Antibiogram of Staphylococcal Isolates and Molecular Characterisation of Coagulase Negative *Staphylococci* obtained from a Hospital in Port Harcourt, Nigeria

ABSTRACT

Difficulty in the treatment and eradication of bacterial infections as a result of the development of resistance to antibiotics has in recent time remained a serious problem confronting public health. This study investigated the antibiogram of Staphylococcal isolates and molecular characterisation of Coagulase Negative Staphylococci (CoNS) obtained from female patients visiting Rivers State University Teaching Hospital (RSUTH) in Port Harcourt, Nigeria. A total of 103 clinical samples were collected and processed using the standard laboratory procedures. Antibiotic susceptibility test of the isolates was performed using Kirby-Bauer disc diffusion method, following CLSI guidelines. Molecular characterisation as well as screening for the detection of antibiotics resistance MecA gene was carried out using Polymerase Chain Reaction (PCR) dependent molecular techniques. From the study, 22.3% *S. aureus* and 1.9% CoNS were recovered. The antibiotics susceptibility test results revealed that all the *Staphylococcus aureus* isolates were 100% resistant to six of the antibiotics tested: Ampicillin, Augmentin, Ceftazidime, Cefuroxime and Cephalexin (β- lactams), 78.3% resistant to Tetracycline and Cotrimoxazole, 73.9% to Vancomycin, 65.2% to Ciprofloxacin, 56.5% to Gentamicin and 47.8% to Erythromycin but 52.2% sensitive to Erythromycin, followed by Gentamicin (34.8%), Ciprofloxacin (17.4%), Vancomycin and Cotrimoxazole (13%) and Tetracycline (8.7%) while Coagulase negative Staphylococci showed 100% resistance to all antibiotics used. Data obtained from the study showed all the isolates displayed multidrug resistance, with MAR indices greater than 0.2. Methicillin (MecA) gene was detected in 66.6% of the isolates while the 16SrRNA gene sequencing revealed two species of CoNS namely *S. saprophyticus* and *S. arlettae*. The study has reported the presence of Coagulase Negative Staphylococci showing resistance to all the tested antibiotics, hence portending alarming public health threats. Strict regulation of antimicrobial usage is therefore vital.

KEYWORDS: Antibiogram, Molecular characterisation, Coagulase Negative *Staphylococci, Staphylococcus aureus,* Females, Hospital, Port Harcourt.

1. INTRODUCTION

The unceasing resistance of bacteria to antibiotics has been a major cause of concern to public health (Gould and Bal, 2013). Antibiotics which were initially effective in the treatment, prevention and control of bacterial infections are now almost of no effect, increasing morbidity rate and lowering predicted life span expectancy. The evolvement of this situation is accelerated by the overuse and misuse of antibiotic as well as lack of development of new drugs (CDC, 2013). The emergence of bacteria that have acquired new resistance mechanisms continues to threaten the ability of healthcare to treat common infections with existing antibiotics. According to a 2016 report from a World Health Organization (WHO) subgroup on antimicrobial resistance, disease caused by antibiotic resistant bacteria could result in 10 million deaths annually by 2050, leading to economic damage as millions of people will be in extreme poverty.

Staphylococci, a group of gram-positive, non-motile, spherical shaped bacteria consisting of 32 species and 8 sub-species are mostly found on the skin and mucous membranes of mammals including humans (Tortora *et al*., 2019). Among these species, *S. aureus, S. saprophyticus* and *S. epidermidis* have been reported to be associated with different clinical syndromes in human. *S. epidermidis* causes hospital-acquired infections, *S. saprophyticus* is linked to urinary tract infections in young sexually active females and *S. aureus* causes superficial skin lesions, osteomyelitis, endocarditis, food poisoning, toxic shock syndrome and Septicaemia while other species such as *S. lugdunensis, S. intermedius S. haemolyticus, , S. schleiferi, S. warneri* are non-pathogenic (Lowry, 1998; Becker *et al.*, 2014). The ability of pathogenic *Staphylococci* to produce the enzyme coagulase, which causes blood clotting, is a frequent way to recognize them. This separates the Coagulase Positive strains of *Staphylococcus* from the Cogulase Negative *Staphylococcal* species (Kloos and Bannerman, 1994). Several types of infections and patterns of antimicrobial susceptibility have been seen among the coagulase-negative species (Abdel-Moein and Zaher, 2020).

