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Review Article **Open Access**

Potentials and limitations of cold-adapted hydrogen producing bacteria: a mini review

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Abstract:

Low-temperature bacteria have potential to produce biohydrogen and are often considered a potential renewable energy generator for the future. However, the bacteria have presented poor hydrogen yield due to slow metabolic rate and prolonged lag phase often caused by their restricted growth temperature limit. The ineffective search for new biocatalysts from cold environments and the application of modification techniques almost jeopardize the economic viability of these strains in the biohydrogen production research. This article examined cold genetic and enzymatic adaptation potentials that led to the continuous expression of novel biocatalysts of biotechnological importance under the following headings; cold-adapted bacteria, biohydrogen-producing bacteria, strategies for adapting to stress in low temperatures, performance of cold-adapted bacteria in biohydrogen production, challenges of cold-adapted bacteria in biohydrogen production and future prospect. Finding new strains and studying their unique properties can improve the efficiency of hydrogen production by cold-adapted bacteria, as this new area has not yet been extensively studied.

Keywords: low-temperature bacteria; cold-adapted bacteria; temperature; mini-review

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Potentiels et limites des bactéries productrices d'hydrogène adaptées au froid: une mini revue

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Résumé:

Les bactéries à basse température ont le potentiel de produire du biohydrogène et sont souvent considérées comme un potentiel générateur d'énergie renouvelable pour l'avenir. Cependant, les bactéries ont présenté un faible rendement en hydrogène en raison d'un taux métabolique lent et d'une phase de latence prolongée souvent causée par leur limite de température de croissance restreinte. La recherche inefficace de nouveaux biocatalyseurs à partir d'environnements froids et l'application de techniques de modification compromettent presque la viabilité économique de ces souches dans la recherche sur la production de biohydrogène. Cet article a examiné les potentiels d'adaptation génétique et enzymatique au froid qui ont conduit à l'expression continue de nouveaux biocatalyseurs d'importance biotechnologique sous les rubriques suivantes; bactéries adaptées au froid, bactéries productrices de biohydrogène, stratégies d'adaptation au stress à basse température, performances des bactéries adaptées au froid dans la

production de biohydrogène, défis des bactéries adaptées au froid dans la production de biohydrogène et perspectives d'avenir. Trouver de nouvelles souches et étudier leurs propriétés uniques peut améliorer l'efficacité de la production d'hydrogène par des bactéries adaptées au froid, car ce nouveau domaine n'a pas encore été largement étudié.

Mots clés: bactéries à basse température; bactéries adaptées au froid; température; mini-revue

Introduction:

Due to its high energy density and friendly by-product (water) when burnt, hydrogen has attracted much attention as a desirable fuel (1). Among the renewable energies, hydrogen is more critical due to its sustainability and possession of 2.75 times the energy density of fossil fuels. Hydrogen can be produced using conventional and fermentation strategies. However, production through fermentation has emerged as an excellent strategy to extract hydrogen from the abundant waste for eco-friendly products (2). The biological hydrogen production processes are considered to be cost-effective due to the high production rate, low energy requirements and simple operation (3). Dark fermentation has exceptional advantages, such as the ability to produce hydrogen from the breakdown of organic waste, thus stabilizing waste with the potential for contamination (4).

Several bacterial strains are used to produce biohydrogen, which is the most promising for renewable energy generation. Cold-adapted bacteria can thrive at low temperatures (20°C and below) and stance unique constraints through flexible structural and conformational changes to proteins and lipids to express highly reactive enzymes (5). The ability of the bacteria ability to grow at low temperatures confers adaptive resilience to stresses including osmotic and low nutrients that affect other thermal species (6). Resistive loads may have enabled their use in industrial production at ambient temperatures (7), guaranteeing low-temperature operation, high productivity, and reduced production costs (8). Given this unique potential, understanding their biotechnological relevance in applied and biological research is fundamental. Furthermore, the widespread use of cold-active enzymes in industrial productions calls for evaluation of cold -adapted bacteria and elucidation of their potentials in renewable energies.

Using cold-adapted bacteria at low temperatures for biohydrogen production saves energy input in bioreactors, which has a significant impact on production costs (7). This new field of research thus underlines the potential of cold-adapted bacteria and, due to their resilience in fermentation, requires a review of their performance in numerous areas. The bacterial strain and its enzymes are currently used as biological tools in anaerobic digestion due to their economic and environmental advantages. However, their capabilities are not fully exploited in fermentative hydrogen production as low hydrogen yield have been recorded in anaerobic fermentation despite vast potential in biotechnological application (9). Therefore, this mini-review is an assessment of the numerous adaptation potentials of cold-active bacteria and their influence on fermentative hydrogen production.

Cold-adapted bacteria:

Cold-adapted bacteria live in cold places and grow well at or below 0-20°C. However, psychrophilic properties allow for rapid growth at temperatures below 15°C than at temperatures above 20°C. Most often, these types of microbiota colonize low-temperature areas like snow, permafrost, sea ice, and glaciers (10). For example, Joshi (6) reported minimal growth below 15°C and optimal growth at 25°C, while Ravi et al., (11) reported an upper growth limit of 37°C. This established the fact that growth temperature range varied between the bacterial species, leading to a division into psychrophilic and psychrotolerant strains (7). In addition, an unquantifiable number of culturable and non-culturable species, including Archaea and Eubacteria, are found in different environments (12).

In this review, cold-adapted bacteria refer to species that originate in a low-temperature environment but have the ability to grow below and above 20°C. These bacterial species adopt unique properties to perform metabolic activities that play important roles in the deep sea, Polar Regions, and frigid Alpines that make up three-quarters of the world. However, the bacteria are ubiquitous and could be found in the sparse cold habitats in temperate regions (13). Their special properties lead to novel catalysts that can improve industrial production techniques (14). Therefore, due to the biodegradability and non-toxicity of their products, they are considered potential reservoirs of biotechnological importance (15).

Biocatalysts are the power of industry due to the demand for dynamism in bioprocessing tools for numerous biochemical processes (8). Therefore, understanding the properties and functions of natural products of cold-adapted bacteria could expand innovative applications to various industries such as food, agriculture, chemicals, and pharmaceuticals (16). In agriculture, their potential has been used to support plant growth and remove waste from

the environment (17). These natural products have shown a wide range of uses in agriculture, medicine, pharmacy, and other fields. The productive abilities are cheap and sustainable sources of products critical to providing basic services to humankind. Therefore, the characteristic splitting of energy into its enthalpy and entropy could be used to increase hydrogen production. Research interests in these bacteria arise from the fact that their enzymes can be used at low temperatures and their thermal stability as well as their rapid and fascinating growth within a short period of time.

Biosynthesis of biocatalyst in psychrophilic and mesophilic states:

Scientists are still studying bacteria mechanisms to gain insight into harvesting biomolecules from cold environments. Cold-adapted bacteria thrive in a broad state (20°C and below) covering cryophilic and psychrophilic temperature ranges and in a finite state in a mesophilic environment (25°C to 37°C). Cold sensations activate histidine kinase to produce aspartate responsible for the transcription of cold genes. It involves proteins transformation in the membrane and the cytoplasm of the cells to create the enzymes needed for the process (18, 19).

Psychrophilic and mesophilic states have opposite influence on the functions of the cold-bacteria and the biosynthesis of biocatalysts. Studying such microbial processes in cold environment will expose salient innovation to improve industrial productivity (20,21). In the psychrophilic state, exposure to interconnected factors such as salinity, alkalinity, high ionic concentration, low nutrient levels, and cryophilic effects drive the reaction to synthesize many adaptable biocatalysts (22,23). This leads to the synthesis of many and new biocatalysts depending on changing environmental stressors. On the other hand, mesophilic state exposes cold-loving bacteria to a harmful state to the cells due to the disruption of cellular processes such as protein synthesis, nucleic acid structure, cold enzymes, and cell division (24). This energy-intensive process redirects the nucleic acids to synthesize more stabilizers to protect the cells instead of synthesizing adaptive biocatalysts. Fig 1 represents the different biocatalysts synthesized by cold-adapted bacteria under psychrophilic and mesophilic conditions.

In the psychrophilic state, more biocatalysts for adaptation to a variety of stressors are expressed than protective proteins. Stressors in the psychrophilic state are intertwined leading to the emergence of new biocatalysts that could be explored for industrial production. Because of the complex interdependent factors that influence the expression many cold genes in the genomes of cold bacteria which trigger the synthesis of unique biocatalysts to adapt to the conditions, a cold environment has become the target of novel biocatalysts of biotechnological importance.

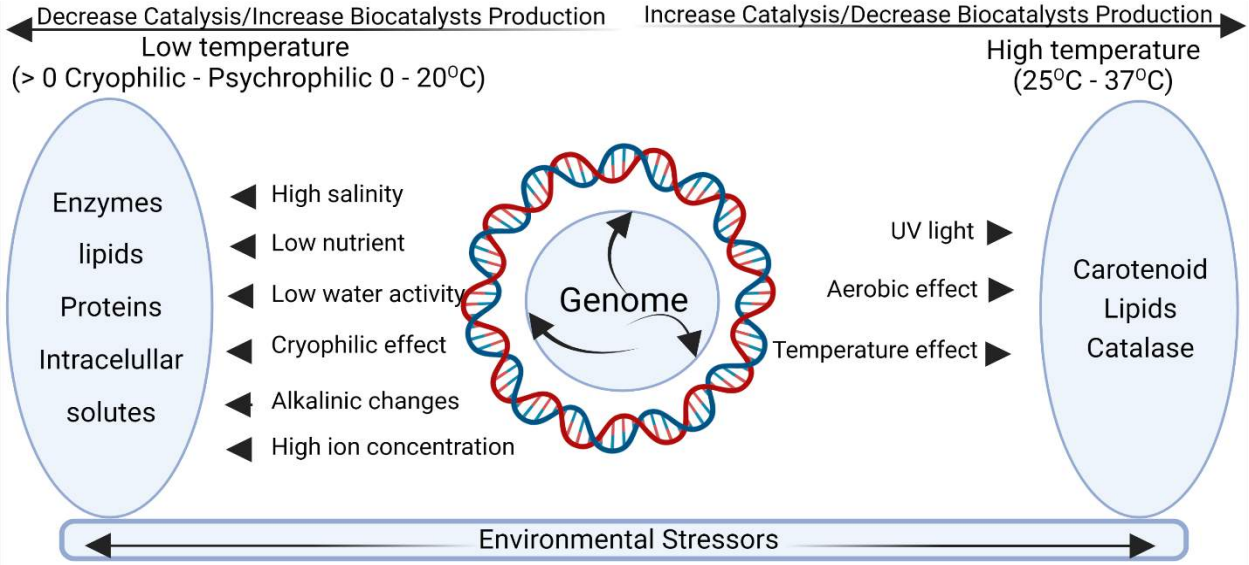

Fig 1: Biocatalysts production by cold adapted bacterial at opposite temperature conditions

Biohydrogen-producing bacteria:

Current routes to hydrogen production rely heavily on fossil fuel conversion and are energy-intensive and expensive. The various methods for hydrogen production are as outlined and classified in Fig 2. In order to reduce the atmospheric impact of the emitted greenhouse gas, more importance is attached to biological pathways with microorganisms as catalysts. Microbial processes are considered safe and inexpensive for hydrogen production because they break down carbohydrates with less energy (25). Several bacteria have been used for biohydrogen production and their catalytic efficiency depends on the temperature limits they can tolerate.

The fermentation processes are the most cost-effective and rely on the use of microorganisms as biocatalysts in the production process (26). However, most bacteria cannot produce hydrogen at temperatures of 20°C due to the inactivation of catalytic activity. This led to exploring production by using cold-active bacteria as a new field of research to boost hydrogen production. An improvement in hydrogen productivity was observed due to the equivalence of the hydrogen produced by some cold-adapted bacteria and that of other thermolabile strains (27,28). However, the efficiency of hydrogen production is challenged by the slow catalytic activity and increased lag time. Therefore, attention is currently focused on fermentative and genetic strategies that can improve their activity to maximize their potential for hydrogen yield.

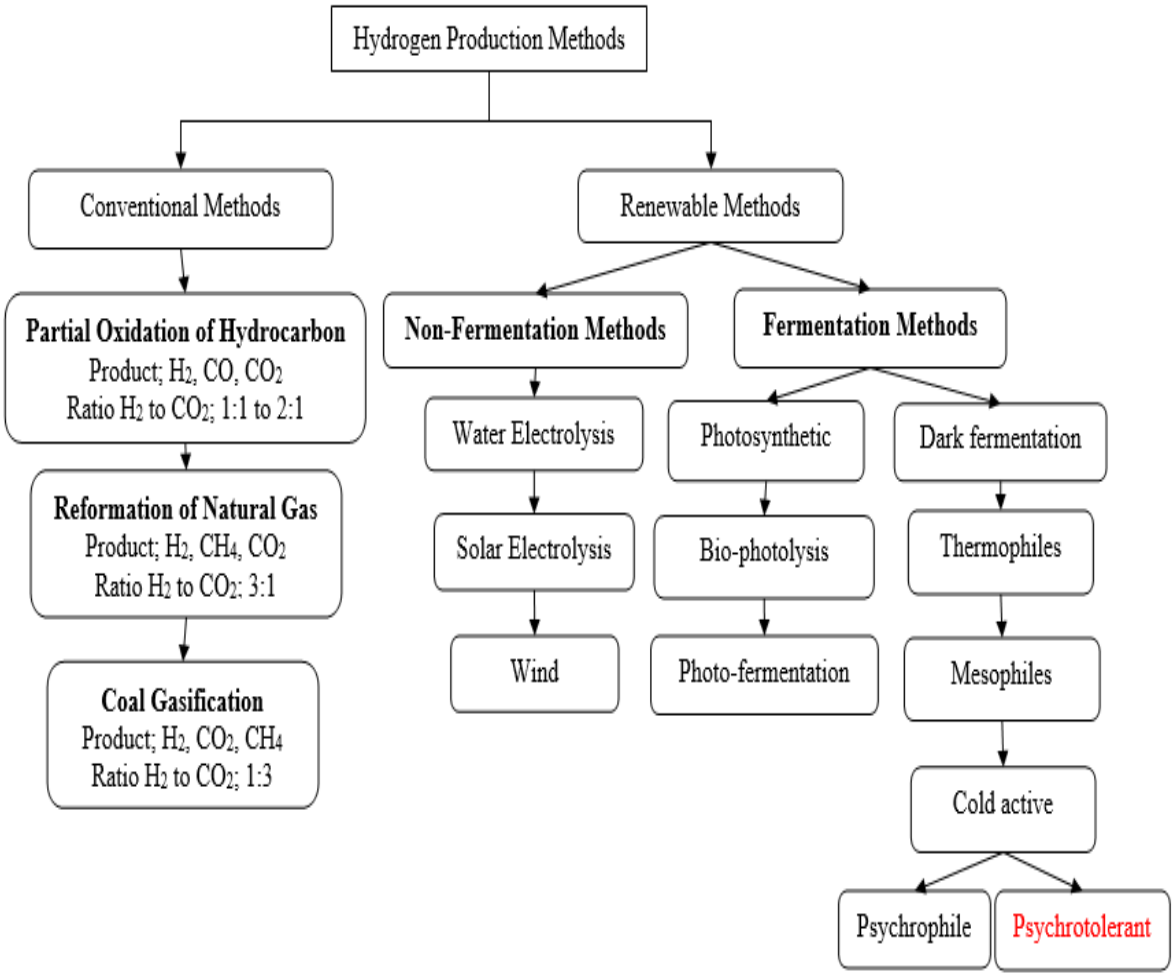

Fig 2: Different hydrogen-producing methods and thermolabile bacteria utilized

Strategies for adapting to stress in low temperatures:

Genetic modification:

Eight independent studies included in this mini-review revealed new genes that encode and are expressed in the cold for new properties at low temperatures. These new biocatalysts with biotechnological potential indicate the existence of limitless properties in these strains for industrial exploration. The genes expressed under cold conditions and the functional proteins and enzymes they encode are summarized in Table 1.

Ijaq et al., (29) examined the genome of *Pseudomonas* sp. Lz4W and discovered genes encoding hypothetical proteins (HPs) whose function has not yet been characterized. The authors discovered that HPs have the function of enhancing membrane stability and the movement of solutes across the membrane in cold areas. The proteins performed this function by distorting the HPs as the temperature rise or fall. It has been observed that at low temperatures, HPs expression increases for the flexibility and survival of the bacteria under cold stress. This finding reveals the hitherto unknown function of HPs in the membrane of cold-adapted bacteria.

Jiang et al., (30) examined the genome of the cold-adapted *Arthrobacter* Z1-20^T, and discovered the abundance of capA genes encoding osmo-protective glycine betaine and cold shock proteins. These novel genes produce clusters and higher copies of lysine as a diamino acid to adapt to cold environments. This study showed that the genes code for essential solutes in addition to proteins for protection in cold environments.

Dai et al., (31) sequenced the genome of *Nesterenkonia* to discover the survival strategies of the bacterium in polar environments. Dissection of the bacterial genome revealed genes encoding for NES-AT protein, which facilitates carbon utilization under nutrient-limited conditions. In addition, the genes that improve glucose metabolism and biofilm production for stress resistance were also found. The study unveils cold-temperature genetic mechanisms for nutrient degradation and how carbon sources are harvested under low-temperature conditions.

Papale et al., (32) studied the genes of cold-adapted *Arthrobacter* sp for making a compound. The authors discovered genes encoding the bphA protein for the production of polychlorinated biophenyls (PCBs) in the cold. This study improved the understanding of PCB secretion by cold-active bacteria and provided the basis for their likely use in cold environments.

Borker et al., (33) reported the detection of genes encoding the production of three enzymes in *Glutamicibacter arilaitensis* LJH19. This bacterium breaks down nocturnal soil compost by secreting amylase, cellulase, and xylanase at low temperatures. Genome analysis revealed 217 unique genes encoding these enzymes and auxin (IAA) in cold environments. The improved germination rate of pea seeds was reported to the IAA, indicating the ability to promote plant growth in cold conditions.

Leng et al., (34) studied the genome of *Planococcus maritimus* XJ11, which produces cold proteases at low temperatures. It was discovered that the bacterium contained genes encoding 21 proteases and 3 serine proteases, adapting the bacterium to low temperature, low salinity, and alkalinity. The potential also allows survival at pH 10 and a temperature of 40°C as the best conditions for catalytic activity. This study showed the production of several enzymes for stability at low temperatures.

In a similar study, a gene encoding glutathione reductase (GR) from *Psychrobacter* sp. ANT206 was cloned into *Escherichia coli*. The genes provided protection against oxidative stress from peroxide (H_2O_2). The rPsGR is a novel gene that is an antioxidant encoding a cold enzyme with high tolerance to both low temperature and high NaCl concentrations (35). The study showed the production of enzymes that confer resistance to low temperatures and osmotic pressure.

Raymond et al., (36) examined and compared the genomes of permafrost bacteria and mesophilic relatives. Cold shock proteins, RNA helicase, and enzymes involved in oxidative stress and carotenoid production are all present in both genomes. However, the permafrost bacterium contains more genes that express appropriate solutes required for osmoregulation in a frosty environment. In various cryophytes, amino acid (AA) changes promote protein flexibility at freezing temperatures by altering the amount of proline, serine, glycine, and aromaticity. This shows that the >1 cold/warm AA ratios previously used for cold adaptation alone were not sufficient. Cryophytes had a larger amount of serine in their proteins than cold-adapted proteins and fewer proline and acidic residues than mesophiles.

Biosynthesis of enzymes and proteins:

Cold-adapted bacteria overcome several challenges of living in cold habitats through a series of synergistic enzyme modifications from the cell envelope to the creation of cryoprotectants and innovative metabolic abilities. Basic research has provided important insights into

how microorganisms thrive under challenging conditions and the mechanisms of action of the numerous adaptive traits, which form the basis for the knowledge-based development of innovative biotechnological tools (37). There are recent advances in enzyme and protein production and diverse potential in different industries but less improved areas of hydrogen energy production (Table 2).

Flegler and Lipski (38) studied a carotenoid in cold-adapted *Arthrobacter* species and discovered a pink bacteriorubin that is rarely produced in cold environments. The biomolecule offers resistance to freeze-thaw conditions and osmotic pressure due to the high NaCl concentration. The function of bacteriorubin produced under freezing conditions is not fully understood but clarified to be produced at low temperatures. This protein was used in the manufacture of a dye and is used in the manufacture of antioxidants.

A similar study was conducted by Kumar et al., (39) on *Mucilaginibacter* sp under freezing conditions (-80°C). The bacterium was reported to have produced exopolysaccharides (EPS) that conferred viability on mesophilic *Escherichia coli*. The produced EPS effectively removes Cu^{2+}, $Fe^{2+,}$ and Mn^{2+} from the contaminated medium. Therefore, EPS has been used as a cold protector and effectively removes harmful ions.

Rios et al., (40) studied keratinase production by a cold-adapted *Pedobacter* sp 3.14.7. This enzyme produced by the bacterium at low temperature, was shown to be a robust additive that improves the thorough removal of blood stains from cotton towels at temperatures below 20°C. The authors also reported the effective ness of cold enzymes added to detergent when washing stains and dirt.

Herrera et al., (41) explained in their discovery how *Acinetobacter baumannii* alters its membrane lipid composition to maintain protein fluidity, permeability and function under cold conditions. The octanoate (C8:0) fatty acid is the only shortest secondary acyl chain reported from a cold bacterium, replacing the C12:0 fatty acid to confer stability in a cold environment. This acyl chain has been used for resistance to many drugs that are difficult to eradicate in healthcare setting. The study provides new insights into how temperature changes under different conditions affect lipooligosaccharides or lipopolysaccharides.

Rathour et al., (42) worked on alkalophilic amylase enzymes from *Shewanella* sp. The authors discovered that the enzyme effectively breaks down 1,4-glycosidic bonds in starch molecules. This potential makes this cold amylase an important enzyme in biotechnological tools, especially in the food industry due to the breakdown of the complex bond.

Govarthanan et al., (43) studied novel cold proteins that stabilize the cell membrane of mesophilic *Escherichia coli* and *Bacillus subtilis* at low temperatures. The work describes the protein and discovers new, unidentified species that were not clarified by previous *in vivo* studies, suggesting new biocatalysts that could have industrial applications. A new mechanism of cold adaptation in psychrophilic *Pseudomonas helmanticensis* was also discovered by Kumar et al., (44). In their results, they reported upregulation of the production of uncharacterized proteins at low temperatures instead of the usual expression of enzymes for proline, polyamines, unsaturated fatty acid biosynthesis, reactive oxygen species (ROS)-neutralizing pathways, and arginine degradation. From this, they concluded that molecular chaperones and cold shock proteins were proteins expressed by these bacteria against cold stress.

Few cold-adapted acetyl xylan esterases (AcXEs) were also discovered, however the processes that enable them to work are still unclear. This enzyme had maximum activity at 30°C and retained over 70% activity at 0°C. It has the ability to deacetylate xylooligosaccharides and xylan. Esterases are flavoring agents in the food industry, and chemical synthesizers and their degradation potential can eliminate wastes (45).

Biocatalysts that support plant growth are also produced by cold-adapted bacteria. Plant growth promoters are hydrolytic enzymes used in industry and found as important bioactive substances in medicine in all eukaryotic genera. Many researchers have reported using these proteins to increase the productivity of rice, grains, vegetables, and legumes. Although the biosynthesis of these substances in a microbial cell performs specific functions in cold environments, they have the ability to promote plant growth at high altitudes.

In contrast to mesophilic and thermophilic proteins, low-temperature expression techniques are more advantageous. As a result, a wide temperature range can be tolerated since the genes are more strongly expressed at low temperatures than at mesophilic temperatures. Because they produce antifreeze proteins and express stress-induced genes, bacteria susceptible to cold stress are better able to survive. All metagenomes examined had genes encoding functional responses to environmental stress, including exopolysaccharides, cold shock proteins, and membrane changes. At low temperatures, enhanced gene expression can be achieved, which has greater functional characteristics

and biotechnological applications.

Performance of cold-adapted bacteria in biohydrogen production:

The search for sustainable, low-cost and environmentally friendly hydrogen producing sources for large-scale production is still ongoing (19). Cold-active bacteria hold unique properties that will bring great transformation and improvement in hydrogen energy generation. Low-temperature anaerobic digestion is energy-efficient and sustainable for biohydrogen production. However, it is a new area that has only recently received the attention it deserves. Therefore, the current performance of the cold-active bacteria in biohydrogen production and their limitations has been discussed in this section of the review as summarized in Table 3.

Production temperature:

Cold-adapted bacteria have demonstrated biohydrogen production at temperatures as low as 4–9°C, saving energy in the substrate conversion process. This operating temperature inhibited methanogenic activities, preventing consumption of the hydrogen produced in the process (39), which highlights the performance of low temperatures in biohydrogen production. Nevertheless, in this temperature range, the thermophiles lose their ability for the production of biogas during sugar fermentation (3). Conversely, the production of biohydrogen at ambient temperatures (25-30°C) has been demonstrated by the same bacterial strain (4,46,47, 48). This operational temperature range has been chosen for its wide range of applications in industrial processes and upholds great potential for future hydrogen generation. The main limitation, however, is that the operating temperature affects the rate of enzymatic catalysis, resulting in high energy consumption and an increased lag time. A gap of 18-25 hours was observed between the start of substrate consumption and hydrogen production (3). The low-temperature conditions not only affect the individual microbes but also change the microbial community structure.

Because thermodynamics plays a large role in maintaining equilibrium, the rate of chemical and biological reaction processes is reduced compared to higher temperatures. Thus, the process requires more energy to achieve similar efficiency (5). This energy-intensive process uses a significant amount of substrate for the catalytic reaction rather than conversion to hydrogen. The scenario impacted negatively on the hydrogen yield of the bacteria, resulting in a low hydrogen yield compared to thermolabile strains. Therefore, the external energy required by the system is a major disadvantage, prompting the search for systems where hydrogen can be produced with a minimal input of energy.

Substrate utilization:

Cold-adapted bacteria have effectively broken-down various carbon sources for hydrogen production. Accordingly, glucose, xylose, fructose, galactose, sucrose and lactose have been used as substrates since they are available in large quantities from synthetic organic sources, which are high hydrogen producing materials. They are a segment of a range of abundant wastes where the production of cold-adapted biohydrogen can be coupled with the use of hemicellulose and lignocellulosic feedstocks.

The bacteria have also demonstrated the ability to hydrolyze organic wastes such as cheese whey, sewage, industrial sludge, wheat straw hydrolysate, and cane molasses for production (40,42,49). In this way, the susceptibility of the bacterium to the inhibitory compounds that are present in various industrial wastes that lead to hydrogen production is demonstrated. The fermentations simultaneously led to hydrogen production, which suggests further application on an industrial scale under room temperature conditions. This indicates the expression of appropriate enzymes by the bacteria to convert the substrates into hydrogen at low temperatures (3). Therefore, using waste to generate hydrogen energy could reduce production costs and make hydrogen gas more accessible and cheaper.

Biohydrogen production by cold bacteria has not yet been fully explored, indicating a new potential area for hydrogen production. Therefore, the possibility of converting many complex organic wastes, which are abundant in the environment, into the production of hydrogen should be considered. This is intended to demonstrate the competence and cost-effectiveness of the technology as well as its competitiveness on the energy market (50). Meanwhile, the activity of their enzymes at low temperatures comes at the expense of substrate affinity, thereby reducing the state of their physical and chemical properties in affinity for the enzyme. This affects early substrate uptake for conversion to hydrogen.

Potential of hydrogen (pH):

In anaerobic fermenters, the bacteria produce hydrogen simultaneously with acetic and butyric acid and other metabolites as by-products. Meanwhile, it has been shown that the acetic acid pathway is more desirable at low

temperatures for high hydrogen yield by cold bacteria due to its influence on the catalytic activities of the enzymes and conversion of the by-product to hydrogen. The gradual accumulation of volatile fatty acids (VFAs) in the medium becomes toxic to the cells, represents a shift in the metabolic pathway and unnecessarily increases pH, leading to a drop in hydrogen yield (4).

The hydrogen yield of cold bacteria is seen to be at its peak at pH 6-7 ranges with many metabolites produced in the medium. However, a high concentration of acetic acid favors high hydrogen yield at low temperatures while undissociated butyric acid metabolites alter the pH and the hydrogen yield (4). The anaerobic system operated with the strain under cold conditions shows a gradual degradation and an increase in VFAs, leading to an enhanced syntrophic relationship within the existing cells (5).

Genomic insights into the cold adaptation of bacteria from low-temperature ecosystems have unique adaptations to survive and sustain their growth and metabolism in a cold environment. To cope with these environmental stresses, and to survive and thrive in low-temperature environments, these bacteria exhibit several mechanisms of physiological adaptation that are not ubiquitous in other thermolabile bacteria. Therefore, this review revealed a consequent shift in survival strategies, including environmental perception and stress response, linking the increase in abundance of many genes to the adaptation of the bacterial community to the extreme environment. In this way, the cold-adapted bacteria have evolved unique adaptive strategies at the gene and protein level to maintain their metabolic activity to survive in harsh cold conditions.

Recent increased interest in cold environments has led to the identification of numerous new products, mainly from microbes. Further bioprospecting of these environments using modern high-throughput techniques such as metagenomics and metabolic engineering will surely lead to the discovery of other novel tools with diverse bioactivities and applications.

Challenges of cold-adapted bacteria in biohydrogen production:

In fermentative hydrogen production, many ecologically abundant wastes have not been commonly employed or converted to hydrogen production by cold-active bacteria. This is because few reports addressing biohydrogen production by cold-active bacteria are available, indicating a new area of research that has not been fully explored. Thus, the use of these bacteria is yet to be a more viable and cost-effective process for hydrogen production.

The catalytic efficiency of their hydrogenases is a high energy-intensive process due to an extensive increase in lag time with enormous energy consumed and a low hydrogen yield. The limited growth temperature range of the bacteria hampered their catalytic process and enzymes may be denatured when the operating temperature is increased beyond normal. Many metabolites produced during production accumulate and become toxic to the hydrogen-producing cells. The metabolites also determine the pathways for hydrogen production and influence the hydrogen yield.

Conclusion and future perspective:

The review shows that many new proteins and enzymes with unique potential for the common cold and its stressors are produced by cold bacterial strains. This points to the possibility of finding innovative biotechnological potentials for industrial applications by searching for cold environments. Therefore, the efficiency of hydrogen production and the yield of cold-adapted bacteria can be improved by this strategy given that hydrogen production is a function of enzymes and the fact that the field is new and has not yet been extensively explored.

The efficiency of biohydrogen production by a microbial strain depends on several parameters, and temperature is considered to be an important parameter that enhances the catalytic reaction. Studies have shown the resistance of many cold enzymes to mesophilic temperatures down to the 40°C range. These underlines the importance of continuously optimizing the parameters of new strains to increase hydrogen production. The high structural similarity between the crystal structures with that of mesophilic enzymes can be exploited by genetic engineering techniques to confer potentials that can improve biohydrogen production (51). This is easy to change since most of the crystals are on the surface of the enzymes. Similarly, amino acid substitution can be used to map gene clusters and proteins to hydrogen production with high fidelity, and an immobilization technique can be used to increase hydrogen productivity by genetically modified strains.

Cold-adapted bacteria and their biosynthetic products have shown effective biotechnological application and economic benefits in many industries. These unique potentials were acquired through adaptation to low-temperature stressors, resulting in the production of enzymes with high activity and stability. The bacteria have shown that in mesophilic and low temperature ranges they can produce hydrogen, which

inhibits other thermolabile strains. But despite their unique properties, the low hydrogen yield of cold-active bacteria threatens the future of large-scale hydrogen power generation. It is evident that new genes expressed at low temperatures and enzyme modifications can affect hydrogen production in the future. Thus, the cold-active bacteria and their enzymes offer a large reservoir of new biotechnological potential that could improve large-scale hydrogen production and should be explored extensively.

Contributions of authors:

MA conceived the study idea; MA and JMN contributed to the writing of the manuscript; MIL and ARS searched the literature databases for publications used for the review; MFA and HM contributed to proofreading the manuscript. All authors approved the final manu -script submitted.

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Table 1: Genes and functions of cold-adapted bacteria encoded under different conditions

Bacteria	Natural habitat	Methods	Annotated genes	No of coded protein	Functional proteins	Functions in cold	Reference
Pseudomonas sp. Lz4W	Oasis	Molecular and proteomic analysis	4,343	18	Hypothetical protein	Peptidoglycan metabolism, organizing the cell barrier, ATP hydrolysis, enhancing normal passage of fluid through the membrane, and catalysis	(22)
Arthrobacter Z1-20^{T}	Soil	16S rRNA	CapA, glycine betaine	N/A	Protein	Flexibility and stability, substrate binding, Osmoprotectant	(23)
Nesterenkonia sp.	Lake	Genome sequencing &Annotation	NES-AT	N/A	Bacteriorhodopsin	Survival under nutrient-limited, osmotic, and ultraviolet conditions, synthesis of biofilm, metabolism of nutrients	(24)
Arthrobacter antarcticus sp.	Lake sediment	Gene amplification and screening	BphA	N/A	BphA protein	Bioremediation of Polychlorobiphenyls (PCBs)	(25)
Glutamicibacter arilaitensis LJH19	Himalayas Valley	Genomic analysis	N/A	217	Amylase, cellulase, xylanase, IAA	The enzymes enhance the adaptation and breakdown of polysaccharides in a cold environment	(26)
Planococcus maritimus XJ11	Shrimp paste	16S rRNA	21	4	Protease & 3 serine proteases	Increasing activity in a wide temperature range of 10 to 40°C Maximum activity in high salinity	(27)
Psychrobacter sp. ANT206	Sea ice	Gene Cloning, Bioinformatics	PsGR	451	Glutathione reductase	High salt tolerance, high substrate affinity, oxidative stress protectant	(28)
Actinotalea sp. KRMCY2	Permafrost cores	Genomic sequence & annotation	4207	3205	Protein	Interexchange of serine and proline, secretion of cold shock proteins and oxidative enzymes	(29)

IAA= Indole acetic acid; NA = Not available

Table 2: Enzymes and proteins produced by cold-adapted bacteria and their biotechnological applications

Cold adapted bacteria	Natural habitat	Enzyme/protein	Specific products	Functions of enzymes/proteins in cold	Biotechnological applications	Reference
Arthrobacter agilis DSM 20550^T *Arthrobacter bussei* DSM 109896T	Cheese	Protein	C^{50} carotenoid Bacterioruberin	Cryoprotectants and NaCl stress protectants	Used in the production of antioxidants, dyes	(31)
Mucilaginibacter sp. ERMR7:07	Proglacial water	Protein	Exopolysaccharide	Cryoprotectants	Biosorption of Cu, Fe, Mn and Zn), production of exopolysaccharide	(32)
Pedobacter sp. 3.14.7	Snowy sheathbills	Keratinase	Metalloprotease	Protection in psychrotolerant environment	Degradation of feathers, Bleaching agents, laundry detergent additive	(33)
Acinetobacter baumannii LOS	Indian Shiwalik Himalayas	Acyltransferase	Octanoate (C8:0)/C12:0	Membrane fluidity and permeability	Used to break down complex polymers such as xylan.	(34)
Shewanella sp. ISTPL2	Lake	α-amylase	N/A	Tolerance of metal ions Tolerant to alkaline medium	α-1,4-glycosidic bonds hydrolysis in starch, Ethanol processing, high-fructose corn syrups	(35)
Shewanella BT05	Brackish water	Solutes	IAA, Siderophore hydrogen cyanide	Stability and flexibility	Solubilization of phosphate, Promote plant growth, bioremediation of pesticide	(36)
Pseudomonas helmanticensis	Soil	Protein	Chaperone, cold shock protein	Cold stress protection	Used in the production of essential materials	(37)
Arcticibacterium luteifluviistationis SM1504T	Arctic seawater	Esterase	SGNH-type acetyl xylan-esterases, Tetramers, His 203 and Ser 32	Polysaccharides and Stabilization	Used in CD4 T cell immune responses and vaccine studies	(38)

Note: All bacteria are isolated from the cold environment. The temp. (ºC) Represents specific or dual temperatures at which the undergo activity

Table 3: Biohydrogen production using different strains of cold-adapted bacteria as inoculum and their hydrogen yield

Cold adapted Bacteria	Natural habitat	Temp. (°C)	pH	Fermentation type	Carbon source	Nitrogen source	Biohydrogen yield	Reference
GA0F bacterium	Glacier sediment	25	7	Anaerobic	CWP, WSH and SCM	Yeast Bacto-tryptone	73.5 ± 10 cm^3 g^{-1}	(44)
Psychrophilic N92	Glacier sediment	29	6.9	Anaerobic	Glucose	$(NH4)_2SO_4$	1.7 mol H_2/mol glucose	(4)
Klebsiella sp. ABZ11	Seawater	30	6.5	Facultative anaerobic	Glucose, sucrose, fructose	Beef extract	3.8 mol/g glucose	(41)
GA0F bacterium	Glacier sediment	26.5	6.2	Anaerobic	Glucose	Tryptone and Yeast	1.93 mol H_2/mol glucose	(39)
Rahnella aquatilis RA9	Demersal lake	20	N/A	Anaerobic	Glucose	Cheese whey	58.1 mL H_2/g CODfed	(42)
Psychrophilic G088	Glacier sediment	20	6.8	Anaerobic	xylose, glucose, fructose, galactose, lactose and sucrose	Tryptone and Yeast	1.7 mol H_2/mol glucose	(3)
Psychrophilic G088	Glacier sediment	25	6.5	Anaerobic	Glucose	Tryptone & yeast extract	1.57 mol H_2/mol glucose	(30)
Sludge strains	Brewery sludge	21	N/A	Anaerobic	Sucrose	N/A	62.6 NmL H_2 g^{-1} sucrose	(37)

Cheese Whey Powder (CWP), Wheat Straw Hydrolysate (WSH), Sugarcane Molasses (SCM)

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Review Article **Open Access**

A review of staphylococcal scalded skin syndrome

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Abstract:

Staphylococcal scalded skin syndrome (SSSS) is characterized by widespread epithelial necrosis and/or superficial blistering of the skin following infection by some toxigenic strains of *Staphylococcus aureus*. The disease primarily affects children under the age of 5 years, but it can also occur in adults. Due to the recent increase in reported cases of SSSS, we have reviewed the epidemiology, pathogenesis, clinical features, diagnosis, treatment, and prevention, including the development of vaccines for *S. aureus* infections. Electronic databases including PubMed, Google Scholar and websites of the Center for Disease Prevention and Control (CDC), and the World Health Organization (WHO), were searched for publications on SSSS written in English language. Our review showed that SSSS is more common in children, amongst whom it carries a mortality rate of <5%, as opposed to mortality rate of >50% in affected adults. Penicillinase-resistant penicillins are recommended for the treatment of SSSS, and administration of fresh frozen plasma (FFP) may aid early recovery. Important staphylococcal vaccine candidates are also highlighted in the review.

