Phenotypic and Molecular Characterization of Multidrug-Resistant *Escherichia Coli* and *Staphylococcus Aureus* Strains Isolated from Clinical and Environmental Samples

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ABSTRACT

Background: Today, antimicrobial resistance (AMR) poses a major threat to humanity, resulting in ineffective treatment of bacterial infections. The study aimed to evaluate phenotypic and molecular characteristics of MDR *E. coli* and *S. aureus* strains isolated from clinical and environmental samples in a hospital, Calabar, Nigeria.

Methods: A total of 240 samples (75.0%, n = 180 clinical samples; 25,0%, n = 60 environmental samples) were collected and cultured. Phenotypic analysis was conducted on 174 *E. coli* and 193 non-duplicate suspected coagulase-positive *S. aureus* strains isolated from burn wound, urine, blood, sputum, ear swab, skin swab, catheter and used cotton wool in University of Calabar Teaching Hospital. Presumptive identification was carried out based on microbiological standards. Molecular characterization techniques, such as PCR, single gene 16S rRNA sequencing, and phylogenetic analysis were employed to confirm the identity of two select strains of *E. coli* and *S. aureus*. Antibiotic susceptibility testing was evaluated using Kirby Bauer disc diffusion method with a total of fifteen (15) antibiotics belonging to five different classes of antibiotics. The demographic characteristics of age and gender were only applicable to specimens from patients, which included 107 females (59.4%) and 73 males (40.5%); while the median age was 30 years.

Results: A total of one hundred and ninety-three (193) *S. aureus* strains were isolated in this study from different clinical specimens and environmental samples. The highest percentage (23.3%) was recovered from burn wound. The least percentage prevalence (1.6%) of *S. aureus* strains were recovered from sputum specimen. *E. coli* isolates recorded highest percentage prevalence (61, 25.4%) from urine specimen, out of a total of 174 isolates obtained from 240 samples collected. The least number of isolates (4, 1.6%) of *E. coli* strains were recovered from skin swab. *S. aureus* strain demonstrated resistance to all the β-lactam drugs tested, gentamycin and streptomycin (aminoglycosides), erythromycin (macrolide), and sulphamethoxazole/trimethoprim (sulphonamide). The organism was sensitive to chloramphenicol and levofloxacin with higher percentage resistance rate (66.66%) compared to 53.33% of *E. coli*. However, *E. coli* was susceptible to augmentin, oxacillin, streptomycin, chloramphenicol, levofloxacin, and ceftazidime but resistant to ampicillin, amoxicillin, oxacillin, gentamycin, amikacin, erythromycin, norfloxacin and sulphamethoxazole/trimethoprim.

Conclusion: This evaluation demonstrates significant occurrence of multidrug-resistant *E. coli* and *S. aureus* strains in the study area, suggesting that the current treatment for these bacterial infections in the region is not effective which is a public health concern.

Keywords: MDR bacteria; inpatient; outpatient, Calabar, Nigeria.

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INTRODUCTION

Escherichia coli strains with the K1 capsular polysaccharide antigen cause approximately 40% of cases of septicaemia and 80% of cases of meningitis (Sabate *et al*., 2018). Different strains of *E. coli* are associated with a number of distinctive diarrheal illnesses. Among these are the enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and Shiga toxin-producing *E. coli* (STEC). Of the STEC, *E. coli* O157:H7 is the prototypic strain (Kiranmayi *et al*., 2011). Each class of *E. coli* has distinct somatic (O) and flagellar (H) antigens and specific virulence characteristics (Sejal and Krilov, 2015).

Neonates, both term and preterm, are susceptible to septicaemia and meningitis. Presentation in the first week after birth (early onset) and particularly in the first 2 days after birth reflects vertical transmission, whereas late-onset infection suggests nosocomial or community acquisition (Naves and Khan, 2010). The corresponding organisms are different; early-onset meningitis is more likely to be caused by group B Streptococci, *E. coli*, and *Listeria monocytogenes*, whereas late-onset meningitis may be caused by other Gram-negative organisms and staphylococcal species (Yan *et al.*, 2022). The *E. coli* pathotype responsible for meningitis and sepsis is called neonatal meningitis–associated *E coli* (Sakr *et al*., 2021). Neonatal meningitis–associated *E. coli* have a K1 capsule that contains sialic acid, which potentiates the bacteria's ability to invade through the blood-brain barrier (Tenaillon *et al*., 2010). Clinical signs of septicaemia include fevers, temperature instability, heart rate abnormalities, respiratory distress, apnea, cyanosis, lethargy, irritability, jaundice, vomiting, diarrhoea, and abdominal distention (Nair *et al*., 2019). Neonates with defects in the integrity of their skin or mucosa or abnormalities of gastrointestinal or genitourinary tracts are at increased risk as well (Montealegre *et al*., 2018).

Uropathogenic *E. coli* (UPEC) strains are responsible for approximately 30% of nosocomial-acquired urinary tract infections (UTIs). Infections in children are often due to blockages in the urinary tract, resulting in pools of stagnant urine (Nair *et al.*, 2019). UPEC can reside in the colon and then be introduced into the urethra. The first step in the development of a UTI is colonization of the periurethral area by enteric pathogens (Sims and Kim, 2011). A variety of virulence factors enable bacteria to ascend into the bladder and kidney. *E coli* possesses pili, hair-like appendages on the cell surface, which improve the bacteria's ability to adhere effectively to the uroepithelium (Sabate *et al*. 2018). Furthermore, UPEC strains contain type 1 fimbriae, which enhance virulence and are involved in initial urethral colonization, and many UPEC strains produce haemolysin, which may be involved in potentiating kidney disease (Sims and Kim, 2011).

