%) from Ogun and 241 (56.2%) were from Osun State. The distribution of participants by facilities from each State is shown in Table 1. With regard to age group distribution, Fig 1 shows the age group 19-30 years as the most represented (n=192, 40.8%) followed by age group 31-40 years (n=125, 26.5%).

Prevalence of bacterial urinary tract infections in the study participants:

Table 2 shows the prevalence of UTIs according to the States. Of the 471 participants, 178 had bacterial UTI by cultures, giving a prevalence of 37.8% in the study. Sixty-six of 117 (56.4%) from Oyo State, 98 of 241 (40.7%) from Osun State, and 14 of 113 (14.1%) from Ogun State had bacterial UTI (x^2=49.113, p<0.0001). Compared to the overall prevalence of 37.8%, the prevalence of UTI in Oyo (56.4%) is significantly higher (OR =2.796, p<0.0001) and that of Ogun (12.4%) is significantly lower (OR=0.1673, p<0.0001), but the prevalence of UTI in Osun is not significantly different (OR=1.286, p=0.2166) from the overall prevalence in the study. Participants in age group 19-30 years had the highest prevalence of UTIs (26.0%, n=50).

Table 1: Frequency distribution of urinary symptomatic and asymptomatic study participants across the healthcare facilities in the three States, southwest Nigeria

State	Facilities	Participants			
		Symptomatic	Asymptomatic	Total (%)	
Oyo	OYHMB	9	41	50	**117 (24.8)**
	UHS	67	0	67	
Ogun	FMC	27	38	55	**113 (24.0)**
	SHH	28	20	48	
Osun	OSHMB	26	105	131	**241 (56.2)**
	OSUTH	48	62	110	
Total (%)		**205 (43.5)**	**266 (56.5)**	**471 (100.0)**	

OYHMB = Oyo State Hospital Management Board; UHS = University Health Service; FMC = Federal Medical Center; SHH = Sacred Heart Hospital; OSHMB = Osun State Health Management Board; OSUTH = Osun State University Teaching Hospital

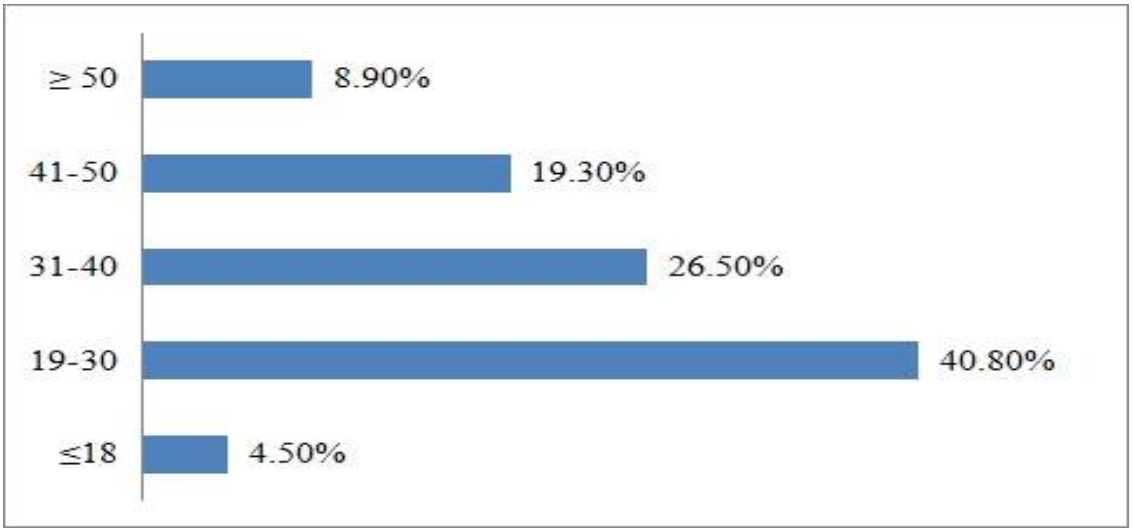


Fig 1: Age distribution of the study participants

Table 2: Prevalence of bacterial urinary tract infection among study participants across the three States, southwest Nigeria

State	Participants		OR (95% CI)	*p* value	x^2	*p* value
	UTI (%)	No UTI (%)				
Oyo (n=117)	66 (56.4)	51 (43.6)	2.796 (1.821-4.293)	<0.0001*	49.113	<0.0001*
Ogun (n=113)	14 (12.4)	99 (87.6)	0.1673 (0.0921-0.304)	<0.0001*		
Osun (n= 241)	98 (40.7)	143 (59.3)	1.286 (0.8842-1.867)	0.2166		
Total (n=471)	**178 (37.8)**	**293 (62.2)**				

UTI = urinary tract infection; x^2 = Chi square; OR= Odd ratio; CI= Confidence interval * = statistically significant

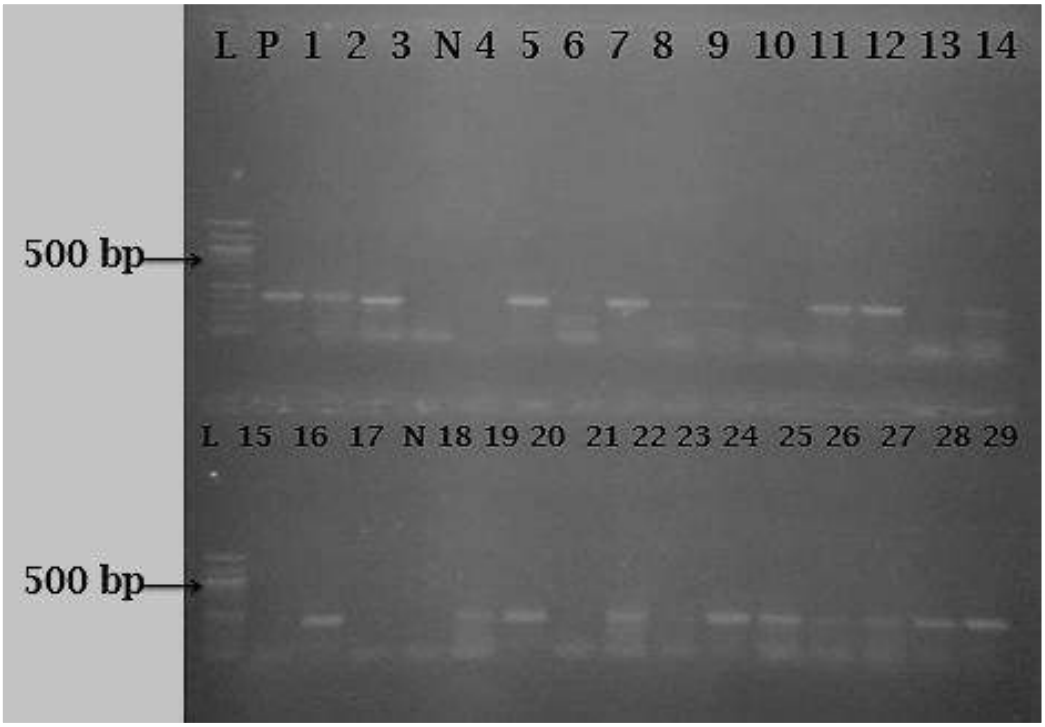
Table 3: Prevalence of urinary tract infections caused by genotypically identified coagulase negative staphylococci across the three States in southwest Nigeria

CoNS	**States**			**Total (n=471)**	x^2	***p* value**
	Oyo (n=117)	**Ogun (n=113)**	**Osun (n= 241)**			
S. saprophyticus	7 (5.9)	3 (2.7)	0	10 (2.1)		
S. lentus	3 (2.6)	1 (0.9)	1 (0.4)	5 (1.1)		
S. xylosus	0	2 (1.8)	0	2 (0.4)		
S. sciuri	0	1 (0.9)	0	1 (0.2)		
S. rodentium	1 (0.9)	0	0	1 (0.2)		
Total	**11 (9.4)**	**7 (6.2)**	**1 (0.4)**	**19 (4.0)**	**18.224**	**<0.0001***

Prevalence of coagulase negative staphylococci UTI in the study:

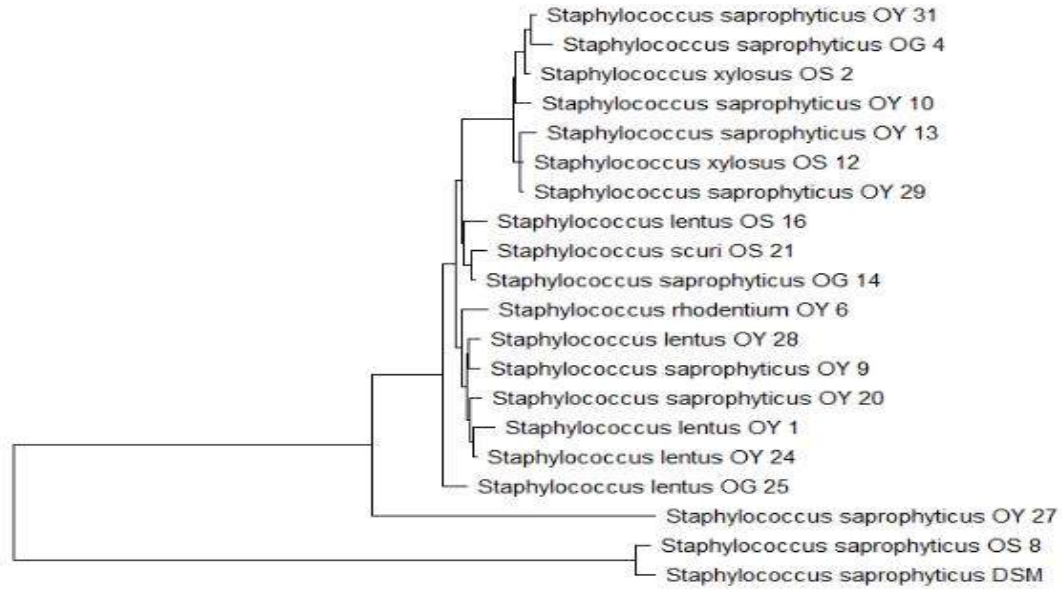
Coagulase negative staphylococci were phenotypically identified in urine samples of 34 (7.2%) of the 471 participants, 29 (14.1%) of which were from 205 symptomatic (case) and 5 (1.9%) from 266 asymptomatic (control) participants (OR=8.601, 95% CI=3.266-22.653, p<0.0001). In term of frequency of isolation, CoNS constituted 19.1% (n=34) of the 178 bacterial pathogens isolated from the urine samples of the participants, 19 (10.7%) of which were genotypically confirmed to be *S. saprophyticus* by PCR and 10 (5.6%) by 16S rRNA sequencing.

Table 3 shows the prevalence of UTI caused by confirmed CoNS by PCR and 16S rRNA sequencing in the study participants. Of the 471 participants, CoNS was identified in 19 patients' samples, giving a prevalence of 4.0%, with 9.4% (n=11) in Oyo, 6.2% (n=7) in Ogun and 0.4% (n=1) in Osun State (x^2=18.224, p<0.0001). Fig 2 shows the *S. saprophyticus* amplified products using the *S. saprophyticus* specific primer. Fig 3 represents the phylogenetic tree of the relatedness of the CoNS isolates.



Lane 1,2,5,7,8,9,11,12,14,16,18,19,21,22,24,25,26,27,28 and 29 at 380bp; Lane L, 1, 4 and N are ladder, positive control and negative control respectively

Fig 2: Agarose gel electrophoresis of amplified PCR amplicons of *Staphylococcus saprophyticus* isolates



OY = Oyo State; OS = Osun State; OG = Ogun State; DSM = Standard *Staphylococcus saprophyticus*
The 19 positive amplicons with *S. saprophyticus* specific-primer further sequenced with 16S rRNA and aligned with the sequences available in GenBank. The strains were established as belonging to the *Staphylococcus* Genus (similarity =95%). Some of the 19 staphylococci were identical to each other. The 19 distinct strains were grouped into 5 *Staphylococcus* species, with the principal species being *S. saprophyticus* of 10 strains, followed by 5 *S. lentus*, 2 *S xylosus,* and 1 each of *S. sciuri*, *S. rodentium*. A control strain, *S. saprophyticus bovis*, gave 98% similarity with *S. saprophyticus* in the GenBank.

Fig 3: Phylogenetic tree of 16S rRNA sequences of coagulase negative staphylococci isolates

Table 4: Antibiotic resistance profiles of genotypically identified coagulase-negative staphylococci isolates from urine samples

Antibiotic	**S. saprophyticus** (%) **(n=10)**	**S. lentus** (%) **(n=5)**	**S. xylosus** (%) **(n=2)**	**S. rodentium** (%) **(n=1)**	**S. sciuri** (%) **(n=1)**	**Total CoNS (%) (n=19)**
Gentamicin	3 (30.0)	3 (60.0)	0	1 (100.0)	0	7 (36.8)
Ciprofloxacin	0	1 (20.0)	0	0	0	1 (5.3)
Levofloxacin	1 (10.0)	1 (20.0)	0	0	0	2 (10.5)
Erythromycin	2 (20.0)	2 (40.0)	0	0	0	4 (21.1)
Clindamycin	9 (90.0)	3 (60.0)	1 (50.0)	1 (100.0)	1 (100.0)	15 (78.9)
Linezolid	1 (10.0)	1 (20.0)	0	1 (100.0)	0	3 (15.8)
Daptomycin	10 (100.0)	3 (60.0)	2 (100.0)	1 (100.0)	1 (100.0)	17 (89.5)
Teicoplanin	1 (10.0)	1 (20.0)	0	0	0	2 (10.5)
Vancomycin	2 (20.0)	1 (20.0)	0	0	0	3 (15.8)
Tetracycline	2 (20.0)	2 (40.0)	0	0	0	4 (21.1)
Tigecycline	0	1 (20.0)	0	0	0	1 (5.3)
Nitrofurantoin	0	0	0	0	0	0
Rifampicin	4 (40.0)	3 (60.0)	0	1 (100.0)	0	8 (42.1)
Penicillin	5 (50.0)	2 (40.0)	0	1 (100.0)	1 (100.0)	9 (47.4)
Oxacillin	10 (100.0)	4 (80.0)	2 (100.0)	1 (100.0)	1 (100.0)	18 (94.7)
Trimethoprim-sulfamethoxazole	10 (100.0)	4 (80.0)	2 (100.0)	1 (100.0)	1 (100.0)	18 (94.7)

Antibiotic resistance profiles of the coagulase negative staphylococci:

The CoNS isolates exhibited high level of resistance to oxacillin (94.7%), sulfamethoxazole-trimethoprim (94.7%), clindamycin (78.9%) and daptomycin (89.5%). All *S. saprophyticus, S. xylosus, S. rodentium* and *S. sciuri* isolates were 100% resistant to daptomycin, sulfamethoxazole-trimethoprim and oxacillin. *S. xylosus, S. rodentium* and *S. sciuri* were also 100% resistant to daptomycin and oxacillin. All *S. rodentium* and *S. sciuri* were

resistant to clindamycin and *S. rodentium* was resistant to gentamicin and linezolid. All *S. saprophyticus, S. xylosus, S. rodentium, S. sciuri* isolates and 3 (60%) of 5 *S. lentus* were multidrug resistant, with resistance to three or more different classes of antibiotics. However, all CoNS isolates were susceptible to nitrofurantoin. All *S. xylosus, S. rodentium* and *S. sciuri* were susceptible to ciprofloxacin and levofloxacin. All *S. rodentium* and *S. sciuri* were sensitive to erythromycin and all *S. xylosus* and *S. sciuri* were susceptible to gentamicin, erythromycin and linezolid (Table 4).

Discussion:

Females of reproductive age are more susceptible to *S. saprophyticus* due to rectal, vaginal and urethral colonization by this organism (22). Genito-urinary colonization by *S. saprophyticus* is also a common feature in female gender as well as changes in the hormonal influences during menstruations, with *Candida* infection which affect the vaginal flora (23). Of the bacterial UTI pathogen isolated in 178 participants in this study, 34 (19.1%) were phenotypically identified as CoNS, 10 (5.6%) of which were genotypically confirmed to be *S. saprophyticus* as the cause of UTI among the selected female participants. *S. saprophyticus* is a prime uropathogen in young women of reproductive age especially below the age of 30 years (13).

In this study, the largest number of participants with urinary complaints recruited into the study were in the age group 21-30 years (40.8%) and lowest were in the age group ≤ 20 years (4.5%). Females in the age group 21-30 years are more prone to UTI and therefore likely to visit healthcare facility with urinary symptoms. This agrees with other researchers who found that young adult women are more susceptible to *S. saprophyticus* colonization and subsequent UTI. Oladeinde et al., (24) reported highest prevalence of UTI in the age group 21-30 years. Onyemelukwe and Nwokocha (10) in Enugu Nigeria also reported 6.3% incidence of *S. saprophyticus* in female urine samples. Farina et al., (3) reported its predominance in sexually active young women. Ekwealor et al., (25) also reported *S. saprophyticus* as a cause of UTIs in sexually active women. The high bacterial colonization of urethral of women within the reproductive age might be due to increased sexual activity in this age group and association of UTI with poor genital hygiene practices (26).

In our study, *S. saprophyticus* were genotypically confirmed in 10.7% of patients whose urine samples were positive for bacterial pathogens. Jhora et al., (27) reported 7.0% frequency of *S. saprophyticus* in UTI. Jombo et al., (28) reported 7.7% incidence from Calabar, Nigeria, Nasiru et al., (29) reported 6.8% incidence from Abuja Nigeria, and Jemikalajah (30) reported 11.1% in Delta State Nigeria, which agree with the rate reported in the present study. However, Vemulapalli and Rao (31) reported a higher frequency of 20.5% for *S. saprophyticus* in India and Alao et al., (32) reported extremely high frequency of 88% for *S. saprophyticus* in southwestern Nigeria, which contrast the finding of our current study. Other researchers from Nigeria have also reported *S. saprophyticus* in urine samples of symptomatic patients (10,28,29).

Stepanovic et al., (33) isolated 5 *S. sciuri* in urine samples of asymptomatic patients. Despite being predominantly associated with animals, members of the *S. sciuri* group have been known to colonize humans (34), and associated with significant infections, most frequently wound infections (35,36). The 16S rRNA sequencing in our study identified 5 species among the 19 *S. saprophyticus* identified by PCR, which included 10 *S. saprophyticus*, 5 *S. lentus*, *S.* 2 *xylosus*, 1 *S. rodentium* and 1 *S. sciuri* from the urine samples of symptomatic patients. We are not unaware that even with well-designed primers, high genomic similarity between *S. saprophyticus* and closely related CoNS species can result in false positives, particularly if the primers target conserved genes such as 16S rRNA, where sequence identity is ≥ 97%. *Staphylococcus lentus*, *S. xylosus*, and other CoNS share significant sequence identity with *S. saprophyticus* in the amplified region. This suggests the need to target more variable or unique genetic markers.

Antimicrobial resistance in CoNS is becoming more common and severe around the globe (37,38). Both human and animal health experts are facing difficulties due to the spread of antibiotic-resistant staphylococci. CoNS in this study exhibited multidrug resistance to the selected antimicrobials tested with high level of resistance to oxacillin (94.7%), sulfamethoxazole-trimethoprim (94.7%), clindamycin (78.9%) and daptomycin (89.5%). Production of β-lactamase was the initial resistance mechanism of staphylococci to β-lactam antibiotics through horizontal transfer of antimicrobial resistance plasmid genes (39). But resistance to oxacillin is mediated by chromosomal *mecA* or *mecC* genes that encodes abnormal penicillin binding proteins (PBPs) with low affinity of binding to β-lactam antibiotics (40).

The relative sensitivity of *S. saprophyticus* (80.0%) to erythromycin in this study agrees with a recent study by Fatholahzadeh et al., (41) who reported 80% susceptibility of *S. saprophyticus* to the same antibiotic. Susceptibility to levofloxacin and nitrofurantoin in this study was 100% in contrast to the study

by Sarzynski et al., (42) who reported 55.6% and 44.4% sensitivity of *S. saprophyticus* to levofloxacin and nitrofurantoin respectively. Agvald-Ohman et al., (43) reported that erythromycin and gentamicin resistance in clinical staphylococci was 48% and 54% respectively, higher than the rates obtained in our study for erythromycin (21.1%) and gentamicin (26.3 %). According to Antoinette et al., (44) clinical isolates in general have increasing levels of antibiotic resistance as a result of recurrent antibiotic exposure.

Conclusion:

Coagulase negative staphylococci, particularly *S. saprophyticus*, are important bacterial pathogen involved in UTIs and should not be discounted as commensals or contaminants. Molecular identification, antibiotic susceptibility, and resistance gene detection are necessary for accurate assessment of clinical significance of CoNS involved in UTI.

Contributions of authors:

OBO participated in the research design and benchwork; COI prepared and revised the manuscript and analysed the data; OBF was involved in manuscript-proofing and data analysis; OEA participated in the research design and supervision. All the authors read and approved the final manuscript**.**

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Conflict of interest:

Authors declare no conflict of interest

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Original Article **Open Access**

Prevalence of β-lactamase-encoding genes in *Salmonella* Typhi isolates from patients attending secondary healthcare facilities in Benue south senatorial district, northcentral Nigeria

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Abstract:

Background: The emergence of multi-drug resistant (MDR) *Salmonella* Typhi poses a major challenge in the treatment of typhoid fever using commonly available antibiotics. However, in many rural and semi-urban communities of low- and middle-income countries (LMICs), routine surveillance for MDR *S.* Typhi is scarcely undertaken. The aim of this study is to determine the prevalence of β-lactamase genes in MDR *S.* Typhi isolated from patients in nine secondary healthcare facilities in Benue south senatorial district, northcentral Nigeria.
Methodology: MDR *S.* Typhi (n=122) isolated from stool samples of patients initially diagnosed with typhoid fever using the Widal agglutination test in a previous study were used in this study. The isolates were confirmed by standard culture and biochemical identification characteristics. Plasmid DNA was isolated from each MDR isolate and selected β-lactam resistant (*bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{CTX-M}$) and carbapenem resistant (*bla*$_{IMP}$) genes were amplified by conventional simplex PCR assay.
Results: A total of 18 (14.8%) MDR *S.* Typhi isolates contained plasmids while on PCR assay, 5 (4.1%) isolates harbored *bla*$_{SHV}$ and 3 (2.5%) harbored *bla*$_{TEM}$, which encodes resistance to β-lactam antibiotics, while none harbored *bla*$_{IMP}$, which encodes resistance to carbapenems or *bla*$_{CTX-M}$, which encodes resistance to many extended spectrum β-lactam antibiotics.
Conclusion: This finding showed that the strains of MDR *S.* Typhi isolates in the study setting are those that harbored *bla*$_{SHV}$ and *bla*$_{TEM}$, which encode resistance to β-lactam antibiotics while strains that harbor *bla*$_{IMP}$ are apparently rare. A larger study across the entire region is required to confirm this finding.

Keywords: *Salmonella* Typhi, multi-drug resistance, *bla*$_{IMP}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, plasmid

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Prévalence des gènes codant pour la β-lactamase dans les isolats de *Salmonella* Typhi provenant de patients fréquentant des établissements de soins secondaires du district sénatorial sud de Benue, dans le centre-nord du Nigéria

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Résumé:

Contexte: L'émergence de *Salmonella* Typhi multirésistante (MDR) pose un défi majeur pour le traitement de la fièvre typhoïde à l'aide d'antibiotiques courants. Cependant, dans de nombreuses communautés rurales et semi-urbaines des pays à revenu faible et intermédiaire (PRFI), la surveillance systématique de *S.* Typhi MDR est rarement mise en œuvre. L'objectif de cette étude est de déterminer la prévalence des gènes de β-lactamases chez les *S.* Typhi MDR isolés chez des patients de neuf établissements de santé secondaires du district sénatorial de Benue Sud, au centre-nord du Nigéria.
Méthodologie: Des souches de *S.* Typhi MDR (n=122) isolées à partir d'échantillons de selles de patients initialement diagnostiqués avec une fièvre typhoïde par le test d'agglutination de Widal lors d'une étude précédente ont été utilisées dans cette étude. Les isolats ont été confirmés par culture standard et identification biochimique. L'ADN plasmidique a été isolé de chaque isolat MDR et les gènes sélectionnés de résistance aux β-lactamines (*bla*$_{SHV}$, *bla*$_{TEM}$ et *bla*$_{CTX-M}$) et aux carbapénèmes (*bla*$_{IMP}$) ont été amplifiés par PCR simplex conventionnelle.
Résultats: Au total, 18 isolats (14,8%) de *S.* Typhi multirésistants contenaient des plasmides. Lors d'un test PCR, 5 isolats (4,1%) étaient porteurs du *bla*$_{SHV}$ et 3 isolats (2,5%) du *bla*$_{TEM}$, codant pour la résistance aux antibiotiques β-lactamines. Aucun n'était porteur du *bla*$_{IMP}$, codant pour la résistance aux carbapénèmes, ni du *bla*$_{CTX-M}$, codant pour la résistance à de nombreux antibiotiques β-lactamines à spectre étendu.
Conclusion: Ce résultat a montré que les souches d'isolats de *S.* Typhi multirésistants étudiées étaient celles qui hébergeaient le *bla*$_{SHV}$ et le *bla*$_{TEM}$, codant pour la résistance aux antibiotiques β-lactamines, tandis que les souches qui hébergeaient le *bla*$_{IMP}$ étaient apparemment rares. Une étude plus vaste couvrant l'ensemble de la région est nécessaire pour confirmer ce résultat.

Mots-clés: *Salmonella* Typhi, multirésistance aux médicaments, *bla*$_{IMP}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, plasmide

Introduction:

Salmonellae are Gram-negative, flagellated, non-spore forming facultative anaerobes (1). They belong to the family Enterobacteriaceae and contain more than 2300 serotypes (2). *Salmonella enterica* is one of the most common causes of human gastroenteritis worldwide. Antibiotics such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole have been used for the treatment of enteric fever for many years (3). Later in the 1980s, the resistance of *Salmonellae* towards multiple drugs was reported (1), caused by the increased use of fluoroquinolones such as ciprofloxacin and the extended-spectrum cephalosporins (4).

The βeta-lactam antibiotics are widely used in the treatment of infections caused by *Salmonella* in both animals and humans (5). This has led to widespread resistance of *Salmonella* to different β-lactam drugs (6). Resistance to β-lactams in *S. enterica* is primarily caused by the production of acquired β-lactamases (1). More than 340 β-lactamases have been described and many of these have been identified in *Salmonella* (7). Different plasmid genes such as *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX}$ and *bla*$_{CMY}$ have been found to encode extended spectrum β-lactamases (ESBL) in *Salmonella* (8). Also, a few variants of the *bla*$_{OXA}$ genes have been identified in *Salmonella* belonging to the *bla*$_{OXA-1}$ group (comprising *bla*$_{OXA-1}$, *bla*$_{OXA-4}$, *bla*$_{OXA-30}$, *bla*$_{OXA-31}$ and *bla*$_{OXA-47}$ genes) and *bla*$_{OXA-2}$ group (comprising *bla*$_{OXA-2}$, *bla*$_{OXA-3}$, *bla*$_{OXA-15}$, *bla*$_{OXA-32}$, *bla*$_{OXA-34}$ and *bla*$_{OXA-53}$ genes) (8).

The past few decades have shown the emergence of multiple antibiotic-resistant *Salmonella*, particularly the ESBL-producing strains, which represents a significant challenge for human medicine (9). This has caused great concern since cephalosporins are drugs of choice for the treatment of salmonellosis in Nigeria and specifically in southern Benue (3). The ESBL-producing bacteria carry a broad range of β-lactamases that hydrolyze a wide spectrum of penicillin and cephalosporin antibiotics, but not carbapenems (10). Many β-lactamases are members of TEM (named after the patient Temoneira), sulfhydryl reagent variable (SHV), CTX-M (active on cefotaxime, first isolated at Munich), and OXA (oxacillinase) families (11). The three enzymes TEM, SHV and CTX-M represent the most prevalent types of ESBL enzymes (11).

Recently, another group of β-lactamases named AmpC have been described, which confers resistance to all β-lactam antibiotics except the fourth generation cephalosporins and carbapenems (7). In contrast to ESBL, the AmpC group is not susceptible to β-lactam inhibitors, such as clavulanic acid, sulbactam and tazobactam (8). In addition, the AmpC group can be encoded by genes located on chromosomes or plasmids (12). Tran et al., (13) reported that *Salmonella* resistance to the extended-spectrum cephalosporins were shown to commonly result from the action of the plasmid AmpC *bla*$_{CMY-2}$ gene.

Identical plasmid-mediated β-lactamase genes have been detected in Enterobacteriaceae from different countries, which could indicate plasmid-borne spread of these genes between countries (1). Nonetheless, there is

paucity of information on the detection of β-lactamase genes among *S*. Typhi isolates in southern Benue, Nigeria. This study was hence conducted to investigate the genetic basis of MDR in *Salmonella* Typhi isolated from stool of patients in secondary healthcare facilities in southern Benue, northcentral Nigeria.

Materials and method:

Study area:

This study was conducted in Benue State secondary healthcare facilities within the nine local government areas (LGAs) of Benue south geographical zone, Nigeria. These LGAs include Otukpo (TKP), Ohimini (OHM), Apa (APA), Agatu (GTU), Obi (OBI), Oju (OJU), Ado (ADO), Okpokwu (OKP), and Ogbadibo (OGB). Benue south geographical zone is in Benue State, northcentral region of Nigeria, with a population of about 1,307,647 people in 2006 (14).

Study design and isolates:

This is a laboratory-based study to determine the prevalence of β-lactamase genes in MDR *S.* Typhi isolates. From a previous study by Adikwu et al., (15), 122 MDR *S*. Typhi isolates were collected, purified, and confirmed on Bismuth Sulfite agar, based on cultural morphology. The previous study had isolated 447 *S.* Typhi from 1,022 stool samples of patients with typhoid fever and positive Widal agglutination test, and antibiotic susceptibility test had been performed on them using the standard disc diffusion method of Kirby-Bauer as described by Kolhatkar and Ochei (16), with the results interpreted using the guideline of the Clinical and Laboratory Standards Institute (17). MDR in the *S.* Typhi isolates was defined as resistance to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole (18).

Plasmid DNA extraction:

Plasmid DNA was isolated from pure overnight cultures using Bio Teke's Plasmid MiniPrep Kit (Beijing & Wuxi, China) following the manufacturer's instructions. The purity of the DNA was assessed using the Nanodrop spectrophotometer. Exactly 0.4µl of extracted plasmid DNA was placed on the spectrophotometer and the ratio of the absorbance at 260 nm to 280 determined. The purity of the DNA is reflected in the OD260:OD280 ratio and must be between 1.8 and 2.0. Values less than 1.8 indicate protein contamination while values above 2.0 indicate RNA contamination. The DNA isolates were preserved at -20^{o}C until further use.

