Original Research Article

Isolation and Molecular Detection of Mec-A Gene in Methicillin Resistant Staphylococcus aureus Colonizing Anterior Nares of School Children in Jos South, Nigeria

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ABSTRACT

Aims: This study investigates and reports the carriage of MRSA in students of some Secondary Schools in Jos South Local Government

Study design: Cross sectional study

Place and Duration of Study: Molecular biology laboratory, Department of Microbiology, National Veterinary Research Institute, Vom, between February 2016 and March 2017.

Methodology: *Staphylococcus aureus* was isolated from the samples using standard cultural and microbiological methods. Antibiotic susceptibility testing was determined as described by the Clinical and Laboratory Standards Institute (CLSI). The detection of MRSA isolates was observed phenotypically using oxacillin and cefoxitin disc diffusion tests. In addition, molecular detection of MRSA *mecA* gene production was carried out using Polymerase Chain Reaction (PCR) method.
Results: All samples isolated (100%) had *Staphlococcus sp*. with 114 (29.6%) isolates being *S. aureus.* Antibiotic resistance in the isolates in decreasing order were as follows: ceftazidime (48.2%), augmentin (37.8%), cefuroxime (35.0%), ceftriaxone (23.7%), ampicillin-sulbactam (21.9%), penicillin G (14.9%), ofloxacin (12.2%), gentamycin (11.4%), erythromycin (8.8%), vancomycin (0.0%). Eighteen of the 114 coagulase positive *S. aureus* isolated (15.8%) were found to be MRSA using the oxacillin disc diffusion test, and thirteen of the MRSA detected (11.4%) identified using cefoxitin disc diffusion test to be also MRSA. The presence of methicillin-resistant gene (*mec A*) was confirmed in four (4) of the 18 MRSA isolates using the PCR technique.
Conclusion: The *S. aureus* isolates were less resistant to ampicillin-sulbactam, erythromycin, gentamycin, ofloxacin, and ceftriaxone, with vancomycin showing no resistance. In addition, *mec A* gene was detected in confirmed MRSA isolates.

Keywords: Staphylococcus aureus; MRSA; antibiotics resistance; susceptibility; gene.

1. INTRODUCTION

Staphylococcus aureus is known to be a major pathogen which causes a wide spectrum of clinical manifestations, including skin and soft tissue Infections, pneumonia, septicemia, endocarditis, Arthritis, gastroenteritis, meningitis, urinary Tract infections, and Toxinoses (such as food poisoning, scalded skin syndrome and toxic shock Syndrome) [1-4]. The drugs of choice for therapy are usually the beta-lactam antibiotics.

Staphylococcus was first identified in 1880 in Aberdeen Scotland by a Surgeon, Sir Alexander Ogston in pus from a surgical Abscess in a knee joint [5]. It is estimated that 20% of human population are long term carriers of *Staphylococcus aureus* which can be found as part of the normal skin flora [6] and in the anterior nares of human and different animals [7,8]. Methicillin Resistant *Staphylococcus aureus* (MRSA) is a strain of the bacterium *Staphylococcus aureus*. It is characterized by antibiotic resistant to methicillin and many other chemotherapeutic agents [9]. MRSA isolates are resistant to the β-lactam antibiotics. Vancomycin is used to treat MRSA infections. Despite the efforts of researchers to help improve the understanding of the pathogenesis of *S. aureus*, there seems to be an increase in staphylococcal infections.

Staphylococcus aureus has a wide host range, and a diverse cellular as well as environmental lifestyles. It has the ability to evolve to highly drug-resistant forms that can cause diseases with epidemic potential [10]. *S. aureus* exhibits its lifestyle in two ways: a commensal asymptomatic state where it is harbored by 20% or more of the general populations at the anterior nares; and the acute state at which it attacks specific tissues and pathogenicity follows consequently. Generally, *S. aureus* causes infections by self- inoculation (a process whereby the organism breaches the natural barrier) or by direct or indirect contact [10].

Antibiotic resistance is a serious public health issue. Some of the factors facilitating antimicrobial resistance are irrational use of antibiotics by health professionals, unskilled practitioners and the public; the ready availability of antibiotics as over the counter drugs, poor quality of antibiotics unhygienic conditions and inadequate surveillance [11, 12]. There is a rise in the prevalence of MRSA globally. It is estimated that MRSA kills more than 40,000 patients every year in the United States alone [13]. Data is unavailable to determine the number of patients that are casualties of MRSA in Nigeria. However, from the studies undertaken in Nigeria and the data from few locations [14-20], it is necessary to contain antimicrobial resistance. Based on the WHO antimicrobial resistance global report on surveillance, *S. Aureus* is a major bacteria of international concern [21]. Some countries have formulated policies and invested in surveillance methods to check this public health issue, however, developing countries need do more work to address antimicrobial resistance [22]. Nigeria has developed a national action plan for antimicrobial resistance, 2017-2022, but its implementation years later is still at the early stages with progress difficult to track. In Nigeria, antibiotics are still bought over the counter, thereby increasing the selective pressure of antibiotics when its reduction is a strategy for containing antimicrobial resistance.