Keywords: staphylococcal scalded skin syndrome, staphylococcal skin infection, staphylococcal vaccines

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Un examen du syndrome de la peau échaudée staphylococcique

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Résumé:

Le syndrome de la peau échaudée staphylococcique (SSSS) est caractérisé par une nécrose épithéliale généralisée et/ou des cloques superficielles de la peau suite à une infection par certaines souches toxigènes de *Staphylococcus aureus*. La maladie touche principalement les enfants de moins de 5 ans, mais elle peut également survenir chez les adultes. En raison de l'augmentation récente des cas signalés de SSSS, nous avons examiné l'épidémiologie, la pathogenèse, les caractéristiques cliniques, le diagnostic, le traitement et la prévention, y compris le développement de vaccins contre les infections à *S. aureus.* Des bases de données électroniques, notamment PubMed, Google Scholar et les sites Web du Centre de Prévention et de Contrôle des Maladies (CDC) et de l'Organisation Mondiale de la Santé (OMS), ont été recherchées pour des publications sur SSSS rédigées en anglais. Notre revue a montré que le SSSS est plus fréquent chez les enfants, parmi lesquels il entraîne un taux de mortalité < 5%, par opposition à un taux de mortalité > 50% chez les adultes affectés. Les pénicillines résistantes à la pénicillinase sont recommandées pour le traitement du SSSS, et l'administration de plasma frais congelé (PFC) peut favoriser une récupération précoce. D'importants candidats vaccins staphylococciques sont également mis en évidence dans l'examen.

Mots-clés: syndrome de la peau échaudée staphylococcique, infection cutanée staphylococcique, vaccins staphylococciques

Introduction:

Staphylococcal scalded skin syndrome (SSSS) is a rare but severe skin infection caused by the exfoliative toxins produced by the bacterium *Staphylococcus aureus.* The disease, also known as Ritter's disease, is named after Gotfried Ritter who published case series of about 300 patients with SSSS in the 1800s (1,2). *Staphylococcus aureus* is a significant cause of serious bacterial infections especially sepsis where it is commonly reported (3,4). It is a significant public health concern due to its potential to cause significant morbidity and mortality, especially in infants and in immunocompromised individuals. The syndrome is characterized by a spectrum of diffuse erythematous rash and skin peeling, which can lead to life-threatening complications (5). It primarily affects children under the age of 5 years, but can also occur in adults (5-10).

The pathogenesis of SSSS is complex and involves the breakdown of desmoglein-1 present in the outer layer of the skin by the exfoliative toxins produced by *S. aureus* (11). This results in the detachment of the top layer of the skin, causing the characteristic blistering and peeling seen in SSSS (1,9-12). Diagnosis of SSSS is challenging and requires a high degree of clinical suspicion as the early stages of the disease can be easily mistaken for other common childhood illnesses such as pemphigus vulgaris and Stevens-Johnson syndrome. Treatment typically involves supportive care, wound management, and antibiotics targeting the underlying *S. aureus* infection (13).

In recent years, there has been an increasing interest in developing effective prevention strategies for SSSS, including the development of vaccines against *S. aureus* infections (1,2,14,15). Despite these efforts, there are still many unanswered questions regarding the pathogenesis, diagnosis, and management of SSSS. This review aims to provide a comprehensive overview of the current aspects of SSSS, including its epidemiology, pathogenesis, clinical features, diagnosis, treatment, and prevention. Additionally, we discuss the current state of research on SSSS, including the development of vaccines for *S. aureus* infections. By consolidating the available knowledge on SSSS, we hope that this review will serve as a valuable resource for clinicians, researchers, and public health professionals working towards better understanding and management of SSSS.

Methodology and Results:

Electronic databases including PubMed and Google Scholar were searched to identify publications on SSSS. Other online sources searched included the Center for Disease Prevention and Control (CDC), and World Health Organization (WHO) websites, as well as grey literatures. The keywords used for the search were "staphylococcal scalded skin syndrome" and "staphylococcal skin infection" and the search period was from January 1, 2010 to December 31, 2022.

Inclusion criteria were original and review publications that provided information on SSSS in humans, case reports of SSSS, epidemiology and genomic sequences of *S. aureus* implicated in scalded skin syndrome (SSS), pathophysiology, and management of SSSS. Publications written in any language other than English, letters to the editors and animal reports were excluded. All duplicate publications were removed, and the full text of selected articles were reviewed by the authors for eligibility, with areas of conflict sorted out by consensus among the authors. A total of seventy publications were finally included for the review.

Discussion:

Epidemiology of SSSS

The incidence of SSSS varies across different geographic regions, but it is estimated to be less than one case per million people per year in most developed countries (5,16-18). The incidence of SSSS may be higher in developing countries due to poor hygiene and living conditions (1,2). SSSS is more common in warmer climates and is more frequently reported during the summer months. SSSS primarily affects children under the age of 5 years, with a peak incidence in infants less than 2 years old (1,2). There is an inverse relationship with age (16,19). SSSS is reported to be more common in males than females, with a male to female ratio of approximately 2:1.

The risk factors for SSSS include underlying skin infections, such as impetigo, and conditions that compromise the immune system such as HIV/AIDS, chemotherapy, or long-term use of immunosuppressive medications (14,16,20-23). Individuals who are colonized with *S. aureus*, but not necessarily infected, are also at an increased risk for developing SSSS (23-25). Newborns and infants are particularly vulnerable to *S. aureus* colonization due to the immaturity of their immune systems and the increased permeability of their skin (2,26). The proportion of individuals who develop SSSS is much lower than those harbouring *S. aureus* primarily because only about 2-5% of *S. aureus* harbour the epidermolytic/exfoliative toxin (ET) *eta* or *etb* genes (27,28).

Outbreaks of SSSS have been reported in neonatal intensive care units and other

hospital settings (5,29). The disease can be spread from person to person through direct contact or by fomites, such as towels or clothing contaminated with *S. aureus* (5,30). Therefore, infection control measures, such as hand hygiene and proper disinfection of equipment and surfaces, are essential to prevent the transmission of *S. aureus* and consequent SSSS in healthcare settings.

Although SSSS is rare, it can be life-threatening and has a significant impact on affected individuals and their families (5,18, 30). It is crucial to maintain a heightened level of awareness and implement effective measures to prevent and manage SSSS, particularly in populations with a higher susceptibility. Such measures should include the adoption of proper wound care practices, timely treatment of any underlying skin infections, and the use of antibiotics to specifically target the underlying *S. aureus* infection. Vaccines against *S. aureus* infections are currently under development and may offer future promise in preventing SSSS (15).

Aetiology of SSSS:

There are three serological forms of staphylococcal ETs (ETA, ETB, and ETD), all of which cleave human desmoglein (31) but only ETA and ETB have been firmly linked to human SSSS (32). In a review, Ladhani et al., (26) suggested that ETB is more frequently isolated than ETA in children with generalized SSSS. The ET comprises of two proteins; ET-A (chromosomal, heat stable) and ET-B (plasmid encoded, heat labile), which act specifically on the zona granulosa of the skin epidermis (33).

Immunocytochemical studies have demonstrated that the toxin binds to the filaggrin group of proteins in keratohyalin granules (34). Most strains of *S. aureus* isolated from patients with an occult or overt infection or colonization with ET–secreting *S. aureus* belong to phage group II and the most common subtypes are 3A, 3B, 3C, 55 and 71 (23). Exotoxin secretion occurs during the bacterial logarithmic growth phase (35).

Risk factors for SSSS:

A suppressed immune system, whether from renal failure, diabetes mellitus (DM) or human immunodeficiency virus (HIV) infection, results in inability to both excrete *S. aureus* exotoxins and to produce antibodies to the ETs (16). Patients undergoing haemodialysis are at risk of SSSS due to infection by *S. aureus* via vascular access port, inability to excrete ETs, and the immunological deficits that accompany renal failure (36,37). In cases of adults without immunosuppression who developed SSSS, it is likely that they contracted strains of *S. aureus* with *etb* gene which encodes virulent exotoxin ETB (38).

Pathogenesis of SSSS:

The pathogenesis of SSSS is complex and involves a number of factors that contribute to the development of the disease with the clinical features being mediated by *S. aureus* ETs. It is estimated that only about 2-5% of all *S. aureus* strains produce ETs (27, 28). ETs are a class of serine protease exotoxins of two major distinct types; ETA and ETB. They are also referred to as epidermolytic toxins, epidermolysins, and exfoliatins (39). These toxins are heat-stable and resistant to proteases and acidic pH, allowing them to survive in harsh environments and cause damage to the skin. The exfoliative toxins C (ETC) and D (ETD) are isoforms which have been observed in other animal models.

The ETA and ETB are encoded by genes carried on *S. aureus* bacteriophage and plasmid respectively (40). These toxin genes can integrate into the bacterial chromosome and transfer the genes between bacteria. The toxin is released following expression of the genes locally by staphylococci, which travels through the body, and then concentrates at the stratum granulosum layer (26,28,39). The toxins are produced during the post-exponential phase of bacterial growth as inactive precursor molecules, which are then activated by proteolytic cleavage by *S. aureus* proteases such as aureolysin and staphopain A.

Once activated, the toxins bind to and cleave the desmoglein 1 (Dsg1) protein. The Dsg1 protein is the primary component responsible for the maintenance of interkeratinocyte adhesion desmosomes that maintain skin cell integrity. The cleavage of Dsg1 thus leads to the detachment of the top layer of the skin, resulting in the characteristic blistering and peeling seen in SSSS. Although the toxins act by a direct effect on the stratum granulosum of the epidermis, the mucosa is never involved (39,41). The toxins ETA and ETB have different mechanisms of action in causing SSSS. ETA is a single-chain toxin that directly cleaves Dsg1 within the skin, while ETB is a two-component toxin that binds to Dsg1 on the surface of skin cells and then triggers cleavage by host proteases (12,32,40,42). Both toxins can cause significant damage to the skin and lead to the formation of blisters, bullae, and exfoliation of the skin.

The severity of SSSS is influenced by various factors, including the amount and type of ETs produced, the patient's immune response, and the patient's age and overall health (11). Infants and young children are more susceptible to SSSS due to their immature immune systems and thinner skin, which is more easily damaged by the ETs (43,44). Immunocompromised individuals such as HIV/AIDS, cancer, or those undergoing chemotherapy, are also at increased risk of developing

severe SSSS (20). It has been postulated that ET targets Dsg1 in the vicinity of the cell membrane ganglioside (GM4), which is mostly ever present in the skin of young children or in adults with peculiar skin diseases (45,46).

Clinical features of SSSS:

The clinical features of SSSS are characterized by a wide spectrum of diffuse erythematous rash and skin peeling, which can lead to life-threatening complications (23,45). The spectrum includes, the localized and the generalized which represent the two broad ends of the observed spectrum. In general, the disease typically begins with the development of a fever, followed by the appearance of a red rash on the skin, which spreads rapidly throughout the body (14,46). The rash is usually accompanied by pain, tenderness, and blistering of the affected area. The characteristic feature of SSSS is the presence of skin peeling, which can occur within hours of the onset of the rash. The skin peeling typically begins around the mouth and eyes, and then spreads to the rest of the body, including the trunk and extremities (23,46). The skin peeling usually begins as small blisters, which then rupture and leave behind raw, denuded skin. The affected skin may appear shiny, red, and moist, and may be tender to the touch. The skin easily detaches by mere rubbing, which is described as Nikolsky's sign (23).

Bullous impetigo, also known as the localized form of SSSS, can occur when the toxin spreads locally around a previously colonized wound in individuals who possess some immunity to the toxin (23,24). This type of impetigo is frequently observed in neonates around the umbilicus, in infants who still have maternal immunity, or in older people who have already have some form of immunity. Although antibodies hinder the distant dissemination of the toxin, they do not prevent its local spread in the colonized or infected region (31,44). Unlike the generalized form, bullous impetigo is confined to a specific area and the blister fluid may contain bacteria and, in some cases, white blood cells (38).

In severe cases of SSSS, the skin peeling can be so extensive that large areas of the body are left with raw, exposed skin. This highly increase the risk of secondary bacterial infections, as well as dehydration and electrolyte imbalances (39,43). Other potential complications of SSSS include sepsis, pneumonia, and kidney failure. The disease is self-limited and wanes within 4 to 5 days in children, which probably parallels the appearance of specific antitoxin immunoglobulins (47,48).

Diagnosis of SSSS:

The diagnosis of SSSS can be challenging, as the early stages of the disease may be easily mistaken for other common childhood illnesses. However, the presence of fever, diffuse erythematous rash, and skin peeling can help to differentiate SSSS from other conditions (14,46). In addition, laboratory investigations, such as blood cultures and skin biopsies, can aid in confirmatory diagnosis and identification of the underlying *S. aureus* infection.

Treatment and prognosis of SSSS:

Patient with SSSS should be promptly isolated, and those at risk of developing SSSS and who may have been exposed to ET-producing *S. aureus* strains will benefit from prompt administration of prophylactic anti-staphylococcal antibiotics (14,26,31). It is important to start empirical antibiotics as early as possible, and penicillinase-resistant penicillins are recommended, as they are effective in treatment of methicillin-sensitive *S. aureus* infection seen in most patients with SSSS (49-51). If the patient has a penicillin allergy, clarithromycin or cefuroxime may be used as alternative. It is still debatable as to whether antibiotics delivered intravenously have superior efficacy than oral administration (52). If a patient is not improving, it is necessary to consider ETs produced by methicillin-resistant *S. aureus* and switch antibiotic to vancomycin (53).

It has been observed that more than 90% of adults above 40 years of age have antibodies against ETA. Therefore, children with SSSS may receive a dose of fresh frozen plasma (FFP) (10 ml/kg) to neutralize exotoxin (54). If appropriately managed, the prognosis of SSSS in children is usually good, with mortality rate less than 5%. In contrast, the mortality rate can be very high in adults (>50%), which is usually associated with an underlying medical condition (14,23).

Prevention of SSSS and efforts at vaccine development:

SSSS can be prevented by adhering to good hygiene practices which include proper and frequent hand hygiene with antibacterial hand soap and observing hand hygiene moments; avoid sharing items such as towels and clothes; keeping fingernails short and clean; using clean and dry beddings and clothing; keeping wounds and cuts clean and covered; preventing contact of infected individuals with child care centers and schools; staphylococcal decolonization with antibacterial creams such as fusidic acid or petroleum jelly under fingernails, and environmental cleaning with antibacterial agents (36,55).

Currently, there are no vaccines available against *S. aureus* although numerous attempts have been made to develop effective vaccines that would activate both humoral and cellular immunity against *S. aureus* infections (56,57). Significant limitations have been ob-

served in translation of vaccine protectivity from preclinical phases to the clinical phase. In addition to vaccines, other novel strategies are being developed in the management of *S. aureus* infections before an efficacious vaccine is available and these include the use of monoclonal antibodies, bacteriophage therapy, antibiotics, and development of new therapeutic proteins such as centyrins (15).

The various techniques that have been employed in the development of *S. aureus* vaccines include the use of recombinant protein or bacterial polysaccharide antigen. This is the most common technique employed as it has been successful in the development of vaccines for other bacterial infections such as *Neisseria meningitidis, Streptococcus pneumoniae* and *Haemophilus influenzae* B (HiB). The use of protein glycol-conjugation is employed to stimulate T-cell dependent B-cell activation and immune response to polysaccharide antigens, which is strong and long-lived (58).

Another technique includes the use of extracellular vesicles (EV) by genetically modifying *S. aureus* using *Escherichia coli* transfected with plasmids encoding five staphylococcal antigens, which are subsequently trafficked into the outer membrane vesicles (OMVs) that are naturally found in Gram-negative bacteria, in order to stimulate the innate and adaptive immune systems (59). Recently, *S. aureus* was also found to secrete extracellular vesicles which have inherent adjuvant ability useful for stimulating innate pro-inflammatory cytokines such as IL-6, IL-12 and TNF-α by dendritic cells (DCs) and dermal fibroblasts in the host, which is therefore being explored as a vaccination technique (60-62). The EVs of *S. aureus* have been proven to stimulate long-lived immunity against subsequent infections through T-cell derived IFN-γ (61,63,64).

The use of whole cell and live attenuated bacteria has also been explored by either physically or chemically inactivating bacteria or using live attenuated bacteria. Recently, an attenuated auxotrophic mutant of MRSA strain 132 was developed which was found to provide high immunogenic protection and produced cross-reactive antibodies against *S. aureus* strains in mice (65). A number of whole cell vaccines have successfully passed the preclinical stages in both mice, bovine and human models. Two whole cell *S. aureus* vaccines, Startvac and Lysigin, were approved for use in mice, while chloroform inactivated *S. aureus* (SA75) and heat-killed (HK) *S. aureus* strain ATCC 12598 passed early phase clinical trials in humans. However, there are limited data on these vaccines in humans as they were not developed further (66,67) possibly because manufacturers prefer to use newer technologies or because whole cell and live attenuated vaccines are associated with more severe adverse reactions.

The use of nucleic acid technique, specifically mRNA has not yet been explored for *S. aureus* vaccine although this has been successful in the development of vaccines against other Gram-positive bacteria such as group A and B streptococci with significant immune response and transgenerational humoral immunity in mice (68). On the other hand, DNA vaccines have shown efficacy in pre-clinical trials in mice infections by stimulating $CD8^+$ T-cell responses (69), although this was not reproducible in humans (70).

Conclusion:

Our review showed that SSSS carries a mortality rate of <5% amongst children <5 years of age, as opposed to a mortality rate of >50% in affected adults. Penicillinase-resistant penicillins are recommended for the empirical treatment of SSSS. Administration of fresh frozen plasma (FFP) to neutralize staphylococcal exotoxins could aid early recovery. Several staphylococcal vaccine candidates are being developed and offer hope for providing specific protection against SSSS in future.

Contributions of authors:

AB conceived the review, designed the outline, wrote the aspects of abstract and conclusion, and reviewed the manuscript; MN searched the literature for relevant publications, wrote the aspects of introduction, epidemiology and clinical Features of SSSS; IJ searched the literature for relevant publications and wrote the materials and methods; TMB searched the literature for relevant publications, reviewed articles, and wrote the aspects of vaccine development against staphylococcal infections; BM searched the literature for relevant publications, reviewed articles, and the wrote the aspects of diagnosis, treatment and prognosis of SSSS.

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Phenotypic characterization and antimicrobial susceptibility profiles of *Vibrio cholerae* isolates during the October 2022 and January 2023 outbreak in North-Kivu province, The Democratic Republic of Congo

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Abstract:

Background: Cholera is an infectious disease characterized by severe watery diarrhea, frequently occurring in outbreaks which affects many communities in the Democratic Republic of the Congo (DRC). At the end of October 2022, a cholera outbreak was declared in the camp of internally displaced people (IDP) of Kanyaruchinya, 20 kilometers north of Goma, the provincial capital of the North-Kivu province in DRC, as well as in other IDP camps and settlements around the city of Goma. The aim of this study was to phenotypically characterize *Vibrio cholerae* isolates associated with this outbreak, and to determinate their antimicrobial susceptibility profiles.
Methodology: Between October 31, 2022 and January 31, 2023, faecal swab samples were collected into Cary-Blair medium from 1604 and 538 patients with clinical signs of cholera at the Kanyaruchinya IDP, and IDPs camps and settlements around the city of Goma, respectively. After enrichment in 1% alkaline peptone water, the samples were cultured on thiosulphate-citrate-bile salt-sucrose (TCBS) agar for isolation and phenotypic characterization of *V. cholerae* O1 using conventional biochemical tests and serotyping technique. Antimicrobial susceptibility of selected isolates was peformed to a panel of 8 antibiotics by the disk diffusion method in accordance with EUCAST and CLSI guidelines.
Results: *Vibrio cholerae* was cultured from 807 samples (50.3%) of 1604 patients from the Kanyaruchinya IDP, and from 206 samples (38.3%) of 538 patients around the city of Goma (p<0.01). All the *V. cholerae* isolates from the Kanyaruchinya IDP (807/807, 100.0%) were serotyped as *V. cholerae* O1 Inaba whereas 136 (66.0%), 67 (32.5%), and 3 (1.5%) *V. cholerae* O1 isolates from around Goma were serotyped as *V. cholerae* O1 Ogawa, *V. cholerae* O1 Inaba, and Hikojima respectively. Antimicrobial susceptibility test on 174 and 62 isolates selected randomly from the 807 and 206 *V. cholerae* isolates from the Kanyaruchinya IDP camp, and from around the city of Goma respectively, showed that all the tested *V. cholerae* O1 isolates were resistant to polymyxin and cotrimoxazole, while being susceptible to tetracycline and azithromycin. All tested *V. cholerae* O1 isolates from Kanyaruchinya IDP camp displayed a unique antimicrobial susceptibility profile characterized by resistance to ampicillin, cotrimoxazole and chloramphenicol, and susceptiblility to ciprofloxacin, norfloxacin, azithromycin, tetracycline and doxycycline. Their counterparts from settlements around the city of Goma displayed a more variable antimicrobial susceptibility profile.
Conclusion: Our results suggest that a single *V. cholerae* O1 Inaba clone probably caused the cholera outbreak in the Kanyaruchinya IDP camp, whereas during the same period, several *V. cholerae* clones (Ogawa, Inaba and Hikojima) were associated with the cholera outbreak around the city of Goma.

Keywords : *Vibrio cholerae* O1, antimicrobial susceptibility, North-Kivu, DRC

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Caractérisation phénotypique et profils de sensibilité aux antimicrobiens des isolats de *Vibrio cholerae* lors de l'épidémie d'Octobre 2022 à Janvier 2023 dans la province du Nord-Kivu, République démocratique du Congo

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Résumé:

Contexte: Le choléra est une maladie infectieuse caractérisée par une diarrhée aqueuse sévère et se manifestant fréquemment par des épidémies qui affectent de nombreuses communautés en République démocratique du Congo (RDC). A la fin du mois d'Octobre 2022, une épidémie de choléra s'est déclarée dans le camp de déplacés internes (CDI) de Kanyaruchinya, situé à 20 kilomètres au nord de Goma, la capitale provinciale de la province du Nord-Kivu en RDC, ainsi que dans d'autres camps de déplacés et des bourgades autour de la ville de Goma. L'objectif de cette étude était la caractérisation phénotypique des isolats de *Vibrio cholerae* associés à cette épidémie et de déterminer leurs profils de sensibilité aux antimicrobiens.
Méthodologie: Entre le 31 Octobre 2022 et le 31 Janvier 2023, des écouvillons fécaux ont été recueillis dans un milieu Cary-Blair respectivement auprès de 1604 et 538 patients présentant des signes cliniques de choléra dans les camps de déplacés de Kanyaruchinya et les CDI les bourgades autour de la ville de Goma. Après enrichissement dans de l'eau peptonée alcaline à 1%, les échantillons ont été cultivés sur de la gélose thiosulfate-citrate-sel-bile-sucrose (TCBS) pour l'isolement et la caractérisation phénotypique de *V. cholerae O1*, en utilisant des tests biochimiques conventionnels et une technique de sérotypage. La sensibilité antimicrobienne des isolats sélectionnés a été évaluée pour un panel de 8 antibiotiques par la méthode de diffusion sur disque, conformément aux directives de l'EUCAST et du CLSI.
Résultats: *Vibrio cholerae* a été isolé à partir de 807 échantillons (50,3%) provenant de 1604 patients du CDI de Kanyaruchinya, et de 206 échantillons (38,3%) provenant de 538 patients autour de la ville de Goma (p<0,01). Tous les isolats *de V. cholerae* du CDI de Kanyaruchinya (807/807, 100,0%) ont été typés comme *V. cholerae O1* Inaba, tandis que 136 (66.0%), 67 (32.5%) et 3 (1.5%) isolats de *V. cholerae O1* des environs de Goma ont été typés comme *V. cholerae* O1 Ogawa, *V. cholerae* O1 Inaba et Hikojima. Les tests de susceptibilité aux antimicrobiens effectués sur 174 et 62 isolats sélectionnés au hasard parmi les 807 et 206 isolats de *V. cholerae* provenant respectivement du CDI de Kanyaruchinya et des environs de la ville de Goma ont montré que tous les isolats de *V. cholerae O1* testés étaient résistants à la polymyxine B et au cotrimoxazole, tout en étant susceptibles à la tétracycline et à l'azithromycine. Tous les isolats de *V. cholerae O1* testés dans le CDI de Kanyaruchinya présentaient un profil de sensibilité antimicrobienne unique, caractérisé par une résistance à l'ampicilline, au cotrimoxazole et au chloramphénicol, et une sensibilité à la ciprofloxacine, à la norfloxacine, à l'azithromycine, à la tétracycline et à la doxycycline. Leurs homologues des bourgades et CDI situés autour de la ville de Goma présentaient un profil de susceptibilité antimicrobienne plus variable.
Conclusion: Nos résultats suggèrent qu'un seul clone de *V. cholerae* O1 Inaba a probablement causé l'épidémie de choléra dans le CDI de Kanyaruchinya, alors qu'au cours de la même période, plusieurs clones de *V. cholerae* (Ogawa, Inaba et Hikojima) ont été associés à l'épidémie de choléra autour de la ville de Goma.

Mots-clés : *Vibrio cholerae* O1, susceptibilité aux antibiotiques, Nord-Kivu, North-Kivu, RDC

Introduction:

Cholera is an acute and life-threatening diarrheal disease caused by a comma shaped Gram-negative bacterium named *Vibrio cholerae* (1,2). The disease, which has been known for centuries, originated in Asia, and has evolved as pandemics (3). The current pandemic (7th), which started in 1961 is caused by *V. cholerae* serogroup O1 or O139, biovar El Tor (4), and has been characterized by large outbreaks in several developing countries (5).

It is estimated that up to near 3 million cases of people are affected annually by cholera, and that this resulted in 21,000 to 143,000 deaths worldwide in 2015 (6). Sub-Saharan Africa has become the leading part of the world with respect to cholera cases (7, 8) as highlighted by the recent large cholera outbreaks in Malawi (9) and in Mozambique (10). However recent outbreaks in Yemen (11) and Haiti (12) are a reminder of the cholera burden on other continents.

Despite the recent decrease in cholera cases worldwide, which has been lauded by the World Health Organization (13), The Democratic Republic of Congo (DRC) has continued to experience multiple cholera outbreaks, essentially in the eastern provinces of the country, a vast territory which has been plagued by civil unrest for the last thirty years resulting in millions of IDP, malnutrition, outbreaks of infectious diseases and a high number of deaths (14). The renewed fighting between the outcast M23 rebellion and the Congolese Armed Forces since April 2022 has aggravated the already volatile situation in the North-Kivu province, and resulted in massive displacements of people in the districts of Rutshuru, Nyiragongo, Masisi around the city of Goma, with the creation of a huge camps for internally displaced people (IDP) in Bulengo, Mugunga, and Kanyaruchinya.

The Kanyaruchinya IDP camp which

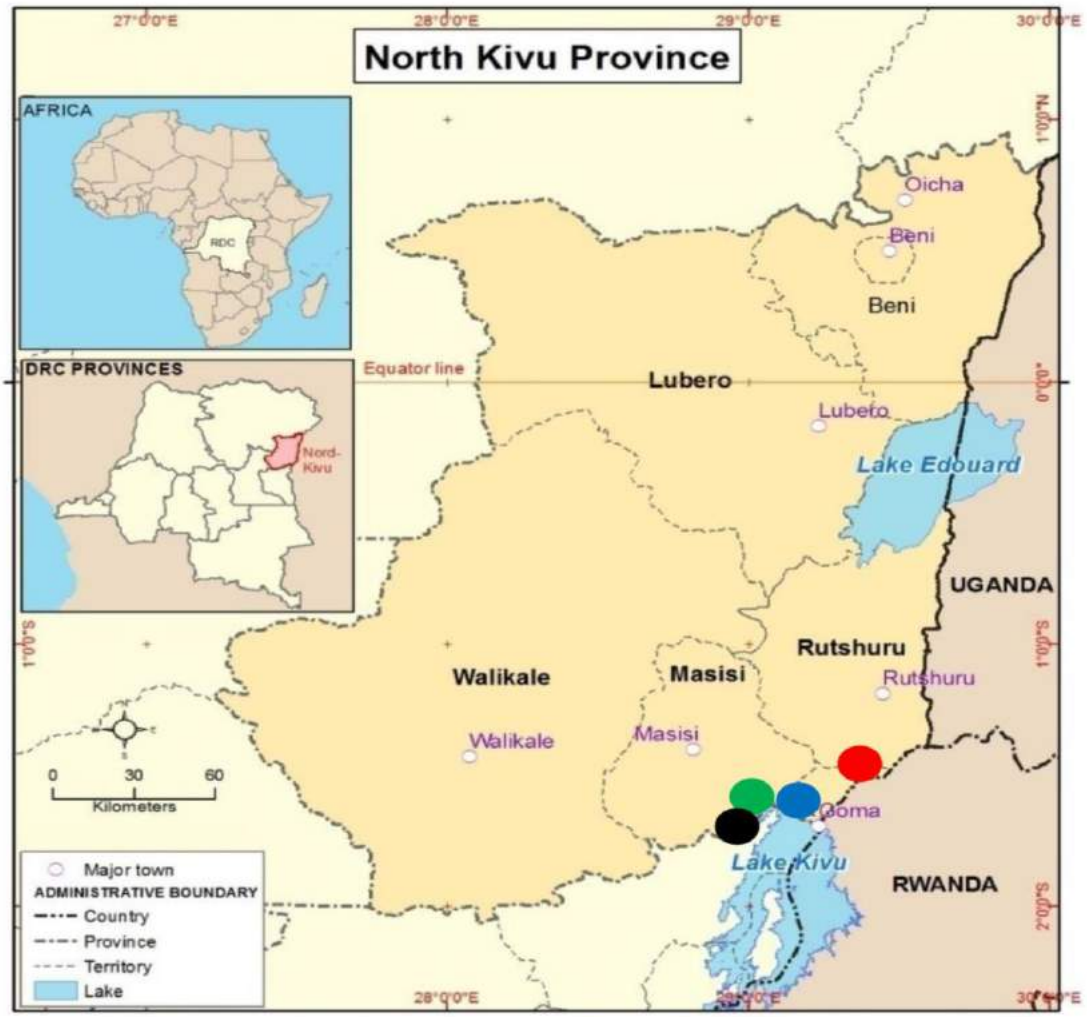
hosted 255,520 people at the end of October 2022 reported the first cases of severe watery diarrhea on October 31, 2022. This triggered the set-up of a cholera treatment center (CTC) for treatment of cases. A total of 3,917 cases were reported with 22 case fatalities. The cholera outbreak was declared as over on January 31 2023. During the same period, a spike in cases of severe watery diarrhea was reported in several settlements around the city Goma, including Bulengo and Mugunga IDP camps. Subsequent to this in suspected cholera cases in Kanyaruchinya IDP, an advanced laboratory for isolation and characterization of *V. cholerae* was set up in the preccint of the IDP camp in the aim of improving the management of this severe cholera outbreak, while the AMI-LABO reach back facility in Goma continued to process samples collected around the city of Goma.

This study describes the phenotypic characteristics and antimicrobial susceptibility profiles of *V. cholerae* O1 isolates from patients suspected with cholera in the Kanyaruchinya IDP camp and in the settlements around the city of Goma between October 31, 2022 and January 31, 2023.

Materials and method:

Study setting:

The districts of Rutshuru and Nyiragongo, which have been the epicentre of protracted civil war since 1996 are located in a range of 20 km to 80 km north of the North-Kivu provincial capital of Goma, which lies on the northern edge of Lake Kivu (Fig 1)



Red circle: Kanyaruchinya IDP; Blue circle: Buhimba settlement; Green circle: Mugunga IDP; Black circle: Bulengo IDP

Fig 1: Map of the North-Kivu province showing the cholera spots between October 31, 2022 and January 31, 2023

Ethical considerations:

Ethical approval for the study was granted by the Comité National d'Ethique de la Santé Publique (CNES) under the reference 6/BUR-CNES/NK/2023. A waiver for written informed consent was obtained from the same body after consultation with the North-Kivu Provincial Healthcare Division (Division Provinciale de la Santé), who both deemed an oral consent sufficient, given the dire circumstances created by the new wave of violence, and the magnitude of the cholera outbreak in the huge camp for IDPs, and around Goma.

The study complied with the World Health Organization (WHO) and international guidelines on investigation during outbreaks (https://apps.who.int/iris/bitstream/handle/10665/250580/9789241549837-eng.pdf). Personal identifiers were removed so that analyses of stored isolates were not traceable to individual patients. Each sample was labelled using a code referring to the date and location of sampling.

Study period, participants and sample collection:

From October 31, 2022 to January 31, 2023, a total of 3,917 patients meeting the clinical case definition of cholera (i. e. an acute watery diarrhea with or without vomiting in a patient with more than one year of age) were admitted in the ward of the cholera treatment center (CTC) of the Kanyaruchinya IDP camp in the North-Kivu province. None of the patients reported having taken antibiotics between symptoms onset and admission in the CTC ward of the Kanyaruchinya IDP camp. During the same period, 538 patients suspected of cholera in the settlements around the city of Goma were assessed for the presence of *V. cholerae* in their stools samples.

Rectal swab specimens were collected from a total of 2142 patients (1604 from the Kanyaruchinya IDP camp, and 538 from the settlements around the city of Goma) upon their arrival at the CTC or at the healthcare center, and before administration of antibiotics. The rectal swabs were put in Carry-Blair medium and immediately shipped to the laboratory for isolation of *V. cholerae*.

Culture isolation and identification of *V. cholerae*:

Laboratory testing was performed by trained personnel following the study protocol. Upon arrival at the laboratory, rectal swabs were incubated in 1% alkaline peptone water broth for 6-8 hours, and subsequently streaked onto thiosulfate-citrate-bile salt sucrose (TCBS) agar and incubated at 37°C for 16–24 hours. Large flattened yellow colonies with opaque centers and translucent peripheries were sub-cultured on nutrient alkaline agar plates overnight.

The colonies were further characterized by Gram-staining and light microscopic examination, and conventional biochemical tests such as oxidase, Kligler's iron agar for fermentation of carbohydrates, Voges-Proskauer and methyl red. Polyvalent O1, Ogawa and Inaba antisera (Becton Dickinson, Erembodegem, Belgium) were used for serotyping of the *V. cholerae* isolates, according to the manufacturer's recommendations.

Antimicrobial susceptibility testing:

Susceptibility of the *V. cholerae* O1 isolates to 8 antimicrobial agents (ampicillin, azithromycin, chloramphenicol, doxycycline, co-trimoxazole, ciprofloxacin, norfloxacin and tetracycline) was determined by the disk diffusion method according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) as updated in January 2023, and the CLSI for antibiotics for which no EUCAST breakpoints were available. *Escherichia coli* ATCC 35218 was used as control for bacterial growth and susceptibility testing.