Amplification of resistance genes:

Genes encoding resistance to β-lactams (*bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{TEM}$), and resistance to carbapenem (*bla*$_{-IMP}$) were amplified by conventional simplex PCR using specific primers (Table 1), and the PCR conditions described by Hasman et al., (19). The conditions and volume of the reaction were determined by the Solis BioDyne ready to load master mix (5x) used.

PCR was performed in a 20µl reaction under the following conditions; initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 30 sec, annealing between 54-66°C for 1 min (the annealing temperature is determined by the primer used), extension at 72°C for 2 min, and a final extension at 72°C for 5 min for a total number of 35 cycles.

Agarose gel electrophoresis:

A check gel was performed on 2% TAE agarose gel and electrophoresed at 70 V for 1 hour to confirm the amplified bands, with 100bp Plus DNA ladder (Transgen Biotech, Cat No. BM 311). The bands were visualized under UV light in a gel documentation system

Table 1: Primers used for study

Target gene	Primer	Sequence	Amplicon Size (bp)	Ref
bla$_{SHV}$	SHV – F	5'- GATGAACGCTTTCCCATGATG -3'	214	(20)
	SHV – R	5' - CGCTGTTATCGCTCATGGTAA -3'		
bla$_{TEM}$	TEM – F	5'- AGTGCTGCCATAACCATGAGTG -3'	431	(20)
	TEM – R	5'- CTGACTCCCCGTCGTGTAGATA -3'		
bla$_{CTX-M}$	CTM-X – F	5'- ATGTGCAGYACCAGTAARGTKATGGC -3'	592	(21)
	CTM-X – R	5'- TGGGTRAARTARGTSACCAGAAYSAGCGG -3'		
	IMP – F	5'– GAAGGCGTTTATGTTCATAC -3'	588	(22)
bla$_{IMP}$	IMP – R	5'- GTATGTTTCAAGAG TGATGC -3'		

Results:

Purity and concentration of plasmids extracted from *Salmonella* Typhi isolates:

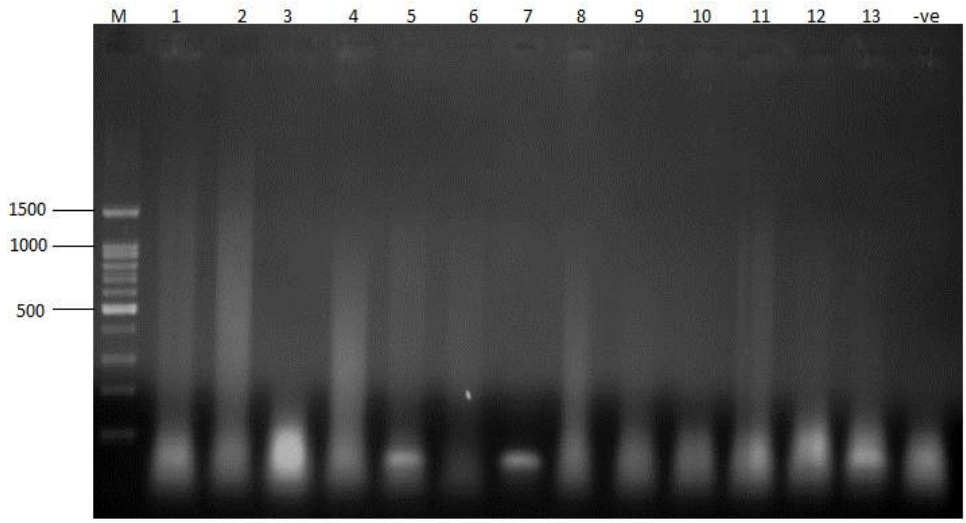
Among the 122 MDR *S.* Typhi isolates, only 18 (14.8%) contained plasmids. The extracted plasmids were pure except for isolate GTU 8 (1.74) as shown in Table 2.

Prevalence of β-lactamase resistance gene in *Salmonella* Typhi isolates:

Among the 122 MDR *S.* Typhi isolates, the β-lactamase resistant genes were detected in 8 of the 18 isolates with plasmids; 0% bla_{IMP} gene (Plate 1), 5 (4.1%) bla_{SHV} gene (Plate 2, 3 and 4), 3 (2.5%) bla_{TEM} gene (Plate 5 and 6), and 0% bla_{CTX-M} gene (Plate 7).

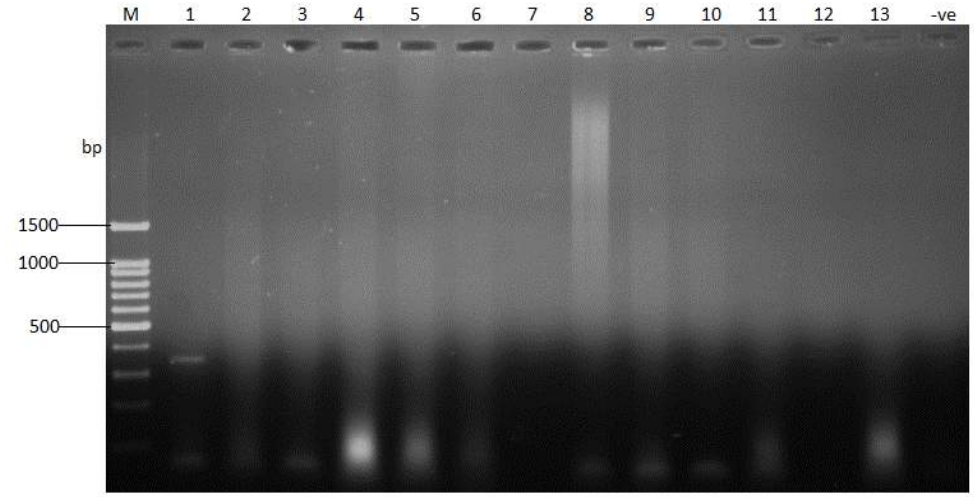
Table 2: Purity and concentration of plasmid DNA extracted from *Salmonella* Typhi isolates

Isolate ID	Nucleic acid concentration (ng/µl)	Absorbance (260:280)
TKP 65	329.1	1.94
TKP 82	833.5	1.98
OHM 27	621.7	1.94
OHM 43	587.3	1.86
APA 5	671.6	1.88
APA 25	661.8	1.95
GTU 8	701.3	1.74
GTU 37	425.6	1.87
OBI 18	393.7	1.96
OBI 38	733.9	1.82
OJU 22	517.8	1.93
OJU 48	658.8	1.97
ADO 16	573.6	1.98
ADO 57	801.7	1.98
OKP 85	606.4	1.88
OKP 100	704.1	1.96
OGB 39	739.6	1.95
OGB 119	583.2	1.99



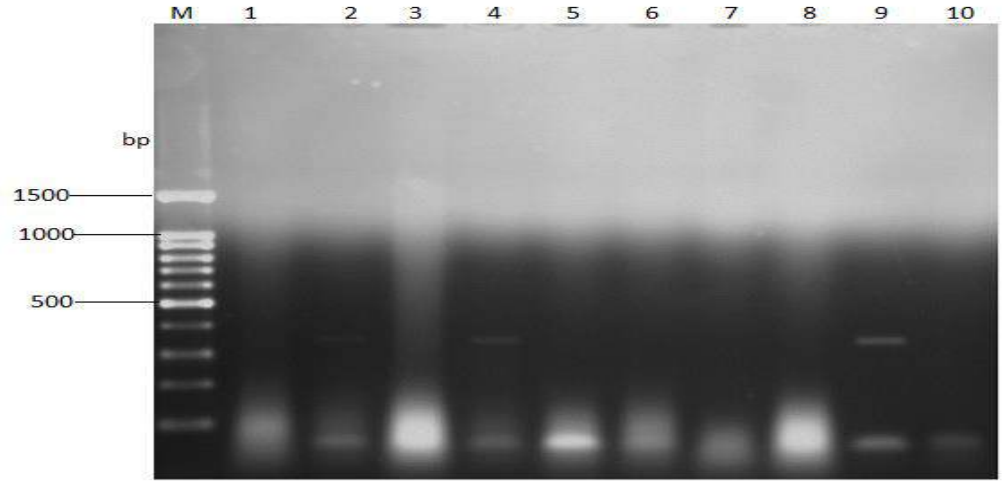
Lane 1 – 13 = Samples; No isolate amplified with bla_{IMP} primer; Lane M: 100bp Molecular DNA ladder; Lane -ve: Negative Control (PCR reaction without DNA); M = Size marker.

Plate 1: Electrophoretograph of amplified bla_{IMP} gene (588bp) in MDR *Salmonella* Typhi isolates



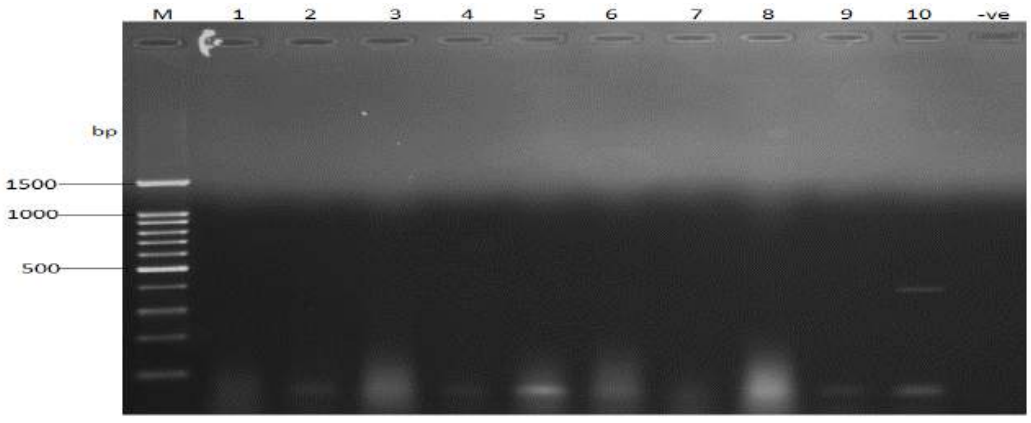
Lane 1 – 13 = Samples; Sample 1 (TKP 46) amplified with *bla*SHV primer; Lane M: 100bp Molecular DNA ladder; Lane -ve: Negative Control (PCR reaction without DNA); M = Size marker

Plate 2: Electrophoretograph of amplified bla_{SHV} gene (214bp) in MDR *Salmonella* Typhi isolates



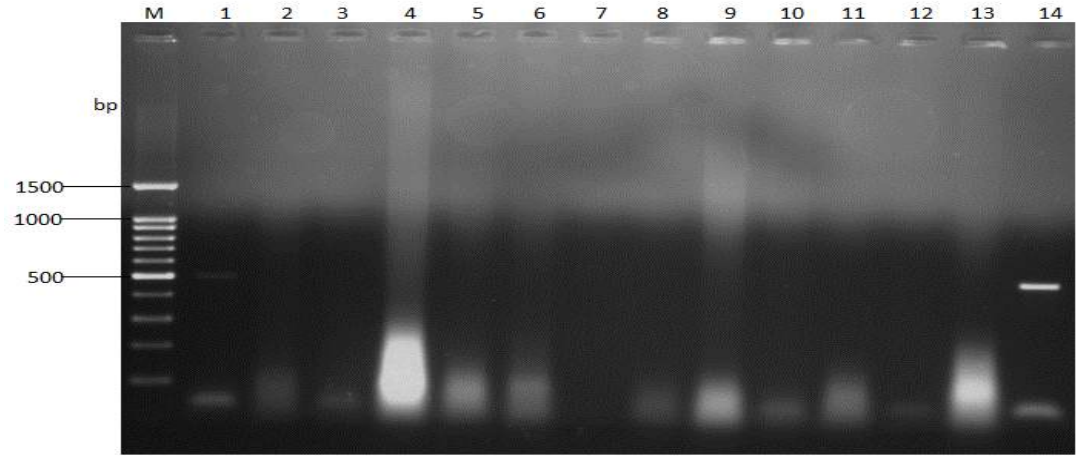
Lane 1 – 13 = Samples; Samples 2 (OBI 66), 4 (OHM 22) and 9 (OJU 51) amplified with bla_{SHV} primer; Lane M: 100bp Molecular DNA ladder; Lane -ve: Negative Control (PCR reaction without DNA); M = Size marker

Plate 3: Electrophoretograph of amplified bla_{SHV} gene (214bp) in MDR *Salmonella* Typhi isolates



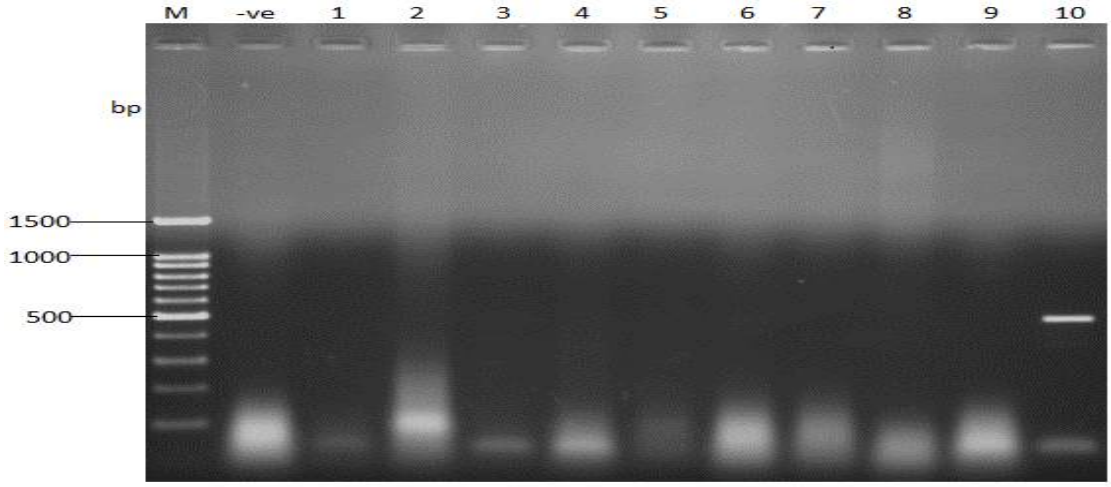
Lane 1 – 13 = Samples; Samples 10 (ADO 7) amplified with bla_{SHV} primer; Lane M: 100bp Molecular DNA ladder; Lane -ve: Negative Control (PCR reaction without DNA); M = Size marker

Plate 4: Electrophoretograph of amplified bla_{SHV} gene (214bp) in MDR *Salmonella* Typhi isolates



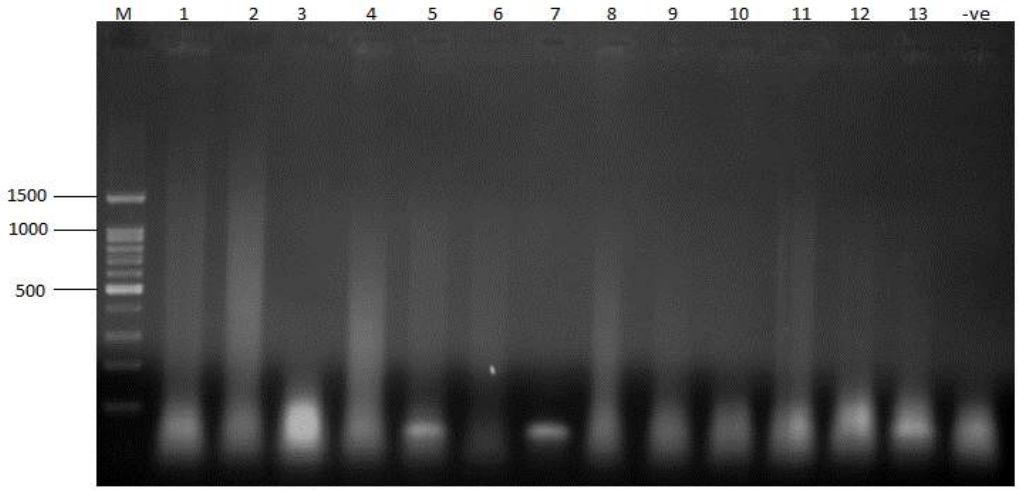
Lane 1 – 13 = Samples; Samples 1 (GTU 5) and 14 (OKP 86) amplified with *bla*$_{TEM}$ primer; Lane M: 100bp Molecular DNA ladder; Lane -ve: Negative Control (PCR reaction without DNA), M = Size marker

Plate 5: Electrophoretograph of Amplified *bla*-$_{TEM}$ gene (431bp) in MDR *Salmonella* Typhi isolates



Lane 1 – 13 = Samples; Sample 10 (OKP 124) amplified with *bla*$_{TEM}$ primer; Lane M: 100bp Molecular DNA ladder; Lane -ve: Negative Control (PCR reaction without DNA). M = Size marker

Plate 6: Electrophoretograph of amplified *bla*$_{TEM}$ gene (431bp) in MDR *Salmonella* Typhi isolates



Lane 1 – 13 = Samples; No isolate amplified with *bla*$_{CTX-M}$ primer; Lane M: 100bp Molecular DNA ladder; Lane -ve: Negative Control (PCR reaction without DNA); M = Size marker.

Plate 7: Electrophoretograph of amplified *bla*$_{CTX-M}$ gene (592bp) in MDR *Salmonella* Typhi isolates.

Discussion:

Enteric fever is endemic due to continued prevalence of *Salmonella* Typhi. The pathogen is transmitted through the fecal-oral route as a result of poor hygiene (1). Antibiotic therapy constitutes the mainstay of the management of enteric fever, however, antibiotic resistance has become a major threat to treatment of enteric fever (15). The purpose of the current study was to detect β-lactamase genes carried by *S.* Typhi isolated from stool of patients.

Amplification of genes by PCR in this study is an indication that some of the MDR isolates harbored bla_{SHV} (4.1%, n=5) and bla_{TEM} (2.5%, n=3) genes which encode resistance to β-lactam antibiotics. They however lacked bla_{IMP} that encodes resistance to carbapenems and bla_{CTX-M}, which encodes resistance to many extended spectrum β-lactam antibiotics. This agrees with the findings of Boisrame-Gastrin et al., (23), Djeffal et al., (24), Eguale et al., (8) and Abdul-Azeez et al., (2). The detection of high resistance rate to β-lactam antimicrobials (27.3%) and the presence of *bla* genes in the present study could be attributed to selective pressure on the commonly used β-lactam antibiotics in the study area. Adikwu et al., (4) reported that amoxicillin and ceftriaxone are frequently employed in the treatment of typhoid fever in Benue South leading to selection pressure. This corroborates the findings of Eguale et al., (8), although they reported a higher detection rate.

The dominant β-lactamase genes detected in the current study were bla_{SHV}. This is consistent with the observed spectrum of resistance to cephalosporin in previous studies (19, 25-27), which all reported resistance to cephalosporins. The finding however disagrees with that of Eguale et al., (8) who reported bla_{TEM} as the dominant beta-lactamase gene. In Africa, bla_{SHV} has been reported from *S.* Typhi isolated from human in Senegal (28), Egypt (29), and children adopted from Mali (23).

Conclusion:

This study showed that some MDR *S.* Typhi isolates harbored bla_{SHV} and bla_{TEM} genes, that encode resistance to β-lactam antibiotics, but lacked bla_{IMP} that encodes resistance to carbapenems. More studies are required to detect other genes encoding resistance to *S.* Typhi in other geographical zones in Benue State in particular and Nigeria at large.

Contributions of authors:

The study was conceptualised and designed by PA, EUU, IOO and CCI; the laboratory acquisition of data was done by PA, DAO, PIA, and MEO; Data analysis was done by PA, DAO, and AAA; the manuscript draft and critical revisions were done by DAO, PA, GAO, GEO, AAA, SOO, MEO, and STS; PA gave the final revision of the manuscript. All authors approved the manuscript submitted for publication.

Conflict of interest:

Authors declare no conflict of interest

Source of funding:

Authors received no external funding

Availability of data:

The data supporting findings of this study are available from the corresponding author upon request.

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Original Article **Open Access**

Genomic characterization of an extensively drug-resistant *Mycobacterium tuberculosis* clinical isolate

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Abstract:

Background: Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, constitutes a predominant infectious disease with high mortality on a global scale, especially in Nigeria. The emergence of extensively drug-resistant (XDR) TB poses a significant challenge to the control of this disease, underscoring the necessity for the identification of underlying genetic mutations to facilitate informed treatment decisions and mitigate transmission.
Methodology: Sputum samples from a total of 199 patients with validated baseline GeneXpert MTB/rifampicin-resistant positive from the northcentral States of Nigeria including Nasarawa, Benue, Kogi, Niger and Abuja in the month of June 2024 were transported to the Zankli TB Reference Laboratory, and processed using NALC-NaOH method, followed by centrifugation. The processed samples were inoculated onto prepared Lowenstein Jensen (LJ) media (solid culture) and Mycobacterial Growth Indicator Tube (MGIT) for liquid culture. Ziehl Nelsen (ZN) stain was performed on the isolates of the positive cultures to ascertain the presence of Acid-Fast Bacilli (AFB) and the Bio-line SD AgMPT64 test was performed to confirm the presence of *M. tuberculosis.* Drug susceptibility testing (DST) was conducted on all positive cultures using the standard proportion method. Long read sequencing was performed on the 2 XDR isolates using the Oxford Nanopore Technology with 50x coverage. The raw reads of the sequenced data were binned into a high-quality metagenome-assembled genome (MAG) via maxbin (V2.2.7), and the quality of the binned metagenome-assembled genome was assessed via checkM (V1.2.3). Resistance genes were discerned through the Comprehensive Antibiotic Resistance Database (CARD) resistance gene identifier, and existing virulence factors were analysed via the VF analyser of the Virulence Factors Database (VFDB), while the phylogenetic tree was constructed via the auto-Multi Locus Species Tree (auto-MLST).
Results: Mutations in *katG* (R463L, A234V, A431V), *rpoB* (D516G, H526T, L511R), *gyrA* (D94N), *murA* (C117D) and *embC* (R738Q) were detected in one of the two sequenced XDR isolates (XMTB2) that passed sequencing quality control. The VFDB identified virulence genes such as *narG*, *narH*, *sapM*, and *narX*, associated with the XMTB2 MAG, while phylogenetic analysis revealed a close phylogenetic connection between the XMTB2 MAG and the well-known Beijing lineage (L2).
Conclusion: The results of this study revealed that the XMTB2 MAG was potentially an XDR isolate that was very virulent because of the presence of many virulence factors identified in it and its close relationship with two of the most widespread and virulent lineages of *M. tuberculosis;* the Beijing lineage (L2) and the Euro-American lineage (L4).

Keywords: *Mycobacterium tuberculosis*; XDR TB; mutation; virulence factors; phylogenetic analysis

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Caractérisation génomique d'un isolat cliniquement résistant de *Mycobacterium tuberculosis*

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Résumé:

Contexte: La tuberculose (TB), causée par *Mycobacterium tuberculosis*, constitue une maladie infectieuse prédominante avec une mortalité élevée à l'échelle mondiale, en particulier au Nigéria. L'émergence de la

tuberculose ultrarésistante (XDR) pose un défi important pour la lutte contre cette maladie, soulignant la nécessité d'identifier les mutations génétiques sous-jacentes pour faciliter des décisions thérapeutiques éclairées et atténuer la transmission.
Méthodologie: Des échantillons d'expectorations provenant d'un total de 199 patients avec un test GeneXpert MTB/rifampicine résistant de base validé et provenant des États du centre-nord du Nigéria, notamment Nasarawa, Benue, Kogi, Niger et Abuja, au mois de juin 2024, ont été transportés au laboratoire de référence de la tuberculose de Zankli et traités par la méthode NALC-NaOH, suivie d'une centrifugation. Les échantillons traités ont été inoculés sur des milieux Lowenstein Jensen (LJ) préparés (culture solide) et sur un tube indicateur de croissance mycobactérienne (MGIT) pour la culture liquide. Une coloration de Ziehl-Neelsen (ZN) a été réalisée sur les isolats des cultures positives afin de vérifier la présence de bacilles acido-résistants (BAAR) et le test Bioline SD AgMPT64 a été réalisé pour confirmer la présence de M. tuberculosis. Un test de sensibilité aux médicaments (TSM) a été réalisé sur toutes les cultures positives par la méthode des proportions standard. Un séquençage à lecture longue a été réalisé sur les deux isolats XDR à l'aide de la technologie Oxford Nanopore avec une couverture 50x. Les lectures brutes des données séquencées ont été regroupées dans un génome assemblé par métagénome (MAG) de haute qualité via maxbin (V2.2.7), et la qualité du génome assemblé par métagénome regroupé a été évaluée via checkM (V1.2.3). Les gènes de résistance ont été identifiés grâce à l'identifiant de gènes de résistance de la base de données complète sur la résistance aux antibiotiques (CARD), et les facteurs de virulence existants ont été analysés via l'analyseur VF de la base de données des facteurs de virulence (VFDB), tandis que l'arbre phylogénétique a été construit via l'arbre d'espèces multi-locus (auto-MLST).
Résultats: Des mutations dans *katG* (R463L, A234V, A431V), *rpoB* (D516G, H526T, L511R), *gyrA* (D94N), *murA* (C117D) et *embC* (R738Q) ont été détectées dans l'un des deux isolats XDR séquencés (XMTB2) qui ont passé le contrôle de qualité du séquençage. Français La VFDB a identifié des gènes de virulence tels que *narG*, *narH*, *sapM* et *narX*, associés au MAG XMTB2, tandis que l'analyse phylogénétique a révélé une connexion phylogénétique étroite entre le MAG XMTB2 et la lignée bien connue de Beijing (L2).
Conclusion: Les résultats de cette étude ont révélé que le MAG XMTB2 était potentiellement un isolat XDR très virulent en raison de la présence de nombreux facteurs de virulence identifiés en lui et de sa relation étroite avec deux des lignées les plus répandues et les plus virulentes de *M. tuberculosis*; la lignée de Beijing (L2) et la lignée euro-américaine (L4).

Mots-clés: *Mycobacterium tuberculosis*; XDR TB; mutation; facteurs de virulence, analyse phylogénétique

Introduction:

Tuberculosis (TB), caused by the pathogen *Mycobacterium tuberculosis*, represents a significant challenge in public health, ranking as a predominant cause of global mortality and engendering considerable health complications. TB is classified as a potentially perilous infectious ailment that primarily affects the pulmonary system. The transmission of TB occurs between individuals through respiratory actions such as coughing and sneezing, which disseminate microscopic aerosolized droplets containing the pathogen into the atmosphere. Infections caused by *M. tuberculosis* result in several million fatalities annually, with this elevated mortality rate being associated with inadequate diagnostic practices and the swift emergence of multidrug-resistant strains of TB (MDR-TB) (1).

By the year 2023, TB re-established itself as the infectious disease accountable for the highest mortality rate globally. During that year, the World Health Organization (WHO) projected 1.25 million deaths among an estimated 10.8 million cases of TB (2). MDR-TB is characterized as an infection resulting from *M. tuberculosis* that exhibits resistance to both rifampicin and isoniazid, whereas XDR-TB is delineated as MDR-TB with supplemental resistance to any fluoroquinolone (FLQ) and a minimum of one second-line injectable pharmacological agent (3).

Extensive drug resistance (XDR) is still a major global public health issue, particularly in nations with high TB prevalence, such as Nigeria. Numerous of these incidents are unreported, infecting people who are at high risk. Public health and the global economy have been significantly impacted by the growth of drug-resistant and XDR *M. tuberculosis* complex (MTBC), as many cases remain unreported or undiscovered in low-resource settings, leading to misdiagnosis and further transmission (4). Inadequate and improper adherence to prescribed treatment regimens by patients constitutes a principal factor contributing to the development of resistance to TB pharmacotherapies (5).

The virulence factors of *M. tuberculosis* cannot be identified exclusively with toxins and other by-products of bacterial metabolic activity that are known to adversely impact the host organism. With the investigation of the properties of *M. tuberculosis*, the quantity of identified virulence factors has steadily increased. This remarkable plasticity within the *M. tuberculosis* genome permits us to classify this bacterium as a "highly successful pathogen" (6).

There is a distinct geographic distribution among the human-adapted lineages of the MTBC, with certain "generalist" lineages such as Lineage (L2) and L4) existing worldwide and others such as L5, L6, and L7 being regionally restricted "specialists." This continent is home to all seven lineages that have evolved to adapt to humans, including the three "specialist" lineages that are unique to Africa (7). The aim of this study was to investigate the genetic determinants of resistance

and virulence in clinical XDR *M. tuberculosis*.

Materials and method:

Study design:

This study is a descriptive cross-sectional design to identify *M. tuberculosis* (MTB) and non-tuberculous *Mycobacteria* (NTM) infection among Xpert MTB/RIF Ultra confirmed positive TB cases.

Ethical approval:

Ethical approval for this study was obtained from the Bingham University Teaching Hospital, Health Research and Ethics Committee Jos, Plateau State, Nigeria (NHREC/21/05/2005/01287). This study used existing laboratory records (secondary data) hence there was no risk of physical harm to the patients. The data were identified for confidentiality and privacy of patients.

Study population:

In June 2024, sputum samples from a total of 199 patients with validated baseline GeneXpert *M. tuberculosis*/RIF positive samples were collected from clinics and hospitals in northcentral Nigeria and submitted to the Zankli TB Reference laboratory, Bingham University Karu, Nasarawa State. Participants were selected based on their confirmed Xpert-positive status at baseline.

Sample collection:

In accordance with the National Guidelines for Sputum Sample Collection, sputum samples were obtained from the participants from Nasarawa, Benue, Kogi, Niger, and Abuja in northcentral regions of Nigeria. The samples were then transported by triple sample packaging in a cold chain to Zankli Reference Laboratory for analysis

Decontamination and culture procedure:

The standardized N-Acetyl L-cysteine (NALC)/Sodium Hydroxide (NaOH) protocol was used to process the sputum specimens in a biosafety level 2+ (BSL 2+) laboratory environment. To mitigate the risk of *Mycobacteria* viability loss, NaOH was neutralized by the addition of phosphate buffer after a 15-minute period. The resultant mixture was centrifuged at 3000g for 15 minutes, following which the supernatant was discarded. The sediment obtained was used for concentrated AFB smear preparation, solid culture inoculation and BACTEC MGIT liquid culture (8,9).

The Ziehl Neelsen (ZN) acid fast staining was performed on prepared smear of the processed samples followed by examination under an oil immersion objective, with the AFB smears being classified as scanty (1-9 AFB/100 fields), 1+ (10-99 AFB/100 fields), 2+ (1-10 AFB/field), or 3+ (> 10 AFB/ field).

A volume of 0.5 mL of the processed sample was inoculated onto prepared LJ slants and incubated aerobically. Similarly, 0.5ml was inoculated into the MGIT tubes and loaded onto the BACTEC-MGIT 960 machine. Weekly observations were conducted over an 8-week period to monitor for growth on the solid media, while the BACTEC-MGIT 960 machine flagged all positive growth. ZN staining was performed on the isolates of the positive cultures to ascertain the presence of AFB, and the MTBC TB Ag MPT64 Rapid assay was performed to confirm the presence of *M. tuberculosis*.

