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Molecular Detection of *mec*A gene among Methicillin Resistant *Staphylococcus aureus* Isolates from Clinical Samples in Sokoto, Nigeria

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ABSTRACT

Methicillin-resistant *S. aureus* (MRSA) infections have become a common problem in hospital and community-acquired infections. This study was aimed at detecting the presence of *mec*A gene among Methicillin Resistant *Staphylococcus aureus* Isolates from clinical samples obtained from 2 major hospitals in Sokoto, Northwestern Nigeria. A total of 95 non duplicate isolates were screened for confirmation using standard microbiological techniques. Antibiotic sensitivity testing was carried out by disc diffusion using Cefoxitin and resistant isolates were tested with different other antibiotics. All MRSA isolates were subjected to molecular analysis by detection of mecA gene based on PCR technique. Showed that, 42/95 (44.2%) were methicillin resistant. The most potent antibiotic was quinupristin/dalfopristin with 83.3% sensitivity followed by rifampicin with 81.0% and clindamycin with a 71.4%. The least activity was shown to be in fluoroquinolone antibiotic ciprofloxacin with 78.6% of the isolates demonstrating resistance followed by, tetracycline and gentamycin with 64.3%, 61.9% and 61.9% respectively. Most of the MRSA isolates were resistant to more than three antibiotics.PCR showed 36(85.7%) harbored the *mec*A gene. Although, polymerase chain reaction is the gold standard for determining Methicillin resistance through the detection of the *mec*A gene, the present study phenotypically established the presence of methicillin resistant *S. aureus* isolates that are *mec*A negative. Therefore, interpretation of MRSA detection strictly based on *mec*A detection should be done with care in the study area.

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INTRODUCTION

Staphylococcus aureus is one of the important pathogens in many countries causing infection in hospitals and the community. It causes a variety of diseases, ranging in severity from boils and furuncles to more serious diseases such as septicemia, pneumonia and endocarditis (Lowy, 1998). Virulent strains of these bacteria are responsible for the majority of hospital acquired infections (HAIs) and can cause severe diseases that are often fatal (Queck *et al*., 2009). Prior to the introduction of penicillin for the treatment of *S. aureus* infections in the 1940s, the mortality rate of individuals with staphylococcal infections was about 80% (Turner *et al*., 2019). However within two years of the introduction of penicillin to medical use, penicillin-resistant strains were discovered. By 1960, about 80% of all *S. aureus* strains were found to be resistant to penicillin (Deurenberg and Stobberingh, 2009). In 1959, methicillin, synthetic penicillin, was introduced. However, by 1960, methicillin resistant *S. aureus* (MRSA) strains were identified, the direct result of *S. aureus* acquiring the *MecA* gene, which encodes for an altered penicillin-binding protein gene (*PBP2a*) (Shopsin and Kreiswirth, 2001). MRSA was first observed among clinical isolates from patients hospitalized in the 1960s, but since the 1990s it has spread rapidly in the community (Lakhundi and Zhang, 2018). Rapid and accurate identification of MRSA is important in helping clinicians choose appropriate antibiotics to treat and prevent spread of these strains.

Laboratory identification of MRSA can be achieved using the disk diffusion method which is widely used by most laboratories, chromogenic agars, the Minimal Inhibitory Concentration (MIC) and more recently molecular methods for detection of *mecA* gene (Kaur *et al*., 2013). The *mecA* gene is highly conserved among the *Staphylococci* species, and so, the importance of PCR in assay of the *mecA* gene as "gold standard" for the detection of methicillin resistance in *Staphylococci* is well established (Ekrami *et al*. 2010; Shariati *et al*.,2010). The 78 Kd penicillin-binding protein (PBP) 2A, which has a modest affinity for beta-lactam antibiotics, is encoded by mecA. The mecA gene product (PBP 2A) is thought to operate as a surrogate enzyme that takes over cell wall synthesis from the regular complement of staphylococcal PBPs, which are inhibited by relatively low (e.g. methicillin) beta-lactam antibiotic doses. While direct biochemical evidence for a transpeptidase activity in PBP 2A is still lacking, transposon inactivation tests have convincingly demonstrated the importance of an intact mecA gene for the production of high-level methicillin resistance. However, it has long been known that an intact mecA and its gene product PBP 2A alone cannot fully govern the resistance phenotype, as all MRSA isolates, regardless of their MIC values, were shown to contain comparable quantities of PBP 2A. Such large differences between cellular levels of PBP 2A and antibiotic MIC values suggested that a factor or variables of unknown nature ('factor X') other than the mecA gene product were also important in phenotypic resistance expression (De Lencastre *et al*., 1994).