Infections with antibiotic-resistant *E. coli* are an increasing concern worldwide, with resistance mediated by extended-spectrum beta-lactamase (ESBL) production. *E. coli* has the ability to secret toxins, polysaccharide and can form biofilm. It can also form biofilm *in-vitro* (Naves and Khan, 2010). These isolates are most often isolated from hospitalized patients but are becoming an increased cause of community-acquired infections as well (Yan *et al*., 2022).

Staphylococcus aureus usually acts as a commensal bacterium, asymptomatically colonizing about 30% of the human population; it can sometimes cause disease (Alabi *et al*., 2016). *S. aureus* is a significant cause of chronic biofilm infections on medical implants, and the repressor of toxins is part of the infection pathway. The organism is responsible for orthopedic implant-related infections, but is also found on cardiac implants, vascular grafts, various catheters, and cosmetic surgical implants (Van *et al*., 2019). After implantation, the surface of these devices becomes coated with host proteins, which provide a rich surface for bacterial attachment and biofilm formation (Sanchez *et al*., 2016). Once the device becomes infected, it must by completely removed, since *S. aureus* biofilm cannot be destroyed by antibiotic treatments (Zago *et al*., 2015). *S. aureus* can lay dormant in the body for years undetected (Yan *et al*., 2022). Once symptoms begin to show, the host is contagious for another two weeks, and the overall illness lasts a few weeks. If untreated, the disease can be deadly. Deeply penetrating *S. aureus* infections can be severe (Gulzar and Zehra, 2019).

□-lactam antibiotics are the first-line treatment for Staphylococcal infections. Alternative drugs are available for treatment but with limitations such as less tissue penetration and efficiency for vancomycin; cost for quinupristin-dalfopristin, tigecycline, daptomycin and linezolid and toxicity for rifampicin (Van *et al*., 2019).

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As a consequent of selective pressure imposed by antimicrobials, *S. aureus* has the capability of developing resistance to drugs with more rapidity. The resistance is chromosome or plasmid mediated and is attributed to transduction, transformation and conjugation (Zago *et al*., 2015).

blaZ gene in the organism mediates the resistance to □-lactam antibiotics i.e. penicillin and its derivatives (Van *et al*., 2019). *mecA* gene which encodes the Phosphate binding protein which may have an altered binding capacity or new Penicillin Binding Protein (PBP2') may be responsible for resistance to methicillin and other □-lactam antibiotics. There are some strains which are named as borderline oxacillin resistant *S. aureus* (BORSA). These strains are □-lactamase hyper-producers and show resistance to oxacillin in absence of mecA or mecC genes expression (Gulzar and Zehra, 2018). Aminoglycosidal resistance may arise because of the mutations in the genes regulating the ribosome leading to the structural changes in the ribosomal proteins which may hinder the binding capacity of the antibiotic and further its action (Andersson *et al*., 2020). Diminished uptake of the antibiotic or modification of antibiotic due to the cellular enzymes such as aminoglycoside acetyltransferases (AAC) or aminoglycoside phosphotransferases (APH) catalysed by a bifunctional protein encoded by aacA-aphD gene may also be responsible for the aminoglycoside resistance (Antonoplis *et al*., 2019). Tetracycline resistance is due to tetK and tetL genes that are located on the plasmid. These genes control the active efflux. The ribosomal protection is mediated by tetO or tetM genes present on transposon or chromosome (Larry *et al*., 2016). Vancomycin intermediate resistance is due to vraSR operon and graS gene (Otto, 2018). Acquisition of Enterococci vanA gene may lead to development of vancomycin-resistant *S. aureus* (VRSA) (Monte *et al*., 2014). Altered peptidoglycan synthesis on exposure to vancomycin may lead to an increase in the thickness of cell wall thus impairing the diffusion of drug into the bacterial cell (Taponem and Pyorala, 2019). Inducible resistance to macrolide antibiotic is commonly due to ermC gene present on the plasmid. Resistance mediated by ermA gene can be due to chromosomal mutations Constitutive resistance is mediated by ermB gene on plasmid (Monte *et al.*, 2014).

S. aureus can be categorized as methicillin-susceptible *S. aureus* (MSSA) or methicillin-resistant *S. aureus* (MRSA). As per Clinical and Laboratory Standards Institute (CLSI), MRSA are those isolates which have minimum inhibitory concentration (MIC) ≥4 μg/mL for methicillin (Gulzar and Zehra, 2018).

Considering the alarming rate of drug resistance development by pathogens, it is essential and imperative to preserve the efficacy of existing drugs through measures to minimize the development and spread of resistant genes, while efforts to develop new treatment options proceed (Otu *et al*., 2021). The pipeline for the development of new antibacterial drugs is now virtually empty, particularly for the treatment of nosocomial infections, and research on treatments to replace antibacterial drugs is still in the early stages (CDC, 2019). This implies that progress in modern medicine, which relies on the availability of effective antibacterial drugs, is now at risk.

The collection of reliable information about antimicrobial resistance (AMR) situation through well-conducted surveillance is essential to inform strategies and prioritize interventions to tackle the problem. AMR surveillance should generate data to support action at all levels: local, national, regional and global (CDC, 2019). Therefore, this study investigated the phenotypic and molecular identities of multidrug-resistant strains of *E. coli* and *S. aureus* isolated from different human clinical and environmental samples in a hospital in Calabar, Nigeria.

METHODOLOGY

Study setting and population

A descriptive cross-sectional study was conducted at the University of Calabar Teaching Hospital, Calabar, Nigeria from May 2024 to November 2024. University of Calabar Teaching Hospital is 410 bed capacity hospital with several clinical service departments and units. The study population composed of all patients (inpatients and outpatients) from whom clinical samples (blood, sputum, urine, ear swab, skin swab and burn wound) and environmental samples (catheter and used cotton wool) were investigated. A total of 240 samples were collected comprising of 30 samples representing each specimen and labeled accordingly. Samples that had incomplete demographic information were not included in the investigation. Informed consent was

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obtained from parents or guardians on behalf of children participants and from each patient involved in the study before enrollment. The study was approved by the University of Calabar Teaching Hospital Ethical Committee with HREC Protocol Assigned Number: UCTH/HREC/33/543. Anonymity and confidentiality of the data were guaranteed.

