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MOLECULAR DETECTION OF SOME DRUG RESISTANT AND VIRULENCE GENES OF METHICILLIN RESISTANT *Staphylococcus aureus* FROM HOSPITALS IN ABIA STATE, NIGERIA

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ABSTRACT
Methicillin resistant Staphylococcus aureus (MRSA) is a major pathogen implicated in healthcare and community associated infections. This study was aimed at determining the antimicrobial resistance pattern and genotypic characteristics of some clinical MRSA isolates from federal medical centre Umuahia and Abia State teaching hospital Aba both in Abia State, Nigeria with respect to resistance and virulence genes. A total of 40 MRSA isolates identified using cefoxitin discs were obtained from different clinical samples. Antibiotic resistance to 15 different antibiotics was determined by Kirby-Bauer disc diffusion method, DNA extraction was by boiling method and gene characterization by Polymerase Chain Reaction (PCR) with appropriate primers. Results obtained showed that all isolates were sensitive to vancomycin while all were resistant to cefoxitin and ceftazidine. Resistance of the organisms to oxacillin, ceftriaxone and augmentin was also high. PCR analysis revealed that 12 (30%) of the total isolates screened possessed mecA gene, 7 (17.5%) harbored the beta-lactamase blaZ gene, 8 (20%) possessed the ermB gene while 4 (10%) carried the pvl gene. The highest incidence of mecA gene was obtained from wound samples while none was obtained from ear swabs. The study establishes a high level of multiple drug resistance among the MRSA isolates and presence of virulence genes in the isolates under study. Therefore, we recommend the infection control department of the various hospitals in the area to screen for MRSA and carry out risk factor analysis to determine accurate measures for controlling the spread of MRSA

KEYWORDS: *Antibiotic resistance, MRSA, resistance genes, virulence genes, PCR*

INTRODUCTION

Staphylococcus aureus is a Gram positive, non-spore forming, non-motile, facultative anaerobic coccus that inhabits the nasal membranes and skin of man and warm-blooded animals. *S. aureus* have also been reported to cause a variety of health challenges, from mild infections such as skin infections and food poisoning, to life threatening infections, such as sepsis, pneumonia, osteomyelitis and infectious endocarditis

(Ibadin *et al.,* 2017). These organisms have the capacity to respond to new antibiotics by developing resistance strategies (Kaur *et al*., 2015) However, Methicillin Resistant *Staphylococcus aureus* (MRSA) are strains of *S. aureus* that has become resistant to the beta-lactam antibiotics which include penicillin (methicillin, oxacillin, dicloxacillin etc.) and the cephalosporins (Kaur *et al*., 2015). They are opportunistic nosocomial pathogens that can cause mild to invasive life-threatening infections (Tong *et al*., 2015). Methicillin resistance is developed in *Staphylococcus* when it acquires the *mecA* gene that encodes for the low-affinity penicillin-binding protein 2a that confers resistance to the β-lactam antibiotics. These *mecA* genes are located on a mobile genetic element region called the Staphylococcal Cassette Chromosome (Katayama *et al*.,2000) MRSA was first reported in 1961, shortly after methicillin became available for treatment of staphylococcal infections (Lowy 2003). Within a short while after discovery, the organism was implicated in several hospital outbreaks in many parts of the world (Lee *et al.,* 2018). The success of MRSA as a pathogen is attributed to its acquisition of virulence factors combined with beta lactam resistance and for most clones' resistance to other classes of antibiotics. In Nigeria, MRSA occurence was first documented by researchers in 1987 with its prevalence pegged at 50.6% (Rotimi *et al*., 1987). Also, subsequent review conducted showed that the MRSA prevalence in Nigeria increased from 18.3% in 2009 to 42.3% in 2013 (Abubakar and Sulaiman, 2018)