Antibiotic resistance occurs when bacteria changes over time (genetic mutation), making antibiotics ineffective and increasing the risk of disease spread. It has been extensively documented that *Staphylococci* isolates in various parts of the world are resistant to regularly used antibiotics (Akortha and Ibadin, 2008; Umolu *et al*., 2002). *Staphylococci* strains possess a range of multi-drug resistance genes on plasmids, which promotes the spread of resistance even between different species (Todar, 2011). Additionally, on the rise is the occurrence of

Methicillin-Resistant *Staphylococcus aureus* (MRSA), which has a very restricted range of available treatments (Onanuga *et al.*, 2005; Nordman and Naas, 2005). The study therefore target the antibiotic susceptibility pattern, molecular characterisation and the resistant genes (MecA) present in Coagulase Negative Staphylococci strains obtained from the study area.

2. MATERIALS AND METHODS

2.1 Specimen Collection

A total of 103 High Vaginal Swab samples were aseptically collected from female patients attending the outpatients department and Antenatal clinic of RSUTH. Samples were analysed in the Microbiology Laboratory of the Hospital immediately after collection.

2.2 Isolation of Organism

Swab samples were inoculated onto Mannitol salt agar medium using streak plate method, inverted and incubated at 37^{o}C for 24 hours (Cheesbrough, 2000).

2.3 Identification of Isolates

After incubation, Isolates were characterised based on their cultural morphology, Gram staining, biochemical properties and Nucleic acid properties. Automated Biometric Identification System (ABIS) online software for bacteria identification was referenced as a standard for bacteria morphological identification (Cheesbrough, 2000).

2.4 Antibiotic Susceptibility Testing

A test tube containing 3-4ml of sterile normal saline was filled with pure isolates. The turbidity of the suspension was then compared with the turbidity standard of 0.5 McFarland of estimated cell count density of 1.5 $X10^{8}$cfu/ml. Sensitivity of isolates to twelve antibiotics Ampicillin (10µg), Meropenem (10µg), Augumentin (30µg), Erytromycin (5µg), Vancomycin (30µg), Tetracycline (30µg), Cotrimoxazole (25µg), Ceftazidime (10µg), Cefuroxime (10µg), Gentamicin (10µg), Cephalexin (15µg) and Ciprofloxacin (5µg) was determined on Mueller- Hinton agar plate using the Kirby-Bauer disk diffusion method (Tendencia, 2004). From a prepared broth containing pure isolates, an aliquot (0.1ml) was sub-cultured onto Mueller- Hinton agar plate using spread plate method, antibiotic disk was picked with sterile forcep and placed on the surface of the agar plate after 10 minutes of inoculation. Antibiotic disk was gently pressed onto the agar surface to ensure firm contact with the agar. Plate was allowed to stand for at least 15 minutes and then incubated in an

inverted position at 35-37^{o}C for 24 hours after which zones of inhibition in diameter (mm) were measured using a meter rule and values were interpreted using the CLSI Zone diameter Interpretative standards as susceptibility (S), intermediate susceptibility (I) or resistance (R) (CLSI, 2016).

2.5 Determination of Multiple Antibiotic Resistance (MAR) Index

Using the formula MAR = a/b, the MAR index was calculated for each isolate, where a, denotes the number of antibiotics to which the test isolate demonstrated resistance and b, denotes the overall number of antibiotics to which the test isolate's susceptibility has been evaluated (Adenaike *et al.,* 2016).

2.6 Molecular Identification of Isolates

All Coagulase Negative Staphylococci isolates obtained were molecularly identified using the following processes.

