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Carriage of Plasmid-mediated β-lactamase genes of *Staphylococcus aureus* isolates from Outpatients and Inpatients of UCTH, Calabar, Cross River State, Nigeria

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Abstract

This study evaluates the carriage of plasmid-mediated β-lactamase genes of *Staphylococcus aureus* isolates from patients of the UCTH, Calabar, Cross River State, Nigeria. 120 swab samples were screened for staphylococci using standard methods. Coagulase-positive staphylococci isolates were subjected to 16S rRNA sequencing for further identification. Disk diffusion method was adopted in testing the susceptibility of all the *S. aureus* strains. β-lactamase activity of *S. aureus* strains was determined using iodometric methods. Plasmid DNA was extracted by alkaline lysis method and agarose gel electrophoresis, while curing was performed in Tryptic Soy Broth (TSB) containing acridine orange. After incubation, the *S. aureus* strains were tested for β-lactamase using iodometric methods. Morphological, biochemical characterization and 16S rDNA sequencing showed thatof the 120 samples collected, 98 (81.7%) yielded staphylococci bacteria, out of which only 9 strains were identified as *S. aureus*. All the *S. aureus* strains were sensitive to ceftriaxone and cefixime and resistant to vancomycin, penicillin and chloramphenicol. There was significant difference ($p \leq 0.05$) in the resistance patterns of the 9 strains of *S. aureus*. All the strains of *S. aureus* showed β-lactamase activity. When these strains were exposed to plasmid-curing agent such as acridine orange, the β-lactamase activity was lost. Susceptibility test against the cured strains using vancomycin, kanamycin and chloramphenicol indicated high sensitivity. The results proved that *S. aureus* isolates besides being β-lactamase producers, are also relatively resistant (plasmid-mediated) to commonly used antibiotics. The study revealed a disturbing pattern of antibiotic resistance phenotypes and β-lactamase production in *S. aureus* isolates.

Keywords: Antibiotic-resistance, β-lactamase, plasmid, iodometric methods.

Introduction

Staphylococcus aureus is a Gram-positive coccal bacterium which belongs to firmicutes, and is frequently found in the human skin and mucous membranes, especially of the nasopharyngeal regions (Ionescu *et al*., 2010). *S. aureus* even though is not always pathogenic, it is the common cause of skin infections (e.g. boils), respiratory disease (e.g. sinusitis), and food poisoning. Disease-associated strains of *S. aureus* often promote infections by producing potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies (Haziz *et al*., 2018).

Before the introduction of penicillin in 1940, 90% mortality had been reported in human patients due to *S. aureus* infections. The advent of penicillin era, recorded remarkable improvement in both recovery and survival rates of infected patients (Shrestha and Suman, 2014). However, not too long after penicillin came into clinical use that the development of resistance against penicillin was reported (Ngwai *et al*., 2016), and *S. aureus* was found to be one of the microbes that developed rapid resistance against the drug that it came in contact with. The ability of *S. aureus* to rapidly develop resistance against antibiotics, including beta-lactam agents (penicillin, cephalosporins and carbapenems), has been attributed to the possession of a gene called *mec* A, located on the staphylococcal cassette chromosome. *Mec* A encodes the production of an altered 75kb penicillin-binding protein 2a (PBP2a) that is responsible for low affinity to all beta-lactam antibiotics and other antimicrobials (Kwon *et al*., 2006; Weese and van Duijkeren, 2010; Dahshen *et al*., 2015).

Today, *Staphylococcus aureus* has become resistant to many commonly used antibiotics. The emergence of resistance has resulted in major clinical crises and has created public health concern globally. The best known mechanism of resistance to beta-lactam drugs is the production of beta-lactamases, which is not chromosomal but rather plasmid-mediated and can be non-inducible or inducible with antibiotics (Nagel *et al*., 2011;Pugazhendhi *et al*., 2020).

A major determinant of most clinically significant antibiotic resistance is genes that are located on extra chromosomal DNA elements called plasmids, some of which can mediate their own transfer by conjugation (Nagel *et al*., 2011). Dissemination of antibiotics resistance genes by horizontal gene transfer is what has led to the rapid emergence of antibiotic resistance among clinical isolates (Yassin *et al*., 2013). Various studies have revealed the extensive roles played by plasmids (Nsofor *et al*., 2013). Plasmids often carry drug resistance genes and can sometimes confer resistance to a number of different drugs. Strains resistant to these antibiotics mediatedby β-lactamases have now spread worldwide (Baqero *et al*., 2014).

