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VARIATIONS IN THE GENOTYPIC AND PHENOTYPIC CHARACTERISTICS OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) FROM STUDENTS ATTENDING POULTRY FARMS IN UMUAHIA, ABIA STATE, NIGERIA

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***Staphylococcus aureus* causes significant epidemiologic and therapeutic challenges with various skin and soft tissue infections, the main forms of disease manifestation. The public health importance of this organism has been heightened by the emergence and spread of species that are resistant to treatment usually referred to as methicillin-resistant *S. aureus* (MRSA). This study was carried out to detect mecA and mecC genes in phenotypically determined MRSA isolates. Nasal swab samples of the subjects were cultured on mannitol salt agar and the isolates were identified as *S. aureus* using a combined morphological and biochemical characteristic. Antibiotic susceptibility profile was performed using the disk diffusion susceptibility method whilst phenotypic detection of MRSA isolates was by Cefoxitin disk diffusion method as per CLSI guidelines. Genomic DNA was extracted from the isolates using commercial kits. The extracted DNA was subjected to multiplex PCR to amplify the 163-bp and 188-bp fragment of the mecA and mecC genes respectively in a Pielter thermal cycler. The susceptibility pattern of MRSA isolates showed that the organisms were highly resistant to Augmentin 29 (93.5%), ceftazidime 18 (58.1%), Streptomycin 19 (76%) whilst high levels of susceptibility were seen for Ofloxacin 27 (87.1%), Levofloxacin 28 (90.3%), and Gentamicin 24 (77.4%). The antibiotic resistance profiles in MRSA isolates were recorded as follows: ciprofloxacin 6 (16.2%), Augmentin 32 (86.4%), erythromycin 13 (35.1%), Ceftazidime 22 (59.4%). Of the 10 MRSA isolates that were subjected to PCR, one isolate was found to harbor the 188-bp of mecC gene whilst mecA was absent from the screened isolates. The detection of mecC MRSA in the present study highlights the diagnostic importance of screening for mecC in mecA negative MRSA. We suggest that surveillance for MRSA should include screening for mecC gene where mecA is not detected in resistant isolates.**

Keywords: PCR, mecA, Antibiotics, MRSA

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a virulent bacterium known for its potentials for causing a wide variety of diseases and global preponderance. There are several reports which suggest that approximately 30% of humans are asymptomatic carriers of *S. aureus* in their nares and/or throat following contact with different animal species (1-3). This bacterium though a normal flora of some parts of the human body has been implicated as the cause of endocarditis, bacteremia as well as skin and soft tissue infections.

The public health importance of this organism has been heightened by the emergence and spread of species that are resistant to treatment usually referred to as methicillin-resistant *S. aureus* (MRSA) (4). The development of these organisms into MRSA follows the acquisition of the mec gene (mecA or mecC) located on mobile genetic element known as *Staphylococcal cassette* chromosome mec (SCCmec) encoding the PBP2a which has low affinity for the β-lactam antibiotics (5, 6). Methicillin-resistant *Staphylococcus aureus* (MRSA) constitute a major public health challenge causing diverse forms of infection in both hospital and in the community setting (7, 8). There has been reports of increasing levels of MRSA across some African and Asian countries with ranges of 43% and 72% been documented in Ethiopia, Cameroun, South Africa and Pakistan (4, 9, 10).

Zoonotic transmission has been reported for mecC-CC130 MRSA after conducting whole genome study (11). Also, there are reports of mecC-positive isolates from Germany, Finland, Sweden and United Kingdom (12, 13). A study in Germany on livestock-associated MRSA recovered 11 mecC positive isolates from 12,691 human subjects (14). Discovery of a new mecA variant (designated as mecC) by some authors have raised concern as these variants are rarely detected by the conventional, confirmatory tests for MRSA (12, 13). MecC-bearing MRSA is a new type of MRSA initially reported in 2011. Many of these organisms have been isolated from animals, especially dairy cattle and have

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the propensity to infect and colonize humans. Close association between animals and humans facilitate transmission of this pathogen through direct/indirect contact or consumption of undercooked animals (15).

Despite these varying reports, there are numerous studies with differences in the result of phenotypic resistance and their corresponding mecA gene presence (16, 17, 18). Phenotypic testing can detect methicillin resistance in *Staphylococcus aureus* using oxacillin or cefoxithin methods. However, some variant mecC strains are difficult to detect using in vitro antibiotics susceptibility testing as many isolates demonstrate marginally elevated resistance (19). There are indications that some isolates designated as MRSA by phenotypic characterization could return a negative PCR result for mecA. This has been attributed to the presence of a mecC strain. In other cases, too, it has been reported that it is also possible to miss rare mecA bearing MRSA if the resistance gene is not expressed at sufficiently high levels *in vitro*.