There is a need for urgent coordination and collaborations to contain antimicrobial resistance in order to avert a major public health disaster. This study focuses on the detection of *mec A* genes in MRSA isolates from nares of school children in Jos South, Plateau state, Nigeria.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Media

Bacteriological media that were used in this study included: MacConkey Agar (MCA), Mueller-Hinton Agar (MHA), Nutrient agar (NA), Luria-Bertani (LB) broth, Mannitol Salt Agar (MSA), Nutrient Broth (NB), Blood Agar medium and Peptone water (PW). All the media were sourced from Oxoid Ltd. (U.K.).

2.1.2 Equipment

The equipment used in this study include: Autoclave (Certoclav, Model SM280E, Surgifriend Medicals, England), Oven (HotboxSize One, Galengkamp, U.K.), Incubator (Model12-140E, Quincy Lab Inc), Refrigerator/Freezer (Model PRN 1313 HCA, BEKO, Germany), Thermocycler (Model TC-312, Techne, England), Gel electrophoresis machine (Max Fill Scie-plas Model HU10 serial no5237), Laminar air flow cabinet (PCR-8 re-circulating laminar flow pre station, Labcaire product 220/240v), Microscope (Model CME 1349522X, Leica, USA), Spectrophotometer (Eppendorf Biophotometer 8.5 mm, Lichtstrahihohe), UV illuminator (VilberbLourmat TFX-35-M serial no NoV02 8104), Centrifuge (Model 5417R: Touch plate Super Mixer, CAT No 1291, Lab-line Instrument Inc USA), Microwave oven (HINARI Life Style 800watts model MX310TCSL), Electronic weighing balance (Model QT 600: Touch plate Super Mixer, CAT No 1291, Lab-line Instrument Inc USA),Vortex machine (Touch plate Super Mixer, CAT No 1291, Lab-line Instrument Inc USA), and Gel Doc system (Biorad, U.K.)

2.1.3 Chemicals and reagents

The chemicals and reagents used in this study included: Carbolfuschin, Crystal violet, Ethanol, Creatinine, Potassium hydroxide and Kovac's reagents, obtained from BDH chemical Ltd, England; Ethidium bromide, Iodine solution, EDTA and Glycerol obtained from Sigma Chemical Ltd, England; and Agarose gel from Schwarz/ Mann Biotech.

2.1.4 Bacteria Isolates

Confirmed *S. aureus* isolates from the anterior nares were obtained and used for this study.

The antibiotic resistance profiles of the isolates are as shown in Table 2.

2.2 Methods

2.2.1 Antibiotic Susceptibility Testing

The antibiotic susceptibility test for *S. aureus* isolates from anterior nares was carried out using the Kirby-Bauer disc diffusion method as modified by the Clinical and Laboratory Standards Institute - CLSI [22]. Briefly, 5 colonies of *S. aureus* isolates were inoculated into 5 ml of Mueller-Hinton broth (MHB) and incubated at 37°C for 24 hours after which the 24-hour MHB was standardized to the turbidity equivalent to 0.5 McFarland standards. The 0.5 McFarland standard was prepared as follows: 99.5 ml of 1% ($\frac{v}{v}$) H_2SO_4 + 0.5ml of 1.172% ($\frac{w}{v}$) $BaCl_2.2H_20$. A sterile cotton swab stick was dipped into the standardized *S. aureus* suspension and streaked on MHA plates. Antibiotics discs were gently placed on the MHA plates using a pair of sterile forceps and the plates were allowed to incubate at room temperature for 1 hour before re-incubating at 37°C for 17 hours. After incubation, the diameters of the zones of inhibition were measured to the nearest millimetre (mm) using a ruler and the result of the susceptibility test was interpreted using susceptibility breakpoint earlier described by CLSI [22].

2.2.2 Phenotypic test for MRSA

All *Staphylococcus aureus* confirmed strains were screened for methicillin resistance by using cefoxitin and oxacillin (oxoid) 30ug and 1ug respectively. The discs were placed at a distance of at least 25mm away from each other.