The production of beta-lactamase encoded in the blaZ gene which is located on the transposable part of the large plasmid within the *S. aureus* bacterial cells confers resistance to various antibiotics (Shrestha and Suman, 2014), and this has invariably increased the importance of *S. aureus* infections. This gene is also said to be easily transferable horizontally from one species of bacteria to another, perhaps because of its strategic location on the plasmid (Lowry, 2003).

The production of β-lactamase normally protects *Staphylococcus aureus* against penicillin and other beta-lactam antibiotics (Ali *et al*., 2004). Beta-lactamase enzymes hydrolyze the beta-lactam bond of both penicillin and cephalosporins, thereby rendering these antibiotics ineffective. Penicillinase resistant beta-lactam antibiotics such as methicillin, nafcillin, oxacillin, vancomycin, cloxacillin, dicloxacillin and flucoxacillin are able to resist degradation by staphylococcal penicillinase (Olowe *et al*., 2007). Nevertheless, there are empirical findings on the development of resistance to a few of these antibiotics including methicillin and vancomycin also known as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA), respectively (Ali *et al*., 2004; Sileshi *et al*., 2018).

Little information on beta-lactamase coagulase-positive *S. aureus* isolation from hospital have been documented in humans in Nigeria (Akindele

et al., 2010; Torimiro *et al*., 2013; Shaikh *et al*., 2015). But there is a dearth of literature on beta-lactamase productions of coagulase-positive *S. aureus* in both outpatients and hospitalized patients, particularly in south-southern region of Nigeria. This study was therefore initiated to determine the carriage of plasmid-mediated beta-lactamase genes of *Staphylococcus aureus* isolates from randomly selected outpatients and inpatients at the UCTH Calabar, Cross River State, Nigeria.

Materials and Methods

Study design and subjects

This investigation was carried out at University of Calabar Teaching Hospital, Calabar, Cross River State, Nigeria, from October, 2018 to September, 2019. The hospital provides diverse services for patients from different parts of the country. Therefore the study design was hospital-based in which outpatients from ear, nose, and throat (ENT) ward and inpatients from the general surgical and orthopaedic wards were captured in the design. Patients with clinical manifestations of surgical site infection having surgical wound with pus discharge, signs of sepsis as well as those diagnosed for surgical site infection from general surgical and orthopaedic wards were enrolled. These patients were hospitalized for various period and were treated with different antibiotics including ceftriaxone, metronidazole, vancomycin, cefixime, oxacillin, gentamicin, chloramphenicol, etc. In addition, patients with acute and chronic otitis media with clinically proven discharge from ENT outpatient ward were randomly recruited. These patients were those that had history of recent treatment with ciprofloxacin, amoxicillin+clavulanic acid, tetracycline, etc., and just a few that had no history of recent antimicrobial therapy. Only patients who volunteered willingness to participate in the study were involved.

Sample collection, isolation and identification of *Staphylococcus aureus*

120 swab samples were aseptically collected from 120 out- and inpatients. The breakdown was as follows: wound swab from those with surgical site infection (i.e., 18 from general surgical ward, and 37 from orthopaedic ward), and 65 ear swab from patients with otitis media showing clinical symptom of ear discharge were collected. The wound site and ear were first cleaned with sterile saline to remove any purulent debris. Sterile cotton swab was moistened with normal saline and rotated three times on the wound surface and ear opening and placed in test tubes containing 10 ml of sterile Trypton Soya Broth (TSB), (BD, Diagnostic Systems, Heidelberg, Germany). The samples were transported in ice packed coolers to the Microbiology Laboratory of the Cross River University of Technology, Calabar, within 1-2 hours of collection and were immediately incubated at 37 °C overnight.

After overnight growth in TSB, loopful of the suspension was evenly streaked into mannitol salt agar (MSA) (Oxoid, Basingstoke, Hampshire, England) and incubated at 37 °C for 24 hours. Bacterial colonies that yielded characteristic small, round, yellow or golden yellow colour with the MSA were selected as presumptive staphylococci. These isolates were subjected to routine morphological identification tests such as gram stain, catalase and coagulase to differentiate from coagulase-negative staphylococci. The isolates from fermented plates were further identified by colony morphology and biochemical characterization- haemolysis, oxidase, IMViC, triple sugar iron agar test, urease test, MacConkey agar, DNase test using standardized protocol (Cowan, 1974; Ochei and Kolhatkar, 2008; Chandra and Mani, 2011). The 16S rRNA sequencing was carried out for further identification of the isolates at species level.