2.6.1 DNA Extraction

The extraction was carried out using an Inqaba South Africa ZR fungal/bacterial DNA micro prep extraction kit. 750 microliters of lysis solution were added to the tube after a heavy growth of the pure culture of the presumed isolates was suspended in 200 microliters of isotonic buffer. The ZR smashing bead lysis tubes were centrifuged at 10,000xg for 1 minute after being processed for 5 minutes at maximum speed in a bead beater with a 2 ml tube holder assembly attached.

Four hundred microliters of supernatant were transferred to and centrifuged at 10,000 g for one minute in a Zymo-Spin IV spin Filter (orange top) that was positioned in a collecting tube. Fungal/bacterial DNA binding buffer was added to the filtrate, increasing the total volume of the filtrate in the collection tubes to 1600 microliters. After that, 800 microliters were put into a collecting tube and spun at 10,000 x g for one minute using a Zymo-Spin IIC column. The collection tube's flow through was thereafter emptied. The remaining volume was combined with the original Zymo-spin. The Zymo-spin IIC was filled with 200 microliters of the DNA Pre-Wash buffer and 500 microliters of the fungal/bacterial DNA Wash Buffer, and then centrifuged at 10,000 g for one minute. This was done using a fresh collection tube. The DNA was eluted by centrifuging the tube at 10,000 x g for 30 seconds after the Zymo-spin IIC column had been transferred to a clean 1.5 microliter centrifuge tube

and 100 microliter of DNA elution buffer had been added to the column matrix. The extremely pure DNA was then maintained at -20 degrees for a further reaction (Carpi *et al*., 2011).

2.6.2 DNA Quantification

The Nanodrop 1000 spectrophotometer was used to measure the isolated genomic DNA. The Nanodrop icon was double clicked to launch the equipment's application. Two ul of sterile, deionized water were used to initialize the apparatus, and regular saline was used to blank it. Two microliters of the extracted DNA were added to the lower pedestal, and the higher pedestal was then lowered to make contact with the lower pedestal. By selecting the "measure" button, the DNA concentration was calculated.

2.6.3 16S rRNA Amplification

The rRNA gene's 16s rRNA region was amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microliters for 35 cycles. The primers at a concentration of 0.5 uM, the extracted DNA as template, and the X2 Dream taq Master mix (taq polymerase, DNTPs, and MgCl) from Inqaba, South Africa were all components of the PCR mixture. The PCR conditions were as follows: 5 minutes of initial denaturation at 95°C were followed by 40 seconds of denaturation at that temperature, 40 seconds of annealing at 52°C, 35 cycles of extension at 72°C, and 5 minutes of final extension at 72°C. The substance was seen using a blue light transilluminator after 30 minutes at 130V and resolution on a 1% agarose gel (Sune *et al.,* 2020).

2.6.4 DNA Sequencing

Inqaba Biotechnological, Pretoria, South Africa, was used to perform the sequencing using a 3510 ABI sequencer and the BigDye Terminator kit. The BigDye® terminator v1.1/v3.1, 0.25 ul of the 10uM Primer PCR primer, 2.25 ul of the 5 x BigDye sequencing buffer and 2 to 10 ng of PCR template per 100 bp were used in the sequencing, which was done at a final volume of 10 ul. The following were the sequencing requirements: There are 32 cycles of 96°C for 10s, 55°C for 5s, and 60°C for 4 minutes.

2.6.5 Phylogenetic Analysis

The retrieved sequences were edited using the bioinformatics program Trace edit, and related sequences were found using the BLASTN method by searching the National Center for Biotechnology Information (NCBI) database. These sequences were aligned with the aid of MAFFT. The evolutionary history was inferred using MEGA 6.0's Neighbor-Joining method. The Jukes-Cantor method was used to calculate the evolutionary distances, and the bootstrap consensus tree constructed from 500 replicates is believed to reflect the evolutionary history of the organisms under study (Felsenstein, 1985).