Isolation and Presumptive identification of bacteria

Clinical specimens were collected using sterile cotton swabs, universal containers, small screw capped bottles, a firmly stopper tube, syringe and a sealed capillary tube (Koneman *et al*., 2005). Each swab was carefully taken from the site of infection and placed in tubes containing readymade media (transport media - nutrient broth) to maintain the swab wet during transportation to laboratory. For the isolation of *Escherichia coli*, specimen was inoculated on MacConkey Agar, Eosin Methylene Blue Agar and Nutrient Agar. Streaked plates were incubated at 37ºC for 24 hours. Bacterial colonies on plates were later Gram stained (Nasreen *et al*., 2009). Characterization of bacterial isolates was carried out based on standard microbiological methods (Koneman *et al*., 2005). For the isolation of *Staphylococcus aureus*, different clinical samples were streaked on mannitol salt agar and blood agar and incubated aerobically for 24 hours at 37°C. The isolates were identified depending on the morphological features on culture media, Gram stain reaction and biochemical tests according to Bergey's Manual (CLSI, 2018).

Molecular identification of two selected strains of bacterial isolates

Genomic DNA extraction procedure

Reagents-

1. 5M NaCl- 29.22 gm for 100 ml (Autoclave)

2. SDS 20%- 10 gm for 50 ml (pH-7.2)

3. CTAB (10%) /NaCl (0.7M) Solution- 4.09 gm for 100 ml NaCl and 10 gm of CTAB (Autoclave)

4. Chloroform: Isoamyl alcohol – 24:1

5. TE (Autoclave)

Procedure

Liquid cultures (1-3 mL) were centrifuged at 4600x *g* for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were added. The mixture was incubated for 1 hour at 37 ºC. After incubation, 5 M NaCl (100 µl) and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and mixed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200 x *g* for 20 min. The aqueous phase was transferred to a new tube, isopropanol (1:0.6) was added and DNA was precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 7200 x *g* for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours. Pellets were resuspended in 50 µl of TE buffer and kept at 4°C (Nishiguchi *et al*., 2000; Trindale *et al*., 2007).

Protocol for bacterial polymerase chain reaction (PCR)

Primers

Forward primer: 27f (5'-AGAGTTTGATCMTGGCTCAG-3')

Reverse primer: 1525r (5'-AAGGAGGTGWTCCARCC-3') (Lane, 1991).

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Cocktail reaction

Ingredient	**Volume** (µl)
Sterile distilled H_2O	6.44
5 U /µl *Taq* Polymerase	0.06
25 mM $MgCl_2$	0.75 (1.5 mM)
10 mM dNTPs	0.25 (250 µM)
10 pM forward primer	0.25 (1 µM)
10 pM reverse primer	0.25 (1 µM)
Template	2
Total volume	12.5

PCR conditions

- 94°C for 2 mins
- 30 cycles of 94°C for 30 secs, 50°C for 60 secs and 72°C for 90 secs
- 72°C for 5 mins
- Stored at 4°C

Post PCR analyses

To make agarose gel, 1.5 g of agarose powder was added into 100 ml of 1X TAE Buffer. Heated in a microwave for 5 minutes. Cooled briefly and 5 µl of GR Green® solution was added. Mixed briefly and poured into a gel tank with well combs. The mixture was left to solidify and PCR products was loaded into each well. Electrophoresis (Gel-Field) was performed at 100V for one hour. Gel was viewed under UV light and pictures were taken. Expected product size: 1,500 bp. Sequencing was done using purified amplicons. Fragments were sequenced using Nimagen, Brilliant Dye TM Terminator sequencing Kit V3.1 BRD3-100/1000 according to manufacturer's instructions. The nucleotides of the isolates were subjected to nucleotide BLAST analysis to reveal their percentage identity/similarities, accession number, highest query cover (%), E value, number of nucleotides and verification of amplification of polymerase chain reaction.

Construction of phylogenetic tree

Phylogenetic trees showing evolutionary relationship of the identified strains of *E. coli* and *S. aureus* obtained from University of Calabar Teaching Hospital, Calabar were constructed using the methods described by Jukes and Cantor (1969), Felsenstein, (1985), Saitou and Nei (1987) and Tamura *et al*. (2013).

Antibiotic susceptibility testing

Antibiotic susceptibility testing (AST) was performed using the Kirby Bauer disc diffusion technique against selected antibiotics including penicillin (ampicillin, cloxacillin, amoxicillin, and augmentin), aminoglycoside (gentamycin, amikacin, streptomycin, and chloramphenicol), macrolide (erythromycin), sulphonamides (septrin – sulphamethoxazole/trimethoprim), cephalosporin (ceftazidime) and fluoroquinolone (ciprofloxacin, levofloxacin, norfloxacin, oxacillin.). The categorization of the isolates into sensitive, intermediate or resistant was done according to the recommendations of the Antibiogram Committee of the French Society of

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Microbiology (CA-SFM) 2020 version 1.2 (Ibrahim *et al.*, 2023). Multi-drug resistance was defined as resistance to at least one antibiotic in three or more antibiotic categories (CLSI, 2020).

Data analysis

The raw data were entered in Microsoft Excel, cleaned, coded and analysed using Statistical Package for Social Sciences (SPSS) version 25.0 (IBM Corp., Armonk, NY, USA). We used frequencies to describe the distribution of sociodemographic, clinical and environmental variables, and resistance profiles of *E. coli* and *S. aureus* to a panel of antibiotics.