Antimicrobial susceptibility testing

Antimicrobial susceptibility test for nine (9) *Staphylococcus aureus* strains was carried out against a panel of 15 antimicrobials using Kirby Bauer disc diffusion method as was described by the Clinical Laboratory Standard Institute, (CLSI) (NCCLS, 2002). The bacterial culture was grown in TSB for 4–5 h at 37 °C and the inoculum size was adjusted with 0.5 McFarland standard. A sterile swab stick was dipped into a testtube containing the inoculum, squeezed against the inside of the test tube to remove excess fluid and then streaked all over the surface of the plate containing Mueller Hinton agar medium (MHA) (Oxoid, Basingstoke, England) to obtain a uniform spread in all direction. The plates were allowed to stand for 10 minutes for the agar surface to dry. Using a cooled previously flamed forceps, the commercially prepared antibiotic discs were picked and placed at least 50mm apart from the edge of the plate to prevent overlapping of the growth inhibition zone. The plates were allowed to stand for few minutes before incubation at 37^{o}C for 18-24 hours. After incubation, the diameters of the zone of growth inhibition were measured in millimetre using a transparent meter rule and interpreted according to the British Society for Antimicrobial Chemotherapy (BSAC) standards (Shiferaw *et al*., 2005). The growth inhibition zones per antibiotic were measured in triplicate and the standard error determined. The following antibiotics with disc (Sensi-Discs, Becton, Dickinson and Company, Sparks, MD) concentrations in micrograms (µg) were used for the study: oxacillin (OX: 10), penicillin G (P: 10), ampicillin (PN: 30), Kanamycin (KA: 20), amoxicillin+clavulanic acid (AMC: 30), ceftriaxone (CRO: 30), chloramphenicol (C: 30), cefuxime (CEF: 30) ciprofloxacin (CIP: 10), erythromycin (E: 10), gentamicin (GM: 10), amoxil (AX; 20), sulphamethoxazole-trimethoprim (SXT: 25), tetracycline (TE: 30), and vancomycin (VAN: 30).

Detection of Beta-lactamase production

Beta-lactamase production test was carried out for all the nine (9) strains showing resistance to one or more beta-lactam antibiotics using the three iodometric methods, as previously described by Devapriya *et al*. (2013).

Filter Paper Method

Soluble starch (0.2g) was added to 100ml of distilled water and dissolved by boiling. After cooling, 1g of penicillin was added. Whatman filter papers were cut into strips (1×5cm) and soaked in the solution. Thereafter, they were air dried for 2 hours. The strips were inoculated with a loop-full of freshly isolated test organism by rubbing the colonies onto the paper strip in a circular manner to cover an area of approximately 5mm in diameter. The inoculated filter paper was covered with a petri dish and incubated for 30 minutes at 37^{o} C. Iodine solution was flooded over the filter paper and the excess solution on the filter paper was drained. The test for beta-lactamase was considered positive when the inoculation site turned colourless within 10 minutes while the rest of non-beta-lactamase producers retained the purple colour.

Tube Method

A solution of penicillin containing 0.5ml volume was dispensed into small test tubes. A loopful of an overnight culture grown on a solid medium of the test organism was suspended in the penicillin solution to give an inoculum size of 10^4 CFU/ml. Two drops of starch indicator were added to the suspension after one hour at room temperature, followed by one drop of iodine reagent. The immediate disappearance of blue colour indicates positive reaction, while persistence of blue colour for longer than 10 minutes shows negative result.

Agar Method

A nutrient agar medium containing 0.2% starch was inoculated with pure colonies of *Staphylococcus aureus.* Penicillin solution was added onto the grown culture plate after overnight incubation at 37^0C. After 15 minutes, the excess penicillin solution was poured off. Iodine solution in 1:5 dilution was added and spread over the growth and later the excess solution was removed by inverting the plate. Reading was taken after 30 minutes at room temperature. The isolate producing beta-lactamase would produce characteristic decolourization around the beta-lactamase producing colonies. Positive and negative control organisms were utilized as controls.