2.6.6 Amplification of MecA Genes

Six of the isolates with the highest MAR index were analysed for the presence of MecA gene. Using the primers MecA F: 5' TGGCTATCGTGTCACAATCG-3' and MecA R: 5'-CTGGAACTTGTTGAGCAGAG-3', the isolates' MecA genes were amplified on an ABI 9700 Applied Biosystems thermal cycler for 35 cycles. The PCR mixture included primers at a concentration of 0.5M, Taq polymerase, DNTPs, and MgCl in addition to 50ng of the extracted DNA as the template. The X2 Dream Taq Master Mix from Inqaba, South Africa, was also used. The following conditions were used for the PCR: Following annealing at 55 °C, 35 cycles of extension at 72 °C for 40 seconds, and final extension at 72 °C for 5 minutes, the first denaturation was performed at 94 °C for 5 minutes. The substance was seen using a blue light transilluminator after 25 minutes at 120V and resolution on a 1% agarose gel (Bell *et al.*, 1998).

3. RESULTS

3.1 Frequency of *Staphylococci* isolates

Staphylococci species were present in 25 (24.3%) cases. *Staphylococcus aureus* predominated in 23 (22.3%) of the samples, while Coagulase Negative Staphylococcal species made up only 2 (1.9%) (Figure 1).

3.2 Antibiotic Susceptibility Test

The antimicrobial susceptibility test results are presented in Table 1 and 2. The results showed that *Staphylococcus aureus* isolates were 100% resistant to six of the antibiotics tested: Ampicillin, Augmentin, Ceftazidime, Cefuroxime and Cephalexin (β- lactams), 78.3% resistant to Tetracycline and Cotrimoxazole, 73.9% to Vancomycin, 65.2% to Ciprofloxacin,

56.5% to Gentamicin and 47.8% to Erythromycin but 52.2% sensitive to Erythromycin, followed by Gentamicin (34.8%), Cipofloxacin (17.4%), Vancomycin and Cotrimoxazole (13%) and Tetracycline (8.7%) while all the Coagulase negative Staphylococci (100%) were resistant to all antibiotics used.

3.3 Multiple Antibiotic Resistance (MAR) Indices

Data obtained showed all of the isolates recovered in this study had MAR index greater than 0.2 (Table 3). The value of the Multiple Antibiotic Resistance Index ranged from 0.6 to 1.0. The most common MAR index for *S. aureus* was 0.8 as 30.4% of the isolates were resistant to 10 of the 12 antibiotics tested, while the least common MAR index was 0.6 as 8.7% of the isolates were resistant to 7 of the 12 antibiotics tested while for CoNS the most common MAR index was 1.0 as the isolates were resistant to all the antibiotics tested.

3.4 Identity of Coagulase Negative *Staphylococci* Isolates Base on 16S rRNA Gene Sequence

The isolate's 16S rRNA gene sequence provided a perfect match during the megablast search for extremely analogous sequences in the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA gene analysis of the isolates demonstrated complete species similarity. It's phylogenetic placement within the *Staphylococcus* sp. was consistent with the distance in evolutionary time calculated using the Jukes-Cantor method which showed a close relationship to *Staphylococcus saprophyticus* and *Staphylococcus arlettae* (Figure 2).

3.5 Presence of MecA Resistant Genes

According to Plate 1, Agarose Gel Electrophoresis results, 4 (66.6%) of the six isolates tested positive for the presence of MecA gene. Lane D represents the 100 bp DNA ladder, while Lanes 1, 2, 4, and 5 are the MecA gene bands at 298 bp.

