Plasmid Profiling and Prevalence of Methicillin-Resistant *Staphylococcus aureus* from Patients in Abakaliki, Nigeria

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Abstract: Methicillin-resistant *Staphylococcus aureus* is a serious global threat. Thus, this research focused on plasmid profile and prevalence of methicillin-resistant *Staphylococcus aureus* isolated from patients in Abakaliki, Nigeria. A total of 454 clinical specimens were obtained and screened for presence of beta-lactamase and methicillin-resistant *Staphylococcus aureus* using nitrocefin sticks; oxacillin and cefoxitin antibiotic disc. Antibiotic susceptibility of the isolates were determined using disc diffusion method according to Clinical Laboratory Standard Institute. Plasmid profiles were analyzed using agarose gel electrophoresis. A total of 41 beta-lactamase producing and 36 methicillin-resistant *Staphylococcus aureus* were detected from the specimens with percentage prevalence's of 23.0 and 20.2 respectively. The isolates were highly resistant to cefoxitin (60.9% to 73.0%), cloxacillin (67.6% to 78.0%), cefotaxime (36.4% to 73.9%) and ceftazidime (32.4% to 52.2%). The isolates had very high percentage susceptibility range to ciprofloxacin (77.1 to 91.3), ofloxacin (79.7 to 87.0) and gentamicin (64.4 to 65.2). All strains of methicillin-resistant *Staphylococcus aureus* were susceptible to vancomycin. Different antibiotic resistance patterns were recorded among the *S. aureus* to other antibiotics. The presence of multiple plasmid DNA was in 32 (18.0 %) clinical isolates. This study reported significant prevalence of MRSA, multiple plasmids and beta-lactamase producing *S. aureus* in clinical specimens. Thus, a serious global problem and public health threat that calls for a strict measure in the choice of drugs used in the treatment of illnesses.
Keyword: Plasmid profile; Prevalence; MRSA; Patients

INTRODUCTION

The emergence of methicillin-resistant *Staphylococcus aureus* is a serious public health problem. This incidence is increasing globally at an alarming rate (David and Daum, 2010; Mishera *et al*., 2013). Methicillin-resistant *Staphylococcus aureus* is nowadays a major cause of life-threatening infections ranging from acute to chronic with significant mortality and morbidity (Blain *et al*., 2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) causes wide range of human infections such as endocarditis, bacteremia, septicaemia, osteomyelitis, septic arthritis, folliculitis, impetigo, cellulitis, necrotizing pneumonia, post-operation infection and toxic shock syndrome (Yunlei *et al*., 2020). These infections can be fatal, invasive and can lead to toxic conditions due to the presence of Panton-Valentine Leukocedine (PVL) gene which is encoded by two contagious genes LUKF-PV and LUKS-PV (Boakas *et al*., 2011; Dhanoa *et al*., 2012). MRSA has developed resistant to multiple classes of antibiotics, thereby complicating the clinical treatment of infection caused by this organism (Blain *et al*., 2010). They harbor their resistant genes in Staphylococcal cassette chromosome mec (SCCmec) and other exotoxin genes has been reported (Cabrera *et al*., 2010; Mamman *et al*., 2012). Researchers have reported the presence of MRSA in some parts of Nigeria with percentage prevalence ranging from 16.5 to 47.4 (Abubakar and Sulaiman, 2018; Ariom *et al*., 2019).

Methicillin-resistant *Staphylococcus aureus* has been reported to be resistant to virtually all available classes of antibiotics including beta-lactam, this is a major threat to patients care and public health (Orji *et al*., 2012; Elom *et al*., 2015). *Staphylococcus aureus* not only produce penicillinase but also have penicillin binding protein (PBPs) with low affinity to beta-lactam drugs (Nestal *et al*., 2007). Otheravailable studies have shown the presence of MRSA from different specimens in clinical isolates across the globe (Amadi *et al*., 2008; Ghebremedhin *et al*., 2009; Nwankwo *et al*., 2010; Mahdi *et al*., 2016).