The risk of colonization by these organisms is elevated amongst children, prison inmates, livestock farmers and persons with frequent visits to healthcare facilities. Previously, *S. aureus* including MRSA was known to be a major cause of nosocomial infections but has now escalated into the community and has sadly also emerged amongst livestock constituting a major public health crisis (14). Based on these considerations, this study was aimed at evaluating MRSA for the presence of mecC genes.

MATERIALS AND METHODS

Collection of samples: A total of 150 samples of nasal swabs were obtained from different apparently healthy students who consented to the investigation.

Isolation of organisms: The specimens (swabs) were inoculated onto mannitol salt agar using the streak plate method of inoculation. Inoculated plates were incubated at 37°C for 24 hours. Upon establishment of growth, bacterial colonies showing typical characteristics of S. aureus (golden yellow pigmentation on mannitol salt agar) resulting from fermentation of mannitol were sub cultured onto freshly prepared nutrient agar plates. The bacterial isolates were identified as S. aureus based on their morphology, Gram-staining, catalase properties, coagulase and DNase tests according to the CLSI guidelines (20). The resulting pure colonies were stored in agar slants for antimicrobial assays.

Antimicrobial susceptibility testing: The identified S. aureus isolates were tested for susceptibility to 9 different antimicrobial agents in the disc diffusion method on Mueller Hinton agar. The antimicrobial agents tested were: augmentin, 30µg (AUG), Levofloxacin, 5µg (LBC), erythromycin, 15µg (ERY), Ceftazidime, 30µg (CAZ), Ofloxacin, 10µg (OFL), gentamicin, 10µg (GEN), Ceftriaxone, 30µg (CRO), Cefotaxime 30µg (CTX) and Ciprofloxacin 5µg (CPR). Discrete colonies from an 18 hours culture of each isolate were suspended into sterile nutrient broth in a tube to achieve a bacterial suspension equivalent to 0.5 McFarland turbidity Standard. A cotton swab was dipped into the bacterial suspension and the swab pressed on the side of the tube to drain excess fluid. The entire surface of the agar plate was then inoculated with the same swab of inoculum, rotating the plate to ensure confluent growth of the bacteria. The antibiotics discs were placed on Mueller Hinton agar plates already seeded with the isolates. The plates were incubated at 35ºC for 24 hours and observed for zones of inhibition, measured using a ruler and recorded. The zones of inhibition produced by the antibiotics against the isolate was used to categorize them as either susceptible, resistant and intermediate status after comparing the zone of inhibition produced by the antibiotics against the isolate with that of a reference guide provided by the CLSI, (21).

Detection of MRSA: A suspension of each isolate was prepared from the colonies from an overnight growth on nutrient agar plate. A suspension of the overnight growth was prepared with sterile saline and the turbidity was adjusted to 0.5 McFarland's standard. A sterile swab was dipped into this suspension and the excess of inoculum was removed by pressing it against the sides of the tube. The swab was then inoculated on Mueller-Hinton agar plate to create a lawn of the organisms. Cefoxitin disks, which are used for methicillin testing, were placed on each inoculated Mueller-Hinton plates. The plates were incubated for 24 hours at 37°C. The diameter of the zone around the disc was measured and the results were interpreted according to the CLSI guidelines. The isolates with a zone of inhibition less than 22 mm were reported as MRSA strains. *Staphylococcus aureus* ATCC 25923 was used as the control strain (21).