Statistical analyses:

Statistical analyses were done using the SPSS 25.0 version software. Chi-square test was used to compare recovery rate of *V. cholerae* from the rectal swabs taken at the CTC of the Kanyaruchinya IDP camp with those taken from patients around Goma city. A p value < 0.05 was considered statistically significant.

Results:

Fig 1 shows a map of the North-Kivu province, with IDP camps and settlements affected by the outbreak. A total of 807 and 206 *V. cholerae* isolates were cultured respectively from the 1604 rectal swabs samples at the Kanyaruchinya IDP camp, and 538 rectal swab samples at the settlements around the city of Goma. The corresponding positivity rates were 50.3% and 38.3% respectively. The difference in culture positivity rates between Kanyaruchinya IDP camp and around the city of Goma was statistically significant (p < 0.01).

All the *V. cholerae* isolates from Kanyaruchinya IDP (n=807, 100.0%) were serotyped as *V. cholerae* O1 Inaba (i. e. agglutinated simultaneously with *V. cholerae* O1 Poly and Inaba antisera) whereas 136/206 (66%), 67/206 (32.5%), and 3/206 (1.5%) *V. cholerae* isolates from settlements around the city of Goma were serotyped as Ogawa (i. e. agglutinated simultaneously with *V. cholerae*

Table 1: Comparative antimicrobial susceptibility profiles of selected *Vibrio cholerae* O1 serotypes isolated from patients from Kanyaruchinya IDP camp and around the City of Goma

Location	**Kanyaruchinya IDP camp**		**City of Goma**					
Serotypes	**Inaba (n=174)**		**Inaba (n=20)**		**Ogawa (n=41)**		**Hikojima (n=1)**	
Antibiotics	**No sensitive (%)**	**No resistant (%)**	**No sensitive (%)**	**No resistant (%)**	**No sensitive (%)**	**No resistant (%)**	**No sensitive (%)**	**No resistant (%)**
Ampicillin	0	174 (100.0)	7 (35.0)	13 (65.0)	14 (34.1)	27 (65.9)	0	1 (100.0)
Ciprofloxacin	174 (100.0)	0	14 (70.0)	6 (30.0)	27 (65.9)	14 (34.1)	1 (100.0)	0
Norfloxacin	174 (100.0)	0	14 (70.0)	6 (30.0)	27 (65.9)	14 (34.1)	1 (100.0)	0
Azithromycin	174 (100.0)	0	20 (100.0)	0	41 (100.0)	0	1 (100.0)	0
Tetracycline	174 (100.0)	0	20 (100.0)	0	41 (100.0)	0	1 (100.0)	0
Doxycycline	174 (100.0)	0	20 (100.0)	0	41 (100.0)	0	1 (100.0)	0
Chloramphenicol	0	174 (100.0)	0	20 (100.0)	0	41 (100.0)	0	1 (100.0)
Cotrimoxazole	0	174 (100.0)	0	20 (100.0)	0	41 (100.0)	0	1 (100.0)

O1 Poly and Ogawa antisera), Inaba (i. e. agglutinated simultaneously with *V. cholerae* O1 Poly and Inaba antisera), and Hikojima (i. e. agglutinated simultaneously with *V. cholerae* O1 Poly; Ogawa, and Inaba antisera) respectively.

Antimicrobial susceptibility tests performed on a total of 236 selected *V. cholerae* isolates [174 selected randomly from the 807 isolates collected at the Kanyaruchinya IDP camp, and 62 from the settlements around the city of Goma (with 41 *V. cholerae* O1 serotype Ogawa, 20 *V. cholerae* O1 Inaba serotype, and 1 *V. cholerae* O1 serotype Hikojima) is shown in Table 1. All the tested *V. cholerae* isolates (100.0%) displayed reduced susceptibility to cotrimoxazole. All the tested from the Kanyaruchinya IDP camp (n=174) were susceptible to ciprofloxacin, norfloxacin, tetracycline, doxycycline, and azithromycin, but displayed reduced susceptibility to ampicillin, and chloramphenicol.

The antimicrobial susceptibility profiles of the 62 *V. cholerae* isolates from patients around the city of Gomawere were slightly different from the patterns of isolates from the Kanyaruchinya, with 35.0% of *V. cholerae* O1 Inaba isolates susceptible to ampicillin, 100% to tetracycline and doxycycline, and 100% resistant to chloramphenicol. With respect to ciprofloxacin, 70% and 30% of isolates were susceptible and resistant respectively. Of the *V. cholerae* O1 Ogawa isolates (n=41), 100% were resistant to chloramphenicol, while the remaining were susceptible to tetracycline, doxycycline, and azithromycin, and 34.1% and 65.9% were susceptible and resistant to ampicillin respectively, while 65.9% and 34.1% of Ogawa isolates were susceptible and resistant to ciprofloxacin respectively.

Discussion:

Since the in influx of Rwandan refugees in DRC in 1994, cholera has become a major healthcare issue in the eastern part of the country, especially in the basins of Lakes Kivu (which includes Goma and its surroundings), and Tanganyika (17). Phylogenomics analyses on *V. cholerae* O1 isolates in eastern DRC provinces in recent studies have shown that a lineage of *V. cholerae* O1 Inaba serotype corresponding to ST515 or AFR10d was the sole lineage associated with cholera outbreaks in the basin of Lake Kivu, whereas the AFR10d coexisted with a ST69 lineage corresponding to AFR10e in the basin of Lake Tanganyika (16,17). In the recent years however, the AFR10d was apparently extinct around Lake Tanganyika (16), whereas no data on isolates in the basin of Lake Kivu were available.

Our daily routine work on *V. cholerae* isolated in settlements around the city of Goma suggest a similar trend to 2021-2022, as only *V. cholerae* O1 Ogawa isolates were characterized in our laboratory (data not shown). Our data show that probably one *V. cholerae* O1 Inaba lineage was responsible of the cholera outbreak in the Kanyaruchinya IDP camp, whereas in settlements around the city of Goma, at least three lineages (*V. cholerae* O1 Inaba, *V. cholerae* O1 Ogawa, and *V. cholerae* O1 Hikojima) co-existed during the cholera outbreak. We hypothetize

that *V. cholerae* O1, Inaba serotype from patients in IDP who left their homes in the Rutshuru district more than fifty kilometers north of Goma, and who found shelter in the Kanyaruchinya IDP camp before pushing further south towards the city of Goma and its sorroundings, might have re-introduced the *V. cholerae* O1 Inaba lineage in these settlements around Lake Kivu where *V. cholerae* O1 Ogawa was already present.

The characterization of isolates of the Hikojima is somewhat startling, and to the best of our knowledge has never been reported prior to this study. The characterization of the three isolates of Hikojima phenotype warrants further investigation as this phenotype has never been characterized in the North-Kivu province. It is expected that future genomic characterization of these Hikojima serotypes will help to get insight into circulation of *V. cholerae* O1 lineages in eastern provinces of DRC and their relationships with other serotypes in the region.

In our study, only 34.1 % of Ogawa isolates were resistant to ciprofloxacin. This finding is intruiging, considering that recent data from Lake Tanganyika basin have shown that Ogawa isolates belonging to the the AFR10e (ST69) lineage displayed reduced susceptibility to ciprofloxacin (16). The difference in culture yield of *V. cholerae* between the Kanyaruchinya IDP camp samples and those from settlements around Goma is difficult to explain, considering the fact that the same teams performed the faecal sampling. Indeed between 50.3% of rectal swabs cultured positive for *V. cholerae* O1 in Kanyaruchinya IDP camp compared to 38.3% from samples collected around Goma, and which is consistent with previous studies in the region (15). One plausible explanation would be the fact that the laboratory had a team at the Kanyaruchinya IDP which process the faecal samples directly without any delay, which was not the case for samples coming from settlements around Goma.

Our study had several limitations. First, due to financial constraints, antimicrobial susceptibility tests could only be completed for 174 and 62 isolates selected randomly out of a total of 807 and 206 isolates from the Kanyaruchinya IDP camp and from the settlements around Goma city respectively, which fall short of the required sample size, as recommended in medical studies (18). The second limitation is the unavailability of minimum inhibitory concentration (MIC) values for all the tested antimicrobial agents as a reference method for confirmation of antimicrobial susceptibility profile of antibiotics.

While no inference on the general anti microbial susceptibility profiles of *V. cholerae* O1 associated with this cholera outbreak can be made due to the above limitations, our findings are worthy of being reported, and make a case for continuous monitoring of *V. cholerae* isolates associated with cholera in DRC. They should also serve as cautions for policy makers in DRC that antimicrobial treatment of *V. cholerae* O1 infection in DRC should take into account the co-existence of several antimicrobial resistance among *V. cholerae* O1 isolates. We are convinced that antimicrobial susceptibility testing in early phase of a cholera outbreak should be used as a way to establish guidelines for antimicrobial treatment of cholera rather than adopting a national antimicrobial policy not adapted to the ever changing features of *V. cholerae* associated with cholera outbreaks in DRC.

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Contributions of authors:

RKSK and PKM designed the study; RKSK wrote the first draft of the manuscript; HKM, JMB and PKM oversaw the acquisition of data; BTM, HKM, JMB and PKM analyzed and interpreted the data; and RKSK and PKM finalized the writing of the manuscript. All authors read and approved the final version of the manuscript.

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Conflicts of interest:

No conflict of interest is declared

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Original Article **Open Access**

Emergence of clinical *vanA*-type vancomycin-resistant *Staphylococcus aureus* isolates in National Orthopaedic Hospital Dala, Kano, Nigeria

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Abstract:

Background: The increasing prevalence of multi drug resistance (MDR) in strains of *Staphylococcus aureus* is a major challenge in the selection of an appropriate therapeutic agents, especially in persistent orthopaedic infections. This study investigated the patterns of antimicrobial resistance and identified the genetic determinants of resistance in *S. aureus* isolates from orthopaedic patients.
Methodology: This was a descriptive cross-sectional study of hospitalized patients at National Orthopaedic Hospital Dala (NOHD), Kano, Nigeria from whom urine samples, and nasal and wound swabs were collected for isolation of *S. aureus*. Samples were cultured on standard media and *S. aureus* isolated and identified using both conventional biochemical tests and a standard rapid diagnostic kit. The antibiotic susceptibility was determined to a panel of 15 antibiotics using the modified Kirby-Bauer disc diffusion method. Vancomycin minimum inhibitory concentration (MIC) of each isolate was determined using vancomycin Epsilon-test strip. *mecA* and *vanA* were detected by multiplex polymerase chain reaction (PCR) assay.
Results: From the total of 134 samples, *S. aureus* was isolated from 36 (26.8%); 10 (7.4%) from urine, 13 (9.7%) from nasal swab, and 13 (9.7%) from wound swab. Thirty-four (94.4%) isolates were phenotypically methicillin (cefoxitin) resistant (MRSA), while 2 (5.6%) isolates were methicillin sensitive (MSSA). Phenotypic resistance rate of the *S. aureus* isolates was highest to gentamicin (94.4%), followed by penicillin (88.8%), cephalosporins and fluoroquinolones (87.4%), while rate was lowest to vancomycin (11.1%, 4/36). Seventeen (47.2%) were MDR, 16 (44.4%) were extensively drug resistant (XDR), and 2 (5.6%) were pan-drug resistant (PDR) *S. aureus* isolates. The *mecA* gene was detected in 4 (11.8%) of the 34 phenotypic MRSA isolates and *vanA* genes in 2 (50.0%) of the 4 phenotypic VRSA isolates.
Conclusion: The detection of *vanA* and *mecA* in clinical *S. aureus* isolates in this study is an indication that clinical VRSA has emerged in MRSA population in Nigeria. This emergence can pose a major threat to primary care-givers and a public health challenge among the daily inhabitants of National Orthopaedic Hospital Dala (NOHD), Kano and the community at large.

Keywords: *Staphylococcus aureus*, *vanA*, *mecA*, MDR, XDR, PDR, orthopaedic

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Émergence d'isolats cliniques de *Staphylococcus aureus* résistants à la vancomycine de type-*vanA* à l'Hôpital National d'Orthopédie de Dala, Kano, Nigeria

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Résumé:

Contexte: La prévalence croissante de la multirésistance aux médicaments (MDR) dans les souches de *Staphylococcus aureus* est un défi majeur dans la sélection d'agents thérapeutiques appropriés, en particulier dans les infections orthopédiques persistantes. Cette étude a examiné les modèles de résistance aux antimicrobiens et identifié les déterminants génétiques de la résistance dans les isolats de S. aureus provenant de patients orthopédiques.
Méthodologie: Il s'agissait d'une étude transversale descriptive de patients hospitalisés à l'Hôpital National Orthopédique de Dala (NOHD), à Kano, au Nigeria, auprès desquels des échantillons d'urine et des écouvillons nasaux et de plaies ont été prélevés pour l'isolement de *S. aureus*. Les échantillons ont été cultivés sur des milieux standard et S. aureus a été isolé et identifié à l'aide d'un test biochimique conventionnel et d'un kit de diagnostic rapide standard. La sensibilité aux antibiotiques a été déterminée sur un panel de 15 antibiotiques en utilisant la méthode de diffusion sur disque de Kirby-Bauer modifiée. La concentration minimale inhibitrice (CMI) de vancomycine de chaque isolat a été déterminée à l'aide d'une bandelette de test de vancomycine Epsilon. *mecA* et *vanA* ont été détectés par un test de réaction en chaîne par polymérase (PCR) multiplex.
Résultats: Sur un total de 134 échantillons, *S. aureus* a été isolé à partir de 36 (26,8%); 10 (7,4%) d'urine, 13 (9,7%) d'écouvillonnage nasal et 13 (9,7%) d'écouvillonnage de plaie. Trente-quatre (94,4%) isolats étaient phénotypiquement résistants à la méthicilline (céfoxitine) (SARM), tandis que 2 (5,6%) isolats étaient sensibles à la méthicilline (MSSA). Le taux de résistance phénotypique des isolats de *S. aureus* était le plus élevé à la gentamicine (94,4%), suivie de la pénicilline (88,8%), des céphalosporines et des fluoroquinolones (87,4%), tandis que le taux était le plus faible à la vancomycine (11,1%, 4/36). Dix-sept (47,2%) étaient multirésistants, 16 (44,4%) étaient extrêmement résistants aux médicaments (XDR) et 2 (5,6%) étaient des isolats de *S. aureus* pan résistants aux médicaments (PDR). Le gène *mecA* a été détecté dans 4 (11,8%) des 34 isolats phénotypiques de SARM et les gènes *vanA* dans 2 (50,0%) des 4 isolats phénotypiques de VRSA.
Conclusion: La détection de *vanA* et *mecA* dans les isolats cliniques de *S. aureus* dans cette étude est une indication que le VRSA clinique est apparu dans la population de SARM au Nigeria. Cette émergence peut constituer une menace majeure pour les dispensateurs de soins primaires et un défi de santé publique parmi les habitants quotidiens de l'hôpital national d'orthopédie de Dala (NOHD), de Kano et de la communauté dans son ensemble.

Mots clés: *Staphylococcus aureus*, *vanA*, *mecA*, MDR, XDR, PDR, orthopédique

Introduction:

Clinically significant bacterial isolates, particularly *S. aureus*, are progressively becoming resistant to almost all antibiotics currently on the market, making them practically ticking time bombs in the near future unless an alternative therapeutic method is offered. This situation poses a threat to antibiotics of last resort. *Staphylococcus aureus* is a member of the human microbiota that has been linked to a variety of illnesses, including mild skin and soft tissue infections (SSTIs) and severe, life-threatening conditions such as infective endocarditis, surgical site infection (SSI), periprosthetic joint infections (PJI), osteomyelitis, and toxic shock syndrome (TSS), in both healthy people and in those with underlying illnesses (1,2).

Despite the difference in the hospital mortality rates for methicillin-susceptible *S. aureus* (MSSA) and the methicillin-resistant *S. aureus* (MRSA) (3), infections caused by MRSA has steadily been on the rise worldwide, resulting in increase consumption of vancomycin (4). The antibiotic 'selective pressure' among overcrowded inhabitants creates an environment that is suitable for the rapid development and efficient spread of numerous multidrug-resistant (MDR) pathogens in both the community and hospital settings (5). This has remained a huge challenge for healthcare professionals.

In the past ten years, there has been an exponential rise in the prevalence of vancomycin-resistant *S. aureus* (VRSA), vancomycin intermediate *S. aureus* (VISA), and heterogeneous vancomycin intermediate *S. aureus* (hVISA) infections. This is supported by *invitro* research that points to multiple vancomycin resistance mechanisms in MRSA, the main ones being decreased permeability and increased cell wall thickness, which reduce vancomycin availability for intracellular target molecules (1). Another type of resistance was brought on by plasmid-mediated vancomycin resistance genes (*vanA*, *vanB*, *vanD*, *vanE*, *vanG* and *vanL*), which may have been acquired from enterococci species (1).

The risk of surgical infections, morbidity, mortality, and financial burden for orthopaedic patients are all known to be greatly increased by MSSA/MRSA colonization and admission into healthcare institutions (6,7). Vancomycin is still regarded as the most effective therapeutic agent against infections caused by MRSA, but its prolonged and extensive usage, dependence on haemodialysis, intensive care units (ICU), and use in patients with indwelling devices have led to the rise of VRSA infections (8).

High frequency of clinical VRSA infec-

tions have been reported in the American continent (1), and the molecular mechanism of resistance in these VRSA strains has been well characterized. In order to establish the hypothesis that "VRSA may emerge more frequently than expected in Nigeria", many researchers have investigated and determines its prevalence using phenotypic methods, with many reporting culture isolations of clinical VISA and VRSA among patients with different infective conditions (9-12). However, only few studies have used molecular methods to investigate the occurrence of VISA and VRSA in Nigeria and none has detected *van* genes in any *S. aureus* isolate (13). The objective of this study is to investigate the patterns of antimicrobial resistance in clinical *S. aureus* isolates from National Orthopaedic Hospital Dala, Nigeria, and identify the genetic determinants of MRSA and VRSA using multiplex PCR approach.

Materials and method:

Study setting and design:

The study setting is National Orthopaedic Hospital Dala (NOHD), Kano located in the north-western region of Nigeria but also a referral hospital for orthopaedic patients from the north-west, north-east, and north-central regions of the country. The hospital has 9 wards and an emergency unit, which can accommodate nearly 2500 inpatient admissions and more than 9000 outpatients per year.

The study design is descriptive cross-sectional conducted between September 2018 and March 2019 on all hospitalized patients in the hospital while excluding patients who declined to participate in the study.

Ethical approval:

The hospital research ethics committee granted permission to conduct the study (NOHD/RET/ETHIC/60). In addition, informed consent of each patient who participated in the study was obtained.

Sample collection, isolation and identification:

Urine sample, nasal and wound swabs, were collected from a total of 134 hospitalized patients according to standard procedures. The specimens were inoculated onto nutrient broth and mannitol salt agar, and incubated aerobically at 37^{o}C for 24 hours.

Suspected staphylococcal colonies on the culture plates were preliminary identified by conventional method of Gram stain, catalase and coagulase tests (Staphytect Plus Test Kit). Standard rapid diagnostic kit (Microgen™ Staph-ID System) and the software probability test results were used to conclusively identify each isolate as *S. aureus*.

Determination of antibiotic susceptibility:

Antibiotic susceptibility test (AST) was performed on pure cultures of the *S. aureus* isolates using the modified Kirby-Bauer disc diffusion method (14). Recommended classes of antibiotics including benzylpenicillin (1µg), amoxicillin-clavulanic acid (30µg), oxacillin (1 µg), cefoxitin (30µg), ceftriaxone (30µg), norfloxacin (10µg) ciprofloxacin (5µg), gentamicin (10µg), erythromycin (15µg), clindamycin (15µg), quinupristin-dalfopristin (15µg), tetracycline (30µg), linezolid (10µg) and trimethoprim-sulfamethoxazole (25µg) discs were used. Vancomycin E-test strip was used to determine vancomycin minimum inhibitory concentration (MIC).

The discs and strips were aseptically placed using sterile forceps on Muller Hinton agar plates that have been inoculated with standardized inoculum of pure colonies of *S. aureus* isolates, and incubated at 37°C for 24 hours. The plates were examined for the presence or absence of zones of inhibition of bacterial growth, and the inhibition zone diameters were interpreted as sensitive or resistance in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline (15).

DNA extraction and PCR amplification:

DNA extraction was performed on *S. aureus* isolates that phenotypically resistant to antibiotic discs. A typical isolate was cultivated in 5ml Luria-Bertani (LB) for 24 hours at 37°C. The genomic DNA (gDNA) of the isolates was extracted with a ZR Fungal/Bacterial DNA Mini Prep™ (USA) using the protocol described by the manufacturer (16).

Amplification of the antibiotic-resistant *vanA* and *mecA* genes was performed in a PCR thermal cycler (Bio-Rad DNA Machine) after an external optimization of the reaction to ensure amplification with specific *vanA* and *mecA* primers (17,18) (Table 1). The PCR master mix contained 1.0 µl each of forward and reverse primers, 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.15 mmol/L dNTP, 1.25 IU Taq DNA polymerase, and 1 L of prepared DNA (0.5g) template, which was added to the final volume. The cycling conditions (denaturation, annealing, and extension) were as previously described by Pournajaf et al., (19).

Results:

Of the total of 134 clinical samples cultured, 78 (58.2%) yielded growth of Staphylococcus isolates. *Staphylococcus aureus* was isolated from 36 (27.6%), *Staphylococcus hyicus* from 1 (0.7%), and coagulase negative staphylococci (CoNS) from 28 (20.8%) (Fig 1).

The percentage resistance of isolated *S. aureus* to the classes of antibiotics tested is shown in Fig 2, with resistance rates exceeding 80% to penicillins, cephalosporins, fluoroquinolones and aminoglycosides, and over

70% resistance rates to macrolide-lincosamide-streptogramin (MLS), tetracycline and sulfamethoxazole. Resistance rates to oxazolidinones (linezolid) exceeded 50% while resistance to glycopeptide (vancomycin) was 11.1% (4/36).

Table 1: Primers used to amplify and detect *mecA* and *vanA* genes

Target gene	Primer name	Oligonucleotide sequence	Amplicon size (bp)	Reference
mecA	MecA-F	GGGATCATAGCGTCATTATTC	527	17
	MecA-R	AACGATTGTGACACGATAGCC		
vanA	VanA-F	CATGAATAGAATAAAAGTTGCAATA	1030	18
	VanA-R	CCCCTTTAACGCTAATACGATCAA		

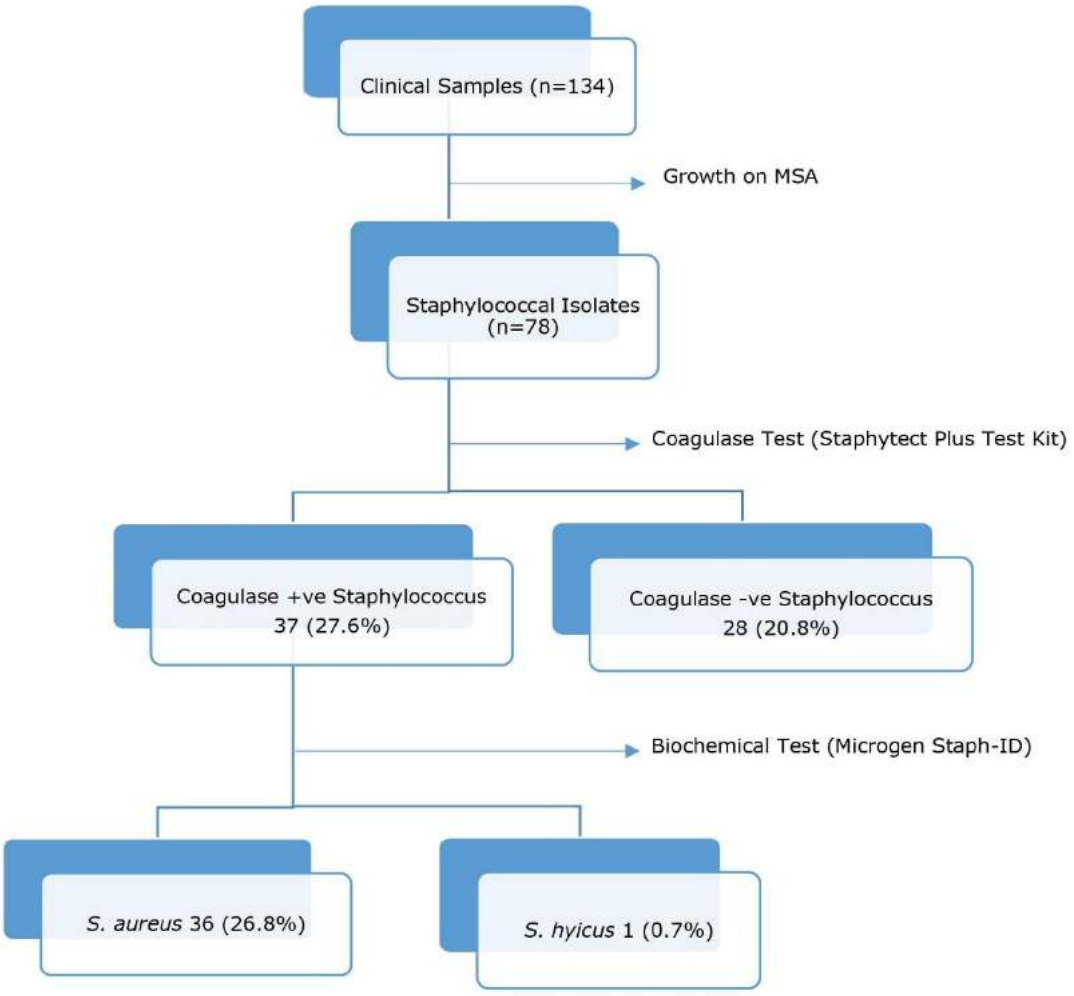


Fig 1: Workflow for isolation and identification of *Staphylococcus aureus*

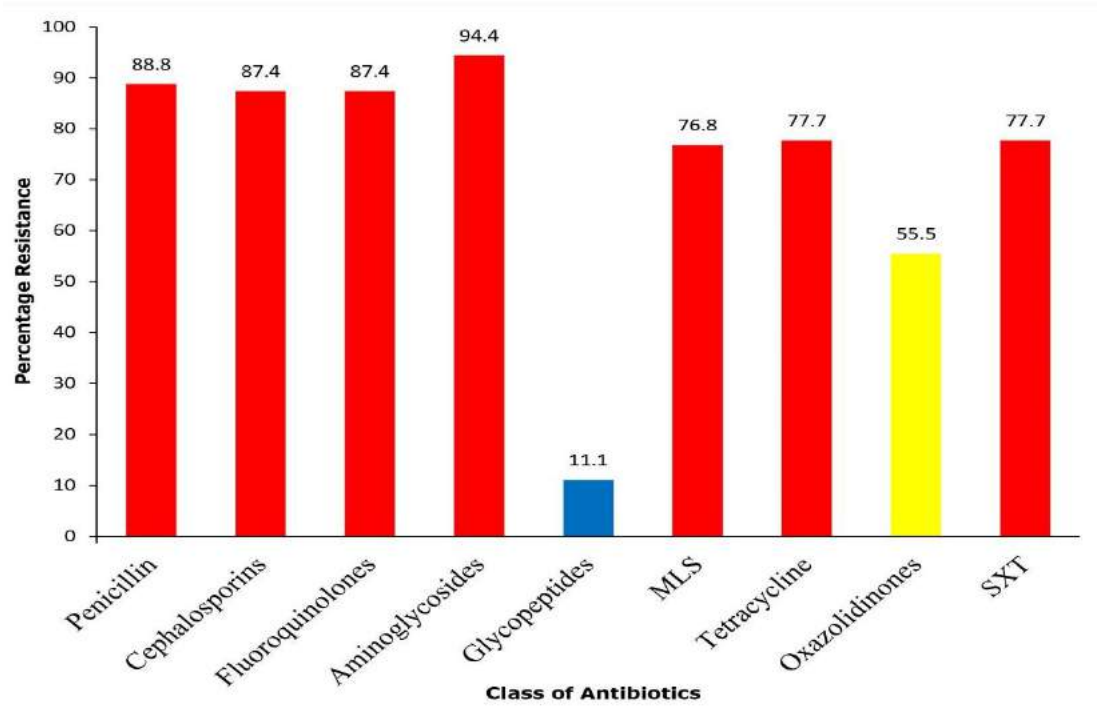


Fig 2: Percentage resistance of isolated *Staphylococcus aureus* to selected antibiotic classes

Table 2: Percentage distribution of isolates by methicillin susceptibility and antibiotic resistance classification

Specimen type	MRSA (%)	MSSA (%)	MDR (%)	XDR (%)	PDR (%)
Urine sample (n=10)	9 (25.0)	1 (2.8)	6 (16.7)	6 (16.7)	1 (2.8)
Wound swab (n=13)	13 (36.1)	0	8 (22.2)	7 (19.4)	1 (2.8)
Nasal swab (n=13)	12 (33.3)	1 (2.8)	3 (8.3)	3 (8.3)	0
Total (n=36)	**34 (94.4)**	**2 (5.6)**	**17 (47.2)**	**16 (44.4)**	**2 (5.6)**

MRSA: methicillin-resistant *S. aureus*; MSSA: methicillin sensitive *S. aureus*; MDR: multi-drug resistant; XDR: extensively drug-resistant; PDR: pan-drug resistant

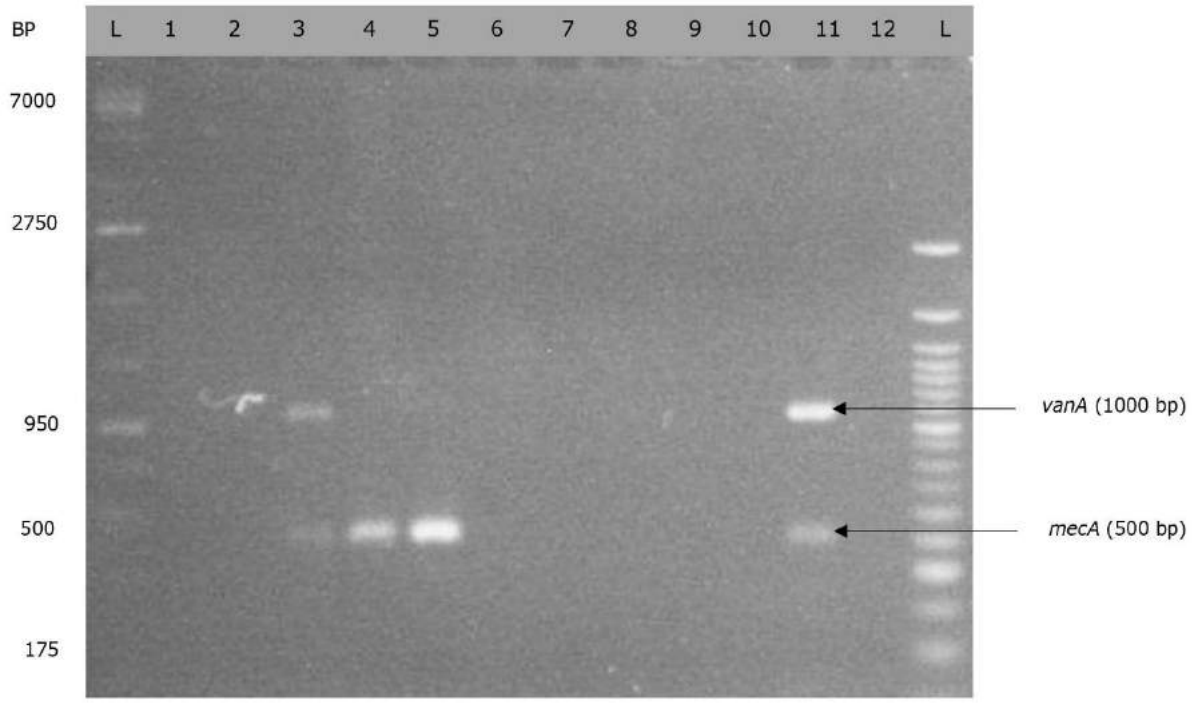
As shown in Table 2, resistance to cefoxitin was used to classify the isolates into MRSA and MSSA according the 2021 EUCAST guideline (15). Among the tested *S. aureus* isolates, 34 (94.4%) were MRSA, and only 2 (5.6%) were MSSA. The isolates were also classified based on their antibiotic resistance profile as described by Magiorakos (20), with 17 (47.2%) being MDR, 16 (44.4%) extensively drug resistant (XDR), and 2 (5.6%) pan-drug-resistant (PDR).

Fig 3 represents the gel electrophoresis pattern of the multiplex PCR amplicons which showed that 4 *S. aureus* isolates tested harbored *mecA* gene while 2 harbored *vanA* gene.

Discussion:

Staphylococcus aureus is one among the bacteria that is most frequently found in orthopaedic patients, especially those with implants, and it typically calls for a challenging and expensive course of therapy. It is the second leading global cause of death for people and can set off a number of infectious diseases (21,22). This has frequently presented its victims with significant economic and health challenges. In this study, we investigated the antibiotic resistance profile of *S. aureus* recovered from hospitalized orthopaedic patients at the NOHD, and reported a prevalence of 26.8%. In a systematic review, a lower prevalence of 18.2% was reported for nasal carriage and urinary tract infection among patients with HIV/AIDS in Nigeria (23). Majority of orthopaedic patients require implants, and since wounds such as burns are frequently left exposed and heal slowly, these circumstances may have contributed to the high prevalence observed.

According to a previous research, *S. aureus* is capable of developing resistance to



Lane 3: N18, Lane 4: U15, Lane 5: U21, Lane 11: W56, Lane L: 100bp Molecular DNA ladder
Keys: N= Nasal, U= Urine, W= Wound, BP= Base pair

Figure 3: Gel electrophoresis of the PCR amplicons of the *vanA* and *mecA* genes from representative resistant *Staphylococcus aureus* isolates

every class of antibiotic now in use and resistance can arise through *de novo* chromosomal gene changes or from the acquisition of horizontally transferred resistance determinants (24). Except for oxazolidinones and glycopeptides, resistance of *S. aureus* isolates was over 70% to almost all of the classes of antibiotics evaluated in this study. The high resistance observed is caused by the ability of these *S. aureus* isolates to horizontally acquire genetic elements, that results in emergence of MRSA strains, which is a serious issue and threat to public health. According to this study, linezolid is becoming a less effective alternative for treating orthopaedic MRSA infections. However, vancomycin still remains a reserve antibiotic under hospital treatment policy and guidelines (25).

MRSA strains are known to cause a variety of infections, including surgical site infection (SSIs), bones and joints infection, bacteraemia, meningitis, pneumonia, endocarditis, and toxic shock syndrome (TSS), some of which have a major impact on patient morbidity, mortality, and financial burden (26). Although, the high level of resistance by some of the isolates is attributed to over expression of penicillin binding protein 2a (PBP2a) due to duplication or enhanced transcription of the *mecA* gene, the high percentage resistance to penicillins and cephalosporins, indicates that MRSA may have become resistant to beta-lactams through acquisition of specific genetic determinant (*mecA*) (24) and other multi-drug resistant genetic determinants.

Despite the efficacy of clavulanic acid in combination with other antibiotics to treat hospital-acquired MRSA infections, resistant strains and those with diminished susceptibility to ceftaroline (a fifth-generation cephalosporin) have been identified (27). Vancomycin and daptomycin, two supposedly 'last-resort' antibiotics, have been made available to clinicians as therapeutic choices as a result of this 'selection pressure' (28). In spite of the fact that daptomycin has broad-spectrum effectiveness against Gram-positive organisms such as MRSA, there are no published randomized control studies or data to support its usage in infections following orthopaedic surgery (25).

Vancomycin is an alternate treatment option for MRSA infections; however, some strains of *S. aureus* (VRSA) have acquired the *vanA* operon, which encodes enzymes that helps them hydrolyze the normal D-ala-D-ala precursors for vancomycin, and to synthesize new D-ala-D-lactate precursors that have reduced binding affinity to vancomycin and other glycopeptides (29). Although *vanA* was detected in only 2 (5.6%) of the 36 *S. aureus* isolated among the participants in this study, these VRSA isolates were resistant to all the prescribed and accessible antibiotics, implying those orthopaedic patients harboring such strains will have fewer or no treatment options available to them, and there is also a high risk

that they can horizontally spread the resistant genes to other bacteria (including *S. aureus*) in the microbiome of the hospital. As such, the hospital MRSA and VRSA strains are particularly a threat to patients, guardians and primary caregivers (nurses and doctors). Detection of these strains in Nigerian hospitals may not be unrelated to increase use of glycopeptides (vancomycin) in recent times, unsanitary conditions, unclean ward beddings, and non-compliance with prescribed antibiotic medications (30). However, vancomycin is not available for prescription in NOHD, and nosocomial preventive measures are usually enforced within the hospital environment.