The increase occurrence of antimicrobial resistance makes the effective treatment and management of infections caused by beta-lactamase and methicillin-resistant *Staphylococcus aureus* highly difficult. There is need to evaluate the epidemiological status of MRSA time to time across the globe. Thus, this study is aimed at plasmid profiling and prevalence of methicillin-resistant *Staphylococcus aureus* from clinical specimens in Abakaliki, Nigeria.

MATERIALS AND METHODS

Study area

This study was carried out Alex Ekwueme Federal University Teaching Hospital Abakaliki in the capital city of Ebonyi State. Ebonyi State is located in the South Eastern part of Nigeria. It shares boundary with Abia, Benue, Cross River and Enugu States. It is between longitude 7°30' and latitude 60°45' E.

Sample collection

A total of four hundred and fifty four (454) specimens were obtained for the purpose of this study. Specimens were collected from wound (53), urine (302) and high vaginal swab (99) of patients attending Alex Ekwueme Federal University Teaching Hospital Abakaliki, from June 2018 to February 2019. Specimens were not duplicated per patient. Wound specimens and high vaginal specimens were collected using sterile swab sticks while clean-catch midstream fresh urine specimens were collected using sterile plastic specimen bottles. These patients have been diagnosed of infections ranging from acute to chronic illnesses. The collected specimens were transported to the Department of Applied Microbiology Laboratory unit, Faculty of Science, Ebonyi State University, Abakaliki within two hours of collection for bacteriological analysis. Then specimens were cultured on mannitol salt agar (MSA) for isolation of *S. aureus*.

Isolation, identification and characterization of the isolates

All the clinical specimens of wound, high vaginal swabs and urine were inoculated on sheep blood agar and mannitol salt agar (MSA; Oxoid, UK) in sterile petri dishes and incubated at 37°C for 18-24 hrs. Pure culture and distinct colony of *S. aureus* was obtained by sub-culturing the isolates on freshly prepared mannitol salt agar. Isolates that displayed yellow golden colonies on MSA were identified as *S. aureus* presumptively. Further identification of *S. aureus* was based on standard microbiological technique which includes; Gram-staining, blood haemolysis test, coagulase test, catalase test, indole test, methyl red test, sugar utilization test, Voges-Proskauer test and colony morphology (Cheesbrough, 2010; Chandrashekhar *et al*., 2012).

Antibiotic susceptibility test

The antibiotic susceptibility patterns of the identified isolates were determined by disc diffusion method as recommended by the Clinical Laboratory Standards Institute (CLSI) guidelines. A 0.5 McFarland's equivalent standard of the test isolates each was inoculated on the surface of Mueller-Hinton agar (Oxoid) plates using sterile swab stick. Antibiotic discs of ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg),vancomycin, (30 µg), cloxacillin (5 µg), cefoxitin (5µg), ciprofloxacin (5µg), ofloxacin (5µg), gentamicin (10µg) and sulphamethoxazole/trimethoprim (25µg)(Oxoid, UK) were placed 30 mm way from each other on the surface of the inoculated agar plates using sterile forceps. The antibiotics were allowed to diffuse for about 10minutes and were incubated at 37^0C for 18-24 hours. The diameters of inhibition zones were measured in millimeter (mm) with rule, recorded and interpreted according to the Clinical Laboratory Standard Institute (CLSI) guidelines (CLSI, 2018).

Detection of beta-lactamase using nitrocefin stick

Staphylococcus aureus isolated in this study were screened for beta-lactamase production using nitrocefin sticks (oxoid, UK).

The nitrocefin sticks were removed from refrigerator, allowed to cool to room temperature (28 ^{0}C±2^{0}C), and the colour coded end was used to touch the colonies and the stick was rotated to pick mass of the cells. Two drops of distilled water were used to moisten the tip of the stick and then allowed for 5-10 minutes and further observed for pink-red colour development upon hydrolysis by beta-lactamase (CLSI, 2018).

