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REVIEW ARTICLE

Molecular Detection of Methicillin Resistance Genes (Mec A; Pvl) in Methicillin Resistant *Staphylococcus Aureus* Isolates from Federal Medical Centre, Yenagoa, Bayelsa State, Nigeria

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Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) is known to cause serious infections that vary widely in severity and susceptibility to antibiotics treatments. The ability of MRSA to resist specific antibiotics is characterized by its potential to express or harbor certain resistance genes. The mecA codes for methicillin resistance by encoding penicillin binding protein 2A (PBP2A) and reveals low affinity for beta-lactams antibiotics such as methicillin. This study was carried out between February and October 2019, to detect mecA and PVL genes in MRSA from clinical samples collected from Federal Medical Centre, Yenagoa, Diete-Spiff Memorial Hospital and Niger Delta University Teaching Hospital (NDUTH), Okolobiri all in Bayelsa state. A total of 250 clinical specimens {(Urine 136(54.4%), wound 49 (19.6%), endocer-vical swab 40 (16%) and High Vagina Swab 25 (10%)} collected at random from the 3 hospitals stated above were immediately taken to Niger Delta University, Department of Medical Laboratory Science Research Laboratory for cultured using selective and general purpose media based on standard bacteriological techniques. Pure culture was standardized with 0.5 Mac Farland turbidity method of standardiza-tion and subjected to antibiotics susceptibility testing by Kirby Buer agar diffusion method with Muller Hinton agar. Genomic DNA was extracted by boiling method. MecA and PVL were amplified in an AB1970 applied biosystem thermal cycler. Of 250 samples collected, a total of 25 (10%) MRSA isolated comprised of 11(44%) from urine 10(40%) from wound, 2(8%) from High vagina swab and 2(8%) from ECS. The highest frequency of MRSA was in urine. The susceptibility pattern of MRSA isolates showed that the organisms was highly resistant to Ampiclox 25(100%), Amoxicillin 21(84%), Streptomycin 19(76%) and was least resistant to Gentamycin 5(20%). Out of the 25 MRSA isolates, none harboured mecA and PVL gene. Statistical analysis of this study reject the null hypothesis at P = 0.05 level of significance. The results obtained showed that the Staphylococcus aureus isolated in this study do not have resistance genes PVL and Mec-A genes, but could have developed another means of resistance ability to these test antibiotics. Further studies on Methicillin Resistant Staphylococcus aureus (MRSA) resistance ability are advised. Continuous monitoring of methicillin resistance pattern of MRSA isolates and the need for high standards of infection control to prevent its transmission and limit its spread is highly recommended.

Keywords: Methicillin Resistance Staphylococcus aureus, Resistance Genes, Antibiotics, Clinical samples, Federal Medical Centre.

1 | INTRODUCTION

Staphylococcus aureus is a Gram-positive, round-shaped bacterium, and a member of the normal flora of the body, frequently found in the nose, respiratory tract, and lower reproductive tract and on the skin. Pathogenic strains of *Staphylococcus aureus* often promote infections by producing virulence factors such as protein toxins and expression of a cell-surface protein that binds and inactivates antibodies. Emergence of antibiotic-resistant strains of *S.aureus* such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem in clinical medicine. Despite much research no approved vaccine for *S. aureus*. Gram-positive bacteria have emerged as important causes of hospital- and community-acquired infections. *Staphylococcus aureus* is a leading cause of nosocomial and community-acquired infections, including bacteremia and surgical wound infections. Approximately 25% of healthy people asymptomatically carry one or more strains of *S. aureus*. Available data on the epidemiology of *S. aureus* indicate that epidemical methicillin-resistant *S. aureus* (MRSA) strains of certain phage types have increased in virulence and have spread rapidly in hospitals. Methicillin resistance was first detected in *S. aureus* in 1961 after this agent was introduced clinically. Over the past 5 decades, there has been a global epidemic of MRSA infection. ref

MRSA infection is usually acquired in hospitals and other health care facilities. This problem is aggravated by the tendency of MRSA for cross-infections. Heavy selection pressures toward acquiring MRSA infection are introduced by the intensive use of antibiotics, particularly cephalosporins and carbapenem, to which organisms are resistant (Loomba *et al*, 2010). Methicillin resistance is a major risk factor for increased morbidity and mortality in *S. aureus* infections. Bacteriuria with *S. aureus* is postulated to occur through a limited number of mechanisms that include catheterization, urologic procedures, or seeding of the genitourinary tract. Bacteremia is associated with bacteriuria in patients infected with *S.aureus*, which suggests that bacteremia is an important precursor for bacteriuria (Chihara *et aI.*, 2010).