BIO-LINE SD Ag MPT64 TB test:

A cumulative volume of 100 µl of broth derived from the BACTEC-MGIT 960 culture was administered to the well of the BIO-LINE SD Ag MPT64 TB rapid kit cassette. The inoculated immunochromatographic cassettes were maintained at ambient temperature for a duration of 20 minutes within a Class II Biosafety cabinet and subsequently evaluated for the presence of both control and test bands. The manifestation of a band in the "C" region validated the integrity of the assay. The additional emergence of a band in the "T" region was interpreted as indicative of the presence of the MPT64 Ag antigen within the test broth culture. The absence of a band in the "C" region was regarded as an indication of an invalid test. The standard reference strain, *M. tuberculosis* complex H37Rv ATCC 27294, was used as positive control.

Drug susceptibility testing (DST):

Drug susceptibility testing (DST) was performed on all positive cultures derived from solid culture utilizing the standard proportion method (10). This methodology encompassed the assessment of the susceptibility of *M. tuberculosis* isolates to first-line anti-TB pharmacological agents (Isoniazid, Rifampicin, and Ethambutol), as well as to the second line fluoroquinolone FLQ (Moxifloxacin) and injectables (Kanamycin, Amikacin and Capreomycin), which facilitated their classification as drug-susceptible TB (DS-TB), MDR-TB, or XDR-TB strains (10).

DNA extraction and next generation sequencing:

Genomic DNA of the two XDR isolates from the DST was extracted using the ZYMO quick-DNA Fungal/Bacterial Miniprep kit according to the manufacturer's instructions. Long-read sequencing was performed using Oxford Nanopore Technology with 50x coverage of the extracted gDNA. Sequencing libraries were generated via SQK-RBK114.96. Sequencing was performed on a GridION (Oxford Nanopore technologies) using an R10.4.1 flow cell with the base calling model r1041_e82_

400 bps_hac_v4.2.0. Reads were randomly subsampled to 50x coverage via Rasusa (V 0.7.1) and assembled via flyer (V 2.9.2-b1786). The assemblies were polished via Medaka (V 1.8.0) and the relevant model (r1041_e82-400 bps_hac_v4.2.0). Annotation was performed via Bakta (V 1.8.1). Taxonomic classification of the raw reads and assemblies was performed via Kraken (V 2.0.9-beta Derrick), and 16S identification was performed via Barnap and Sina. The assemblies were analysed and visualized via Quast and Bandage (V 0.8.1). The raw reads of the sequenced data were binned into a high-quality metagenome-assembled genome (MAG) via maxbin (V 2.2.7), and the quality of the binned MAG was assessed via checkM (V 1.2.3).

Bioinformatic analysis:

All antibiotic resistance genes were identified via the Comprehensive Antibiotic Resistance Database (CARD) resistance gene identifier (11). Existing virulence factors were investigated via the VF analyser tool of the Virulence Factor Database (VFDB) (12). For phylogenetic tree analysis, the auto-locus species tree (auto-MLST), which automates all steps, was constructed via de novo method, which allows the user to choose from which additional species can be added to construct a detailed phylogenetic tree and can also choose which genes to use for MLST.

In this study, 20 simultaneous genomes of *M. tuberculosis* in the FASTA were downloaded from the NCBI database. The multiple sequence files, including the query sequenced genome, were uploaded as inputs to the server. Since all the steps are automated by the server, they were left as defaults, and an email address was submitted for notification about the auto-MLST job that was performed.

Results:

Rifampicin-resistant isolates identified:

The number of GeneXpert MTB/rifampicin-resistant (RR) sputum samples of patients received at the Zankli TB reference laboratory for the month of June 2024, were 199. Among the samples, 151 (76.0%) were positive for MTB isolates by culture, while 48 (24.0%) were negative for MTB isolates on culture, with 22 (11.0%) of the isolates being MDR while only 2 (1.0%) isolates were XDR strains (Table 1).

Table 1: Frequency of MTB isolates by culture among GeneXpert MTB/rifampicin-resistant positive samples

MTB isolates	Number (%)
Culture positive	151 (76.0)
Culture negative	48 (24.0)
MDR isolate	22 (11.0%)
XDR isolate	2 (1.0%)
Total	**199**

MDR = Multidrug-resistant; XDR = Extensively drug-resistant

Drug sensitivity test results of the 2 XDR isolates:

Out of the two XDR isolates from the DST, the first isolate (XMTB1) was resistant to all the tested drugs except moxifloxacin, while the second isolate (XMTB2) was resistant to all the tested drugs except isoniazid, with ethambutol, having no effect on the isolate (Table 2).

Resistance genes detected in the XMTB2 MAG:

The resistance genes in the second *M. tuberculosis* isolate that passed the sequencing quality control (XMTB2 MAG) were identified via the CARD resistance gene identifier (Table 3). The mechanisms involved include antibiotic target alteration, antibiotic inactivation, antibiotic efflux and antibiotic target replacement.

Table 2: Drug sensitivity test results of the two XDR-isolates

S/N	Isoniazid (0.2 µg/ml)	Rifampicin (40 µg/ml)	Ethambutol (2 µg/ml)	Kanamycin (30 µg/ml)	Amikacin (30 µg/ml)	Capreomycin (40 µg/ml)	Moxifloxacin/ Levofloxacin (2 µg/ml)
Isolate 1	R	R	R	R	R	R	S
Isolate 2	S	R	-	R	R	R	R

S/N: Serial number; R= Resistant, S= Sensitive; - = No effect; XDR = Extensively drug resistant

Table 3: List of antibiotic resistance genes in the XMTB2 MAG

Resistance gene	Protein	Drug class	Resistance mechanism
erm(37)	erythromycin ribosome methylase	macrolides, lincosamides, streptogramins	antibiotic target alteration
*bla*C	Beta-lactamase	cephalosporins, cephamycin, carbapenems	antibiotic inactivation
Nickel/cobalt **t**ransporter gene	putative nickel/cobalt transporter	fluoroquinolones, aminoglycosides, isoniazid	antibiotic efflux
e*fp*A	putative efpA	Rifamycin, isoniazid	antibiotic efflux
aac(2')-Ic	aminoglycoside 2'-N-acetyltransferase	Aminoglycosides	antibiotic inactivation
*murA (i*ntrinsic)	UDP-N-acetylglucosamine enolpyruvcfl transferase	Fosfomycin	antibiotic target alteration
embC mutant	Arabinosyltransferase	Ethambutol	antibiotic target alteration
katG mutant	Catalase peroxidase variant model	Isoniazid	antibiotic target alteration
gyrA mutant	Gyrase A variant model	Fluoroquinolones	antibiotic target alteration
rpoB mutant	RNA polymerase	Rifamycin	antibiotic target alteration, antibiotic target replacement
23S rRNA mutant	rRNA gene variant model	Aminoglycoside	antibiotic target alteration

Table 4: Chromosomal mutations in antibiotic resistance genes in the XMTB2 MAG

Resistance gene	SNP	Initial amino acid	Final amino acid
murA (intrinsic)	C117D	Cysteine	Aspartic acid
embC mutant	R738Q	Arginine	Glutamine
katG mutant	R463L	Arginine	Leucine
	A234G	Alanine	Glycine
	A431V	Alanine	Valine
	G300W	Glycine	Tryptophan
gyrA	D94N	Aspartic acid	Asparagine
rpoB	D516G	Aspartic acid	Glycine
	H526T	Histidine	Threonine
	L511R	Leucine	Arginine
23S rRNA	A2145G	Alanine	Glycine

SNP = single nucleotide polymorphism

Chromosomal mutations of antibiotic resistance genes in the XMTB2 MAG:

Chromosomal mutations of antibiotic resistance genes in the second XDR *M. tuberculosis* isolate that passed the sequencing quality control (XMTB2 MAG), were identified via the CARD resistance gene identifier (Table 4).

Virulence factor class and related genes:

Virulence factors, including both the proteinaceous and non-proteinaceous factors of the XMTB2 MAG were also identified (Table 5).

Phylogenetic tree of XMTB2 genome constructed via an auto-multi locus species tree:

The phylogenetic tree of XMTB2 MAG was constructed via autoMLST. The relationships were analysed based on mash distances to the query genome, in which all the strains were found to belong to either the popular and virulent Beijing lineage (L2) or the Euro-American lineage (L4), which indicates that XMTB2 MAG belongs to either of the two lineages (Fig 1)

Table 5: Virulence factor class and related genes

Virulence factor class	Virulence factors	Related genes
Amino acid and purine metabolism	Glutamine synthesis	*glnA1*
	Leucine synthesis	*leuD*
	Lysine synthesis	*lysA*
	Proline synthesis	*proC*
	Purine synthesis	*purC*
	Tryptophan synthesis	*trpD*
Anaerobic respiration	Fused nitrate reductase	*narX*
	Nitrate reductase	*narG*
		narH

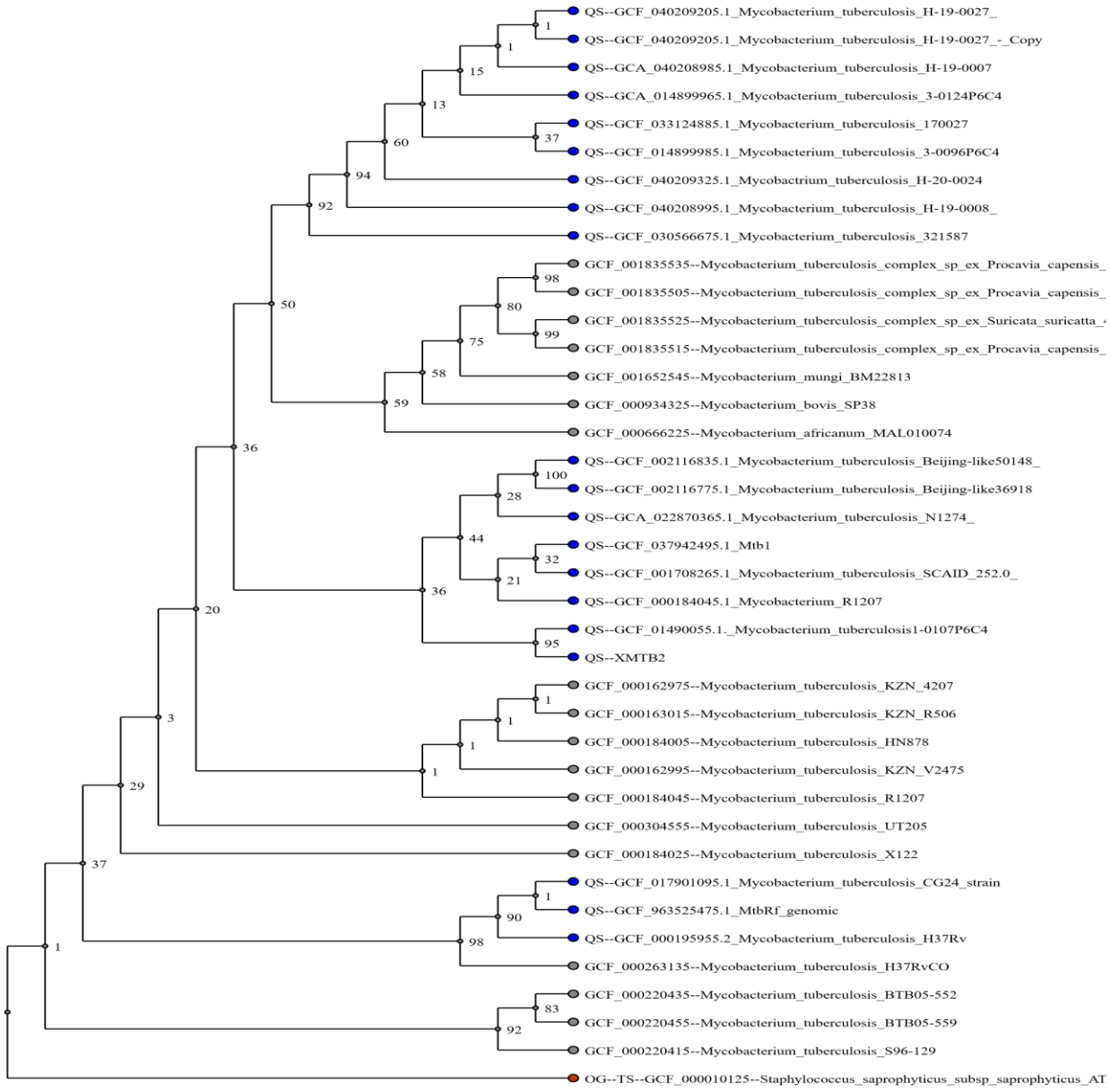

Fig 1: Phylogenetic tree of XMTB2 MAG

Discussion:

Extensively drug-resistant TB (XDR-TB) represents a significant public health concern. The anticipated effectiveness of recently endorsed anti-TB pharmacological agents for the management of DRTB, as well as the relative proportions of primary resistance cases compared with acquired resistance cases within the global context of XDR-TB, has not been unequivocally documented (13). The high number of negative sputum samples (24.0%) among the 199 patients with GeneXpert MTB/rifampicin-resistant TB means these samples proved to be negative by culture despite being GeneXpert positive from various clinics and hospitals. This disparity may be attributed to a probable lack of compliance in GeneXpert testing from most facilities at the local level or poor sample collection and shipment. Only 1% of the isolates were XDR, which signifies that XDR TB is not common in the region. This phenomenon agrees with the study of Trisakul et al., (13), which stated that although phenotypic DSTs are routinely employed in laboratory analyses, the challenge of accruing an adequate number of XDR samples for such testing is exacerbated by the scarcity of cases. Consequently, the procurement of a comprehensive inventory of XDR phenotype strains for the investigation of resistance to critical anti-TB drugs presents a significant challenge.

The DST of the two isolates selected for whole-genome sequencing (WGS) revealed that the first isolate was resistant to both the first- and second-line oral antitubercular drugs tested, with the exception of the second-line fluoroquinolone (moxifloxacin or levofloxacin). Despite showing sensitivity to isoniazid and failing to react to ethambutol, the second isolate was resistant to both the second-line oral fluoroquinolones and the three injectable antituberculous drugs. This discrepancy was resolved by WGS and agrees with the findings of Olawoye et al., (4), who reported that two of their isolates were XDR by WGS despite flagging them as pre-XDR and MDR by the BACTEC MGIT 960 machine.

The evolution of antibiotic resistance genes detected in the XMTB2 MAG may be linked to practices such as incomplete treatment, inappropriate dosing, inappropriate prescribing practices, and poor quality of the antibiotics used in therapy of TB. These genes confer resistance to various antibiotics, which is one of the very important concerns in TB treatment. These genes, such as *erm37* and *blaC*, encode resistance to macrolides, and cephalosporins or carbapenems, respectively. This finding aligns well with the study performed by Sandgren et al*.,* (14) who identified *erm37* as one gene that encodes resistance to macrolides through methylation of RNA on the ribosomes. This association with isoniazid and rifampicin resistance is also enhanced by the mutations in *katG* and *rpoB* genes respectively, confirming that the XMTB2 MAG belongs to a potentially XDR strain.

This finding however contrast with what was described by Hazbón et al*.,* (15) concerning *katG* mutations and designated the R463L mutation as a polymorphism not associated with isoniazid resistance, and Wang et al. (16), who referred to *katG* Arg463Leu, *gyrA* Glu21Gln, *gyrA* Ser95Thr, and *gyrA* Gly668Asp as mutations that are characteristic of specific lineages, and have been documented as natural polymorphisms that serve as valuable indicators for the evolutionary characterization of the genome.

Many distinct chromosomal changes in the XMTB2 MAG illustrate single nucleotide polymorphisms (SNPs) which include variations at one position in a DNA sequence. D516G and H526T are encoded by the *rpoB* gene and change the drug interaction site on the RNA polymerase, hence being highly associated with rifampicin resistance. This finding agrees with the study of Sandgren et al., (14), which established D516G and H526T among the most frequent mutations in the *rpoB* gene that confer resistance to rifampicin. In the *gyrA* gene, the D94N mutation alters the DNA gyrase enzyme, hence contributing to fluoroquinolone resistance (15). Rifampicin resistance has been identified as being linked to a critical region known as the rifampicin resistance determining region (RRDR), specifically within codon positions 507-533, where numerous researchers have documented the existence of both prevalent and novel mutations in the *rpoB* gene, particularly within the hotspot region and in areas outside of the hotspot but still contained within the *rpoB* gene (17).

In contrast to other pathogenic organisms, such as *Vibrio cholerae* or *Corynebacterium diphtheriae*, *M. tuberculosis* does not employ toxins or enzymes, which are conventionally recognized virulence determinants; rather, a multitude of virulence-associated genes serve to fulfil this role (18). Phagosome arrest was one of the virulent factor classes identified in the XMTB2 MAG isolate in our study. The virulence factors obtained were nucleoside diphosphate kinase and tyrosine phosphatase, which were encoded by the *ndK* and *ptpA* genes, respectively. This is in agreement with the study of Wong et al. (19) whom tested *M. tuberculosis* H37Rv (Rv2234) in a THP-1 model and discovered that *ptpA* dephosphorylates the VPS33B protein of macrophages, the key regulator of membrane phagosome fusion to lysosomes.

The *M. tuberculosis* complex is composed of seven discrete phylogenetic lineages

that are endemic to various geographical regions worldwide, thereby substantiating the hypothesis that these strain types exhibit specific adaptations to individuals with diverse genetic backgrounds (20,21). The geographical distribution and dissemination of these lineages are characterized by heterogeneity, with lineage 2 demonstrating particularly significant mobility, as indicated by recent transmission from Asia to Europe and Africa (22). The phylogenetic tree constructed via auto-MLST shows that the first genome observed with query genome GCA_014899965.1 *M. tuberculosis* 3-0124P6C4 was sourced from a human sample, specifically the lung, and it was collected by Hinduja National Hospital, India. The strain belongs to L2, which is popularly known as the Beijing lineage (23,24).

M. tuberculosis R1207 serves as a prototypical member of the R86 cluster within the Beijing lineage of *M. tuberculosis* and was procured as a clinical isolate from the Western Cape region of South Africa. This specific strain is classified as MDR, with resistance to isoniazid and rifampicin but susceptible to ethambutol (24,25). SCAID 252.0 is a clinical sample that was geographically found at Karagandy, Kazakhstan, and was submitted by the Scientific Centre for Anti-infectious Drugs. This particular strain was extracted from the sputum of a patient diagnosed with MDR-TB who was involved in the second phase of a clinical trial of the novel pharmaceutical agent FS-1. The sputum sample was obtained prior to the administration of the initial dosage (during the screening phase). Drug susceptibility assessments on the strain conducted on both solid and liquid media indicated resistance to first-line anti-TB agents as well as to certain second-line anti-TB medications. This strain underwent sequencing to investigate the phenomenon of reversion of sensitivity, which was identified during clinical trials and subsequently verified through experimentation involving guinea pigs (26).

The query strain KZN4207, is a wild-type drug-sensitive strain that belongs to the lineage 4 (4.5). KZN 4207 belongs to F15/ LAM4/KZN and was isolated from the sputum of a human sample at KwaZulu Natal Province in South Africa (25,27). The KZN R506 strain is an XDR human sample found in the KwaZulu Natal province of South Africa (24,28). HN878 is a representative of the Beijing lineage and was obtained as a clinical isolate in the USA in 1990. HN878 is considered to be hyper virulent, although it is not drug resistant but rather pan susceptible (24,25).

The XDR-MTB isolate sequenced in our study (XMTB2) is a highly virulent strain that belongs to either the Beijing lineage (L2) or the Euro-American lineage (L4). The occurrence of the Beijing lineage in Nigeria is not surprising, considering that it was discovered in Benin Republic, which shares a western border with Nigeria, and the continuous growth of Nigeria-China economic connections over the past few decades. Another study indicated a degree of association with HIV. The East Asian or Beijing lineage is well known for its increased virulence, rapid illness development, and MDR, which sets it apart from other MTBC lineages (4).

Our study has some limitations. First, the study was limited by the relatively small number of isolates that underwent sequencing, as only two XDR isolates formed the basis of this study. Of these, one relatively failed quality control, thereby limiting the anticipated information from the sequenced genomes. It is likely that a larger sample size of XDR isolates will reveal more information on genomic characteristics and determinants of resistance in XDR *M. tuberculosis*. Secondly, the study did not consider the demographic aspects of the XDR isolate especially the HIV status which could have revealed more information especially with respect to treatment.

Conclusion:

This study investigated the determinants of antibiotic resistance in XDR *M. tuberculosis* isolate, the virulence factors and the phylogenetic relationship with other *M. tuberculosis* strains. Although it was difficult to obtain an XDR isolate, the results of this study proved that the XMTB2 MAG was potentially an XDR isolate that is very virulent because of the presence of many virulence factors identified in it and its close relationship with two of the most widespread and virulent lineages of *M. tuberculosis,* the Beijing lineage (L2) and the Euro-American lineage (L4).

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Contribution of authors:

DI Conducted the laboratory work and analysed data and writing of the first draft of the manuscript. OOA conceptualized, designed and supervised the study. OOA also analysed some data and participated in writing the manuscript. DMA proof read and edited the manuscript. TTE participated in the laboratory work and writing of the manuscript. All authors approved the final copy of the manuscript.

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The dataset of this study is available from the corresponding author on reasonable request. The sequenced data in the study were successfully uploded to the NCBI SRA database with the BioProject accession number PRJNA 1256495

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Original Article **Open Access**

Prevalence of bacterial pathogens and molecular detection of metallo-β-lactamases in Gram-negative bacteria from mobile phones of healthcare workers in a Nigerian tertiary hospital

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Abstract:

Background: The increasing usage of mobile phones in healthcare settings is cause for concern because these gadgets could be used to disseminate harmful bacteria. This study aimed to detect the presence of bacterial pathogens and carriage of metallo-β-lactamase (MBL) genes in Gram-negative bacteria from mobile phones of healthcare workers (HCWs) and medical students at the Olabisi Onabanjo University Teaching Hospital, Ogun State, Nigeria.
Methodology: This cross-sectional study was conducted from February to April 2022. Mobile phones of one hundred and twenty-one HCWs and medical students were swabbed with sterile cotton swabs and inoculated in peptone water overnight, and then sub-cultured on selective media (eosin methylene blue agar, cetrimide agar, *Salmonella-Shigella* agar, and mannitol salt agar). Bacterial isolates were identified using the standard bacteriological method. Antibiotic susceptibility testing was done using the Kirby-Bauer disc diffusion method. The phenotypic presence of MBL and MBL genes was verified using the combined disc method and polymerase chain reaction, respectively.
Results: Ninety-seven bacteria isolates were identified from mobile phones of 72 participants, giving a prevalence of 59.5%, with 65 (67.0%) Gram-positive and 32 (33.0%) Gram-negative isolates. *Staphylococcus aureus* was the most frequent isolate (n=49, 50.5%) and were resistant to azithromycin (63.3%) and ceftazidime (69.4%), but sensitive (100%) to imipenem. Carbapenem resistance (resistance to imipenem and meropenem) was reported in 28.6% (2/7) of *Pseudomonas aeruginosa*, 25.0% (2/8) of *Klebsiella pneumoniae* and 33.3% (1/3) of *Klebsiella oxytoca* isolates. Coagulase-negative staphylococci exhibited the highest resistance to ceftazidime (75%), while *P. aeruginosa* exhibited the highest resistance to aztreonam and amoxicillin/clavulanic acid. The bla_{VIM} was detected in 2 of the 32 (6.3%) Gram-negative isolates.
Conclusion: Carbapenem-resistant Gram-negative bacteria isolates carrying $bla_{VIM.}$ MBL were isolated from mobile phones of HCWs, which could be a source for the spread of multidrug-resistant nosocomial infections.

Keywords: antibiotic resistance, bla_{VIM}, bacterial contaminants, mobile phones, health workers

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Prévalence des agents pathogènes bactériens et détection moléculaire des métallo-β-lactamases chez les bactéries à Gram négatif à partir des téléphones portables du personnel soignant d'un hôpital tertiaire Nigérian

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Résumé:

Contexte: L'utilisation croissante des téléphones portables dans les établissements de santé est préoccupante, car ces appareils pourraient être utilisés pour disséminer des bactéries nocives. Cette étude visait à détecter la présence de bactéries pathogènes et le portage des gènes de métallo-β-lactamase (MBL) chez des bactéries à Gram négatif à partir des téléphones portables de professionnels de santé et d'étudiants en médecine du centre hospitalier universitaire Olabisi Onabanjo, dans l'État d'Ogun, au Nigéria.
Méthodologie: Cette étude transversale a été menée de février à avril 2022. Les téléphones portables de cent vingt et un professionnels de santé et étudiants en médecine ont été prélevés avec des cotons-tiges stériles et inoculés dans de l'eau peptonée pendant une nuit, puis repiqués sur des milieux sélectifs (gélose au bleu de méthylène éosine, gélose au cétrimide, gélose *Salmonella-Shigella* et gélose au mannitol). Les isolats bactériens ont été identifiés par la méthode bactériologique standard. Français Les tests de sensibilité aux antibiotiques ont été effectués en utilisant la méthode de diffusion sur disque de Kirby-Bauer. La présence phénotypique des gènes MBL et MBL a été vérifiée en utilisant respectivement la méthode du disque combiné et la réaction en chaîne par polymérase.
Résultats: Quatre-vingt-dix-sept isolats de bactéries ont été identifiés à partir des téléphones portables de 72 participants, ce qui donne une prévalence de 59,5%, avec 65 (67,0%) isolats Gram positifs et 32 (33,0%) isolats Gram négatifs. *Staphylococcus aureus* était l'isolat le plus fréquent (n=49, 50,5%) et était résistant à l'azithromycine (63,3%) et à la céftazidime (69,4%), mais sensible (100%) à l'imipénème. Une résistance aux carbapénèmes (résistance à l'imipénème et au méropénème) a été signalée chez 28,6% (2/7) des isolats de *Pseudomonas aeruginosa*, 25,0 % (2/8) de *Klebsiella pneumoniae* et 33,3 % (1/3) de *Klebsiella oxytoca*. Les staphylocoques à coagulase négative présentaient la plus forte résistance à la ceftazidime (75 %), tandis que *P. aeruginosa* présentait la plus forte résistance à l'aztréonam et à l'amoxicilline/acide clavulanique. Le bla_{VIM} a été détecté chez 2 des 32 isolats Gram négatif (6,3%).
Conclusion: Des isolats de bactéries Gram négatif résistantes aux carbapénèmes et porteuses du bla_{VIM} ont été isolés à partir de téléphones portables de professionnels de santé, ce qui pourrait favoriser la propagation d'infections nosocomiales multirésistantes.

Mots-clés: Résistance aux antibiotiques, bla_{VIM}, contaminants bactériens, téléphones portables, agents de santé

Introduction:

Hospital-acquired infection (HAI) is recognized as a global public health issue (1), and the leading cause of poor patient care outcomes worldwide (2). In many countries, HAIs are responsible for increased length of hospital stay, mortality, morbidity, and healthcare costs (3). In hospitals, healthcare providers may acquire and disseminate infection to patients (4,5). Previous studies have shown that mobile phones used by healthcare workers are contaminated with microorganisms (6,7), that could spread from these healthcare workers' cellphones to their hands or the other way around (8).

Mobile devices have become common in healthcare facilities, resulting in a surge in the creation of software for medical applications (9). Many applications are now available to help healthcare providers with a variety of vital tasks, including access to electronic health record maintenance and data collection (10). Apart from using mobile phones in hospitals for registration and medical treatments, the device is also employed for improved information and time management, communications, care of patients as well as management, therapeutic decision-making, and medical training and education (11,12). As more cellular phones are utilized in healthcare settings, there is an increasing risk of bacterial transmission from smartphones to the hospital environment (13).

Objects with frequent hand contact such as mobile devices could act as reservoirs for pathogens, allowing them to transfer to the hands of healthcare providers before being transmitted to patients (8). These objects are being discovered to act as carriers for infectious agents including *Escherichia coli, Staphylococcus aureus, Acinetobacter,* and *Pseudomonas* species (4,14). Healthcare professionals have been reported to place little focus on frequent hand disinfection and have poor hand washing practices, which expose them and other people and their mobile phones to bacterial colonization (8). Previous studies from Africa have documented the prevalence of bacterial contaminants of mobile phones of healthcare workers (15-17). Researchers from Nigeria have also reported the presence of bacterial contaminants on the mobile phones of healthcare workers (5,12,18).

Metallo-β-lactamases (MBL) are now recognized to be among the most significant resistance mechanisms because of their ability to hydrolyze an array of β-lactam antibiotics, including carbapenems (19). MBL genes are carried on highly mobile genetic elements, allowing for quick spread (20), and are not usually inhibited by therapeutic serine-lactamase inhibitors such as clavulanic acid and

sulfones. They confer multidrug resistance (MDR) profile against a wide range of therapeutically significant β-lactams and other antimicrobial drugs (19,21).

The presence of an MBL-positive isolate in a hospital setting is not only a therapeutic issue but also a major worry for infection control and prevention. They offer considerable hazards, particularly because of their role in unsuspected spread inside healthcare facilities and their capacity to be involved in horizontal MBL gene exchange with other infectious agents in hospitals (22). MBLs have been found in a variety of Gram-negative bacilli from the family Enterobacteriaceae (23), as well as in some non-glucose-fermenters such as *Pseudomonas aeruginosa* and *Acinetobacter* species (21,24,25).

Many researchers from different countries (26,27), including Nigeria (21) have reported the presence of MBL genes in isolates recovered from patients and environments (hospital and community). Although MBL has been discovered in a range of systemic infections and environmental settings in Nigeria, there is no report of MBL genes in Gram-negative isolates from mobile phones. Therefore, this study determines the presence of metallo-beta-lactamase genes in Gram-negative isolates from mobile phones of healthcare workers and students at the Olabisi Onabanjo University Teaching Hospital, Sagamu, Ogun State, Nigeria.

Materials and method:

Study setting, design, participants, and sample collection:

This was a cross-sectional study of 121 volunteer healthcare workers in 18 departments of the Olabisi Onabanjo University Teaching Hospital, Sagamu, Ogun State, Nigeria in February 2022. Sterile swab sticks moistened with normal saline were used to swab the surface and back of each participant's mobile phone, and the swabs were immediately transported to the Pharmaceutical Microbiology Laboratory of the Hospital for microbiological analysis.

Isolation and identification of bacterial isolates from mobile phones:

The swab sticks were used to inoculate sterile peptone broth in test tubes and incubated at 37°C for 24 hours. A loopful of inoculum from the peptone broth was streaked on eosin methylene blue agar, cetrimide agar, *Salmonella-Shigella* agar, and mannitol salt agar, and incubated at 37°C for 24 hours. Distinct colonies were picked and stored in nutrient agar slant at 4°C until further use.