DNA extraction using kit: Deoxyribonucleic acid (DNA) was extracted from bacterial colonies sub-cultured on sterile Nutrient broth. DNA Extraction from broth samples was performed using NORGEN DNA extraction Kit (Model 24700, (NORGEN, Canada) following the manufacturer's instructions. According to this method, genomic DNA was extracted using 200 µl of broth sample (containing approximately 3×106 cells transferred using a micropipette in an aseptic condition into a 1.5 µl tubes. The tube was centrifuged at 200 × g (approximately 2000 rpm) for 10 minutes. The supernatant was discarded. This was followed by the addition of 200 µl Digestion Buffer A to cell pellets. Then it was subjected to gentle mixing and addition of RNase to the cell suspension. Following this 12 µl of Proteinase K was also added to the suspension. The mixture was vortexed gently to homogenize the contents. The suspension was then incubated at 55ºC for 1 hour. After incubation, 200 µl of Buffer K was added to the lysate. Followed by another process of mixing by vertexing. Then 100% ethanol was added and solution further mixed before DNA Binding to the Column. Exactly 800 µl of the mixture from above was added to a spin column in a Collection Tube and centrifuge at 14,000 x g for 2 minutes. Flow through from the Collection Tube was discarded and this particular step was repeated with the remaining filtrate. Then 500 µl of Wash Solution A was thereafter added to the Spin Column in a new Collection tube and centrifuge again at 14,000 x g for 1 minute after which 500 µl same Wash solution A was added to the Spin Column and centrifuge at 10,000 x g for 1 minute in order to completely dry the column. The Spin Column was transferred into a clean 1.7 ml Elution tube and 200 µl of DNA Elution Buffer B was added directly to the center of the resin bed. This was centrifuge 14,000 × g for 2 min to elute the DNA. The template DNA was aliquoted and used for conventional PCR to identify resistance genes (22).

PCR reaction: Detection of *Staphylococcus* species and detection of resistance genes, mecA and mecC were performed by Polymerase Chain Reaction. The extracted DNA was subjected to PCR to amplify the 163-bp and 188-bp fragment of the mecA and mecC genes respectively using the following primer sequences as presented in Table 1 below.

The PCR reaction was carried out using the Solis Biodyne 5X FIREPol Master mix. PCR was performed in a reaction mixture of 25 µl, and the reaction concentration was brought down from 5X concentration to 1X concentration containing 1X Master mix buffer (Solis Biodyne), 1.5 mM MgCl2, 200 µM of each deoxynucleoside triphosphates (dNTP, Solis Biodyne), 25 pmol of each primer (Stab Vida, Portugal), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5 µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in a Techne Prime thermal cycler. The program was set at an initial denaturation of 95°C for 15 minutes followed by 35 cycles of 30 seconds at 95°C; 1 minute as indicated in the primer table and 1 minute 30 seconds at 72°C. This was followed by a final extension step at 72°C for 10 minutes.

Multiplex PCR Amplification Multiplex PCR was adopted in this study by putting the primers and each isolate in one reaction tube such that there will be simultaneous amplification. This involved the use of 5X FIREPol master mix (Solis Biodyne, Estonia) brought down to 1X concentration. The final reaction volume was 20 µl comprising of 1X Blend Master mix buffer (Solis Biodyne, Estonia), 2.0 mM MgCl2, 200 µM of each deoxynucleoside

Table 1: Primers Sequences.

Genes	Primer sequence (5′-3′)	Annealing Temperature (ºC)	Product size (bp)	References
Staph 756	F- AACTCTGTTATTAGGGAAGAACA R- CCACCTTCCTCCGGTTTGTCACC	58	756	22
mecA	F- TGGTATGTGGAAGTTAGATTGGGAT R- CTAATCTCATATGTGTTCCTGTATTGGC	52	163	23
mecC	F- CATTAAAATCAGAGCGAGGC R- TGGCTGAACCCATTTTTGAT	52	188	23

triphosphates (dNTP) (Solis Biodyne, Estonia), 20 pmol of each primer (Jena, Germany), 2 unit of FIREPol DNA polymerase (Solis Biodyne, Estonia), Proofreading Enzyme, 5 µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in an Pielter thermal cycler (MJ Research Series, USA) for an initial denaturation of 95°C for 5 minutes followed by 35 amplification cycles of 30 seconds at 95°C; 1 minute at 58°C and 1 minute 30 seconds at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80 V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. A 1000 bp DNA ladder was used as DNA molecular weight standard (23)

RESULTS

The overall prevalence of S. aureus colonization in this study was 45.3% (68/150). Of the total 68 S. aureus isolates, 31 (45.5%) were found to be phenotypically methicillin-resistant and 54.4% (n=37) were Methicillin susceptible. Among the MRSA isolates, high levels of susceptibility were seen for Ofloxacin (87.1%, n=27), Levofloxacin (90.3% n=28), and Gentamicin (77.4% n=24). On the other hand, the highest resistance was recorded against Ceftazidime (58.1%, n=18), augmentin (93.5%, n=29) and ceftriaxone (45.1%, n=14). Resistance against erythromycin was at 41.9% (n=13). The antibiotic resistance profiles in MRSA isolates were recorded as following: ciprofloxacin 16.2% (n=6), Augmentin 86.4% (n=32), erythromycin 35.1% (n =13), Ceftazidime 59.4% (n=22). Of the 10 MRSA isolates that were subjected to PCR, to amplify the 163-bp fragment of the mecA gene and 188-bp of mecC gene, as shown on Figure 2, one isolate was found to harbor the 188-bp of mecC gene whilst mecA was absent from the screened isolates.