The isolates with inhibition zone diameter of<10mm for oxacillin and <21 mm for cefoxitin were termed methicillin resistant where as those showing a zone diameter of ≥13mm for oxacillin and ≥22mm for cefoxitin were termed methicillin sensitive Clinical and laboratory standard institute[22].

2.2.3 Molecular detection of MRSA *mec A* genes

Isolates that were MRSA positive were screened to detect the presence of *mec A* gene.

2.2.4 DNA extraction

The DNA extraction was performed by the boiling method as described previously [23]. Following purification on MacConkey agar, bacterial

DNA was isolated from a 24-h culture in Luria-Bertani broth prepared according to the manufacturers' protocol. The bacterial cells were harvested by centrifugation at 3200 rpm in a microcentrifuge for 2 min at room temperature and the supernatant was discarded. The harvested cells were re-suspended in 1 ml of sterile normal saline and the micro-centrifuge tubes were placed in the vortex for 5 sec. Centrifugation was carried out at 3200 rpm for 1 min and the supernatant was discarded. 0.5 ml of sterile normal saline was added to the pellets and the tubes were vortexed for 5 sec after which they were heated in the block heater at 90°C for 10 min. immediately after heating, rapid cooling was done by transferring the tubes into the freezer for 10 min. Cell debris was removed after centrifugation was done at 3200 rpm for 1 min and 300 µl of the supernatant was transferred into a sterile 2 ml Eppendorf tube as DNA and stored at -10°C until use.

Estimation of the concentration, purity and yield of the DNA sample was accessed using the absorbance method (the measurement of absorbance) with the spectrophotometer (Nanodrop 1000, InqabaBiotectm, South Africa). For DNA concentration, absorbance readings were performed at 260 nm (A260) and the readings were observed to be within the instrument’s linear range (0.1 – 1.0). DNA purity was estimated by calculating the A260/A280 ratio and this was done by the spectrophotometer’s computer software (where A260/A280 ratio ranges from 1.7 – 1.9).

2.2.5 DNA amplification of *mec A* genes

The technique of [23] was used for the polymerase chain reaction (PCR) procedure in this study. The primers (sense) *mec*A1 5’-AAAATCGATGGTAAAGGTTGGC-3’ and (antisense) *mec*A2 5’ AGTTCTGCAGTACCGGATTTGC-3’ (Inqaba, South Africa) were used to detect the presence of *mec* A gene in the methicillin–resistant isolates by yielding a PCR product of 533 bp [23]. The master mix for PCR was performed in a 50 µl reaction mixture containing 5 µl of a 10X PCR buffer, 1.5µl of a 200 µM concentration of each deoxynucleoside triphosphate (dNTP), 2.5 µl of a 25 mM MgCl2, 0.25 µl of a 1.25 unit Taq polymerase (Biolab, South Africa), 1 µl of a 10pM of each primer and 0.25 µl of a 2.5 µl of DNA template. DNA amplification was carried out using GeneAmp^R PCR System9700 (Applied Biosystems, U.S.A) for 50 cycles in 50 µl of reaction mixture as follows: initial denaturation at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute with a final extension at 72°C for 5 minutes, then kept at 4°C.

2.2.6 Agarose gel electrophoresis

PCR products (10 µl) was electrophorised in 1.5% agarose gel. (The gel containing 5 µl of 10mg/ml ethidium bromide solution) at a 100 volts for 60 minutes. 50 bp DNA marker (Fermenter^R) was used as the molecular size marker. The Amplicons were visualized under automatic molecular imaging transiluminator (Gel DOC^{TM} XR+ BIORAD, U.S.A) to check for the presence of the DNA PCR products at 533bp and the image was stored in the computer.

3. RESULTS AND DISCUSSION

3.1 Age and Sex Distribution of the Study Population

A total of 385 students were enrolled for this study, out of which 173 were male and 212 were female, with male to female ratio of 1.6:2. as shown on table 1.

Table 1. Age and Sex Distribution of the Study Population

Schools	Age (10-15yrs)	Age (16-21yrs)	Female (%)	Male (%)
GSS Rantya	46 (11.9)	33 (8.6)	31 (8.1)	48 (12.5)
GSS Kufang	99 (25.7)	110 (28.5)	126 (32.7)	83 (21.6)
Sacred Heart	74 (19.2)	23 (5.9)	55 (14.2)	42 (10.9)
Total	219 (56.9)	166 (43.1)	212 (55.0)	173 (45.0)

3.2 Antimicrobial Resistance Profile

The antimicrobial resistance in the *S. aureus* isolates from anterior nares of school children are shown in Table 1. The resistance in the isolates were as follows ceftazidime (48.2%), augmentin (37.8%), cefuroxime (35.0%), ceftriaxone (23.7%), ampicillin-sulbactam (21.9%), penicillin G (14.9%), ofloxacin (12.2%), gentamycin (11.4%), erythromycin (8.8%), vancomycin (0.0%).