Methicillin-resistant *Staphylococcus aureus* (MRSA) belong to a group of gram-positive bacteria that is genetically different from other strains of *Staphylococcus aureus*. MRSA is responsible for several difficult treatments of infections in humans. MRSA is any strain of *S. aureus* that has developed resistance, through horizontal gene transfer and natural selection, multiple drug resistance to beta-lactam antibiotics. (B-lactam antibiotics are a broad spectrum group which includes; some penams – penicillin derivatives such as methicilllin and oxacillin, and cephems such as the cephalosporins (Gurusamy *et al*., 2013). Strains unable to resist these antibiotics are classified as methicillin-susceptible *S. aureus*, or MSSA. Survey in Nigeria has shown that resistance of *Staphylococcus aureus* to ampicillin ranges between 0 to 95.6% with the least resistance in Oyo State and the greatest resistance in Cross River State. (Akindolire *et al*., 2016) Similarly, the coagulase negative staphylococci (CONS) showed 100% resistance to ampicillin in Kano and Borno, both in the North-East and North-West of Nigeria (Pius *et al*., 2016). The *Staphylococcus* species showed variable degrees of resistance to the aminoglycosides. However, there was less resistance to amikacin than to gentamicin in studies in which both antimicrobials were tested (Akindolire *et al*., 2016).

The aim of this study is to detect the presence of mecA gene in *Staphylococcus aureus* using molecular technique (Polymerase Chain Reaction). The specific objectives are to isolate and characterize *Staphylococcus aureus* from clinical specimen; and determine the susceptibility pattern of the isolated methicillin resistant *Staphylococcus aureus* to different classes of antibiotics, and detect the presence of mecA and PVl gene in *Staphylococcus aureus* using

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Polymerase Chain Reaction technique.

2 | MATERIALS AND METHODS

Study Area

The study was carried out in the Federal Medical Center (FMC) Yenagoa, Niger Delta University Teaching Hospital, NDUTH, Okolobiri and Diette-Koki Memorial Hospital all in Yenagoa Local Government Area, the state capital of Bayelsa. Yenagoa is geographically located at lat 4°55‘29‘N, Long 6°15‘51‘E. It has an area of 706km^2 and populated by different ethnic groups across the country.

Ethical Clearance

Ethical clearance was obtained from the ethical committee of the hospital

Sample Size

Taro Yamane formula was used (See appendix)

Sample size using the Taro Yamane method,

$n = N/1 + Ne2$

Where n= sample size required

N = population size

e = allowable error

Assuming N = 670 and e = 0.5, therefore

n = 670/ 1 + 200 (0.05)

n = 250 (minimum sample size)

Statistical analysis

Using the Chi-square formula;

$X2 = (O - E)\,2 \qquad E$

Sample collection and Processing

Two hundred and fifty (250) clinical samples obtained include Urine 136 (54.4%), Wound 49(19.6%), Endocervical swab 40 (16%) and High Vaginal Swab 25(10%) from Federal Medical Centre (FMC), Yenagoa, Diete-Spiff Memorial Hospital and Niger Delta University Teaching Hospital (NDUTH), Okolobiri all in Bayelsa state and immediately taken to Niger Delta University, department of Medical Laboratory Science Research Laboratory for culture using blood agar, Mannitol Salt agar, Nutrient agar and Muller Hinton agar for antimicrobial susceptibility testing.