Detection of methicillin resistant *Staphylococcus aureus* (MRSA)

This was done using disc diffusion method according to Clinical and Laboratory Standard Institute (CLSI) guidelines. Mueller-Hinton agar plate wasprepared according to its manufacturer's specification. Colonies of the isolated bacteria were grown in 5 ml of nutrient broth. The turbidity of the broth culture containing the isolates was adjusted to 0.5 McFarland standards and inoculated using swabbed method onto the prepared Mueller-Hinton agar plate. Antibiotic discs of cefoxitin and oxacillin were placed onto the inoculated agar plates for the detection of MRSA. The plate was then incubated at 37^{0}C for 24 hours. Diameter zone of inhibition was measured, recorded and interpreted according to CLSI guidelines (CLSI, 2018).

Plasmid DNA extraction

S. aureus isolates grown for 24 hours in 5 ml of LB broth (Merck, Germany)were harvested by centrifuging 1.5 ml of each culture in microcentifuge tubes for 5 minutes at 6000 rpm (revolutions per minute). The plasmid DNA was extracted using Zyppy Plasmid Miniprep Kit (Zymo Research, Epigenetics, USA) according to the manufacturer's instruction (Amoakoa *et al*., 2016).

Agarose gel Electrophoresis

Plasmid DNA was determined in a 1% (w/v) agarose gel prepared by dissolving 1.0 g of agarose (Bio-Rad) in 100 ml of1 X Tris-acetate-ethylene diamine tetraacetate (TAE; pH 8.0) buffer. The mixture was heated for 3 min in a microwave oven. After cooling, ethidium bromide (EtBr; 1 ml/ml) was added to the molten gel, which was then pouredin a gel casting tray and then allowed to solidify. After solidification, combs were removed and the gel was carefully placed in the electrophoresis tank containing1X TAE buffer (40 mMTris, 20 mM acetic acid, and 100 mM EDTA pH 8.0).The plasmid DNA detection were prepared by mixing 5µl of plasmid DNA extract with 2µl of 6 X DNA loading dye (Fermentas). For each run, 5 µl of Lambda DNA/HindIII Marker 3 (2.5kb; Thermo Fisher Scientific) was added to one of the wells to estimate the band sizes and 5 µl of negative control, comprised of Sigma water (Nuclease free water) was added to another well. Then 5 µl of the extracted plasmid DNA of each isolates were carefully loaded into the remaining wells. Agarose gel electrophoresis was performed at 80 V; 400 mA (mini Ampere) for 60 minutes. Gels were visualized and photographed using a gel documentation system (Gel Doc 2000; Bio-Rad) (Amoakoa *et al*., 2016).

Data Analysis

Data generated from this research were analyzed using statistical package for social sciences (SPSS) version 16.0 software. One-way ANOVA and Tukey *post hoc* test were used to determine the prevalence base on the patient demographicdata. Results were taken as significant where *p* value is less than 0.05 ($p < 0.05$).

RESULTS

Out of 178 *S. aureus* isolated, 23 (12.9 %); 37 (20.8 %) and 118 (66.3 %) were obtained from wound; high vaginal and urine specimen respectively. A total of 57 (32.0 %) and 121 (68.0 %) of the isolates were identified from male and female patients respectively. There was significant difference in the prevalence of *S. aureus* at ($p < 0.05$) in relation to specimens source (Table 1). Distributions of *S. aureus* were recorded according to occupations of the patients; students and artisans had the highest number of the isolates. Statistical analysis showed a significant occupational influence in the *S. aureus* prevalence at ($p< 0.05$) (Table 2).

The result showed that 41 (23.0 %) isolates were beta-lactamase producers (Table 3) while 36 (20.2 %) were found to be MRSA. There was significant difference in the prevalence of MRSA at ($p < 0.05$) Table 4). Plasmids were identified in 66 (37.1 %) and multiple plasmid DNA was equally observed in 32 (18.0 %) among the isolates. *S. aureus* isolates showed different range of resistant and susceptibility to different classes of antibiotics tested. Beta-lactamase producing *S. aureus* were highly resistant to beta lactam drugs with increasing percentage range; ceftriaxone (27.0 to 43.5), ceftazidime (32.4 to 52.2) and cefotaxime (36.4 to 73.9). Notably, all the MRSA strain was susceptible to vancomycin, although, some of the strains of *S. aureus* were vancomycin resistant which ranged from 4.3 to 16.1 percent (Table 5).