Isolation and electrophoretic pattern of plasmid DNA

Plasmid extraction was carried out using alkaline lysis method as described by Marko *et al*., (1982). Agarose gel electrophoresis of plasmid DNA from 9 strains of *Staphylococcus aureus* was carried out in line with the Standard Protocol Methods (Sambrook and Russell, 2001).

Curing of the plasmid

All the 9 beta-lactamase positive strains of *S. aureus* were incubated in Tryptic Soy Broth (TSB) containing acridine orange at 37 °C for 24 h. After incubation, the strains were streaked onto medium consisting 0.3% yeast extract, 3% TSB and 1.5% agar-agar. After 24 h of incubation, the isolates were tested for beta-lactamase activity based on the three iodometric techniques.

The susceptibility testing for cured strains were carried out on MH agar plates with and without penicillin (1%) and incubated for 48h at 37^oC and observed for growth. Antibiotic susceptibility test was performed with antibiotics that showed resistant previously with vancomycin, Kanamycin and chloramphenicol. The plasmid evaluation was again performed and assayed for the presence of plasmid.

Data analysis

One way analysis of variance was used to compare the difference in the level of resistance amongst the different strains of *Staphylococcus aureus*. The difference between the means was considered significant at $p \leq 0.05$.

Results

The study has revealed a number of very fascinating results.

Table 1 shows the results of prevalence of staphylococciin the 120 samples collected from both outpatients and inpatients in the UCTH Calabar. In a summary, of the 120 samples, 46 (38.3%) Gram positive and coagulase positive Staphylococci were isolated, of which only 9 strains were identified as *Staphylococcus aureus* based on morphological, biochemical characterisation and 16S rRNA sequencing.

Table 1: Carriage and prevalence of staphylococci from different sampling groups

Study group	Total	Staphylococci					Total	%
	COPS	No.	%	CONS	No.	%		
General Surgery Ward	18	12	66.6		4	22.2	16	88.8
Orthopedic Ward	37	10	27		20	54.1	30	81.1
ENT Ward (Otitis Media)	65	24	37		28	43.1	52	80.0
Total	**120**	**46**	**38.3**		**52**	**43.1**	**98**	**81.7**

Key: COPS: Coagulase-positive staphylococci, CONS: Coagulase-negative staphylococci, ENT: Ear, Nose and Throat

Table 2: Antibiotic susceptibility profile of Gram-positive *Staphylococcus aureus* strains

	Mean zones of inhibition measured in millimeters								
Antibiotic	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**
Vancomycin	10±1.5(R)	09±1.2(R)	11±1.4(R)	10±1.8(R)	12±1.5(R)	08 ±1.2(R)	09±1.5(R)	11±1.2(R)	12±1.8(R)
Chloramphenicol	11±1.4(R)	10±1.2(R)	11±1.4(R)	12±1.4(R)	14±1.2(I	10±1.7(R)	12±1.5(R)	11±1.7(R)	14±1.4(I)
Amoxi.+Clav. Acid	21±1.7(S)	15±1.4(I)	20±1.5(S)	21±1.2(S)	19±1.5(S)	22±1.8(S)	11±1.4(R)	20±1.2(S)	21±1.7(S)
Erythromycin	24±1.4(S)	15±1.5(I)	14±1.5(I)	12±1.8(R)	16±1.4(I)	14±1.2(I)	16±1.5(I)	15±1.7(I)	16±1.5(I)
Ampicillin	20±1.4(S)	25±1.2(S)	23±1.7(S)	20±1.8(S)	26±1.2(S	25±1.2(S)	24±1.4(S)	19±1.2(S)	15±1.5(I)
Penicillin	10±1.2(R)	11±1.2(R)	11±1.8(R)	09±1.4(R)	08±1.2(R)	11±1.4(R)	12±1.2(R)	08±1.5(R)	09±1.8(R)
Kanamycin	11±14(R)	13±1.8(I)	14±1.2(I)	12±1.5(R)	13±1.7(I)	14±1.4(R)	14±1.5(I)	15±1.5(I)	11±1.8(R)
Ciprofloxacin	26±1.2(S)	26±1.8(S)	24±1.2(S)	20±1.5(S)	26±1.7(S)	23±1.4(S)	15±1.5(I)	12±1.5(R)	25±1.8(S)
Cefixime	29±1.2(S)	31±1.5(S)	24±1.2(S)	20±1.5(S)	21±1.7(S)	22±1.4(S)	27±1.7(S)	26±1.7(S)	30±1.2(S)
Amoxi	25±1.5(S)	12±1.5(R)	25±1.4(S)	26±1.7(S)	24±1.8(S)	23±1.4(S)	25±1.7(S)	29±1.2(S)	26±1.4(S)
Oxacillin	21±1.5(S)	19±1.2(S)	12±1.5(R)	20±1.7(S)	14±1.8(I)	21±1.4(S)	20±1.7(S)	22±1.8(S)	28±1.4(S)
Ceftriaxone	21±1.5(S)	19±1.4(S)	24±1.5(S)	25±1.5(S)	23±1.7(S)	25±1.2(S)	28±1.5(S)	26±1.8(S)	27±1.4(S)
Tetracycline	14±1.5(I)	25±1.4(S)	22±1.2(S)	21±1.5(S)	15±1.7(I)	29±1.2(S)	26±1.5(S)	18±1.2(S)	29±1.8(S)
Sulphamethox.+Trim.	18±1.4(S)	19±1.2(S)	18±1.5(S)	22±1.5(S)	29±1.7(S)	20±1.4(S)	20±1.5(S)	14±1.8(I)	22±1.8(S)
Gentamycin	26±1.4(S)	24±1.2(S)	21±1.5(S)	16±1.5(I)	26±1.7(S)	19±1.4(S)	23±1.4(S)	22±1.8(S)	27±1.8(S)