Identification and Characterization of *Staphylococcus aureus*

Pure bacteria cultures were characterized and identified on the basis of (a) morphology and staining reaction (b) cultural characteristics and biochemical reactions. The characterization was also based on the criteria contained in CRC Handbook of Microbiology Bergeys manual of Determinative Bacteriology. Official Method of Analysis and Laboratory Methods in Food and Diary Microbiology.

Antibiotics Susceptibility Testing

Antibiotic susceptibility testing of the isolate was carried out by Kirby Buer agar diffusion method using commercially available discs. The antibiotics used include Ciprofloxacin (CPX), Amoxillin (AM), Chloramphenicol (CH), Norfloxacin (NB), Gentamycin (GN), Streptomycin (S), Rifampicin (RD), Ampiclox (APX), Erythromycin (E) Levofloxacin (LEV). Muller Hinton agar was flooded with standardized (with 0.5 Mac Farland turbidity method) broth culture and allowed to dry, then the antibiotics discs were placed on the surface and incubated at 37C for 24hrs. Sensitivity/susceptibility to a particular antibiotic is indicated by a clear zone of inhibition around the disc.

Molecular Analysis

DNA Extraction (Boiling Method)

Overnight broth culture of the bacterial isolate in Luria Bertani (LB) transferred into 1.5ml eppendorf tube and was spun at 14000rpm for 3 min in a micro-centrifuge. The supernatant was discarded and 1000μl of 0.5% normal saline was added to the sediment and was vortexed on el tech XH-B vortexer. The cells were re-suspended in 500ul of normal saline and heated at 950C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20^{o}C for other downstream reactions.

DNA quantification and Purification by NANO Drop Spectrophotometer

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software

of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microlitres of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

16S rRNA

The 16s rRNA region of the rRNA gene of the isolates were 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 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5uM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator.

Amplification of Mec *A* genes

The Mec A genes from the isolates were amplified using mecA-F (5′-CAAGATATGAAGTGGTAAATGGT-3′), and mecA-R (5′-TTTACGACTTGTTGCATACCATC-3′).

Primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master Mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4uM and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 51ºC for 40 seconds; extension, 72ºC for 50 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator for a 533bp product size.

Amplification of pVLgene

The pVL genes from the isolates were amplified using the staphp VL F: GCTGGACAAAACTTCTTGGAATAT-3'andstaphp VL R:5'-GATAGGACACCAATAAATTCTGGATTG-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master Mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4uM and 25ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 60ºC for 40 seconds; extension, 72ºC for 50 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 25 minutes and visualized on a blue light transilluminator for a 1100bp product size.

3 | RESULTT

Null hypothesis: there is no significant difference in the potency of test antibiotics use on MRSA at P = 0.05.

Research hypothesis: there is a significant difference between the potency of test antibiotics use on MRSA at P = 0.05.

KEY: CPX – Ciprofloxacin, NB – Norfloxacin, CN – Gentamycin, AMX – Amoxicillin, S – Streptomycin, RD – Rifampicin, E – Erythromycin, CH – Chloramphenicol, APX – Ampiclox, LEV – Levofloxacin

4 | DISCUSSION

Out of the 250 available clinical specimens collected, 170(68%) were from females and 80(32%) from males; this shows the frequency at which women visit the Hospital and how health conscious they are when compared with their male counterparts; this is in line with the study of Bertakis *et al*., (2000) which shows that women are more likely to visit the Doctor and utilize health care service than men. The total number of Methicillin resistant *Staphylococcus aureus* isolated was 25 with females 17(68%) pre-

TABLE 1: Distribution of specimen bygender

SPECIMEN	MALE (%)	FEMALE (%)	TOTAL (%)
Urine	43 (31.6)	93 (68.4)	136 (54.4)
Wound swab	37 (76)	12 (24)	49 (19.6)
High vaginal swab	-	35 (100)	35 (14.0)
Endocervical swab	-	30 (100)	30 (12.0)
Total	80 (32)	170 (68)	250 (100)

TABLE 2: Distribution of Methicillinresistant Staphylococcus aureusisolates by age and gender