Table 1: Prevalence of *S. aureus* in clinical specimens

Specimen source	Sample size	Number of Isolates (%)	No. positive for males (%)	No. positive for females (%)	Percentage occurrence
Wound swab	53	23 (43.4)	14 (7.9)	9 (5.0)	12.9
High vaginal swab (HVS)	99	37 (37.4)	0 (0.0)	37 (20.8)	20.8
Urine	302	118 (39.1)	43 (24.2)	75 (43.1)	66.3
Total	454	**178 (39.2)**	**57 (32.0)**	**121(68.0)**	**100.0**

Table 2: Distribution of *S. aureus* according to occupations of the patients

Specimen source	Students	Farmers	Traders	Artisans	House wives	Civil servants	Public servants
Wound swab	7	7	3	4	2	0	0
High vaginal swab	10	7	6	5	4	3	2
Urine	40	16	18	22	6	8	12

Table 3: Occurrence of plasmids and beta-lactamase among *Staphylococcus aureus* in relation to sample type

Specimen source	Number of isolates	Number of Isolates with plasmid (%)	Number of Isolates with double plasmid (%)	Beta-lactamase positive Isolates (%)
Wound swab	23	10 (43.5)	3 (13.0)	5 (21.7)
High vaginal swab (HVS)	37	14 (37.8)	6 (16.2)	7 (18.9)
Urine	118	42 (35.6)	23 (19.5)	29 (24.6)
Total	**178**	**66 (37.1)**	**32 (18.0)**	**41 (23.0)**

Table 4: Prevalence of methicillin-resistant *Staphylococcus aureus* in relation to sample type

Specimen source	Number of isolates tested	MRSA positive (%)	MRSA negative (%)
Wound swab	23	3 (13.0)	20 (87.0)
High vaginal swab (HVS)	37	6 (16.2)	31 (83.8)
Urine	118	27 (22.9)	91 (77.1)
Total	**178**	**36 (20.2)**	**142 (79.8)**

Table 5: Antibiotic resistance profiles of the *Staphylococcus aureus* isolates in relation to sample type

Antibiotics	Wound Isolates (n=23)		HVS Isolates (n= 37)		Urine Isolates (n=118)	
	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
VAC	22 (95.7)	1 (4.3)	34 (91.9)	3 (8.1)	99 (83.9)	10 (16.1)
CN	15 (65.2)	8 (34.8)	24 (64.9)	13 (35.1)	76 (64.4)	42 (35.6)
CRO	13 (56.5)	10 (43.5)	27 (73.0)	10 (27.0)	81 (68.6)	37 (31.4)
OB	7 (30.4)	16 (69.6)	12 (32.4)	25 (67.6)	26 (22.0)	92 (78.0)
FOX	9 (39.1)	14 (60.9)	10 (27.0)	27 (73.0)	40 (33.9)	78 (66.1)
CAZ	11 (47.8)	12 (52.2)	25 (67.6)	12 (32.4)	65 (55.1)	53 (44.9)
OFL	20 (87.0)	3 (13.0)	29 (78.4)	8 (21.6)	94 (79.7)	24 (20.3)
CIP	21 (91.3)	2 (8.7)	31 (83.8)	6 (16.2)	91 (77.1)	27 (22.9)
CTX	6 (26.1)	17 (73.9)	20 (54.1)	17 (45.9)	75 (63.6)	43 (36.4)
SXT	15 (65.2)	8 (34.8)	20 (54.1)	17 (45.9)	62 (52.5)	56 (47.5)

Key:S- susceptible, R- resistant, ceftazidime (CAZ; 30 µg), cefotaxime (CTX; 30 µg), ceftriaxone (CRO; 30 µg), vancomycin (VAC; 30 µg), cloxacillin (OB; 5 µg), cefoxitin (FOX; 5µg), ciprofloxacin (CIP; 5µg), ofloxacin (OFL; 5µg), gentamicin (CN; 10µg) and sulphamethoxazole/trimethoprim (SXT;25µg).