RESULTS

Sociodemographic characteristics of patients

A total of 240 samples, (75.0%, n = 180 clinical samples; 25,0%, n = 60 environmental samples) were collected and cultured. The demographic characteristics of age and gender were only applicable to specimens from patients, which included 107 females (59.4%) and 73 males (40.5%); the median age was 30 years. Most samples were collected from patients aged 25–34 years (30.5%), 0-14 (22.2%), 15-24 (12.2%) and those aged 55 years and above (18.3%) (table 1).

Percentage (%) prevalence of clinical bacterial isolates per specimen

A total of one hundred and ninety-three (193) *S. aureus* strains were isolated in this study from different clinical specimens and environmental samples. The highest percentage (23.3%) recovered from burn wound. The least percentage prevalence (1.6%) of *S. aureus* strains were recovered from sputum specimen while 41 (17.0%) isolates were recovered from skin swab and 32 (13.3%) were isolated from used cotton wool (table 2). *E. coli* isolates recorded highest percentage prevalence (61, 25.4%) from urine specimen, out of a total of 174 isolates obtained from 240 specimens collected. The least number of isolates (4, 1.6%) of *E. coli* strains were obtained from skin swab specimen while 42 (17.5%) isolates were recovered from catheter and 24 (10.0%) isolates were obtained from used cotton wool. The result is presented in table 2.

Table 1: Sociodemographic characteristics of patients (n = 240)

Variables	Frequency (n)	Percentage (%)
Age bracket		
0-14	40	22.2
15-24	22	12.2
25-34	55	30.5
35-44	15	8.3
45-54	15	8.3
55 and above Gender	33	18.3
Female	107	59.4
Male	73	40.5

Median age = 30 years.

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Table 2: Percentage (%) prevalence of clinical bacterial isolates per specimen (n = 240)

S/N	Specimen	*S. aureus*	*E. coli*
1.	Sputum	4 (1.6)	11 (4.5)
2.	Blood	20 (8.3)	5 (2.0)
3.	Ear swab	17 (7.0)	19 (7.9)
4.	Urine	11 (4.5)	61 (25.4)
5.	Skin swab	41 (17.0)	4 (1.6)
6.	Burn wound	56 (23.3)	8 (3.3)
7.	Catheter	12 (5.0)	42 (17.5)
8.	Used cotton wool	32 (13.3)	24 (10.0)
Total		**193 (100)**	**174 (100)**

Presumptive identification of bacterial isolates from samples

Table 3 shows the morphological and biochemical characterization of clinical bacterial isolates obtained from UCTH, Calabar. The isolates were presumptively differentiated into *Staphylococcus aureus* and *Escherichia coli* and confirmed by molecular analyses using 16S rRNA gene sequence. Gel image of polymerase chain reaction (PCR) of clinical bacterial isolates showing number of base pairs in shown in Plate 1. The two labelled bands with varying base pairs represent the two suspected bacterial isolates.

Characteristics of partial 16S ribosomal RNA sequence of bacterial isolates

The characteristics of partial 16S ribosomal RNA sequences of the two organisms obtained from UCTH, Calabar using nBLAST (n – nucleotides; basic local alignment search tool) on GenBank is represented in Table 4. According to the result, the 16S rRNA gene sequence data of *Staphylococcus aureus* exhibited 99.74% homology with other strains obtained from database. The number of nucleotides is 1,521 with accession number MZ802727. The homologuy of *Escherichia coli* isolate is 99.33% in comparison with other strains of *E. coli* in the database, with accession number MZ802725. One thousand five hundred and four (1,504) nucleotides of *E. coli* were sequenced. The sequences of the two bacterial isolates have been submitted to the National Centre for Biotechnology Information (NCBI) GenBank.

Table 3: Presumptive identification of *S. aureus* and *E. coli* from clinical and environmental samples

Parameter	Isolate 1 (*S. aureus*)	Isolate 2 (*E. coli*)
Morphology	Cocci in clusters	Rod shape
Gram staining	Gram positive	Gram negative
Motility	Nonmotile	Motile
Indole test	-	+
Methyl red	+	+
Voges-Proskauer test	+	-
Coagulase test	+	-
Catalase test	+	+

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Citrate test	NC	-
Growth on EMB agar sheen	NC	Green metallic
Pigmentation on Nutrient agar	Golden yellow	Grayish white
Growth on MacConkey's agar Mannitol fermentation	Pink colony+	Pink colony+

Phylogenetic trees of clinical bacterial isolates

Figure 1 presents the phylogenetic tree of *Staphylococcus aureus* isolate (in red) showing evolutionary relationship with other strains available in the GenBank database. The optimal tree with the sum of branch length = 0.00432251 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 12 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1524 positions in the final dataset. The phylogenetic tree of *Escherichia coli* isolate (in red) showing evolutionary relationship with other strains available in the Genbank database is shown in Figure 2. The optimal tree with the sum of branch length = 0.004439245 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 13 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1505 positions in the final dataset.

Antibiotic susceptibility of clinical bacterial isolates

To determine the MDR status of the two strains of *S. aureus* and *E. coli*, a panel of 15 antibiotics were screened against the organisms using disc diffusion assay. Interestingly, there were variations in the resistance pattern of the isolates. *S. aureus* strain demonstrated resistance to ampicillin, amoxicillin and augmentin (penicillins), gentamycin and streptomycin (aminoglycosides), erythromycin (macrolide), septrin (sulphonamide), and ceftazidime (cephalosporin). The organism was sensitive to chloramphenicol and levofloxacin and intermediate to amikacin, norfloxacin and ciprofloxacin. *S. aureus* demonstrated higher percentage resistance (66.66) to the antibiotics. It was observed that *Escherichia coli* had percentage resistance of 53.33. It was resistant to ampicillin, amoxicillin, and cloxacillin (penicillins), gentamycin and amikacin (aminoglycosides), erythromycin (macrolide), norfloxacin (fluoroquinolone), and septrin (sulphonamide) but sensitive to augmentin, oxacillin, strept--omycin, chloramphenicol, levofloxacin, and ceftazidime. The organism was intermediate to ciprofloxacin. The result is represented in table 5.

