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*mec*A Gene Profile of Methicillin-Resistant *Staphylococcus aureus* Isolates from Clinical Sources in Port Harcourt, Nigeria

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Abstract: *Staphylococcus aureus* strains are responsible for a range of acute to chronic infections in humans and other animals. There is scanty information about the genetic background of *S. aureus* strains in Rivers State, Nigeria. The aim of this study was to determine the occurrence of MRSA among *S. aureus* isolates as well as detect the presence of *mec*A gene among methicillin-resistant *Staphylococcus aureus* isolates in Port Harcourt, Nigeria. Two hundred and five (205) non duplicate *Staphylococcus aureus* previously isolated from human sources were randomly collected from three health facilities- University of Port Harcourt Teaching Hospital, Braithwaite Memorial Specialist Hospital and De-Integrated Laboratories- all located in Port Harcourt, Nigeria, for this study from August, 2012 to July, 2013. Isolates were grouped as hospital in-patient (Hospital-acquired – Nosocomial; n = 76) and out-patient cases (community-acquired; n = 129). Isolates were reconfirmed following standard laboratory protocols and stored in duplicate - one set at +4°C (for phenotypic detection of MRSA) and another set at -70°C for molecular analysis. Using the disk diffusion method, detection of MRSA was carried out with 1µg of oxacillin (OXOID) placed on Mueller-Hinton agar with 4% NaCl supplementation). *Molecular Analyses* were carried out on all ORSA strains as follows- Bacterial genomic DNA extraction and PCR amplification for detection of 16S rRNA and *mec*A genes. Amplified products were analyzed using 2.0% agarose gel electrophoresis and subsequently visualized on a UV trans-illuminator. About twelve percent (12.2%) of the 205 *Staphylococcus aureus* studied were resistant to oxacillin. MRSA detection was significantly higher in in-patient isolates (23.7% of 76) than out-patient (5.4% of 129) *S. aureus* ($p = 0.00031$). Urine samples accounted for majority of the isolates (52 of 205) but MRSA detection was highest in Wound swabs (9 of 48 isolates. Of the 25 MRSA, *mec*A gene was detected in 17, being significantly higher in in-patient MRSA (14) than out-patient MRSA (3) ($p<0-05$). This study has established the presence of the methicillin resistance encoding gene- *mec*A, among MRSA isolates in Port Harcourt and that this gene is largely responsible for the MRSA phenotype. Study further establishes that these MRSA are more frequent in the Hospital environment. Further studies on molecular epidemiology of *S. aureus* are recommended in this region. Improved infection control measures in the healthcare facilities as well as sustained surveillance of methicillin-resistant *S. aureus* in this region are also advocated.

Keywords: *Staphylococcus aureus*, MRSA, *mec*A Gene

1. Introduction

Staphylococcus aureus strains are responsible for a wide range of acute to chronic infections and conditions in humans and other animals, ranging from mild skin infections to more serious and invasive infections such as sepsis, pneumonia, endocarditis, deep-seated abscesses, food poisoning and toxic shock syndrome [1]. It has been reported that about 20% of the human population are long term carriers of *S. aureus* [2].

The introduction of the antibiotic- penicillin in the early 1940s [3] and the successive introduction of streptomycin, tetracycline, chloramphenicol and erythromycin was, in each case, rapidly accompanied by the emergence of resistant organisms [4].

During the early 1960s, introduction of the semisynthetic β-lactamase-resistant penicillins, such as methicillin and oxacillin, brought about a general decline in the prevalence of multiple-resistant *S. aureus* [4], but by the late 1960s to early 1970s, however, strains resistant to the β-lactamase-resistant penicillins were isolated with increasing frequency [5]. Continuing to this day, there has been a growing incidence of hospital-associated (nosocomial) and also community-acquired infections caused by strains of *S. aureus,* especially the methicillin-resistant *S. aureus* (MRSA), which have gained worldwide notoriety as hospital 'superbugs' and that are resistant to multiple antibiotics [6-8]. Virtually all MRSA produce an additional penicillin-binding protein, PBP2a or PBP2' which confers resistance to all B-lactam agents [9]. PBP2a is encoded by the *mec*A gene [10]. Additional genes, which are also found in susceptible isolates, can affect the expression of methicillin resistance in *S. aureus,* resulting in heterogeneity of resistance and making detection of resistance difficult [11-12].