Although *mec*A has been detected among clinical MRSA isolates obtained from various animal species within the study area, there is paucity of information on the prevalence of mecA gene among human MRSA isolates in Sokoto. Therefore, the aim of the paper is to detect the presence of *mec*A gene from MRSA isolated from clinical samples.

MATERIALS AND METHODS

Bacterial isolates: Isolates used in this study were obtained from Usmanu Danfodiyo University Teaching Hospital and Specialist Hospital in Sokoto, North Western Nigeria. All isolates were identified by their colony morphology, Gram staining and biochemical tests such as catalase test, coagulase test using both slide and tube methods and deoxyribonuclease test (Garcia, 2010; UKSMI, 2014). The isolates were examined for methicillin resistance by using Cefoxitin disk (30µg) diffusion test, Resistance to other Antibiotics and Polymerase Chain Reaction (PCR).

Inoculum for Antibiotic Susceptibility Testing was prepared by the direct colony suspension method as the method recommended by the Clinical Laboratory Standards Institute for testing *Staphylococci* for potential methicillin resistance (CLSI, 2012).

Cefoxitin disk diffusion test

Cefoxitin disk 30µg (Oxoid, UK) was used on Mueller Hinton agar (Oxoid, UK). The inoculum turbidity was adjusted to 0.5 McFarland and the agar plates inoculated, inverted and incubated at 35°C for 24h. After the prescribed period of incubation the zone of inhibition was measured using a metre rule (against transmitted light) and the results (zones ≤21mm indicate resistance) interpreted using the CLSI 2014 [14] guidelines.

Antibiotic Susceptibility Testing

Standard inoculum was prepared by making a direct saline suspension of isolated colonies selected from an 18-hour agar plate incubated at 37^{O}C. The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland (1-2 x 10^8 CFU/ml). It was then observed, using adequate light to visually compare the inoculum tube and the 0.5 McFarland standards against a card with a white background and contrasting black lines. Antibiogram was done in accordance to Clinical and Laboratory Standard Institute (CLSI). Commercially prepared antibiotic discs were placed on the inoculated Mueller Hinton agar 25mm away from each other. The plate was then incubated at 35° C for 18-24 hours after which the zones were read using the interpretation chart provide by clinical laboratory standard institute (CLSI,2020). Resistance to at least three (3) antibiotics of different classes was considered multi drug resistance.

Molecular Analysis of the *S. aureus* Isolates.

DNA extraction from the clinical bacterial isolates was done using Qiagen (USA) DNA extraction kit following Manufacturer's protocol. The extracted DNA was PCR amplified using

27F **(**5'-**AGAGTTTGATCMTGGCTCAG**-3') and 907R (5'**-CCGTCAATTCMTTTRAGTTT**-3') targeting the 16S rRNA as described by Jiang *et al*., [16]. Briefly, a total of 25µL volume of PCR reaction mixture comprsing 3 µL of genomic DNA, 12.5 µL of Mastermix (BioLabs New England), 5.5 µL nuclease free H_2O as well as 1 µL each of 16S rRNA primers (27F and 907R) were mixed together and incubated in a thermocycler following manufacturer's instruction. In addition, *mecA* gene was amplified with the following primers: *mecA*-f: (5'-**AAAATCGATGGTAAAGGTTGGC**-3'); *mecA*-r: (5'-**AGTTCTGCAGTACCGGATTTGC**-3') with **533bp** using the same kit. In all cases negative controls were included. DNA amplification was carried out for 40 cycles according to the following protocol: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min with a final extension at 72 °C for 5 min. The PCR products were then analyzed in 2% (w/v) agarose gels stained with ethidium bromide and visualized under UV light (Murakami *et al.*, 1991).