The erythromycin ribosome methylase (*erm*) genes are responsible for macrolide, lincosamide, and streptogramin B (MLSB) antibiotic resistance. They primarily encode proteins that methylate adenine residues A2058/ 2059 in the peptidyl transferase region of 23S rRNA domain V. (Yao *et al*., 2019). The clinical efficacy of traditional macrolides such as erythromycin and azithromycin used in the treatment of MRSA infections have been reduced as a result of quick transmission and broad antibiotics resistance spectrum of *erm* genes (Yao *et al*., 2019)

PVL is a bi-component exotoxin transmitted by bacteriophages that is encoded by two genes, *lukF-PV* and *lukSPV* (Coombs *et al*., 2020) The genes code for the production of cytotoxin associated with furunculosis, a severe necrotizing haemorrhagic pneumonia, necrotizing fasciitis and other lesions that involve the skin and mucosa (Yu *et al*., 2013). PVL genes are carried by nearly every Community Associated Methicillin Resistant *Staphylococcus aureus* (CA-MRSA) strain as well as a small proportion of clinical Methicillin Susceptible *Staphylococcus aureus* (MSSA) strains. MRSA (and other *S. aureus*) infections evoke a strong response from the immune system, with neutrophils providing the primary defense (Rigby and DeLeo, 2012) and PVL has been identified to form pores in the membranes of leukocytes, causing their lysis. Production of PVL is increased *in vitro* by β-lactam antibiotics through transcriptional activation (Dumitrescu *et al*., 2007; Stevens *et al.,* 2007).

The global emergence and spread of MRSA harbouring multi-resistance and virulence genes limits the effectiveness of therapeutic options for staphylococcal infections and worsens their clinical outcomes. Meanwhile, quite a number of investigations have reported that *S. aureus* is among the most frequently encountered nosocomial pathogen in Nigeria, however, data on the molecular features and gene distribution of this pathogen is very limited thus necessitating this research.

METHODS

A total of 40 MRSA isolates obtained from a previous study (Ifediora *et al*., 2019) were analyzed for the presence and/or absence of targeted virulence and antibiotics resistant genes (mecA***, erm B, blaZ and pvl*** **genes**). The MRSA isolates were obtained from different health centers in Abia State. The ethical consent was obtained and necessary protocols adhered to. All isolates emanated from clinical samples comprising of 15 urine samples, 10 wound samples, 7 high vaginal swab samples, 5 urethral swab samples, 2 nose swab samples and 1 blood sample. Identification of *S. aureus* was confirmed on the basis of Gram staining, catalase tests, coagulase, DNAse, yellowish pigmentation on Mannitol salt agar and hemolysis on blood agar.

Antimicrobial Susceptibility Testing by Disc Diffusion Method

S. aureus isolates were tested for antibiotics resistance using the Kirby-Bauer disk diffusion method Discrete colonies of isolates on nutrient agar plates were emulsified in 3 – 4 ml of sterile physiological saline and the turbidity adjusted to 0.5 McFarland standard. The surface of Mueller Hinton agar (MHA) in the plate was inoculated with the bacterial suspension using sterile swab sticks. The inoculated plates were allowed to dry for 10 minutes before the antibiotic discs were applied aseptically to the surface of the agar. After 30 minutes of applying the discs, the plates were inverted, and incubated at 35^{o}C. The following antibiotic discs from Oxoid were used: oxacillin (1 µg), cloxacillin (5 µg), clindamycin (2 µg), erythromycin (15 µg), gentamycin (30 µg), ciprofloxacin (5 µg), azithromycin (15 µg), levoflaxacin (5 µg), vancomycin (30 µg), and ceftazidime (30 µg), cefuroxime (30 µg), ceftriaxone (30 µg), ofloxacine (5 µg), and Augmentin (30 µg). The diameter of the zone of inhibition of each isolate to the tested antibiotics was measured in millimeters with a ruler and compared to the Clinical Laboratory and Standards Institute guideline for interpretation. (CLSI, 2013). Methicillin resistance was detected using cefoxitin disc (30 µg). Isolates were considered resistant or sensitive according to CLSI criteria (CLSI, 2013)

DNA Extraction

DNA extraction was carried out by boiling method using the following protocol; five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) medium was spun at 14,000rpm for 3 min. The cells were re-suspended in 500µl of normal saline and heated at 95^{0}C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14,000rpm. The supernatant containing the DNA were transferred to a 1.5ml Eppendorf tubes and stored at -20^{o}C for further tests (Queipo-Ortuño *et al*., 2008).