About 50% of the *S. aureus* isolates recovered from all the samples (urine, nasal and wound swabs) were both MDR and XDR, indicating that the isolates in this study may have arisen from previous antibiotics exposure or may have picked up resistant genes from other organisms such as vancomycin resistant enterococci (VRE) in the environment, which are known to be reservoirs of antimicrobial resistance (AMR) genes that are carried on plasmids, transposons and integrons. In contrast to our study, 90.2% and 71.6% of the *S. aureus* isolated from urine and wound samples of sick patients respectively in several Nigerian teaching hospitals were MDR (9). The type of infectious diseases, the locales, or community pressure on the antibiotics tested could all be contributing factors to variations in MDR rates. Although, 37.1% MDR, 13.8% XDR and 0% PDR were reported among bacterial strains in the study by Basak et al., (31), these rates were still slightly lower than the rates reported in our current study.

The emergence of VRSA and occurrence of XDR and PDR *S. aureus* strains in our study constitute significant financial burden to orthopaedic patients (and/or their guardians) who require implants and gradual recovery process. There are limited number of alternatives for potent antibiotic medications required to combat the threats of these strains in Nigerian hospitals. Unfortunately, this high prevalence of pathogenic MDR *S. aureus* strains may cause NOHD, a referral center for orthopaedic patients, to eventually develop into a center for nosocomial infections.

Conclusion:

This study reports the emergence of clinical *vanA*-type VRSA in NOHD, Kano, Nigeria and found that the majority of the *S. aureus* isolated from orthopaedic patients were MDR and XDR strains from their *in vitro* resistance to EUCAST-recommended antibiotics.

The detection of *vanA* and *mecA* in a few *S. aureus* isolates raises the probability of horizontal transmission to other isolates and increased risk of nosocomial infection in the NOHD. Although there are ongoing research studies on developing novel therapeutic approaches that may be effective against VRSA strains, for now, clinicians are limited to very few treatment options.

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Contributions of authors:

AU, AGO and OBO were involved in study conceptualization; AU and ODA were involved in data acquisition; AU, ODA and KM were involved in analysis of data; AU, KM, AGO and OBO were involved in drafting and critical review of the manuscript. All the authors approved the final manuscript submitted for review.

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Conflicts of interest:

Authors declared no conflict of interest

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Original Article **Open Access**

Emergence of New Delhi metallo-β-lactamase-1 (NDM-1) producing Enterobacterales from water sources: an impending public health challenge in Adamawa-north senatorial zone, Nigeria

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Abstract:

Background: The emergence of New Delhi metallo-beta-lactamase-1 (NDM-1) among Enterobacterales in water sources has raised a major public health concern and constitute critical threat to human health as these organisms exhibits high level of resistance to available potent antibiotics. The aim of this study is to detect the presence of NDM-1 gene among carbapenem resistant Enterobacterales (CRE) isolates from water sources.
Methodology: A total of 256 water samples were collected from randomly selected hand-dug wells (128 samples) and river/stream (128 samples) for each of dry and rainy seasons in four out of the five local government areas (LGAs) of Adamawa-north senatorial zone, Nigeria. The water samples were filtered using membrane filtration technique and the filters introduced into appropriate bacteriologic media for bacterial growth. The bacterial isolates recovered were identified by both phenotypic and molecular protocols. Phenotypic carbapenem (imipenem) resistance was determined by disc diffusion test, bla_{NDM-1} gene was detected by specific polymerase chain reaction (PCR) test, and plasmid DNA was extracted and electrophoresed by standard procedure.
Results: Of the 256 water samples analyzed for bacteria growth, 300 bacterial isolates of the order Enterobacterales were recovered. Of these, only 45 (12.6%) isolates were phenotypically resistant to carbapenem (imipenem) antibiotic and bla_{NDM-1} gene was detected in 30 (66.7%) of these. While bla_{NDM-1} gene was detected in all the isolates of *Klebsiella oxytoca, Klebsiella variicola, Enterobacter aerogenes, Enterobacter hormaechei, Enterobacter asburiae, Citrobacter freundii,* and *Morganella morganii* that were resistant to imipenem, other isolates harbored bla_{NDM-1} gene in varying proportion. Most of the isolates positive for bla_{NDM-1} also harbored R-plasmids.
Conclusion: Emergence of carbapenem resistance mediated by NDM-1 gene in Enterobacterales isolated from water sources constitutes an emerging public health challenge with potential transmission to humans, thereby complicating the treatment of infections caused by these resistant pathogens in man. As such, the urgent need for antimicrobial surveillance and stewardship is of utmost importance.

Keywords: Enterobacterales; NDM-1; carbapenem; water sources; Adamawa; Nigeria

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Émergence de New Delhi métallo-β-lactamase-1 (NDM-1) produisant des Entérobactéries à partir de sources d'eau: un défi de santé publique imminent dans la zone sénatoriale nord d'Adamawa, au Nigeria

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Résumé:

Contexte: L'émergence de la métallo-bêta-lactamase-1 de New Delhi (NDM-1) parmi les entérobactéries dans les sources d'eau a soulevé un problème majeur de santé publique et constitue une menace critique pour la santé humaine, car ces organismes présentent un niveau élevé de résistance aux puissants antibiotiques disponibles. Le but de cette étude est de détecter la présence du gène NDM-1 parmi les isolats d'entérobactéries résistantes aux carbapénèmes (CRE) provenant de sources d'eau.
Méthodologie: Un total de 256 échantillons d'eau ont été prélevés dans des puits creusés à la main (128 échantillons) et des rivières/ruisseaux (128 échantillons) sélectionnés au hasard pour chacune des saisons sèches et pluvieuses dans quatre des cinq zones de gouvernement local (LGA) d'Adamawa-zone sénatoriale nord, Nigeria. Les échantillons d'eau ont été filtrés à l'aide de la technique de filtration sur membrane et les filtres ont été introduits dans des milieux bactériologiques appropriés pour la croissance bactérienne. Les isolats bactériens récupérés ont été identifiés par des protocoles phénotypiques et moléculaires. La résistance phénotypique au carbapénème (imipénème) a été déterminée par un test de diffusion sur disque, le gène *bla*$_{NDM-1}$ a été détecté par un test de réaction en chaîne par polymérase (PCR) spécifique et l'ADN plasmidique a été extrait et soumis à une électrophorèse selon la procédure standard.
Résultats: Sur les 256 échantillons d'eau analysés pour la croissance bactérienne, 300 isolats bactériens de l'ordre Enterobacterales ont été récupérés. Parmi ceux-ci, seuls 45 (12,6%) isolats étaient phénotypiquement résistants à l'antibiotique carbapénème (imipénème) et le gène *bla*$_{NDM-1}$ a été détecté dans 30 (66,7%) d'entre eux. Alors que le gène *bla*$_{NDM-1}$ a été détecté dans tous les isolats de *Klebsiella oxytoca, Klebsiella variicola, Enterobacter aerogenes, Enterobacter hormaechei, Enterobacter asburiae, Citrobacter freundii* et *Morganella morganii* qui étaient résistants à l'imipénem, d'autres isolats abritaient le gène *bla*$_{NDM-1}$ dans différentes proportions. La plupart des isolats positifs pour *bla*$_{NDM-1}$ hébergeaient également des plasmides R.
Conclusion: L'émergence de la résistance aux carbapénèmes médiée par le gène NDM-1 chez les entérobactéries isolées des sources d'eau constitue un défi de santé publique émergent avec une transmission potentielle à l'homme, compliquant ainsi le traitement des infections causées par ces pathogènes résistants chez l'homme. À ce titre, le besoin urgent de surveillance et de gestion des antimicrobiens est de la plus haute importance.

Mots clés: Enterobacterales; NDM-1; carbapénème; sources d'eau; Adamawa; Nigeria

Introduction:

The order Enterobacterales consist of a large group of Gram-negative bacteria that are found in the large intestine of humans and other warm-blooded animals, mostly as endogenous microbiota. Implicated mostly in community and hospital-acquired infections, the most common therapeutic option for infections involving Enterobacteriaceae was the β-lactam antibiotics. However, with the emergence of multi-drug resistant Enterobacteriaceae, carbapenem, a broad -spectrum antibiotic became an ideal and last line of therapeutic option for the treatment of infections involving them (1). Many mechanisms for carbapenem resistance have been documented. The most prominent among them is the production of different classes of carbapenemases (2,3).

Carbapenemases are classified as β-lactamase enzymes that belong to Ambler molecular classes A, B and D (2,4) and are able to hydrolyse the entire β-lactam antibiotics, including monobactams, extended spectrum cephalosporins and carbapenem (2,3). Among the most reported carbapenemases is the New Delhi metallo-β-lactamase-1 (NDM-1), which is encoded by carbapenem resistance determinant, *bla*$_{NDM}$ (5). The development and introduction of antibiotics for therapy came with a lot of prospects and hope. However, the occurrence of new resistant markers, notably NDM-1, incapacitated the potentials of β-lactams as sure therapeutics for infections involving organisms that harbored such resistance markers. New antibiotic resistance markers are evolving every now and then due to mutation (6) and selective pressure in the use of antibiotics which consequently constitute a threat to therapy (4,7).

New Delhi metallo-β-lactamase-1 (NDM-1), a relatively newly described metallo-β-lactamase (MBL), can hydrolyze all β-lactams including carbapenems except monobactam. It was first identified in *Klebsiella pneumoniae* and *Escherichia coli* isolated from a Swedish patient who was hospitalized in India in 2008 (8). Since then, it has spread all over the world (9-11). Members of the order Enterobacterales harboring *bla*$_{NDM-1}$ constitute clinical and public health significance as the gene encoding this enzyme is found on transmissible plasmid, as such resistance traits can be easily transferred from one bacterium to another (12,13).

The detection of *bla*$_{NDM-1}$ gene in pathogens from water sources (14,15) and other environmental samples (16,17) in some parts of the world suggest that this gene is not only limited to clinical pathogens but is also present in our local environment (12). Moreso, the presence of antibiotic resistance genes in water sources meant for domestic purposes could serve as a vital reservoir for the spread of antibiotic resistance to human pathogens (18). Thus, this study intends to bring to limelight the

presence of *bla*$_{NDM-1}$ gene among CRE from water sources in a pristine environment devoid of carbapenem usage.

Materials and method:

Study area:

The study area was Adamawa-north senatorial zone commonly known as Mubi region. Mubi region comprises of five local government areas (LGAs) namely; Madagali, Michika, Mubi south, Mubi North and Maiha with a land size of 4,493.815 km^2 and a population of 682,026. Mubi zone is located between latitudes 9^o 30' and 11^o 00'N of the equator and between 13^o 00' and 14^o 00E of the Greenwich Meridian. The area has a tropical wet and dry climate, the dry season last for minimum of six months (November to March), while the wet season spans between May and October. The mean annual rainfall ranges from 700 to 1050mm. Some of the major ethnic groups in the region include Fali, Gude, Fulani, Marghi, Kilba, Nzanyi, Mudang, Zilwo, among others (19).

Sampling plan:

From each ward, water from four wells were chosen at random and sampled in duplicate in both dry and rainy seasons. A river/ stream was also selected from each LGA for sampling. For each river/stream, two samples were collected at random (at upstream and downstream) in quadruples for both dry and rainy seasons.

Sample collection:

A total of 256 water samples from two sources (well and river) were analysed for bacterial growth. From these, 128 water samples were from 32 hand dug wells (64 water samples each for dry and rainy season) and another 128 samples were from 4 rivers/streams (64 water samples each for dry and rainy season). Each water sample was collected in 500ml sampling bottles, labelled appropriately and transported in an ice-cold box to the Microbiology Laboratory of the Department of Biological Science Technology, Federal Polytechnic Mubi, Adamawa State for analysis (20).

Isolation and identification of bacterial isolates:

Bacteria were isolated by membrane filtration technique using, a sterile 47mm, 0.45µm mixed cellulose ester (MCE) membrane filter (Merck, Bangalore). At the end of the filtration, sterile forcep was used to pick the filter unto the surface of MacConkey agar (MCA) and replicated on Salmonella-Shigella agar (SSA), eosin methylene blue (EMB) and deoxycholate citrate agar (DCA). The plates were incubated at 35-37^{o}C for 18-24 hours.

After Gram stain, each discrete bacterial colony was identified to species level by biochemical tests such as Simmon's citrate test, triple sugar iron (TSI) agar and oxidase test before they were confirmed with Microgen GN A kit and 16S rRNA gene sequencing. The identified bacterial colonies were sub-cultured and stored in nutrient agar slant for further use.

Screening for carbapenemase production:

The isolates were screened for carbapenem resistance and hence possible carbapenemase producers according to CLSI guidelines (21). In this method, 10 µg imipenem antibiotic discs (Oxoid, UK) were placed on the surface of Mueller Hinton Agar (MHA) (Oxoid, UK) plates inoculated with each of the isolate and then incubated at 37°C for 24 h. After incubation, the zone of inhibition ≥ 23 mm in diameter was considered sensitive while the zone of inhibition ≤19mm in diameter was taken as resistance and was considered as suspected carbapenemase producers (21,22).

Molecular detection of New-Delhi metallo-beta-lactamase (*bla*$_{NDM-1}$) gene:

Phenol chloroform method was used for DNA extraction according to manufacturer's instructions (ThermoFisher Scientific). Dried extracted DNA was dissolved in 50 µl of DNAse-free water and kept at -20°C for further use. Moreover, 5 µl of each DNA sample was checked for integrity on 1% agarose gel (23).

The conventional polymerase chain reaction (PCR) was used to amplify genes encoding NDM carbapenemase**.** Specific primer set (F-GGGCAGTCGCTTCCAACGGT, R-GTAGTGCTCAG TGTCGGCAT) targeting 475 bp was used to detect *bla*$_{NDM-1}$ coding gene in 45 bacterial isolates resistant to imipenem in separate PCR reactions (24). PCR profile included an initial denaturing 5 min at 94ºC, then 35 cycles of 94ºC for 50s, 62ºC for 45s and 72ºC for 60s then terminate at 72ºC for 10mins. The PCR was carried out in a Gene Amp 9700 PCR System Thermocycler (Applied Biosystem Inc., USA) using the appropriate profile as designed for each primer pair.

Five microlitres (5µl) of the PCR product was electrophoresed in 2% agarose gel containing 5 µl of 10 mg/ml ethidium bromide and ran at 100V for 45mins. A 1 kb plus DNA marker was used as molecular size marker. The PCR amplicons were visualized under ultraviolet (UV) transilluminator in a gel documentation system (25,26).

Plasmid DNA isolation:

The QIAGEN Plasmid Purification mini kit was employed to isolate plasmid DNAs from the

selected bacterial isolates (27) on the principles of alkaline lysis method (28).

Data analysis:

Simple percentage was used to tabulate the frequencies of the Enterobacterales

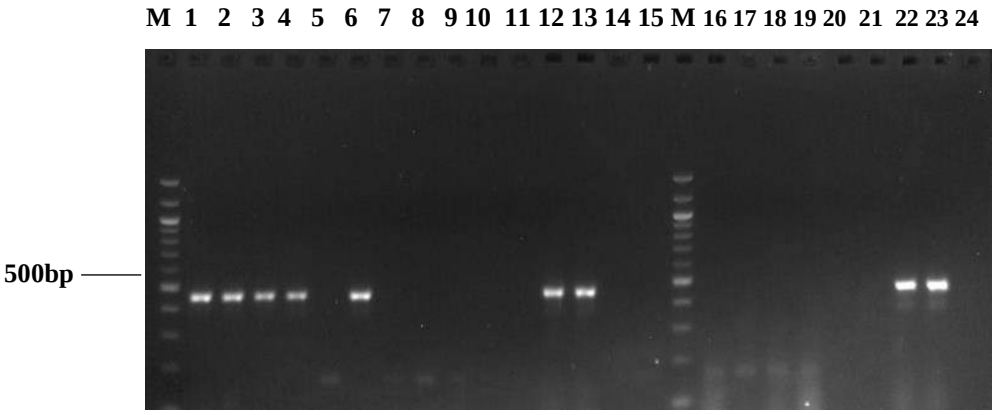
Results:

From the 256 water samples analyzed for bacteria growth, 300 enteric bacterial isolates belonging to two families (Enterobacteriaceae and Morganellaceae), 7 genera and 14 species were identified. Of these, only 45 isolates were phenotypically resistant to carbapenem (imipenem) antibiotic accounting for 15.0% prevalence rate.

From these 45 isolates, *bla*$_{NDM-1}$ was detected in 30 accounting for prevalence of 66.7%. *bla*$_{NDM-1}$ gene was detected in all the isolates of *K. oxytoca, K. variicola, E. aerogenes, E. hormaechei, E. asburiae, C. freundii,* and *M. morganii* that were phenotypically resistant to imipenem. *bla*$_{NDM-1}$ was also detected in *K. pneumoniae* (66.7%, 4/6)*, E. coli* (50.0%, 6/12), *E. gergoviae* (50.0%, 1/2)*,* and *P. mirabilis* (50%, 2/4) that were phenotypically resistant to imipenem (Table 1). Moreover, most of the isolates (63.3%, 19/30) carrying *bla*$_{NDM-1}$ also harbored R-plasmids (Table 1, Plate 2).

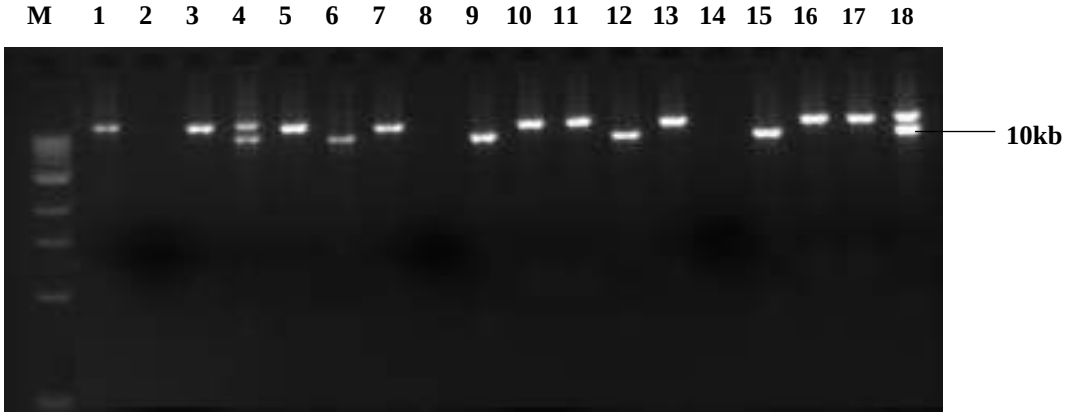
Table 1: Prevalence of NDM-1 gene among carbapenem (imipenem) resistant Enterobacterales

Bacterial species	No of isolates (%)	No of isolates resistant to imipenem (%)	No of imipenem-resistant isolates with *bla*$_{NDM-1}$ (%)	No of *bla*$_{NDM-1}$ positive isolates with R-plasmid (%)
Escherichia coli	63 (21.0)	12 (19.1)	6 (50.0)	2 (33.3)
Klebsiella pneumoniae	52 (17.3)	6 (11.5)	4 (66.7)	2 (50.0)
Klebsiella oxytoca	36 (12.0)	4 (11.1)	4 (100)	3 (75.0)
Klebsiella variicola	1 (0.33)	1 (100)	1 (100)	0
Enterobacter aerogenes	24 (8.0)	1 (4.2)	1 (100)	0
Enterobacter cloacae	30 (10.0)	2 (6.7)	0	0
Enterobacter ludwigii	1 (0.33)	1 (100)	0	0
Enterobacter hormaechei	11 (3.7)	3 (27.3)	3 (100)	3 (100)
Enterobacter asburiae	2 (0.7)	1 (50.0)	1 (100)	1 (100)
Enterobacter gergoviae	12 (4.0)	2 (16.7)	1 (50.0)	1 (100)
Proteus mirabilis	27 (9.0)	4 (14.8)	2 (50.0)	1 (50.0)
Citrobacter freundii	11 (3.7)	1 (9.1)	1 (100)	1 (100)
Morganella morganii	25 (8.3)	6 (27.3)	6 (100)	5 (83.3)
Salmonella enterica	5 (1.7)	1 (20.0)	0	0
Total	**300**	**45 (15.0)**	**30 (66.7)**	**19 (63.3)**



Band size approximately 475bp indicates the presence of the *bla*$_{NDM-1}$ gene. Lane M= Molecular weight marker, Lane 1-4, 6, 12, 13, 22 and 23 are positive for bla$_{NDM-1}$. Lane 5, 7-11, 14-21, and 24 are negative for bla$_{NDM-1}$

Plate 1: Agarose gel electrophoresis of PCR products of *bla*$_{NDM-1}$ gene in selected Enterobacterales isolates



M= Molecular weight marker. Lane 2, 8 and 14: No plasmid band. All other lanes have plasmid band. Lane 4 and 18 have 2 plasmid bands each. Lanes 1,3, 4, 5, 7, 10, 11, 13, 16, 17 and 18 have plasmid bands with MW >10kb

Plate 2: Agarose gel electrophoresis of R-plasmids in selected Enterobacterales isolates

Discussion:

Resistance to imipenem and discovery of the *bla*$_{NDM-1}$ gene among imipenem-resistant Enterobacterales encountered in this study was unexpected. This is because this class of antibiotic was not in use in the entire study area during the period of carrying out this research. This was so significant because the study area was a pristine environment in which selective pressure may not have been responsible for the organism's resistance to carbapenem (imipenem) antibiotic and subsequent detection of the *bla*$_{NDM-1}$ gene. A similar phenomenon was observed and reported by Adenipekun et al., (29) in Lagos although this was from clinical samples. According to the authors, carbapenem is rarely used in clinical practice in Nigeria except for emergencies or conditions that were critical, and they concluded that detection of NDM-1 in Lagos may be connected to its geographical position; a border, vibrant and buoyant State that attracts all works of life from around the globe.

The observation that antibiotic resistance genes from bacteria are usually pervasive in natural environments, as well as places believed to be fallow (30,31), was evident in this study because the NDM-1 gene was discovered in an environment which was considered alien in the used of carbapenem. From the few data available in Nigeria, detection of *bla*$_{NDM-1}$ gene was reported most often from clinical specimens (22,23). A study however reported the detection of *bla*$_{NDM-1}$ gene from recreational beaches in Lagos (32). An additional study reported the detection of *bla*$_{NDM-1}$ gene in pharmaceutical wastewaters from Lagos and Ogun States (16), while another study reported the detection of

bla$_{NDM-1}$ from the soil of hospital environment in Akwa-Ibom State and Abuja, Federal Capital Territory, Nigeria (17). In Oghara, Delta State, Nigeria, *bla*$_{NDM-1}$ was also detected in some water sources (33). In spite of these, there is paucity of information on prevalence of *bla*$_{NDM-1}$ gene on surface and groundwater sources in Nigeria. As such, the finding of this study may serve as baseline data for epidemiological surveillance in the study area and the country at large.

bla$_{NDM-1}$ gene code for the synthesis of an enzyme, New-Delhi metallo-β-lactamases-1 (NDM-1) that hydrolyses a broad range of antimicrobials, including carbapenems among others (8), which are last resort antibiotics for therapy of infections involving Gram-negative bacteria, especially ESBL-producing isolates (4). Carbapenem resistance may also be as a result of weak attachment of carbapenems to penicillin-binding proteins, and increasing expression of multidrug efflux pumps by the bacteria (34-36). Bacteria carrying *bla*$_{NDM-1}$ gene are frequently called 'superbugs' because infections involving them are severe and most times not easily cured (18).

The detection of *bla*$_{NDM-1}$ gene in pristine environment like ours and in water sources call for concern. This is because of the ability of organisms harboring NDM-1 to exhibit traits of multiple drug resistance. It has been reported previously that bacteria carrying NDM-1 gene tends to express, in addition to carbapenemases, other unrelated genes such as those encoding enzymes like ampicillinase C (AmpC), cephalosporinases, and modifying enzymes for aminoglycosides, macrolides, sulfamethoxazole, and fluoroquinolones (8,37). Moreso, because these water sources are often used for domestic and agricultural activities, the threat of transmission of 'superbug' to humans, as well as the transfer of NDM-1 encoding genes to strains of other Enterobacteriaceae species is imminent. Most importantly, pipe-borne water supply is unavailable in the entire study area and the available and dependable water sources were contaminated with 'superbugs' even before the introduction of antecedent antibiotics.

What would the future hold for these communities in terms of treatment option? This is so significant and worrisome because in Nigeria and other developing countries, antibiotic surveillance and stewardship is often relegated to the background. The problem is further compounded by the fact that most of the Enterobacterales isolates carrying the *bla*$_{NDM-1}$ gene also harbored resistance-plasmids. This corroborates reports from previous studies (12,38, 39). Studies have shown that the gene encoding the production of NDM-1 enzyme is often localized on mobile plasmids which facilitates the dissemination of the gene rapidly between bacteria and different environments (13,40). Moreso, it was reported that plasmids harboring *bla*$_{NDM-1}$ gene also bear several other resistance genes, thereby making these organisms superbugs (40), allowing limited or no therapeutic options (10). The extensive and inappropriate antibiotic usage in humans and animal husbandry including their subsequent spread into the ecosystem may have quickened the evolution, selection, and or the horizontal transmission of antibiotic resistance plasmids in bacterial populations as seen in this study (38).

Conclusion:

The findings from this study area shows the presence of Enterobacterales with plasmid-borne *bla*$_{NDM-1}$ gene from water sources. Resistance to carbapenem antibiotics mediated by New Delhi metallo-lactamase-1 (NDM-1) constitutes an emerging challenge in the treatment of bacterial infections. As such, the urgent need for antimicrobial surveillance and stewardship is of utmost importance.

Contributions of authors:

MYT designed the study protocols and wrote the first draft of the manuscript. Authors OIE and EAO supervised and corrected the draft, while authors ROO and FJ gave professional and scientific advice, and also corrected the draft. All authors read and approved the final manuscript.

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Original Article **Open Access**

The influence of exposure to various concentrations of five antimicrobial agents on intracellular cytotoxin B production in *Clostridioides difficile*

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Abstract:

Background: *Clostridioides difficile* is an important cause of healthcare-associated diarrhea. Several anti-microbial agents are known to promote *C. difficile* infection (CDI). The impact of various concentrations of ampicillin (AMP), cefotaxime (CTX), clindamycin (CC), metronidazole (MTZ) and vancomycin (VAN) on intra-cellular cytotoxin B production was investigated in this study.
Methodology: Six clinical strains of *C. difficile* were grown at minimum inhibitory concentration (MIC) and sub-MIC concentrations of these antibiotics. Inoculum standardization was performed by Miles and Misra method. Intracellular toxin B production was detected using Vero cell cytotoxicity assay in sonicated cultures on days 1, 2, 3, 4, 5 and 7 days of incubation.
Results: There was a heterogeneous relationship between antibiotic exposure and the intra-cellular toxin production by the toxigenic strains. Clinical strains of *C. difficile* when exposed to MIC and sub-inhibitory concentrations of certain antibiotics produced high cytotoxin levels. All toxigenic isolates produced increased levels of cell-bound cytotoxin after exposure to antibiotics but there was no consistent pattern and the response to different doses varied considerably. Metronidazole was the most potent inducer of cell-bound cytotoxin followed by cefotaxime and clindamycin. Vancomycin induced the least amount of cytotoxin activity.
Conclusion: The effects of sub-inhibitory concentration of antibiotic that predispose to *C. difficile* infection may partially suppress the normal gut flora, allowing colonization and growth of *C. difficile*, and may affect the level of toxin produced.

Keywords: *Clostridioides difficile*, antibiotic exposure, intracellular cytotoxin B.

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L'influence de l'exposition à diverses concentrations de cinq agents antimicrobiens sur la production de cytotoxine B intracellulaire chez *Clostridioides difficile*

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Résumé :

Contexte: *Clostridioides difficile* est une cause importante de diarrhée nosocomiale. Plusieurs agents antimicrobiens sont connus pour favoriser l'infection à *C. difficile* (ICD). L'impact de diverses concentrations d'ampicilline (AMP), de céfotaxime (CTX), de clindamycine (CC), de métronidazole (MTZ) et de vancomycine (VAN) sur la production de cytotoxine B intracellulaire a été étudié dans cette étude.
Méthodologie: Six souches cliniques de *C. difficile* ont été cultivées à une concentration minimale inhibitrice (CMI) et à des concentrations sous-CMI de ces antibiotiques. La standardisation de l'inoculum a été réalisée par

la méthode de Miles et Misra. La production intracellulaire de toxine B a été détectée à l'aide d'un test de cytotoxicité sur cellules Vero dans des cultures soniquées aux jours 1, 2, 3, 4, 5 et 7 jours d'incubation. **Résultats:** Il existe une relation hétérogène entre l'exposition aux antibiotiques et la production intracellulaire de toxines par les souches toxigènes. Les souches cliniques de *C. difficile* lorsqu'elles étaient exposées à des concentrations de CMI et sous-inhibitrices de certains antibiotiques produisaient des niveaux élevés de cytotoxine. Tous les isolats toxicogènes ont produit des niveaux accrus de cytotoxine liée aux cellules après exposition aux antibiotiques, mais il n'y avait pas de tendance constante et la réponse aux différentes doses variait considérablement. Le métronidazole était l'inducteur le plus puissant de la cytotoxine liée aux cellules, suivi du céfotaxime et de la clindamycine. La vancomycine a induit le moins d'activité de cytotoxine.
Conclusion: Les effets de la concentration sous-inhibitrice d'antibiotique qui prédisposent à l'infection à *C. difficile* peuvent supprimer partiellement la flore intestinale normale, permettant la colonisation et la croissance de *C. difficile*, et peuvent affecter le niveau de toxine produite.

Mots-clés: *Clostridioides difficile*, exposition aux antibiotiques, cytotoxine intracellulaire B

Introduction:

Clostridioides difficile is a major cause of healthcare-associated diarrhoea. Infections caused by this organism, called *C. difficile* infection (CDI), may manifest as asymptomatic carrier, mild self-limiting antibiotic-associated diarrhea (AAD), severe antibiotic-associated colitis (AAC) or pseudomembranous colitis (PMC). CDIs are sometimes complicated by ileus, toxic megacolon and gut perforation with peritonitis leading to potentially fatal consequences. The major virulence factors of most pathogenic *C. difficile* are the two large molecular weight toxins; toxin A (TcdA, an enterotoxin) and toxin B (TcdB, a cytotoxin).

A wide range of antimicrobial agents has been implicated in the development of CDI. This is because they deplete the normal gut flora which normally provide colonization resistance and thus allow the overgrowth of *C. difficile* and production of potent toxins (1). Almost all antimicrobial agents can predispose to the development of CDI. However, cephalosporins, clindamycin and the new generation fluoroquinolones are known agents most often predisposing to CDI (2).

Toxin production varies among different toxigenic strains and it is influenced by different growth and environmental factors such as temperature, glucose, biotin limitation and amino acid concentrations. A previous study has shown that sub-inhibitory concentrations of clindamycin and cephaloridine, unlike tetracycline, stimulate enterotoxin production (3). In addition, the antibiotics are known to increase toxin A production. Drummond and colleagues (4) demonstrated that exposure of *C. difficile* to sublethal concentration of various antibiotics such as vancomycin, metronidazole, amoxicillin, clindamycin, cefoxitin and ceftriaxone has no consistent relationship between growth and toxin A production. Unlike clindamycin, sub-MIC levels of metronidazole, linezolid and vancomycin increased the transcription rate of toxin A and B genes (5). Emerson and colleagues (6) using microarray technology analyzed the transcriptional responses of *C. difficile* 630 strain to environmental shock and to the growth in the presence of sub-inhibitory concentrations of certain antibiotics such as amoxicillin, clindamycin and metronidazole. They found that amoxicillin and clindamycin increased the transcription of ribosomal protein genes and changed the transcription of genes encoding surface-associated proteins, while exposure of *C. difficile* to sub-inhibitory concentration of metronidazole resulted in minor changes in the transcription patterns.

The aim of this study was to investigate the effects of various concentrations of antibiotics known to predispose to CDI and, also, of those used in its therapy on the production of intracellular *C. difficile* toxin B in Vero cell line and to quantify the cytotoxic effects on cell bound cytotoxin B.

Materials and method:

Bacterial strains*:*

The six *C. difficile* strains used in this study were; local strains KM233D ribotype 078, and KM34A ribotype 097 (local toxigenic strains isolated from the stool samples of patients with PMC); strain KM175 ribotype 039 (a local non-toxigenic strain isolated from the stool of a patient with suspected AAD); strain KA11 ribotype 017 (toxin A-negative/toxin B-positive strain isolated from the stool of a patient with AAD); strain KM362C ribotype 046 (a local toxigenic strain isolated from a patient with AAC) and a UK strain, ribotype 001, isolated from stool of a patient involved in an outbreak of CDI in the UK (obtained from Professor BI Duerden and Dr JS Brazier of Anaerobe Reference Unit, Cardiff, UK).

All the strains were tested for their susceptibility to the five antibiotics by E-test (AB Biodisk, Solna, Sweden) on blood agar according to manufacturer's instructions. PCR ribotyping was previously performed (7). All the strains were stored at -70ºC until further testing.

Antimicrobial agents:

The following antibiotic powders were used; ampicillin (AMP), clindamycin (CC), ce-

fotaxime (CTX), metronidazole (MTZ) and vancomycin (VAN) (all from Sigma Pharmaceuticals). They were prepared in sterile distilled water as a 10x solution with reference to the highest concentration required.

Inoculum standardization:

The number of viable bacteria in the inoculum was determined by a modified Miles and Misra method (8). An inoculum containing 10^{11} CFU/ml which produced the highest cytotoxic activity was then used throughout the cytotoxin detection experiments.

Cytotoxin detection assay:

Strains were seeded onto blood agar and incubated anaerobically for 48 h at 37ºC. Then, a loopful of each strain was inoculated into 10ml of sterile liver broth (Oxoid Ltd, Basingstoke, Hampshire, UK), and incubated anaerobically overnight. This was diluted 1 in 10 corresponding to 10^{11} CFU/ml after which it was exposed to different concentrations of the test antibiotics i. e. MIC, 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 MICs.

Doubling dilutions were then made in 10ml of freshly prepared sterile Brain Heart Infusion (BHI) broth (Difco, Becton and Dickinson Company, Sparks, MD, USA) in 6 different bottles per concentration containing the specific concentrations of the test antibiotics. An inoculum of 100 µl containing approximately 10^{11} CFU/ml of the inoculated liver broth was added to each bottle. An antibiotic-free broth culture was used as control for each strain for baseline comparison. The inoculated broth cultures were incubated at 37ºC for 1, 2, 3, 4, 5, and 7 days anaerobically. At the end of each incubation period, cell-bound cytotoxin was assayed.

Cell-bound cytotoxin detection*:*

After incubation of each bottle of the broth cultures for each period (1 to 5, and 7 days), 5ml of the broth cultures was centrifuged at 3,500xg for 10 min and the sediment washed twice with 3ml of 50mM PBS (pH 7.0). The washed sediment was sonicated with an ultrasonic homogenizer (Labsonic U, B. Braun, Model 1254, Melsungen AG, W. Germany) for 5 min. The cellular debris was removed by centrifugation at 3,500 g for 10 min. The supernatant was filtered through a sterile membrane filter with 0.45µm pore size (Millex-AH, Millipore, Carrigtwchill, Co. Cork, Ireland) and assayed for cytotoxicity.

Assay for cytotoxicity:

Cytotoxicity was assayed as previously described (9) in a microliter plate using Vero cell line and dilution series from 1:40 to 1:5120. The highest dilution resulting in complete rounding of the cells was taken as the number of cytotoxic units (CU)/50µL sample (10) and the results were expressed as CU/ml. At the end of 7th day incubation, the mean CUs/ml were calculated and tabulated. Analysis of the data was done by calculating the mean of the cytotoxic activities produced on all post-exposure days to various antibiotics.

Results:

The MICs of the five antibiotics tested against the 6 *C. difficile* strains investigated are shown in Table 1. The inoculum at which cell-bound cytotoxin production was maximal below the neat broth i. e. 10^{11}CFU/ml was used throughout the experiments (data not shown). The non-toxigenic strain, ribotype 039, did not induce cell-bound cytotoxin regardless of the presence or absence of any of the 5 antibiotics tested. There was no remarkable difference in the cytotoxic activity between 24 and 48 h incubation. Therefore, the data shown are those after 24 h incubation.