Key: S=Sensitive; R=Resistance; I=Intermediate; ± =Standard error, I-IX= strains of *S. aureus*

The result in Table 2 shows that isolates of bacterial strains were variously susceptible to many antibiotics that were tested against them. It was also found that there were variations in the resistance pattern of the strains for each antibiotic. Thus, all the 9 isolates of *Staphylococcus aureus* strains exhibited resistance to penicillin, and to the polypeptide antibiotic vancomycin. On the contrary, 8 strains showed sensitivity to semisynthetic antibiotic ampicillin while 7 strains showed sensitivity to oxacillin. Intermediate sensitivity was seen in 7 strains for the macrolide antibiotic, erythromycin. All the strains showed sensitivity to the third generation cephalosporins, cefixime and ceftriaxone. Seven strains were sensitive to amoxicillin+clavulanic acid, ciprofloxacin and tetracycline. Only one strain was found to show intermediate susceptibility to amoxicillin+clavulanic acid, ampicillin, ciprofloxacin, oxacillin, sulphamethoxazole+ trimethoprim, and gentamycin. Seven *Staphylococcus aureus* strains showed resistance to chloramphenicol, two strains showed intermediate sensitivity to the same antibiotic. Each aminoglycoside antibiotics such as erythromycin, oxacillin, vancomycin, kanamycin and chloramphenicol showed varying resistance patterns. Eight strains were sensitive to gentamycin and one strain showed intermediate sensitivity. Four strains exhibited resistance to kanamycin while 8 strains were sensitive to sulphamethoxazole+trimethoprim.

All the 9 strains of *Staphylococcus aureus* investigated for β-lactamase production showed positive results for beta-lactamase elaboration in all the three iodometric methods as shown in Table 3.

Electrophoretic results of the plasmid isolation from the 9 *Staphylococcus aureus* strains revealed that all the strains harboured plasmid in their cells. The molecular size of the plasmid DNA was calculated to be ~14 kb. In this study, λ DNA (Eco RI /Hind III double restriction enzymes or digest) was used as DNA ladder or marker DNA.

Table 3: Use of iodometric methods to detect Beta-lactamase production in *Staphylococcus aureus* strains

	***Staphylococcus aureus* strains**									
Iodometric Method	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**%**
Tube	+	+	+	+	+	+	+	+	+	100
Agar	+	+	+	+	+	+	+	+	+	100
Filter Paper	+	+	+	+	+	+	+	+	+	100

%=Percentage detection of beta-lactamase, I-IX= strains of *S. aureus*

Table 4 shows the detection of beta-lactamase activity of plasmid cured strains subjected to the three iodometric test methods. The results indicated no colour change after the addition of iodine, thus providing evidence of no beta-lactamase activity.