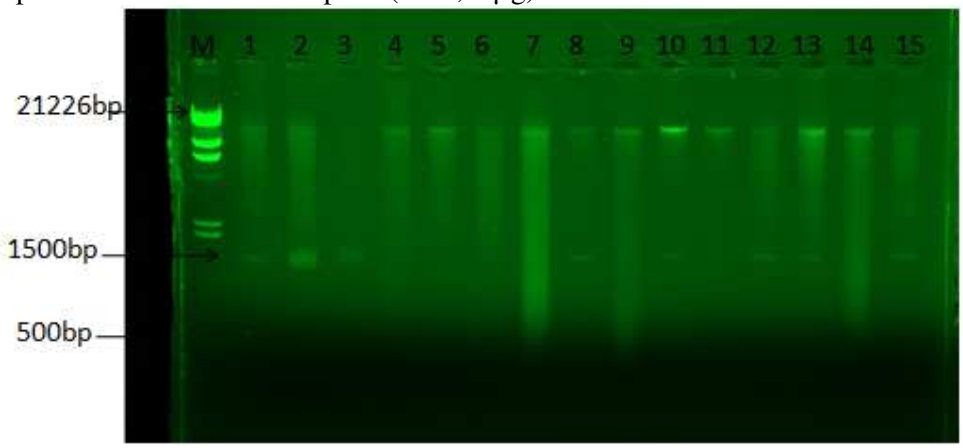

Figure 1: Gel electrophoretic separation profile of plasmid DNAs isolated from Methicillin Resistant *S. aureus* isolates.Lane M = 2.5kb HindIII Marker 3, Lane 1-15 =*S. aureus* plasmid amplicons.

DISCUSSION

In this study 178 (39.2) *S. aureus* were isolated from 454 clinical specimen collected from urine, wound and high vaginal swab of patients diagnosed of wound and urinary tract infection. However, 41 beta-lactamase and 36 methicillin-resistant *Staphylococcus aureus* were detected with percentage prevalence of 23.0 and 20.2 respectively. The methicillin-resistant *Staphylococcus aureus* percentage prevalence of 20.2 in this study is considered significant. Thus, it falls within the range reported by previous studies of other researches where they observed the prevalence in Nigeria to be 20.0 % (Adesoji *et al*., 2019), 26.6 % in Kathmandu Nepal (Regmi *et al*., 2020), 31.8%in Saudi Arabia (Ali *et al*., 2020) and 21.0 % to 30 % (Gorwitz *et al*., 2006) although slightly higher than our observation. Similar prevalence of 22.6 %, 43.3 %, 28.6 %, 28.0 % and 30.4% have been reported from studies done in Ebonyi, Kano, Bauchi and Ogun respectively (Ariom *et al*., 2019, Iroha *et al*., 2015, Nwankwo *et al*., 2010,Ghamba*et al*., 2012), they are little higher than the present result. The emergence of methicillin-resistant *Staphylococcus aureus* strains is due to the acquisition and insertion of mobile; genetic elements into the chromosomes of susceptible strains. Abubakar and Sulaiman, 2018 reviewed that the prevalence of MRSA infection in hospitals has increased and rate was reported as 16.5% in 2010 to 42.3% in 2013. This observation is in line with 20.2 % prevalence of MRSA reported in this present research. Other studies have reported higher rates of MRSA from clinical specimen in Nigeria to range from 34.7 % to 79% (Onemu and Ophori, 2013; Olowe *et al*., 2013). The abuse of methicillin antibiotics by patient and inappropriate use of antibiotics in the hospital could be responsible for the higher prevalence of MRSA recorded in patients evaluated. The varying percentage prevalence observed by several authors of other researches indicated that the prevalence of MRSA differs from one study area to another as a result of infectious control measures applicable.