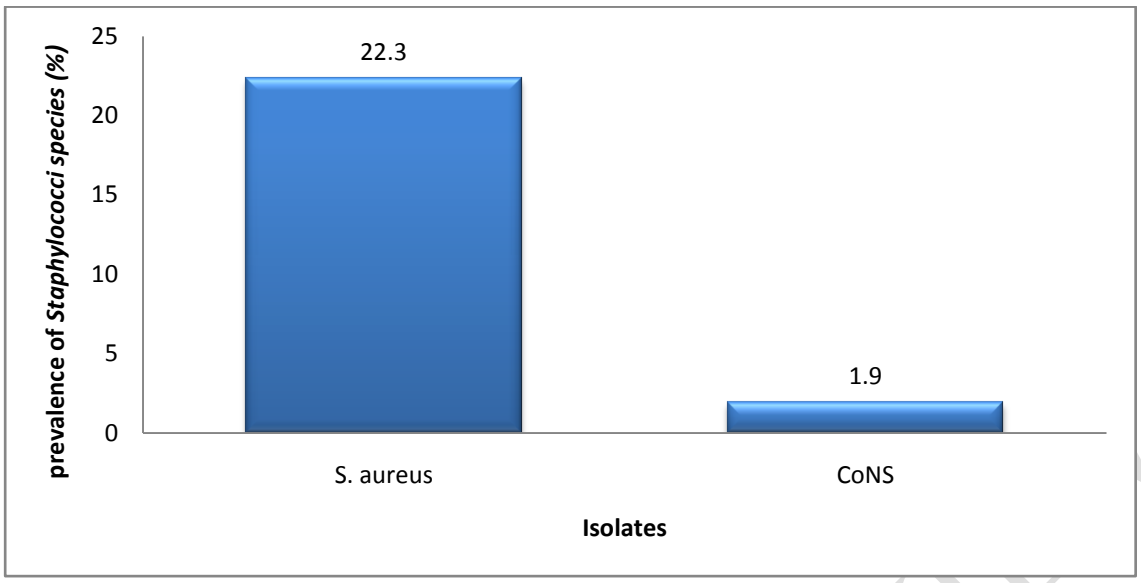

Figure 1: Frequency of *Staphylococci* Isolates recovered from the study (%)

Table 1: Antibiotic Susceptibility Pattern of *Staphylococcus aureus* (n=23)

Antibiotics (Conc.)	**Resistant n (%)**	**Intermediate n (%)**	**Susceptible n (%)**
Ampicillin (10µg)	23(100)	0(0)	0(0)
Meropenem (10 µg)	23(100)	0(0)	0(0)
Augumentin (30 µg)	23(100)	0(0)	0(0)
Erythromycin (5 µg)	11(47.8)	0(0)	12(52.2)
Vancomycin (30 µg)	17(73.9)	3(13)	3(13)
Tetracycline (30 µg)	18(78.3)	3(13)	2(8.7)
Cotrimoxazole (25 µg)	18(78.3)	2(8.7)	3(13)
Ceftazidime (10 µg)	23(100)	0(0)	0(0)
Cefuroxime	23(100)	0(0)	0(0)

(10 µg)			
Gentamicin (10 µg)	13(56.5)	2(8.7)	8(34.8)
Cephalexin (15 µg)	23(100)	0(0)	0(0)
Ciprofloxacin (5 µg)	15(65.2)	4(17.4)	4(17.4)

Table 2: Antibiotic Susceptibility Pattern of Coagulase Negative *Staphylococci* (n=2)

Antibiotics (Conc.)	**Resistant n (%)**	**Intermediate n (%)**	**Susceptible n (%)**
Ampicillin (10µg)	2(100)	0(0)	0(0)
Meropenem (10 µg)	2(100)	0(0)	0(0)
Augumentin (30 µg)	2(100)	0(0)	0(0)
Erythromycin (5 µg)	2(100)	0(0)	0(0)
Vancomycin (30 µg)	2(100)	0(0)	0(0)
Tetracycline (30 µg)	2(100)	0(0)	0(0)
Cotrimoxazole (25 µg)	2(100)	0(0)	0(0)
Ceftazidime (10 µg)	2(100)	0(0)	0(0)
Cefuroxime (10 µg)	2(100)	0(0)	0(0)