Table 1: Minimum inhibitory concentrations (µg/ml) of selected antibiotics against clinical strains of *Clostridioides difficile*

Antibiotics/breakpoints (µg/ml)	**MIC (µg/ml) of antibiotics against strains**					
	078	**039**	**097**	**017**	**046**	**001**
Clindamycin (4)	8.0	>256	>256	>256	4.0	8.0
Metronidazole (8)	0.25	0.06	0.06	0.25	0.25	0.25
Vancomycin (4)	0.5	1.0	1.0	1.0	0.5	2.0
Ampicillin (8)	0.5	0.5	1.0	1.0	1.0	2.0
Cefotaxime (32)	>256	>256	>256	>256	>256	>256

MIC=Minimum inhibitory concentration

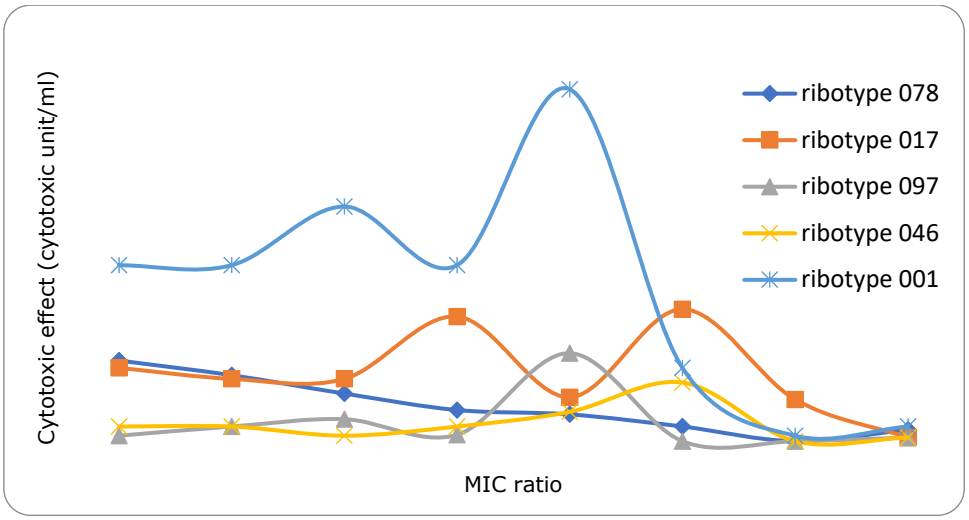

Fig 1: Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to cefotaxime by all test strains

Cell-bound cytotoxic activity post exposure to cefotaxime (CTX):

For strain KM233D (ribotype 078), the effects of exposure to CTX on the cell-bound cytotoxic activities were not very remarkable as shown by the mean of cytotoxic unit/ml (CU/ml) in Fig 1. There was no increase in cytotoxin production at the MIC value. The increase observed at ½ MIC was marginal, 1.3x the control, but this rose gradually from 117.3 CU/ml (2.3x) at ¼ MIC to 352.0 CU/ml (7.0x) at 1/64 MIC. For cell-bound cytotoxin of strain KA11 (ribotype 017), the effect of exposure to CTX was considerably higher. It was 182.3 CU/ml and 320.0 CU/ml (11.3 and 20 times the control) at the MIC and 1/64 MIC, respectively. The greatest increase was noted at ½ and 1/8 MICs with production of cytotoxin measuring 576.1 (36.0x) and 544.0 CU/ml (34.0x), respectively.

Cefotaxime failed to induce cytotoxin production in strain KM34A (ribotype 097) at the MIC and ½ MIC but induced high level cytotoxin production of 384.2 CU/ml (24-fold) at 1/4 MIC and thereafter gradually waned off through 1/16 MIC (6x) to 1/64 MIC (1.5x). The antibiotic also did not induce cyto-toxin production in strain KM362C (ribotype 046) at the MIC but the maximum induction occurred at ½ MIC (256.0 CU/ml; 16-fold increase) then declined gradually to 64 CU/ml (4 x) each at 1/32 MIC and 1/64 MIC. With the UK ribotype 001, maximal induction occurred at ¼ MIC; 1532.1 CU/ml versus 64.0 CU/ml for the control which was a 24-fold increase. Another surge occurred at 1/16 MIC with the production of 1024.0 CU/ml (16-fold increase).

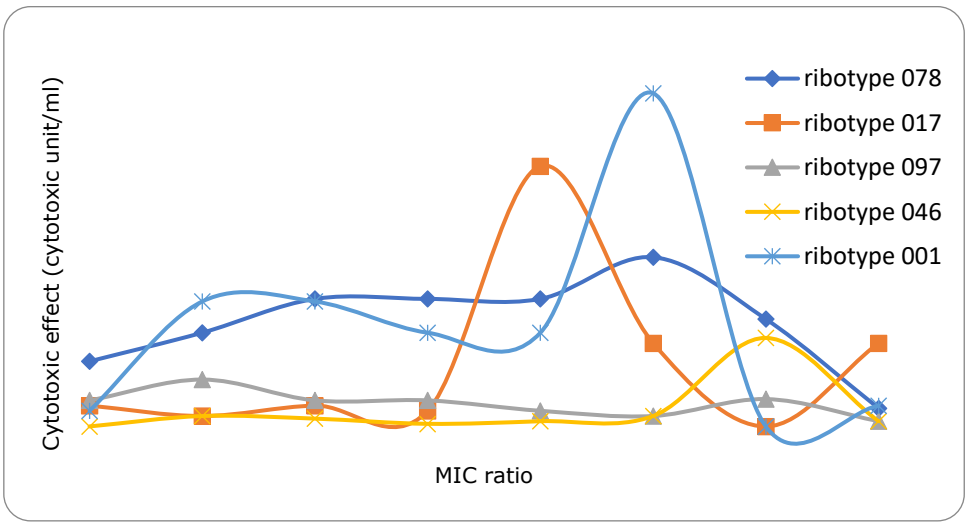

Fig 2: Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to ampicillin by all test strains

Cell-bound cytotoxic activity post exposure to ampicillin (AMP):

The quantified cytotoxin content of the sonicated cell effluents of the tested strains before and after exposure to ampicillin is shown in Fig 2. AMP did not induce cell-bound cytotoxin at MIC and ½ MIC in any of the strains. For strain KM233D (ribotype 078), the maximum cytotoxin production occurred at 1/16 with production of 520.0 CU/ml (9.3-fold increase over the control level). Thereafter production dropped to 392.0 CU/ml each at 1/32 and 1.64 MICs.

There was no apparent change in the cell-bound cytotoxic activity in KA11 (017), except at 1/4 MIC where the rise in production was 3.1-fold higher (800.0 CU/ml) than the control. Cell-bound cytotoxin production by strain KM34A (097) was almost in a linear fashion reaching a maximum of 80.0 CU/ml (a 5-fold increase) at 1/16 MIC.

With strain KM362C (046), cytotoxin production was maximal (272 CU/ml) at the ¼ MIC level (17-fold increase) and declined linearly to 24 CU/ml (1.5-fold increase) at 1/64 MIC. Cell-bound cytotoxin production was highest at ¼ MIC in the UK strain, ribotype 001; 1024.0 versus 64 CU/ml in control (16-fold increase).

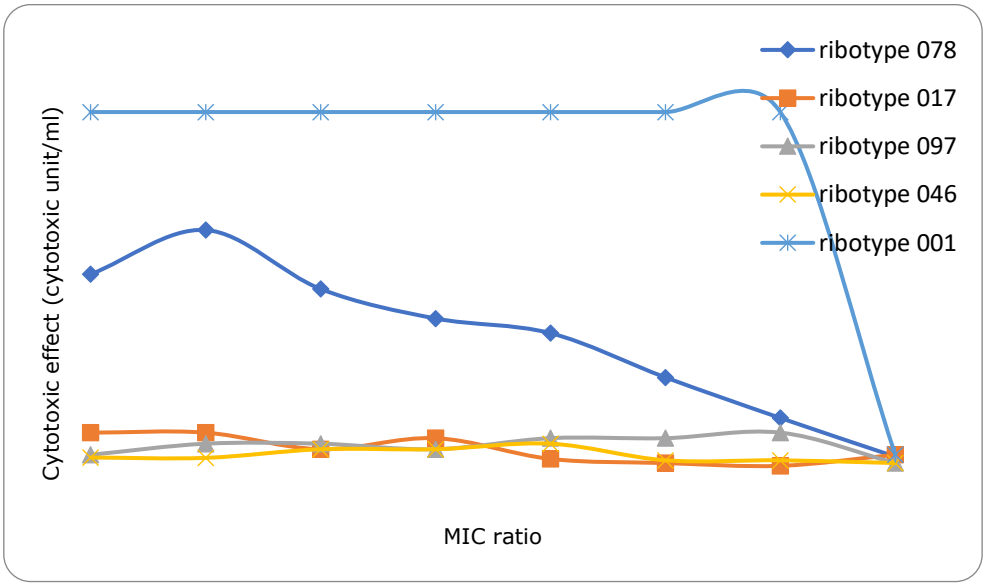

Fig 3: Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to metronidazole by all test strains

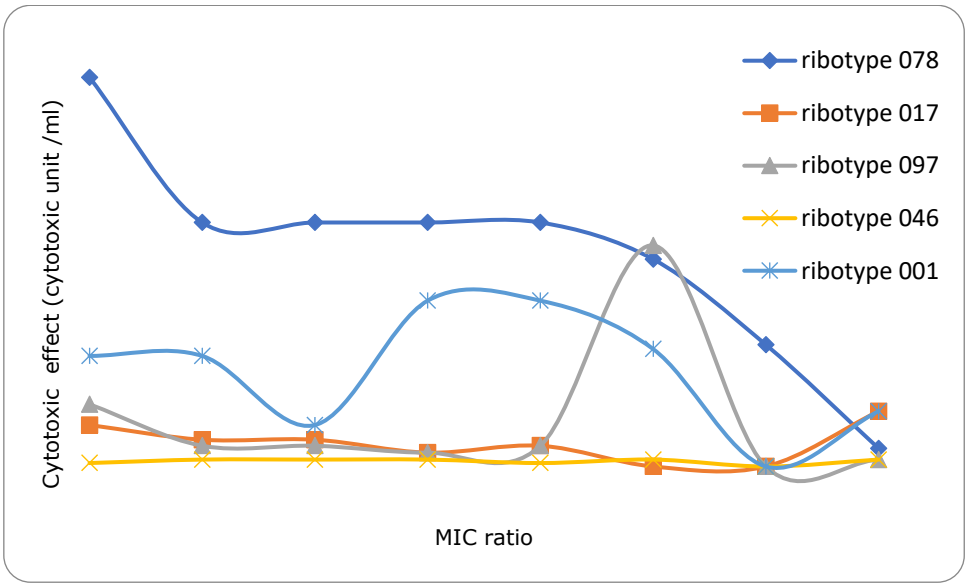

Fig 4: Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to vancomycin by all test strains

Cell-bound cytotoxic activity post exposure to metronidazole (MTZ):

Fig 3 shows the amount of cell-bound cytotoxic unit (CU/ml) produced by the strains post-exposure to MTZ. There was a linear increase in the cell-bound cytotoxin production by strain KM233D (ribotype 078), in the presence of MTZ from 277.3 CU/ml at ½ MIC (4.8-fold increase), through 853.0 CU/ml at 1/16 MIC (14.7-fold) to 1365.3 CU/ml at 1/64 MIC (23.5-fold). For strain KA11 (017), cell-bound cytotoxin production was not as dramatic as there was only 2.5-fold (160.0 CU/ml) increase at 1/16 MIC reaching maximum level of 192.0 CU/ml at 1/64 MIC (3-fold increase).

Strain KM34A (097) yielded maximum production of 192.0 CU/ml at ¼ MIC declining to 128.0 CU/ml at 1/64 MIC. For strain KM362C (046), the maximum production was 128 CU/ml (8-fold increase) at 1/8 MIC. With the UK strain (ribotype 001), post-exposure production was massive with 2048.0 CU/ml (32-fold increase) at all concentrations.

Cell-bound cytotoxic activity post exposure to vancomycin (VAC):

As shown in Fig 4, the maximum cell-bound cytotoxin production by strain KM233D (ribotype 078) was 565.3 CU/ml (13.5-fold increase) which occurred post-exposure to VAC at 1/16, 1/32 and 1/64 MICs. There was no cytotoxin production at the MIC and ½ MIC. At ¼ MIC it was 282.6 CU/ml (6.7-fold rise). Strain KA11 (017) produced a relatively low level constitutive cytotoxin at all MIC and sub-MICs. VAC induced maximum production of cell-bound cytotoxin (512.0 CU/ml; 32-fold increase) at ¼ MIC and lowest (32.0 CU/ml; 2-fold) at 1/16 MIC in strain KM34A (097).

Post-exposure of strain KM362C (046) did not result in any appreciable cytotoxin production at all concentrations. Exposure of the UK strain (001) did not yield appreciate increase in cytotoxin production. At ½, ¼, and 1/64 MICs, the production was 272.0 (2.1-fold increase), 384 (3-fold) and 256.0 (2-fold) CU/ml, respectively.

Cell-bound cytotoxic activity *post exposure to* clindamycin (CLIN):

Fig 5 shows the cell-bound cytotoxic activities after exposure to clindamycin for the different strains. Cell-bound cytotoxic activity of strain KM233D (ribotype 078) after exposure to clindamycin at the MIC, was about 17.9-fold higher than the control. Then it increased gradually reaching a maximum of 32-fold at 1/16 MIC (1088.0 CU/ml). For ribotype KM11 (017), post-exposure to CLIN induced high cell-bound cytotoxin production at the MIC (272.0 CU/ml; 17-fold higher than the control), 264.0 CU/ml (16.5-fold increase) at 1/8 MIC and 256.0 CU/ml (16-fold) at 1/32 MIC. With strain KM34A (097), the cytotoxic activities were each 272 CU/ml (17-fold) at the MIC, ½, 1/8, and 1/32 MICs, and 288.0 (18-fold) at 1/16 MIC. Strain KM362C (046) induced 64 CU/ml (4-fold increase) at 1/8 MIC, 48 CU/ml (3-fold increase) at MIC, ¼ and 1/32 MICs.

Exposure of UK strain (001) to clindamycin induced cell-bound cytotoxin production at all concentration but maximally at 1/4 and 1/16 MICs of 192 CU/ml (3-fold).

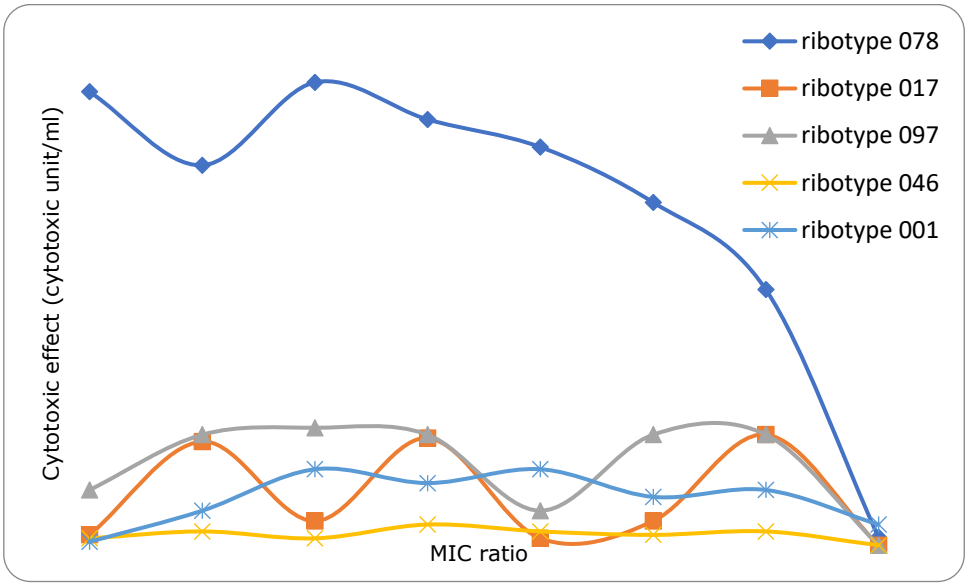

Fig 5: Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to clindamycin by all test strains

Discussion*:*

This study focused on the effects of MIC and sub-inhibitory concentrations of 5 different antibiotics, including those used for treatment of CDI and those known to predispose to CDI occurrence, on the production of cytotoxin B by 6 different strains of *C. difficile*. The study showed clearly that there is a heterogeneous relationship between antibiotic exposure and toxin B production intracellularly by the toxigenic strains of *C. difficile*. The non-toxigenic strain from a symptomatic patient with diarrhea, ribotype 039, did not produce toxin B inside the cell in the absence or presence of any antibiotic. This may be related to the absence of the genes that encode the toxin or the absence of other virulence factors.

The results showed that certain strains, when exposed to the MIC and sub-inhibitory concentrations of certain antibiotics, are capable of producing high level of cytotoxin compared to the antibiotic-free control. This may be due to the stress that an organism experiences in the presence of antibiotics. Early reports suggested that the stress may induce extracellular toxin production. For example, Onderdonk et al., (11) reported that raised temperature leads to higher cytotoxin production and toxin production increased in the presence of sub-inhibitory concentration of vancomycin and penicillin. A study by Karlsson and colleagues (12) showed that temperature may act as a controlling factor for the expression of toxin A and TcdD. Therefore, toxin production may be enhanced by environmental stress. Emerson et al., (6) showed that exposure of *C. difficile* to environmental stress such as heat shock and acid shock, lead to upregulation of certain genes that allow the vegetative form of *C. difficile* to tolerate this type of environmental stress. In addition, *C. difficile* respond to oxidative stress by upregulation of electron transporters (6). The same investigators found that exposure to amoxicillin and clindamycin increased the transcription of ribosomal protein genes and altered transcription of genes encoding surface-associated proteins, while minor changes in the transcription occurred after exposure to metronidazole (6).

In this study, strains KM233D, KA11 and UK strain, ribotypes 078, 017 and 001, respectively were the most responsive to antibiotic induction of cytotoxin production. KA11 (ribotype 017) was an isolate from an AAD patient while KM233D (078) was an isolate from a patient with PMC. UK strain, (ribotype 001) was a strain associated with CDI outbreak in the UK. Surprisingly, metronidazole was the most potent inducers of cytotoxin in our hands. It provoked an increase in the cell-bound toxin in all toxigenic strains. This was followed by cefotaxime and clindamycin which produced almost the same level of cytotoxin. Ampicillin and vancomycin induced the least amount of cytotoxic activity on all the strains. Metronidazole and ampicillin induced cell-bound cytotoxin for all toxigenic isolates at various degrees especially ribotypes 078 and 001. Although ribotypes 078 and 097 strain were isolated from patients with PMC, metronidazole and ampicillin induce more cell-bound cytotoxin in ribotype 078 rather than 097. Vancomycin induced *C. difficile* ribotype 078 and 001 to produce a large amount of intracellular cytotoxin. This may indicate that vancomycin increases cytotoxin release from within the cell rather than enhances synthesis for *C. difficile* ribotypes 078 and 001.

CDI is not only enhanced by usage of antibiotics but also by physiological changes that affect the pathogenicity of *C. difficile* (13). Previously, Onderdonk et al., (14); Honda et al., (3) and Drummond et al., (4) reported that certain antibiotics enhance synthesis of toxin A and/or cytotoxin B, which are the main virulence factors. Onderdonk et al., (14) in 1981, compared the effect of clindamycin and its metabolites in a hamster model of *C. difficile*-associated colitis and did not find any correlation between clindamycin potency and $AACD_{50}$ (toxin lethal to 50% of the animals). Other investigators described a human gut model of *C. difficile* infections and they demonstrated that *C. difficile* germinate and produce cytotoxin in response to clindamycin (15), cefotaxime with and without its metabolites, desacetylcefotaxime (16), metro -nidazole (17) and fluoroquinolones such as ciprofloxacin and levofloxacin (18). Recently, Baines et al., (19) demonstrated that vancomycin reduced the vegetative forms and cytotoxin titers of epidemic strains of *C. difficile* but did not have any anti-spore activity. In addition, the same researchers showed that vancomycin was more effective than metronidazole in reducing *C. difficile* PCR ribotype 027 numbers and cytotoxin titer (19). In contrast, germination and cytotoxin production was not observed in the human gut model after exposure to piperacillin-tazobactam or tigecycline (20,21)

There appears to be no correlation between the severity of symptoms and level of cytotoxin produced in Vero cell line. For example, cefotaxime induced more cytotoxic effect in Vero cells from ribotype 017 which caused only AAD in contrast to ribotype 097 that was associated with PMC. Moreover, ampicillin induced more cytotoxic effects on Vero cells by ribotype 017 isolated from a patient with diarrhea (AAD) than ribotype 046 isolated from a patient with colitis (AAC)

and ribotype 097 isolated from a PMC case. This suggests that the quantity of the cytotoxin produced is not enough to explain the gut pathology caused by the different strains of *C. difficile.*

Our results showed that there is a general fluid relationship between antibiotics and toxin production by *C. difficile*. All isolates showed increase in cytotoxin production after exposure to antibiotics but there was no consistent pattern and the response to different doses varied considerably. Antibiotics that are used for treatment and those that precipitate the disease have different effects on different *C. difficile* isolates, therefore the relationship may be complex. The effects of sub-inhibitory concentration of antibiotic that predispose to CDI development may suppress the normal gut flora partially and allow colonization and growth of *C. difficile* and may affect the level of toxin produced.

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Contributions of authors:

WJ conceptualized the study, carried out the laboratory work, and wrote the initial manuscript; BD and VOR conceptualized the study research, supervised and corrected the final manuscript draft. All authors approved the final manuscript submitted.

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Authors declare no conflict of interest

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Original Article **Open Access**

Prevalence and risk factors for hepatitis C virus infection among HIV positive patients at the Lagos University Teaching Hospital, Nigeria

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Abstract:

Background: Worldwide, an estimated 58 million people have chronic hepatitis C virus (HCV) infection, with about 1.5 million new infections occurring per year. About 2.3 million people living with HIV globally have serological evidence of past or present HCV infection. The aim of this study was to determine the prevalence of active HCV infection and associated risk factors among HIV positive patients attending the HIV clinic, Lagos University Teaching Hospital (LUTH), Idi-Araba, Lagos, Nigeria.
Methodology: A cross sectional study was conducted to determine the prevalence of and risk factors for HCV infection among randomly selected HIV positive patients at the LUTH HIV clinic. Socio-demographic, clinical and laboratory data were collected from the participants using a structured questionnaire. Blood samples were collected and tested for HCV antibodies with an enzyme linked immunosorbent assay (CTK Biotech USA) and HCV RNA was detected using reverse transcriptase polymerase chain reaction assay.
Results: One hundred and ninety-five HIV infected participants were recruited into the study of which 134 (68.7%) were females and 61 (31.3%) were males. The mean age of participants was 40.1±7.8 years. Of the 195 participants, 5 tested positive for antibody to HCV, giving a seroprevalence rate of 2.6% (95% CI = 0.8-5.9%). Of the 5 seropositive participants, HCV RNA was detected in 1 (20.0%), giving a prevalence of 0.5% (1/195) for active HCV infection. The seroprevalence of HCV in males of 4.9% (3/61) and females of 1.5% (2/134) was not significantly different (OR=3.41, 95% CI=0.56-20.98%, p=0.18). The mean $\log_{10}$ HIV viral load was significantly higher among participants seropositive for HCV (5.1±0.9 log copies/ml) than those seronegative (2.7±1.2 log copies/ml) ($p < 0.001$). The mean duration of antiretroviral therapy was significantly lower among participants seropositive for HCV (2.6±1.3 years) than those seronegative (5.6±3.1 years) (p=0.004). The seroprevalence of HCV was significantly higher in those with CD4 count <350 cells/mm^3 (8.5%) than those with CD4 count >350cells/mm^3 (p=0.02). The seroprevalence of HCV in the HIV-positive participants was significantly associated with sexual partners (p=0.0473), with highest seroprevalence in those with ≥ 3 sexual partners (OR=11.625, 95% CI=1.049-128.83). Other risk factors were not significantly associated with seroprevalence of HCV (p>0.05), while risk factors associated with active HCV infection could not be evaluated with the only one HCV RNA positive participant
Conclusion: Although the prevalence of active HCV infection in HIV infected individuals in this study was apparently low (0.5%), screening with HCV antibody test and confirmation with HCV RNA PCR assay are recommended.

Keywords: Hepatitis C virus; HIV; HCV RNA; prevalence; risk factors

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Prévalence et facteurs de risque de l'infection par le virus de l'hépatite C chez les patients séropositifs à l'Hôpital Universitaire de Lagos, Nigéria

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Résumé:

Contexte: Dans le monde, environ 58 millions de personnes sont infectées par le virus de l'hépatite C (VHC) chronique, avec environ 1,5 million de nouvelles infections par an. Environ 2,3 millions de personnes vivant avec le VIH dans le monde ont des preuves sérologiques d'une infection passée ou présente par le VHC. Le but de cette étude était de déterminer la prévalence de l'infection active par le VHC et les facteurs de risque associés chez les patients séropositifs fréquentant la clinique du VIH, Hôpital Universitaire de Lagos (LUTH), Idi-Araba, Lagos, Nigeria.
Méthodologie: Une étude transversale a été menée pour déterminer la prévalence et les facteurs de risque de l'infection par le VHC chez des patients séropositifs sélectionnés au hasard à la clinique LUTH HIV. Des données socio-démographiques, cliniques et de laboratoire ont été recueillies auprès des participants à l'aide d'un questionnaire structuré. Des échantillons de sang ont été prélevés et testés pour les anticorps anti-VHC avec un test immuno-enzymatique (CTK Biotech USA) et l'ARN du VHC a été détecté à l'aide d'un test de réaction en chaîne par polymérase par transcriptase inverse.
Résultats: Cent quatre-vingt-quinze participants infectés par le VIH ont été recrutés pour l'étude, dont 134 (68,7%) étaient des femmes et 61 (31,3%) étaient des hommes. L'âge moyen des participants était de 40,1±7,8 ans. Sur les 195 participants, 5 ont été testés positifs pour les anticorps anti-VHC, ce qui donne un taux de séroprévalence de 2,6% (IC à 95%=0,8-5,9%). Sur les 5 participants séropositifs, l'ARN du VHC a été détecté chez 1 (20.0%), ce qui donne une prévalence de 0,5% (1/195) pour l'infection active par le VHC. La séroprévalence du VHC chez les hommes de 4,9% (3/61) et les femmes de 1,5% (2/134) n'était pas significativement différente (OR = 3,41, IC à 95%=0,56-20,98%, p=0,18). La charge virale VIH moyenne log10 était significativement plus élevée chez les participants séropositifs pour le VHC (5,1±0,9 log copies/ml) que chez les séronégatifs (2,7±1,2 log copies/ml) (p<0,001). La durée moyenne du traitement antirétroviral était significativement plus faible chez les participants séropositifs pour le VHC (2,6±1,3 ans) que chez ceux séronégatifs (5,6±3,1 ans) (p=0,004). La séroprévalence du VHC était significativement plus élevée chez les personnes ayant un nombre de CD4 < 350 cellules/mm^3 (8,5%) que chez celles ayant un nombre de CD4 > 350 cellules/mm^3 (p=0,02). La séroprévalence du VHC chez les participants séropositifs était significativement associée aux partenaires sexuels (p=0,0473), la séroprévalence la plus élevée chez ceux ayant ≥ 3 partenaires sexuels (OR=11,625, IC à 95%=1,049-128,83). Les autres facteurs de risque n'étaient pas associés de manière significative à la séroprévalence du VHC (p>0,05), tandis que les facteurs de risque associés à l'infection active par le VHC n'ont pas pu être évalués avec le seul participant positif à l'ARN du VHC.
Conclusion: Bien que la prévalence de l'infection active par le VHC chez les personnes infectées par le VIH dans cette étude soit apparemment faible (0,5%), le dépistage avec le test d'anticorps anti-VHC et la confirmation avec le test PCR de l'ARN du VHC sont recommandés.

Mots clés: Virus de l'hépatite C; VIH; ARN du VHC; prévalence; facteurs de risqué

Introduction:

Hepatitis C virus (HCV) infection is a major public health problem and a leading cause of liver related morbidity and mortality. Worldwide, an estimated 58 million people have chronic HCV infection, with about 1.5 million new infections occurring per year (1). Of the 58 million persons living with HCV infection globally in 2019, an estimated 15.2 million (21%) knew their diagnosis, and of those diagnosed with chronic HCV infection, around 9.4 million (62%) had been treated with direct acting antivirals by the end of 2019 (1). The World Health Organisation (WHO) estimated that in 2019, approximately 290,000 people died from HCV infection, mostly from cirrhosis and hepatocellular carcinoma (1).

According to recent global estimates in 2021, 38.4 million people were living with the human immunodeficiency virus (HIV), with 1.5 million new infections, and 650,000 people died of AIDS related illness (2). About 85% of people living with HIV knew their HIV status in 2021 and 28.7 million were accessing antiretroviral therapy (2). The prevalence of HIV in Nigeria is 1.4% with 1.9 million people living with HIV. In 2020, there were 86,000 new infections and 49,000 AIDS related deaths (3).

Both HCV and HIV share common routes of transmission which makes the risk of co-infection common. About 2.3 million people (6.2%) of those living with HIV globally have serological evidence of past or present HCV infection (1). In Nigeria, the prevalence of HIV/HCV co-infection among people living with HIV age 15-64years was 1.1%; 1.2% among females and 0.8% among males (3).

Co-infection with HIV has an impact on the natural course of HCV infection. There is increased HCV viral load in patients who are co-infected with HIV compared with those with HCV alone. In addition, there is accelerated progression of liver disease, liver fibrosis, cirrhosis, liver failure and rapid progression to hepatocellular carcinoma (4). This is more pronounced in those with CD4 count less than 200cells/mm^3 (5). This accelerated progression can be delayed with the use of antiretroviral therapy such that co-infected patients with negligible HIV viral load take longer to develop cirrhosis than those with high HIV viral load (6).

Screening for HCV is done using a serological test for antibodies to HCV. Once an individual acquires HCV infection, the antibodies persist for life even though the body may have spontaneously cleared the virus (7). If the test is positive for anti-HCV antibodies, a nucleic acid test for HCV ribonucleic acid

(RNA), which is the is the 'gold standard' test for the diagnosis of chronic HCV infection (8) is needed to confirm chronic infection and the need for treatment. This test is important because about 30% of people infected with HCV spontaneously clear the infection by a strong immune response without the need for treatment (1). Antiviral medicines can cure more than 95% of persons with hepatitis C infection, but access to diagnosis and treatment is low. At present, there is no licensed effective vaccine against HCV. Prevention is mainly by reducing the risk of exposure to the virus and this will depend on the route of transmission of HCV.

In many HIV clinics in Nigeria, only screening tests for HCV antibody are performed and PCR for the detection of HCV RNA is not done routinely because they are expensive and not readily available. Most studies on hepatitis c virus infection in Nigeria have been limited to using HCV antibody test to determine prevalence with very few studies able to confirm active or chronic HCV infection using HCV RNA. This study aims to assess the prevalence and risk factors for active HCV infection in HIV positive patients using RT-PCR to detect HCV RNA.

Materials and method:

Study location and population:

This study was conducted at the Lagos University Teaching Hospital (LUTH) HIV clinic. This clinic has been operational since 2004. It implements free treatment of HIV/AIDS with antiretroviral drugs with support from the President's Emergency Plan for AIDS Relief (PEPFAR) in accordance with national guidelines. There is on-site HIV screening, counselling, provision of medication, follow-up, monitoring and evaluation of all patient activities. Over 20,000 adults and 1,200 children had been enrolled into the treatment programme. The HIV clinic has a dedicated laboratory that determines HIV viral load and $CD4^+$ cell count.

The study population consisted adult HIV-positive patients attending the HIV clinic. The inclusion criteria were HIV infected patients ≥18 years of age, who gave informed consent to participate in the study. Those who were not willing to participate were excluded.

Study design and participant selection:

This was a cross-sectional study to determine prevalence of HCV infection in HIV positive patients. Participants were chosen by simple random sampling on each clinic day. A list of all patients who had clinic appointment on each day was compiled and a simple random sample of 5 patients were chosen using a computer-generated list of random numbers with Microsoft Excel software. Active HCV infection was defined as positive anti-HCV antibody test and positive HCV RNA by reverse transcriptase polymerase chain reaction (RT-PCR) assay.

Ethical approval:

Ethical approval was obtained from the Health Research and Ethics committee of Lagos University Teaching Hospital (LUTH). Informed consent form was signed by those who agreed to participate in the study.

Data collection

Socio-demographic, clinical and laboratory data were collected from the participants using a structured questionnaire. Data on the risk factors such as blood transfusion, multiple sexual partners, smoking, alcohol use, sexual orientation, use of condoms, intravenous drug use, CD4 count, HIV viral loads, antiretroviral therapy and were retrieved from the case notes of the patients.

Sample collection and HCV antibody testing:

Five millilitres of blood were collected into an EDTA bottle. Blood was then spun in a centrifuge for 20 minutes at 2000 revolutions per minute. A sterile pipette was used to transfer plasma into a plain sterile cryo-tube. Plasma was stored at -70^{o}C until testing. Processing of specimen was performed at the virology laboratory of the central research laboratory, College of Medicine, University of Lagos. Plasma was tested for the qualitative detection of IgG antibodies to HCV using a solid phase enzyme linked immunosorbent assay (CTK Biotech USA) according to manufacturers' instruction.

Polymerase chain reaction (PCR) assay for HCV RNA detection:

HCV RNA was extracted from blood samples of all participants using the QIAamp1 Viral RNA Kit (QIAGEN, Valencia, CA), following the manufacturer's instructions. The 5'NC region of the HCV genome was cDNA amplified by a nested reverse transcriptase polymerase chain reaction (RT-PCR), using QIAGEN® One Step RT-PCR (QIAGEN, Valencia, CA), according to the manufacturer's instruction.

The primer sequences, *ACTGTCTTCA CGCAGAAAGCGTCTAGCCAT* as the outer forward primer, *CGAGACCTCCCGGGGCACTCGC AAGCACCC* as the outer reverse primer, *ACG CAGAAAGCGTCTAGCCATGGCGTTAGT* as the inner forward primer; and *TCCCGGGGCACTC GCAAGCACCCTATCAGG* as the inner reverse primer were used for detection of 5' NC region of HCV genome with nested RT-PCR (9). For the first round, the reaction mixture containing 5 µl 5× PCR buffer with $MgCl_2$ (Qiagen), 1 µl dNTP mix (0.4 mM), 1.5 µl of outer forward and reverse primers, 1µl Qiagen one step RT-PCR enzyme mix, 5 µl of template and RNAse free water up to 20 µl. The cycling conditions were as follows; 50^{o}C for 30 mins,

then 95°C for 15 mins, followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 60 seconds and final extension at 72°C for 10 mins. The second round was carried out like the first round with the inner set of primers and the cycling conditions were as follows; 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 45 seconds, 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

The PCR products were electrophoresed on 1.5% agarose gel, stained with SYBR green. The gel was visualized in ultraviolet (UV) transilluminator and photographed using a camera. The expected PCR product size was 251 bp for the inner primer set.

Data analysis:

Prevalence data and 95% confidence intervals were calculated. Categorical variables were compared using the Chi-square or Fisher's exact test while continuous variables were compared using the Students' *t*-test to evaluate the association between the prevalence of HIV/HCV co-infection and associated risk factors. P value < 0.05 was considered to be statistically significant.

Results:

Socio-demographic and clinical characteristics of the study participants:

One hundred and ninety-five HIV infected participants were recruited into the study, of which 134 (68.7%) were females and 61 (31.3%) were males. The age of the participants ranged from 21-69years, with a mean age of 40.1±7.8 years, and median age of 39 years. The mean age of the female participants was 38.4±7.9 years while the mean age of the males was 44.1±9.4 years. Majority of the participants were in the age group 31-40 (45.3%) and most of the participants were married (70.8%, 138/195), while 19% were single. About 42.8% of them had secondary level education, while 41.7% had tertiary level education (Table 1).

The mean age of participants at first sexual intercourse was 20.6±4.0 years. Most of the participants (71.9%, 138/195) had one sexual partner and only 2.6% had three or more sexual partners. The predominant sexual orientation of the participants was heterosexual (99.4%). Majority of the participants (63.5%) used condoms, did not smoke cigarettes (93.8%), did not drink alcohol (89.7%) and did not abuse intravenous drug (95.8%). History of blood transfusion was reported in 18.9% of the participants (Table 1). The use of antiretroviral therapy was reported by 89.5% of the participants, with mean duration of antiretroviral therapy being 5.5±3.1 years. The median $CD4^+$ count was 491 cells/mm^3 (IQR: 334 - 696, range: 98 - 1363), and the mean log_{10}HIV viral load was 2.7 ± 1.3 log copies/ml.