MRSA has caused problems in most hospitals worldwide and increasing numbers have been reported in a number of countries. There has been a progressive increasing rate in methicillin resistance in United State of America from 5% in 1981 to 52% in 2005 [13]. There have also been significant increases in methicillin resistance in clinical strains of *S. aureus* isolates between 1999 and 2002 in European countries, particularly Belgium, Germany, Ireland, the Netherlands and the United Kingdom [14]. MRSA prevalence varied widely, ranging from <1% in Northern Europe to >40% in Southern and Western Europe [14].

The threat posed by such antibiotic-resistant pathogens to patient health and to the community at large is quite overwhelming and improving our understanding of the genetic nature of the global *S. aureus* population, and of the mechanisms responsible for the acquisition and spread of the major antibiotic resistance genes within the species, is of clear public health importance.

However, while data concerning the prevalence and genetic background of *S. aureus* in many countries around the world have been broadly reported, similar information is still very limited in Nigeria, moreso in Rivers State. Information on the prevalence and nature of the major antibiotic resistance genes (*mec*A inclusive) in *S. aureus* isolates from Port Harcourt is either insufficient or unavailable, inspite of the established fact that MRSA is a significant health problem worldwide. The aim of this study is to determine the occurrence of MRSA among *S. aureus* isolates as well as detect the presence of *mec*A gene among methicillin-resistant *Staphylococcus aureus* isolates in Port Harcourt, Rivers State in the Niger Delta region of Nigeria.

2. Materials and Methods

2.1. Study Area/ Collection of Specimens

Two hundred and five (205) non duplicate clinical isolates of *Staphylococcus aureus,* were collected between August, 2012 and July, 2013, from three health facilities-University of Port Harcourt Teaching Hospital, Braithwaite Memorial Specialist Hospital and De-Integrated Laboratories-all located in Port Harcourt, Rivers State of Nigeria, were used in this study. Isolates were previously cultivated from different specimens such as Urine, Blood, High Virginal Swab, Endo-cervical Swab, Intra- cervical Swab, Wound swab, Ear Swab, Eye Swab, Semen and other body fluids. Isolates were also grouped as Hospital in-patient or Out-patient isolates according to the criteria as prescribed by the Centers for Disease Control and Prevention [15].

2.2. Specimen Processing/ Identification Tests/Biochemical Tests

Reconfirmation of isolates were done using colonial morphology on DNase agar plate, Mannitol salt agar plate (yellow colonies showing Mannitol fermentation and non-yellow (mannitol negative) colonies), Gram stain, Catalase and Coagulase (bound / free) tests following standard protocols [16]. All confirmed isolates were stored in duplicate, one set at +4°C (later sub-cultured to carry out phenotypic characterization) and another set frozen in tryptic soy broth containing 10% glycerol and stored at -70°C for molecular analysis.

2.3. Detection of MRSA

Detection of MRSA by disk diffusion method as described by Kirby and Bauer (1966) [17] were performed on all isolates with 1µg of oxacillin (Oxoid, UK) per disk placed on Mueller-Hinton (MH) agar with 4% NaCl supplementation. Briefly, inocula of bacteria were prepared and matched to 0.5 McFarland turbidity standards. Sterile swab stick was dipped into the bacteria suspension and used to streak the MH agar, after which the 1µg of oxacillin disc was placed on the surface of MH agar plate and incubated at 37°C for 24 hrs. The zone of inhibition around the discs were measured with ruler and interpreted using the interpretation chart as prescribed by CLSI (2009) [18]. Organisms showing inhibition zone sizes equal to or lesser than 10mm were interpreted as resistant to oxacillin. Organisms with a zones equal to or greater than 12 mm were interpreted as susceptible while those with an inhibition zone of 11-12 mm were interpreted as intermediate. *S. aureus* strains NCTC 6571 and NCTC 12493 were used as susceptible and resistant controls respectively.