Gentamicin (10 µg)	2(100)	0(0)	0(0)
Cephalexin (15 µg)	2(100)	0(0)	0(0)
Ciprofloxacin (5 µg)	2(100)	0(0)	0(0)

Table 3: MAR Indices of *Staphylococci* Isolates (n=25)

	Number (%)	
MAR Index	***S. aureus* (n=23)**	**CoNS (n=2)**
0.1	0(0)	0(0)
0.2	0(0)	0(0)
0.3	0(0)	0(0)
0.4	0(0)	0(0)
0.5	0(0)	0(0)
0.6	2(8.7)	0(0)
0.7	4(17.4)	0(0)
0.8	7(30.4)	0(0)
0.9	6(26.1)	0(0)
1.0	4(17.4)	2(100)

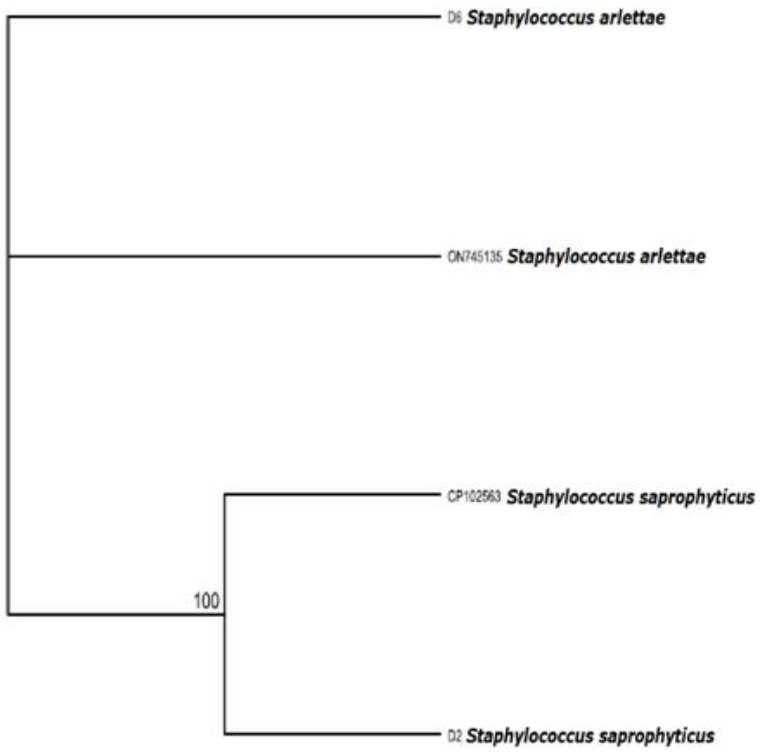

Figure 2: Phylogenetic tree displaying distance in evolutionary time between Coagulase Negative *Staphylococci* isolates

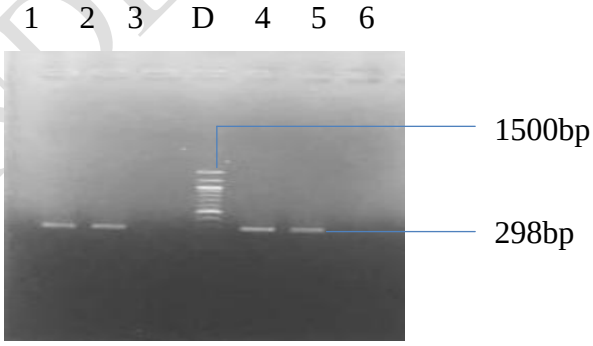

Plate 1: Amplified Products of MecA Genes from Isolates

4. DISCUSSION

Staphylococcus species evolved unique defence and ways to withstand various antibiotics. The only mechanisms of resistance that bacteria employ are the enzymatic degradation of drugs, structural change of the target, and usage of efflux pump (Lowry, 2003). According to the susceptibility test, *Staphylococcus* species obtained were completely resistant to every type of β-lactam antibiotics utilized in the study, including penicillin (Ampicillin and Augmentin), carbapenems (Memopenem), and cephalosporins (Ceftazidime, Cefuroxime and Cephalexin), suggesting that all of the isolates were Methicillin-Resistant *Staphylococcus* (MRSA). The findings of Oyagade and Oguntoyin in 1997 and Uwaezuoke and Aririatu in 2004 that MRSA was implicated in majority of *Staphylococcal* infections are supported by this investigation.