MALE(%) Age Range (years)	Isolate Total	FEMALE (%) Isolate Total
1-10	-	1 1
11-20	1 1	2 2
21-30	2 2	11 11
31-40	1 1	3 3
41-50	3 3	-
51-60	-	-
61-70	-	-
71-80	1 1	-
Total	8 (10%)	17 (10%)

TABLE 3: Distribution ofMethicillin resistant Staphylococcusaureus isolate by specimens

SPECIMEN	S.aureus (%)
Urine	14 (56)
Wound swab	7 (28)
High vaginal swab	2 (8)
Endo Cervical Swab	2 (8)
Total	25 (100)

TABLE 4: Statistical analysis showing observed and expectedvalue for Chi square value determination. X^2= $(O-E)^2/E$

Antibiotics	Observed(O)	Expected(E)	O-E	(O-E)2	X2= (O-E)2 E	Σx2
CPX	60	42	18	324	7.71	
	28	42	-14	196	4.67	
CN	80	42	28	1444	34.38	
AMX	16	42	-26	676	16.09	
	24	42	-18	324	7.71	
RD	76	42	34	1156	27.52	
E	32	42	-10	100	2.38	
CH	40	42	-2	4	0.10	
APX	0	42	-42	1764	42	
LEV	64	42	22	484	11.52	154.08