Bacteria were identified to species level by Gram staining and conventional biochemical test schemes that included oxidase, indole, Voges-Proskauer, methyl red, citrate, urease, triple sugar iron (TSI), hydrogen sulfide and gas production for Gram-negative enteric bacteria, and catalase, coagulase and deoxyribonuclease tests, as well as haemolysis patterns on blood agar for Gram-positive bacteria isolates (28).

Antibiotic susceptibility testing:

The antibiotic susceptibility test (AST) of each isolate was determined by the Kirby-Bauer disc diffusion technique to selected antibiotics including amoxicillin-clavulanic acid (20/10µg), cefoxitin (30µg), cephalexin (30µg), ceftazidime (30µg), cefotaxime (30µg), cefepime (30µg), aztreonam (30µg), amikacin (30 µg), azithromycin (15µg), ciprofloxacin (5µg), imipenem (10µg), and meropenem (30µg).

The surface of Mueller Hinton (MH) agar plate was inoculated with bacterial suspension from overnight culture growth standardized to 0.5 McFarland turbidity standard, using a sterile cotton swab. The antibiotic discs (Mast, UK) were aseptically placed on the inoculated agar surface with sterile forceps and allowed to diffuse in the agar medium for one hour. The culture plates were incubated at 37°C for 24 hours (29).

Zones of growth inhibition observed were measured in millimeters and isolate was classified as sensitive, intermediate, or resistant in accordance with the Clinical and Laboratory Standards Institutes (CLSI) guideline (30).

Combined disc test (CDT) for detection of MBL:

Standardized inoculum (0.5 McFarland standard) of each carbapenem-resistant Gram-negative isolate in the AST was inoculated onto a sterile MH agar plate. Two each of imipenem and meropenem antibiotic discs were placed on the surface of the agar medium. Then, 10µL of 750µg ethylene diamine tetra-acetic acid (EDTA) was added to one disc of imipenem and one disc of meropenem followed by incubation at 37°C for 18 hours. If the zone of growth inhibition of the imipenem-EDTA disc or meropenem-EDTA was greater than 7mm than that of the imipenem disc or meropenem alone, the isolate was regarded as metallo-β-lactamase positive (31).

Molecular detection of MBL genes:

DNA extraction:

The bacterial chromosomal DNA was extracted using the boiling technique as described by Diagbouga et al., (32). Briefly, a culture of tryptic soy agar (TSA) was inoculated in 2 ml Luria Bertani (LB) broth and incubated for 18-24 hours. The LB broth was centrifuged at 10,000 rpm/min for 10 minutes. Bacterial cells were suspended in 500µL phosphate buffer (100 mM, pH 7) to weaken the

cell membranes. The cells were then submerged in a boiling water bath at 100°C for 15 minutes to remove genetic materials. The DNA was precipitated using 250 µL of 100% alcohol, washed twice with 1000 µL of 70% alcohol, and resuspended in 100 µL sterile water.

***PCR assay*:**

The PCR total volume of 25µL consists of 12.5µL of 2x master mix (Inqaba, South Africa), 1µL of DNA, 1µL of forward and reverse primers (10µmol/L) (Table 1), and 9.5µL of molecular grade water. The tubes were placed into a thermal cycler instrument (Applied Biosystems). The following thermal cycling conditions were employed to detect *bla*$_{IMP,}$ *bla*$_{NDM,}$ and *bla*$_{VIM}$; initial denaturation for 5 minutes at 94°C, was followed by 30 cycles of denaturation at 94°C for 30 seconds and annealing at 52°C for *bla*$_{IMP}$, 60°C for *bla*$_{NDM}$ and 55°C for *bla*$_{VIM}$ for 30 seconds and extension at 72°C for 30 seconds, followed by a 5-minute extension at 72°C (33).

The PCR products were stained with ethidium bromide (0.5µg/ml) and electrophoresed on 1.5% agarose gel alongside a DNA ladder (100 bp or 1 kb plus ladder) and viewed under UV light in the gel documentation system.

Statistical analysis:

For data analysis, the Statistical Package for Social Sciences version 22.0 (Chicago, USA) was used. Categorical variables were presented as percentages and frequencies in the study.

Ethical approval:

The study protocol was approved by the Human Research Ethics Committees of Olabisi Onabanjo University Teaching Hospital, Sagamu, Ogun state (OOUTH/ HREC/486/2022AP).

Results

Distribution of samples among health workers:

Table 2 presents the distribution of the 121 study participants across various departments/units of the hospital and their respective gender demographics. The frequency of the female participants is 62.8% (n=76) while that of the male participants is 37.2% (n=45). The medical records department had the highest number of participants (n=17 with 11 females and 6 males), followed by social works, general wards, and accident and emergency units, with 12 participants each (Table 3).

The highest number of samples was obtained from mobile phones of other health workers (67.0%, n=81), while nurses contributed the second highest at 13.2% (n=16).

Table1: List of primers used for the detection of metallo-β-lactamase genes

Primer	Sequence (5' to 3')	Gene	Amplicon size (bp)	Reference
IMP-F	GGAATAGAGTGGCTTAAYTCTC	*bla*$_{IMP}$	188	Ellington et al., (33)
IMP-R	CCAAACYACTASGTTATCT			
VIM-F	GATGGTGTTTGGTCGCATA	*bla*$_{VIM}$	390	Ellington et al., (33)
VIM-R	CGAATGCGCAGCACCAG			
NDM-F	GGGCAGTCGCTTCCAAGGT	*bla*$_{NDM}$	475	Deshpande et al., (34)
NDM-R	GTAGTGCTCAGTGTCGGCAT			

Table 2: Distribution of participants with respect to gender demographics across hospital departments/units

Department/unit	Gender		Total (%)
	Male (%)	Female (%)	
Social Works	6	6	12
Medical Laboratory Science	4	7	11
Surgery	4	6	10
Microbiology	3	5	8
Paediatric Infectious Disease	3	1	4
Laundry	5	3	8
Parasitology	2	3	5
Male Medical Ward	3	2	5
Female Medical Ward	-	1	1
Tailoring	-	4	4
Medical Records	6	11	17
Assessment Department	1	1	2
General Outpatient Department	-	5	5
Orthopaedics and Trauma	-	1	1
Pharmacy	-	2	2
Cash unit	-	2	2
General Ward	5	7	12
Accident and Emergency	3	9	12
Total	**45 (37.2)**	**76(62.8)**	**121 (100.0)**

Table 3: Distribution of participants with respect to categories of healthcare works across hospital departments/units

Department/unit	Categories of healthcare workers					Total (%)
	Doctor	Nurse	Pharmacists/ technicians	Other health workers	Students	
Social Works	-	-	-	11	1	12
Medical Laboratory Science	5	-	-	6	-	11
Surgery	-	8	-	2	-	10
Microbiology	-	-	-	8	-	8
Paediatric Infectious Disease	-	-	-	4	-	4
Laundry	-	-	-	8	-	8
Parasitology	-	-	-	5	-	5
Male Medical ward	-	2	-	1	2	5
Female Medical ward	-	1	-	-	-	1
Tailoring	-	-	-	4	-	4
Medical Records	-	-	-	12	5	17
Assessment Department	-	-	-	2	-	2
General Outpatient Department	-	2	2	1	-	5
Orthopaedics and Trauma	-	-	-	1	-	1
Pharmacy	-	-	2		-	2
Cash unit	-	-	-	2	-	2
General Ward	-	-	-	8	4	12
Accident and Emergency	3	3	-	6	-	12
Total	**8 (6.6)**	**16 (13.2)**	**4 (3.3)**	**81 (66.9)**	**12 (9.9)**	**121 (100.0)**

Table 4: Frequency of bacterial isolates from mobile phones of healthcare workers

Bacterial species	No of bacterial isolates	Percentage
Staphylococcus aureus	49	50.5
Coagulase negative staphylococci	16	16.5
Escherichia coli	14	14.4
Pseudomonas aeruginosa	7	7.2
Klebsiella pneumoniae	8	8.3
Klebsiella oxytoca	3	3.1
Total	**97**	**100.0**

Isolation/identification of bacterial isolates:

Swab samples were positive for bacterial growth from mobile phones of 72 (59.5%) participants. Ninety-seven bacteria were isolated from these, including 65 (67.0%) Gram-positive isolates and 32 (33.0%) Gram-negative isolates.

Staphylococcus aureus was the most frequent isolate (n=49, 50.5%) followed by coagulase-negative staphylococci (n=16, 16.5%). Others include *E. coli* (n=14, 14.4%), *K. pneumoniae* (n=8, 8.3%), *P. aeruginosa* (n=7, 7.2%) and *K. oxytoca* (n=3, 3.1%) (Table 4).

Antibiotic resistance profiles of bacterial isolates:

Table 5 shows the antibiotic resistance profiles of bacterial isolates obtained from the mobile phones of the healthcare workers and medical students. *S. aureus* was resistant to both azithromycin (63.3%) and ceftazidime (69.4%) but sensitive to imipenem.

Carbapenem resistance (resistance to imipenem and meropenem) was observed in 28.6% (2/7) of *P. aeruginosa*, 25.0% (2/8) of *K. pneumoniae* and 33.3% (1/3) of *K. oxytoca*. *E. coli* species were sensitive to meropenem, ceftazidime, and cefepime. Coagulase-negative staphylococci isolates exhibited the highest resistance to ceftazidime (75%), while *P. aeruginosa* exhibited the highest resistance to aztreonam and amoxicillin/clavulanic acid.

The antibiotic resistance profiles of the multidrug-resistant bacterial isolates from the mobile phones of the healthcare workers and students are displayed in Table 6. Each isolate displays a unique resistance profile, indicating varying levels of multidrug resistance. The frequency of MDR was significantly higher among Gram-negative bacterial isolates (19/32, 59.4%) than among Gram-positive bacterial isolates (24/65, 36.9%) (OR=2.5, 95%CI= 1.1-5.9, p=0.05).

Table 5: Antibiotic resistance of bacterial isolates from mobile phones of healthcare workers and students

Antibiotics	***S. aureus* (n=49)**	**CoNS (n=16)**	***E. coli* (n=14)**	***P. aeruginosa* (n=7)**	***K. pneumoniae* (n=8)**	***K. oxytoca* (n=3)**
Imipenem	-	1 (6.3)	-	2 (28.6)	2 (25.0)	1 (33.3)
Cefepime	14 (28.6)	4 (25.0)	-	2 (28.6)	3 (37.5)	1 (33.3)
Augmentin	5 (10.2)	3 (18.8)	9 (64.3)	6 (85.7)	4 (50.0)	2 (66.7)
Amikacin	1 (2.1)	3 (18.8)	2 (14.3)	3 (42.9)	2 (25.0)	1 (33.3)
Azithromycin	31 (63.3)	10 (62.5)	ND	ND	ND	ND
Meropenem	3 (6.1)	1 (6.3)	-	2 (28.6)	2 (25.0)	1 (33.3)
Ceftazidime	34 (69.4)	12 (75.0)	-	3 (42.9)	1 (12.5)	-
Cefotaxime	8 (16.3)	4 (25.0)	3 (21.4)	2 (28.6)	4 (50.0)	2 (66.7)
Ciprofloxacin	7 (14.3)	6 (37.5)	4 (28.6)	4 (57.1)	5 (62.5)	1 (33.3)
Cefoxitin	10 (20.4)	4 (25.0)	10 (71.4)	6 (85.7)	7 (87.5)	3 (100.0)
Cephalexin	26 (53.1)	12 (75.0)	13 (92.9)	7 (100.0)	7 (87.5)	3 (100.0)
Aztreonam	ND	ND	4 (28.6)	4 (57.1)	4 (50.0)	1 (33.3)

ND- Not determined; CoNS-Coagulase-negative staphylococci

Phenotypic and molecular detection of MBL genes:

The result of phenotypic and molecular detection of MBL genes in 5 carbapenem-resistant Gram-negative bacterial isolates is shown in Table 7. Among the Gram-negative bacterial isolates, MBL enzyme was detected in 15.6% (5/32). The *bla*$_{VIM}$ MBL gene was detected in 6.3% (2/32) (Fig 1), while *bla*$_{IMP}$ and *bla*$_{NDM}$ were not detected in any isolate.

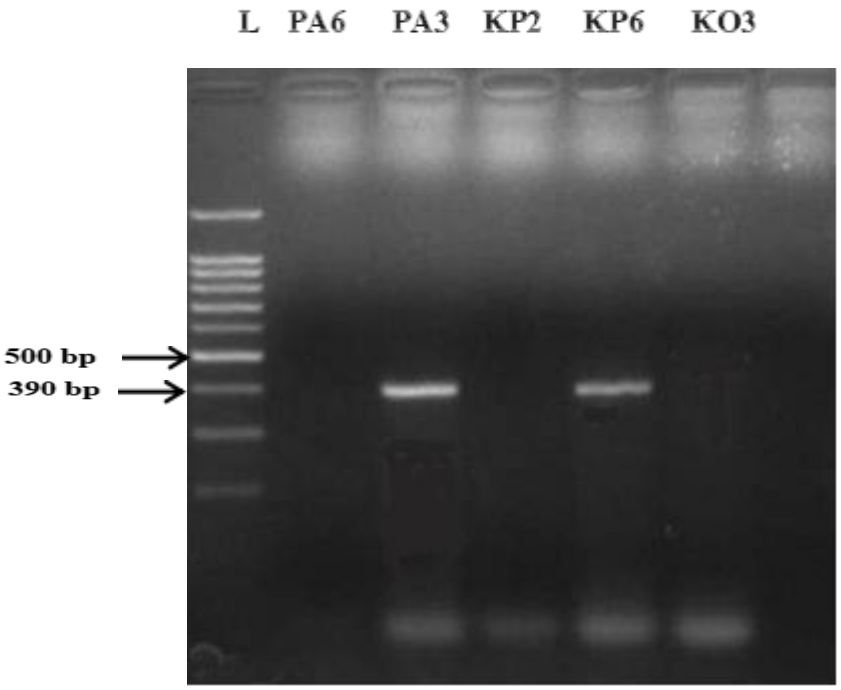
Table 6: Multidrug resistance profiles of bacterial isolates from mobile phones of healthcare workers

Category	**Isolate types**	**No of isolates**	**No of MDR isolates**	**% MDR isolates**
Gram-positive	*Staphylococcus aureus*	49	18	36.7
	Coagulase-negative staphylococci	16	6	37.5
Subtotal		**65**	**24**	**36.9**
Gram-negative	*Pseudomonas aeruginosa*	7	6	85.7
	Escherichia coli	14	6	42.9
	Klebsiella oxytoca	3	2	66.7
	Klebsiella pneumoniae	8	5	62.5
Subtotal		**32**	**19**	**59.4**
Overall Total		**97**	**43**	**44.3**

Table 7: Phenotypic and molecular detection of MBLs in carbapenem-resistant Gram-negative isolates

Isolates	**Phenotypic MBL (zone of inhibition in mm)**				**MBL genes**		
	IMP	**IMP+EDTA**	**MEM**	**MEM+EDTA**	***bla*$_{IMP}$**	***bla*$_{VIM}$**	***bla*$_{NDM}$**
P. aeruginosa (PA3)	10	32	8	28	-	+	-
P. aeruginosa (PA6)	0	29	0	26	-	-	-
K. pneumoniae (KP2)	8	32	0	28	-	-	-
K. pneumoniae (KP6)	8	30	0	28	-	+	-
K. oxytoca (KO3)	0	28	0	27	-	-	-

IMP-imipenem; MEM-meropenem; EDTA: Ethylene diamine tetra-acetic acid; MBL-metallo-β-lactamase



L = 100 bp ladder; PA3 = *P. aeruginosa*; PA6 = P. *aeruginosa*; KP2 = *K. pneumoniae*; KP6 = *K. pneumoniae*; KO3 = *K. oxytoca*

Fig 1: Amplification of *bla*$_{VIM}$ genes in Gram-negative bacteria

Discussion:

Mobile phones of healthcare workers are potential sources of nosocomial infections as they are extremely susceptible to pathogenic bacteria in healthcare settings (5, 36). Contaminated hands of HCWs play a significant role in the spread of HAIs (15). The prevalence of bacterial contaminants on mobile phones was 59.5% in this study, which is lower than 80.0% and 94.6% reported in other Nigerian studies (12,37), but comparable to the study of Akinyemi et al., (18) who reported a prevalence of 62.0%. In a study conducted in Ghana, Olu-Taiwo et al., (17) reported an overall prevalence of 83.3% of bacterial contaminants on mobile phones.

This study identified *S. aureus,* coagulase-negative staphylococci*, E. coli, P. aeruginosa, K. pneumoniae,* and *K. oxytoca* in swab samples from mobile phones. Other studies have reported the presence of these bacterial pathogens including *Enterobacter* species, *Acinetobacter baumannii, Bacillus* species, *Streptococcus* species and *Proteus* species (12,16,17). *S. aureus* was the most frequent isolate (50.5%), followed by coagulase-negative staphylococci (16.5%) in our study, similar to other studies in Nigeria that have reported *S. aureus* to be the most frequent isolate (5,37), but differ from Nwankwo et al., (12) who reported *S. epidermidis* to be the most frequent bacterial isolate.

The most important health challenge in treating patients is pathogen resistance to multiple antibiotics (38). In this study, 36.9% and 59.4% of Gram-positive and Gram-negative bacteria respectively, were resistant to three or more classes of antibiotics. This demonstrates that unless essential norms and controls for phone usage and disinfection in healthcare settings are enforced, mobile devices will worsen the burden of nosocomial infections caused by antimicrobial resistant pathogens (5). The overall prevalence of multi drug resistance among the isolates in this study was 43.3%. This contrasts the study by Bodena et al., (15), who reported higher levels of multidrug resistance. In our study, *S. aureus* and CoNS were susceptible to imipenem and amikacin but resistant to ceftazidime, cephalexin, and azithromycin. *P. aeruginosa* and *K. pneumoniae* were resistant to amoxicillin-clavulanate, ciprofloxacin, cefoxitin, cephalexin, and aztreonam. Nwankwo et al., (12) also reported a high level of antibiotic resistance of *P. aeruginosa* isolates from the mobile phones of health workers.

Among the several mechanisms of carbapenem resistance, the production of MBL is of great significance due to its strong carbapenem hydrolyzing capacity, ability to hydrolyze all beta-lactam antibiotics except aztreonam, and resistance to beta-lactamase inhibitors (39). Although the carriage of carbapenem-resistance gene among Gram-negative isolates was low (6.3%) in our study, it is of great concern considering that it is a 'last-

resort' antibiotic and the likelihood of transmission from healthcare personnel to susceptible patients in the hospital and vulnerable people in the community. This is because MBL-encoding genes are typically found on mobile genetic elements that also carry genes that encode resistance to other antibiotics, leading to multidrug resistance (40).

There have been reports of *bla*$_{VIM}$ and *bla*$_{NDM}$ in Gram-negative bacteria isolated from patients' samples in Nigeria (21), and from environmental contexts (41), but no reports of *bla*$_{VIM}$ and *bla*$_{NDM}$ in mobile phone isolates. To the best of our knowledge, this is the first report of *bla*$_{VIM}$ in Gram-negative bacterial isolates recovered from mobile phones of Nigerian healthcare personnel.

Conclusion:

Our findings revealed the presence of the carbapenem-resistant gene (*bla*$_{VIM}$) in Gram-negative isolates on healthcare workers' mobile phones and the extensive contamination of these phones with various bacterial pathogens linked to nosocomial infections in healthcare facilities. This study emphasizes the significance of mobile phones as a reservoir for bacteria containing clinically significant antibiotic-resistant genes.

To mitigate the risk of cross-contamination and the spread of resistant pathogens, implementing routine cleaning protocols can help ensure that these devices do not contribute to healthcare-associated infections.

Contributions of authors:

FOB and FTI were involved in collection and processing of data; FOB, AOM and OOB were involved in analysis of data and interpretation of results; FOB, FTI, AOM, and OOB were involved in manuscript writing. All authors approved the final manuscript submitted for publication.

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Conflict of interest

No conflict of interest is declared

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Original Article **Open Access**

Environmental contamination rates by extended-spectrum β-lactamase producing *Acinetobacter baumannii* in selected hospitals in southwest Nigeria

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Abstract:

Background: *Acinetobacter baumannii* poses significant threat in healthcare facilities due to its innate ability to persist on harsh environmental surfaces for extended periods and its common resistance to multiple antibiotics, through mechanisms such as extended-spectrum β-lactamase (ESBL) production. Healthcare-associated (HAI) *A. baumannii* infections are a growing concern in low-and-middle-income countries (LMICs), with environmental surfaces serving as important reservoirs for transmission. However, there is limited information on *A. baumannii* contamination rates of environmental surfaces in healthcare facilities. This study determined the contamination rates and antimicrobial susceptibility profiles of *A. baumannii* from high-touch surfaces in selected hospitals in Akoko area of Ondo State, southwest Nigeria.
Methodology: A total of 204 environmental samples were collected by swabbing selected surfaces (48 door-knobs, 120 bedframes, and 36 handwashing basins) of 4 selected hospitals (hospital A, B, C and D). Samples were cultured on MacConkey agar plates and incubated aerobically at 37°C for 24 hours. Colonies isolated from the samples were identified as *A. baumannii* based on cultural morphology and conventional biochemical test scheme. The susceptibility of the isolates to selected antibiotics was determined by the Kirby Bauer disc diffusion method in line with the Clinical and Laboratory Standards Institute (CLSI) guidelines, and ESBL production in selected isolates was detected by the double disk synergy test (DDST). Multi-drug resistance (MDR) in an isolate was defined as resistance to three or more antibiotic categories. Differences in contamination rates between the hospital sites and touch surfaces were compared using Pearson's Chi squared test, with $p<0.05$ considered as statistical significance.
Results: *Acinetobacter baumannii* was isolated from 13.7% (28/204) of the environmental samples, with the highest contamination rate recorded for samples collected from handwashing basins (33.3%, 12/36) while bed frames had the lowest *A. baumannii* contamination rate (4.2%, 5/120) ($x^2=24.372$, $p<0.0001$). Hospital site D had the highest *A. baumannii* contamination rate of 19.5% (10/51) while hospital site A had the lowest rate of 7.8% (4/51) ($x^2=4.305$, $p=0.2303$). The *A. baumannii* isolates were commonly resistant to the third-generation cephalosporins (39.3% to ceftazidime, 32.1% to ceftriaxone and 28.5% to cefotaxime), with 7 of 11 (63.6%) cephalosporin-resistant isolates positive for ESBL production. Four (14.3%) *A. baumannii* isolates were MDR. `
Conclusion: The isolation ESBL-producing and MDR *A. baumannii* from environmental surfaces in the selected hospitals in this study underscores the importance of stringent infection control practices and environmental surveillance in resource-limited settings to ensure patient safety.

Keywords: *Acinetobacter baumannii*, ESBL, MDR, high-touch surface, hospital, contamination

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Taux de contamination environnementale par *Acinetobacter baumannii*, producteur de β-lactamases à spectre étendu, dans certains hôpitaux du sud-ouest du Nigéria

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Résumé:

Contexte: *Acinetobacter baumannii* représente une menace importante dans les établissements de santé en raison de sa capacité innée à persister sur des surfaces environnementales difficiles pendant de longues périodes et de sa résistance fréquente à de multiples antibiotiques, grâce à des mécanismes tels que la production de β-lactamases à spectre étendu (BLSE). Les infections nosocomiales à *A. baumannii* constituent une préoccupation croissante dans les pays à revenu faible ou intermédiaire (PRFI), les surfaces environnementales constituant d'importants réservoirs de transmission. Cependant, les informations sur les taux de contamination par A. baumannii des surfaces environnementales des établissements de santé sont limitées. Cette étude a déterminé les taux de contamination et les profils de sensibilité aux antimicrobiens d'*A. baumannii* sur des surfaces fréquemment touchées dans des hôpitaux sélectionnés de la région d'Akoko, dans l'État d'Ondo, au sud-ouest du Nigéria.
Méthodologie: Au total, 204 échantillons environnementaux ont été prélevés par écouvillonnage de surfaces sélectionnées (48 poignées de porte, 120 cadres de lit et 36 lavabos) dans quatre hôpitaux sélectionnés (hôpitaux A, B, C et D). Les échantillons ont été cultivés sur gélose MacConkey et incubés en aérobiose à 37°C pendant 24 heures. Les colonies isolées des échantillons ont été identifiées comme appartenant à *A. baumannii* sur la base de leur morphologie et d'un protocole de tests biochimiques conventionnels. La sensibilité des isolats aux antibiotiques sélectionnés a été déterminée par la méthode de diffusion sur disque de Kirby Bauer, conformément aux directives de l'Institut des normes cliniques et de laboratoire (CLSI), et la production de BLSE dans les isolats sélectionnés a été détectée par le test de synergie à double disque (DDST). Français La multirésistance aux antibiotiques (MDR) dans un isolat a été définie comme une résistance à trois catégories d'antibiotiques ou plus. Les différences de taux de contamination entre les sites hospitaliers et les surfaces de contact ont été comparées à l'aide du test du Chi carré de Pearson, avec $p<0,05$ considéré comme statistiquement significatif.
Résultats: *Acinetobacter baumannii* a été isolé de 13,7% (28/204) des échantillons environnementaux, le taux de contamination le plus élevé étant enregistré pour les échantillons prélevés dans les lavabos (33,3%, 12/36) tandis que les cadres de lit présentaient le taux de contamination par *A. baumannii* le plus faible (4,2%, 5/120) ($x^2=24,372$, $p<0,0001$). Le site hospitalier D présentait le taux de contamination par *A. baumannii* le plus élevé, soit 19,5% (10/51), tandis que le site hospitalier A présentait le taux le plus faible, soit 7,8% (4/51) ($x^2=4,305$, $p=0,2303$). Les isolats d'*A. baumannii* étaient généralement résistants aux céphalosporines de troisième génération (39,3% à la céftazidime, 32,1% à la céftriaxone et 28,5% à la céfotaxime), avec 7 des 11 isolats résistants aux céphalosporines (63,6%) positifs à la production d'ESBL. Quatre isolats d'*A. baumannii* (14,3%) étaient multirésistants.
Conclusion: L'isolement d'*A. baumannii* producteurs de BLSE et multirésistants à partir de surfaces environnementales dans les hôpitaux sélectionnés dans cette étude souligne l'importance de pratiques rigoureuses de contrôle des infections et de surveillance environnementale dans les environnements à ressources limitées pour garantir la sécurité des patients.

Mots-clés: *Acinetobacter baumannii*, BLSE, multirésistant, surface fréquemment touchée, hôpital, contamination

Introduction:

Acinetobacter baumannii is an aerobic Gram-negative coccobacillus opportunistic bacterium, known to cause a wide range of infectious diseases including meningitis, urinary tract infection, bacteraemia, wound infections and pneumonia in susceptible host (1). It is a common cause of healthcare-associated infections (HAIs) with high mortality rate (2). Prolonged hospital stays, weakened immunity, overuse of broad-spectrum antibiotics, comorbidities such as diabetes mellitus and burns, invasive procedures, and presence of indwelling catheters or mechanical ventilators are common risk factors for acquiring *A. baumannii* infections (3,4).

The propensity of *A. baumannii* to develop resistance to multiple antibiotics further complicates the challenges posed by *A. bau mannii* infection. Hard-to-treat multidrug-resistant strains of *A. baumannii* have been widely reported globally prompting the World Health Organisation to designate carbapenem-resistant *A. baumannii* as one of the most critical priority pathogens for which new therapeutics are urgently needed (5).

Acinetobacter baumannii infections are mostly HAIs with community-acquired infections (CAIs) not commonly reported (6). Its ability to survive harsh environmental conditions in hospital environments and persist on surfaces for extended periods makes it a common HAI pathogen (7). Thus, environmental contamination plays a significant role in the epidemiology of healthcare-acquired *A. baumannii* infections.

Healthcare-associated infections generally constitute a significant public health challenge causing prolonged hospital stays and sometimes death (8). HAIs are major health challenge in low-and-middle-income countries (LMICs), with an estimated prevalence of 19.2% compared to 12% in high-income countries (5). The less stringent infection control measures in LMICs due to the limited resources are largely responsible for the higher prevalence of HAIs in LMICs compared to high-income countries.

The hospital environment can act as reservoir of HAI pathogens such as *A. baumannii*. Contact with contaminated surfaces is a major means of acquiring healthcare-associated *A. baumannii* infection therefore it is important to identify potential hospital surfaces that can serve as reservoirs for *A. bau-*

mannii. This is crucial in preventing the exposure of susceptible patients to *A. baumannii* infection.

Although the hospital environment has been variously implicated as the reservoir for MDR HAI pathogens, there is dearth of information on the persistence of pathogenic microorganisms in the hospitals in sub-Saharan Africa. This study investigated the contamination of surfaces by *A. baumannii* in clinical areas of selected hospitals, southwest Nigeria.

Materials and method:

Study design:

This is an environmental survey of surfaces of acute medical facilities in southwest Nigeria. Surfaces in selected publicly (sites A, B and D) and privately (site C) owned acute medical facilities were sampled for contamination with *A. baumannii*. Surfaces sampled include high-touch surfaces such as bed frames, doorknobs and hand-washing basins in the clinical areas of the selected healthcare facilities. Sampling of surfaces was done three times on different occasions.

Sampling of hospital surfaces:

Samples were collected from the selected surfaces with sterile swabs, which were labelled and moistened with sterile distilled water under aseptic conditions before use. Selected surfaces were then severally swabbed at different spots to ensure adequate coverage. Sampling of selected surfaces was done in triplicates at each sampling using different swabs. The swabs were transported on ice parks to the laboratory for enrichment and isolation of *A. baumannii.*

Isolation of *A. baumannii*:

The cotton buds from the swabs were aseptically cut and transferred into 5 ml sterile distilled water which was vigorously vortexed at maximum speed for 1 minute. Samples from the surfaces were enriched by transferring 1ml of the sample suspension into 9ml of sterile trypticase soy broth (Oxoid, UK). The inoculated broths were incubated for 24 hours at 37^{o}C, after which 200µL was inoculated onto Blood and Mac- Conkey agar plates (LAB M, UK) prepared according to the manufacturer's instructions. The inoculated plates were incubated for 24 hours at 37^{o}C.

Colonies showing cultural characteristics similar to *Acinetobacter* on MacConkey agar were sub-cultured onto fresh agar to obtain pure culture of selected colonies. The pure cultures of selected isolates were identified as *A. baumannii* based on their colony morphology, Gram staining reaction and biochemical tests including catalase, oxidase, motility, citrate, indole, methyl red, urease, and oxidation-fermentation (OF) reaction (9).

Antibiotic susceptibility test:

The susceptibility of *A. baumannii* isolates to commonly used antibiotics was determined by the Kirby-Bauer disc diffusion test. Briefly, standardised inoculum (0.5 McFarland standard) was prepared from overnight cultures of the isolates and inoculated on freshly prepared Mueller–Hinton (MH) agar (LAB M, UK) using sterile swabs. Antibiotic discs (Abteck, UK) were aseptically placed on the inoculated MH agar after 30 minutes. Antibiotics used include ceftazidime (30µg), ceftriaxone (30µg), cefotaxime (30µg), gentamicin (10 µg), meropenem (10µg), ciprofloxacin (5µg) and amikacin (30µg).