The result of antibiotic susceptibility pattern showed a high resistance of *Staphylococcus aureus* to the test antibiotics; 100% to Methicillin, 78.3% to Tetracycline and Sulfonamides, 73.9% to Glycopeptides, 65.2% to Fluoroquinolones, 56.5% to Aminoglycosides and 47.8% to Macrolides. This demonstrates that widely used antibiotics like Ampillicin, Augmentin and cefuroxime are no longer effective in the treatment of vaginal *Staphylococcal* infection in this area and as such the use of these antibiotics should be discouraged.

Other researchers have reported high resistant rate of Coagulase Negative Staphylococci specifically *S. saprophyticus* to some antibiotics (Jhora *et al.*, 2011; Nandihal and Chikkaraddi, 2021) but 100% resistance to all tested antibiotics has rarely been recorded. The 100% resistance displayed by *S. saprophyticus* and *S. arlettae* in the study is of serious public health concern as treatment of Staphylococcal infection caused by CoNS will be limited to only a few more potent antibiotics which might not readily be available as such increasing the rate of morbidity of these infections.

All *Staphylococcus* isolates obtained had a MAR index greater than 0.2. This implies that there is significant antibiotic abuse in this setting due to frequent self-medication, which is mostly linked to insufficient dosage and noncompliance with therapy as well as the accessibility of antibiotics to consumers over the counter with or without a prescription (James *et al.*, 2017).

The molecular analysis provide detailed information of organisms nucleic acid sequence, confirming Coagulase negative Staphylococcal isolates recovered from the study to be *S.*

saprophyticus and *S. arlettae.* This also has aided the identification of *S. arlettae,* a Staphylococcal species rarely found in the female genital tract and its distinction from *S. saprophyticus* which possesses identical biochemical characteristics. These species are common cause of uncomplicated urinary tract infections (UTIs) especially in young sexually active females (Ravi *et al.,* 2022). The presence of these organisms suggest that women in this region are predispose to factors or engage in practises such as multiple sexual involvement, history of vaginal infection, sharing of underwear, douching, use of public toilets, damp underwear, harsh soap/deodorants and unsafe vagina wash that encourage it proliferation (Oscar *et al.,* 2019; Kurewa *et al.,* 2010).

Genes for drug resistance can be found on the chromosome or in plasmids. Due to its ability in efficiently transferring genetic material both horizontally and vertically between different bacterial species, conjugative plasmid is primarily accused for the emergence of resistance (Grohman *et al*., 2003). The current investigation indicated that 66.6% of isolates had Methicillin A (MecA) genes, this might be responsible for the high resistance of isolates to all β-lactam antibiotics as MecA genes code for the production of abnormal penicillin binding protein which has low affinity for binding β-lactams (Lakhundi and Zhang, 2018). This supports the findings of Hafeez and Aslam (2016) who found that a high proportion of MecA gene was present in vaginal swab samples. This indicates that the microbial population is rapidly evolving multidrug resistant strains, which can be linked to the frequent and careless use of antibiotics that is a widespread practice in Nigeria and other poor nations (Olayinka *et al*., 2004).

CONCLUSION

This research revealed high resistance rates of *Staphylococcus* species to drugs that are frequently used to treat Staphylococcal infections. Women of reproductive age, that engages in frequent and inappropriate use of antibiotic as in the case of self-medication and incomplete treatment have a high likelihood of acquiring resistant strains of *Staphylococcus.* The study therefore, establish the need for strict monitoring of antibiotic usage and antibiotic resistance surveillance to ensure that antibiotic potency is retained and to prevent bacteria from developing total resistance to the last resort antibiotics.

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