RESULTS

Table 1 presents the prevalence of MRSA among the isolates collected from the two hospitals, out of the 35 confirmed *S.aureus* isolates from Specialists Hospital 20 (57.1) were confirmed as MRSA using Cefoxitin disc diffusion. **Table 2** presents the antibiogram pattern of the MRSA isolates to the different antibiotics. The most potent antibiotic was quinupristin/dalfopristin with 83.3% of all the tested isolates being sensitive and an inhibition zone of ≥19mm, it is followed by rifampicin with 81.0% inhibition zone of ≥20mm and clindamycin with a 71.4% sensitivity rate and inhibition zone of ≥21mm. The least activity was shown to be in fluoroquinolone antibiotic ciprofloxacin for which 78.6% of the isolates demonstrated phenotypic resistance with an inhibition zone of ≤15mm. **Figure 1** shows the electrophoretic gel image for DNA extracted, all the wells demonstrated the presence of the DNA, although some showed a thicker band indicating that the concentration of the DNA is higher in those samples. This gel was done to confirm, that the extraction was a success and also to determine the amount of the template that will be needed for the amplification processes.

Figure 2 shows the electrophoretic gel image for 16S (976 bp product) and *mecA* gene (533 bp product). The gel indicates that 16S has been amplified in the samples. However for the *mecA* gene, all the isolates were amplified with the exception of samples u73, u75 and the negative control which were not amplified as demonstrated by the appearance of bands.

Table 1. Prevalence of MRSA among the *S. aureus* isolates collected

Hospital	MSSA	MRSA	TOTAL
Specialist Hospital	15(42.9)	20(57.1)	35
UDUTH	38(63.3)	22(36.7)	60
Total	**53(55.8)**	**42(44.2)**	**95(100)**

P-value = 0.053, χ^{2} = 3.758

Table 2. Susceptibility pattern of the Methicillin resistant *Staphylococcus aureus* isolates to different antibiotics

Antibiotic	Disc content (µg)	Antibiogram of the isolates (mean zone of inhibition)		
		Sensitive	Intermediate	Resistant
Rifampicin	5	34(81.0)	-	8(19.0)
Ciprofloxacin	5	7(16.7)	2(4.8)	33(78.6)
Clindamycin	2	30(71.4)	5(11.9)	7(16.7)
Teicoplanin	2	27(64.3)	6(14.3)	9(21.4)
Tetracycline	30	11(26.2)	5(11.9)	26(61.9)
QD	15	35(83.3)	1(2.4)	6(14.3)
Erythromycin	15	11(26.2)	4(9.5)	27(64.3)
Gentamycin	30	10(23.8)	6(14.3)	26(61.9)