All the plates were subsequently incubated at 37°C for 18 hours after which the diameter of the zones of inhibition observed around each disc was measured and the results were interpreted as being resistant, intermediate or susceptible to the antibiotics using the CLSI guidelines (10). Isolates resistant to antibiotics belonging to three or more classes of antibiotics were classified as multidrug resistant (MDR) isolates.

Detection of extended spectrum β-lactamase (ESBL) production:

The double disc synergy test (DDST), as described by Kaur et al., (11) with slight modification, was used to phenotypically confirm ESBL production in A. *baumannii* isolates that were resistant to third-generation cephalosporins in the antibiotic susceptibility test. Briefly, cefotaxime (30µg) and ceftazidime (30µg) discs were placed at 1.5cm from the centre of amoxicillin (20 µg)/clavulanic acid (10µg) disc placed at the centre of a freshly prepared MH agar plate that have been seeded with standardised (0.5 McFarland standard) inoculum of selected *A. baumannii* isolate. The MH plate was incubated at 37^{o}C for 24 hours and isolate showing enhanced zone of inhibition around the cephalosporin discs towards the amoxicillin-clavulanic acid disc was interpreted as being positive for ESBL production.

Data analysis:

Descriptive statistic was used to describe the number of samples contaminated with *A. baumannii* and number of isolates resistant to tested antibiotics as percentages. Differences in the contamination rates between the different hospitals was compared by Pearson's Chi squared test with $p<0.05$ considered to be statistically significant.

Results:

A total of 204 environmental samples were collected from different surfaces in the 4 selected hospital sites; 12 from doorknobs, 9 from handwash basins and 30 bedframe

samples. The frequency of samples with *A. baumannii* contamination is shown in Table 1. A total of 28 isolates were identified as *A. baumannii* based on their cultural morphology, Gram staining reaction and biochemical characteristics.

Samples collected from hospital site D had the highest *A. baumannii* contamination rate (19.5%, 10/51) while hospital site A had the lowest *A. baumannii* contamination rate of 7.8% (4/51) (x^2=4.305, p=0.2303). Overall, handwashing basins had the highest *A. baumannii* contamination rate of 33.3% (12/36) while bedframes had the lowest contamination rate of 4.2% (5/120) (x^2= 24.372, p<0.0001).

The susceptibility of *A. baumannii* isolates to selected antibiotics is shown in Table 2. Most of the *A. baumannii* isolates were susceptible to amikacin (89.3%) and ciprofloxacin (85.7%) while ceftazidime (at 60.7% susceptible) was the least active against the isolates. Four (14.3%) of the 28 isolates were MDR isolates based on resistance to antibiotics in three or more classes. Seven (64.0%) of 11 *A. baumannii* isolates resistant to third-generation cephalosporins (ceftazidime) evaluated for ESBL production by DDST were confirmed to be ESBL producers (Fig 1).

Table 1: Frequency distribution of environmental samples contaminated with *Acinetobacter baumannii* in selected hospital sites

Surfaces sampled	**Hospital sampling site**				**Total (%)**
	Site A (%)	**Site B (%)**	**Site C (%)**	**Site D (%)**	
Doorknobs (n=12 each)	2 (16.7)	3 (25.0)	2 (16.7)	4 (33.3)	11 (22.9)
Wash hand basin (n=9 each)	2 (22.2)	4 (44.4)	3 (33.3)	3 (33.3)	12 (33.3)
Bed frames (n=30 each)	0	2 (6.7)	0	3 (10.0)	5 (4.2)
Total (n=51 each)	**4 (7.8)**	**9 (17.6)**	**5 (9.8)**	**10 (19.5)**	**28 (13.7)**

Table 2: Frequency of resistance in *Acinetobacter baumannii* isolated from hospital environment to selected antibiotics

Antibiotics	**Hospital sampling site**				**Total (%) (n=28)**
	Site A (%) (n=4)	**Site B (%) (n=9)**	**Site C (%) (n=5)**	**Site D (%) (n=10)**	
Ceftazidime (30µg)	2 (50.0)	3 (33.3)	2 (40.0)	4 (40.0)	**11 (39.3)**
Gentamicin (10µg)	1 (25.0)	2 (22.2)	1 (20.0)	2 (20.0)	**6 (21.4)**
Meropenem (10µg)	1 (25.0)	3 (33.3)	2 (40.0)	3 (30.0)	**9 (32.1)**
Ceftriaxone (30µg)	0	3 (33.3)	2 (40.0)	4 (40.0)	**9 (32.1)**
Cefotaxime (30µg)	1 (25.0)	3 (22.2)	2 (40.0)	3 (30.0)	**8 (28.5)**
Ciprofloxacin (5µg)	0	2 (22.2)	0	2 (20.0)	**4 (14.3)**
Amikacin (30µg)	0	1 (11.1)	1 (20.0)	1 (10.0)	**3 (10.7)**

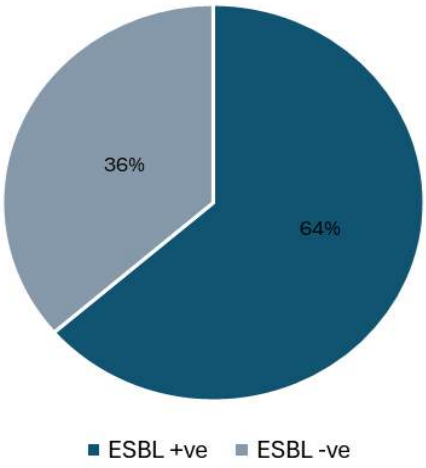

Fig 1: Frequency distribution of ESBL-producing and non-ESBL producing *Acinetobacter baumannii* isolates to third generation cephalosporins

Discussion:

Contaminated environmental surfaces have long been recognised as significant exogenous sources of a wide range of HAI pathogens, such as *A. baumannii*. The ability of *A. baumannii* to persist for extended periods on these surfaces increases its transmission potential in healthcare facilities. Non-adherence to strict infection control practices in resource-limited countries, due to various constraints, further heightens the risk of transmitting HAI pathogens from environmental reservoirs. Multi--drug resistance in *A. baumannii* complicates the management of HAI infections caused by this organism, as therapeutic options are limited. This presents a substantial challenge in resource-limited countries because of limited access to antimicrobial agents. This study determined the prevalence of ESBL-producing MDR *A. baumannii* on the surfaces of selected healthcare facilities in resource-limited settings to assess the risks of acquiring *A. baumannii* HAI in the studied area.

Acinetobacter baumannii was isolated from the selected surfaces in all the medical facilities in this study indicating common *A. baumannii* surface contamination in the medical facilities in the studied region. *A. baumannii* has been previously isolated from environmental surfaces in medical facilities in Nigeria (12,13). The potential for *A. baumannii* to persist on environmental surfaces may be attributed to the ability of *A. baumannii* to survive the ambient conditions in the region. A wide range of pathogenic organisms are known to persist for extended periods at ambient conditions in tropical regions. For instance, Akinbobola et al., (14) reported that common HAI pathogens including A. *baumannii*, *Escherichia coli* and *Pseudomonas aeruginosa* can persist on commonly used hospital fabrics for a significant period in tropical ambient conditions. Additionally, *A. baumannii* is known to survive harsh environmental conditions due to its innate ability to tolerate desiccation and form protective biofilm biomass (15,16).

Of all the surfaces sampled in the study, handwashing basins were most contaminated by *A. baumannii.* This can be attributed to the potential of moisture to enhance the survival of *A. baumannii* on harsh environmental surfaces (14). Likewise, the hands of healthcare workers are important routes for the dissemination of HAI pathogens such as *A. baumannii* in healthcare facilities (17,18). Thus, the relatively higher contamination of handwashing basins with *A. baumannii* compared to other surfaces in this study can also be attributed to the potential shedding of *A. baumannii* from contaminated hands of healthcare workers during hand washing. Handwashing basins have been previously identified as common reservoirs of *A. baumannii* infection outbreaks in healthcare facilities (19,20). The frequent contamination of doorknobs also a high touch surface with *A. baumannii* in the healthcare facilities studied further reveal the potential for *A. baumannii* dissemination through contaminated hands of healthcare workers and patients.

The widespread resistance to antibiotics in *A. baumannii* isolates is a major challenge in the treatment of *A. baumannii* infections. *A. baumannii* strains isolated from surfaces in this study were resistant to different antibiotics tested. The common resistance to third generation cephalosporin antibiotics such as ceftazidime, cefotaxime and ceftriaxone observed in this study can be attributed to widespread ESBL production in Gram-negative bacteria like *A. baumannii* isolates (21). Most of the *A. baumannii* isolates (~64%) resistant to third-generation cephalosporins in this study produced ESBL further indicating the widespread occurrence of ESBL in *A. baumannii* isolates. This has significant implica-

tions for the treatment of *A. baumannii* infections in LMICs like Nigeria due to the limited availability of relatively expensive alternatives.

Conclusion:

In conclusion, results from this study demonstrated common contamination of hospital surfaces with MDR *A. baumannii* indicating a potential risk of transmission to susceptible patients. Common resistance to multiple antibiotics in the *A. baumannii* isolates further complicates the threat posed by the contamination of sampled surfaces in the selected healthcare facilities. This underscores the need to develop robust and effective infection control measures to prevent contamination of surfaces with pathogenic organisms. A critical component of such infection measures should include cost-effective continuous surveillance of environmental contamination with pathogenic bacterial species and adherence to established infection control measures to prevent the widespread dissemination of pathogens in hospital environments.

This study provides important information on the contamination of surfaces in sampled medical facilities with antibiotics resistant *A. baumannii* strains. However, the study is only limited to four medica facilities and the identification of *A. baumannii* strains based on cultural and biochemical characterristics could have underestimated the level of contamination, Therefore, further studies involving more medical facilities in the studied area employing molecular based identification will help in further quantifying the potential risk of *A. baumannii* contamination of surfaces in medical facilities in the studied district.

Contributions of author:

The study was solely conceptualised by the authors who conducted the study, generated the data, wrote and edited the manuscript for publication.

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Conflicts of interest

No conflict of interest is declared

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Knowledge and practices of hepatitis E virus disease prevention among pig handlers in selected local government areas of Enugu State, Nigeria

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Abstract:

Background: Hepatitis E virus (HEV) is a zoonotic pathogen with significant public health implications, particularly among pig handlers who are at increased risk due to occupational exposure. Despite the growing pig farming industry in Enugu State, Nigeria, awareness and preventive practices related to HEV remain poorly understood. This study assessed the knowledge and preventive measures of HEV among pig handlers in Enugu State, identifying key demographic factors influencing awareness and hygiene practices.
Methodology: A cross-sectional study was conducted among pig handlers in three Local Government Areas (Nsukka, Enugu South, and Aninri), representing the three senatorial zones of Enugu State, Nigeria. Random sampling was used to recruit participants, and data were collected with structured questionnaires administered by investigators between December 2023 and March 2024. Statistical analysis was done with SPSS version 27 and results included descriptive statistics and multivariable logistic regression to determine associations between sociodemographic factors and HEV knowledge and preventive practices.
Results: Of the total 228 enrolled pig handlers, only 29.8% had good knowledge of HEV while 70.2% had poor knowledge. Educational level was a strong predictor of HEV knowledge, with poor knowledge of HEV being significantly higher among participants with secondary-level education (aOR=4.1, 95% CI=1.79–9.59, p=0.005). Gender was equally associated with HEV knowledge, with good knowledge of HEV being significantly lower among females compared to males (aOR=0.39, 95% CI=0.217-0.710, p=0.001). Age also influenced knowledge, with poor knowledge of HEV being significantly higher in older participants (41–50 years) (aOR=3.20, p=0.004) compared to younger handlers (≤30 years). Conversely, good preventive practices were significantly higher among females compared to males (aOR=2.47, 95% CI=1.418-4.329, p=0.001), and among married individuals (aOR=0.39, p=0.010) and those aged 31–40 years (aOR=0.027, 95% CI=0.07-0.54, p=0.004), while poor preventive practices/measures were significantly higher among participants with no formal education (aOR= 28.75, 95% CI=5.285-156.385), p=0.001).
Conclusion: This study highlights a critical gap in HEV knowledge and preventive practices among pig handlers in Enugu State, Nigeria. While education, gender and age significantly influenced HEV knowledge levels, preventive practices were significantly higher among female gender, married individuals and those with high educational levels. These findings underscore the need for targeted health education programs, improved biosecurity measures, and enhanced surveillance to mitigate HEV transmission risks in pig farming individuals and communities. These insights contribute to global HEV research and support policy recommendations for zoonotic disease prevention in occupational settings.

Keywords: Hepatitis E virus, Pig handlers, Zoonosis, Knowledge, Practice, Enugu State, Nigeria

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Connaissances et pratiques en matière de prévention de l'hépatite E chez les porcins dans certaines zones administratives locales de l'État d'Enugu, au Nigéria

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Résumé:

Contexte: Le virus de l'hépatite E (VHE) est un agent pathogène zoonotique ayant des implications importantes pour la santé publique, en particulier chez les porcins qui présentent un risque accru en raison de leur exposition professionnelle. Malgré la croissance de l'élevage porcin dans l'État d'Enugu, au Nigéria, la sensibilisation et les pratiques préventives liées au VHE restent mal comprises. Cette étude a évalué les connaissances et les mesures préventives du VHE parmi les manutentionnaires de porcs de l'État d'Enugu, en identifiant les principaux facteurs démographiques influençant la sensibilisation et les pratiques d'hygiène.
Méthodologie: Une étude transversale a été menée auprès de manutentionnaires de porcs dans trois zones de gouvernement local (Nsukka, Enugu Sud et Aninri), représentant les trois zones sénatoriales de l'État d'Enugu, au Nigéria. Un échantillonnage d'aléatoire a été utilisé pour recruter les participants, et les données ont été collectées à l'aide de questionnaires structurés administrés par les enquêteurs entre décembre 2023 et mars 2024. L'analyse statistique a été réalisée avec SPSS version 27 et les résultats comprenaient des statistiques descriptives et une régression logistique multivariée pour déterminer les associations entre les facteurs sociodémographiques et les connaissances et les pratiques préventives du VHE.
Résultats: Sur un total de 228 manutentionnaires de porcs inscrits, seuls 29,8% avaient une bonne connaissance du VHE, tandis que 70,2% avaient une faible connaissance. Français Le niveau d'éducation était un bon prédicteur de la connaissance du VHE, la faible connaissance du VHE étant significativement plus élevée chez les participants ayant un niveau d'éducation secondaire (aOR=4,1, IC à 95%=1,79-9,59, *p*=0,005). Le sexe était également associé à la connaissance du VHE, la bonne connaissance du VHE étant significativement plus faible chez les femmes que chez les hommes (aOR=0,39, IC à 95%=0,217-0,710, *p*=0,001). L'âge a également influencé la connaissance, la faible connaissance du VHE étant significativement plus élevée chez les participants plus âgés (41-50 ans) (aOR=3,20, *p*=0,004) que chez les plus jeunes (≤ 30 ans). Français À l'inverse, les bonnes pratiques préventives étaient significativement plus élevées chez les femmes que chez les hommes (aOR=2,47, IC à 95%=1,418-4,329, *p*=0,001), et chez les personnes mariées (aOR=0,39, *p*=0,010) et celles âgées de 31 à 40 ans (aOR=0,027, IC à 95%=0,07-0,54, *p*=0,004), tandis que les mauvaises pratiques/mesures préventives étaient significativement plus élevées chez les participants sans éducation formelle (aOR=28,75, IC à 95%=5,285-156,385, *p*=0,001).
Conclusion: Cette étude met en évidence une lacune critique dans les connaissances et les pratiques préventives sur le VHE parmi les manutentionnaires de porcs dans l'État d'Enugu, au Nigéria. Alors que l'éducation, le sexe et l'âge ont significativement influencé les niveaux de connaissances sur le VHE, les pratiques préventives étaient significativement plus élevées chez les femmes, les personnes mariées et celles ayant un niveau d'éducation élevé. Ces résultats soulignent la nécessité de programmes d'éducation sanitaire ciblés, de mesures de biosécurité améliorées et d'une surveillance renforcée pour atténuer les risques de transmission du VHE chez les éleveurs porcins, tant au niveau individuel que communautaire. Ces informations contribuent à la recherche mondiale sur le VHE et étayent les recommandations politiques en matière de prévention des zoonoses en milieu professionnel.

Mots-clés: Virus de l'hépatite E, Porcins, Zoonose, Connaissances, Pratique, État d'Enugu, Nigéria

Introduction:

Hepatitis E virus (HEV) causes hepatitis E, which is an inflammation of the liver (1). Hepatitis E is estimated by the World Health Organization (WHO) to have contributed to about 44,000 fatalities in 2015, or 3.3% of all deaths from viral hepatitis (2). Severe viral hepatitis is the most common clinical sign of hepatitis E virus infection, and in certain people, it can result in severe liver failure (3). Additionally, the illness is commonly linked to mortality and extrahepatic symptoms (2,3). In Nigeria (4), HEV infection has been found to be a primary cause of acute sporadic hepatitis that is enterically transferred, particularly in the adult demographic. The primary cause of both acute and chronic liver infections and inflammation is viral hepatitis. Hepatitis A, B, C, D, E, F, and G viruses can cause acute liver illness.

The faecal-oral pathway is one way that this enteric hepatitis E virus might spread. Human HEV strains and those recovered from pigs, cows, chickens, rabbits, rats, and fish in developed nations have been found to share genetic similarities in developing nations (5). Animals are a major source of HEV infection and contribute to the increased prevalence of the disease (6). Farm- ers, animal butchers, veterinarians, and per- sons handling animal products or consuming raw or uncooked meat from deer, wild boar, and pigs infected with HEV are among the groups most at risk (4,6). Contact with pigs may increase the risk of contracting a zoonotic illness because they are the primary reservoir of zoonotic HEV and the most significant reservoir for HEV (5,6). The nucleotide sequ- ence of the first animal strain of HEV was iden- tified after it was isolated from pigs in the mid- western United States and ever since then (6), swine HEV has been found in every swine-pro- ducing nation on the planet. Swine HEV sequ- ences are classified as either genotype 3 or genotype 4.

HEV outbreaks typically occur in countries and settings with low resources such as those with poor access to water, inadequate health care, and poor sanitation and hygiene. Water that is fit for drinking is typically contaminated by flooding or the disposal of faeces during the rainy season. The most prevalent way that outbreaks occur in rural, underdeveloped areas is through a water channel that traverses contaminated soil (4,6,7).

Research has demonstrated that HEV can result in chronic illness due to the virus's clinical persistence in immunocompromised

individuals and those receiving immunosuppressive therapy (6,7). Similarly, newer finding (8) has shown that HEV can primarily infect pregnant women and cause serious and deadly infections. This study was therefore aimed to determine the knowledge and disease prevention practices of hepatitis E virus among pig handlers in Enugu State, Nigeria.

Materials and method:

Study area:

This study was conducted in three Local Government Areas (LGAs) (Nsukka, Enugu South and Aninri) from the 3 senatorial zones (Enugu North, Enugu East and Enugu West respectively) of Enugu State, Nigeria. These LGAs were chosen based on the availability of large cluster of pig farms and the willingness of the leadership (gatekeepers) of these farm clusters consenting to participate in this study.

Enugu State is comprised of 261 political wards and shares borders with several neighboring States; Benue and Kogi to the north, Ebonyi to the east and southeast, Abia to the south, and Anambra to the west. The State was named after its capital and largest city, Enugu, and spans approximately 8,161 km² in area. According to 2023 estimates, the State is home to over 5.4 million residents, with a population density of about 1,300 people per square kilometer (9). The State was known for civil service, trading and all forms of agriculture, and recently witnessed surge in number of pig farms due to quest for animal protein, therefore making it a good setting to conduct this research.

Study design and participants:

The study used a descriptive cross-sectional questionnaire design to investigate pig handlers in Enugu State, Nigeria. To reduce non-response bias and avoid disrupting the respondents' work, investigators administered the questionnaire in person to those who gave informed consent after explaining the purpose of the study.

Participants were selected by simple random sampling and data collected between the period December 2023 and March 2024, with participation limited to pig and pig waste handlers and butchers aged 18 years and above. Participants who declined consent were excluded.

Ethical consideration:

Ethical approval with reference number MH/MSD/REC21/492 was obtained from the Health Research and Ethics Committee of Enugu State Ministry of Health Enugu State. In addition, another ethical approval with reference MOA/VED/EA/VOL1/1/25 was obtained from Enugu State Ministry of Agriculture and Agro-Industralization. Informed consent was obtained from each participant prior to interview-administration of questionnaires.

Sample size calculation:

The sample size was calculated using the formula of Lwanga and Lemeshow (11) suitable for descriptive cross-sectional study; $n=Z^2(1-P)/d^2$, where n=expected sample size, Z=standard normal deviation at 95% confidence interval (1.96), P=anticipated prevalence of 20% for 'good knowledge' of HEV from a previous study by Fasakin et al., (10), and d=margin of error at 6% (0.06). Considering a possible 10% attrition from poorly filled questionnaire (missing data), the minimum sample size was 190 participants, but we increased the number of participants recruited to 228.

Study data collection tool:

Information was collected from the study participants using a pre-tested questionnaire that was administered by the research interviewers. The survey tool was divided into five sections for information collection; socio-demographic, knowledge of HEV, HEV disease prevention practices, and vaccination strategies. Three professionals in the field of public health education evaluated the questionnaire and were validated after conducting a pilot with 30 pig handlers, with a Cronbach alpha coefficient score of 0.80.

Scoring system of participants' knowledge and practice:

Knowledge of HEV was assessed using 8 stem questions and some having multiple responses which results to 10 questions. Each correct answer was allotted score "one" (1) and every wrong answer was allotted "zero" (0). The results of the total score were 10 and "good knowledge" of HEV was graded using half of the maximum point (> 5) while "poor knowledge" was put at a cut-off value of < =5. HEV preventive practices/measures were also scored similarly, by assessing 10 stem questions which resulted in a maximum score of 10 points. Each correct answer was scored (1) point while wrong answer was scored (0). Participants whose scores were between 0 to 5 were scored as having "poor preventive measures" while those whose scores were between 6 to 10 were scored as having "good preventive measures".

Statistical analysis:

The Statistical Package for Social Sciences (SPSS) version 27 (IBM, United States) was used for data entry and analysis. The demographic information of the respondents and the analyses of their HEV knowledge and preventive behaviors were conducted using descriptive statistics. Frequencies and percentages were also used to express categorical variables.

Significant differences between the variable groups were determined using the Chi-square and Fisher Exact tests as appropriate. Binary logistic regression was used to establish associations between independent variables and levels of HEV knowledge and preventive practices. Associations with *p*-value less than 0.05 were considered statistically significant.

Results:

Demographic characteristics of study participants:

A total of 385 pig handlers who worked in piggeries were approached to participate in the study, but only 228 consented and completed the study questionnaire, which gave 59% response rate. Table 1 shows the summary of the socio-demographics and job characteristics of the participants. The mean age of the participants was 43.42±16.1 years, and 84 (36.8%) were aged 51 years and above while 109 (47.4%) participants were males and 120 (52.6%) were females.

Ninety-four (41.3%) of the participants had at least primary level education, while majority (n=174; 76.3%) were married and 108 (47.4%) have worked longer than 6 years in pig farm as animal handlers. Only 54 (23.7%) participants reported to have received certain levels of formal training on pig handling or farm cleaning and 210 (92.1%) of the participants consume pork as main source of animal protein (Table 1).

Table 1: Frequency distribution of Participants' Social Demographic characteristics

Variables	Frequency	Percent
Age group (years)		
≤ 30	60	26.3
31 – 40	30	13.2
41 – 50	54	23.7
≥51	84	36.8
Mean age (±SD) (years)	43.42±16.123	
Gender		
Male	108	47.4
Female	120	52.6
Highest education attained		
No formal education (NFE)	12	5.3
Primary	94	41.2
Secondary	68	29.8
Tertiary	54	23.7
Marital status		
Single	54	23.7
Married	174	76.3
Received any form of training for this job		
Yes	54	23.7
No	174	76.3
Use of stream as source of water supply at home		
Yes	126	55.3
No	102	44.7
Consume pork		
Yes	210	92.1
No	18	7.9
Years in pig farm		
<1	24	10.5
1 – 5	66	28.9
6 – 10	108	47.4
>10	30	13.2

Participants' knowledge of HEV:

Table 2 shows that only 28.9% (n=66) of the participants had "good knowledge" of HEV while majority (70.2%, n=162) had "poor knowledge" of the virus. However, on bivariate analysis, factors significantly associated with knowledge of HEV among the participants were age, marital status, educational level and gender (p<0.05). Further analysis of the table using binary logistic regression shows that among the age groups, those aged 41-50 years were three times more likely (aOR=3.2; 95% CI=1.211–8.456; p=0.010) to have poor knowledge of HEV than age group over 51 years. Those aged ≤30 years and those aged 31-40 years showed no statistical association with levels of knowledge.

Educational level had a significant association with knowledge levels. Participants with secondary education had four times odd of poor knowledge compared to those with tertiary education (aOR=4.1, 95% CI=1.791–9.597, p=0.005). This result suggests that those with lower education were more likely to have poor knowledge of HEV while those with tertiary education had good knowledge levels.

Participants' preventive practices/measures against HEV:

Table 3 presents the preventive practices/measures by the study participants against HEV infection. Among the 147 participants (64.5%) with good preventive practices, males comprised 53.7%, while females accounted for 74.2%. However, poor preventive practices/measures against HEV was twice as high in males compared to females (aOR=2.47, 95% CI=1.418–4.329, p=0.001). Analysis of age groups revealed that poor preventive practices /measures against HEV was significantly lower among participants aged 31–40 years (aOR= 0.027, 95%CI=0.027–0.548, p=0.004), compared to other age groups who did not show statistically significant associations with preventive practices (p>0.05). Marital status also played a role in HEV preventive measures. Single individuals were twice as likely to engage in poor practices compared to those who were married (aOR=2.009, 95% CI=1.078–3.743, p=0.037), indicating that married participants adhered more strictly to preventive measures.

Educational attainment showed significant association with HEV preventive practices. Participants with no formal education demonstrated the highest likelihood of poor preventive measures (aOR=28.75, 95% CI= 5.285–156.385, p=0.001), followed by those with primary education (aOR=10.624, 95% CI=4.489–25.168, p<0.05). Conversely, those with secondary education were significantly less likely to have poor preventive practices (aOR=0.174, 95%CI=0.035–0.858, p<0.05). This underscores the importance of higher education in improving health literacy, hygiene awareness, and access to preventive resources.

However, pork consumption (p=1.00), formal training in pig farming (p=0.214), and years spent in pig farming (p=0.106) were factors not significantly associated with HEV preventive practices among the participants.

Table 2: Multivariable logistic analysis of factors with associated with overall HEV knowledge of the participants

Variable	Total (n=228)	Poor knowledge (n=160, 70.2%)	Good knowledge (n=66, 29.8%)	*p* value	aOR (95% CI)
Age group (years)					
≤ 30	60	36 (60.0)	24 (40.0)		0.60 (0.298 – 1.209)^{ns}
31 – 40	30	18 (60.0)	12 (40.0)	0.004	0.60 (0.251 – 1.433)^{ns}
41 – 50	54	48 (88.9)	6 (11.1)		3.20 (1.211 – 8.456)*
≥ 51	84	58 (69.0)	26 (31.0)		R
Gender					
Male	108	64 (59.3)	44 (40.7)	0.001	0.39 (0.217 – 0.710)*
Female	120	96 (80.0)	24 (20.0)		R
Marital status					
Single	54	30 (55.6)	24 (44.4)	0.010	0.39 (0.210 – 0.754)*
Married	174	130 (74.7)	44 (25.3)		R
Highest education attained					
NFE	12	10 (83.3)	2 (16.7)	0.005	4.0 (0.799 – 20.017)^{ns}
Primary	94	63 (67.0)	31 (33.0)		1.6 (0.817 – 3.234)^{ns}
Secondary	68	57 (83.8)	11 (16.2)		4.1 (1.791 – 9.597)*
Tertiary	54	30 (55.6)	24 (44.4)		R

NFE=No formal education; * =significant at p-value <0.05; ns = not significant; R= Reference group; aOR = adjusted Odd ratio; CI = Confidence Interval

Table 3: Multivariable logistic analysis of factors associated with overall HEV preventive practices among the participants

Variable	Poor practice (n=81, 35.5%)	Good practice (n=147, 64.5%)	*p*-value	aOR (95% CI)
Gender				
Male (n=108)	50 (42.3)	58 (53.7)	0.001	2.47 (1.418 - 4.329)*
Female (n=120)	31 (25.8)	89 (74.2)		R
Age group (years)				
≤ 30 (n=60)	26 (43.3)	34 (56.7)	0.004	1.307 (0.668 - 2.571)^{ns}
31 – 40 (n=30)	12 (30.0)	28 (70.0)		0.027 (0.027 - 0.548)*
41 – 50 (n=54)	22 (40.7)	32 (59.3)		1.175 (0.583 - 2.369)^{ns}
>50 (n=84)	31 (36.9)	53 (63.1)		R
Marital status				
Single (n=54)	26 (48.1)	28 (51.9)	0.037	2.009 (1.078 - 3.743)*
Married (n=174)	55 (31.6)	119 (68.4)		R
Highest education attained				
NFE (n=12)	10 (83.3)	2 (16.7)	0.001	28.75 (5.285 - 156.385)*
Primary (n=94)	61 (64.9)	33 (35.1)		10.624 (4.489 - 25.168)*
Secondary (n=68)	2 (2.9)	66 (97.1)		0.174 (0.035 - 0.858)*
Tertiary (n=54)	8 (14.8)	46 (85.2)		R
Consume pork				
No (n=18)	6 (33.3)	12 (66.7)	1.000	
Yes (n=210)	75 (35.7)	135 (64.3)		
Received formal farm operational training				
No (n=174)	58 (33.3)	116 (66.7)	0.214	
Yes (n=54)	23 (42.6)	31 (57.4)		
Years in Pig farm				
< 1 (n=24)	6 (25.0)	18 (75.0)		
1 – 5 (n=66)	24 (36.4)	42 (63.6)	0.106	
6 – 10 (n=108)	45 (41.7)	63 (58.3)		
>10 (n=30)	6 (20.0)	24 (80.0)		

NFE=No formal education; * =significant at *p*-value <0.05; ns = not significant; R= Reference group; aOR = adjusted Odd ratio; CI = Confidence Interval

Discussion:

This study was aimed at assessing the knowledge and practices related to the prevention of HEV among pig handlers in Enugu State, Nigeria. Our findings indicate that only 29.8% of the enrolled pig handlers in Enugu State had good knowledge of HEV while 70.2% had poor knowledge of HEV. This finding is consistent with very low awareness of HEV reported by Mbachu et al., (12), where 98.6% of their subjects had poor knowledge of HEV. Similar finding was reported in Ogbomoso Oyo state Nigeria (13) where only 1% of the pregnant women enrolled had knowledge of HEV. Another study in Malaysia (14) reported that only 4.5% of their study participants who were food handlers had knowledge of HEV.