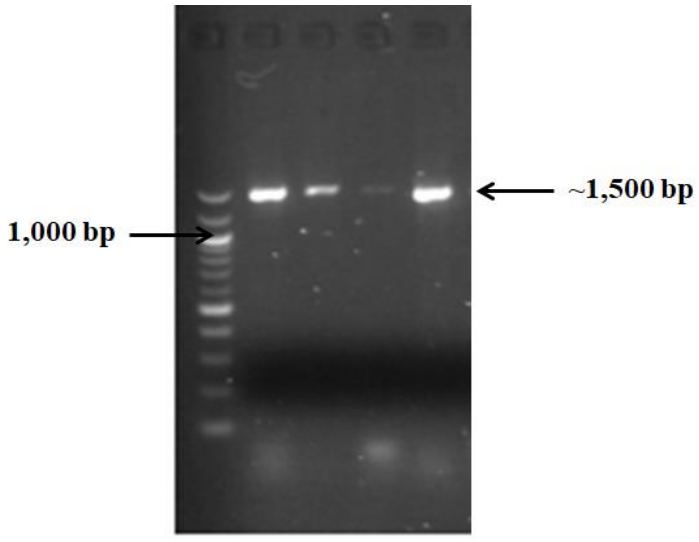

Plate 1: Gel image of polymerase chain reaction (PCR) of clinical bacterial isolates

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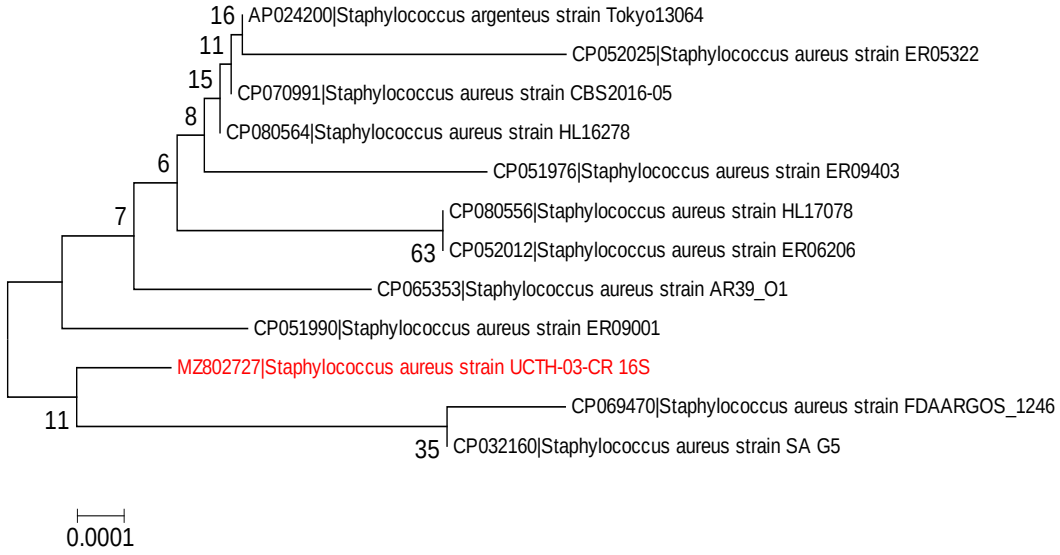

Figure 1: Phylogenetic tree showing evolutionary relationship of *Staphylococcus aureus* obtained from UCTH – Nigeria (in red) with other strains available in the GenBank database.

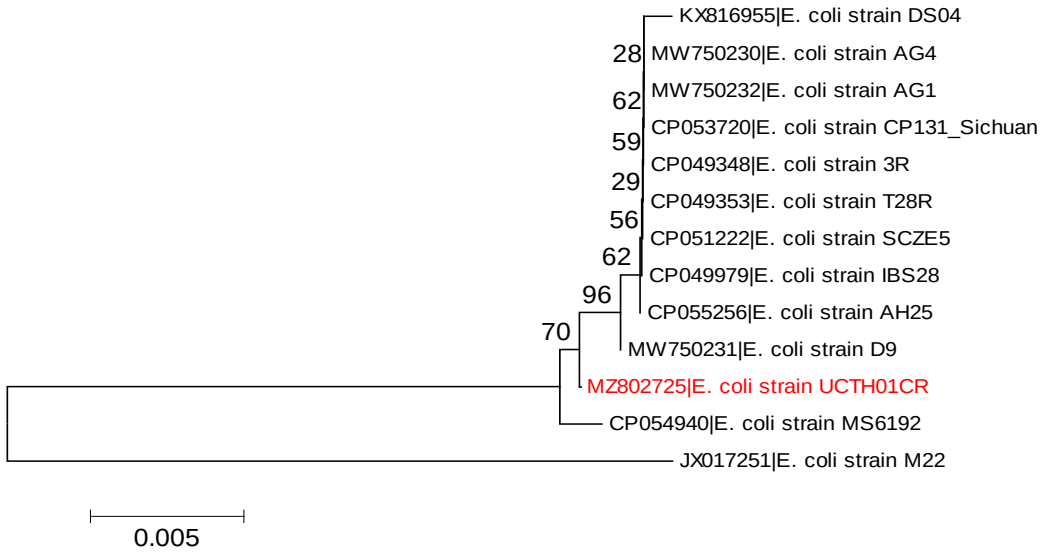

Figure 2: Phylogenetic tree showing evolutionary relationship of *Escherichia coli* obtained from UCTH – Nigeria (in red) with other strains available in the GenBank database

Table 4: Characteristics and properties of partial 16S ribosomal RNA sequences of two bacterial isolates obtained at the UCTH, Cross River State, Nigeria using nBLAST on GenBank

Sample organism	Strain	Accession number	No of nucleotides	Highest nBLAST identity (%)	E value	Alignment score	Highest query coverage (%)
Escherichia coli	UCTH-01-CR	MZ802725	1,504	99.93	0.00	≥200	100
Staphylococcus aureus	UCTH-03-CR	MZ802727	1,521	99.74	0.00	≥200	99

n – nucleotide; BLAST – basic local alignment search tool; % - percentage.