DNA Quantification

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 µl of sterile distilled water and blanked using normal saline. Two micro-litres 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.

Amplification of mecA, erm B, blaZ and pvl genes

All the 40 isolates were analyzed for antibiotics resistance genes: *mecA, ermB, blaZ* genes and the *pvl* virulence genes using the appropriate primers as shown on Table 1 on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 µl for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix (by Thermo Fisher Scientific), the primers at a concentration of 0.4µM and 50ng of the extracted DNA as template. The PCR conditions are summarized on Table 2. The product was resolved on a 1% agarose gel at 130V for 25 minutes and visualized on a blue light transilluminator.

Table 1: Sequences and amplified product size of primers used in polymerase chain reaction assay

Genes	Oligonucleotide sequences (5! 3!)	Product size (bp)
MecA	MecA-F (5′-CAAGATATGAAGTGGTAAATGGT-3′) mecA-R (5′-TTTACGACTTGTTGCATACCATC-3′).'	533
Pvl	staphpVL F: GCTGGACAAAACTTCTTGGAATAT-3' staphpvl R5'-GATAGGACACCAATAAATTCTGGATTG-3'	1100
Blaz	blaz F: 5'-TAAGAGATTTGCCTATGCTT-3' blaz R: 5'-TTAAAGTCTTACCGAAAGCAG-3'	300
ErmB	ErmB F: 5' GCA TTC ATC ATC AAT CAA AAT G -3' ermB R: 5'-CTA TAA CCT TCT GTG CTT TGC A-3'	198

Table 2: Cyclic polymerase chain reaction conditions of different primer sets

Genes	Initial Denaturation	No of cycles	Denaturation	Annealing temp	Extention	Final Extention
MecA	95ºC for 5min	35cycles	95ºC for 30s	51ºC for 40s	72ºC for 50s	72ºC for 5min
Pvl	95ºC for 5min	35cycles	95ºC for 30s	60ºC for 40s	72ºC for 50s	72ºC for 5min
BlaZ	95ºC for 5min	35cycles	95ºC for 30s	48ºC for 40s	95ºC for 30s	95ºC for 30s
ErmB	95ºC for 5min	35cycles	95ºC for 30s	56ºC for 40s	95ºC for 30s	95ºC for 30s

RESULTS

The result of the study revealed that most of the examined MRSA isolates were highly resistant to majority of the tested antibiotics as shown in Table 3. The resistance rates of ceftazidime and cefoxitin was 100%, ceftriaxone, oxacillin, augmentin and cloxacillin were 97.5%, 92.5%, and 90% respectively. Whereas all the MRSA isolates were found to be susceptible to vancomycin (100%), followed by

clindamycin (85%) and erythromycin (57.5%).

Out of the 40 MRSA isolates, the most predominant gene obtained was *mecA* gene with a prevalence of 12 isolates, representing 30% (Figure 1). A total of 7 (17.5%) of the isolates had the *blaZ* gene (Figure 2), while the *ermB* gene was observed in 8 (20%) of the isolates (Figure 3). Additionally, only 4 (10%) isolates possessed the *pvl* gene with amplicon size observed at 1100bp (Figure 4). Interestingly, all 4 MRSA that harboured the *pvl* gene in the current study were obtained from wound, HVS and urine samples. The prevalence of virulence and antibiotics resistant genes among the MRSA isolates is shown in Figure 5, with the highest prevalence occurring in wound samples and none from ear swabs. It was further observed that while no isolate possessed all 4 genes, 2 isolates had the combinations of the 3 genes.