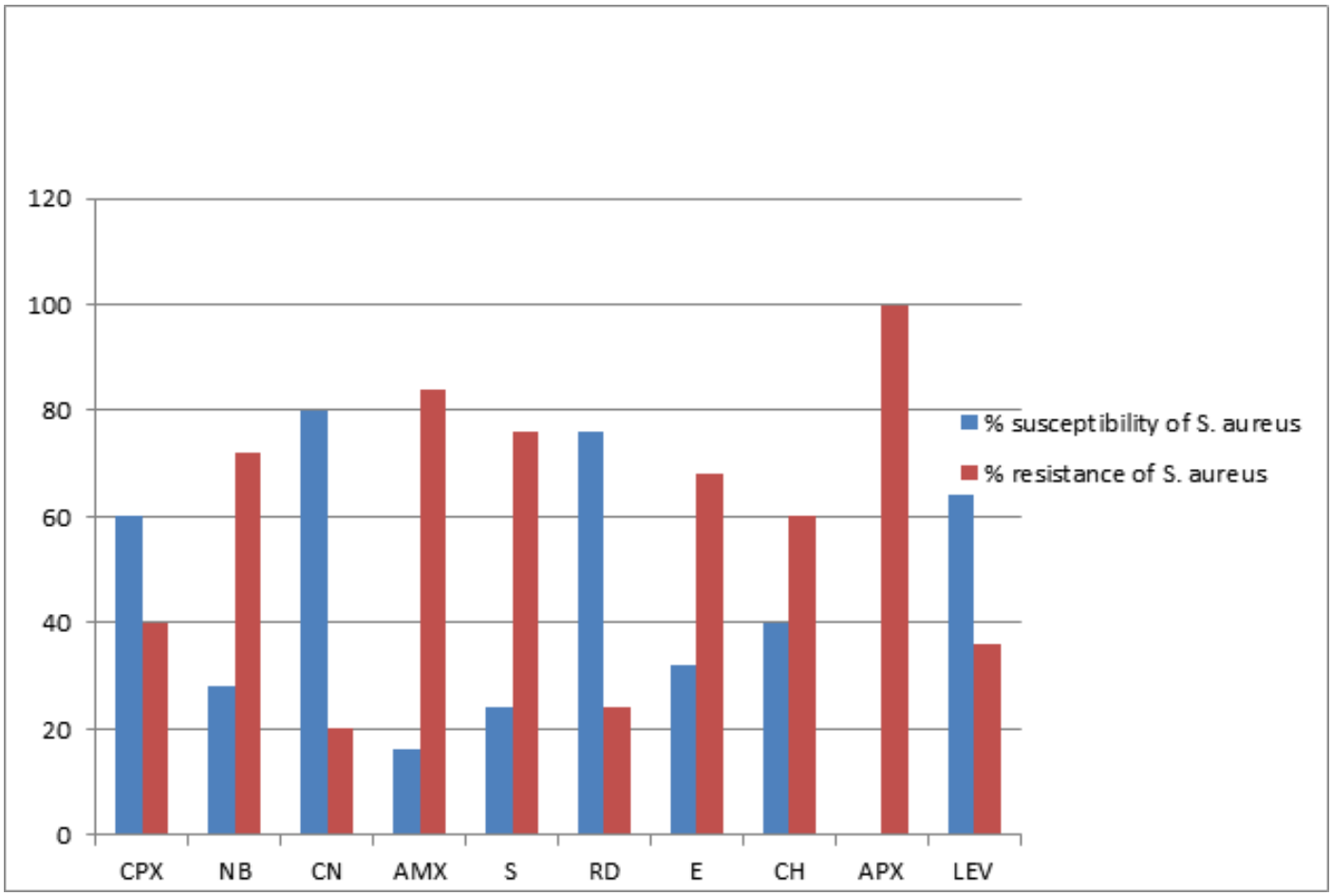

FIGURE 1: Bar chart showing AntibioticsSusceptibility Pattern of Methicillin Resistance Staphylococcus aureus

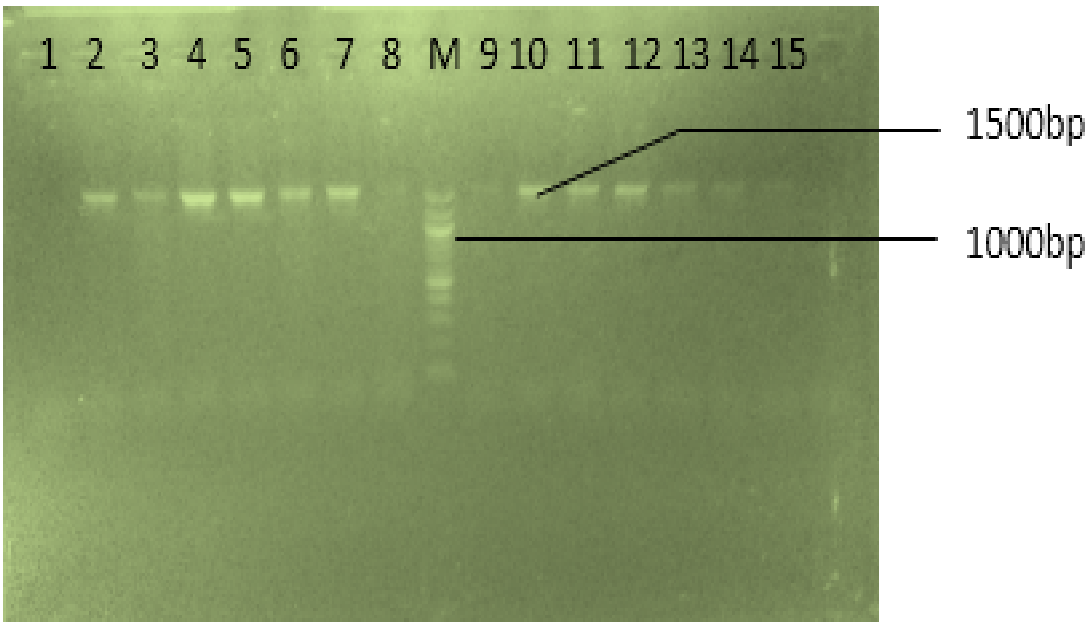

FIGURE 2: Agarose gelelectrophoresis of the 16SrRNA gene of some selected bacterial isolates. Lane 2– 15 represents the 16SrRNA gene bands (1500bp). Lane M represents the 100bpMolecular ladder.

pondence over males 8(32%). The highest number of isolates were from male 3(37.5%) who falls between the age group of 41-50years, this could be because persons within this age range could come in contact with this organism due to their daily routine physical activities. The highest number of isolates from females 11(64.7%) was obtained between the age range of 21 and 30years and this could be due to their sexual lifestyle in agreement with the study of Ani and Mgbechi, (2008) which stated that high sexual activity, multiple sex partners and physical activities are predisposing factors to staphylococcal infections.