The reason why relatively high percentage of our study participants had good knowledge of HEV compared to these other studies could be attributed to an oral report that some of the pig handlers had during interventional education by the State Ministry of Agriculture following an outbreak of African swine fever mentioned HEV. This trend is suggestive of uniform poor knowledge of the virus in Africa and Asia. Limited awareness of HEV among pig handlers and the general public can heighten the risk of zoonotic transmission, particularly through direct contact with pigs or consumption of undercooked pork. Poor hygiene practices and inadequate disposal of pig waste may lead to environmental contamination, increasing the likelihood of community outbreaks. Additionally, insufficient knowledge contributes to underreporting and delayed diagnosis, ultimately weakening public health efforts.

Our study found that participants aged 41–50 years have significantly higher odds (aOR=3.20, *p*=0.004) of poor knowledge compared to those aged ≤30 years. Similar review studies in Nigeria, Ethiopia, and Sudan (15) have found that higher proportion of younger individuals (≤30 years of age) tend to have good knowledge of HEV than their older counterparts. The reason for this could be due to higher educational exposures, cultural belief and social media influence. Our finding however contrast studies in Europe and the USA (15,16), which reported that higher proportion of older adults (≥50 years of age) often have good knowledge due to accumulated health awareness programs.

This study shows that good knowledge of HEV was significantly higher among males than females (aOR=0.39, *p*=0.001), which is consistent with study in Ghana, West Africa where men have higher health literacy due to greater access to formal education (17). However, studies in high-income countries (UK, Canada) show no significant gender difference, which could likely be due to equal access to health education (18). Our study indicates

that larger proportion of single (unmarried) individuals have good knowledge of HEV than married individuals, and vice versa (aOR= 0.39, p=0.010), in keeping with studies from Ethiopia and Uganda (19,20) which reported that married individuals often have lower health literacy, possibly due to family responsibilities, limiting access to health information.

In our study, we found that educational level was significantly associated with HEV knowledge as reported in many other studies such as the Malaysian study (14), which reported that those with tertiary education had higher knowledge than those with lower level education. Oladipo and colleagues (13) also reported that those with higher education had better knowledge of HEV than those with lower education level. The reason for this could be due to the fact that individuals with higher education are more likely to have information on HEV due to improved health literacy, greater access to reliable information, and increased exposure to public health messages. A study in Ekiti State, Nigeria (4) reported consistently low awareness of HEV among antenatal attendees with lower educational background, despite 96% having at least a secondary-level education. This suggests that educational attainment did not play a significant role in differentiating knowledge levels. The reason for this could be traced to the fact that the Ekiti study participants did not have those whose educational status was higher than secondary school. Our study found that having a secondary level education is significantly associated with good knowledge (aOR=4.1, p=0.005). Similar studies in Kenya and South Africa (21) confirm that acquisition of higher education improves HEV awareness. In developed countries, tertiary education is the strongest predictor of health knowledge (18).

With respect to HEV preventive practices, higher proportion of male participants had significantly poor preventive practices of HEV compared to females (aOR=2.47, p= 0.001). Therefore, females were more likely to engage in HEV hygiene and preventive measures in their respective farms. This finding is consistent with other studies in Nigeria, Ethiopia, and Uganda which reported that women generally adopt HEV preventive hygiene practices, particularly waste handling and general farm sanitation (10,19,20). Higher proportion of our participants aged 31–40 years had signifi- cantly good preventive practices while higher proportion of our older participants aged > 50 years had poor preventive practices (aOR=0.027, p=0.004). This is in keeping with a study in Europe (15,16) where it appears that younger pig farm workers (those <35 years) tend to adopt HEV preventive measures than their older counterparts. The reasons for this could be due to procedural awareness, risk perception and educational but similar findings were reported in Niger Republic (22), Saudi Arabia (23) and India (24).

In our study, higher number of participants who were single (unmarried) had significantly poor preventive practices (aOR=2.009, p=0.037) than married participants who were more likely to engage in good hygiene and preventive measures against HEV in the pig farms. The reason for this disparity is not particularly apparent but may be due to social, economic, and psychological benefits of marriage which translates into more consistent health-protective behaviors in the HEV prevention on pig farms. Higher proportion of participants with no formal education (NFE) had significantly poor preventive practices (aOR=28.75, p=0.001), followed by those with primary education (aOR=10.624, p= 0.001), while lower proportion of those with secondary education had significantly poor preventive practices (aOR=0.174, p=0.001). This could be related to the fact that workers with low level education have limited health literacy, poorer access to information, and fewer resources, leading to poor preventive practices among them. In contrast, those with secondary education are better informed and empowered to adopt effective HEV prevention measures.

To the best of our knowledge, this study is the first in southeast Nigeria to specifically target pig handlers, an occupational group at heightened risk for HEV zoonotic transmission due to close contact with infected animals, exposure to fecal matter, and contaminated water. Consequently, our study offers valuable insights into a vulnerable and often overlooked population. However, several limitations must be considered. Relying on self-reported data introduces the possibility of recall bias or social desirability bias. Furthermore, selection bias may have occurred, as only those pig handlers who were at the respective farms and consented were enrolled. However, some eligible individuals chose not to participate and were excluded. Nonetheless, we believe these limitations were minimized by the inclusion of pig handlers from all three senatorial zones of Enugu State, providing a fairly representative sample of the State.

Conclusion:

This study highlights a critical gap in both knowledge and preventive practices regarding HEV infection among pig handlers in Enugu State, Nigeria. Only 29.8% of the participants had good knowledge of HEV, leaving a majority with poor knowledge of the virus. This deficient understanding, consistent with findings from other regions in Nigeria and parts of Asia, poses a heightened risk for zoonotic transmission, particularly given the suboptimal hygiene and biosecurity practices ob-

served on pig farms. Demographic factors play a significant role in shaping HEV knowledge and practices. Younger handlers, those with higher education, and married individuals generally exhibited better knowledge and consistently adopted more effective preventive strategies.

These findings underscore the design and implantation of tailored educational programs and targeted public health interventions to improve HEV awareness and biosecurity on pig farms in Enugu State Nigeria. Enhancing health literacy especially among less educated and older farm workers together with reinforcing proper hygiene practices can reduce HEV transmission risks. In addition, integrating these preventive measures within existing agricultural and public health frameworks is crucial to safeguarding the health of both pig handlers and the broader community.

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Contributions of authors:

ENJ, OFN were responsible for study design, ENJ, CMC and CNV actively collected data, CMC did data analysis. ENJ, OFN, CMC and CNV performed data interpretation, drafted the manuscript and all the reviews. All authors read and approved the final version of the manuscript.

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

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Data for the study are available upon a reasonable request from the corresponding author.

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Original Article **Open Access**

In vitro antimicrobial activities of two medicinal plant extracts against selected urinary pathogens and their synergistic effects with synthetic antibiotics

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Abstract:

Background: Antibiotic resistance is currently a major global health concern. Multidrug-resistant (MDR) bacteria are emerging and spreading rapidly, threatening the ability to treat common infectious diseases such as urinary tract infection (UTI). The objective of this study is to determine the *in vitro* antimicrobial activities of two medicinal plants (*Euphorbia hirta* and *Senna alata*) and their synergistic effects with synthetic antibiotics.
Methodology: Aqueous and methanol extracts of the two plants were done using Soxhlet and cold maceration techniques. The antimicrobial activities of the extracts were assessed against selected clinical microbial isolates (*Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Candida albicans*) using the agar well diffusion while Kirby-Bauer disc diffusion was used for antimicrobial susceptibility testing of the isolates to some selected synthetic antibiotics as comparator. The minimum inhibitory concentration (MIC) of the extracts against the isolates was determined by the broth dilution method, and the minimum bactericidal concentration (MBC) was determined by sub-culturing samples from MIC tubes with no visible growth onto plain agar to determine the lowest concentration that resulted in 99.9% microbial kill. Synergy of synthetic anti-microbials and the plant extracts was determined by the modified disk diffusion test. Data were analyzed using SPSS version 25.0. The Student's *t*-test was used to compare the mean zone diameter of inhibition (ZDI) of triplicate tests for each of the extract on the isolates, and $p<0.05$ was considered statistically significant.
Results: For *E. hirta*, methanol extracts exhibited significantly higher inhibitory activity ($p<0.05$) than aqueous extracts, particularly against *P. aeruginosa* (mean ZDI: 20.3±0.577mm vs 7.7±0.577mm) and *C. albicans* (mean ZDI: 20.7±1.155mm vs 6.0±0.577mm), with MIC values of 6.25mg/ml and 25.0mg/ml respectively. Similarly, for *S. alata*, methanol extracts exhibited higher inhibitory activity than aqueous extracts especially against *S. aureus* (mean ZDI: 21.3±1.528mm vs 6.0±1.155mm), but low inhibitory activity was observed against *K. pneumoniae* (mean ZDI: 7.0±1.000mm vs 6.0±0.577mm). Synergy testing showed enhanced antimicrobial activities when aqueous and methanol extracts were combined with synthetic antibiotics especially imipenem, producing significantly larger mean ZDI against *S. aureus* (45.6±0.577mm vs 48.3±0.577mm for *S. alata* and 38.3 ± 1.15mm vs 41.3±1.15mm for *E. hirta*). The combined extracts of the two plants also demonstrated significantly higher mean ZDI for methanol compared to aqueous extracts against *E. coli* (24.0±1.00mm vs 6.3±0.577mm), *K. pneumoniae* (20.3±0.577mm vs 6.7±1.155mm) and *C. albicans* (21.0±1.155mm vs 6.3±0.577mm).
Conclusion: The synergistic interactions between plant extracts and synthetic antibiotics in this study suggests a potential application in treatment of infections caused by antibiotic-resistant pathogens.

Keywords: *Euphorbia hirta, Senna alata*, Antibiotics, Antimicrobial activities, Synergistic.

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Activités antimicrobiennes *in vitro* de deux extraits de plantes médicinales contre certains pathogènes urinaires et leurs effets synergétiques avec des antibiotiques de synthèse

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Résumé:

Contexte: La résistance aux antibiotiques est actuellement un problème de santé mondial majeur. Les bactéries multirésistantes (MDR) émergent et se propagent rapidement, menaçant la capacité à traiter des maladies infectieuses courantes telles que les infections urinaires (IU). L'objectif de cette étude est de déterminer les activités antimicrobiennes in vitro de deux plantes médicinales (*Euphorbia hirta* et *Senna alata*) et leurs effets synergétiques avec les antibiotiques de synthèse.
Méthodologie: Des extraits aqueux et méthanoliques des deux plantes ont été réalisés par Soxhlet et macération à froid. Français Les activités antimicrobiennes des extraits ont été évaluées contre des isolats microbiens cliniques sélectionnés (*Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* et *Candida albicans*) en utilis333ant la diffusion en puits d'agar tandis que la diffusion sur disque de Kirby-Bauer a été utilisée pour les tests de sensibilité antimicrobienne des isolats à certains antibiotiques synthétiques sélectionnés comme comparateur. La concentration minimale inhibitrice (CMI) des extraits contre les isolats a été déterminée par la méthode de dilution en bouillon, et la concentration minimale bactéricide (CMB) a été déterminée par sous-culture d'échantillons de tubes CMI sans croissance visible sur de la gélose ordinaire pour déterminer la concentration la plus faible qui a entraîné une élimination microbienne de 99,9 %. La synergie des antimicrobiens synthétiques et des extraits de plantes a été déterminée par le test de diffusion sur disque modifié. Les données ont été analysées à l'aide de SPSS version 25.0. Français Le test *t* de Student a été utilisé pour comparer les diamètres moyens de la zone inhibitrice des tests en triple pour chacun des extraits sur les isolats, et $p<0,05$ a été considéré comme statistiquement significatif.
Résultats: Pour *E. hirta*, les extraits au méthanol ont montré une activité inhibitrice significativement plus élevée ($p<0,05$) que les extraits aqueux, en particulier contre *P. aeruginosa* (ZDI moyen: 20,3±0,577mm contre 7,7±0,577mm) et *C. albicans* (ZDI moyen: 20,7±1,155mm contre 6,0±0,577mm), avec des valeurs de CMI de 6,25mg/ml et 25,0mg/ml respectivement. Français De même, pour *S. alata*, les extraits au méthanol ont montré une activité inhibitrice plus élevée que les extraits aqueux, en particulier contre *S. aureus* (ZDI moyen: 21,3±1,528mm contre 6,0±1,155 mm), mais une faible activité inhibitrice a été observée contre *K. pneumoniae* (ZDI moyen: 7,0±1,000mm contre 6,0±0,577mm). Les tests de synergie ont montré des activités antimicrobiennes améliorées lorsque les extraits aqueux et au méthanol étaient combinés avec des antibiotiques synthétiques, en particulier l'imipénème, produisant un ZDI moyen significativement plus élevé contre *S. aureus* (45,6±0,577mm contre 48,3±0,577mm pour *S. alata* et 38,3±1,15mm contre 41,3±1,15mm pour *E. hirta*). Les extraits combinés des deux plantes ont également montré un ZDI moyen significativement plus élevé pour le méthanol par rapport aux extraits aqueux contre *E. coli* (24,0±1,00 mm contre 6,3±0,577mm), *K. pneumoniae* (20,3±0,577mm contre 6,7±1,155mm) et *C. albicans* (21,0±1,155mm contre 6,3±0,577mm).
Conclusion: Les interactions synergétiques entre les extraits de plantes et les antibiotiques de synthèse dans cette étude suggèrent une application potentielle dans le traitement des infections causées par des agents pathogènes résistants aux antibiotiques.

Mots-clés: *Euphorbia hirta, Senna alata*, Antibiotiques, Activités antimicrobiennes, Synergique.

Introduction:

Antibiotic resistance is currently a major global health concern. Multidrug-resistant (MDR) bacteria are emerging and spreading rapidly, threatening the ability to treat common infectious diseases such as urinary tract infection (UTI) (1). UTI represents one of the most common infectious diseases encountered in medical practice today affecting all age groups from neonates to geriatrics (1). It is estimated that about 35% of healthy women suffer symptoms of UTI at some stages in their life. About 5% of women each year suffer from painful and frequent urination (2). The incidence of UTI is greater in women compared to men which may be either due to anatomical predisposition or urothelial mucosal adherence to the mucopolysaccharide lining or other host factors (3).

The extended spectrum β-lactamase (ESBL)-producing bacteria identified among the members of the family Enterobacteriaceae, are increasingly causing UTI both in hospitalized patients and outpatients (4,5). The increasing drug resistance among these bacteria has made therapy of UTI difficult. Indiscriminate use of antibiotics is considered to be one

of the main reasons for emergence of drug-resistance. According to the World Health Organization (WHO), drug-resistant infections could cause 10 million deaths each year by 2050 and significant damage to the global economy (6). Thus, it is very crucial to identify products with antimicrobial properties that could be utilized to develop novel and efficient antibiotics.

Plant products have been used since ancient times to treat diseases and the practice is continued till today. It is estimated that about 80% of the population from developing countries uses medicines derived from plant species (7). As plant-derived medicines are safer than synthetic alternatives (8), their usage will continue to grow in the future. It is expected that by the year 2050, the value of international trade of medicinal plants and their products would reach 5 trillion USD (9). Plants produce a variety of compounds that are not used in their primary metabolism but increase their ability to survive environmental challenges. These secondary metabolites such as alkaloids, phenols, terpenes and others are bioactive and provide defense against herbivores and microbes (10). Different parts of plants such as the leaf, root, flower, fruit, peel, bark, seed, stem, rhizome, etc can be used for therapeutic purposes as they contain high amounts of bioactive compounds with therapeutic potential (11).

Nigeria is a tropical country with a rich variety of medicinal plants. It is estimated that there are several plant species which are expected to have therapeutic benefits (12). Over the last decade, numerous studies have been conducted to analyze the antibacterial properties of eclectic plants. However, a more diverse range of medicinal plants needs to be analyzed extensively to further enhance knowledge. Thus, this study is designed to evaluate the antimicrobial properties of some plants in Nigeria that are used as herbal medicine treatment of many diseases, including *Senna alata* and *Euphorbia hirta.*

Senna alata (also known as ringworm bush or *Cassia alata*) and *E. hirta* (commonly known as 'Tawa-Tawa') have attracted attention due to their wide range of biological activities. Both plants are traditionally used in folk medicine for various ailments, and recent studies have suggested that their extracts may exhibit potent antimicrobial effects, particularly against UTI-causing pathogens (13). In this study, *in vitro* antimicrobial activities of the two medicinal plants and their synergistic eff with synthetic antibiotics were determined.

Materials and method:

Study area and design:

The study was an evaluation of the *in vitro* antimicrobial activities of two medicinal plants (*S. alata* and *E. hirta*) on selected urinary pathogens and their synergistic effects with selected synthetic antibiotics carried out in Medical Microbiology and Parasitology Laboratory of Al-Hikmah University, Ilorin, and University of Ilorin Teaching Hospital, Ilorin, Nigeria.

Ethical clearance for the study:

Ethical approval with reference number (UITH/CAT/189/21^{B}/626) was obtained from University of Ilorin, Teaching Hospital, Nigeria prior to the commencement of the study.

Plant collection, authentication and pulverization of the plant leaves:

Fresh leaves of *E. hirta and S. alata* were collected from the premises of Kwara State stadium Ilorin, Nigeria. The plants were identified and authenticated at the herbarium unit of the Department of Plant Biology, Faculty of Life Sciences, University of Ilorin, where they were given herbarium voucher number UILH/001/1511/2024 and UILH/001/1059/ 2024 respectively. The leaves were washed thoroughly separately and air dried in shade for two weeks. Dried leaves were ground into a coarse powder using an industrial blender.

Preparation of the plant extract:

Methanol and aqueous extracts of *E. hirta* and *S. alata* leaves were prepared using Soxhlet extraction and maceration methods respectively (14). For the methanol extracts, 100g of powdered leaves were placed in the Soxhlet apparatus thimbles and extracted with 500 ml of methanol through continuous solvent reflux until exhaustion. The resulting extracts were concentrated using a rotary evaporator at 50°C and then stored at 4°C.

For the aqueous extraction, 100g of ground leaves were soaked in 200 ml distilled water for 72 hours at room temperature with occasional stirring. After filtration, the extracts were frozen at -20 °C and lyophilized at -50°C under vacuum to obtain dry powders. The dried powder was stored at 4°C for further microbiological and phytochemical analyses. The percentage yield of the plant extract was calculated using the formula; Weight of the extract (g) / Weight of the plant × 100 (15).

Preparation of extract stock solution:

The extract stock solution was prepared by dissolving 2g of each dry plant extract in 10 ml of dimethyl sulfoxide (DMSO) (200 mg/ml) for methanol extract and 2g of each dry plant extract in 10 ml of sterile distilled water (200 mg/ml) for aqueous extract (16)

Test microbial isolates:

The clinical isolates of *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Candida albicans* were obtained from the Medical Microbiology laboratory of the University of Ilorin

Teaching Hospital, Ilorin, Nigeria. The isolates were sub-cultured on Blood and MacConkey agar plates (bacteria) and Sabouraud dextrose agar (fungi) and incubated at 37°C for 24 hours (16).

Determination of antimicrobial activity of the plant extracts on selected pathogens:

The antimicrobial activity of the plant extracts on the selected microbial pathogens was determined by the agar well diffusion method. Each of the isolate suspension was prepared by taking a few colonies from overnight culture plate with sterilized wire loop and inoculating into normal saline. The turbidity of the bacterial suspension was adjusted to get the equivalence of 0.5 McFarland standards (~1.5×10^8 CFU/ml).

A sterile swab stick was used inoculate the microbial suspension onto the Mueller Hinton (MH) agar plate and properly spread to ensure the entire surface was covered with the inoculum. Four wells of 6 mm size each were bored in the inoculated media with sterile cork-borer. Each well was filled with 50μL of the plant extracts. DMSO was used as negative/ solvent control while positive controls (gentamicin 10μg and nitrofurantoin 100μg for bacteria and 10μg fluconazole for fungi) were tested along the test isolates by the disk diffusion method.

The extracts and DMSO were allowed to diffuse into the agar well for 30 minutes at room temperature and the plates incubated for 24 hours at 37◦C. After incubation, plates were observed for the formation of a clear zone around the well which corresponds to the antimicrobial activity of the extracts and DMSO. The of zone diameter of inhibition (ZDI) was observed and measured in mm. Three replicates of the experiment were carried out and the mean (and standard deviation) of the tests were calculated (15,16).

Determination of minimum inhibitory and bactericidal concentrations of the extracts:

The two-fold broth (tube) dilution assay was used to determine the minimum inhibitory concentration (MIC) of the plant extracts against the microbial pathogens. A total of 13 test tubes were prepared for the procedure. Tube 1 contained 2 ml of the undiluted plant extract from the extract stock solution (200mg/ml), while tubes 2 to 10 each contained 1 ml of nutrient broth.

A serial dilution (1 in 2) of the extract was carried out from tube 1 to tube 10 to achieve varying concentrations (200 mg/ml in tube 1 to 0.391 mg/ml in tube 10). Subsequently, 1 ml of microbial inoculum was added to each of the 10 tubes. Tubes 11, 12, and 13 served as controls (inoculum control, plant extract control, and broth control respectively). All the tubes were incubated for 24 hours, after which the tube with the lowest concentration of the extract showing no turbidity was recorded as the MIC.

The minimum bactericidal concentration (MBC) was determined by sub-culturing MIC tubes that showed no visible growth onto plain nutrient agar plate to determine the concentration that resulted in complete (99.9%) microbial kill (16).

Antimicrobial susceptibility test of synthetic antibiotics against bacteria pathogens:

The susceptibility of the same clinical microbial isolates to selected synthetic antibiotics was determined by the disk diffusion method of Bauer and Kirby (16). Six broad-spectrum antibiotics commonly prescribed by physicians in Nigeria for UTI; amoxicillin-clavulanic acid (augmentin AUG 30μg), imipenem (IMI 10µg), nitrofurantoin (NIT 100µg), ceftazidime (CAZ 30µg), cefoxitin (FOX 30µg), and gentamicin (GN 30µg) were selected for testing against bacteria, and fluconazole (FCN 10 µg) against *C. albicans*.

A sterile swab stick was used to inoculate standardized suspension of the microbial isolate onto the MH agar plate and properly spread to ensure the entire surface was covered. The antibiotic discs were then placed on the surface of the bacterial lawn and the plates were incubated at 37°C for 24 hours. After incubation, the zone of inhibition was measured and compared with the CLSI standard chart to determine sensitivity or resistance of the microorganisms to the antibiotics (16).

Antimicrobial effects of combined synthetic antibiotics and plant extracts:

The antimicrobial effects of combination of both synthetic antibiotics and plant extracts on bacterial and fungi isolates were determined by the disc diffusion test. Standardized inoculum of overnight culture of each isolate was prepared and inoculated separately on MH agar plates using sterile swab.

Approximately 50µL of each medicinal plant extract was incorporated into each of the selected synthetic antimicrobial discs; amoxicillin-clavulanic acid (augmentin AUG 30μg), imipenem (IMI 10µg), nitrofurantoin (NIT 100 µg), ceftazidime (CAZ 30µg), cefoxitin (FOX 30µg), and gentamicin (GN 30µg) against bacteria, and fluconazole (FCN 10 µg) against *C. albicans*.

The plates were incubated at 37°C for 24 hours and the inhibitory zone diameters were measured and recorded for the combined extracts and synthetic antibiotics, and compared with the inhibitory zone diameters recorded for the individual (uncombined) synthetic antibiotic and plant extract, to determine if there are synergistic, antagonistic or indifference activities.

Antimicrobial effects of combined extracts of the two plants on microbial pathogens:

Antimicrobial activity of the combined extracts of *E. hirta* and *S. alata* on the microbial isolates was determined by agar well diffusion method. A sterile swab stick was used to inoculate standardized suspension of the overnight culture of each organism onto MH agar plate and properly spread to ensure the entire surface of the agar was covered with the inoculum. Four wells of 8 mm each were bored into the inoculated media with sterile cork-borer. Each well was labeled and filled with 100 µL of the mixture of extracts of the two plants. DMSO was used as negative/solvent control while positive control (gentamicin 10μg and nitrofurantoin 100μg for bacteria and 10μg fluconazole for fungi) were tested on the isolates by the disk diffusion method.

The extracts and DMSO were allowed to diffuse into the agar for 30 minutes at room temperature and incubated for 24 hours at 37◦C. After incubation, plates were observed for the formation of a clear zone around the well which corresponds to the antimicrobial activity of plant extracts/DMSO. The zone diameter of inhibition (ZDI) was observed, and measured in mm. Three replicates of the experiment were carried out and the mean (and standard deviation) were calculated (15,16).

Statistical analysis of data:

Data were analyzed using SPSS version 25.0. The Student's *t*-test was used to compare the mean zone diameter of inhibition (mean ZDI) of triplicate tests for each of the extract on the isolates, and $p<0.05$ was considered statistically significant.

Results:

The extraction yields of the plants showed that the highest yield was obtained from methanol extract of *S. alata* of 7.9% while the least yield was from aqueous extract of *E. hirta* of 5.2% (Table 1). Table 2 highlights the antimicrobial activities of the aqueous and methanol extracts of *E. hirta* and *S. alata* plants.

For *E. hirta*, methanol extracts exhibited significantly higher inhibitory activity than aqueous extracts ($p<0.05$), particularly against *P. aeruginosa* (mean ZDI: 20.3±0.577mm and 7.7±0.577mm respectively) and *C. albicans* (mean ZDI: 20.7±1.155mm and 6.0± 0.577mm respectively). Similarly, the methanol extract of *S. alata* outperformed the aqueous extract, producing significantly larger ($p<0.05$) inhibition zones against *S. aureus* (mean ZDI: 21.3±1.528mm and 6.0±1.155mm respectively) and *E. coli* (mean ZDI: 15.3 ± 0.577 mm and 7.0±1.000 mm respectively). However, both extracts exhibited comparable and limited activity against *K. pneumonia*e (mean ZDI: 7.0±1.000mm and 6.0±0.577mm respectively).

Table 3 shows the antimicrobial susceptibility of the conventional antibiotics against selected bacterial and fungi pathogens. The antibiotic with the highest inhibitory activity was imipenem on *E. coli* (ZDI: 35mm), followed by ceftazidime on *K. pneumoniae* (ZDI: 30mm). However, all the bacterial uropathogens showed low susceptibility (high resistance) to nitrofuratoin with ZDI ranges of 6-10 mm. *Candida albicans* on the other hand was susceptible to fluconazole with ZDI of 26mm.

Table 1: Percentage extract yields of *Euphorbia hirta* and *Senna alata* medicinal plants

Plants	Solvents	Weight of plants/g	Weight of extracts/g	% yield
Euphorbia hirta	Aqueous	100	5.2	5.2
	Methanol	100	6.4	6.4
Senna alata	Aqueous	100	6.0	6.0
	Methanol	100	7.9	7.9

Table 2: *In vitro* antimicrobial activity of aqueous and methanolic extract of *Euphorbia hirta and Senna alata* plants on selected bacteria and fungi pathogens

Organisms	Plants	Zone of inhibition (mm)		*t*-value	*p* value
		Aqueous (Mean ± SD)	Methanol (Mean ± SD)		
Staphylococcus aureus	*Euphorbia hirta*	11.0± 1.000	10.7 ± 0.577	1.000	0.423
	Senna alata	6.0 ± 1.155	21.3 ± 1.528	-10.094	0.010**
Escherichia coli	*Euphorbia hirta*	6.0 ± 1.528	18.0 ± 1.000	-8.000	0.015**
	Senna alata	7.0 ± 1.000	15.3 ± 0.577	-12.500	0.006**
Klebsiella pneumoniae	*Euphorbia hirta*	6.0 ± 1.155	15.0± 0.577	-15.588	0.004**
	Senna alata	6.0 ± 0.577	7.0 ± 1.000	-0.500	0.667
Pseudomonas aeruginosa	*Euphorbia hirta*	7.7 ± 0.577	20.3 ± 0.577	-19.000	0.003**
	Senna alata	7.0 ± 1.000	10.3 ± 0.577	-5.000	0.038**
Candida albicans	*Euphorbia hirta*	6.0 ± 0.577	20.7± 1.155	-16.252	0.004**
	Senna alata	6.0 ± 1.155	10.5± 1.000	-13.000	0.006**

** statistically significant at $p < 0.05$; mm=millimeter

Table 3: Antimicrobial susceptibility of some selected antimicrobials on selected urinary bacteria and fungi pathogens

Antimicrobials	Zone diameter of inhibition (mm) of microbial pathogens				
	S. aureus	*K. pneumoniae*	*E. coli*	*P. aeruginosa*	*Candida albicans*
GN (10µg)	15	26	7	26	-
IMI (10µg)	25	27	35	30	-
AUG (30µg)	16	6	9	28	-
CAZ (30µg)	13	30	8	10	-
FOX (30µg)	6	6	21	8	-
NIT (100µg)	6	6	10	6	-
FCN (10µg)	-	-	-	-	26

GN = Gentamicin, IMI = Imipenem, AUG= Amoxicillin-clavulanic acid, CAZ = Ceftazidime, FOX = Cefoxitin, NIT =Nitrofurantoin, FCN = Fluconazole

Tables 4 and 5 respectively showed the synergistic effects of *S. alata* and *E. hirta* plant extracts with some selected synthetic antibiotics. Imipenem showed the strongest synergistic activity with methanol and aqueous extracts of *S. alata* against *S. aureus* with mean ZDI of 48.3±0.577 mm and 45.6±0.577 mm respectively, followed by *P. aeruginosa* with respective mean ZDI of 47.6±0.577 mm and 39.0±1.000 mm, and the least, *K. pneumoniae* with respective mean ZDI of 19.3± 0.577 mm and 31.0±1.000 mm. The ZDI values of the extracts of *S. alata* combined with synthetic antibiotics are higher than the ZDI of the synthetic antibiotics without the extracts for *S. aureus* (ZDI: 25mm) and *P. aeruginosa* (ZDI: 30mm) in Table 3.

Ceftazidime exhibited the weakest synergistic activity with methanol and aqueous extracts of *S. alata* plant against *K. pneumoniae* (with respective mean ZDI of 11.0±1.000 mm and 31.6±0.577 mm), followed by *P. aeruginosa* (with respective mean ZDI of 24.6±0.577 mm and 10.3±0.577 mm), and the least, *S. aureus* (with respective mean ZDI of 11.6 ± 0.577 mm and 9.3±1.155 mm) (Table 4). Fluconazole also exhibited weak or no synergistic activity with methanol and aqueous extracts of *S. alata* against *C. albicans* with respective mean ZDI of 21.3±1.155 mm and 20.3±0.577 mm, which is less than the ZDI of 26 mm produced by fluconazole without the extracts (Table 3), indicating possible antagonism.