Table 3 Antibiotics resistance pattern of MRSA to selected antibiotics

Antibiotic	**Sensitive No. (%)**	**Resistance No. (%)**
Vancomycin	40(100)	0(0)
Gentamycin	20(50)	20(50)
Clindamycin	34(85)	6(15)
Erythromycin	23(57.5)	17(42.5)
Levofloxacin	10(25)	30(75)
Cefuroxine	5(12.5)	35(87.5)
Ofloxacine	8(20)	32(80)
Azithromycin	20(50)	20(50)
Ceftriaxone	1(2.5)	39(97.5)
Ciprofloxacine	12(30)	28(70)
Augmentin	4(10)	36(90)
Cloxacillin	4(10)	36(90)
Ceftazidine	0(0)	40(100)
Oxacillin	3(7.5)	37(92.5)
Cefoxitin	0(0)	40(100)

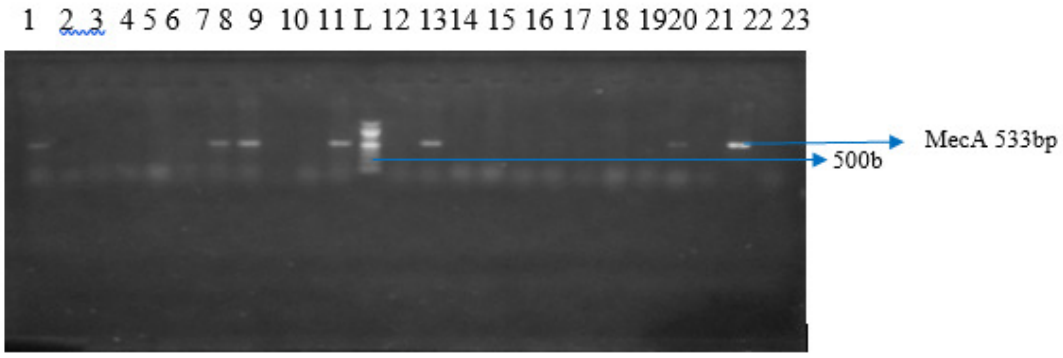

Fig. 1: Agarose gel electrophoresis of the *Mec A* gene of the *S. aureus* isolates. Lane 1,7,811,13,20,23 showing *mecA* at 533bp. Lane L represents the 1000bp molecular ladder

Fig. 2: Agarose gel electrophoresis showing the amplified *blaZ* gene bands. Lanes 1,6,8,10,17 and 20 showing the amplified bands at 300bp while lane L represents the 1000bp molecular ladder

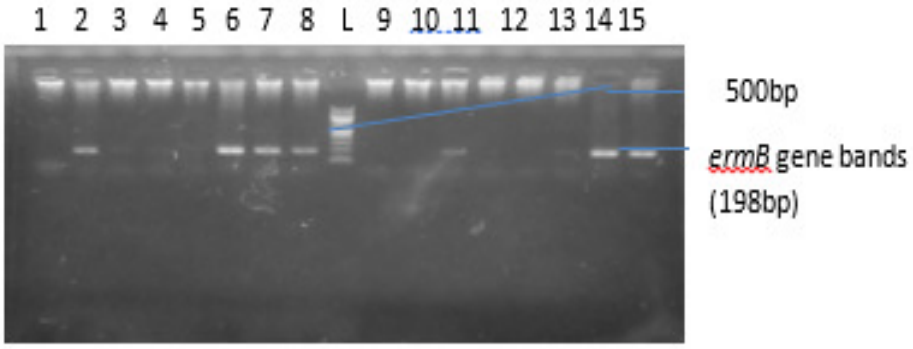

Fig. 3: Agarose gel electrophoresis of the *erm*B gene of the *S. aureus* isolates. Lane 2,6-8, 11 15, showing the *ermB* gene band at 198bp Lane L represents the 1000bp molecular ladder