Urine specimen harbored the highest frequency of MRSA in this study with percentage prevalence of 22.9, followed byhigh vaginal specimen with 16.2 % and least percentage was observed from wound with 13.0. This observation has been previously reported by another research where they recovered 71.4% of the MRSA from urine and 28.6 % in wound specimen (Adetayo *et al.,* 2014). However, the extensive use of prosthetic devices such as urinary catheterization on patient with urinary tract and high vaginal infection could be attributed to the prevalence of MRSA as indicated in our study. The presence of MRSA in wound is a possible indication of breach in the skin epithelium and mucosal barriers thereby exposing it to possible bacterial infection.

In this study, 41 (23.0 %) were equally identified to be beta-lactamase producing *S. aureus*. This—observation is in agreement with the report of Ariom *et al.,* 2019 who reported beta-lactamase producing *Staphylococcus aureus* prevalence rate of 38.1. Plasmids were identified in 66 (37.1 %) and multiple plasmid DNA was equally observed in 32 (18.0 %) among the isolates. The molecular weight of the plasmid ranged from 1500 bp to 21226 bp (Figure 1). The resistant ability of beta-lactamase and MRSA has been attributed to the presence of plasmids that carry genetic determinant and as a result of chromosomal mutational behavior such as alteration in penicillin binding protein to beta-lactam (Gould *et al*., 2012; Ibe *et al*., 2014). Plasmids have the ability to mediate the production of drug inactivating enzymes such as beta-lactamase; an enzyme that inactivates β-lactam rings in β-lactam antibiotics and this has indicated that plasmid encoded antibiotic resistant encompasses most classes of antibiotics currently in clinical use (Esimone *et al*., 2010).

In this present study, beta-lactam antibiotics resistance was observed with percentage frequency ranged for ceftazidime (32.4 to 52.2), cefotaxime (36.4 to 73.9) and

ceftriaxone (27.0 to 43.5). This observation is in agreement with the work of Adesoji *et al*., 2019 who reported resistance rate of ceftazidime (83.3 %) and ceftriaxone (24.2 %) from *S. aureus* isolated from hospital in Nigeria. Resistance frequency to quinolones was very low, ciprofloxacin (8.7 to 22.9) and ofloxacin (13. 0 to 21.6) percent.

This is in agreement with the report that MRSA were 94.7 % susceptible to ciprofloxacin (Ariom *et al*., 20 19). However, other antibiotics such as sulph amethoxazole and trimethoprim and gentamicin (aminoglycoside) had resistance rate of 35.6 % and 47.5 % respectively. All the MRSA recovered in this study were 100 percent resistant tocloxacillin and cefoxitin. Notably, these MRSA isolates were 100 percent susceptible to vancomycin and this is in accordance with the report, that vancomycin is the drug of choice for the treatment of infections due to MRSA (Aligholi *et al*., 2008). Moreover, vancomycin resistant *S. aureus* was identified in this study, but there are not MRSA producers and this agreed with the study where they observed presence of vancomycin-resistant *Staphylococcus aureus* isolates from clinical specimen (Azimian *et al*., 2012).

CONCLUSION

In conclusion, this study reported significant prevalence of MRSA, multiple drug resistant *S. aureus*, presence of multiple plasmids andbeta-lactamase producing *S. aureus* in clinical specimens. Therefore, a serious global and public health threat that calls for continuous vigilance for MRSA and a strict measure in the choice of antibiotic use in the treatment of illnesses. However, the MRSA isolates were completely sensitive to vancomycin. Vancomycin remains the first empirical choice of treatment for MRSA infection and should be used based on the physicians and laboratory diagnosis directives. New antibiotics should be developed to curtail the global threat posed by MRSA and multiple drug resistant organisms. Thus, there is need for molecular studies to detect the clones, resistance genes, monitoring the epidemiology of MRSA and multiple drug resistant *S. aureus* in the hospital under study.

CONFLICT OF INTEREST

The authors have not declared conflict of interests

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