Similar to *S. alata*, imipenem showed the strongest synergistic activity with methanol and aqueous extracts of *E. hirta* plant against *P. aeruginosa* (with respective mean ZDI of 43.3±1.15 mm and 42.7±1.15 mm), followed by *S. aureus* (with respective mean ZDI of 41.3±1.15 mm and 38.3±1.15 mm), *K. pneumoniae* (with respective mean ZDI of 40.3±0.58 mm and 40.0±1.00 mm), and the least *E. coli* (with respective mean ZDI of 20.0±1.00 mm and 20.7±1.53 mm) (Table 5). The ZDI values of the extracts of *E. hirta* combined with synthetic antibiotics are higher than the ZDI of the synthetic antibiotics without the extracts for *S. aureus* (ZDI: 25mm) and *P. aeruginosa* (ZDI: 30mm) in Table 3.

However, fluconazole exhibited weak synergistic or indifference activity with methanol and aqueous extracts of *E. hirta* against *C. albicans* with respective mean ZDI of 30.3± 0.58 mm and 26.7±1.15 mm, which was just slightly higher than the ZDI of 26 mm produced by fluconazole without the extracts (Table 3) for the methanol extract, but equivalent to that produced by aqueous extract, indicating possible indifference activity.

Table 4: Synergistic effect of *Senna alata* plant with selected synthetic antimicrobials on urinary bacteria and fungi pathogens

Antimicrobial	Organism	Zone of inhibition (mm)		*t*-value	*p*-value
		Aqueous (Mean ± SD)	Methanol (Mean ± SD)		
GN	*S. aureus*	13.5 ± 0.61	22.3 ± 1.53	-9.23	0.0009**
	K. pneumoniae	15.0 ± 1.17	25.0± 1.00	-12.01	0.0005**
	E. coli	16.2 ± 1.18	21.9 ± 1.23	-4.26	0.0112
	P. aeruginosa	25.0 ± 1.00	31.1 ± 0.61	-10.21	0.0003**
IMI	*S. aureus*	45.6 ± 0.577	48.3 ± 0.577	-4.000	0.057
	K. pneumoniae	31.0 ± 1.000	19.3 ± 0.577	35.000	0.001**
	E. coli	32.7 ± 0.577	31.3 ± 1.528	1.512	0.270
	P. aeruginosa	39.0 ± 1.000	47.6 ± 0.577	-13.000	0.006**
AUG	*S. aureus*	19.3 ± 1.155	20.7 ± 0.577	-4.000	0.057
	K. pneumoniae	40.3 ± 0.577	38.0 ± 1.000	7.000	0.020**
	E. coli	31.0 ± 1.000	33.6 ± 0.577	-3.024	0.094
	P. aeruginosa	21.3 ± 1.155	27.3 ± 0.577	-10.392	0.009**
CAZ	*S. aureus*	9.3 ± 1.155	11.6 ± 0.577	-7.000	0.020**
	K. pneumoniae	31.6 ± 0.577	11.0 ± 1.000	62.000	0.000**
	E. coli	15.3 ± 0.577	14.0 ± 0.000	4.000	0.057
	P. aeruginosa	10.3 ± 0.577	24.6 ± 0.577	-43.000	0.001**
FOX	*S. aureus*	23.6 ± 0.577	21.0 ± 1.000	4.000	0.057
	K. pneumoniae	30.3 ± 17.616	27.3 ± 1.155	0.285	0.803
	E. coli	18.3 ± 0.577	14.0 ± 1.000	13.000	0.006**
	P. aeruginosa	21.3 ± 1.528	25.0 ± 1.000	-3.051	0.093
NIT	*S. aureus*	11.3 ± 0.59	12.6± 0.63	0.00	1.0000
	K. pneumoniae	7.0 ± 1.15	7.0± 1.15	0.47	0.6769
	E. coli	14.0 ± 0.58	14.3 ± 0.58	-2.17	0.1012
	P. aeruginosa	12.4 ± 0.45	16.1 ± 0.72	-7.83	0.0013**
FCN	*Candida albicans*	20.3 ± 0.577	21.3 ± 1.155	-1.000	0.423

GN = Gentamicin, IMI = Imipenem, AUG = Amoxicillin-clavulanic acid, CAZ = Ceftazidime, FOX = Cefoxitin, NIT =Nitrofurantoin, FCN = Fluconazole; ** mean significance at *p*-value < 0.05; SD: Standard deviation

Table 5: Synergistic effects of *Euphorbia hirta* with selected synthetic antimicrobials on urinary bacterial and fungi pathogens

Antimicrobials	Organism	Zone of inhibition (mm)		*t*-value	*p*-value
		Aqueous (Mean ± SD)	Methanol (Mean ± SD)		
GN	*S. aureus*	11.7 ± 0.58	20.3 ± 1.53	-9.19	0.0008**
	K. pneumoniae	12.0 ± 1.00	21.0 ± 1.00	-11.02	0.0004**
	E. coli	16.7 ± 1.15	20.7 ± 1.15	-4.24	0.0132**
	P. aeruginosa	21.0 ± 1.00	27.7 ± 0.58	-10.00	0.0006**
IMI	*S. aureus*	38.3 ± 1.15	41.3 ± 1.15	-3.18	0.0335**
	K. pneumoniae	40.0 ± 1.00	40.3 ± 0.58	-0.50	0.6433
	E. coli	20.7 ± 1.53	20.0 ± 1.00	0.63	0.5614
	P. aeruginosa	42.7 ± 1.15	43.3 ± 1.15	-0.71	0.5185
AUG	*S. aureus*	21.0 ± 1.00	19.7 ± 0.58	2.00	0.1161
	K. pneumoniae	40.3 ± 0.58	18.0 ± 1.00	33.50	0.0000**
	E. coli	25.3 ± 0.58	25.7 ± 0.58	-0.71	0.5185
	P. aeruginosa	20.7 ± 0.58	25.3 ± 1.15	-6.26	0.0033**
CAZ	*S. aureus*	10.3 ± 0.58	9.0 ± 1.00	2.00	0.1161
	K. pneumoniae	18.0 ± 1.00	23.7 ± 0.58	-8.50	0.0011**
	E. coli	6.7 ± 1.15	6.3 ± 0.58	0.45	0.6779
	P. aeruginosa	10.3 ± 0.58	34.0 ± 1.00	-35.50	0.0000**
FOX	*S. aureus*	21.0 ± 1.00	23.0 ± 1.00	-2.45	0.0705
	K. pneumoniae	27.3 ± 1.15	22.3 ± 1.53	4.52	0.0106**
	E. coli	11.3 ± 0.58	5.7 ± 0.58	12.02	0.0003**
	P. aeruginosa	31.0 ± 1.00	30.3 ± 0.58	1.00	0.3739
NIT	*S. aureus*	10.7 ± 0.58	10.6 ± 0.58	0.00	1.0000
	K. pneumoniae	6.6 ± 1.15	6.3 ± 0.58	0.45	0.6779
	E. coli	13.3 ± 0.58	14.3 ± 0.58	-2.12	0.1012
	P. aeruginosa	10.6 ± 0.58	14.3 ± 0.58	-7.78	0.0015**
FCN	*Candida albicans*	26.7 ± 1.15	30.3 ± 0.58	-4.92	0.0079**

GN = Gentamicin, IMI = Imipenem, AUG = Amoxicillin-clavulanic acid, CAZ = Ceftazidime, FOX = Cefoxitin, NIT =Nitrofurantoin, FCN = Fluconazole; ** mean significance at *p*-value < 0.05; SD: Standard deviation

The antimicrobial activity of the combined extracts of *E. hirta* and *S. alata* on the pathogens in Table 6 showed that methanol extracts of the combined plants exhibited higher inhibitory activity than aqueous extracts against most of the tested pathogens, producing significantly higher ZDI against *E. coli* (mean ZDI: 24.0±1.000 mm), *K. pneumoniae* (mean ZDI: 20.3±0.577 mm), and *C. albicans* (mean ZDI: 21.0±1.155 mm), compared to the lower ZDI of the aqueous extracts of 6.3± 0.577 mm, 6.7±1.155 mm and 6.3±0.577 mm for the respective pathogens. There was no difference in the inhibitory activity of the combined methanol and aqueous extracts of the two plants against *S. aureus* (mean ZDI: 19.3±0.577 and 16.0±0.577 mm respectively) and against *P. aeruginosa* (mean ZDI: 20.3 ±0.577 and 20.7±0.577 mm, respectively).

Table 7 presents the MIC values of *S. alata* extracts against the selected pathogens, with methanol extracts producing lower MIC values (range 6.25–50 mg/ml) than aqueous extracts (25-100 mg/ml). The lowest MIC values with methanol extracts of *S. alata* were produced against *C. albicans* (6.25 mg/ml) and *S. aureus* (12.5mg/ml), followed by MIC values of 50 mg/ml against *P. aeruginosa*, *E. coli* and *K. pneumoniae*. However, high MIC of 100 mg/ml was produced with aqueous extracts of *S. alata* against *K. pneumoniae*, *P. aeruginosa* and *S. aureus*.

The methanol extracts of *E. hirta* produced lower MIC values (range 6.25–50 mg/ml) than aqueous extracts (range 50-100 mg/ml). The lowest MIC values with methanol extracts of *E. hirta* were produced against *P. aeruginosa* (6.25 mg/ml) and *C. albicans* (12.5 mg/ml), followed by *K. pneumoniae* (25 mg/ml), *E. coli* (25 mg/ml) and *S. aureus* (50 mg/ml). Comparatively, aqueous extracts of *E. hirta* produced high MIC values against *P. aeruginosa* (25 mg/ml), *E. coli* (50 mg/ml), *S. aureus* (50 mg/ml) and *K. pneumoniae* (100 mg/ml).

Table 8 presents the MBC values of *E. hirta* against some selected pathogens. The MBC values for methanol extracts range from 25 mg/ml against *P. aeruginosa* to 100 mg/ml against *S. aureus* while MBC values for aqueous extract range from 100 mg/ml against *C. albicans* to 200 mg/ml against *S. aureus.* For *S. alata*, the MBC values of the methanol extracts range from 25 mg/ml against *C. albicans* to 100 mg/ml against *K. pneumoniae* while the MBC values of the aqueous extracts range from 100 mg/ml against *C. albicans* to 200 mg/ml against *K. pneumoniae.*

Table 6: *In vitro* antimicrobial activity of aqueous and methanolic extracts of combined *Euphorbia hirta* and *Senna alata* plants on selected urinary bacteria and fungi pathogens

Organism	**Zone of inhibition (mm)**		***t*-value**	***p*-value**
	Aqueous (Mean ± SD)	**Methanol (Mean ± SD)**		
Staphylococcus aureus	16.0 ± 0.577	19.3 ± 0.577	-8.000	0.015**
Escherichia coli	6.3 ± 0.577	24.0 ± 1.000	-26.500	0.001**
Klebsiella pneumoniae	6.7 ± 1.155	20.3 ± 0.577	-41.000	0.001**
Pseudomonas aeruginosa	20.7 ± 0.577	20.3 ± 0.577	1.000	0.423
Candida albicans	6.3 ± 0.577	21.0± 1.155	-43.000	0.001**

mean significance at p-value < 0.05; mm = millimeter

Table 7: Minimum Inhibitory Concentration (MIC) of *Euphorbia hirta* and *Senna alata* extract against selected urinary bacteria and fungi pathogens

Organism	***Euphorbia hirta***		***Senna alata***	
	Aqueous Extract	**Methanolic Extract**	**Aqueous Extract**	**Methanolic Extract**
Staphylococcus aureus	50 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml
Klebsiella pneumoniae	100 mg/ml	25 mg/ml	100 mg/ml	50 mg/ml
Escherichia coli	50 mg/ml	25 mg/ml	50 mg/ml	50 mg/ml
Pseudomonas aeruginosa	25 mg/ml	6.25 mg/ml	25 mg/ml	25 mg/ml
Candida albicans	50 mg/ml	12.5 mg/ml	50 mg/ml	6.25 mg/ml

Table 8: Minimum Bactericidal Concentration (MBC) of *Euphorbia hirta* and *Senna alata* extract against selected urinary bacteria and fungi pathogens

Organism	***Euphorbia hirta***		***Senna alata***	
	Aqueous Extract	**Methanolic Extract**	**Aqueous Extract**	**Methanolic Extract**
Staphylococcus aureus	200 mg/ml	100 mg/ml	200 mg/ml	100 mg/ml
Klebsiella pneumoniae	200 mg/ml	100 mg/ml	200 mg/ml	100 mg/ml
Escherichia coli	200 mg/ml	100 mg/ml	100 mg/ml	100 mg/ml
Pseudomonas aeruginosa	100 mg/ml	25 mg/ml	200 mg/ml	100 mg/ml
Candida albicans	100 mg/ml	50 mg/ml	100 mg/ml	25 mg/ml

Discussion:

The extraction yield of the leaves of the medicinal plants analyzed in this study showed that the highest yield was obtained from methanol extracts of *S. alata* (7.9%) with methanol extracts of *E. hirta* producing a lower yield of 6.4%. The yields with aqueous extracts were correspondingly lower than the methanol extracts for the two plants. This variation is primarily attributable to the nature of the solvent used, which significantly affects the solubility and extractability of phytochemicals in the plants. Polar solvents such as water are more selective for hydrophilic compounds, whereas solvents such as methanol, with intermediate polarity, can extract a broader range of both polar and non-polar constituents, resulting in higher yields. These findings align with previous studies (17,18) that reported similar solvent-dependent variations in extractive values of medicinal plants. Similarly, Tourabi et al., (19) reported that the extraction yield of methanol on medicinal plants generally exceeded that of aqueous extracts due to the superior solvent penetration ability of methanol.

Methanol extracts of *E. hirta* in our study demonstrated significantly higher antimicrobial activity than aqueous extracts, particularly against *K. pneumoniae*, *E. coli*, *C. albicans* and *P. aeruginosa.* Similarly, methanol extracts of *S. alata* exhibited higher inhibitory activity against *S. aureus* and *E. coli*. These findings are consistent with those of previous studies (20,21) which reported that methanol extracts of medicinal plants exhibited higher antimicrobial activity than aqueous extracts. The higher antimicrobial activity of methanol extracts could be attributed to greater solubility of the active phytochemicals in methanol, leading to enhanced activity (22).

The study on the combined antimicrobial effects of *E. hirta* and *S. alata* shoed both synergistic and antagonistic interactions against various pathogens. The methanolic extract of *S. alata* exhibited antagonistic effects when combined with *E. hirta* against *S. aureus*, as the inhibition zone decreased from 21 mm (for *S. alata* alone) to 19 mm in the combination. Conversely, synergistic effects were observed against *E. coli* and *K. pneumoniae*, with inhibition zones increasing from 18 mm to 24 mm and 15 mm to 20 mm, respectively. These findings contrast with previous studies (16,23), which reported strong synergistic interactions across all tested combinations of medicinal plant extracts and no antagonistic effects. The observed variation may be attributed to differences in the specific plant species used, extraction methods, phytochemical compositions, or microbial strains used in the studies. Adal et al., (24) similarly reported that *S. alata* extracts were effective against both Gram-positive and Gram-negative bacteria, particularly when using organic solvents. Their findings aligns with the results of the current study regarding antimicrobial activity of *S. alata* extracts against *S. aureus* and *P. aeruginosa*.

The *in vitro* antimicrobial susceptibility of the selected pathogens to the antimicrobial agents commonly used for the treatment UTIs showed significant insights into the effectiveness of the various antibiotics against these uropathogens in our study. Imipenem was identified as the most potent antibiotic against all tested pathogens, which is consistent with findings from other studies that emphasize its effectiveness against multidrug-resistant bacteria (25,26). However, the growing concern of resistance to carbapenems, particularly in hospital settings, has been documented in other studies, indicating that while imipenem remains effective, its use should be monitored closely due to potential resistance development (27).

The patterns of the pathogen susceptibility observed align with previous research, highlighting the challenges in managing UTIs due to varying resistance patterns (28). Imipenem and gentamicin demonstrated minimal activity against *S. aureus*, with zone diameter of inhibition (ZDI) of 25 mm and 15 mm respectively, consistent with findings of Rahim et al., (29) who reported significant resistance of *S. aureus* to these antibiotics. *K. pneumoniae* were largely susceptible to imipenem (ZDI: 27mm) and gentamicin (ZDI: 26mm), reinfor-

cing their effectiveness as treatment options (30,31). Similarly, ceftazidime (ZDI: 30 mm) showed strong activity against *K. pneumoniae* as observed in other studies (32). *E. coli* demonstrated the highest susceptibility to imipenem (ZDI: 35 mm), further confirming its potency against this common uropathogen. *P. aeruginosa* exhibited susceptibility to gentamicin (ZDI: 26 mm) and imipenem (ZDI: 26 mm), supporting the literature that identifies these antibiotics as first-line treatments (36). Fluconazole showed high inhibitory activity against *C. albicans* (ZDI: 26 mm), although susceptibility to fluconazole has been reported to vary by strain of *Candida* and location (37). Overall, imipenem was the most potent antibiotic against all tested bacterial pathogens, consistent with other studies, highlighting its role against multidrug-resistant strains (38). However, the increasing concern of carbapenem resistance, particularly in the hospital settings, underscores the need for careful monitoring of its use (39).

Synergistic testing of the plant extracts combined with conventional antibiotics evaluated in this study revealed notable synergistic interactions, particularly with *S. alata* extracts. Among the antibiotics tested, imipenem exhibited the highest synergistic activity when combined with both the methanolic and aqueous extracts of *S. alata*, especially against *S. aureus*, where the inhibition zones markedly increased from 25mm (for imipenem disc alone) to 48 mm and 46 mm, respectively. Amoxicillin-clavulanic acid also displayed substantial synergistic effects in combination with *S. alata* extracts with inhibition zones increasing from 6-28 mm (for amoxicillin-clavulanic acid alone) to 19-40 mm when combined with the plant extracts. Gentamicin showed mild synergism ranges from 13-31 mm compared to 15-26 mm. Similarly, cefoxitin demonstrated synergism with inhibition zones ranging from 6-20 mm alone and increasing to 19-30 mm when combined with *S. alata* extracts.

In contrast, ceftazidime demonstrated weaker synergistic or indifference effects across all tested bacterial pathogens, with inhibition zones ranging from 8-30 mm for ceftazidime disc alone, and 9-31 mm when combined with *S. alata* extract. Nitrofurantoin also exhibited weak synergistic effects, with inhibition zones increasing slightly from 6-10 mm to 7-16 mm across all pathogens. The anti-fungi drug, fluconazole, when combined with *S. alata* extracts, exhibited antagonistic interaction against *C. albicans*, as the inhibition zone decreased from 26 mm to 21 mm.

Synergistic activity was also observed when imipenem was combined with both methanolic and aqueous extracts of *E. hirta*, particularly against *P. aeruginosa* (inhibition zone of 43 mm for both extracts) and *S. aureus* (41 mm and 38 mm for methanol and aqueous extracts respectively), but no synergistic effect was observed against *E. coli*, with inhibition zones of 20 mm and 21 mm, respectively. Interestingly, amoxicillin-clavulanic acid in combination with *E. hirta* also showed consistent synergism across all tested pathogens, with inhibition zones ranging from 18-40 mm. Cefoxitin showed inhibition zones of 6-31 mm, nitrofurantoin 6-14 mm, gentamicin 11-28 mm, and ceftazidime 6-34 mm when combined with *E. hirta* extracts. Contrastingly to *S. alata* extracts, fluconazole combined with *E. hirta* extracts showed enhanced synergistic activity against *C. albicans*, with inhibition zones increasing from 26 mm to 30 mm. These findings are consistent with those reported by Alam et al., (40) and Acharjee et al., (41) who in separate studies reported enhanced antimicrobial activities when plant extracts were combined with standard antibiotics. The observed synergistic interactions highlight the therapeutic potential of integrating medicinal plant extracts with conventional antibiotics as a strategy to enhance antimicrobial efficacy and mitigate the global issue of antibiotic resistance.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) results further reinforce the potency of methanol extracts over aqueous extracts. The methanol extracts of *E. hirta* methanol extract produced lower MIC values, ranging from 6.25 mg/ml against *P. aeruginosa* to 50mg/ml against *S. aureus*, whereas aqueous extracts ranged from 25mg/ml to 100mg/ml. These results agree with the study by Mehmood et al., (42), who reported similar trends in lower MIC values for methanol extracts of medicinal plants. Similarly, methanol extract of *S. alata* produced MIC values as low as 6.25 mg/ml against *C. albicans* but as high as 50mg/ml for *K. pneumoniae* and *E. coli*, contrasting with aqueous extracts which produced very high MIC values of up to 100 mg/ml.

These findings agree with the study of other researchers (42,43), who reported that methanol extracts of medicinal plants tend to produce lower MIC and MBC values compared to aqueous extracts. The MBC test showed that methanol extracts of both *E. hirta* and *S. alata* exhibited stronger bactericidal effects compared to aqueous extracts, with MBC of the methanol extract of *E. hirta* ranging from 25mg/ml against *P. aeruginosa* to 100mg/ml against *S. aureus*, while aqueous extracts ranged from 100mg/ml for *C. albicans* to 200 mg/ml for *S. aureus*. Similarly, methanol extract of *S. alata* produced MBC values that ranged from 25 mg/ml for *C. albicans* to 100 mg/ml for *K. pneumoniae*, whereas the aqueous extracts ranged from 100mg/ml for *C. albicans* to 200mg/ml for *K. pneumoniae*. These findings agree with previous studies, demonstrating the superiority of methanol ex-

tracts in extracting bioactive compounds responsible for antimicrobial activity. For example, Ghaffar and Perveen (44) reported that methanol extracts of medicinal plants often produce lower MBC values due to their ability to dissolve both polar and non-polar phytochemicals, unlike aqueous extracts that mainly extract polar compounds. Furthermore, Mosallaie et al., (45) reported that phenolics and alkaloids, commonly extracted by methanol, are crucial contributors to bactericidal activity, supporting our findings in this study.

Our study has some limitations. First, the study was entirely an *in vitro* evaluation, which may not fully reflect the complex interactions that occur within a living organism. The observed antimicrobial and synergistic effects may differ *in vivo* due to factors such as bioavailability, metabolism, and host immune system. Second, our study utilized crude methanol and aqueous extracts, which contain a mixture of several phytochemicals. This may mask the individual effect of specific bioactive compounds, and difficulty in identifying the potential synergistic or antagonistic interactions among the phytochemicals.

Conclusion:

Nevertheless, this study highlights the significant antimicrobial properties of both methanol and aqueous extracts of *S. alata* and *E. hirta*. The methanol extracts consistently exhibited higher extraction yields and greater antimicrobial activities compared to the aqueous extracts. Furthermore, the observed synergistic interactions with antibiotics such as imipenem underscore the therapeutic potentials of these medicinal plants, especially in the context of combating antimicrobial resistant pathogens.

Contributions of authors:

BRT carried out the research and wrote the draft of the manuscript; DZA, MZK, SHB, SHU and YF collected the samples and prepared them for extraction; OBO supervised the project and revised the manuscript; EAH co-supervised the project and revised the manuscript; AM and MTO carried out the statistical analysis. All authors read and approved the manuscript submitted for publication.

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Original Article **Open Access**

Phytochemical screening, *in vitro* anti-fungal activities, and time-kill kinetics of methanol extracts of *Nauclea latifolia* against selected dermatophytes

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Abstract:

Background: Dermatophytosis is a highly contagious superficial mycosis of the genera *Trichophyton, Epidermophyton* and *Microsporum* that affects both man and animal. The different plant parts of *Nauclea latifolia* are known to have ethnomedicinal uses. The objective of this study is to access the phytochemical components of *N. latifolia* plant parts and to investigate the anti-fungal activities and time-kill kinetics of the plant parts against isolated dermatophyte species.
Methodology: The pulverized parts (root, stem, leaf, fruit) of authenticated *N. latifolia* plant were extracted using cold maceration method for 48 hours in methanol. The extracts were then concentrated almost to dryness and the phytochemical constituents assessed. The preliminary screening of the anti-dermatophytic activities of the extracts was determined by the agar disc diffusion technique on selected dermatophytes, consisting of two isolates of *Microsporum ferrugineum* (strains 1 and 2), two isolates of *Trichophyton mentagrophytes* (strains 1 and 2), one isolate of *Microsporum audouinii* and one isolate of *Microsporum canis.* The minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and time-kill kinetics of the extracts were determined using standard methods on the selected dermatophytes.
Results: The pulverized stem bark of the plant had higher yield of 12.73% when compared to other plant parts (leaf; 4.44%, root; 1.53%, and fruit; 4.16%). All extracts of the plant parts contain flavonoids and tannins, with the stem bark and fruit extracts containing tannins in higher concentrations. The leaf and root extracts of the plant contained saponin, but this is absent in the stem bark and fruit extracts. There were no zone diameters of inhibition produced by different concentrations of the root and leaf extracts against the selected dermatophytes while the mean zone diameters of inhibition produced by the stem bark and fruit extracts was appreciably low (range 2.0±00-10±2.00mm) at 500mg/ml of the extracts. The MICs and MFCs were the same for stem bark and fruit extracts at 62.5mg/ml for *T. mentagrophyte* strain 2 and *M. ferrugineum* strain 2, and at 250mg/ml for *M. audouinii*. Similarly, the MICs and MFCs were the same for the stem bark extracts at 125mg/ml for *T. mentagrophyte* strain 1. In the time-kill assay, reduction in the count of the viable cells occurred within 0-1 hour, and at 8 hours, there were no viable cells.
Conclusion: Crude methanol extracts of *N. latifolia* stem bark and fruit exhibited fungicidal activity against the selected dermatophytes, albeit at high concentration above what is considered to indicate potential efficacy of an extract for further therapeutic exploration. Nevertheless, the time-kill assay result holds promise that the stem bark and fruit extracts of the plant potentially possess anti-dermatophytic activity that could justify the plant use for treating dermatophyte infections.

Keywords: Dermatophyte, phytochemicals, methanol extraction, time-kill kinetics, *Nauclea latifolia*

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Criblage phytochimique, activités antifongique *in vitro* et cinétique de destruction temporelle des extraits méthanoliques de *Nauclea latifolia* contre certains dermatophytes

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Résumé:

Contexte: La dermatophytose est une mycose superficielle très contagieuse des genres *Trichophyton*, *Epidermophyton* et *Microsporum* qui touche aussi bien l'homme que l'animal. Les différentes parties de la plante *Nauclea latifolia* sont connues pour leurs usages ethnomédicaux. L'objectif de cette étude est d'identifier les composants phytochimiques de parties de *N. latifolia* et d'étudier leurs activités antifongique et leur cinétique d'élimination contre des espèces de dermatophytes isolées.
Méthodologie: Les parties pulvérisées (racine, tige, feuille, fruit) de *N. latifolia* authentifiée ont été extraites par macération à froid pendant 48 heures dans du méthanol. Les extraits ont ensuite été concentrés presque jusqu'à siccité et leurs constituants phytochimiques ont été évalués. Le criblage préliminaire des activités anti-dermatophytiques des extraits a été déterminé par la technique de diffusion sur disque d'agar sur des dermatophytes sélectionnés, constitués de deux isolats de *Microsporum ferrugineum* (souches 1 et 2), de deux isolats de *Trichophyton mentagrophytes* (souches 1 et 2), d'un isolat de *Microsporum audouinii* et d'un isolat de *Microsporum canis*. La concentration minimale inhibitrice (CMI), la concentration minimale fongicide (CMF) et la cinétique de tuer le temps des extraits ont été déterminées à l'aide de méthodes standard sur les dermatophytes sélectionnés.
Résultats: L'écorce de tige pulvérisée de la plante présentait un rendement supérieur de 12,73% à celui des autres parties de la plante (feuilles; 4,44%, racines; 1,53% et fruits; 4,16%). Tous les extraits de parties de la plante contiennent des flavonoïdes et des tanins, les extraits d'écorce de tige et de fruits contenant des concentrations plus élevées de tanins. Les extraits de feuilles et de racines de la plante contenaient de la saponine, mais celle-ci est absente dans les extraits d'écorce de tige et de fruits. Français Il n'y avait pas de diamètres de zone d'inhibition produits par les différentes concentrations des extraits de racines et de feuilles contre les dermatophytes sélectionnés tandis que les diamètres moyens de zone d'inhibition produits par les extraits d'écorce de tige et de fruit étaient sensiblement faibles (plage de 2,0±00 à 10±2,00 mm) à 500mg/ml des extraits. Les CMI et les CMF étaient les mêmes pour les extraits d'écorce de tige et de fruit à 62,5mg/ml pour la souche 2 de *T. mentagrophyte* et la souche 2 de *M. ferrugineum*, et à 250mg/ml pour *M. audouinii*. De même, les CMI et les CMF étaient les mêmes pour les extraits d'écorce de tige à 125mg/ml pour la souche 1 de *T. mentagrophyte*. Dans le test de tuer le temps, la réduction du nombre de cellules viables s'est produite dans un délai de 0 à 1 heure, et à 8 heures, il n'y avait plus de cellules viables.
Conclusion: Des extraits méthanoliques bruts d'écorce de tige et de fruit de *N. latifolia* ont montré une activité fongicide contre les dermatophytes sélectionnés, bien qu'à une concentration supérieure à celle considérée comme indiquant l'efficacité potentielle d'un extrait pour une exploration thérapeutique ultérieure. Néanmoins, les résultats du test de temps de destruction laissent entrevoir une activité anti-dermatophytique potentielle des extraits d'écorce de tige et de fruit de la plante, justifiant ainsi son utilisation pour le traitement des infections dermatophytiques.

Mots-clés: Dermatophyte, composés phytochimiques, extraction au méthanol, cinétique de temps de destruction, *Nauclea latifolia*

Introduction:

Dermatophyte fungal diseases are part of the frequently occurring infectious diseases in the universe and amongst the most commonly diagnosed skin infections in Africa in which the dermatophyte spectrum and the clinical symptoms are completely different from the ones seen in other parts of the world, probably because African environment is hot and humid giving it the main reason for the high prevalence (1). They are contagious and are caused by group related fungi in the genera of *Trichophyton, Microsporum* and *Epidermophyton* (2). Infection with any of the disease-causing dermatophyte species result in dermatophytosis and affects both man and animal with grave economic impacts (3,4).

Resistance and the after effects from the usage of common topical and systemic antifungal medications for dermatophytosis emphasizes the necessity for novel therapies (5). *Nauclea latifolia* is a multi-stemmed evergreen woody plant that can reach a height of 200m, commonly found in the Savannah forests and wet tropical rainforest zones of West and Central Africa (6). The phytochemical components displayed positive results in healing many diseases through different processes such as antioxidative, anti-secretory, anti-inflammatory, anti-ulcerogenic and anti-hypertensive effects (7).