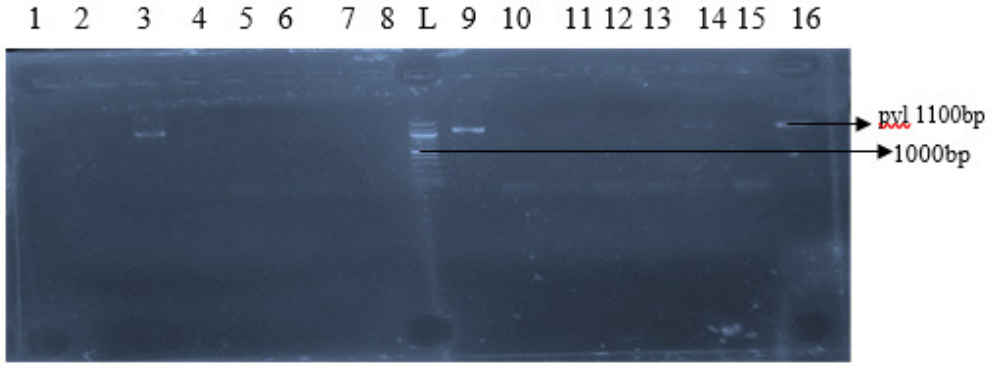

Fig. 4: Agarose gel electrophoresis of the pVL gene of 16 *S. aureus* isolates. Lane 3, 9 and 16 showing the pVL gene band at 1100bp. Lane L represents the 1000bp molecular ladder

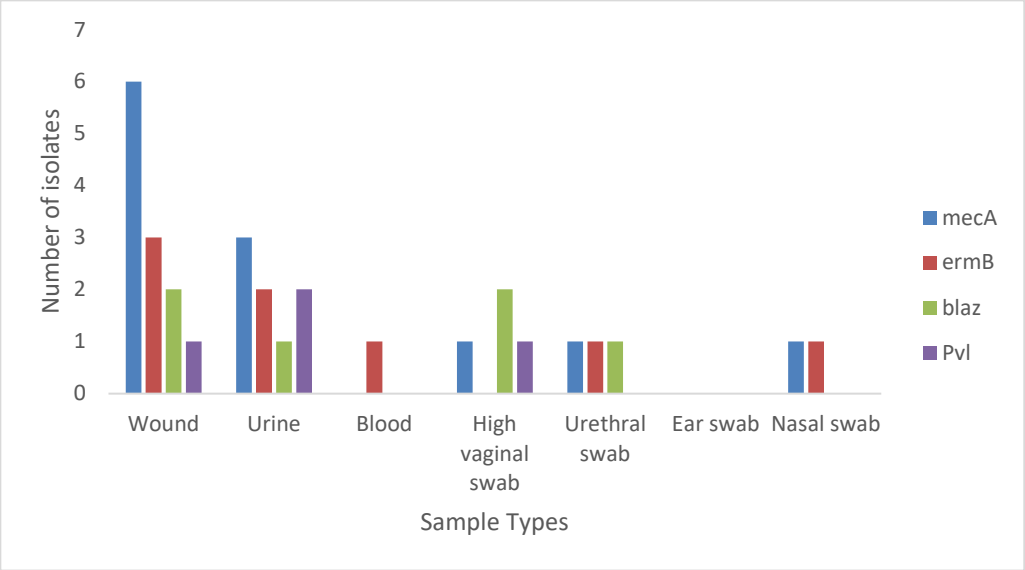

Fig. 5: Prevalence of genes among the clinical samples

DISCUSSION

The high level of multiple drug resistance shown by the MRSA isolates obtained in this study is of great concern. Majority of the MRSA isolates showed resistance to more than 4 antibiotic classes, indicating the presence of strong selective pressures from antibiotics used in the study area. Resistance to beta lactam drugs including cephalosporins, augmentin and oxacillin used in this study is not surprising. This is consistent with the observation that clinical staphylococcal isolates are resistant to a large number of commonly prescribed antimicrobial agents and to beta lactams in particular (Olukoya *et al*., 2005). However, the MRSA isolates in this study exhibited excellent susceptibility to vancomycin, and the finding is in line with results from previous studies in Nigeria and other parts of the world (Fayomi *et al.,* 2011; Firoozeh *et. al.,* 2020).