2.4. Molecular Analysis

2.4.1. Bacterial Genomic DNA Isolation

Staphylococcus aureus genomic DNA extraction was carried out on all isolates expressing the phenotypic resistance to oxacillin (Methicillin- Resistant *Staphylococcus aureus* (MRSA) using the method as described by Oviasogie and Agbonlahor (2013) [19]. Briefly, purified isolates were pelleted and suspended in 180µl of ATL buffer. The isolates were purified on nutrient agar plates (Oxoid, UK) and pellets

were harvested and suspended in 180 µl of lysis buffer ATL containing 200ng/M of lysostaphin; 20mM Tris Hel; pH 8.0; 2mM EDTA; 1.2% Triton and incubated at 37°C for 30 minutes. 20µl proteinase K was added. The mixture was vortexed and incubated at 56°C for 3 hours, with occasional vortexing in between. The tube was briefly centrifuged to remove drops from side of the tube. 200µl of buffer A1 was then added, pulse-vortexed for 15secs and incubated at 70°C for 10mins and briefly centrifuged. To the mixture, 200µl of ethanol (96-100%) was added and pulse-vortexed for 15secs and centrifuged briefly. The spin column (supernatant) was transferred into a 2ml collection tube and centrifuged at 8000rpm for 1min. To this, 500µl of buffer AW1 was added and centrifuged at 8000rpm for 1min. The spin column was then transferred into another clean 2ml collection tube to which 500µl of buffer AW2 was added and centrifuged at 14000rpm for 3mins. The spin column was similarly pipette into a fresh 2ml collection tube and finally centrifuged at 8000rpm for 1min. The product constituted the extracted double stranded DNA of the isolates which was stored on ice until ready for PCR amplification.

2.4.2. PCR Master Mix and Amplifications

Into an eppendolf tube, 20µl each of both forward and reverse primers were added and kept on ice. To this mixture, 15µl of DNA polymerase, 5µl Deoxyribonucleic acid triphosphate (DNTPs) and 20µl of buffer 2B (sodium acetate and acetic acid) were added. The mixture was vortexed for 1min. Then 10µl of co-factor (magnesium chloride) was added to the mixture. This constituted the DNA master mix.

PCR amplifications for detection of 16S rRNA and *mec*A genes were carried out as follows.

2.4.3. Detection of mecA Gene in MRSA Isolates

DNA which had been previously extracted was used for amplification. A volume of 20µl PCR reaction mixture consisting of 10µl of PCR mix and 10µl of the extracted DNA was used for PCR. A 380-bp fragment of the *mec*A gene was amplified using the primers; *mec*A- F: 5' CAA GAT ATG AAG TGG TAA ATG GT - 3' and *mec*A- R: 5' TTT ACG ACT TGT TGC ATA CCA TC- 3′. The mixture was briefly centrifuged and tubes were immediately loaded into the PCR machine (M J Research PTC 200 Peltier Thermal Cycler-Biodirects, USA), which was programmed with the following conditions: an initial denaturation step for 5 minutes at 94°C, 39 cycles of amplification were performed as follows: denaturation at 94°C for 30 seconds, annealing at 62°C for 45 seconds and DNA extension at 72°C for 60 seconds, followed by an additional cycle of 10 minutes at 72°C to complete partial polymerizations.