In this study, the incidence of Methicillin resistant *Staphylococcus aureus* in relation to the clinical specimens showed that urine (56%) had the highest frequency followed by wound (28%), then Higher vaginal swab (16%) and this is in agreement with the study of Agbagwa and Ifeanacho (2005) which stated that urinary tract infection (UTI) is one of the major reason people visit the hospital.

The Antibiotics Susceptibility pattern showed that the most potent antibiotic to the study of Methicillin resistant *Staphylococcus aureus* was Gentamycin (80%), followed by Rifampicin (76%), Norfloxacin (64%), Ciprofloxacin (60%) and the least poetent was Chloramphenicol (40%), Erythromycin (32%), Streptomycin (24%), Amoxicillin (16%), but the organism was resistant to Ampiclox 0% susceptibility; this revealed multidrug resistance and can be due to the prolonged use of antibiotics, presence of genes like *mecA*, PVL in concordance with the work done by Kuhl *et al*., 1978 which stated that the genes mecA is believed to be the major cause to Methicillin and Methicillin-like resistance.

Though several genes associated with antibiotic resistance in *Staphylococcus aureus* are known, in this study the genes of interest were *mecA* gene and PVL responsible for Methicillin resistance that codes for the Penicillin Binding Protein 2A and play a role in the skin and soft-tissue infections and severe necrotizing pneumonia caused by these bacteria respectively. These strains exhibit increased expression of chromosomes to produce; α hemolysin, a pore forming toxin that lyses many types of eukaryotic cells, as well as α-type phenol-soluble modulins (PSMs), amphipathic peptides that recruit, activate and destroy leucocytes (Deleo *et al*., 2010).

The *mecA*and PVL gene were not detected from the isolates that displayed a phenotypic Methicillin resistance, this can be due to the presence of other resistant genes which encode for resistant to Methicillin and Methicillin-like antibiotics such as mecC gene in line with study of Kim *et al*., 2012 which stated that due to *mecC*-encoded PBP2A/2' Methicillin-sensitive *S.aureus*(MSSA) confers high minimum inhibitory concentration (MIC) values against a range of beta lactams; also blaZ gene in line with the study by Duran *et al*., 2012 which confers high level of resistance to multi-drugs antibiotic including beta lactams. A previous study in Nigeria reported the complete absence of five major SCCmec types and mecA genes as well as the gene product of PBP2a in isolates which were phenotypically MRSA suggesting a probability of hyperproduction of β-lactamase as a cause of the phenomenon (Olayinka *et al*., 2009).

Statistical analysis showed that there is a significant difference in the efficacy of the various antibiotic used on MRSA in this study at P>0.05.

5 | CONCLUSION

In this study, isolation of *Staphylococcus aureus* from clinical specimens, microbiological characterization, susceptibility testing of *Staphylococcus aureus, mecA* and PVL gene amplification by Polymerase Chain Reaction were carried out. Of the twenty five isolates, none harbored mecA or PVL gene but were still resistant; these isolated MRSA maybe encoded by a different gene. Thus, early detection could reduce morbidity and mortality and also reduce the development of multi-drug antibiotic resistance.

Recommendation

Personal hygiene should be taken seriously; **R**egular hand-washing should be a habit Aseptic management of leions and use of catheters and proper use of prescribed antibiotics**; S**earch for new therapeutic alternative and policies to control use of antibiotics and hospital-acquired infections should be a constant habit for Health Care Practitioners.

Acknowledgement

We acknowledge the staff and the management of Federal Medical Centre, Bayelsa State for permitting us to collect the samples.

Authors' contributions

OAO & SON designed and conducted the experiment; **OMO** analyzed the data

Ethical Approval

The individual patients' consents were sought and approved. The ethical committee of the Federal Medical Centre, Yenagoa approved the collection of the sputum samples.

Competing Interest: None

Source of Funding: Self funding

(1–11)

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