While the cured strains inoculated in MHA without penicillin showed growth, the MHA with penicillin showed no growth. Antibiotic susceptibility tests performed with the cured strains using Vancomycin, Kanamycin and Chloramphenicol showed that (Table 5), the strains were susceptible to the three antibiotics, and the strains that showed intermediate sensitivity before curing, became highly sensitivity with wider zones of inhibition.

Table 4: Use of iodometric methods to detect Beta-lactamase production in *Staphylococcus aureus* strains after plasmid is cured

	***Staphylococcus aureus* strains**									
Iodometric Method	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IV**	**%**
Tube	-	-	-	-	-	-	-	-	-	0
Agar	-	-	-	-	-	-	-	-	-	0
Filter Paper	-	-	-	-	-	-	-	-	-	0

%=Percentage detection of beta-lactamase, I-IX= strains of *S. aureus*

Table 5: Sensitivity profile of plasmid cured *Staphylococcus aureus* strains

	Mean zones of inhibition measured in millimetres								
Antibiotic	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**
Vancomycin	25±1.5(S)	23±1.2(S)	26±1.4(S)	29±1.8(S)	25±1.5(S)	23±1.2(S)	22±1.5(S)	28±1.2(S)	23±1.8(S)
Chloramph--enicol	24±1.4(S)	27±1.2(S)	30±1.4(S)	28±1.4(S)	24±1.2(S)	26±1.7(S)	24±1.5(S)	23±1.7(S)	27±1.4(S)
Kanamycin	19±1.7(S)	25±1.4(S)	27±1.5(S)	18±1.2(S)	20±1.5(S)	22±1.8(S)	21±1.4(S)	27±1.2(S)	23±1.7(S)

± = Standard error; S=Sensitivity; I-IX = Strains of *S. aureus*

Discussion

The objective of the current study was to determine the carriage of plasmid-mediated beta-lactamase genes of *Staphylococcu saureus* isolates from randomly selected outpatients and inpatients at the University of Calabar Teaching Hospital, Calabar, Cross River State, Nigeria. According to the results obtained, the overall prevalence of coagulase-positive staphylococci amongst the patients in the three wards used in the study was 46 (38.3%) out of 120 samples collected. Of this number, 9 strains were identified as *Staphylococcus aureus* based on morphological, biochemical characteristics and 16S rRNA sequencing. Over the past decades, staphylococcal infections have been prevalent with different rates in various hospital and communities in both developed and under developed nations of the world (Devapriya *et al*., 2013;Gaurav*et al*., 2019). The adaptive ability of *S. aureus* accounts for its ubiquity and survival strategies.The result of this study is consistent with the findings of a similar investigation conducted in the southeast of Nigeria by Chijioker *et al*. (2016), who reported isolation rate of 37.8% from 424 clinical samples evaluated. Also, similar prevalence of *S. aureus* was reported from patients with pus/wound discharge at Gondar University hospital in north Ethiopia (Muluye *et al*., 2014).

Development of resistance to antibiotics by *Staphylococcus aureus* has become a major public health concern worldwide most probably due to the fact that they are very often associated with hospital and community-acquired infections. However, these bacteria have been found to exhibit a great deal of versatility in their behaviour towards antibiotics as some strains have been observed to evolve resistance to most commonly used antibiotics. All the 9 isolates of *Staphylococcus aureus* strains showed resistance to penicillin and the polypeptide antibiotic vancomycin. On the contrary, 8 strains showed sensitivity to semisynthetic antibiotic ampicillin and 7 strains were sensitive to oxacillin. Intermediate sensitivity was seen in 7 strains for the macrolide antibiotic, erythromycin. All the strains of *S. aureus* showed sensitivity to the third generation cephalosporins, cefixime and ceftriaxone while seven strains weresensitive to amoxicillin+clavulanic acid, ciprofloxacin and tetracycline.