PCR for 16S- rRNA Gene

DNA which had been previously extracted was used for amplification. A volume of 20µl PCR reaction mixture consisting of 10µl of PCR master mix and 10µl of the extracted DNA was used for PCR. An 886-bp fragment of the 16S- rRNA gene was amplified using the primers; 16S-1- F: 5' -GTGCCAGCAGCCGCGGTAA-3' and 16S-2 –R: 5'-AGACCCGGGAACGTATTCAC-3'. After an initial denaturation step for 5 minutes at 94°C, 29 cycles of amplification were performed as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and DNA extension at 72°C for 60 seconds, followed by an additional cycle of 10 minutes at 72°C to complete partial polymerizations.

Electrophoresis and visualization of Gel

Amplified products were analyzed using horizontal 2% Agarose gel electrophoresis as follows: 10µL of DNA molecular size marker (100-1500bp) mixed in 2µL loading dyes (ethidium bromide) was introduced into the first well. Then, 10µL of samples and 2µL of loading dyes were introduced into the other wells of Agarose. Electrophoresis was run at 90V for 60mins and the products were viewed under 302nm UV trans-illuminatior (Alpha Innotech Corporation). Photographs of the separated bands in agarose gels were taken with in-built camera on the Alpha imager.

2.5. Data Analysis

All data were analyzed using the Chi square and t-tests. In addition, SPSS version 17.0 statistical package was employed. P-values of <0.05 were accepted as significant.

3. Results

Two hundred and five (205) non-duplicate isolates of *Staphylococcus aureus* cultivated from different clinical specimens between August, 2012 and July, 2013, were used in this study (Table 1). Among these, 165 isolates were collected from UPTH, Port Harcourt, 29 isolates from BMSH, Port Harcourt, and 11 isolates from De-Integrated Medical Laboratories, Port Harcourt in Nigeria (Table 2).

The distribution of *S. aureus* according to the specimen type / site of isolation showed that urine accounted for 53 (25.9%), followed by wound swab (23.4%). Specimen distribution of MRSA showed that wound swab accounted for the highest (9 of 25 isolates) (Table 1).

The oxacillin disc susceptibility testing showed that 25 (12.2%) out of 205 isolates of *S. aureus* were resistant to oxacillin (Table 3).

MRSA detection was significantly higher in in-patient isolates (23.7% of 76) than out-patient (5.4% of 129) *S. aureus* ($p = 0.000318$) (Table 3).

Detection of *mecA* gene by PCR was carried out on the 25 oxacillin- resistant *S. aureus* (ORSA) isolates. The result of PCR shows that 17 (68%) out of the 25 isolates contained *mecA* gene as indicated by the amplification of 380 bp expected product (Table 4 and Figure 1), being significantly higher in in-patient MRSA (14) than out-patient MRSA (3) ($p<0.05$).

There was detection of 16S rRNA targets in all 25 oxacillin- resistant *S. aureus* (ORSA) isolates.

Among the 165 isolates collected from UPTH, 21 (12.7%) were resistant to oxacillin (ORSA) by the disc diffusion method, while *mecA* gene was detected in 15 (71.4%) out of the 21 MRSA. Similarly, of the 29 isolates collected from BMSH, 3 (10.3%) were resistant to oxacillin (ORSA) by the

disc diffusion method, while *mecA* gene was detected in 2 (66.7%) out of the 3 MRSA (Table 3). There was no association between frequency distribution of MRSA and the source of the isolates ($p > 0.05$).

Table 1. *Distribution of Staphylococcus aureus isolates according to Sample type.*

Specimen	No. (% of total isolates)	MSSA	MRSA	MRSA (%)
Wound swab	48 (23.4)	39	9	18.8
HVS	31 (15.1)	30	1	3.2
Pus	7 (3.4)	6	1	14.3
ICS	8 (3.9)	6	2	25.0
Blood	13 (6.3)	8	5	38.5
Abscess	5 (2.4)	5	0	0.0
Urine	53 (25.9)	50	3	5.7
Ear swab	4 (2.0)	4	0	0.0
Eye swab	7 (3.4)	6	1	14.3
US	6 (2.9)	4	2	33.3
Semen	17 (8.3)	16	1	5.9
ECS	4 (2.0)	4	0	0.0
Sinus swab	2 (1.0)	2	0	0.0
TOTAL	205	180	25	

Legend
MRSA - Methicillin Resistant *Staphylococcus aureus,* MSSA - Methicillin Susceptible *Staphylococcus aureus,* HVS - High vaginal swab, ICS - Intra cervical swab, US- Urethral swab, ECS-Endo cervical swab.