Table 2. Antimicrobial Resistance and Sensitivity pattern of *Staphylococcus aureus* isolates (n=114)

Antibiotic	Resistance (%)	Sensitivity (%)
Augmentin	43 (37.8)	71 (62.2)
Ceftazidime	55 (48.2)	59 (51.8)
Ceftriaxone	27 (23.7)	87 (76.3)
Cefuroxime	40 (35.0)	74 (65.0)
Penicillin G	17 (14.9)	97 (85.1)
Erythromycin	10 (8.8)	104 (91.2)
Gentamycin	13 (11.4)	101 (88.6)
Ofloxacin	14 (12.2)	100 (87.8)
Ampicilin-Sulbactam	25 (21.9)	89 (78.1)
Vancomycin	0 (0)	114 (100)

3.3 Biochemical Test for Isolates

Out of the 385 *Staphylococci species,* 114 were confirmed by biochemical tests to be coagulase positive *(Staphylococcus aureus)* while 271 were coagulase negative giving 29.6% and 70.4% respectively as represented in the pie chart as shown in Figure 1.

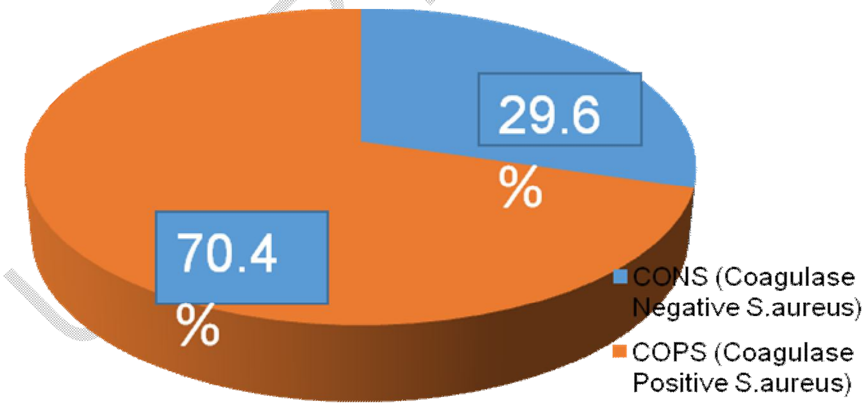

Fig. 1: Coagulase positive and negative *S. aureus* aong the isolates.

3.4 Phenotypic Confirmation of MRSA

Out of the 114 coagulase positive *Staphylococcus aureus* isolated, eighteen (18) were found to be MRSA using oxacillin disc diffusion test which gave a prevalence of 15.8%

among *S.aureus* isolates and overall prevalence among study population of 4.7%. Only thirteen (13) of the MRSA detected were identified using cefoxitin disc diffusion test to be also MRSA, thereby giving a prevalence of MRSA among *S.aureus* isolates 11.4% and overall of 4.7% as shown in table 3.

Table 3. Frequency of MRSA among the coagulase positive *staphylococcus aureus* isolates (n=114)

Confirmatory Test	Number	Frequency
	114	29.6
MRSA (oxacillin disc)	R 18	15.8
	S 96	84.2
MRSA (cefoxitin disc)	R 13	11.4
	S 101	88.6
Overall Prevalence (n= 385)	18	4.7

Key: **R**=Resistant **S**=Sensitive

3.5 Molecular Detection of *mec A* Genes

The presence of methicillin-resistant gene was confirmed using PCR technique. Four(4) of the 18 MRSA isolates were confirmed *mec A* positive as evidenced by the ampilifiaction of *mec A* gene at the specific amplicon of 533bp fig: 2. Hence, the *mec A* gene isolation rate among *staphylococcus aureus* isolates(n=114) was 3.51% and an overall (n=385) was 1.04% as shown in Table 4.

Table 4. The isolation rate of *mec A* gene among the *S. aureus*.