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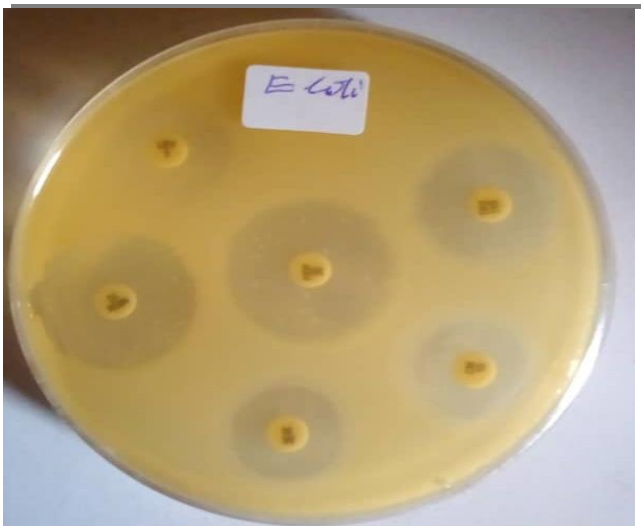

Plate 2: Antibiotic susceptibility test of *E. coli strain* Plate 3: Antibiotic susceptibility test of *S. aureus strain*

Table 5: Antibiotic susceptibility of clinical bacterial isolates

Mean zones of inhibition (mm) and standard deviation			
S/N	Antibiotic agent	*S. aureus*	*E. coli*
1.	Ampicillin	10.33 ± 0.72(R)	12.66 ± 0.72(R)
2.	Amoxicillin	12.00 ± 0.50(R)	12.00 ± 0.50(R)
3.	Cloxacillin	11.66 ± 0.72(R)	10.33 ± 0.20(R)
4.	Oxacillin	12.33± 0.20(R)	28.66 ± 0.72(S)
5.	Augmentin	10.33 ± 0.20(R)	24.66 ± 0.20(S)
6.	Gentamycin	10.33 ± 0.72(R)	10.33 ± 0.98(R)
7.	Streptomycin	12.66 ± 0.72(R)	21.66 ± 0.72(S)
8.	Amikacin	15.33 ± 0.72(I)	13.00 ± 0.50(R)
9.	Chloramphenicol	17.66 ± 0.98(S)	29.66 ± 0.72(S)
10.	Ciprofloxacin	15.00 ± 0.50(I)	15.33 ± 0.20(I)
11.	Levofloxacin	17.66 ± 0.72(S)	28.66 ± 0.72(S)
12.	Norfloxacin	14.33 ± 0.72(I)	12.00 ± 0.50(R)
13.	Erythromycin	12.66 ± 0.72(R)	11.66 ± 0.72(R)
14.	Septrin	12.00 ± 0.50(R)	12.33 ± 0.20(R)
15.	Ceftazidime	12.66 ± 0.20(R)	19.66 ± 0.72(S)
Percentage (%) resistance		**66.66**	**53.33**
R = Resistance; I = Intermediate; S = Sensitive; % = Percentage; ± Standard Deviation			

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DISCUSSION

The widespread misuse of antibiotics, especially in developing countries, has contributed significantly to the evolving resistance profile of microorganisms, evidenced by increasing occurrences of antibiotic resistance among bacterial populations especially in hospital settings (Van *et al*., 2019; Otu *et al*., 2021). Additionally, resistance rates are typically higher in developing countries as compared to developed countries (Antonoplis *et al*., 2019; Yan *et al*., 2022). Consequently, it is imperative that local surveillance of common pathogenic organisms and their antibiograms be implemented to advise the current use of antibiotics. This is essential in the formulation of prescribing policies based on local statistics. This study provides important data on current antimicrobial resistance pattern of *E. coli* and *S. aureus* strains isolated from hospital in Calabar, southern Nigeria.

Result obtained in this study through phenotypic evaluation and molecular characterization using 16S rRNA gene sequencing, identified the clinical bacterial isolates as *E. coli* and *S. aureus* (Plate 1, Table 3 and 4). The phylogenetic trees showing the evolutionary relationship of the clinical bacterial isolates are presented in this study as a means of tracing these organisms to their evolutionary ancestors (Figures 1 and 2). The gene sequence mentioned is widely regarded as the most reliable and extensively studied taxonomic marker molecule for identifying bacteria (Abdullah *et al*., 2023). DNA sequencing was conducted on the 16S rRNA fragments of both isolates. A 1000-base pair PCR product of *E. coli* and 1500-base pair PCR product of *S. aureus* was recognised. The query cover of 100% and identity of 99.93% of *E. coli* and 99% query cover with an identity of 97.83% of *S. aureus* on NCBI were used to create phylogenetic trees that revealed evolutionary connection to other strains of the same species observed in the GenBank. Significantly, each individual isolate in gene bank formed a cluster with each isolate exhibiting a 100% similarity. The isolates obtained from the GenBank were all identified as belonging to the expected genera of *E. coli* and *S. aureus*. Phenotypic assays, when used together with host information (because of their high specificity for certain hosts), are likely sufficient for routine diagnostic purposes. Accurate identification at the species level is crucial for various research purposes, such as assessing prognosis, conducting epidemiological surveys, and guaranteeing exact therapeutic management (Ibrahim *et al*., 2023). These bacterial pathogens have been isolated from diverse hospitals worldwide and reported to have also been aetiologic agents of community- and hospital-acquired infections (Ramirez-Castillo *et al*., 2018; Onyeka *et al*., 2021; Abdoulaye *et al*., 2022).