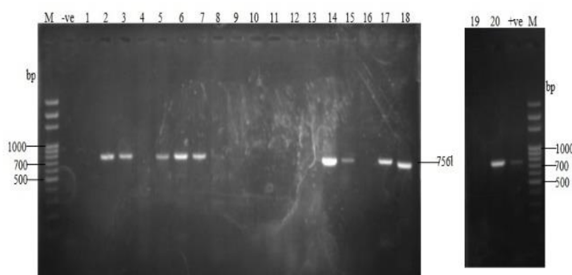

Figure 1: Agarose Gel electrophoresis of 16S rRNA amplification bands corresponding to the expected band sizes of 756 bp for *Staphylococcus aureus* isolates.

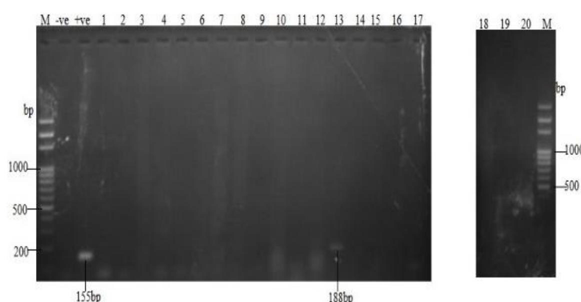

Figure 2: Multiplex PCR of mecA and mecC genes; M: 100 bp DNA ladder; -ve/+ve: Control; Lane 4-15: Negative sample for mecA and mecC genes; Lane 16: samples positive for mecC gene and negative for mecA gene.

Table 2: Antibiotics Susceptibility Profile of the Methicillin Resistant *S. aureus* Isolates.

Antibiotics	No. (%) Susceptible	No. (%) Intermediate	No. (%) Resistant
Ceftriaxone	11 (35.4)	6 (19.3)	14 (45.2)
Gentamicin	24 (77.4)	3 (9.7)	4 (12.9)
Ofloxacin	27 (87.1)	0 (0.0)	4 (12.9)
Ceftazidime	8 (25.8)	5 (16.1)	18 (58.1)
Erythromycin	12 (38.7)	6 (19.3)	13 (41.9)
Levofloxacin	28 (90.3)	0 (0.0)	3 (9.7)
Augmentin	0 (0.0)	2 (6.4)	29 (93.5)
Cefotaxime	14 (45.2)	4 (12.9)	13 (41.9)
Ciprofloxacin	21 (67.7)	4(12.9)	6(19.3)

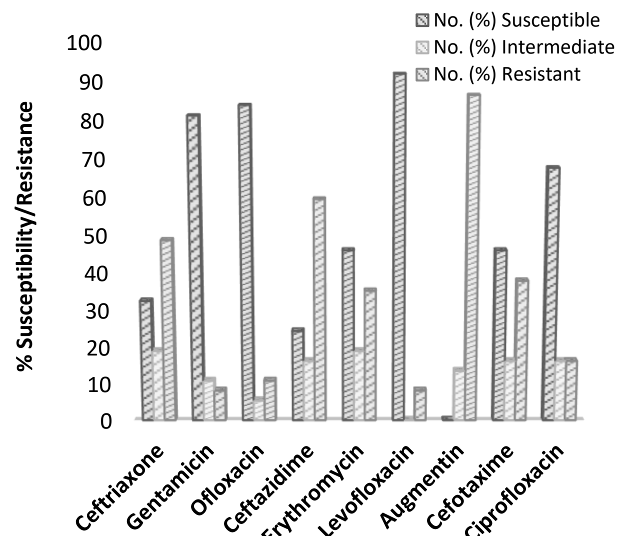

Figure 3: Drug Susceptibility Profile of the Methicillin *Susceptible S. aureus* Isolates.

DISCUSSION

Staphylococcus aureus is one of the most clinically important multi-drug-resistant threats worldwide, according to the global priority pathogens list of antibiotic-resistant bacteria (24). It presents significant epidemiologic and therapeutic challenges. Antibiotic resistance profiles of the MRSA isolates collected in this study are consistent with earlier studies investigating S. aureus globally (25-27).