	Number	Frequency (%)
mec A gene prevalence aong *S. aureus* isolates (n=114)	4	3.51
Overall *mec A* gene prevalence among students (n=385)	4	1.04

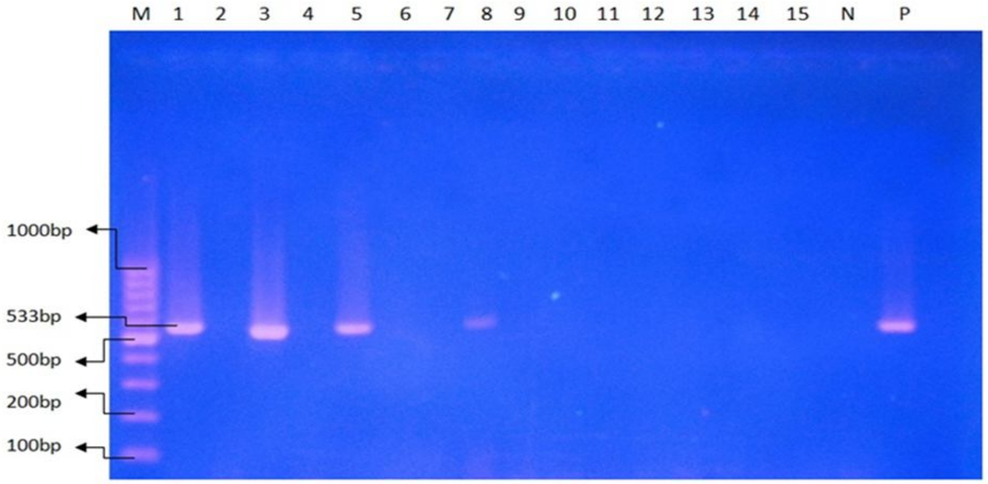

Fig 2: PCR showing four amplicons produced by four isolates at 533bp With a 100bp molecular marker.

3.6 Discussion

Antibiotic resistance has been described as one of the paramount microbial threats of the 21st century [24]. *Staphylococcus aureus* has always been a stumbling block for antimicrobial chemotherapy, and the introduction of new classes of antimicrobial agents is usually followed by the emergence of resistant forms of this pathogen [25]. Methicillin-resistant *Staphylococcus aureus* (MRSA) has been proven to be a globally- spread nosocomial pathogen of the 20th century [26].Therefore, surveillance of the antimicrobial susceptibility patterns of *S. aureus* is of utmost importance in understanding new and emerging resistance trends, and in the management of both hospital and community-acquired infections. In the antimicrobial resistant profile, it has been shown that Ceftazidine exhibited the highest resistance pattern, while Vancomycin showed no resistance. Vancomycin can therefore be used for the treatment of *staphylococcus aureus* as well as other antibiotics such as Ampicillin, Gentamycin, Ofloxacin, and Ceftriaxone which showed low resistance.

In the antibiotic susceptibility testing it was observed that Erythromycin had the highest number of isolates sensitive to it i.e., 104 isolates giving a percentage of 91.2% as opposed to Ceftazidime with the lowest number of isolates 59 (51.8%; n=114). Data on MRSA strains in most sub-Saharan African countries, cast shadow on the public health implication and awareness of the organism. Information from this study intends to provide the baseline data, for eventual appreciation on the need for cautious approach and intervention measures.

In this study, the MRSA prevalence of 4.7% (phenotypic) and 1.04 % (genotypic) may be considered to be low, especially as there isn't any previous epidemiological data on MRSA in this area among school children. Although, MRSA prevalence are known to vary with geographical location, type of health institutions, studied population and method of detection employed. However, our MRSA prevalence may be considered very low, when compared with similar study in Ibadan, Southwestern Nigeria with a prevalence level of 20.3% [27], as both phenotypic and molecular methods were employed. Previous studies on MRSA in the southwestern Nigeria with same detection methods, reported prevalence of less than 2% [24, 28]. Oxacillin disc diffusion method was the earliest method of detection for methicillin resistance expression. Because of low specificity and sensitivity, cefoxitin disc diffusion was introduced by CSLI, that is known as a good surrogate marker of *mec A* gene detection [29, 30]. Earlier MRSA prevalence reported from Nigeria were based on phenotypic methods using oxacillin.

4. CONCLUSION

A low percentage of secondary school children in Jos are carriers of multi-drug resistant *Staphylococcus aureus*. The *S. aureus* isolates were more resistant to augmentin (37.8%), cefuroxime (35.0%), ceftriaxone (23.7%), ampicillin-sulbactam (21.9%), penicillin G (14.9%), ofloxacin (12.2%), gentamycin (11.4%), erythromycin (8.8%), vancomycin (0.0%). This implies that the antibiotics could be useful in the MRSA infections.

CONSENT

Not applicable

ETHICAL APPROVAL

Not applicable

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