Table 2. *Distribution of Isolates based on Area (Location).*

	UPTH	BMSH	DE-INTEGRATED LABS
No. of isolates screened	165	29	11
MRSA (%)	21 (12.7)	3 (10.3)	1 (9.1)
In-patient	16 (76.2)	2 (66.7)	0 (0)
Out-patient	5 (23.8)	1 (33.3)	1 (100)
MSSA (%)	144 (87.3)	26 (89.7)	10 (90.9)
In-patient	52 (36.1)	6 (23.1)	0 (0)
Out-patient	92 (63.9)	20 (76.9)	10 (100)

Table 3. *Prevalence of MRSA among S .aureus isolates in Port Harcourt.*

Category	Number screened	MRSA (%)	MSSA (%)
Total no of *S. aureus* screened	205	25 (12.2)	180 (87.8)
Out- patient	129	7 (5.4)	122 (94.6)
In-patient	76	18 (23.7)	58 (76.3)
p-value		0.000318	0.179371

Table 4. *Distribution of mecA Gene among MRSA.*

Population screened	No. screened	No. positive (%)	No. negative(%)	P value
MRSA	25	17 (68)	8 (32)	
In-patient	18	14 (77.8)	4 (22.2)	<0.05
Out-patient	7	3 (42.9)	4 (57.1)	
UPTH	21	15 (71.4)	6 (28.6)	
BMSH	3	2 (66.7)	1 (33.3)	
DE-INTEGRATED	1	0 (0)	1 (100)	