Prevalence of Hepatitis C in HIV infected participants:

Of the 195 HIV infected participants, 5 tested positive for antibody to hepatitis C virus with a seroprevalence rate of 2.6% (95% CI=0.8-5.9%). Of these 5, HCV RNA was detected in 1 (20.0%), giving a prevalence of 0.5% (1/195) for active HCV infection among the study participants. The seroprevalence of HCV in males was 3/61 [4.9% (95% CI=1.0-13.7%)] and in females, it was 2/134 [1.5% (95% CI= 0.2-5.3%)], but the difference was not statistically significant (OR=3.41, 95% CI =0.56-20.98, p=0.18). The median CD4 count was 261 cells/mm^3 (IQR: 236-630) and mean log_{10}HIV viral load was 5.1±0.9 log copies/ml.

HCV RNA was detected in only 1 of the 5 participants who were seropositive for HCV. The prevalence of active HCV infection was therefore 0.5% (95% CI=0.01-2.82%). This patient was a 30-year-old male with 3 or more sexual partners, who had been on antiretroviral therapy for 5 years, had previous history of blood transfusion, did not smoke, drink alcohol or use IV drugs. His $CD4^+$ cell count was 333 cells/mm^3 and HIV viral load was 4.3 log_{10}copies/ml.

Risk factors for hepatitis C infection in HIV positive patients:

The 5 participants who were seropositive for HCV were compared to those seronegative for HCV using a bivariate analysis (Table 2). The mean log_{10} HIV viral load was significantly higher among participants seropositive for HCV (5.1±0.9 log copies/ml) than those seronegative (2.7±1.2 log copies/ml) (p<0.001). The mean duration of antiretroviral therapy was significantly lower among participants seropositive for HCV (2.6 ± 1.3 years) than those seronegative (5.6 ± 3.1 years) (p=0.004). The seroprevalence of HCV was significantly higher in those with $CD4^+$ cell count < 350 cells/mm^3 (8.5%) compared to those with $CD4^+$ cell count <350 cells/mm^3 (p=0.02).

The seroprevalence of HCV in the HIV-positive participants was significantly associated with sexual partners (p=0.0473), with highest seroprevalence in those with ≥ 3 sexual partners (OR=11.625, 95% CI=1.049-128.83). The participants seropositive for HCV were younger than those seronegative (34.8± 6.6 years vs 40.3±8.8 years), but the difference was not statistically significant (p=0.17). The seroprevalence of HCV was highest in the age group 21-30 years (9.1%), followed by age group 31-40 years (2.3%) and age group 41-50 years (1.6%). However, this difference was not statistically significant (p=0.34). Since there was only one HIV infected patient

Table 1: Socio-demographic characteristics of HIV infected participants attending the HIV clinic of the Lagos University Teaching Hospital, Idi-Araba, Nigeria

Characteristic	Frequency	Percentage (%)
Gender		
Male	61	31.3
Female	134	68.7
Age group (years)		
21 – 30	22	11.5
31 – 40	87	45.3
41 – 50	61	31.8
51 – 60	16	8.3
61-70	6	3.1
Marital status		
Single	37	19.0
Married	138	70.8
Widowed	17	8.7
Divorced	2	1.0
Separated	1	0.5
Education level		
None	5	2.6
Primary education	25	12.9
Secondary education	83	42.8
Tertiary education	81	41.7
Number of sexual partners		
None	39	20.3
One partner	138	71.9
Two partners	10	5.2
Three or more partners	5	2.6
Sexual orientation		
Heterosexual	163	99.4
Homosexual	1	0.6
Condom use		
Yes	115	63.5
No	66	36.5
Smoking		
Yes	12	6,2
No	182	95.8
Alcohol intake		
Yes	20	10.3
No	174	89.7
Blood transfusion		
Yes	36	18.8
No	155	81.2
IV Drug abuse		
Yes	7	4.2
No	159	95.8
Antiretroviral therapy		
Yes	170	89.5
No	20	10.5
CD4 (cells/mm^3) count categories		
<350	47	26.3
350 – 500	45	25.1
>500	87	48.6
Median CD4 count (cells/mm^3)	491 (IQR: 334 – 696)	
Mean age (years)	40.1 ± 8.7	
Mean age sexual intercourse (years)	20.6 ± 4.0	
Mean duration of ART (years)	2.6±1.3	
Mean $\log_{10}$ HIV viral load (copies/ml)	2.7±1.3	

with active HCV infection (HCV RNA positive), analysis of the risk factors for active HCV infection could not be performed.

Discussion:

The seroprevalence of HCV infection in HIV infected patients was estimated in a population of adults consisting predominantly of females. The seroprevalence of HCV in HIV positive patients in this study was 2.6%. This relatively low prevalence may be explained by the low frequency of documented high-risk behavior in this cohort of HIV-infected patients. However, this seroprevalence is higher than the 1.1% national prevalence of HCV in HIV/HCV co-infected patients reported in the National AIDS Indicator Survey (NAIIS) (3), but the rate is similar and consistent with findings from Abuja where HCV seroprevalence of 2.3% was reported in HIV infected patients (10,11). Nevertheless, the seroprevalence is lower than the HIV/HCV prevalence reported in many studies such as 13.5% from Nasarawa, 11.3% from Jos, 4.8% from Ibadan, 5.8% from Lagos, 5.7% from Rwanda and 11.3% from Cameroun (12-17). The difference in sample size, socio-demographic

Table 2: Bivariate analysis of risk factors for hepatitis C virus seroprevalence in selected HIV positive patients at the HIV clinic, Lagos University Teaching Hospital, Idi-Araba, Nigeria

Variable	Number anti-HCV negative (%) (n=190)	Number anti-HCV positive (%) (n=5)	P value
Gender			
Male	58 (95.1)	3 (4.9)	0.18
Female	132 (98.5)	2 (1.5)	
Age group (years)			
21 – 30	20 (90.9)	2 (9.1)	0.34
31 – 40	85 (97.7)	2 (2.3)	
41 – 50	60 (98.4)	1 (1.6)	
51 – 60	16 (100.0)	0	
61 – 70	6 (100.0)	0	
Marital status			
Single	35 (94.6)	2 (5.4)	0.77
Married	135 (97.8)	3 (2.2)	
Widowed	17 (100.0)	0	
Divorced	2 (100.0)	0	
Separated	1 (100.0)	0	
Educational status			
None	5 (100.0)	0	0.76
Primary education	25 (100.0)	0	
Secondary education	80 (96.4)	3 (3.6)	
Tertiary education	79 (97.5)	2 (2.5)	
Sexual partners			
None	37 (94.9)	2 (5.1)	0.047*
1	136 (98.6)	2 (1.4)	
2	10 (100.0)	0	
3 or more	4 (80.0)	1 (20.0)	
Sexual orientation			
Heterosexual	158 (96.9)	5 (3.1)	0.85
Homosexual	1 (100.0)	0 (0)	
Condom use			
Yes	112 (97.4)	3 (2.6)	1.0
No	65 (98.5)	1 (1.5)	
Smoking			
Yes	12 (100.0)	0	0.77
No	177 (96.3)	5 (2.7)	
Alcohol			
Yes	20 (100.0)	0	1.0
No	169 (97.1)	5 (2.9)	
Blood transfusion			
Yes	34 (94.4)	2 (5.6)	0.24
No	152 (98.1)	3 (1.9)	
IV Drug abuse			
Yes	7 (100.0)	0 (0)	1.0
No	156 (98.1)	3 (1.9)	
Antiretroviral therapy			
Yes	165 (97.1)	5 (2.9)	1.0
No	20 (100.0)	0	
CD4 (cells/mm^3) count categories			
<350	43 (91.5)	4 (8.5)	0.02*
350–500	45 (100.0)	0	
>500	86 (98.8)	1 (1.2)	
Mean age ± SD (years)	40.3±8.8	34.8±6.6	0.17
Mean age at first sexual intercourse	20.5±4.0	22.5±4.9	0.33
Mean duration of ART	5.6±3.1	2.6±1.3	0.004*
Mean log_{10} HIV viral load (copies/ml)	2.7±1.2	5.1±0.9	<0.0001*

* = statistically significant; SD = Standard deviation

factors, risk behaviours and types of exposure may account for the variation in the prevalence rates in the Nigerian studies. In comparison with this study, the vast majority of other studies in Nigeria which showed a higher seroprevalence of HCV in HIV infected patients predate this study, which may suggest improvement in preventive measures on HCV such as improvement in sexual education, screening of blood for transfusion, and injection safety, which are the main routes of transmission of HCV in Nigeria.

All patients in this study were tested for HCV-RNA using RT-PCR assay and only one patient tested positive. The patient also tested positive for HCV antibody, giving HCV-RNA detection rate of 20% of those who tested positive for antibodies to HCV, and low prevalence of active HCV infection of 0.5% among the entire participants in this study. Very few studies in Nigeria have reported prevalence of active HCV infection by HCV RNA detection in HIV infected patients. The difference between the HCV seroprevalence rates using ELISA and active infection by RT-PCR reported in this study may be due to clearance of the HCV in some patients (past HCV infection) or false positive antibody result or fluctuation of HCV viraemia in those with chronic HCV infection.

The prevalence of active HCV infection in this study is lower than the prevalence of 8.2% reported by Agwale et al., (18) who

tested 146 HIV positive patients in Northern Nigeria for HCV RNA and HCV genotype and a prevalence of 6% reported in Jos (19). In the study by Agbaji et al., (19), 79 of 262 (30.2%) patients who tested positive to HCV antibody had HCV viraemia. These findings highlight the importance of HCV-RNA confirmatory testing to determine those who have active HCV infection among those who are HCV seropositive. Studies comparing HCV antibody tests to HCV-RNA suggest that false positive antibody tests may be common in Africa (20). However, due to limited capacity for molecular virology, the tests are expensive and mostly not available. Therefore, routine confirmation of seropositive HCV infection by RT-PCR is not done.

The risk factors for active HCV infection in HIV positive patients could not be determined in this study because only one patient had HCV viraemia. Therefore, risk factors were assessed for only those seropositive for HCV. The only patient who had HCV viraemia was a 30-year-old male, who had 3 or more sexual partners, had been on antiretroviral therapy for 5 years, and had previous history of blood transfusion, but no history of smoking, alcohol consumption or IV drug abuse. The $CD4^+$ count of this patient was 333 cells/mm^3 and his HIV viral load was 4.33 log copies/ml.

The HIV infected patients seropositive for HCV in the study had lower $CD4^+$ counts than those seronegative, and those with $CD4^+$ count <350/mm^3 had the highest HCV seropositive rate. This implies that HCV infection is associated with immune suppression in HIV positive patients. Some authors have shown a decline in $CD4^+$ cell count associated with HIV/HCV infection (13,14,21). The mean log_{10} HIV viral load was significantly higher among patients seropositive for HCV (5.1±0.9 copies/ml) than those seronegative (2.7±1.2copies/ml), which suggests that HIV disease tend to progress in those who have HIV/HCV co-infections. There are very few studies in Nigeria that have assessed the association between HCV infection and viral load in HIV positive patients. In a study of 17,882 patients in Jos by Ladep et al., (13), there was a significantly higher HIV viral loads in patients with HIV/HCV co-infection compared to those with HIV mono-infection. It has been demonstrated that there is more rapid HIV disease progression in HIV/HCV co-infected patients compared to those who were HIV-infected mono-infected (22).

More than 80% of the HIV positive patients in this study were on antiretroviral therapy but the mean duration of antiretroviral therapy was significantly lower among patients seropositive for HCV (2.6±1.3 yrs) than those seronegative for HCV (5.6±3.1 years). Suppression of HIV disease progression with antiretroviral therapy may lead to possible recovery of $CD4^+$ cells and decrease viral load, which can delay the progression of HCV liver disease (23).

The limitations of the study include the low number of patients who tested positive for the HCV antibody and HCV RNA. A future study involving large number of patients with positive HCV antibody and HCV RNA test should be conducted to determine the true prevalence and risk factors of active HCV infection in HIV positive patients.

Conclusion:

In conclusion, the seroprevalence of HCV in this study was 2.6% while the prevalence of active HCV infection was 0.5%, and only one in five (20.0%) of those seropositive for HCV had active HCV infection. This implies that the other four patients (80.0%) may have had past infection or false positive antibody test. The HIV viral load was significantly higher while the $CD4^+$ count was significantly lower in those seropositive for HCV. The mean duration of antiretroviral therapy was significantly lower among participants seropositive for HCV. However, the risk factors of active HCV infection could not be assessed since only one patient was HCV RNA positive. Nevertheless, the findings of this study underscores the need for screening of HIV positive patients with HCV antibody and confirmation by detection of HCV RNA.

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Contributions of authors:

OPO conceptualized the study, developed study methodology, collected data, performed data analysis and wrote the manuscript; SOB and OSA supervised the study. All authors approved the manuscript submitted.

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Author declares no conflict of interest

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Original Article **Open Access**

Seroprevalence of transfusion transmissible infections by ELISA in donors testing negative with rapid ICT in Asokoro District Hospital, Abuja, Nigeria

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Abstract:

Background: Blood transfusion saves lives, but it is associated with many complications which include transfusion transmissible infections (TTIs). The major objectives of this study were to determine; the prevalence of TTIs in the blood donated at Asokoro District Hospital, Abuja, Nigeria from 01 January to 31 December 2019; investigate the accuracy of rapid immunochromatographic (ICT) tests for preliminary TTIs screening of blood donors' samples and confirmed by ELISA test; and establish baseline data that will provide the impetus for improvement in equipment, infrastructure, and health system management, with the ultimate goal of ensuring safer blood transfusion practice.
Methodology: This was a retrospective cross-sectional study of records of blood donors at the Blood Bank of Asokoro District Hospital, a quasi-tertiary centre situated at Abuja, Federal Capital Territory (FCT), northcentral Nigeria, between 01 January to 31 December 2019. The socio-demographic biodata (age and gender), blood parameters (PCV and blood groups), and TTIs test results (by rapid ICT kit and ELISA) of donors were manually extracted from the record books of the hospital's blood bank. Only donor blood samples that initially tested negative for TTIs met the inclusion criteria. The data were entered into the spreadsheet of Microsoft Excel, and analysed with the Statistical Package for the Social Sciences (SPSS) for Windows version 23.0. Chi square test was used to determine association between variables and *p* value less than 0.05 was considered statistical significance.
Results: A total of 1400 blood samples of donors, received within the study period, met the inclusion criteria of testing negative for TTIs on rapid ICT kit test. The mean age (± SD) of the donors was 35.6±6.7 years with age range of 18-67 years. The median age was 35 years, and the predominant age group was 30-39 years, accounting for 55.4% (775/1400). Majority of the donors (97.1%, 1359/1400) were males. In terms of ABO blood group distribution, group O Rh D positive was the commonest, followed by group A Rh D positive, while the least is group AB Rh D positive. The overall prevalence of TTIs by ELISA test was 4.9% (68/1400). This prevalence was highest for HCV (1.9%, 26/1400), followed by HIV (1.2%, 17/1400), HBV (0.9%, 13/1400) and syphilis (0.9%, 12/1400).
Conclusion: The detection of TTIs by ELISA test from false negative donor samples preliminarily screened with rapid ICT kit highlighted by our study speaks to the unreliability of rapid ICT kits in screening of blood donors for TTIs. There is need therefore for health authorities in Nigeria and other LMICs to ensure widespread availability of highly sensitive blood screening methods such as ELISA to the point where it will be possible to enforce legislation against the use of the less accurate rapid ICT screening kits.

Keywords: transfusion transmissible infections; seroprevalence; ELISA; rapid ICT kit; blood donors

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Séroprévalence des infections transmissibles par transfusion par ELISA chez les donneurs testés négatifs avec des TIC rapides à l'hôpital du district d'Asokoro, Abuja, Nigeria

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Résumé:

Contexte: La transfusion sanguine sauve des vies, mais elle est associée à de nombreuses complications, notamment les infections transmissibles par transfusion (ITT). Les principaux objectifs de cette étude étaient de déterminer; la prévalence des ITT dans le sang donné à l'hôpital du district d'Asokoro, Abuja, Nigeria du 1er janvier au 31 décembre 2019; enquêter sur l'exactitude des tests immunochromatographiques rapides (ICT) pour le dépistage préliminaire des ITT des échantillons de donneurs de sang et confirmés par un test ELISA; et d'établir des données de référence qui donneront l'élan nécessaire à l'amélioration de l'équipement, de l'infrastructure et de la gestion du système de santé, dans le but ultime d'assurer une pratique plus sûre de la transfusion sanguine.
Méthodologie: Il s'agissait d'une étude transversale rétrospective des dossiers des donneurs de sang à la banque de sang de l'hôpital du district d'Asokoro, un centre quasi-tertiaire situé à Abuja, Territoire de la Capitale Fédérale (FCT), centre-nord du Nigeria, entre le 1er janvier et le 31 décembre 2019. Les biodonnées socio-démographiques (âge et sexe), les paramètres sanguins (PCV et groupes sanguins) et les résultats des tests ITT (par kit ICT rapide et ELISA) des donneurs ont été extraits manuellement des registres de la banque de sang de l'hôpital. Seuls les échantillons de sang de donneurs initialement testés négatifs pour les ITT répondaient aux critères d'inclusion. Les données ont été saisies dans le tableur de Microsoft Excel et analysées avec le progiciel statistique pour les sciences sociales (SPSS) pour Windows version 23.0. Le test du chi carré a été utilisé pour déterminer l'association entre les variables et une valeur de p inférieure à 0,05 a été considérée comme une signification statistique.
Résultats: Un total de 1400 échantillons de sang de donneurs, reçus au cours de la période d'étude, répondaient aux critères d'inclusion de test négatif pour les ITT sur le kit de test rapide ICT. L'âge moyen (± ET) des donneurs était de 35,6±6,7 ans avec une tranche d'âge de 18 à 67 ans. L'âge médian était de 35 ans et la tranche d'âge prédominante était de 30 à 39 ans, représentant 55,4% (775/1400). La majorité des donneurs (97,1%, 1359/1400) étaient des hommes. En termes de distribution des groupes sanguins ABO, le groupe O Rh D positif était le plus courant, suivi du groupe A Rh D positif, tandis que le groupe AB Rh D positif était le moins important. La prévalence globale des ITT par test ELISA était de 4,9% (68/1400). Cette prévalence était la plus élevée pour le VHC (1,9%, 26/1400), suivi du VIH (1,2%, 17/1400), du VHB (0,9%, 13/1400) et de la syphilis (0,9 %, 12/1400).
Conclusion: La détection des ITT par test ELISA à partir d'échantillons de donneurs faussement négatifs préalablement sélectionnés avec un kit ICT rapide mis en évidence par notre étude témoigne du manque de fiabilité des kits ICT rapides dans le dépistage des ITT chez les donneurs de sang. Il est donc nécessaire que les autorités sanitaires du Nigéria et d'autres PFR-PRI garantissent la disponibilité généralisée de méthodes de dépistage sanguin très sensibles telles que l'ELISA au point qu'il sera possible d'appliquer la législation contre l'utilisation des kits de dépistage rapide des TIC moins précis.

Mots clés: infections transmissibles par transfusion; séroprévalence; ELISA; kit TIC rapide; donneurs de sang

Introduction:

Blood transfusion is a very important aspect of management of patients with various medical and surgical conditions. Although blood transfusion saves lives, it is also associated with many complications which include transfusion transmissible infections (TTIs). Unsafe blood transfusion increases the risk of TTIs (1). Blood safety remains a big issue in transfusion medicine. The World Health Organisation (WHO) recommends that all blood donors should be screened for TTIs principally human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and syphilis caused by *Treponema pallidum* (1).

TTIs constitute an unacceptably high public health burden. HBV and HCV caused 1.1 million deaths globally in 2019, while HIV was estimated to cause 680,000 deaths globally in 2020, with an estimated 7.1 million new cases (2). In sub-Saharan Africa, access to safe and

adequate blood transfusion services has remained a challenge (3). The National Blood Service Commission (NBSC) is the Nigerian government institution charged with the responsibility of providing safe blood and blood products, and regulating blood transfusion services nationwide. According to NBSC data, 90% of the total blood donation in Nigeria is sourced from commercial donors (4). Commercialised blood donation raises serious ethical and safety concerns, and recipients of blood from commercial donors run the risk of contracting TTIs such as HIV, HBV, HBC and syphilis (1).

The threats posed by TTIs are high, and so accurate diagnosis of infectious agents in donor blood is a major strategy for safe blood. In developing countries like Nigeria, the rapid diagnostic immuno-chromatographic techniques (rapid kits), which are easier to use and inexpensive, are widely used for donor blood screening rather than the Enzyme-Linked Immunosorbent Assay (ELISA) testing. ELISA testing is costly and requires higher technology and trained personnel, but they are more accurate for the diagnosis of TTIs (5).

In as much as rapid tests are being used to detect TTIs in many blood banks in Nigeria to overcome poor funding and equipment constraints, they raise major concerns on their accuracy to diagnose TTIs in the blood donors. Therefore, this retrospective study was done to determine the prevalence of TTIs in the blood donated at Asokoro District Hospital, Abuja, Nigeria from 01 January to 31 December 2019; investigate the accuracy of rapid kit tests for four TTIs namely HIV, HCV, HBV and syphilis in blood donors' samples subjected to confirmatory testing using ELISA technique; and establish baseline data that will hopefully provide impetus for improvements in health system management, equipment and infrastructure that will ultimately ensure safer blood transfusion practice

Materials and method:

Study location:

The study was conducted at Asokoro District Hospital Blood Banking Service (ADHBBS). Asokoro District Hospital is a quasi-tertiary centre situated in Abuja, Federal Capital Territory (FCT), Nigeria. It serves as a referral centre for cases from primary and secondary healthcare facilities in the FCT, and other neighbouring states such as Kogi, Nasarawa and Niger States. The ADHBBS provides TTI-tested blood and blood products. All prospective donors are screened for TTIs with rapid kits and only those who test negative to all four TTIs are allowed to donate. The donor samples are then further tested by ELISA and only donor blood whose samples are confirmed negative for all four TTIs are retained in the blood bank for use. All the procedures conducted by the ADHBBS are in strict compliance with the guidelines in the Nigerian National Blood Policy issued by the National Blood Service Commission (6).

Study design and population:

This is a retrospective cross-sectional study of records of blood donors at ADHBBS from 01 January to 31 December 2019. Donors are predominantly voluntary non-remunerated and family replacement donors. The socio-demographic biodata (age and gender), blood parameters (PCV and blood groups), and TTIs test results (rapid kit and ELISA) of the donors were manually extracted from the record books of the ADHBBS. Only donor blood samples that initially tested negative for these TTIs (HIV, HCV, HBV and syphilis) met the inclusion criteria. Cases with missing or incomplete biodata and test results were excluded.

Ethical consideration:

Ethical clearance was obtained from the Medical Ethics Committee of Asokoro District Hospital with approval number; FCTA/HHSS/HMB/ADH/114/22.

Screening methods:

Rapid tests for TTIs were conducted using lateral flow immuno-chromatographic (ICT) assay for qualitative detection of antibodies. The following rapid kits were used during the study period; Abbot Determine HIV-1/2, Atlas Medicals Rapid Test Strips (IgG and IgM) for *Treponema pallidum* (VDRL), HCV and HBsAg. ELISA tests were carried out using fourth generation kit (ADALTIS/EIAgen) for *in-vitro* diagnostic screening in human serum and plasma, to detect antibodies to HBsAg, HCV, syphilis, HIV-1, HIV-2 and HIV-1 p24 antigen. Diagnostic sensitivity and specificity rates for these tests hover between ≥ 99.7% and 100%. Detection limit for p24-antigen was 1 IU/ml, based on the WHO 1st International Reference code 90/636 (7). The manufacturers' instructions, as stated in the inserts of all the test kits, were strictly followed in the performance of these tests.

Data analysis:

The data retrieved from the blood bank record book were entered using the spreadsheet of Microsoft Office 365 version of Excel (Microsoft Corporation, Redmond, Washington, USA), and exported to the Statistical Package

for the Social Sciences (SPSS) for Windows, version 23.0 (SPSS Inc. Chicago, Illinois, USA) for analysis. Chi square test was used for determining association between variables and *p* value less than 0.05 was considered statistical significance.

Results:

A total of 1400 blood samples of donors, received within the study period met the inclusion criteria of testing negative for TTIs on rapid kit testing. The mean age (± SD) of the participants was 35.6 ± 6.7 years with a range of 18-67 years while the median age was 35 years. The preponderance of blood donors was in the 30-39 years age group, accounting for 55.4% (775/1400) while the least (0.4%, 6/1400) was in the <20 years age group. Majority of the donors, accounting for 97.1% (1359/1400), were males (Table 1).

With respect to ABO blood group distribution of donors, group O Rhesus D positive, accounting for 61.1% (855/1400), was the commonest, followed by group A Rhesus D positive (17.9%, 250/1400), while AB Rh D positive (0.1%, 1/1400) was the least common (Table 2). The prevalence of HIV among donors using ELISA was 1.2% (17/1400) while that of HBV, HCV and syphilis were 0.9% (13/1400), 1.9% (26/1400), 0.9% (12/1400) respectively (Fig 1, Table 3).

Table 1: Socio-demographic characteristics of blood donors in Asokoro District Hospital, Abuja, Nigeria

Variables	**Gender**		**Total (%)**
	Male (%)	**Female (%)**	
Age range (years)	18-67	21-43	18-67
Mean age ± SD (years)	35.7±6.6	29.6±5.2	35.6±6.7
Median age (years)	35	29	35
95% CI	35.4-36.1	27.9-31.3	35.2-35.9
Age group (years)			
<20	6 (0.4)	0	6 (0.4)
20-29	219 (15.6)	23 (1.6)	242 (17.3)
30-39	758 (54.1)	17 (1.2)	775 (55.4)
40-49	339 (24.2)	1 (0.1)	340 (24.3)
≥50	37 (2.6)	0	37 (2.6)
Total	**1359 (97.1)**	**41 (2.9)**	**1400 (100.0)**

Table 2: ABO Blood group distribution of donors

Blood group	**Frequency**	**Percent**
AB+	1	0.1
A-	10	0.7
A+	250	17.9
B-	14	1.0
B+	219	15.6
O+	855	61.1
O-	51	3.6
Total	**1400**	**100.0**

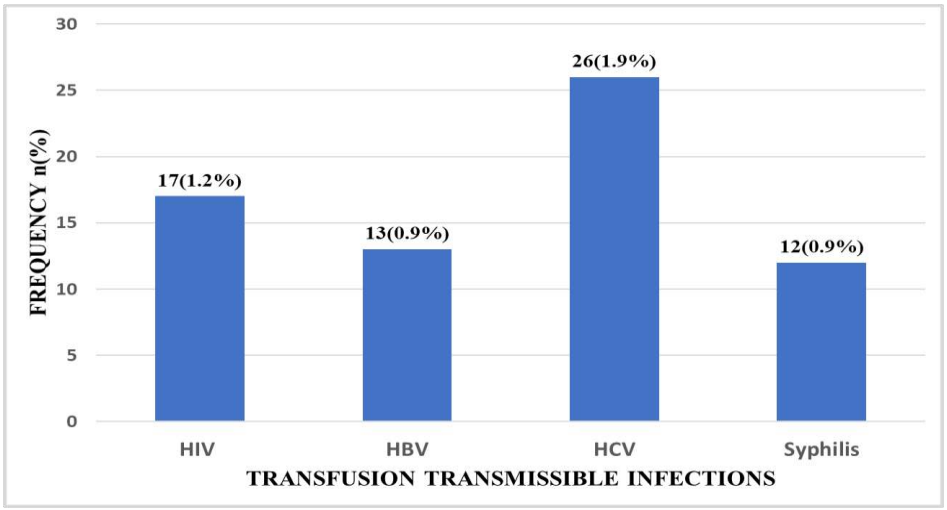

Fig 1: Prevalence of TTIs with ELISA in donors who tested negative with rapid ICT kits

Table 3: Prevalence of transfusion transmissible infections by ELISA test with respect to age group of donors with false negative rapid ICT kits

Age group (years)	HIV (%)		HBV (%)		HCV (%)		Syphilis (%)	
	Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
<20	0	6 (100.0)	0	6 (100.0)	0	6 (100.0)	0	6 (100.0)
20-29	2 (0.8)	240 (99.2)	4 (1.3)	238 (98.3)	5 (2.1)	237 (97.9)	4 (1.7)	238 (98.3)
30-39	10 (1.3)	765 (98.7)	4 (0.5)	771 (99.5)	17 (2.2)	758 (97.8)	6 (0.8)	769 (99.2)
40-49	5 (1.5)	335 (98.5)	5 (1.5)	335 (98.5)	2 (0.6)	338 (99.4)	2 (0.6)	338 (99.4)
≥50	0	37 (100.0)	0	37 (100.0)	2 (5.4)	35 (94.6)	0	37 (100.0)
Total	**17 (1.2)**	**1383 (98.7)**	**13 (0.9)**	**1387 (99.1)**	**26 (1.9)**	**1374 (98.1)**	**12 (0.9)**	**1388 (99.1)**
Chi square	1.056		4.302		6.212		2.527	
p value	0.901		0.376		0.184		0.640	

HIV: Human immunodeficiency virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus

The prevalence of TTIs by ELISA test was not significantly associated with age group of the donors (p>0.05) for all TTIs, although age groups 20-29, 30-39, and 40-49-years were most frequently affected, except for HCV in which age group >50 years had the highest prevalence of 5.4% (2/37) while the prevalence in age group <20 years was 0% for all the TTIs (Tables 3).

Discussion:

Transfusion transmissible infections poses a major challenge to safe blood transfusion services especially in sub-Saharan Africa. Most laboratories and blood bank service providers in developing countries still rely on the use of rapid kits for pre-donation screening of blood donors due to the unavailability of ELISA testing facilities in most of these blood banks (8). The mean age of the participants of 35.6±6.7 years in our study is similar to the findings of Buseri et al., (8) in southwest Nigeria, while the age range of 18-67 years is similar to the reports of studies done by Buseri et al., (8), Khan et al., (9) in northwest Frontier Province, India, and Mukhtar et al., (10) in northwest Nigeria, who reported age range of 18-64 years, 18-61 years and 18-60 years respectively. Majority (55.4%, 775/1,400) of the donors in our study were in the 30-39 years age group, findings similar to the studies of Buseri et al., (8) and Mukhtar et al., (9). The reasons for this are unclear but could be related to the hospital policy that encourages blood donation from spouses of women who attend

the antenatal clinic. Majority of the blood donors were males (97.1%, 1,359/1,400) and this is similar to reports of studies done by Mukhtar et al., (10), Egah et al., (11) and Nwokediuko et al., (12) with 98%, 95% and 91.8% being males respectively.

After ELISA testing, the prevalence of HIV infection in donor samples in this study was 1.2% (17/1400), while the prevalence of HBV, HCV and syphilis were 0.9% (13/1400), 1.9% (26/1400), and 0.9% (12/1400) respectively. These findings highlight the reduced accuracy of rapid kit screening. This discordance between the rapid kit test and ELISA was also reported in studies done by Al-Matary et al., (13) in Yemen, Agrawal et al., (14) in India and Erhabor et al., (15) in northwest Nigeria. Of the four TTIs, the prevalence of detecting false negative ICT test was highest for HCV (1.9%, 26/1400), which may indicate that accuracy of rapid HCV ICT kits is particularly low, but could also be due to the fact that HCV has not received adequate attention in Nigeria as awareness, screening, and vaccination programs, which are in place for both HIV and HBV infections, are largely absent for HCV infection. Although the high prevalence of HCV infection was accounted for by age group >50 years (5.4%, 2/37), there was no statistically significant association between age group of donors and prevalence of all the TTIs (p>0.05). The use of rapid kit tests for HBV and the other TTIs is clearly associated with high false negatives and a probable risk of increase TTIs in transfused blood to recipients. This is not surprising as HBV markers in surface antigen negative blood donors were found to contain the viral core antigen in the study done by Salawu et al., (16).

The ABO blood group distribution of donors showed a preponderance of O Rhesus D positive blood group with 61.1% (855/1400), a finding which agrees with several studies in Nigeria (17,18,19), confirming that O Rhesus D positive blood group is the commonest blood group in Nigerians. The major limitation of our study is that rapid kits from only a few manufacturers were tested against the fourth generation ELISA and as such, our findings only speak to the accuracy of the rapid kits made by these specific manufacturers and may not necessarily reflect on the accuracy of rapid kits by other manufacturers. Another limitation is the retrospective nature of our study design which may be associated with incomplete or inaccurate data. In addition, there was no 'gold standard' test (e. g. PCR) to truly compare the diagnostic performance (sensitivity, specificity, predictive values, likelihood ratio etc) of the two screening methods (rapid ICT kit and ELISA) for the four TTIs.

Conclusion:

The significant detection of false negative donor samples with ELISA highlighted by our study speaks to the unreliability of rapid kit screening in the prevention of TTIs. There is need therefore for health authorities in Nigeria and elsewhere to accelerate the widespread availability of highly accurate blood screening methods like ELISA to the point where it will be possible to enforce the legislation against the use of the less accurate rapid kit screening.

Future studies on diagnostic accuracy of these tests should be conducted using a 'gold standard' test and the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) tool as guide. Local research and development efforts should also be funded to enable affordable highly accurate alternatives to ELISA. Furthermore, the high prevalence of HCV infection deduced from this study should be combated by aggressive awareness and screening campaigns

Contributions of authors:

CGN contributed to study concept, design, definition of intellectual content, literature search, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing and manuscript review; YDO contributed to data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing and manuscript review; EOS contributed to the study concept, design, definition of intellectual content, data analysis, statistical analysis, manuscript preparation, manuscript editing and manuscript review; KNE made substantial contributions to the study concept, design, definition of intellectual content, literature search, manuscript preparation, manuscript editing and manuscript review; BA contributed to the study concept, design, definition of intellectual content, manuscript editing and manuscript review; and AFA made substantial contributions to concept, design, manuscript editing and manuscript review.

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Conflict of interest:

Authors declare no conflict of interest

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Original Article **Open Access**

Prevalence and antifungal susceptibility pattern of oral candidiasis among HIV-infected patients in a Mission Hospital, southeast Nigeria

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Abstract:

Background: Oral candidiasis is an infection that follows colonization of oral cavity by *Candida* species mostly *Candida albicans.* About 90% of HIV-infected persons develop this disease during the course of HIV infection and could serve as early sign of HIV-related immunodeficiency. Treatment involves the use of antifungal drugs. The objectives of this study are to determine the prevalence of oral candidiasis and the susceptibility of isolated *Candida* species to available antifungal agents among selected HIV-infected patients in a mission hospital, southeast Nigeria.
Methodology: This was a descriptive cross-sectional study of 150 consecutively selected HIV-infected patients attending the Heart-to-Heart clinic of Iyi-Enu mission hospital Ogidi, Anambra State, Nigeria, between December 2022 and February 2023. Demographic information of each participant was obtained using structured questionnaire. Five milliliters of whole blood were drawn from the antecubital vein of each participant for $CD4^+$ estimation. Mouth specimens were collected using two sterile cotton swabs for microscopy and culture on Sabouraud Dextrose Agar, and *Candida* species were identified after subculture on CHROMAgar. Antifungal susceptibility testing was performed by Kirby-Bauer disk diffusion method using fluconazole, clotrimazole, ketoconazole, and nystatin disks, and results interpreted according to the guidelines of the Clinical and Laboratory Standards Institute.
Results: A total of 98 (65.3%) HIV-infected participants were positive for oral candidiasis, with 4 species of *Candida* isolated; *Candida albicans* (62.2%), *Candida glabrata* (18.4%), *Candida tropicalis* (12.2%) and *Candida krusei* (7.1%). Fifty-nine (60.2%) of the 98 participants had $CD4^+$ cell count < 200, 33 (33.7%) had counts in the range of 200-399, and 6 (6.1%) had counts in the range of 400-499 cells/μL (p=0.001). The prevalence of candidiasis was not significantly different between the female (67.0%, 65/97) and male (62.3%, 33/53) participants (p=0.6598), but the prevalence was significantly higher (p<0.05) in participants age group 21-30 years (80.7%, 42/52), divorced (100%, 1/1) and married (75%, 45/60), those with primary school level education (73.7%, 42/57), civil servants (85.7%, 18/21), and those who performed mouth hygiene once daily (71.9%, 69/96). Nystatin (77.6%, 76/98) showed the highest while fluconazole and ketoconazole (68.4%, 62/98) showed the lowest *in vitro* antifungal activity
Conclusion: Oral candidiasis is prevalent among HIV-infected patients in the study population, with evidence of *in vitro* resistance of the *Candida* isolates to available antifungal drugs. Proper diagnosis, susceptibility testing and treatment of infection will be helpful in managing oral candidiasis infection among HIV infected patients.