The gold standard for identifying the presence of MRSA is the detection of *mecA* gene by polymerase chain reaction. Expression of *mecA* gene yields an altered penicillin binding protein, PBP2a, which has a reduced affinity for β- lactam antibiotic binding. In this study 12(30%) out of 40 *S. aureus* isolates were confirmed as methicillin resistant *S. aureus* by the detection of *mecA* gene. Similarly, there are reported cases of detection of *mecA* gene in MRSA isolates from other parts of the country, which include the following: in Benin City, Nigeria, 4 isolates of *S. aureus* representing 11% were confirmed to carry *mecA* gene according to molecular technique (Obasuyi, 2013). Okon *et al*., (2013) reported the detection of 12.5% MRSA from clinical specimens from six tertiary hospitals in Northeastern Nigeria. However, Olowe *et al.* (2013) reported a higher prevalence of 19.2% MRSA from clinical isolates in Medical Microbiology Laboratory of University Teaching Hospital, Ado-Ekiti.

In the present study out of 40 isolates which were phenotypically identified as

MRSA only 12(30%) possessed the *mecA* gene while the rest were *mecA* negative. This phenomenon of mecA negative strains may be attributed to the production of modified PBPs 1 and 2 with decreased affinities for β lactamase, production of a new β lactamase, over production of PBP4 or increased β lactamase production (Khorvash *et al*., 2008).

There are various reports of prevalence among the *erm* gene from various countries but paucity of data from Nigeria. Findings by David *et al*. (2018) among Nigerian patients listed *ermB, ermC, msrA* and *msrB* genes as the genes responsible for erythromycin resistance. The high prevalence of *ermB* gene in this study agrees with the findings of Jarajireh *et al*. (2016) who detected high frequency of *ermB* in both (hospital-acquired) HA-MRSA and (community-acquired) CA-MRSA but is in contrast with the study in Brazil by Coutinho *et al.* (2010) who reported low frequency of the *ermB* gene.

The prevalence of *pvl* toxin genes in this study was 10% and the isolates were from wound, HVS and urine samples. The prevalence of *pvl* toxin gene in this study is in line with work of Orji *et al*. (2016) who reported a prevalence of 10.7% for *pvl* gene among *S. aureus* of nosocomial origin in a local hospital in Nigeria. Similar result was also obtained by Sahar *et al.* (2013), where studies from Algeria and Tunisia reported higher *pvl* prevalence while investigations from South Africa and Zambia reported the lowest prevalence (Mulemba *et al*., 2017). *PVL* -positive MRSA is more frequently reported with skin and soft tissue infections (SSTIs), and community-associated clones. This diversity of *pvl* gene carriage prevalence among various MRSA strains around the world might be explained by the strong association between *pvl* gene carriage with certain *mecA* gene subtype (Types IV & V) and the distinct geographical distribution of *mecA* subtypes as proposed by various recent studies (David and Daum, 2010).

The acquisition and occurrence of these genes among *Staphylococcus aureus* can constitute a serious challenge in disease management and infection control in resource limited country like Nigeria. It is important therefore, to carry out more studies on the routes of acquisition of these genes and hence improve control.

CONCLUSION

The findings obtained are evidence of high prevalence of antibiotic resistance among MRSA from patients in Abia State, Nigeria. The corresponding increase in *erm* and *pvl* genes is an indication that there is need for increased surveillance in the healthcare to reduce the transmission of resistant strains. Also, the absence of *mecA* gene in some phenotypic MRSA observed in this study indicates that there may be other factors other than the production of altered PBP2a that may be responsible. There is also the need for further studies to identify these factors that cause the resistance phenomenon observed phenotypically.

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