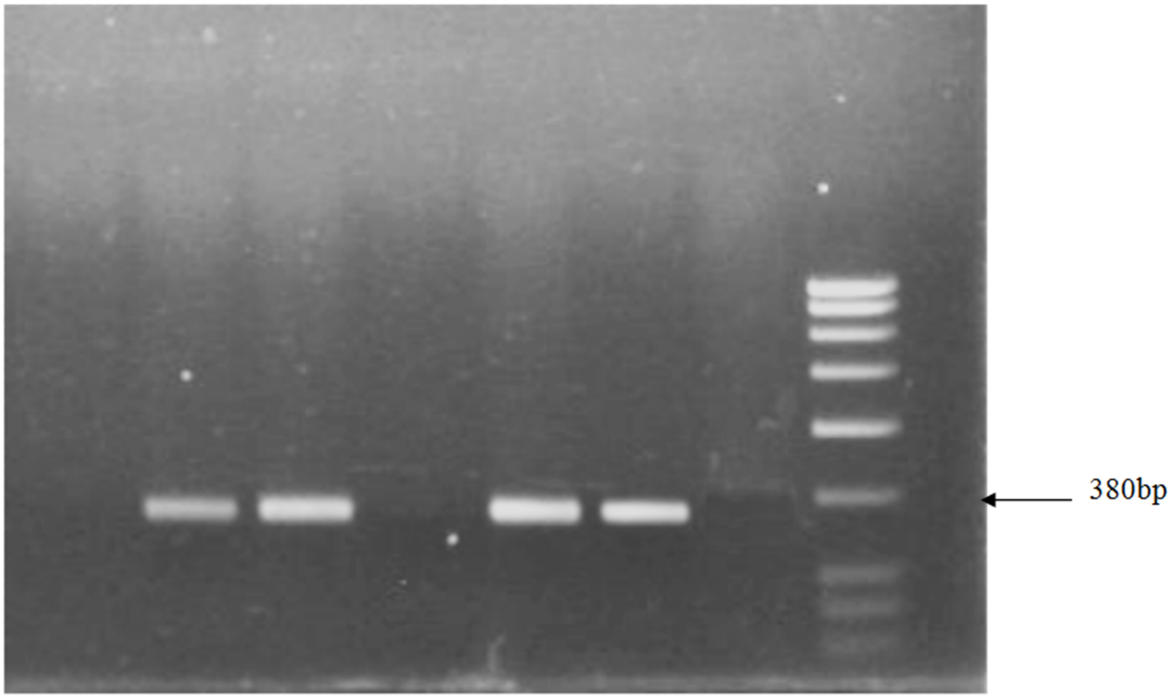

Figure 1. *Representative Agarose Gel Electrophoresis of PCR Product for Detection of mecA Gene among Oxacillin- Resistant S. aureus isolates.*

Legend
Lanes 2, 3, 5 and 6 (Left to Right) are positive for *mecA* as indicated by 380 bp PCR product, Lanes 1, 4 and 7 (Left to Right) are negative for *mec*A. Lane 8: molecular weight size marker (Ladder).

4. Discussion

A number of investigations have indicated that *S. aureus* is the main etiological agent of many infections in Nigeria [20-21]. However, in many studies, identification of *S. aureus* isolates have been based on phenotypic methods and few data exists on the characterization of *S. aureus* isolates using molecular methods [22-24]. Continual surveillance of the occurrence and changes in types of *S. aureus*, clonal identities, and their geographic spread is essential for the establishment of adequate infection control programmes.

In this study, 12.2% of *S. aureus* isolates were resistant to oxacillin (methicillin) (Table 3). This is comparable to the 12.5% MRSA rate detected in tertiary hospitals in North-Eastern Nigeria [24], 13.1% MRSA rate detected at the University of Abuja Teaching Hospital, [25] and 15% rate in a study involving eight African hospitals and Malta [26]. The detection rate in this study is higher than those reported from some other countries- France (6%), Ireland (5%), and United Kingdom (2%), [27], but less than 20.6% and 47.8% reported from South-western Nigeria [28-29], 69% reported in Zaria, northern Nigeria [30] and 77% at Ebonyi State University Teaching Hospital, Abakaliki [31]. Also, relatively high prevalence rates have been reported in similar studies outside Nigeria; 38.6% in Delhi [32] and 83% in Pakistan [33].

It has been reported that in disc diffusion tests, hyper-producers of penicillinase may show small methicillin or oxacillin zones of inhibition, whereas most true methicillin /oxacillin-resistant isolates give no zone [34]. Some hyper-producers of penicillinase give no zone, particularly with oxacillin, and will therefore be falsely reported as MRSA [34]. This probably accounts for the higher rates of detection compared to those reported from some other countries- France (6%), Ireland (5%), and United Kingdom (2%), [27].

Conversely, the relatively low rate of MRSA in this study might be connected to improved infection control programme in the study facilities or due to improved consciousness on abuse of antibiotics in this locality by both health practitioners and in the community since emergence of resistant strains has been largely due to antibiotic abuse.

While the collection of *S. aureus* did not specifically determine community versus nosocomial isolates, it could be generally expected that most out-patient isolates would be community-acquired while most in-patient isolates would be nosocomial. In this study, MRSA was significantly higher in in-patients (23.7%) than out-patients (5.4%) ($p<0.05$) (Table 3) and this is expectedly so because of established MRSA risk factors, such as recent hospitalization, surgery, residence in a long-term care facility, receipt of dialysis or presence of invasive medical devices [35]. Similar higher in-patient prevalence rates compared to out-patient, have been reported from different regions in Nigeria; In Jos, 43% rate was reported of which 81% was from in-patients [36], 28.6% in Kano (62% of this was from in-patient) [37], 34.7% in Ilorin (70.6% of this was from in-patients) [1]. This is a further confirmation that MRSA is an acclaimed nosocomial pathogen worldwide.