Phytochemicals are substances in plants that occur naturally and play an important role in defense mechanisms to protect them from diseases (8). *Nauclea latifolia* has served as herbal medicine for treating different ailments and diseases (9). Bekoe et al., (10) reported that the usage of *N. latifolia* in treating viral diseases, hypertension, stomach pain, helminthiasis, back pain, diabetes, fever, cancer, gastric ulcer, malaria, measles, diarrhoea and conjunctivitis have been well established. It is therefore, imperative to assess the anti-fungal properties of *N. latifolia* extracts on dermatophytes, with the aim of providing alternative therapeutics of dermatophytosis.

Materials and method:

Study area and design:

The study was conducted at Nnamdi Azikiwe University, Awka, Anambra State, Nigeria as an evaluation of the qualitative phytochemical constituents of methanol extracts of *N. latifolia* plant parts (leaf, stem bark, root and fruit), and determination of *in vitro* anti-dermatophyte activity and time kill kinetics of the extracts against selected dermatophytes obtained from dermatophytic lesions of primary school pupils (with consent from their parents).

Ethical approval:

Ethical approval to carry out the study was obtained from Nnamdi Azikiwe University Teaching Hospital (NAUTH) Ethical Committee

Plant collection, identification, preparation and extraction:

Nauclea latifolia plant was identified and authenticated by a Botanist at Botany Department of Nnamdi Azikiwe University, Awka, Nigeria. Fresh mature leaves, roots, stem barks and fruits of the plant were collected and separately dried under shade. They were pulverized separately and stored in brown bottles in dry environment until ready for extraction. Pulverized materials of *N. latifolia* leaves (170 g), roots (130g), stem barks (150g), fruits (143g), were extracted using cold maceration method for 48 hours in methanol.

Methanol extracts of the plant parts were collected, and then concentrated almost to dryness (MeOH fraction) under vacuum at 45 ± 5^0C using rotary evaporator. The resultant fractions were separately stored at 4^0C. The crude methanol extracts were screened for phytochemical constituents. The percentage yield was calculated using the formula as shown below; percentage yield = weight of extract/weight of pulverized materials of *N. latifolia* x 100 (11).

Phytochemical analysis:

Qualitative phytochemical analysis was carried out using the methods of Trease and Evans (12) to determine the presence of the different phytochemicals in the leaves, roots, stem barks and fruits of the tested plant.

Preparation of stock solution:

The stock solutions were prepared by dissolving 1000mg of the plant part in 2 ml of DMSO for a final concentration of 500 mg/ml to be obtained and used for the preliminary screening of the anti-dermatophyte activities of the extracts. This was done for each of the plant part extract. For determining the MICs, stock solution of the extract of plant was prepared by dissolving 10,000 mg of the extract in 2 ml of DMSO to obtain a final concentration of 5000mg/ml. These were transferred to screw capped bottles and kept at 4°C until ready for use.

Test dermatophyte organisms:

These were dermatophyte organisms (six isolates) previously identified from clinical samples collected from primary school pupils whom their parents consented, and consist of two isolates of *Microsporum ferrugineum*, two isolates of *Trichophyton mentagrophytes*, one isolate of *Microsporum audouinii* and one isolate of *Microsporum canis.* These organisms were maintained by monthly sub-culturing on Sabouraud Dextrose Agar (SDA) at 25-27°C.

Inoculum standardization of test organisms:

All the test isolates were inoculated onto SDA plates and incubated for 7–10 days at a temperature of 25°C to obtain young, actively growing cultures consisting of mycelia and conidia. Mycelial disc of 5 mm in diameter, cut from the periphery of the 7–10-day-old cultures, was aseptically inoculated into tubes containing Sabouraud dextrose broth. The tubes were incubated at 25 °C for 2-3 days. Following incubation, the tubes were placed on a vortexing machine and vortexed for about 15-20 min to properly disperse the cells in the broth.

The organism concentration in the tubes was standardized by adjusting to concentration which was about 10^4 CFU/ml from 10^8 CFU/ml already produced from standardizing to 0.5 MacFarland standard. The control used in time kill kinetics was dimethylsulphoxide which did not contain any dermatophyte.

Preliminary testing of the extracts for anti-dermatophyte action:

The preliminary testing of the extracts for anti-dermatophyte activities was carried out by the agar well diffusion procedure using Onyegbule et al., (13) with slight modifications. Dilutions of 250, 125, 62.5, 31.25, and 15.125mg/ml were prepared from the 500mg/ml stock solution of the extracts by doubling dilution of the extracts. Molten SDA (20ml) was poured into sterile petri dishes (90 mm) and left to gel. The aseptically prepared agar plates with holes (6 mm) made in them using a sterile metal cork-borer were swabbed with standardized concentrations (10^4 CFU/ml) of cultures of test isolates grown in Sabouraud dextrose broth.

A 20µl volume of the different dilutions of each extract and the controls were put in each hole under aseptic condition and kept at room temperature for about 30 minutes to allow the extracts and controls to diffuse into the agar medium and incubated accordingly. Ketoconazole (50µg/ml) was utilized as positive control and dimethylsulphoxide (DMSO) as the negative control. The plates were incu-

bated for 24-28 hours at room temperature for fungal isolates and the inhibition zones diameters (IZDs) were measured and recorded. The cork borer (6mm) size was subtracted from the figures recorded for the IZDs to get the actual figure. The process was conducted for three times and the mean IZDs calculated and recorded.

Determination of MIC of extracts on isolates:

The MIC of the extract of the plant parts on test isolates was carried out by agar dilution method using the method described by Ali-Shtayeh and Suheil (14). The extract stock solution (5000 mg/mL) was further diluted in a 2-fold serial dilution to obtain the following extract concentrations; 2500, 1250, 625, 312.5, 156.25, 78.26, 39.12, 19.56 and 9.78mg/ml. The agar plates were made by pouring 9ml of the molten double strength SDA into sterile Petri dishes that contained 1 ml of the different dilutions of the extract to make the final extract concentrations of 500, 250, 125, 62.5, 31.25, and 15.625, 7.826, 3.912, 1.956 and 0.978 mg/ml respectively.

The test isolates which were grown overnight in broth were adjusted to McFarland 0.5 standard and streaked onto the agar plates (containing dilutions of the extract) surface. The SDA plates were incubated at room temperature (25-27^{o}C) for 5-7 days, after which all plates were checked for growth. Control plates, which contained no plant extract, were also made with the test. The minimum dilution (concentration) of the extracts completely inhibiting the growth of each organism was regarded as the MIC.

Determination of MFCs of extracts on isolates:

The MFC of the extracts was carried out by subculturing portions of the agar from the plates that showed no growth in the MIC tests as described by Ali-Shtayeh and Suheil (14). The agar parts were plated into plates containing freshly prepared plain SDA which were incubated at room temperature (25-27^{o}C) for 5-7 days and checked every day for fungal growth. The absence of growth at the conclusion of incubation period implies total cell death. The minimum concentration of the extracts that produced total cell death was considered as the MFC.

Determination of fungicidal action of extracts on isolates by time-kill kinetics:

Determination of fungicidal action of the extracts on test isolates by time-kill kinetics was performed using the procedure of Adeniyi et al*.,* (15) with slight modifications**.** Standardized concentrations (10^4 CFU/mL) of logarithmic phase culture of test isolates were prepared. An appropriate quantity of the extract was added to a sterile test tube containing Sabouraud dextrose broth, and 1ml of the standardized test culture was added to 9 ml of the extract-broth mixture to give a microbial concentration 10^3 CFU/mL and a concentration equal to the MFC of the extract.

Sterile molten SDA was poured into sterile Petri plates and allowed to set. 0.1ml of the extract-broth-culture mixture was put onto the agar and spread with a sterile spreader. This is to give control time 0 minutes count. Samples were taken after 1, 4, 8, and 24 hours intervals. The procedure was done in triplicate to ensure accuracy. The agar plates were incubated at 25-27^{O}C for 5-7days and observable colonies were counted. Each of the organisms was also grown in tubes containing broth with no added plant extract (control) and samples taken at the indicated time intervals and treated. Control plates were similarly incubated. The numbers of colony forming units (CFU) were counted after the period of incubation.

Data analysis:

The Student's *t*-test was used to compare the mean inhibition zone diameter produced by the plant part extracts against the dermatophytes, with the Statistical Packages for the Social Sciences (SPSS) version 26.0. The level of significance was set at $p<0.05$.

Results:

Extract yield of pulverized *N. latifolia* plant:

The percentage extract yield of the pulverized *N. latifolia* stembark was highest at 12.73%, followed by leaf (4.44%), fruit (4.16%) and roots (1.53%) (Table 1).

Phytochemical constituents of *N. latifolia* plant extracts:

Tannin was observed to be in high quantities in stem bark and fruit but moderate in root and leaf. Flavonoid was present in high quantities in all the plant parts (leaf, root, stem bark and fruit). Alkaloid, steroid and terpenoid were absent in all the parts of the plant. Saponin was absent in stem bark and fruit but present in moderate quantities in root and leaf (Table 2).

Table 1: Percentage extract yield of the pulverized *Nauclea latifolia* plant parts

Extract	Pulverized materials (g)	Yield (g)	% yield
Leaf	170	7.55	4.44
Root	150	2.29	1.53
Stem bark	130	16.55	12.73
Fruit	143	5.95	4.16

Table 2: Phytochemical constituents of *Nauclea latifolia* plant Extracts.

Phytochemical constituents	Extracts			
	Leaf	Root	Stem bark	Fruit
Alkaloid	-	-	-	-
Saponin	++	++	-	-
Steroid	-	-	-	-
Terpenoid	-	-	-	-
Flavonoid	+++	+++	+++	+++
Tannin	+++	+++	++++	++++

+ = low, ++ = moderate, +++ = high, ++++ = very high

Anti-dermatophyte activities of *N. latifolia* extracts:

The mean (±SD) inhibition zone diameters at different concentrations of the extracts of the plant parts against the selected dermatophytes are shown in Fig 1 for the stem bark and Fig 2 for the fruit. In Fig 1, the crude methanol extract of *N. latifolia* stem bark against the test dermatophytes at the least concentration of 62.5mg/ml, produced mean zone diameter of inhibition of 1 mm to *T. mentagrophyte* strain 2, 3 mm to *M. ferrugineum* strain 1 and 2 mm to *M. ferrugineum* strain 2*.* At 125 mg/ml, it was 2 mm to *T. mentagrophyte* strain 1, 3 mm to *T. mentagrophyte* strain 2*,* 6 mm to *M. ferrugineum* strain 1 and 5 mm to *M. ferrugineum* strain 2.

At concentration of 250 mg/ml, it was 4mm to *T. mentagrophyte* strain 1*,* 6mm to *T. mentagrophyte* strain 2, 9mm to *M. ferrugineum* strain 1 and 8 mm to *M. ferrugineum* strain 2. At the highest concentration of (500 mg/ml,) the extract produced mean zone diameter of 7 mm to *T. mentagrophyte* strain 1, 9 mm to *T. mentagrophyte* strain 2, 3 mm to *M. audouinii*, 2 mm to *M. canis*, 12 mm to *M. ferrugineum* strain 1 and 10 mm to *M. ferrugineum* strain 2.

In Fig 2, the mean inhibitory zone diameters (mm) produced by the crude methanol extract of *N. latifolia* fruits against the test dermatophytes at the least concentration of 62.5 mg/ml, was 2 mm to *T. mentagrophyte* strain 2, 3 mm to *T. ferrugineum* strain 1 and 2 mm to *M. ferrugineum* strain 2. At the concentration of 125 mg/ml, it was 3mm to *T. mentagrophyte* strain 1, 4 mm to *T. mentagrophyte* strain 2, 6 mm to *M. ferrugineum* strain 1 and 5 mm to *T. ferrugineum* strain 2. At the concentration of 250 mg/ml the extracts produced 5 mm to *T. mentagrophyte* strain 1, 7 mm to *T. mentagrophyte* strain 2, 9 mm to *M. ferrugineum* strain 1 and 8 mm to *M. ferrugineum* strain 2.

At the highest concentration of 500 mg/ml, it produced 8 mm to *T. mentagrophyte* strain 1, 10 mm to *T. mentagrophyte* strain 2, 2 mm to *M. audouinii*, 2 mm to *M. canis*, 12 mm to *M. ferrugineum* 1 and 10 mm to *M. ferrugineum* strain 2. The positive control (ketoconazole at 50 µg/ml) produced mean inhibition zone diameter of 28 mm to *T. mentagrophyte* strain 1, 30 mm to *T. mentagrophyte* strain 2*,* 10 mm to *M. audouinii,* 8 mm to *M. canis,* 36 mm to *M*. *ferrugineum* strain 1 and 32 mm to *M*. *ferrugineum* strain 2. The negative control (DMSO) produced no inhibition zone diameter to the selected dermatophytes.

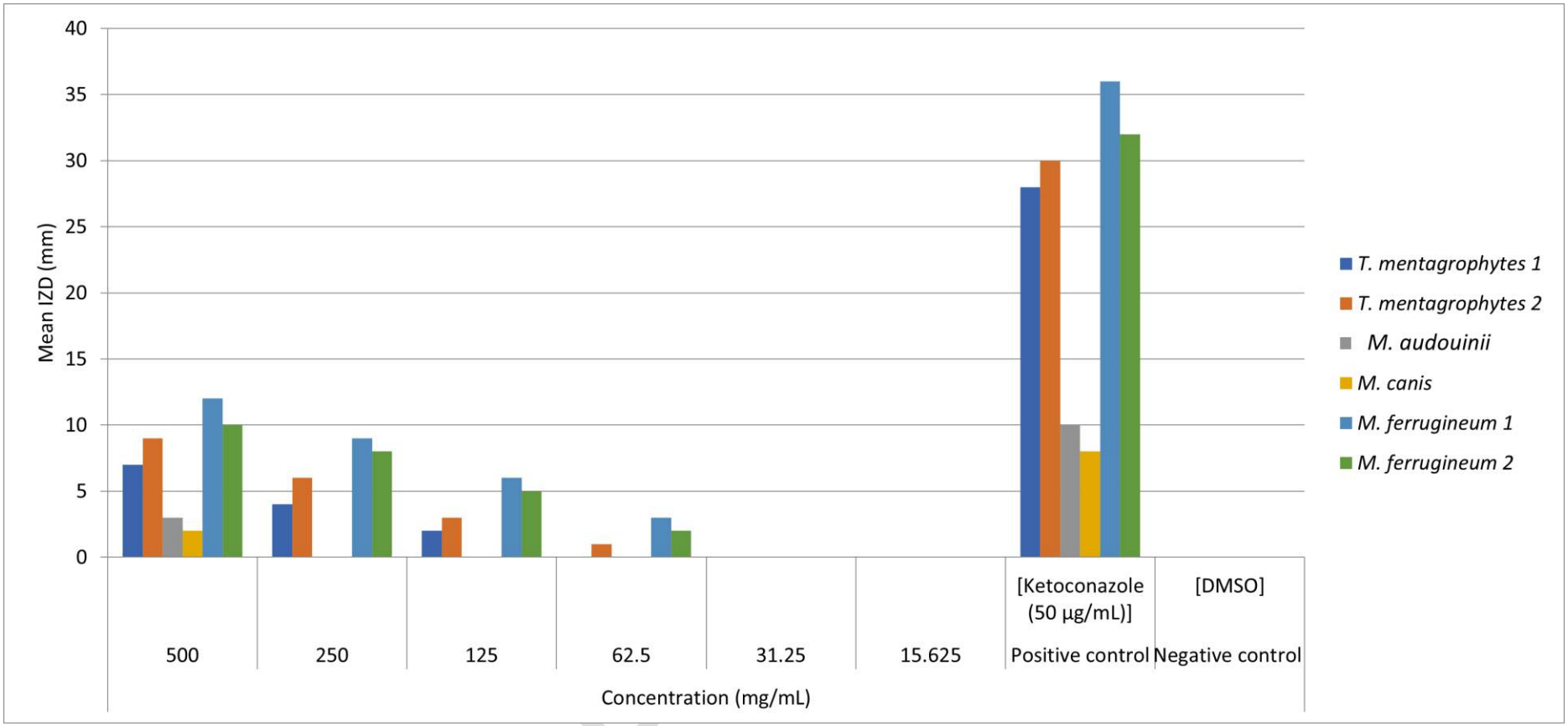

Fig 1: Mean inhibition zone diameters (IZDs) in millimeters (mm) produced by crude methanol extract of *Nauclea latifolia* stem barks and the control against selected dermatophytes

GALE

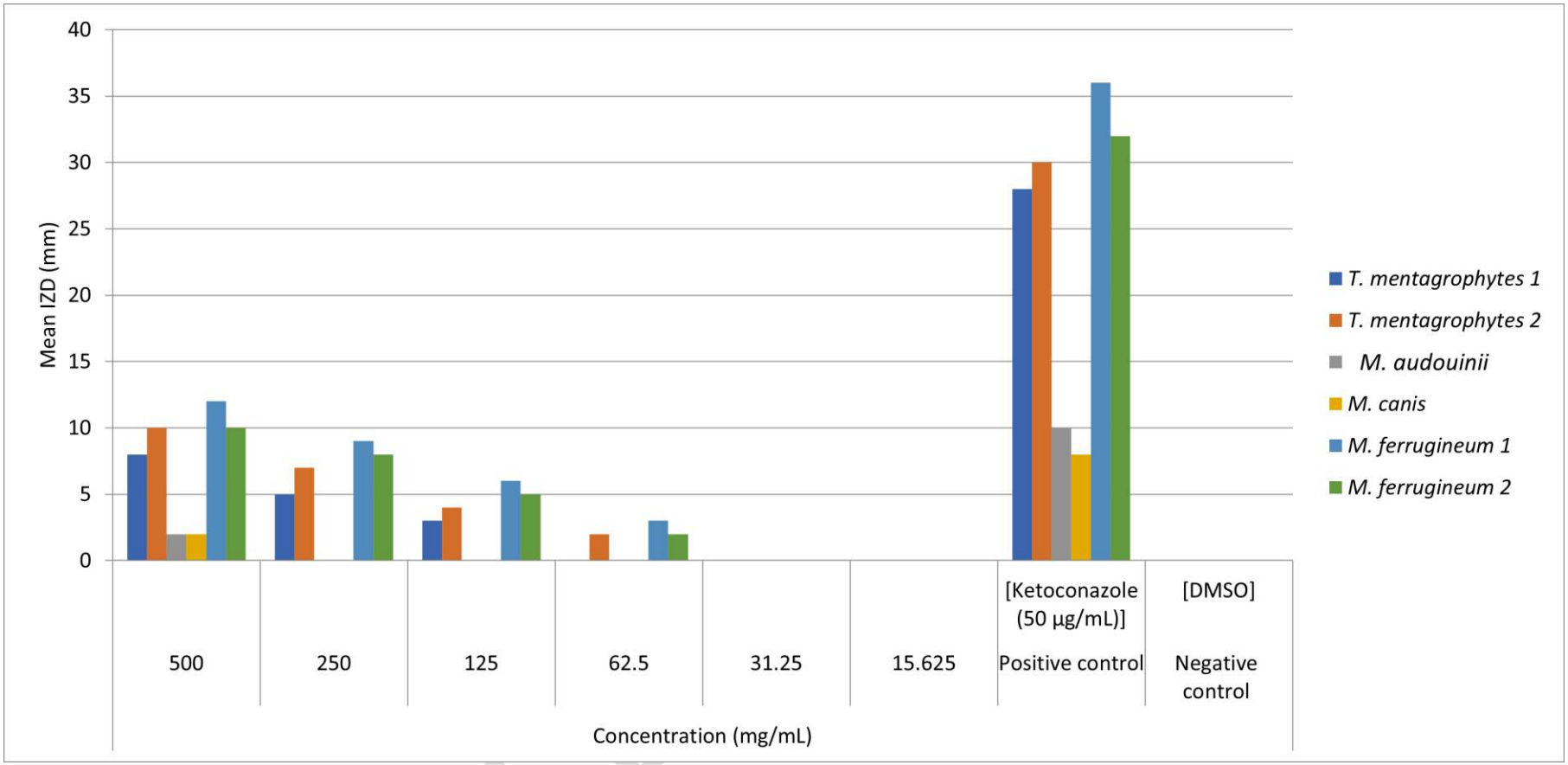

Fig 2: Mean inhibition zone diameters (IZDs) in millimeters (mm) produced by crude methanol extract of *Nauclea latifolia* fruits and the controls against selected dermatophytes

GALE

Table 3 shows the comparative mean (±SD) inhibition zone diameter of the stem bark and fruit extracts of the plant at 500 mg/ml concentration (the concentration at which the extracts exhibited some antimicrobial activities against the selected dermatophytes). The results showed that there were no significant differences in the antimicrobial activities of the two plant parts at 500 mg/ml concentration against the selected dermatophytes (*T. mentagrophytes* 1and 2; *M. ferrugineum* 1 and 2; *M. audouinii* and *M. canis*) (p>0.05). The leaf and root extracts were not considered because even at this high concentration, the extracts did not give any zone of inhibition against the dermatophytes.

Minimum inhibitory and fungicidal concentrations of the plant extracts:

The leaf and the root extracts of *N. latifolia* had no antimicrobial activity on all the selected dermatophytes. The minimum inhibitory concentration (MIC) of the stem bark and fruit extracts of *N. latifolia* against *M. audouinii* and *M. canis* was 250 mg/ml while against *T. mentagrophyte* strain 2, the MIC was 62.5 mg/ml. For *M. ferrugineum* strain 1; the MIC was 31.25 mg/ml, and *M. ferrugineum* strain 2 it was 62.5 mg/ml.

The minimum fungicidal concentration (MFC) was the same as the minimum inhibitory concentration (MIC) of the fruit and stem barks extracts against *T. mentagrophyte* strain 2 (62.5mg/ml) and *T. ferrugineum* strain 2 (62.5mg/ml), *T. mentagrophyte* strain 1 stem bark extracts (125mg/ml) and *M. audouinii* stem bark extract (250 mg/ml). The MFC of the stem bark extract against *M. canis* was 500 mg/ml and for *M. ferrugineum* strain 1 was 62.5 mg/ml. The MFC of the fruit extract against *T. mentagrophyte* strain 1 was 125 mg/ml, *M. audouinii* 500 mg/ml, *M. canis* 500 mg/ml and *M. ferrugineum* strain 1 62.5 mg/ml (Table 4).

Table 3: Comparison of mean inhibition zone diameter of the stem bark and fruit extracts at 500mg/ml concentration

Organisms	**Means (±SD) zone diameter (mm) of inhibition**		***t*-test**	***p*-value**
	Stem bark	**Fruit**		
Trichophyton mentagrophytes 1	7.00±1.00	8.00±1.00	-1.225	0.288
Trichophyton mentagrophytes 2	9.00±1.00	10.00±2.00	-0.775	0.482
Microsporum audouinii	3.00±0.00	3.00±1.00	<0.001	1.000
Microsporum canis	2.00±0.00	2.00±0.00		
Microsporum ferrugineum 1	8.67±4.16	12.00±2.00	-1.250	0.279
Microsporum ferrugineum 2	10.00±2.00	10.00±2.00	<0.001	1.000

Table 4: Minimum inhibitory and fungicidal concentrations of the extracts on selected dermatophytes

Test dermatophyte isolates	**MICs of extracts (mg/ml)**		**MFCs of extracts (mg/ml)**	
	Stem bark	**Fruit**	**Stem bark**	**Fruit**
Trichophyton mentagrophytes 1	125	62.5	125	125
Trichophyton mentagrophytes 2	62.5	62.5	62.5	62.5
Microsporum audouinii	250	250	250	500
Microsporum canis	250	250	500	500
Microsporum ferrugineum 1	31.25	31.25	62.5	62.5
Microsporum ferrugineum 2	62.5	62.5	62.5	62.5

MIC = Minimum inhibitory concentration; MFC = Minimum fungicidal concentration

Time kill assay:

Results of the time kill assay shown in Tables 5 and 6 indicated that within 0-1 hour, there was drastic reduction in the number of viable cell count. Within the succeeding 1–4 hours, there was also reduction but at the 8th hour, all the dermatophyte isolates were dead. The control showed an increase in the viable cell count of each of the selected dermatophyte even after the 8th hour (Table 7).

Table 5: Fungicidal action of crude methanol extract of *Nauclea latifolia* stem barks incorporated into Sabouraud dextrose broth against selected dermatophytes

Test dermatophyte isolates	Concentration (mg/ml)	Incubation period (hours)	0	1	4	8	24
Trichophyton mentagrophytes 1	125	Viable Cell Count ($x10^4$CFU/mL)	301	102	32	0	0
Trichophyton mentagrophytes 2	62.5		356	141	20	0	0
Microsporum audouinii	250		246	72	22	0	0
Microsporum canis	500		296	88	16	0	0
Microsporum ferrugineum 1	62.5		322	112	34	0	0
Microsporum ferrugineum 2	62.5		282	198	21	0	0

Table 6: Fungicidal action of crude methanol extract of *Nauclea latifolia* fruits incorporated into Sabouraud dextrose broth against selected dermatophytes

Test dermatophytes isolates	Concentration (mg/ml)	Incubation period (hrs)	0	1	4	8	24
Trichophyton mentagrophytes 1	125	Viable Cell Count ($x10^4$CFU/mL)	264	82	11	0	0
Trichophyton mentagrophytes 2	62.5		291	69	8	0	0
Microsporum audouinii	500		272	91	19	0	0
Microsporum canis	500		265	96	13	0	0
Microsporum ferrugineum 1	62.5		332	121	25	0	0
Microsporum ferrugineum 2	62.5		278	83	16	0	0

Table 7: Viable cell counts of selected dermatophytes in Sabouraud dextrose broth without incorporation of extracts

Test dermatophyte isolates	Incubation period (hrs)	0	1	4	8	24
Trichophyton mentagrophytes 1	Viable Cell Count ($x10^4$CFU/mL)	353	728	1222	1748	2386
Trichophyton mentagrophytes 2		421	879	1325	1932	2658
Microsporum audouinii		242	644	1080	1620	2324
Microsporum canis		286	682	1040	1688	2232
Microsporum ferrugineum 1		341	779	1125	1871	2654
Microsporum ferrugineum 2		222	642	1082	1620	2128

Discussion:

Medicinal plants have since been recognized as notable source for treating variety of diseases and might be regarded as precursor of contemporary medicine (16,17,18). In this study, pulverized *N. latifolia* stem bark yielded the highest extract percentage, followed by the leaf, fruit and root, and contained tannin, flavonoids, and saponin. This result agrees with that of Malami et al., (19) on the leaves extract of *N. latifolia* containing flavonoids, tannins and saponins, and also with the study of Adepoju et al., (20) on the stem bark of *N. latifolia* containing tannin. However, the methanol extracts of the leaf, root, stem bark, and fruit in this study did not contain alkaloids, steroids and terpenoids that were present in the study of Malami et al., (19) and Adepoju et al., (20). If these were present in the extracts in our study, they might have been so minimal such that the crude methanol extraction method we used could not qualitatively detect them. In the study of ethanol and methanol leaf extracts of *N. latifolia* and phytochemical testing of metabolites by Olusola (21), the author did not detect tannin but detected saponin, flavonoid and alkaloid. It could be possible that *N. latifolia* plant part contains alkaloid, flavonoid, saponin, terpenoids, tannins and steroid as reported by Odeyemi et al., (22) in the qualitative phytochemical testing of the methanol stem bark extract of *Napoleona imperialis*.

Although, *N. latifolia* leaves and roots have been acclaimed to possess medicinal and antimicrobial properties, the methanol extracts in our study did not exhibit any antifungal activity or inhibitory properties at all the extract concentrations against the selected dermatophytes in the preliminary agar well diffusion assay. However, extracts of the stem bark and fruit of the plant inhibited the growth of *M. ferrugineum* strains 1 and 2, and *T. mentagrophyte* strain 2 at high extract concentration of 62.5mg/ml (62,500µg/ml), which is 1250 times higher than the control ketoconazole concentration of 50µg/ml, producing zone diameter of inhibition of 1-10mm, compared to the zone diameter produced by ketoconazole of 10-36mm against the selected dermatophytes. At a further higher extract concentration of 500mg/ml (500,000µg/ml), all the selected dermatophytes were inhibited by the extracts of the two plant parts, with mean zone diameter of inhibition that ranged from 2.0±00 to 10.0±2.0mm. At this extract concentration there was no significant difference in the inhibition activity of the methanol extracts of the two plant parts ($p>0.05$) on the selected dermatophytes. The anti-dermatophytic activities of these extracts may therefore appeared to be insignificant for further therapeutic exploration.

The minimum inhibitory concentrations (MICs) of the methanolic extracts of *N. latifolia* stem barks and fruits against the selected dermatophytes ranged from 31.25 mg/ml for *M. ferrugineum* strain 1 to 250 mg/ml for *M. audouinii* and *M. canis*. The MICs of the methanolic extracts of *N. latifolia* stem barks against *T. mentagrophyte* strain 1 and 2 were different, and although they are the same dermatophyte species, their genetic composition might be different, to account for this disparity. This similarly applied to the methanolic extracts of the stem barks and fruit of *N. latifolia* against *M. ferrugineum* strain 1 and 2. It was also observed that the MICs of the methanolic extracts of both the stem barks and fruits of *N. latifolia* were the same against each of the selected dermatophytes except for *T. mentagrophyte* strain 1. Higher concentrations of the methanolic extract of the stem barks and fruits (250-500 mg/ml) were required to completely kill (fungicidal) *M. canis* and *M. audouinii*.

The time-kill kinetic study revealed a progressive reduction in the viable cell count at 0-1 hour, 1-4 hours, and at the 8th hour, there was no viable cell, while with the control, the dermatophytes were still multiplying at the 8th hour. This was a similar observation in the previous study of methanol extracts of various plant parts (stem bark, leaves, seed and root) of *N. imperialis* by Odeyemi et al., (23), which reported anti-fungal activity, with the time-kill kinetics showing no viable dermatophyte at the 8th hour.

Conclusion:

Our study showed that crude methanol extracts of the stem bark and fruits of *N. latifolia* exhibited anti-fungal property, but at a much higher extract MIC values than what is generally considered to indicate potential efficacy of an extract or isolated compound from an extract for further therapeutic exploration (24). Nevertheless, with no viable cells at 8th hour in the time-kill kinetic assay for all the selected dermatophytes, the stem bark and fruit parts of the plant may potentially possess anti-dermatophytic activities that justifies the usage in traditional medicine. In this study however, the methanol extract of the leaf and root parts of the plant do not have any inhibitory activity against the selected dermatophytes.

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Contribution of authors:

Both authors contributed to the conception and design of the study. Preparation of the materials and the analysis were performed by OO. The whole work was supervised by EIB. The first draft of the manuscript was written by OO and proofread by EIB. Both authors commented on previous versions of the manuscript, read and approved the final manuscript.

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