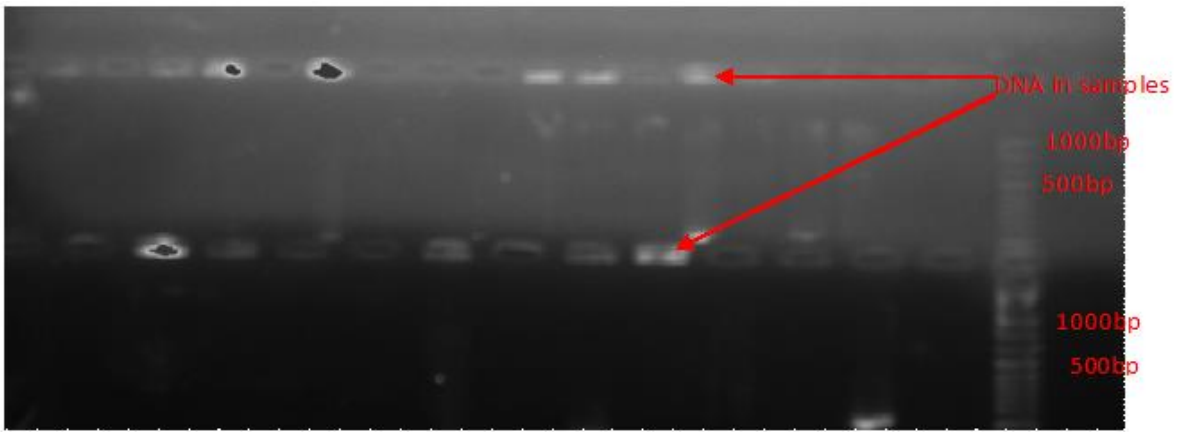

Figure 1. Electrophoretic gel image showing presence of DNA

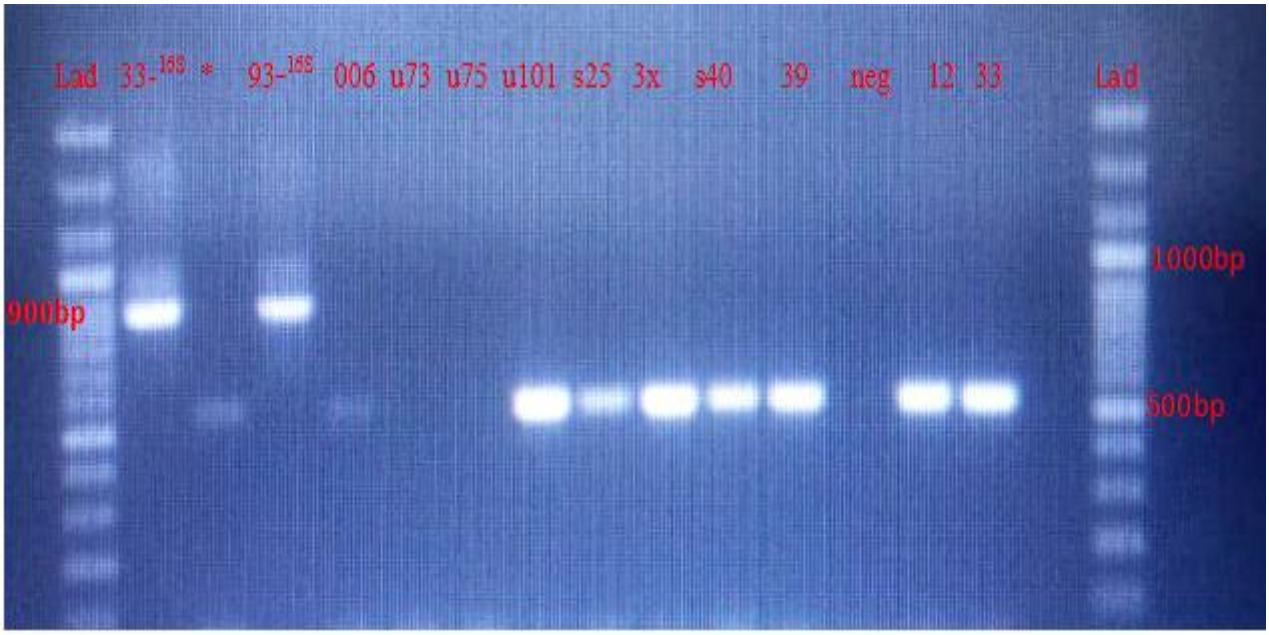

Figure 4.5. Amplified 16s rRNA (976bp) and *mecA* gene products (533bp) from MRSA

KEY

L: 1kb plus molecular ladder
Lane 2 and 4: 16s rRNA
Lane 3, 5-12, 14 and 15: samples
Lane 6 and 7: mecA negative samples
Lane 13: negative control (PCR premix only)

DISCUSSION

Oxacillin or methicillin-resistant *Staphylococcus aureus* (MRSA) isolates are among the major pathogens causing infections in the world, leading to the emergence of increasingly virulent and multiresistant strains. Detection of MRSA is important for patient care and appropriate utilization of infection control resources. Methicillin-resistant *S. aureus* is a significant pathogen that has emerged over the last four decades, causing both nosocomial and community-acquired infections. Rapid and accurate detection of methicillin resistance in *S. aureus* is important for the use of appropriate antimicrobial therapy and for the control of nosocomial spread of MRSA strains (Breves *et al.,* 2015). Although several reports on the molecular detection of mecA gene among MRSA isolates obtained from various animal species exist in Northwestern Nigeria (Gaddafi *et al*., 2021; Musawa *et al*., 2020), there is paucity of information on such studies among human MRSA isolates obtained within the study area. Therefore, the aim of this study is to detect mecA gene among clinical MRSA isolates obtained from some hospitals in Sokoto, Northwestern Nigeria.