Keywords: oral candidiasis, prevalence, *Candida albicans*, antifungal susceptibility test, HIV patient

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Prévalence et profil de sensibilité aux antifongiques de la candidose buccale chez les patients infectés par le VIH dans un hôpital de mission, au sud-est du Nigeria

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Résumé:

Contexte: La candidose buccale est une infection qui fait suite à la colonisation de la cavité buccale par des espèces de *Candida*, principalement *Candida albicans*. Environ 90% des personnes infectées par le VIH développent cette maladie au cours de l'infection par le VIH et pourraient constituer un signe précoce d'immunodéficience liée au VIH. Le traitement implique l'utilisation de médicaments antifongiques. Les objectifs de cette étude sont de déterminer la prévalence de la candidose buccale et la sensibilité des espèces isolées de *Candida* aux agents antifongiques disponibles chez des patients sélectionnés infectés par le VIH dans un hôpital de Mission, au sud-est du Nigeria.
Méthodologie: Il s'agissait d'une étude transversale descriptive de 150 patients infectés par le VIH sélectionnés consécutivement et fréquentant la clinique Heart-to-Heart de l'hôpital Iyi-Enu Ogidi, État d'Anambra, Nigéria, entre décembre 2022 et février 2023. Informations démographiques de chaque participant a été obtenue à l'aide d'un questionnaire structuré. Cinq millilitres de sang total ont été prélevés de la veine antécubitale de chaque participant pour l'estimation des $CD4^+$. Des échantillons de bouche ont été prélevés à l'aide de deux cotons-tiges stériles pour la microscopie et la culture sur Sabouraud Dextrose Agar, et les espèces de *Candida* ont été identifiées après sous-culture sur CHROMAgar. Les tests de sensibilité aux antifongiques ont été effectués par la méthode de diffusion sur disque de Kirby-Bauer en utilisant des disques de fluconazole, de clotrimazole, de kétoconazole et de nystatine, et les résultats ont été interprétés conformément aux directives du Institut des Normes Cliniques et de Laboratoire.
Résultats: Un total de 98 (65,3%) participants infectés par le VIH étaient positifs pour la candidose buccale, avec 4 espèces de *Candida* isolées; *Candida albicans* (62,2%), *Candida glabrata* (18,4%), *Candida tropicalis* (12,2%) et Candida krusei (7,1%). Cinquante-neuf (60,2%) des 98 participants avaient un nombre de cellules $CD4^+ < 200$, 33 (33,7%) avaient un nombre de cellules entre 200 et 399 et 6 (6,1%) avaient un nombre de cellules entre 400 et 499/µL (p=0,001). La prévalence de la candidose n'était pas significativement différente entre les femmes (67,0%, 65/97) et les hommes (62,3%, 33/53) participants (p=0,6598), mais la prévalence était significativement plus élevée (p<0,05) dans participants tranche d'âge 21-30 ans (80,7%, 42/52), divorcés (100%, 1/1) et mariés (75,0%, 45/60), ceux ayant un niveau d'études primaires (73,7%, 42/57), les fonctionnaires (85,7%, 18/21) et ceux qui ont effectué une hygiène buccale une fois par jour (71,9%, 69/96). La nystatine (77,6%, 76/98) a montré l'activité antifongique la plus élevée tandis que le fluconazole et le kétoconazole (68,4%, 62/98) ont montré l'activité antifongique *in vitro* la plus faible.
Conclusion: La candidose buccale est prévalente chez les patients infectés par le VIH dans la population étudiée, avec des preuves de résistance *in vitro* des isolats de *Candida* aux médicaments antifongiques disponibles. Un diagnostic, des tests de sensibilité et un traitement appropriés de l'infection seront utiles dans la gestion de l'infection par la candidose buccale chez les patients infectés par le VIH.

Mots-clés: candidose buccale, prévalence, *Candida albicans*, test de sensibilité aux antifongiques, patient VIH

Introduction:

Oral candidiasis is a common infection of the tongue and other oral mucosa caused mostly by *Candida albicans* (1). Other non-*albicans* species of *Candida* that have been implicated in this infection include *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Candida guilliermondii*, *Candida parapsilosis*, *Candida pseudotropicalis*, *Candida stellatoidea* and *Candida lusitaniae* (2,3). Oral candidiasis can occur in immune competent (4) as well as in immunocompromised patients (1). *Candida* species colonizes about 40-60% of oral cavity of immune competent individuals and 62-93% of HIV-infected patients (5). It has been reported that more than 90% of HIV patients develop the candidiasis during the course of infection (6), and data shows that about 9.5 million HIV-patients suffer from oral candidiasis (7).

The increased incidence of oral candidiasis in association with HIV/AIDS epidemic att-

racted the attention of many researchers and has remained the most common opportunistic infection among HIV/AIDS patients (4,8). Oral candidiasis could serve as early sign of HIV related immunodeficiency and is commonly observed in patients with $CD4^+$ counts less than 200 cells/µl (5,8,9). There are many clinical presentations and classifications of oral candidiasis. Vila et al., (4) simply categorized oral manifestations into three broad groups; acute, chronic and chronic mucocutaneous candidiasis syndrome. Several clinical forms can occur in the oral cavity and in multiple sites at the same time.

Acute pseudomembranous candidiasis also referred to as oral thrush accounts for a third of cases of oral candidiasis (10) and is the most common type among patients with HIV/AIDS/cancer, and those receiving immunosuppressive therapies (11). It usually presents as multifocal curdy yellow-white plaques throughout the oral mucosa. Chronic and recurrent infection is frequent among HIV/AIDS patients which can progress to esophageal candidiasis with difficulty in swallowing (4).

HIV/AIDS emerged as global crisis since 1981 and its prevalence is still on the increase in many countries especially in the developing ones (12). Sub-Saharan Africa countries bear excessive share of the global HIV burden with 23 million people living with HIV/AIDS in the region (13), out of the estimated 35.3 million people living with HIV/AIDS globally (14). It has been reported that high HIV load, $CD4^+$ count, type of antiretroviral (ART) medication, non-availability or non usage of highly active antiretroviral therapy (HAART) contribute to factors associated with oral candidiasis in HIV patients (7,15). Poor immunologic response by the patients, living in resource limited locales and development of antifungal resistance pose serious problems amongst the patients (16).

Infection with *Candida* has been related with some virulent factors. These include its ability to attach to the epithelial cell walls, persistent within epithelial cells, induction of tumor necrosis factor, proteinases, ability to form mycelia, germ tube formation (11), and morphological switching (4). A major biological characteristic of *C. albicans* is its ability to form pathogenic biofilms which is an important factor in its ability to cause infection (17,18). Once biofilm is formed, the expression of *Candida* virulence factors increases and its susceptibility to antimicrobials and phagocytosis decreases exceedingly (19).

Oral candidiasis can be diagnosed by identifying clinical signs and symptoms, direct examination of oral sample smear for the presence of *Candida* species, positive culture and serological tests (20). Treatment involves the use of antifungal agents like azoles (clotrimazole, itraconazole, miconazole, ketoconazole, fluconazole), polyenes (nystatin and amphoteri cin B), 5-flourocytosine, allylamineestrocarbonates, morpholine, and caspofungin (21). The choice of antifungal drug depends on the type of lesion and the patients' immunological status. The antifungal agents target the cell membrane, cell wall and nucleic acids of *Candida*. HIV/AIDS patients are usually treated topically as well as systemically. Patients with relapsing candidiasis are treated with antifungals that have the least risk of resistance development or selection of resistant strains (22). The widespread use of these antifungal agents has led to an increase in antifungal resistance. Azole drugs especially fluconazole have been implicated greatly in the development of resistant strains (23).

Oral candidiasis in HIV/AIDS patients remains a major cause of morbidity, and negatively affect the quality of life of such persons. Controlling opportunistic *Candida* species associated with oral candidiasis through proper species identification and susceptibility testing of isolates to common antifungal drugs before administration remains the best way of managing infected individuals. The objective of this study therefore, is to determine the prevalence of oral candidiasis among HIV-infected patients and the susceptibility of isolated *Candida* species to the available antifungal agents in a Mission Hospital, southeast Nigeria.

Materials and method:

Study design:

This is hospital-based descriptive cross-sectional study conducted at the Heart-to-Heart clinic in Iyi-Enu mission hospital Ogidi, Anambra State, Nigeria, from December 2022 to February 2023 (3 months). The Heart-to-Heart clinic of the hospital is a clinic that manages HIV/AIDS outpatients and inpatients.

Ethical clearance:

Informed consent of the study participants and the approval of the ethical committee of Iyi-Enu Mission Hospital were obtained.

Study participants:

Included in the study are male and female participants aged ≥ 15 years with positive HIV status, who have not taken any anti-

fungal drug within two weeks prior to enrolment and sample collection.

Sample size and participant selection:

The sample size was estimated using the Leslie Kish formula (24), which gave the calculated minimum number of participants as 150, who were then recruited using consecutive sampling method over the period of study.

Socio-demographic and clinical data collection:

A structured questionnaire was used to collect information on gender, age, marital status, educational level, socio economic status, oral hygiene care and length of HIV infection of the participants.

Specimen collection:

Five milliliter (5mls) of whole blood was drawn from the antecubital vein of each participant into EDTA anti-coagulated bottle for $CD4^+$ estimation. Oral swabs were also collected from each of the participant using two sterile cotton swabs. The specimens were transported in an ice pack to the laboratory of the Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka for analysis.

$CD4^+$ cell estimation:

The $CD4^+$ count was determined using the Partec Cytoflow Counter II (Partec Germany) and correlated with presence or absence of candidiasis. The counts were categorized according to WHO guideline as severe (< 200 cells/µL), low (200-349 cells/µL), moderate (350-499 cells/µL) and high (≥500 cells/µL) (25).

Microscopy and culture of swab samples:

Swab samples were examined microscopically on clean grease free glass slide using 10x and 40x objective lens for presence of pus cells, oval to round clusters of budding yeast cells and branching pseudohyphae (7).

The second cotton swab was aseptically inoculated onto Sabouraud Dextrose Agar (SDA) impregnated with 0.05mg/ml chloramphenicol and incubated aerobically at 35-37^{o}C for 24 hours for observation of colonies (26). Thereafter, discrete colonies were emulsified in 1ml sterile distilled water and a loopful inoculated on CHROMAgar (Biotech, India) medium. This was incubated for 24 hours at 25^{o}C for identification of various species of *Candida* based on color, with light green for *C. albicans*, cream color for *C. glabrata*, pale pink for *C. krusei* and dark blue for *C. tropicalis* (27,28).

Antifungal sensitivity test:

Susceptibility testing was carried out on the *Candida* isolates using the Kirby-Bauer disk diffusion method and interpretation according to the Clinical and Laboratory Standards Institute guidelines (29). The antifungal drugs tested include; fluconazole (25µg), clotrimazole (10µg), ketoconazole (10µg) and nystatin (50 µg) (Rosco diagnostic company, Neosensitabs, Denmark).

Twenty-four hours old inoculum suspension of each *Candida* isolate was prepared and adjusted to the turbidity of 0.5 McFarland standards, and then seeded on sterile Mueller Hinton agar supplemented with 2% glucose and 0.5µg/ml methylene blue. The plate was dried at ambient temperature for 15min, and the discs containing the different antifungal agents were aseptically placed on the agar lawn. The agar plate was incubated for 24 hrs at 35^{o}C. The diameter of zone of inhibition was measured in millimeter. The AST was carried out on each isolate in duplicate and the mean value was used for susceptibility interpretation as sensitive, moderately sensitive and resistant in accordance with CLSI and the manufacturers guidelines.

Statistical analysis:

Data were described in frequency and percentages. The Pearson Chi-square test was used to determine association of oral candidiasis with gender, age, marital status, education level, occupation, oral hygiene and length of HIV infection. P value < 0.05 was considered statistically significant at 95% confidence interval.

Results:

The demographic characteristics of the 150 HIV-infected participants is shown in Table 1. Majority of the participants were females (64.7%). Fifty-two (34.7%) participants are in the age group 21-30 years and 16.0% are ≤ 20 years of age. Most are single (59.3%) while 0.7% are divorced. Seventy-one (47.3%) had secondary level education, 37.3% are traders while 14% are civil servants.

Of the 150 HIV-infected participants, 98 (65.3%) had oral candidiasis, with 4 species of *Candida* identified; *C. albicans* (62.2%), *C. glabrata* (18.4%), *C. tropicalis* (12.2%), and *C. krusei* (7.2%) (Table 2). Fifty-nine (60.2%) of the 98 HIV-infected patients with oral candidiasis had $CD4^+$ count <200 cells/µl, 33 (33.7%)

had their $CD4^+$ cell count in the range 200-349 cells/µl while 6 (6.1%) had theirs in the range 350-499 cells/µl. There was a statistically significant association between $CD4^+$ cell count and oral candidiasis (p=0.001).

The prevalence of oral candidiasis with respect to socio-demographic and clinical data is as presented in Table 3. The prevalence of oral candidiasis of 67.0% (65/97) in the female was higher than 62.3% (33/53) in the male participants, but this rate was not significantly different (p=0.6598). However, the prevalence of oral candidiasis was significantly higher in participants age group 21-30 years (80.7%, 42/52) compared to other age groups (p= 0.008), and also in divorced (100%, 1/1) and married (75%, 45/60) participants (p=0.028), in participants with primary school level education (73.7%, 42/57, p=0.0014), civil servants (85.7%, 18/21, p=0.0482), and in participants who performed mouth hygiene once daily (71.9%, 69/96, p=0.0388). The prevalence of oral candidiasis was not significantly associated with duration of HIV infection when analysed with Chi square test for trend (x^2=0.4882, p= 0.4847) (Table 3).

The antifungal susceptibility pattern showed that *C. albicans* was most susceptible to all the four antifungal drugs while *C. krusei* was the most resistant. Nystatin (77.6%, 76/ 98) was the drug with the highest *invitro* antifungal activity while fluconazole and ketoconazole (68.4%, 62/98) had the lowest *in vitro* antifungal activity (Table 4).

Table 1: Socio-demographic and clinical characteristics of HIV-infected participants in a Mission Hospital, southeast Nigeria

Characteristics	Frequency	Percentage (%)
Gender		
Female	97	64.7
Male	53	35.3
Age group (years)		
<20	24	16.0
21-30	52	34.7
31-40	41	27.3
>40	33	22.0
Marital status		
Single	89	59.3
Married	60	40.0
Divorced	1	0.7
Educational level		
Primary	57	38.0
Secondary	71	47.3
Tertiary	22	14.7
Occupation		
Students	41	28.0
Traders	57	37.3
Civil servants	21	14.0
Self-employed	31	20.7
Oral hygiene practice		
Brush teeth once daily	96	64.0
Brush teeth twice daily	54	36.0
Duration of HIV infection (months)		
≤6	19	12.7
7-24	36	24.0
25-35	44	29.3
≥36	51	34.0

Table 2: Frequency of *Candida* species isolated from HIV-infected participants in a Mission Hospital, southeast Nigeria

Candida species	Frequency	Percentage (%)
Candida albicans	61	62.2
Candida glabrata	18	18.4
Candida tropicalis	12	12.2
Candida krusei	7	7.2
Total	**98**	**100.0**

Table 3: Prevalence of oral candidiasis in HIV-infected participants in a Mission Hospital, southeast Nigeria with respect to socio-demographic and clinical characteristics

Characteristic variables	No tested	No positive (%)	x^2	OR (95% CI)	*p* value
Gender					
Female	97	65 (67.0)	0.1635	1.231 (0.6123-2.475)	0.6598
Male	53	33 (62.3)			
Age group (years)					
≤20	24	12 (50.0)	11.842	NA	0.008*
21-30	52	42 (80.7)			
31-40	41	21 (51.2)			
>40	33	23 (69.7)			
Marital status					
Single	89	52 (58.4)	4.851	NA	0.028*
Married	60	45 (75.0)			
Divorced	1	1 (100.0)			
Educational level					
Primary	57	42 (73.7)	13.091	NA	0.0014*
Secondary	71	49 (69.0)			
Tertiary	22	7 (31.8)			
Occupation					
Students	41	21 (51.2)	7.896	NA	0.0482*
Traders	57	37 (64.9)			
Civil servants	21	18 (85.7)			
Self-employed	31	22 (71.0)			
Oral hygiene practice					
Brush teeth once daily	96	69 (71.9)	4.268	2.203 (1.098-4.418)	0.0388*
Brush teeth twice daily	54	29 (53.7)			
Duration of HIV infection (months)					
≤6	19	7 (36.8)	0.4882^+	NA	0.4847
7-24	36	26 (72.2)			
25-35	44	37 (84.1)			
≥36	51	29 (56.9)			

x^2= Chi square; OR = Odds Ratio; CI = Confidence Interval; * = statistically significant; + = Chi square by trend; NA = Not Applicable

Discussion:

Oral candidiasis continues to pose a major problem in HIV-infected individuals especially in sub-Saharan Africa where about 23 million people live with HIV/AIDS (15). Mahajan et al., (6) reported that more than 90% of HIV patients develop oral candidiasis during the period of infection. It could serve as early sign of HIV related immunodeficiency and is often observed in patients with $CD4^+$ counts less than 200 cells/µl (5,8,9). As observed in this study, HIV participants with $CD4^+$ count < 200 cells/µl had oral candidiasis prevalence of 60.2% while those with $CD4^+$ counts in the range of 350-499 cells/µl had 6.1%, with a significant association (p=0.001) between oral candidiasis and $CD4^+$ count. Our finding agrees with the reports of Ambe et al., (7), Berberi et al., (30), Holiwala et al., (31) and Erfaningejad et al., (32) who in their various studies reported significant associations between $CD4^+$ cell count < 200 cells/µl and high prevalence of oral candidiasis.

A wide range of prevalence of oral candidiasis (11-90%) have been reported among HIV/AIDS patients (7,29,33). The high prevalence of 65.3% reported in our study agrees with prevalence rates of 60.0%, 69.0% and 65.5% reported by some researchers respectively (33,34,35). While higher prevalence of 80% and 79.4% were reported in Cote d'Ivoire (28) and Ghana (36), some other researchers have reported lower prevalence rates of 29-50% (5,7,32,37,38), and prevalence as low as 9.7% have also been reported in Jos, Nigeria (39). These differences in prevalence of oral candidiasis among HIV patients could be as a result of geographic areas sampled, cultural behaviors and the diagnostic methods used.

Candida albicans accounted for 62.2% of the *Candida* species identified in this study, while non-*albicans Candida* such as *C. glabrata* (18.4%), *C. tropicalis* (12.2%) and *C. krusei* (7.2%) accounted for 37.8%. The isolation of *C. albicans* as the most prevalent species may be associated with its many pathogenic factors (32), and its ability to cause infection with low colony count (12). The high prevalence of *C. albicans* in our study agrees with the results of other researchers (7,31,37,40,41) but higher prevalence of ≥ 80% has been reported in South Africa (42), Ghana (28) and Ivory Coast (36).

Table 4: Antifungal susceptibility profile of the oral *Candida* species isolated HIV-infected participants in a Mission Hospital, southeast Nigeria

***Candida* species**	**Susceptibility**	**Antifungal drugs (%)**			
		Fluconazole	**Ketoconazole**	**Nystatin**	**Clotrimazole**
Candida albicans (n=61)	Sensitive	47 (77.0)	47 (77.0)	52 (85.3)	50 (82.0)
	Moderately sensitive	5 (8.2)	4 (6.6)	1 (1.6)	3 (4.9)
	Resistant	9 (14.8)	10 (16.4)	8 (13.1)	8 (13.1)
Candida glabrata (n=18)	Sensitive	6 (33.3)	7 (38.9)	10 (55.6)	8 (44.4)
	Moderately sensitive	1 (5.6)	2 (11.1)	1 (5.6)	2 (11.1)
	Resistant	11 (61.1)	9 (50.0)	7 (38.8)	8 (44.4)
Candida tropicalis (n=12)	Sensitive	6 (50.0)	6 (50.0)	9 (75.0)	7 (58.3)
	Moderately sensitive	2 (16.7)	2 (16.7)	0	2 (16.7)
	Resistant	4 (33.3)	4 (33.3)	3 (25.0)	3 (25.0)
Candida krusei (n=7)	Sensitive	3 (42.9)	2 (28.6)	5 (71.4)	2 (28.6)
	Moderately sensitive	0	0	0	4 (57.1)
	Resistant	4 (57.1)	5 (71.4)	2 (28.6)	1 (14.3)
Total (n=98)	Sensitive	62 (63.3)	62 (63.3)	76 (77.6)	67 (68.4)
	Moderately sensitive	8 (8.2)	8 (8.2)	2 (2.0)	11 (11.2)
	Resistant	28 (28.5)	28 (28.5)	20 (20.4)	20 (20.4)

Candida tropicalis, *C. glabrata* and *C. parapsilosis* have emerged as *Candida* species of health concern over the past few decades and cause disease in 20-40% of immunocompromised persons (7). Their emergence can be attributed to improved identification methods, type of patients' disease and antifungal drugs used (43). In our study, *C. glabrata* (18.4%) was the most frequent non-*albicans Candida* isolated, which agrees with the findings of Erfaninejad et al., (32), Ewumwem et al., (37) and the meta-analysis review done in sub-Saharan Africa over a period of 10 years (44). In contrast, higher prevalence of *C. tropicalis* have been reported in Brazil (45) and China (46). Factors such as sample size, immune status and life style of patients, oral hygiene and high-risk behaviors may contribute to the differences in *Candida* species isolated and their prevalence (40).

As observed in our study, the prevalence of oral candidiasis was higher in female (67.0%) than in male (62.3%) HIV participants but this was not statistically significant (p= 0.6598). Our observation supports the reports of higher prevalence in females by other researchers (34,36,40,46). The lower prevalence in males may be attributed to their apparent low interest in attendance of health clinics for HIV testing and counseling until the disease becomes symptomatic (40). Ignorance of HIV infection, and the mythical belief in southeast Nigeria that young men who prosper in business ventures are usually mystically killed through native poisoning, may have created fear in these men that lead them to not voluntarily test for their HIV status early in the course of HIV infection. Contrarily, Suryana et al., (5) reported significantly higher prevalence of oral candidiasis in HIV infected males.

Oral candidiasis occurred significantly more in HIV-infected patients in age group 21-30 years (80.7%, p=0.008) in our study. This age group represents sexually active youths and it has been suggested that lack of awareness of the risk factors associated with HIV and opportunistic infections may have predisposed them to oral candidiasis (34). However, other

researchers have reported higher prevalence of candidiasis among age group ≥ 30 years (5, 30,37). Analysis of the prevalence of oral candidiasis with respect to level of education showed that the prevalence was significantly higher among HIV participants with primary school level education (73.7%), which may be attributed to apparent low level of personal and oral hygiene in this group of poorly educated people. The prevalence was also significantly higher among civil servants (85.7%) and self-employed (71.0%) than other occupational groups (p=0.0482). The reasons for the higher prevalence of oral candidiasis in these occupational groups of HIV-infected patients is not clear.

Our study also reported significantly higher prevalence of oral candidiasis among HIV patients who perform oral hygiene (tooth brushing) only once a day (71.9%) compared to those who performed it twice a day (29.6%) (p=0.0388). This observation agrees with the study of Wang (7), who also reported high prevalence of candidiasis among patients who brushed once daily, implying that good oral hygiene will be an important means of managing oral candidiasis. Oral candidiasis can be acquired at any stage of HIV infection and could serve as early sign of HIV related immunodeficiency (5,8). Although, the prevalence of oral candidiasis was higher among participants who have had HIV infection for a period of 25-35 months (84.1%) and 7-24 months (72.2%), there was no significant difference on Chi square test for trend analysis (x^2=0.4882, p= 0.4747) between length of infection of HIV infection and oral candidiasis, indicating that length of time of HIV infection is not a factor in acquiring oral candidiasis.

The widespread use of antifungal drugs has increased the rate of antifungal resistance by *Candida* species which poses a major challenge in the treatment and management of oral candidiasis (47). The antifungal susceptibility pattern carried out in our study showed that *C. albicans* was the most susceptible to all the antifungal drugs tested while *C. krusei* was the most resistant. This result agrees with study of Wang (7), who also reported *C. albicans* as the most sensitive and *C. krusei* and *C. glabrata* as most resistant to seven antifungal drugs tested in his study. *Candida glabrata* and *C. krusei* have been reported to be less susceptible to antifungal drugs and infections caused by them tend to be more difficult to treat (47). Of the four antifungal agents tested in our study, nystatin (77.6%) was the most active *in vitro* while fluconazole (63.3%) and ketoconazole (63.3%) were the least active. This observation agrees with the results of Wang (7) and Hodiwala et al., (31), who in their separate studies reported nystatin as the most active antifungal agent against oral *Candida* isolates. In contrast, a research study in Cameroon (48) reported ketoconazole (85.5%) as the most active and nystatin (68.1%) as the least active of the ten antifungal drugs tested. In a systematic review and meta-analysis of comparative efficacy of antifungal agents used in the treatment of oral candidiasis among HIV-infected adults in 2021 (49), fluconazole was ranked the most effective antifungal against oral *Candida.*

The emergence of antifungal resistant *C. albicans* and non-*albicans Candida* is of serious concern to healthcare workers all over the world (46). In our study, the resistance rate of *Candida* species to fluconazole and keto -conazole is 28.6% which agrees with 24.6% and 24.0% resistance rates to fluconazole reported by Wang (7) and Osaigbovo et al., (47) respectively. Resistance rate as high as 90.0% have also been reported to flucona- zole by *C. albicans* (32). Fluconazole is usually the first line treatment agent of choice because of its favorable pharmacokinetic profile, low toxicity, availability, minimal drug interaction and minimal adverse effects (50), but *Candida* strains resistant to fluconazole have recently emerged (41). The antifungal drugs tested in our study can be obtained over-the-counter in Nigeria, a practice that has encouraged self medication and may have contributed to the resistance of *Candida* species observed in our study.

Conclusion:

Oral candidiasis was highly prevalent among HIV-infected participants in this study. Age group, marital status, educational level, occupation, and oral hygiene are significant factors associated with oral candidiasis in por HIV-infected participants. Nystatin was the most active *in vitro* antifungal drug tested, but emergence of non-*albicans Candida* is also of great concern. Proper identification of *Candida* species and susceptibility testing to antifungal drugs before administration will be helpful in managing oral candidiasis among HIV-infected patients.

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Contributions of authors:

CCE conceived and designed the study, and wrote the manuscript, CJN collected and processed the samples, CGA, NVA and ANM carried out the literature search and critical review of the manuscript, CMO perform statistical analysis of the data and reviewed the manuscript. All authors read and approved the final manuscript.

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Short Communication **Open Access**

Antibiotic susceptibility profiles of Gram-negative bacterial uropathogens in a tertiary hospital, southwest Nigeria

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Abstract:

Background: Increasing rates of antibiotic resistance have made it necessary to regularly monitor antibiotic susceptibility patterns of gram negative bacterial uropathogens in order to optimize antibiotic therapy for urinary tract infections. The aim of this study was to analyze the antibiotic susceptibility patterns of Gram-negative bacterial uropathogens in Babcock University Teaching Hospital, Ilishan-Remo, southwest Nigeria.
Methodology: This study was a retrospective review of the Medical Microbiology Laboratory records of the hospital to analyze the *in vitro* antibiotic susceptibility patterns of Gram-negative urinary bacterial isolates between May 2016 and April 2022. The bacteria were isolated and identified from routine urine samples using standard bacteriological methods. *In vitro* antibiotic susceptibility test (AST) to amoxicillin-clavulanate, piperacillin-tazobactam, ceftriaxone, ceftazidime, nitrofurantoin, ciprofloxacin and meropenem was routinely performed by the modified Kirby-Bauer disk diffusion test and susceptibility break points determined using the Clinical and Laboratory Standards Institute (CLSI) guidelines.
Results: A total number of 3,549 urine samples were processed during the period of review, and 808 (22.8%) samples yielded positive bacterial cultures. Of the 808 isolates, 604 (74.8%) were Gram-negative bacteria. The most frequently isolated Gram-negative bacteria were *Escherichia coli* (41.9 %) and *Klebsiella* spp (27.5%) while *Pseudomonas* spp and *Proteus* spp accounted for 4.3% and 1.0% of all isolates respectively. Meropenem had the highest *in vitro* antibacterial activity (74.3% to 90.3% of isolates were sensitive) for all isolates. Overall, *E. coli, Klebsiella* spp., and *Proteus* spp. showed high resistance rates to amoxicillin-clavulanate (65.3% to 97.1%).
Conclusion: Effective antimicrobial stewardship programs must be in place in order to ensure the appropriate use of antibiotics for treating urinary tract infections.

Keywords: urinary tract infections; uropathogens; antibiotic resistance; *Escherichia coli*

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Profils de sensibilité aux antibiotiques des uropathogènes bactériens à Gram négatif dans un hôpital tertiaire du sud-ouest du Nigeria

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Résumé:

Contexte: L'augmentation des taux de résistance aux antibiotiques a rendu nécessaire la surveillance régulière des schémas de sensibilité aux antibiotiques des uropathogènes bactériens à Gram négatif afin d'optimiser l'antibiothérapie des infections des voies urinaires. Le but de cette étude était d'analyser les profils de sensibilité aux antibiotiques des uropathogènes bactériens à Gram négatif à l'hôpital universitaire de Babcock, à Ilishan-Remo, dans le sud-ouest du Nigeria.
Méthodologie: Cette étude était un examen rétrospectif des dossiers du laboratoire de microbiologie médicale de l'hôpital pour analyser les schémas de sensibilité aux antibiotiques in vitro des isolats de bactéries urinaires à Gram négatif entre mai 2016 et avril 2022. Les bactéries ont été isolées et identifiées à partir d'échantillons d'urine de routine à l'aide de méthodes bactériologiques classiques. Un test de sensibilité antibiotique in vitro (AST) à l'amoxicilline-acide clavulanique, à la pipéracilline-tazobactam, à la ceftriaxone, à la ceftazidime, à la nitrofurantoïne, à la ciprofloxacine et au méropénème a été systématiquement effectué par le test de diffusion sur disque de Kirby-Bauer modifié et les seuils de sensibilité ont été déterminés en utilisant les directives de l'Institut des Normes Cliniques et de Laboratoire (CLSI).
Résultats: Au total, 3,549 échantillons d'urine ont été traités au cours de la période d'examen, et 808 échantillons (22,8%) ont produit des cultures bactériennes positives. Sur les 808 isolats, 604 (74,8%) étaient des bactéries Gram-négatives. Les bactéries Gram-négatives les plus fréquemment isolées étaient *Escherichia coli* (41,9%) et *Klebsiella* spp (27,5%) tandis que *Pseudomonas* spp et *Proteus* spp représentaient respectivement 4,3% et 1,0% de tous les isolats. Le méropénem avait l'activité antibactérienne *in vitro* la plus élevée (74,3% à 90,3% des isolats étaient sensibles) pour tous les isolats. Dans l'ensemble, *E. coli*, *Klebsiella* spp et *Proteus* spp ont montré des taux de résistance élevés à l'amoxicilline-acide clavulanique (65,3% à 97,1%).
Conclusion: Des programmes efficaces de gestion des antimicrobiens doivent être en place afin d'assurer l'utilisation appropriée des antibiotiques pour le traitement des infections des voies urinaires.

Mots clés: infections des voies urinaires; uropathogènes; résistance aux antibiotiques; *Escherichia coli*

Introduction:

Urinary tract infections (UTIs) are common infections in both the community and hospital setting (1). In 1997, UTIs accounted for about 7 million visits to outpatient clinics, 1 million visits to emergency departments, and 100,000 hospitalizations annually in the United States of America (2). About 35% of healthcare associated infections are UTIs and UTIs are the second most common cause of bacteremia in hospitalized patients (3,4). UTIs also exert adverse economic impacts on patients (5), for example, the annual cost to the health care system of the United States attributable to community-acquired UTI alone is estimated to be approximately $1.6 billion (6).

Majority of UTIs are caused by bacteria (7). Amongst bacteria Gram-negative bacteria are the most prevalent uropathogens (7), and, of these, *Escherichia coli* accounts for 70% to 90% of cases (8). Also, the management of UTIs, particularly uncomplicated UTIs, has involved the use of antibiotics such as amoxicillin-clavulanate, nitrofurantoin, cephalosporins, fluoroquinolones, trimethoprim-sulfamethoxazole and others. (9). However, increasing rates of antibiotic resistance are making many of these antibiotics ineffective resulting in considerable morbidity, mortality and increased healthcare costs (10). In addition, the rates of antibiotic resistance are higher in low-and-middle-income countries (LMICs) with high levels of inappropriate antibiotic use (11).

Studies conducted in Nigeria have shown high rates of antibiotic resistance among Gram-negative uropathogens (12,13) and this is associated with higher morbidity and mortality (14). The problem posed by antibiotic resistance is further worsened by the paucity of research and development in new antibiotics and lack of access to effective antibiotics in Africa and other developing parts of the world (15).

It is therefore imperative to ensure regular institutional and national surveillance of antibiotic susceptibility patterns of common bacterial uropathogens, to improve clinical decision making and optimize antibiotic use (16). The objective of this study therefore was to analyze the bacterial and antibiotic susceptibility profiles of Gram-negative bacterial uropathogens in Babcock University Teaching Hospital, with the aim of providing data that will guide empiric antibiotic therapy of UTIs in the hospital.

Materials and method:

Study setting:

The study was conducted in the department of medical microbiology and parasitology, Babcock University Teaching Hospital, a 240-bed tertiary centre located in Ilishan-Remo, Ikenne Local Government, southwest Nigeria. The hospital is dedicated to teaching, research and specialist services and serves Ogun State and neighboring States in southwest Nigeria.

Study design:

This was a retrospective study that involved a review of the medical microbiology laboratory records to analyze the antimicrobial susceptibility profiles of Gram-negative bacterial urinary isolates obtained between May,

2016 and April, 2022.

Isolation and antibiotic susceptibility pattern of bacterial isolates:

Routine processing of urinary samples in the laboratory during the period of the review involved macroscopic and microscopic examination. Subsequently urinary samples were inoculated into Cystine Lactose Electrolyte Deficient (CLED) and Blood agar plates and incubated aerobically at 35-37^{o}C for 18-24 hours.

Isolates were identified by conventional biochemical tests and antimicrobial susceptibility testing (AST) was performed using the modified Kirby-Bauer disk diffusion method. The susceptibility break points were determined using the Clinical and Laboratory Standards Institute (CLSI) guidelines (17).

Data analysis

Data analysis was done using IBM SPSS software version 20.0 Descriptive statistics were used to analyze isolates based on frequency and AST patterns.

Ethical considerations:

Ethical approval for the study was obtained from Babcock University Health Research and Ethics Committee (Number: BUHREC 679/21). As data were retrospectively obtained from the laboratory records and did not involve contact with patients nor recruitment of patients, informed consent was not deemed necessary. However, privacy and confidentiality of patients' data were protected in accordance with the Declaration of Helsinki.

Results:

In the 6-year period under consideration, a total number of 3,549 urine samples were processed in the medical microbiology laboratory and 808 (22.8%) samples yielded positive cultures. Of the 808 isolates, 604 (74.8%) were Gram-negative bacteria (Table 1). The most frequently isolated Gram-negative bacteria were *Escherichia coli* (56.1 %) and *Klebsiella* spp (36.8%) while *Pseudomonas* spp and *Proteus* spp accounted for 5.8% and 1.3% of isolates respectively (Fig 1).

Most (90.3%) of the *E. coli* isolates were sensitive to meropenem (Table 2). In addition, *E. coli* isolates also showed sensitivity of 61.1% and 58.4% to ciprofloxacin and nitrofurantoin respectively (Table 2). The *E. coli* isolates however exhibited resistance to amoxicillin-clavulanate (69.6%), ceftriaxone (49.9%), piperacillin-tazobactam (46.9%), ceftazidime (44.5%) and ciprofloxacin (31. 6%) (Table 2).

Furthermore, the *Klebsiella* isolates exhibited sensitivity of 82.9% to meropenem but resistance to amoxicillin-clavulanate (65.3%), piperacillin-tazobactam (55.0%), ciprofloxacin (41.9%), ceftazidime (40.5%) and nitrofurantoin (40.5%) (Table 2). However, *Pseudomonas spp* showed sensitivity of 74.3% and 57.1% to meropenem and piperacillin-tazobactam respectively but showed resistance to piperacillin-tazobactam (42.9%), ciprofloxacin (42.9%) and ceftazidime (40%), (Table 2). Also, *Proteus* spp exhibited resistance to ceftriaxone (87.5%), amoxicillin-clavulanate (75.0%) and ceftazidime (62.5%) but sensitivity of 87.5% to meropenem and 50.0% to ciprofloxacin (Table 2).

Table 1: Frequency of bacteria uropathogens in Babcock University Teaching Hospital, Ilishan Remo, Nigeria

Microbial isolate	Frequency	Percentage
Gram negative		
Escherichia coli	339	41.9
Klebsiella spp	222	27.5
Pseudomonas spp	35	4.3
Proteus spp	8	1.0
Gram positive	124	15.3
Fungi (*Candida* spp)	80	10.0
Total	808	100

Discussion:

Gram-negative isolates were the most frequently (74.8%) isolated bacteria in this retrospective study. These findings are similar to previous studies done in India (18) in which Gram-negative isolates accounted for 90.32% of isolates. The most frequently isolated Gram -negative isolate was *E. coli* (56.1%). Again, this finding is similar to other studies done in Bangladesh (19), Chad (20), Ethiopia (21) and India (22) in which *E. coli* was the most frequently isolated Gram-negative bacteria from urinary tract infections.