Tests based on the detection of the *mec*A gene using PCR or DNA hybridization is considered the "gold standard" for methicillin resistance [38]. In this study, PCR for detection of *mecA* gene showed that 17 (68%) of the 25 isolates previously categorized as MRSA by disk diffusion method harboured the *mecA* gene as indicated by the amplification of 380 bp expected product (Table 4 and Figure 1).

Studies have shown that, whereas most of MRSA isolates contain *mecA* gene, some other strains of MRSA show absence of *mecA* [39]. Moreover, false-positive and false-negative results have also been reported with PCR [40-41]. Some of these resulted from *mecA* drop-outs in the staphylococcal cassette chromosome *mec* (SCC*mec*) which have been observed in some populations and which may also account for regional differences [41]. In a study of 98 *S. aureus* isolates, Qureshi *et al., (2012)* [42] reported that, based on disk diffusion method, 78 were classified as methicillin- resistant, while 20 were classified as MSSA. However, the *mec*A gene was detected in 89 (87.22%) of the total study isolates (MRSA & MSSA). Qureshi *et al., (2012)* [42] further reported that, although there was complete concordance between *mec*A gene detection and those of disk diffusion method in 78 isolates, they noted that out of the 20 isolates which were previously categorized as MSSA by disk diffusion method, 11 showed amplification of *mec*A gene by PCR. In another study, out of 194 *S aureus* isolates, 40 (20.6%) were MRSA using 10 µg methicillin disc, but PCR analysis showed that *mecA* gene was present in 43 (22.2%) of the 194 *S. aureus* isolates [29]. Martin-Lopez *et al.* (2002) [43] recorded 96.5% of MRSA by detection of *mecA* gene, while in a multi-centre study in South-Western Nigeria, Adesida *et al.* (2005) [44] reported a prevalence of 1.4% resistance to methicillin by the detection of the *mecA* gene using PCR and this is far lower than the prevalence in this study.

Although, the expression of *mecA* gene is considered an important mechanism of methicillin resistance in staphylococci, other mechanisms alone or in combination, have been detected in staphylococcal strains [42]. Studies have also shown that apart from *mecA* gene, PBP4 and *ica* gene cluster can also encode resistance in MRSA [45-46]. Other identified factors include hyper-production of β-lactamase, production of normal PBP with altered binding capacity, and /or other, as yet unidentified, factors [42]. Furthermore, a novel *mec*A homologue, mecALGA251, encoded in a new SCCmec element, designated type XI, and subsequently named *mec*C, among human and bovine MRSA isolates in the UK and Denmark has been described [47-48]. This *mec*A homologue, is reportedly not detectable by routine *mec*A-specific PCR approaches or PBP2a slide agglutination tests suggesting that it may represent a public health threat because phenotypic and genotypic tests seem unable to detect this new resistance mechanism. Such observations can help explain the discrepant findings in this study which revealed 8 *mec*A- negative MRSA isolates.

*mec*C MRSA have now been isolated in small numbers from humans and a wide range of other host species in several European countries [47-49]

Viewed from the perspective of clinical practice, this discrepant finding among tests suggests a cautious use of a single method for determining methicillin resistance in staphylococci, as this can lead to erroneous results and patients receiving inappropriate medications.

5. Conclusion

This study has established the presence of the methicillin resistance encoding gene- *mec*A, among MRSA isolates in Port Harcourt and that this gene is largely responsible for the MRSA phenotype. Study further establishes that these MRSA are more frequent in the Hospital environment. Further studies on molecular epidemiology of *S. aureus*, especially strain clonality, are recommended in this region. There is also the need for improved infection control measures in the healthcare facilities as well as sustained surveillance of methicillin-resistant *S. aureus* in this region.

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