Results obtained from this study showed that 42(44.2%) of the isolates obtained were resistant to methicillin resistant based on cefoxitin disc diffusion method. This positivity rate is higher than findings of Adetayo *et al.,* (2014), Abdullahi and Iregbu (2018) and Breves *et al.,* (2015), who reported a prevalence of 30.4% in Ibadan, 26.9% in Abuja and 31.4% in Brazil respectively. The result from this study is however similar to what was reported by Adeiza *et al.,* (2020) in Sokoto with 46.9%, Ariom *et al.,* (2019) in Abakaliki with 43.4% and Samson and Anthony (2013) in Benin City with 79%, indicating that MRSA is ever increasing. This is further corroborated by findings of Abubakar and Sulaiman (2018), who in a systematic review of MRSA infections in Nigeria reported an increase from 18.3% (2009) to 42.3% (2013). It is clear that MRSA has become a global nosocomial pathogen with attendant therapeutic problems and warrant urgent infection awareness, considering the common practice of unregulated sale of antimicrobial agents and movement of people which may result in rapid dissemination.

Polymerase chain reaction has been considered as the gold standard for determining resistant genes being them highly conserved among Staphylococcal species. Of the 42 MRSA isolates analysed to detect the presence or otherwise of *mecA* gene using PCR, 36(85.7%) were found to harbour the gene. This in agreement with findings of Breves *et al.,* (2015); Adetayo *et al.,* (2014); Murakami *et al.,* (1991) and Mahmood and Flayyih (2014) who all detected *mecA* gene in *S. aureus* isolates. PCR based assays are considered as the gold standard for the detection of MRSA due to the heteregenous resistance by various phenotypic detection methods displayed by many clinical isolates. Genotypic methods are more accurate in detecting MRSA as compared to conventional antimicrobial susceptibility methods (Mahmood and Flayyih 2014). The failure to detect the presence of the *mecA* gene by PCR in isolates that phenotypically exhibit methicillin resistance have been well documented in the literature. Ibadin *et al*. (2017) in a study conducted in the ancient town of Benin attributed the

phenomenon to hyperproduction of the β-lactamase enzyme in *mecA* negative isolates. The authors in the study reported up 6% of MRSA lacking the *mecA* gene, comparatively 4.8% methicillin-resistant coagulase negative staphylococci lacked the gene (Ibadin *et al.,* 2017).

In an earlier study (Elhassan *et al.,* 2015) alterations in the sequence of amino acids present in the protein binding proteins cascade in PBPs 1, 2, and 3 have been suggested to be the basis of the resistance phenotype. These include three amino acid substitutions present in PBPs 1, 2, and 3. A myriad of other reasons have been postulated in an attempt to explain such findings. These include the suggestion of technical problems like the presence of PCR amplification inhibitors that lead to false negative PCR reaction. Point mutations and deletion in the *mecA* gene or any of the variety of gene regulatory elements (fem factors, *mecI* and *mecR1* regulatory genes) involved in *mecA* expression or repression have been identified as credible explanations. The emergence of new variants of the *mecA* gene (e.g. *mec*C gene) is another reason that could explain the absence off the *mecA* gene in some isolates as seen in this study. Since the PCR primers used in the amplification of the target gene are only specific to *mecA* other genetic variants of this gene will not be detected (Dhungel *et al.,* 2021)

CONCLUSION AND RECOMMENDATIONS

Although, polymerase chain reaction is the gold standard for determining Methicillin resistance through the detection of the *mec*A gene, the present study phenotypically established the presence of methicillin resistant *S. aureus* isolates that are *mec*A negative. Therefore, we recommend that;

I. Interpretation of MRSA detection strictly based on *mec*A detection should be done with care in the study area.
II. Cefoxitin disc diffusion test can be used as an alternative to PCR for detection of MRSA in resource constraint settings.
III. There is the need to re-evaluate policies on antibiotics use both within and outside hospitals in Sokoto metropolis. A viable and practicable antibiotics stewardship program needs to be put in place.

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CONFLICT OF INTEREST

None declared.

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