Only one strain was found to show moderate susceptibility to amoxicillin+clavulanic acid, ampicillin, ciprofloxacin, oxacillin and sulphamethoxazole+trimethoprim. While seven *Staphylococcus aureus* strains exhibited resistance to chloramphenicol, two strains showed moderate resistant to the same antibiotic. Eight strains were sensitive to gentamycin and one strain showed intermediate sensitivity. Four strains exhibited resistance to kanamycin while 8 strains were sensitive to sulphamethoxazole+trimethoprim. The antibiotic susceptibility patterns are presented in Table 3. The results showed variation in the resistance pattern of the 9 strains of *S. aureus* to different antibiotics. This could be due to localized infections with different strain of *S. aureus* at the different sites of specimen collection and the fact that the patients were exposed to different types of antimicrobials before this study was conducted. Another possible reason could be the difference in level of previous exposure to antimicrobial agents, which might not present the same concentration of antibiotic at the infection site for all patents due to difference in body weights, degree of infection and dosages. This observation agrees with the findings of Sileshi *et al*. (2018), who evaluated the antimicrobial resistance profile of *Staphylococcus aureus* isolated from patients with infection at Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia.

Penicillin inhibits synthesis of bacterial peptidoglycan which of course, is the major cell wall component that gives rigid mechanical stability due to its highly cross linked lattice wall structure in the bacterium (Dahshen *et al*., 2015). In this study, 9 isolates of *Staphylococcus aureus* strains from clinical specimens were highly resistant to penicillin; and the resistance may be due to the excessive rational or irrational use of the penicillin. The inherent weakness of penicillin is because of the attack of ring nucleus by beta-lactamase produced in *Staphylococcus aureus* (Esimone *etal*., 2006). Resistance to beta-lactam antibiotics by *S. aureus* have been reported in several empirical studies (Devapriya *et al*., 2013; Sunday *et al*., 2019; Gaurav *et al*., 2019).

Also in this study, all *S. aureus* strains were found to be resistant to vancomycin. The development of resistance occurs through different mechanisms such as chromosomal mutations or through plasmids, and it may also be due to the linkage of resistance genes from old antibiotics to the latest ones which most often are available in different markets and thus commonly sold over the counter (Summers, 2002). *Staphylococcus aureus* strains from clinical samples have been found to be vancomycin-resistant (Naseer and Jayaraj, 2010). However, 100% sensitivity to vancomycin in multidrug resistant *Staphylococcus aureus* has also been reported by Pikala *etal*. (2007).

Of particular concern is that 8 strains in this study were sensitive to ampicillin and 7 strains were sensitive to oxacillin contrary to reports from previous studies where resistance to ampicillin and oxacillin was observed to be 82.2% and 88.5% of *S. aureus* isolates from patients with surgical site infection at Debre Markos Referral Hospital, northwest Ethiopia, respectively, (Kahsay *et al*., 2014). On the other hand, it is in agreement with the report, which asserts that 90% sensitivity to ampicillin and oxacillin was recorded in *S. aureus* strains isolates from patients admitted to Felege Hiwot referral Hospital, North Ethiopia (Mulu *et al*., 2012). Such difference could be attributed to variation in patient hospital stay, level of infection control practices by health facilities, and previous exposure of patient to antimicrobials (Harrop *et al*., 2012).

In this investigation, seven strains showed resistance to chloramphenicol while 4 strains were resistant to Kanamycin. *S. aureus* has been shown to be resistant to chloramphenicol in other studies (Salimnia and Brown, 2005). Kanamycin resistance has proven to be due to a plasmid encoded determinant (Storrs *etal*., 2010). Very unlike the report of the previous study where, erythromycin was said to have proved effective antibiotic against *Staphylococcus aureus* (Hassam *etal*., 2013),in this study erythromycin moderate sensitivity strains were observed which may have made the treatment difficult. It was also observed in this investigation that all the strains were

sensitive to the third generation cephalosporins ceftriaxone and cefixime. Gaurav *et al*., (2019) also reported the sensitivity of isolates of *Staphylococcus aureus* strains from patients to ceftriaxone and cefixime. Statistical analysis revealed that there was significant difference ($p \leq 0.05$) in the resistance patterns of the 9 strains of *S. aureus* isolated from the patients.