The bulk of the screened patients fell within the age range of 0-34 years, with female subjects making up the most vulnerable demographic gender. According to the survey, this particular group, along with youngsters and the old (55 and above) have been reportedly identified as being very vulnerable (Alabi *et al*., 2016). The exposure of these age groups can be attributed to their daily routine activities, as they are regularly exposed to environments that are contaminated.

In our study, 174 (72.5%) *E. coli* were isolated from various clinical and environmental samples, among which the highest prevalence (61, 25.4%) was obtained from urine and 42 (17.5%) were environmental isolates recorded from catheter. The least prevalence (4, 1.6%) from clinical specimens was obtained from skin swab samples. In comparison to other investigations conducted globally with rates of prevalence ranging from 44 to 91%, the detection rates observed in this study are proportionately lower (Ghanem and Haddadin, 2018; Vurayai *et al*., 2022; Umar *et al*., 2023). The difference in the detection rates may be due to changes in sampling locations, variations in techniques used, or primarily caused due to a lower prevalence of *E. coli* in tested environments. Among the environmental isolates, a larger portion was detected from catheters (42. 17.5%). Studies conducted previously support our findings, as similar isolates have been well known to colonize inanimate hospital surfaces (Vurayai *et al*., 2022). High rates of bacterial prevalence in hospital environments are a well-established route for contracting hospital acquired infections (HAIs) (Yan *et al*., 2022). Overcrowding, lack of adequate beds combined with patients placed on hospital floors and hallways, and inadequate sanitary practices among the facilities, all present an increased risk of resistant bacterial transmission and contraction of HAIs among the patients (Shahida *et al*., 2016).

In addition, result revealed that there was more *S. aureus* (56, 23.3%) from burn wound swab specimens than any other sampling source with a total percentage prevalence of 193 (60.4%) out of 240 samples analysed. The least *S. aureus* organisms (4, 1.6%) were isolated from sputum specimen. Ibanga *et al*. (2020) also recorded

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very high *S. aureus* number from wound swabs. This high number of isolates is suggestive of the exposed nature of the sampling point. This is supported by other findings presented by Nimmo *et al*. (2011), who found more *Staphylococcus* isolates on exposed body surfaces than the internal parts. Uncovered wounds have sticky surfaces and the skin is continuously exposed; it is therefore easy for such high microbial numbers to be recorded. Again, recent studies found that 5.2% (Joshua *et al*., 2022), 18.8% (Gumaa *et al*., 2021), and 86% (Abdul-Aziz *et al*., 2022) of the contamination was identified in wound samples collected from the same institutions. Other localities within Nigeria with higher occurrence rates include, Kwara (100%) (Bale *et al*., 2019), Abuja (78.7%) (Adeiza *et al*., 2020), and Sokoto (85.7%) (Umar *et al*., 2023). The differences in prevalence can be attributed to many factors, such as demographics, specimen categories, and research period (Garoy *et al*., 2019). The increased occurrence rate can be ascribed to causes such as excessive occupancy in healthcare institutions, inadequate sanitization equipment, communal areas, and insufficient hand hygiene by healthcare providers. The presence of *S. aureus* in these clinical specimens and environmental samples is a cause for concern in terms of public health and clinical practice. It impedes the healing process, worsens the severity of lesions and other infections, and can act as a source for further infections caused by pathogens (Abdul-Aziz *et al*., 2022).

The alarming increase in the rates of antimicrobial resistance (AMR) is a global cause for concern, and the World Health Organization (WHO) has listed beta-lactamase producing bacterial organisms on its critical priority list requiring the formulation of new treatments options (Shrivastava *et al*., 2018). Nigeria, alongside other African countries, is contained within the WHO regions which are burdened with the high risk of antimicrobial resistance (Hoque *et al*., 2022). Several strategies to combat AMR rely on a periodic surveillance system, assessment of threats and an improvement in healthcare policies developed from implementing the findings.

One of our findings indicated that *E. coli* was resistant to ampicillin, amoxicillin and cloxacillin, which is consistent with other studies, revealing that penicillins are widely used in clinical and environmental settings (Nair *et al*., 2019; Sakr *et al*., 2021). Penicillins are widely accessed without prescription and are usually inappropriately used, which might contribute to the observed resistance to these drugs (Sabate *et al*., 2018). The resistance of *E. coli* to penicillins could also be facilitated by the presence of AmpC β-lactamases encoded by the chromosome of *E. coli* (Hoque *et al*., 2022). Additionally, our study revealed that the *E. coli* isolate was also resistant to sulfamethoxazole/trimethoprim (septrin). Our findings corroborate reports from other studies in which *E. coli* isolates were found to be highly resistant to sulfamethoxazole/trimethoprim (Olson *et al*., 2009; Mbanga *et al*., 2023). The overuse and misuse of sulfamethoxazole/trimethoprim may have contributed to the resistance of *E. coli* to this drug combination. However, the resistance of *E. coli* to sulfamethoxazole/trimethoprim has been reported even in individuals who have never used the drug combination (Somorin *et al*., 2022).