The MRSA isolates from the current study showed 41.9% and 45.2% resistance towards erythromycin and ceftriaxone, respectively, which was higher than previously reported rates of 39% and 23%, respectively (28). Ciprofloxacin resistance was seen in 19.3% of the MRSA. However, higher resistance rate (36.7%) was observed for ciprofloxacin (28) as compared to the resistance rate of 14% in the current study. On the contrary, resistance to erythromycin ranging between 66.66% - 67.9 % has been observed by other researchers (29, 30). It was observed however, that 87.1%, 90.3% and 77.4% of the total MRSA isolate

obtained in this study were susceptible to Ofloxacin, Levofloxacin and Gentamicin. The susceptibility of the isolates to the flouroquinolones and gentamicin is in concordance with an earlier study (31, 32). The observations from this investigation alerts us about the rapidly increasing resistance to commonly used antibiotics and raises concerns towards the efficacy of last-resort antibiotics.

The results of the antibiotics susceptibility testing raise a disturbing concern as a greater percentage of the isolates was resistant to the tested antibiotics. This study highlights the need to take MRSA with utmost seriousness. Uncontrolled use of antimicrobials in poultry processes requires concerted interventions to curb increasing morbidity due to MRSA. Replacement of antibiotics with indigenous medicinal plants in the feeds and water of poultry has been suggested as a measure of addressing this issue. Again, proper hand washing is paramount in trying to reduce transmission of this category of bacteria.

Polymerase chain reaction (PCR) confirmed 10 out of 20 (50.0%) isolates to be *S. aureus*. This observation also agrees with those of other researchers (33) in which Polymerase chain reaction (PCR) confirmed 217 out of 403 (53.8%) isolates to be *S. aureus*. The mecA gene was not detected from the isolates that displayed a phenotypic Methicillin resistance, probably due to the presence of other resistant genes which encode for resistant to Methicillin such as mecC gene. This study reports the isolation of an MRSA isolate carrying mecC gene along with resistance to several antibiotics corroborating earlier studies by Kim (34). This finding indicates that the true prevalence of mecC carrying MRSA may be underestimated as only a limited number of isolates were assayed. The findings in the present study showed low frequency of detection of mecA gene prompting the search for other intrinsic factors that may contribute to the observed phenotypic expression of MRSA as well as inclusion of mecC while assaying for methicillin resistance.

All MRSA isolates in this study were negative for mecA while one out of the ten (0.1%) amplified isolates were positive for mecC, suggesting some sort of variation in the mecA genes of earlier phenotypically identified MRSA which is in accordance with a previous study (35). This observation is also in line with a related study as reported by other researchers (1) where neither mecA nor pvl genes were recovered from 45 phenotypically positive MRSA isolates. Similarly, 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 hyper-production of β-lactamase as a cause of the phenomenon (36). The absence of mecA in MRSA strains has also been reported by many authors worldwide (17, 37). S. aureus isolates bearing the MecC gene has not only been detected in animal species but also have been detected in humans though less frequently. Peterson et al. (38) reported that mecC constituted 1.5%, with an increasing frequency reaching 1.9% and 2.8% in 2010 and 2011, respectively in Denmark (38). In Germany, 1604 (collected in 2004 to 2005) and 1603 (collected in 2010 to 2011) MRSA isolates were analyzed and found one isolate from each sampling period harbored mecC gene (39). Methicillin-resistant *S. aureus* (MRSA) was isolated and identified by the presence of mecC, in 27% of their isolates (33). Numerous other studies have reported *S. aureus* isolates that exhibited phenotypic resistance to methicillin but were found to be negative for the mecA gene (40, 41). Detection of mecA gene by molecular methods such as PCR is a gold standard for confirmation of MRSA isolates, however, with several reports of conflicting absence of mecA gene in phenotypically identified MRSA. It is worth noting that other genes as observed from this study may be associated with the observed phenotypes globally reported.

CONCLUSION

A mecC gene positive isolates represent a potential public health problem, and highlights the need for surveillance program and monitoring of animal and environmental reservoirs for the presence and evaluation of mecC gene carrying *S. aureus* strains. This study suggests for inclusion of screening of other resistance traits in mecA negative MRSA isolates. Further large-scale studies that will compare the antibiotic resistance profile of mecC MRSA and mecA MRSA is advocated. The detection of mecC-MRSA in the present study highlights the diagnostic importance of screening for mecC in mecA-negative MRSA.

COMPETING INTERESTS

No Conflict of Interest Indicated by the authors.

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