All the nine (9) strains of *Staphylococcus aureus* assayed for beta-lactamase production showed positive results for beta-lactamase activity in all the three iodometric test methods. Beta-lactamases are enzymes that have the ability to hydrolyze the β-lactam ring of the β-lactam antibiotics (Olowe *et al*., 2007), thereby inactivating the antibiotics, and thus bring about therapeutic failure. Beta-lactamase production is a very important cause for antimicrobial resistance to beta-lactam drugs in bacteria. The production of these enzymes enhances the ability of *S. aureus* to resist a wide range of antibiotics including some cephalosporins (Tassew *et al*., 2016). *Staphylococcal* beta-lactamase is present in plasmids and may be either non-inducible or inducible in the presence of antibiotics (Maddux, 1991). Tarfour *et al*., (2005) had reported earlier that beta-lactamase activity in *S .aureus* and *E. coli* isolates from patients in a hospital were 8.8 % and 11%, respectively. In another scientific reports, *S. aureus* isolated from various clinical samples were said to have exhibited beta-lactamase activity, and also demonstrated multiple drug resistance (Salimnia and Brown, 2005; Haziz *et al*., 2018).

Electrophoretic pattern of plasmid from the 9 isolates of *Staphylococcus aureus* strains showed that all the strains harboured plasmid whose molecular size of the plasmid DNA was found to be ~21 kb when λ DNA (Eco RI /Hind III double digest) was used as DNA ladder or DNA marker.

The cured strains which were subjected to iodometric tube test method, showed no colour change after the addition of iodine, thereby confirming the absence of beta-lactamase activity. The cured strains inoculated in MHA without penicillin showed growth whereas MHA with penicillin showed no growth. This study had also established that antibiotic susceptibility tests for the cured strains which were performed with Vancomycin, Kanamycin and Chloramphenicol, proved that all those antibiotics for which the strains showed resistance before curing, had high susceptibility (Table 5). This means that beta-lactam resistance was lost during curing process, thus confirming that the beta-lactamase production is plasmid-mediated. The resistance patterns also revealed the presence of multidrug resistant plasmids in the bacterial isolates. Plasmid isolation and agarose gel electrophoresis for the cured strains also indicated that the strains had lost their plasmid during curing. Different strains of *Staphylococcus aureus* possessed plasmids of different molecular weights. In this study the isolated plasmid was ~14 kb. The presence of plasmids is invariably an indication that the antibiotic resistance is plasmid-mediated. It could be suggested therefore that complete plasmid genome sequencing will help in offering better explanation and thus broaden the understanding of the actual mechanism involved in this action. In earlier published works, multidrug resistant *S. aureus* isolates possessed ~23 kb plasmid (Rahman *et al*., 2005) and ~13 kb plasmid (Pugazhendhi *et al*., 2020).

Plasmid curing was carried out with acridine orange for the isolated of *Staphylococcus aureus* strains, and this agrees with an earlier similar study in which *Streptococcus* and *Micrococcus* plasmids were cured using acridine orange (Dhanarani *etal*., 2009), In another study, the loss of antibiotic resistance and plasmid were said to be interrelated. The loss of 6.4- and 3.4-kb plasmid in *Bacteroide fragilis* C68c was attributed to antibiotic resistance (Nakano *et al*., 2004).

The study has also shown negative result for beta-lactamase activity when cured strains of *Staphylococcus aureus* were tested. Antibiotics which were ineffective or moderately effective initially, turn out to become very active after the strains were cured, implying therefore that cured plasmid strains produced sterile culture on media containing penicillin.

All the experiments have proved that a determinant that was present in the strains before curing, and which was the reason for the exhibition of multidrug resistance by the strains, was lost during the process of curing. Therefore, it can be inferred that this factor, which is actually responsible for the antibiotic resistance, was midwifed by a plasmid.

Conclusion

The present study indicates that the *Staphylococcus aureus* strains isolated from the patients are found to be multi-drug resistant and harbour plasmids. Antibiotic resistant bacteria can spread widely in hospital settings except adequate control measures are put in place to checkmate these constantly-evolving organisms. Plasmid-mediated antibiotic resistance is a global health challenge as resistant bacteria spread from one host to the other by horizontal gene transfer. The acquisition of these genes makes treatment for these opportunistic pathogens difficult. Vancomycin, which is considered the last drug easily prescribed for Staphylococcalinfections also showed high resistance, further compounding the treatment problems. It has therefore become very imperative to discourage the unnecessary and indiscriminate use of antibiotics for almost every infection without professional and proper diagnostic practices.

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Ethics approval and consent to participate

Study protocol was approved by the Ethical Committee of Cross River State Ministry of Health, Calabar. Individual verbal informed consent was obtained from all adult participants and the parents or guardians of all children who participated in the study following adequate explanation about the purpose of the study.

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