Our study also revealed resistance of *E. coli* to levofloxacin and intermediate to ciprofloxacin. This finding agrees with reports from other studies that found that *E. coli* had developed resistance to quinolones (Stapleton *et al*., 2020; Jalil and Atbee, 2022; Tchesnokova *et al*., 2023). These resistance patterns reported in our study and comparable studies could be due to the misuse of quinolones for the treatment of urinary tract infections and respiratory tract infections (Patel and Goldman, 2016; Tang and Zhao, 2023). The resistance of *E. coli* to quinolone antibiotics could be due to the occurrence of chromosomal mutations or plasmid-mediated quinolone resistance (Werner *et al*., 2011). Furthermore, *E. coli* may also harbour chromosomally encoded AmpC β-lactamases that are capable of hydrolysing cephalosporins, especially when overexpressed (Somorin *et al*., 2023). Unfortunately, even after a reduction in prescriptions of fluoroquinolones like ciprofloxacin, there appears to be a decrease in the susceptibility *E. coli* to ciprofloxacin (Tchesnokova *et al*., 2023). It was also observed that *E. coli* was resistant to erythromycin (macrolide) and gentamycin and amikacin (aminoglycosides). Our findings disagree with those reported in other studies where *E. coli* was 95% sensitive to erythromycin (Sabate *et al*., 2018) and 90%–100% susceptible to amikacin (Kuti *et al*., 2018). This variation in results could be attributed to indices such as level of infection control practices by health facilities, and previous exposure of patient to antimicrobials and patient hospital stay.

The World Health Organisation (WHO) has identified *S. aureus* as a high-priority organism (Hutchings *et al*., 2019) because it is an opportunistic pathogen that can infect both humans and animals, causing a variety of

illnesses. *S. aureus* commonly acquires resistance to antibiotics that are currently available on the pharmaceutical market (Ibrahim *et al*. 2023). *S. aureus* isolate in our study was resistant to all the β-lactam antibiotics (ampicillin, amoxicillin and augmentin) tested, which is similar to the results reported by Andrianarivelo *et al*. (2017) in Madagascar and by Salem *et al*. (2016) in Mauritania. Resistance of *S. aureus* to gentamicin and streptomycin (aminoglycosides) in our study has also been reported in different studies conducted in Uganda (Kitara *et al*.,2011), Burkina Faso (Koinam *et al*., 2017) and Morocco (Elhamzaoui *et al*., 2009). The best anti-staphylococcal activities were exhibited by chloramphenicol and levofloxacin, for which the isolate was susceptible with wider zones of inhibition. These findings are in concordance with results from studies conducted in Madagascar (Andrianarivelo *et al*., 2017) and Burkina Faso (Koinam *et al*., 2017). In our study, resistance of *S. aureus* to macrolide (erythromycin) was detected. The study by Ojulong *et al*. (2009) in Uganda reported high resistance rate to erythromycin but absolute resistance (100%) of *S. aureus* isolates to erythromycin was reported by Onwubiko *et al*. (2011) in a study conducted in Kano, Nigeria.

Multidrug resistant (MDR) microbial strains are defined to be resistant to at least three different groups of antibiotics (CLSI, 2020). Our result shows that both isolates demonstrated MDR with *S. aureus* having higher percentage resistance (66.66%) while that of *E. coli* was 53.33%. The isolates mostly expressed resistance against antimicrobial groups like β-lactams, aminoglycosides, macrolides, and folate pathway antagonists, as seen in Table 5. The presence of high rate of MDR *S. aureus* in clinical and environmental samples poses a significant threat to public health due to its wide-ranging consequences, such as treatment failure, recurrent infections, increased morbidity and mortality rates, heightened usage and spread of antibiotic-resistant genes, restricted availability of effective antibiotics, prolonged and complex hospital stays, excessive healthcare costs, and reduced societal productivity (Hutchings *et al*., 2019). *S. aureus* is a priority pathogen that is commonly associated with multidrug-resistant (MDR) infections (Ibrahim *et al*. 2023). In Nigeria, the prevalence of MDR *S. aureus* ranges from 13% to 82% (Sunday *et al*., 2020). On a global scale, it plays a major role in causing infections that have been linked with healthcare settings and those acquired within the population (Garba *et al*., 2024)

Furthermore, multidrug-resistant *E. coli* is a primary concern due to their role in nosocomial infections which are not easily treatable, particularly in countries where access to effective antibiotics may be difficult (Ibrahim *et al*., 2023). This high rate of resistance to multiple antibiotic classes by *E. coli* strain may indicate the selection of co-resistance. There is a lack of information available on such high incidences of MDR *E. coli* in hospital environments and clinical samples in Nigeria. However, our results are comparable to studies previously conducted worldwide (Montealegre *et al*., 2018). The excessive use of antibiotics, transfer of resistance genes, and gradual accumulation of mutations are all factors that may explain the high prevalence of antibiotic resistance (Mbanga *et al*., 2023). In addition, the increased resistance may result from excessive use of these antibiotics frequently sold and used within the country (Somorin *et al*., 2022). Our findings suggest that multidrug-resistant *E. coli* and *S. aureus* strains are widely disseminated within hospital environments and their patients, and may include extended spectrum beta-lactam resistant isolates which are a significant hindrance for physicians who face a scarcity of therapeutic options available for treatment (Jalil and Atbee, 2022).

CONCLUSION

The phenomenon of antibiotic resistance is a reality in Nigeria. Our study reports high antibiotic resistance expressed by *E. coli* and *S. aureus* isolates, with high prevalence of MDR genes circulating in the hospital environment, which could serve as evidence for treatment failure of these bacterial infections within the locality. Therefore, to achieve the best possible therapeutic effectiveness, it is essential to explore new and alternative treatment options and appropriate control measures must be designed and implemented to reduce the prevalence of MDR bacteria in the country.

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Contributions of Authors

The lead author designed the study, wrote the framework, supervised all experimental protocol and validation of the original version submitted for publication. The other authors managed the samples collection and implemented laboratory procedures and interpretation of results.

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Conflict of Interest

No conflict of interest is declared

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