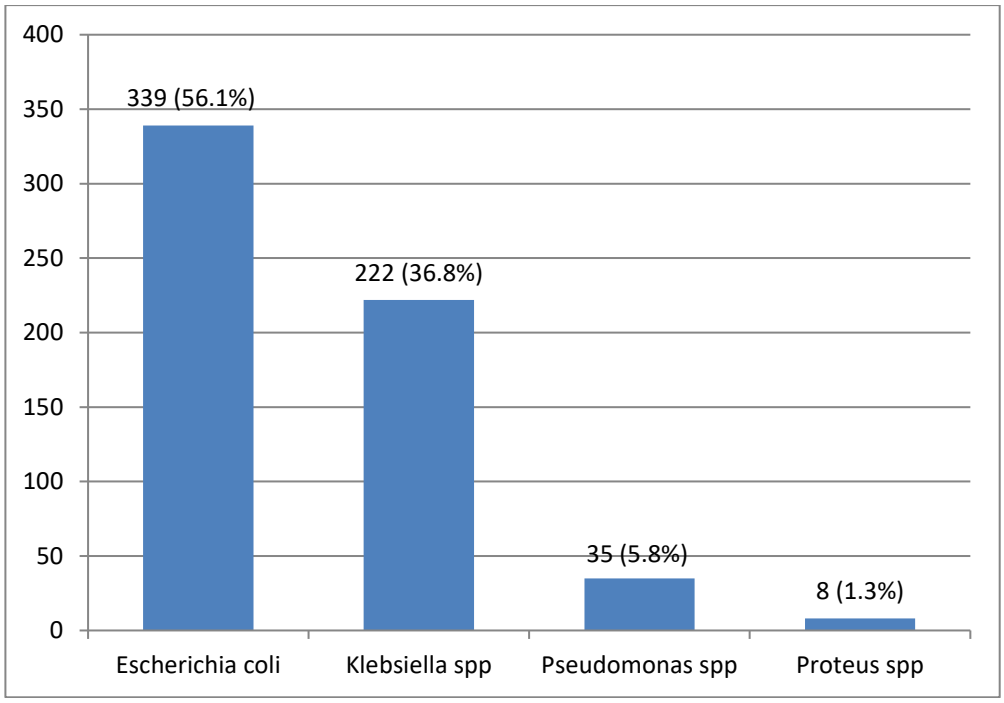

Fig 1: Frequency of Gram-negative bacterial uropathogens in Babcock University Teaching Hospital, Ilishan Remo, Nigeria

Table 2: Antibiotic susceptibility patterns of Gram-negative bacterial uropathogens in Babcock University Teaching Hospital, Ilishan Remo, Nigeria

Antibiotics/isolates/ Susceptibility breakpoint	***Escherichia coli* (%) (n=339)**			***Klebsiella* spp (%) (n=222)**			***Pseudomonas* spp (%) (n=35)**			***Proteus* spp (%) (n=8)**		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Amoxicillin-clavulanate	63 (18.6)	41 (12.1)	235 (69.3)	55 (24.8)	22 (9.9)	145 (65.3)	16 (45.7)	5 (14.3)	14 (40.0)	1 (12.5)	1 (12.5)	6 (75.0)
Piperacillin-tazobactam	161 (47.5)	20 (5.9)	158 (46.6)	80 (36.0)	20 (9.0)	122 (55.0)	20 (57.1)	0	15 (42.9)	3 (37.5)	2 (25.0)	3 (37.5)
Ceftriaxone	112 (33.0)	59 (17.4)	168 (49.6)	NT	NT	NT	NT	NT	NT	1 (12.5)	0	7 (87.5)
Ceftazidime	157 (46.3)	32 (9.4)	150 (44.3)	106 (47.8)	26 (11.7)	90 (40.5)	16 (45.7)	5 (14.3)	14 (40.0)	2 (25.0)	1 (12.5)	5 (62.5)
Nitrofurantoin	199 (58.7)	10 (2.9)	130 (38.4)	111 (50.0)	21 (9.5)	90 (40.5)	20 (57.1)	0	15 (42.9)	NT	NT	NT
Ciprofloxacin	208 (61.4)	25 (7.4)	106 (31.2)	100 (45.0)	29 (13.1)	93 (41.9)	18 (51.4)	2 (5.7)	15 (42.9)	4 (50.0)	1 (12.5)	3 (37.5)
Meropenem	307 (90.5)	7 (2.1)	25 (7.4)	184 (82.9)	7 (3.1)	31 (14.0)	26 (74.3)	2 (5.7)	7 (20.0)	7 (87.5)	0	1 (12.5)

S-sensitive; I = intermediate; R = resistant; NT = not tested

Our study showed that meropenem had the highest *in vitro* antibacterial activity (isolates sensitivity of 74.3% to 90.3%) for all isolates. This is similar to a study done in Somaliland in which 95.9% of urinary tract isolates were sensitive to meropenem (23). In another study from Japan, 100% of the Gram-negative urinary tract isolates were sensitive to meropenem (24). The highest sensitivity to meropenem was shown by *E. coli* (90.3%) while the lowest sensitivity was shown by *Pseudomonas* spp (74.3%). Similarly in a study from the USA, *E. coli* isolates from the urinary tract showed a sensitivity of ≥ 99.4% to meropenem (25). Overall, *E. coli, Klebsiella* spp and *Proteus* spp isolates showed high resistance to amoxicillin-clavulanate (65.3% to 97.1%). However, in a study from the Czech Republic, lower resistance rates to amoxicillin-clavulanate of 12.6%, 14.3% and 38.8% were respectively reported for *Proteus* spp, *E. coli* and *Klebsiella* spp (26).

The high sensitivity to meropenem should not encourage its use as a first line antibiotic to treat UTIs. This is because meropenem is an antibiotic on the Watch Group of the AWaRe antibiotic categorization by the World Health Organization (WHO) for purpose

of antimicrobial stewardship (27). Antibiotics in this group have higher resistance potential and should be prioritized as key targets of anti -biotic stewardship programs and monitoring (27). Since meropenem is intravenously adm-inistered, this makes it a wrong choice for out-patients with uncomplicated UTIs. Therefore, the use of meropenem as a first line empiric antibiotic agent for treating UTIs should be discouraged in order to avoid the emergence of resistance.

However, the urinary isolates in this study have shown high resistance to most of the antibiotics that can be administered orally such as amoxicillin-clavulanate, nitrofurantoin and ciprofloxacin. It is therefore expedient for the hospital's antimicrobial stewardship (AMS) program to regularly monitor local antibiotic susceptibility patterns of urinary isolates and also draw up guidelines for empiric antibiotic therapy for UTIs. Furthermore, strict infection prevention and control (IPC) protocols should be adhered to, in order to prevent the spread of multidrug resistant pathogens in the hos-pital.

The limitations of the study included the fact that it was laboratory based and did not involve review of the medical records of patients, therefore, correlation of our *in vitro* findings with clinical UTIs was not possible.

Conclusion:

Rising rates of antibiotic resistance have made it necessary to regularly monitor anti-biotic susceptibility patterns of Gram-negative bacterial uropathogens. This will help to opti-mize antibiotic therapy for UTIs. In addition, strict IPC protocols must be in place to prevent the spread of multidrug resistant pathogens in the hospital.

Conflict of interests:

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in wri-ting this article.

Contributions of authors:

IIO conceptualized and designed the study, and produced the manuscript draft; IIO and EE analyzed the data; EE and HNO contributed to drafting of the manuscript; CJE and TOO reviewed the manuscript. All authors approved the manuscript for submission.

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Data availability:

The data that support the findings of this study are openly available in Mendeley Data at https://data.mendeley.com/datasets/nd4w9wnmzc/1

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Case Report **Open Access**

Isolation of *Globicatella sanguinis* from a neonate with sepsis using BacT/Alert and VITEK-2 compact system at Federal Teaching Hospital, Katsina, northwest Nigeria: A case report

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Abstract:

Neonatal sepsis is a significant cause of neonatal morbidity and mortality, predominantly in developing countries. The bacterial causes of neonatal sepsis and their antimicrobial susceptibility patterns are however dynamic. *Globicatella sanguinis* is a streptococcus-like bacterial agent capable of causing serious infection in humans that has been rarely isolated from clinical samples, and is an uncommon pathogen that is difficult to identify. Identification based on phenotypic methods alone can misidentify many bacteria, and this may affect precise antibiotic treatment. We report the isolation of a rare bacterial pathogen, *G. sanguinis* from a three-hour-old preterm female neonate (28 weeker, extremely low birth weight) with sepsis and respiratory distress syndrome (RDS) at Federal Teaching Hospital Katsina, Nigeria, and to the best of our knowledge, one of the very few reported cases all over the world. Blood sample was aseptically collected from the neonate and cultured on BacT/Alert automated system (BioMérieux, Mercy-Etoile, France). A rare bacterium was identified from a positive culture, and *in vitro* susceptibility test using VITEK-2 compact system showed the isolate to be sensitive to gentamicin, cefuroxime, ceftriaxone, and ceftazidime. Despite antibiotic treatment and other standards of care, on day 9 of admission, the baby developed apnea and all resuscitative measures proved abortive. In a developing country like Nigeria where child mortality due to infection is high, the inclusion of advanced technologies such as improved VITEK-2 compact system, PCR, MALDI-TOF MS, and next-generation sequencing, could play a significant role in its reduction.

Keywords: *Globicatella sanguinis*; neonate; sepsis; respiratory distress syndrome

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Isolement de *Globicatella sanguinis* d'un nouveau-né atteint de septicémie à l'aide du système compact BacT/Alert et VITEK-2 au Federal Teaching Hospital de Katsina, au nord-ouest du Nigéria: à propos d'un cas

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Résumé :

La septicémie néonatale est une cause importante de morbidité et de mortalité néonatales, principalement dans les pays en développement. Les causes bactériennes du sepsis néonatal et leurs profils de sensibilité aux antimicrobiens sont cependant dynamiques. *Globicatella sanguinis* est un agent bactérien de type streptocoque capable de provoquer une infection grave chez l'homme qui a rarement été isolé à partir d'échantillons cliniques, et est un agent pathogène

rare et difficile à identifier. L'identification basée sur des méthodes phénotypiques seules peut mal identifier de nombreuses bactéries, ce qui peut affecter un traitement antibiotique précis. Nous rapportons l'isolement d'un agent pathogène bactérien rare, *G. sanguinis* chez un nouveau-né prématuré de trois heures (28 semaines, poids de naissance extrêmement faible) atteint de septicémie et de syndrome de détresse respiratoire (SDR) à l'hôpital fédéral d'enseignement de Katsina, au Nigéria, et à notre connaissance, l'un des très rares cas signalés dans le monde. L'échantillon de sang a été prélevé de manière aseptique sur le nouveau-né et cultivé sur le système automatisé BacT/Alert (BioMérieux, Mercy-Etoile, France). Une bactérie rare a été identifiée à partir d'une culture positive, et un test de sensibilité *in vitro* utilisant le système compact VITEK-2 a montré que l'isolat était sensible à la gentamicine, au céfuroxime, à la ceftriaxone et à la ceftazidime. Malgré un traitement antibiotique et d'autres normes de soins, au jour 9 de l'admission, le bébé a développé une apnée et toutes les mesures de réanimation se sont avérées avortées. Dans un pays en développement comme le Nigeria où la mortalité infantile due à l'infection est élevée, l'inclusion de technologies de pointe telles que le système compact VITEK-2 amélioré, la PCR, la SM MALDI-TOF et le séquençage de nouvelle génération pourrait jouer un rôle important dans sa réduction.

Mots-clés: *Globicatella sanguinis*; nouveau-né; état septique; syndrome de détresse respiratoire

Introduction:

Neonatal sepsis is a substantial cause of neonatal morbidity and mortality, particularly in developing countries. The microbial agents implicated in neonatal sepsis and their antimicrobial susceptibility patterns are however dynamic (1). Neonatal sepsis is a clinical condition comprising of non-specific symptoms and signs of infection, accompanied by bacteremia in the first 28 days of life. It is a systemic infection occurring in the neonatal period (2). Bacterial infection is mostly implicated in about three-quarters of the million neonatal mortality each year, all over the world. (3), this is because the neonatal period is the most vulnerable period of life due to susceptibility to infectious agents, as neonates are deficient in both humoral and cell-mediated immunity. They also produce immunoglobulins at a much lower proportion when compared to older children and adults (4).

Neonatal sepsis can be categorized as early-onset sepsis (between 0 to 3 days of life) or late-onset sepsis (from day 4 or later). Early-onset sepsis is commonly connected with the acquisition of microbes from the mother, and onset is mostly immediate in premature babies. Infection can arise through hematogenous, transplacental spread from an infected mother or more commonly, through ascending infection from the vaginal. The baby may acquire organisms that colonize the genitourinary tract of the mother as it passes through the birth canal during delivery. The common bacterial causes of early-onset sepsis include group B streptococcus (GBS), *Escherichia coli*, coagulase negative staphylococcus, *Haemophilus influenzae*, and *Listeria monocytogenes* (5). Early-onset sepsis is about 10 to 20 times more likely in premature and very low birth weight babies (6).

Late-onset sepsis occurs after 4 days of life and it is characteristically acquired from the environment. Bacteria that have been implicated include coagulase-negative staphylococcus, *Staphylococcus aureus, Escherichia coli, Klebsiella, Pseudomonas, Enterobacter,* group B streptococcus, *Serratia*, *Acinetobacter,* and anaerobes. The skin, respiratory tract, conjunctivae, gastrointestinal tract, and umbilicus of neonate might become colonized through interactions with the environment or caregivers (5).

Empirical antibiotic treatment initiated to lessen the deadly outcomes of sepsis in neonates is based on the knowledge of common pathogens encountered in a given locality, as well as their sensitivity patterns (3). In developed countries, the most common organisms causing neonatal sepsis are Group B streptococci, *E. coli,* and *L. monocytogenes* while Gram-negative bacteria and coagulase-negative staphylococci are the most implicated in developing countries (7). But in recent years the emergence of rare bacterial pathogens is being witnessed (8,9) which may or may not be covered by empirical antibiotic treatment.

The 'gold standard' for the diagnosis of sepsis in neonates is a positive culture from a disinfected site, including blood, cerebrospinal fluid (CSF), or urine (10). Here we report the isolation of a rare bacterial pathogen, *Globicatella sanguinis,* a streptococcus-like agent from a three-hour-old preterm neonate with sepsis, and to the best of our knowledge one of the few reported cases so far, in Nigeria.

Case report:

A 3-hour-old female preterm neonate was referred to Federal Teaching Hospital (FTH), Katsina, on account of prematurity, difficulty in breathing, and inability to feed. The neonate was born at home to a 20-year-old primigravida by spontaneous vaginal delivery at about 28 weeks of gestation, with a birth weight of 750 grams. Pregnancy was unbooked and the duration of labor was about seven hours with spontaneous premature rupture of membrane (PROM) for about half an hour prior to labor.

On examination, the neonate was pink in room air, lethargic, afebrile, anicteric, acya-

nosed, not dehydrated, and had no pedal edema. Respiratory rate was 48cpm with oxygen saturation (SPO_2) of 67% on continuous positive airway pressure (CPAP). Temperature was 33.2^{o}C (hypothermia) and PCV was 48%. An assessment of preterm extremely-low-birth-weight (ELBW) 28-weeker with suspected sepsis was made based on clinical features of lethargy, inability to feed, respiratory distress, and hypothermia.

Approximately 2 milliliters of blood were aseptically collected from the peripheral vein of the neonate into an appropriately labeled Bact/Alert paediatric bottle, which was then moved into a closed flask and instantly transported to the laboratory for analysis. Baby was placed on empirical antimicrobial combinations of gentamicin and cefuroxime, as well as other supportive management, in line with the hospital neonatal unit protocol, pending the outcome of the blood culture.

Bacterial detection with BacT/Alert blood culture system:

Inoculated bottles were placed in BacT/Alert microbial detection system, which utilizes a colorimetric sensor and reflected light to detect the presence of carbon dioxide (CO_2) produced when bacteria (if present in the blood sample) grow and metabolize the substrates in the culture medium, with the color of the gas-permeable sensor installed in the bottom of each culture bottle changing to yellow (11).

The bottles were incubated aerobically and continuously observed for the presence of microbial growth for 5 days. Culture-positive bottle was removed for bacterial identification by Gram stain smears and sub-cultures on Blood and MacConkey agar plates. The procedures for loading and unloading the culture bottles into the BacT/Alert machine were done in line with the manufacturers' user-instruction manual.

Bacterial identification and antibiotic susceptibility test using VITEK-2 compact system:

The bacteria inoculum was prepared from pure colonies of the isolates on the agar plate and the turbidity adjusted to 0.5 McFarland standard using the Vitek-2 Densi-Check instrument. Bacterial identification with Vitek-2 compact system was performed according to manufacturer's instructions using the 64-well card. The computerized Vitek-2 compact system (30-card capacity) uses a fluorogenic technique for microbial identification and a turbidimetric technique for susceptibility testing to generate minimum inhibitory concentration (MIC) data with a 64-well card, barcoded with data on card type, expiration date, lot number, and unique card identification number (12,13).

The bacterial culture suspension was inoculated into the appropriate card using an integrated vacuum device inside the filling chamber within 30 minutes of inoculum preparation. A test tube holding the suspension was placed in a distinct rack (cassette) and the identification card was placed in the adjacent slot while implanting the transfer tube into the corresponding suspension tube. The cards were later transferred into the loading chamber where they were sealed and incubated in a rotating carousel at 37^{o}C. Upon loading the cassette, the machine handles all successive steps for inoculation and interpretation with regards to identification and susceptibility test, and the AST results were available for bacteria isolate in less than 18 hours. Eight pre-installed antibiotics (gentamicin, ciprofloxacin, cefuroxime, ceftriaxone, ceftazidime, augmentin, meropenem, and vancomycin) were tested against the isolate.

Globicatella sanguinis was identified by the system, sensitive to gentamicin, cefuroxime, ceftriaxone and ceftazidime. Despite antibiotic treatment and other standards of care given, on day 9 of admission, the baby developed apnea and all resuscitative measures proved abortive. For religious and cultural reasons, autopsy was not performed.

Discussion:

Globicatella sanguinis is an uncommon pathogenic Gram-positive coccus that has been occasionally reported as an unusual cause of infections of the bloodstream, central nervous system, endocardium, and urinary tract in man. The other member of the genus, *G. sulfidifaciens*, is not a known pathogen in humans (9). The clinical and epidemiological implications of *G. sanguinis* remain to be determined, even though published data suggest its opportunistic nature and the ability to cause serious infections. (8). The case in this study is one of the very few reported cases of isolation of *G. sanguinis* from a neonate in Nigeria and all over the world (8,14,15,16). Its clinical implication in humans was documented on some rare occasions where it was isolated from urine, blood, and cerebrospinal fluid in cases of urinary tract infections, bacteremia, and meningitis respectively (17,18).

Globicatella sanguinis is a facultatively anaerobic, alpha-haemolytic, and catalase negative bacterium. Its identification and characterization with conventional laboratory tests are problematic, as there exists various descriptions of biochemical reactions for the same species (19). The colony morphology of the strains

makes it difficult to distinguish from aerococci, viridans streptococci, and enterococci. Although ability to grow in 6.5% NaCl and failure to grow at 10°C helps to differentiate *Globicatella* from viridans streptococci and enterococci respectively, it is hard to distinguish it from *Aerococcus* (20). Standardized systems such as Rapid ID 32 Strep or API 20 Strep were recognized to identify alpha-hemolytic streptococci (21) but these are not readily available in our setting.

In view of the growing emergence of multi-drug-resistant bacteria, the rational use of antibiotics based on accurate and reliable laboratory results becomes an absolute necessity. This can only be achievable through accurate identification of pathogens as well as treatment based on sensitivity patterns. There is limited knowledge on antibiotic susceptibility patterns as far as this rare bacterium is concerned, all over the world, however, the *in-vitro* antibiotic susceptibility based on VITEK-2 compact system in this case report shows that the bacterium is sensitive to gentamicin, cefuroxime, ceftriaxone and ceftazidime. The neonate was placed on empirical combination of gentamicin and cefuroxime, as well as other supportive management, and may have died from complications of RDS. RDS is significantly associated with poor outcomes of neonatal sepsis (22) .

Although more evaluation of the presence and nature of *G. sanguinis* in uninfected persons is needed, this bacterium may be part of the lower gastrointestinal or urogenital microbiome of the mother, with the potential to cause disease in vulnerable hosts. Its low prevalence may be partly due to the difficulty in laboratory identification and characterization by phenotypic methods, hence the recognition of this pathogen will be enhanced as advanced technologies such as improved VITEK-2 compact system, MALDI-TOF MS, and next-generation sequencing techniques emerge into common use.

Conclusion:

In this report, we highlight the fact that identification solely based on phenotypic methods can misidentify many bacteria, and this may affect precise antibiotic treatment. Due to problems with identification and characterization, and the fact that there is a small number of reported cases, the pathogenic significance of *G. sanguinis* is still only partly known. The bacterium is hardly encountered in medical laboratories, hence technicians, microbiologists as well as clinicians are not adequately familiar with the phenotypic characteristics and identification peculiarities. To this end, the pathogen may be unnoticed when isolated or simply reported as unidentified streptococcus-like isolate.

In developing countries where child mortality due to infection is high, inclusion of advanced technologies such as improved VITEK-2 compact system, PCR, MALDI-TOF MS, and next -generation sequencing, could play a significant role in its reduction.

Contributions of authors:

OHK, SMB, YSA, SA, and ABT conceived and developed the idea of the case report. OHK performed the laboratory work. All authors discussed the results and contributed to the final manuscript.

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Case Report **Open Access**

Isolated axillary tuberculous lymphadenitis in a Nigerian female: A case report with review of the literature

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Abstract:

Tuberculosis (TB) is a chronic granulomatous infectious pulmonary and systemic disease caused mostly by members of the *Mycobacterium tuberculosis* complex (MTBC). It has variable clinical presentation and is a major cause of morbidity and mortality in the middle-and-low-income-countries (LMICs). Isolated axillary tuberculous lymphadenitis (ATL) is rare and is defined as the presence of axillary tuberculous lymphadenitis in the absence of previous or active pulmonary TB or evidence of extrapulmonary TB elsewhere. We present a case of isolated ATL in a 54-year-old HIV-negative Nigerian woman, whose diagnosis was made using histological evaluation that demonstrated typical Langhan's giant cells and caseous necrosis, with the detection of mycobacterial DNA by GeneXpert TB test. Isolated ATL is a diagnostic enigma but should be considered in young and middle-aged women in TB endemic regions presenting with enlarged axillary lymph nodes in the absence of foci of infections or malignancy. Sex difference in immunological response to infection may account for this unique presentation among the female gender.

Keywords: isolated axillary tuberculosis; *Mycobacterium*; histopathology; case report

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Lymphadénite tuberculeuse axillaire isolée chez une femme Nigériane: à propos d'un cas avec revue de la littérature

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Résumé:

La tuberculose (TB) est une maladie pulmonaire et systémique infectieuse granulomateuse chronique causée principalement par des membres du complexe *Mycobacterium tuberculosis* (MTBC). Il a une présentation clinique variable et est une cause majeure de morbidité et de mortalité dans les pays à revenu intermédiaire et faible (PRFI). La lymphadénite tuberculeuse axillaire (LTA) isolée est rare et se définit comme la présence d'une lymphadénite tuberculeuse axillaire en l'absence de tuberculose pulmonaire antérieure ou active ou de signes de tuberculose extrapulmonaire ailleurs. Nous présentons un cas d'ATL isolé chez une femme nigériane de 54 ans, séronégative, dont le diagnostic a été posé à l'aide d'une évaluation histologique qui a démontré des cellules géantes typiques de Langhan et une nécrose caséeuse, avec la détection d'ADN mycobactérien par le test GeneXpert TB. L'ATL isolée est une énigme diagnostique mais doit être envisagée chez les femmes jeunes et d'âge moyen dans les régions d'endémie tuberculeuse présentant des ganglions lymphatiques axillaires hypertrophiés en l'absence de foyers d'infection ou de malignité. La différence entre les sexes dans la réponse immunologique à l'infection peut expliquer cette présentation unique parmi le sexe féminin.

Mots clés: tuberculose axillaire isolée; *Mycobactérie*; histopathologie; rapport de cas

Introduction:

Tuberculosis (TB) is a chronic hypersensitive granulomatous infectious pulmonary and systemic disease, caused mostly by members of the *Mycobacterium tuberculosis* complex (MTBC) (1). It is a major cause of morbidity and mortality, particularly in low-and-middle-income countries (LMICs), where chronic debilitating illness, particularly the acquired immune deficiency syndrome (AIDS), poverty and overcrowding are the major risk factors (1,2). The disease severity also varies from indolent, asymptomatic disease to severe disease depending on the causative organism and the immune status of the individual (1). Its pattern of organ involvement also varies, with pulmonary tuberculosis acounting for majority of cases, and extra-pulmonary TB accounting for 7-30% of cases of which 17-33% of these cases present with tuberculous lymphadenitis (3).

The causative organism is mainly spreads from person to person through airborne droplets containing organisms from an active case to a susceptible host; and rarely by drinking milk contaminated by *Mycobacterium bovis* implicated in oropharyngeal and intestinal TB (1). The pathogenesis involves the infection of macrophages, with subsequent development of cell-mediated immunity resulting in formation of granuloma, tissue destruction and caseous necrosis (1).

The diagnosis of pulmonary TB is made from clinical presentations of patients, chest X-rays, computerized tomographic (CT) scan, sputum smear microscopy and culture, molecular methods, histological evaluation of tissues and use of biological markers (4). In rare cases, extrapulmonary TB may present without classical features of TB and in uncommon locations, resulting in diagnostic difficulties. Unilateral axillary TB has been identified as one of such TB diseases which may present with atypical manifestations. We therefore report such unusual case of axillary TB lymphadenitis in a 57-year-old Nigerian female seronegative for the human immunodeficiency virus (HIV).

Case report:

A 54-year-old Nigerian female patient presented in a private hospital with the complaint of swelling in the left axillary region, dating back to 14 months prior to presentation. The enlargement was insidious in inception but progressive. There was no associated pain or tenderness and no history of cough, loss of appetite, night sweat or chronic weight loss. There was also no previous history of TB or exposure to person with active TB. There was no history of nipple discharge, ulceration, or retraction or breast mass or mastalgia and no associated history of infection on the chest, neck or left upper limb.

Physical evaluation revealed an apparently healthy female patient. There was no evidence of pallor, icterus, cyanosis, digital clubbing, or pedal edema. Examination of the left axilla showed multiple, firm, mobile, matted, multinodular axillary lymph nodes, altogether measuring 8x6x4 cm. There were no enlarged lymph nodes in the contralateral axilla. Basic investigations such as complete blood count, liver function and renal function tests were normal. Serological tests for HIV I and II were negative. Chest X-ray showed clear lung fields (Fig 1). Fine needle aspiration biopsy of the axillary mass was however inconclusive.

She subsequently had excision biopsy of the left axillary mass, which showed multiple matted lymph nodes, measuring 8x6x4cm (Fig 2A). The biopsy sample was stored in 10% buffered formalin solution and submitted for histological evaluation. The lymph nodes were firm to hard in consistency and cut surface showed gritty sensation with area of caseous necrosis (Fig 2B).

Light microscopic evaluation showed caseous necrosis with numerous Langhan's type giant cells, some lymphocytes, variable fibrosis, and residual lymphoid tissues (Fig 3A and 3B). The presence of *Mycobacterium* DNA in the tissue was confirmed by GeneXpert test. A diagnosis of axillary tuberculous lymphadenopathy (ATL) was made and patient was subsequently referred to special TB treatment centre for continuation of management.

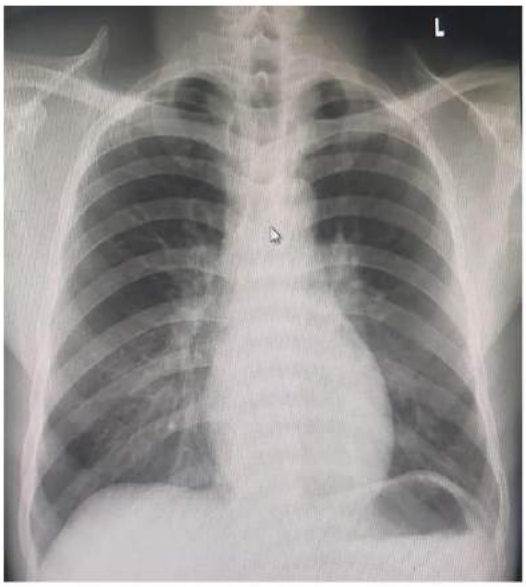

Fig 1: Chest X-ray of the patient (normal finding with no evidence of tuberculosis)

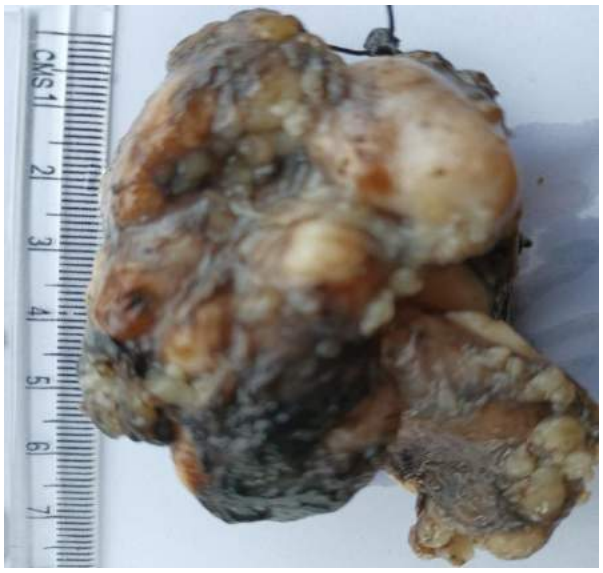

Fig 2A: Gross morphology showing matted axillary lymph nodes (8x6x6cm)

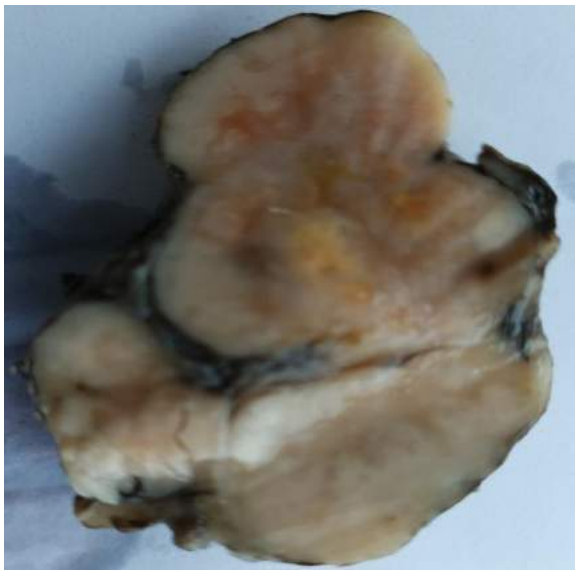

Fig 2B: Cut section through the lymph nodes with cheese-like appearance

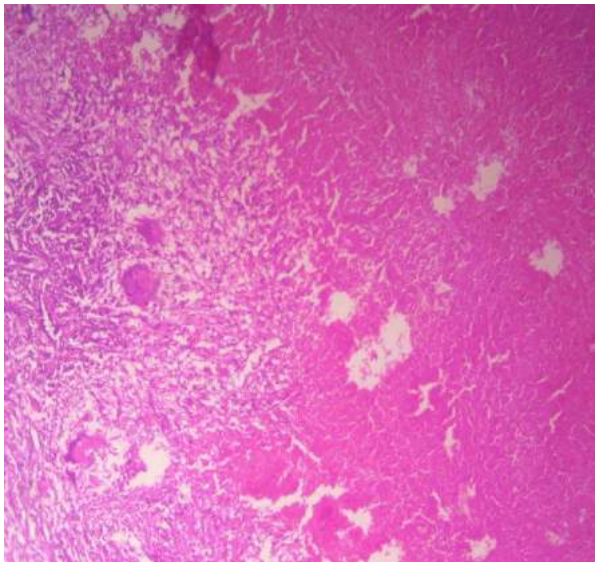

Fig 3A: Histological section of the lymph node showing caseous necrosis, variable fibrosis, lymphocytic, macrophage and langhan's giant cell infiltrates

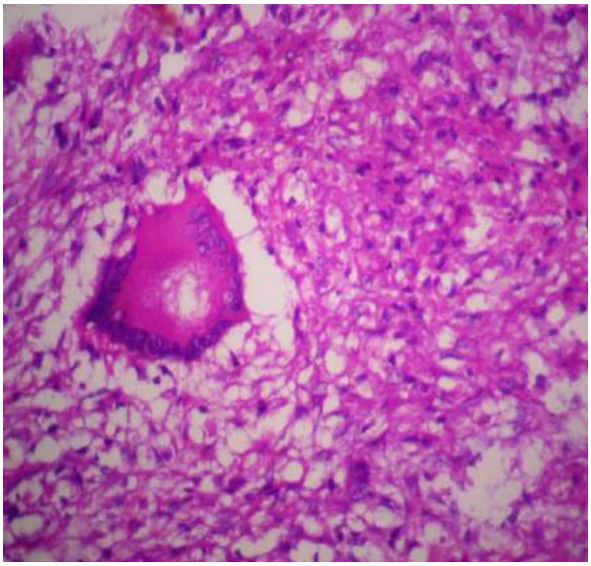

Fig 3B: Higher magnification showing multi-nucleated Langhan's giant cell characteristic of tuberculous lymphadenitis

Discussion:

Tuberculous lymphadenitis is relatively common and is second most common extra-pulmonary manifestation of TB with the most common location being cervical lymph nodes (5,6,7). Other documented sites of tuberculous lymphadenitis in decreasing order of frequency are supraclavicular, axillary, mesenteric, porta hepatis, perihepatic and inguinal regions (4,8,9). While TB lymphadenitis may be a local manifestation of systemic disease and therefore easy to diagnose, isolated TB lymphadenitis is relatively uncommon, and therefore presents with unique diagnostic challenges.

Isolated axillary TB lymphadenitis is extremely rare and is described in a patient with enlarged axillary lymph nodes without previous or active pulmonary TB or evidence of extrapulmonary TB elsewhere (7,10). In the case reported, systemic TB was immediately considered after diagnosis of tuberculous lymphadenitis was made. However, the absence of radiological features on chest X-ray excluded pulmonary TB. From the literature, only six such cases have been documented, affecting only women within the age of 21-69 years, with predilection for left axilla (3,4,7, 8,10,11, 12). This pattern of presentation is however poorly understood. Jayabal and Arumugam (4) attributed it to the direct communication of left

axillary lymphatics from the left thoracic duct or lymphatic supply from the left upper limb.

This female gender predilection for isolated ATL also elicits curiosity. Interestingly studies have shown that adult females have stronger humoral and adaptive immune response against infectious diseases (13). This may likely account for the gender difference in clinical presentation of isolated ATL. There is an overlap between the age group affected and the peak age for breast cancer (14), which is also likely to present with axillary lymph node enlargement secondary to metastasis. Incidentally, our patient did not have any symptom or sign in keeping with breast cancer, which excluded breast cancer as the possible initiator.

Other likely causes of asymptomatic axillary lymph node enlargement are mastitis, regional infective causes, post-vaccination lym phadenitis, silicon induced granulomatous lym phadenitis, metastatic carcinoma or lymphoma (9). In our patient, there was no evidence of infection in the region drained by the left axillary lymph node. There was also no history of vaccination or silicon implant in ipsilateral breast, and no constitutional symptom of TB or history suggestive of immunodeficiency. Lymphoma was also excluded after history taking and basic laboratory investigations.

Histological diagnosis is critical in diagnosis of TB in resource poor countries. Prior to such evaluation, radiological evidence of presence of foci of calcifications on the lymph nodes are clues to possibility of TB lymphadenitis (11), but absence of such positive clues made the diagnosis difficult. Isolation of *Mycobacterium* DNA using GeneXpert from the biopsy sample further supported the diagnosis of TB in this patient.

Conclusion:

In conclusion, isolated axillary tuberculous lymphadenitis is a diagnostic enigma but should be considered in absence of infections or malignancy in TB endemic areas. Histological evaluation of such lymph nodes is key to such diagnosis. Sex difference in immunological response to infection may account for this unique presentation among females (13).

Contribution of authors:

UOJ wrote the section on discussion and performed the laboratory investigations; YDG did the literature review and wrote the section on introduction; and EO managed the patient and provided the clinical summary.

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Conflict of interest:

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