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In Vitro Activities of 7 Antimicrobial Agents Against Methicillin Resistant *Staphylococcus aureus* Isolates From Northwestern Nigerian Hospitals

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Abstract

Background: Staphylococcus aureus (*S. aureus*) is an important pathogen in human infections which is implicated in a wide variety of infections, from mild skin infections to more serious and invasive infections. Methicillin resistant *S. aureus* (MRSA) is one of the major causes of nosocomial infection in hospital setting. *Objectives:* The study was aimed at determining the distribution of *mec*A gene among the *S. aureus* isolates from eight health institutions in Northwestern Nigeria. *Methods:* A total of 100 isolates of *S. aureus* were collected from various clinical specimens. Antibiotics susceptibility testing including methicillin sensitivity testing, beta lactamase testing and PCR for detection of *mec*A gene were carried out on all the 100 isolates of *S. aureus*. *Results:* More than half of the total number of isolates 57 (57.0%) were recovered from wound swabs and urine samples. While the remaining 43 (43.0%) were recovered from ear swabs, high vaginal swabs, urethral swabs blood culture, sputum and semen samples.Male preponderance and age range of 21-30 years were the most important demographic findings.All the 100 (100%) strains were MRSA using 10µg methicillin and 5µgoxacillin discs. PCR analysis showed that *mec*A gene was present only in 5 (5.0%) of the 100 *S. aureus* isolates tested. Furthermore, all the 100 isolates were β-lactamase producers. That there were strong associations between isolation site of specimens / nature of specimens (X^2= 16.74; $p < 0.05$) All the isolates were susceptible to vancomycin. Vancomycin, fusidic acid, rifampicin and gentamicin were found to be the most effective drugs against the *S. aureus* isolates among the antibiotics used. *Conclusion:* The prevalence of *mec*A gene in *S. aureus* in Northwestern Nigeria is 5.0% and *mec*A gene detection is a good predictor of methicillin resistance in *S. aureus* in Nigeria, hence use as a method of detection of MRSA. On the basis of this finding; establishment of molecular diagnostic laboratory in secondary and tertiary health units is urgently required. Control of MRSA infection is essential, and it can be achieved by proper implementation of hospital control measures.

Keywords

*Staphylococcus aureus, mec*A Gene, MRSA, Antimicrobial Agents, Northwestern Nigeria

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1. Introduction

Staphylococcus aureus has emerged as one of the most important human pathogens, being over the past several decades a leading cause of hospital and community acquired infections (Lowy, 1998). It is associated with variety of clinical infections including septicemia, pneumonia, wound sepsis, septic arthritis, osteomylitis and post – surgical toxic shock syndrome with substantial rates of morbidity and mortality (Boyce, 1997; Casgrove *et al.,* 2003; Shopsin and

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Kreiswirth, 2003). One of the reasons for the success of this human pathogen is its variability, occurring at patterns within regions and countries. Though, the organism poses no major problem on human skin, but can cause infection under the skin such as boil or inside the organs such as lung where it causes pneumonia (Boyce, 1997; Casgrove *et al.,* 2003; Shopsin and Kreiswirth, 2003).The organism has been reported as the most common cause of nosocomial infections in the United State of America from 1990 to 1996 (CDC, 1996). Several studies in Nigeria indicated that *Staphylococcus aureus* is among the most frequently isolated bacteria in Nigeria (Ako – Nai *et al.,* 1991; 1995; 2002; Oni *et al.,* 1997; Unackukwu *et al.,* 2005).

Methicillin-resistant *Staphylococcus aureus*(MRSA) has also been noted as one of the main pathogens of public health importance. It was first discovered in the United Kingdom in 1961 and is now widespread, particularly in the hospital setting where it is commonly termed a super bug (Jerons, 1991). The term methicillin resistant is historically used to describe resistance to any of this class of antimicrobials. Today in USA approx 35% of hospital strains of *S. aureus* are resistant to methicillin (or other penicillin antibiotics). And in recent years the emergence of vancomycin resistant *S. aureus*(VRSA) has caused additional concern. Non methicillin – resistant strains of *Staphylococcus aureus* are called methicillin susceptible *Staphylococcus aureus* (MSSA) (Aires de Sousa *et al.,* 1998). Patients with MRSA infections have been noted with worse clinical and economical outcome compared with patients with MSSA infections (Melzer *et al.,* 2003). A study by Chang *et al.,* (2004) indicated that bacterial meningitis caused by MRSA was associated with a mortality rate of 56% compared to a mortality rate of 135 in a patient group with meningitis cause by MSSA. Also in some Cohort studies of patients with MRSA bacteria, higher mortality rates increased morbidity; longer hospital stay and higher cost compared with patients with MSSA bacteria have reported (Blot *et al*., 2002; Melzer *et al*., 2003; Kopp *et al*., 2004). In addition, MRSA infections are particularly difficult to treat if they are at anatomical sites, where antibiotic penetration is reduced (Duckworth, 2003).

Disc diffusion, broth and agar dilutions tests are routinely used to detect MRSA in the laboratory. The detection of MRSA is not a clear cut test is more difficult than other antibiotics susceptibility tests due to the heterogeneity of the strains as most of the of organisms are susceptible to low concentrations of β-lactam antibiotics, which complicates the detection of strains resistant to low levels of antibiotic. It is in view of this that *mec*A gene detection has been used as an alternative way of detecting or confirming MRSA either by use of DNA probe, commercially available fluorescence test (Ieven *et al.,* 1995), latex agglutination test (Cavassini *et al.,*1999), and PCR (Rohrer *et al*., 2001) which provide accurate results than standard susceptibility tests. PCR has been used for detection of MRSA by amplifying mecA gene in *S. aureus* in many laboratories especially in developed countries like USA, UK. It is in view of this, the study attempted to provide a rapid and simple method of detecting *mec*A gene in *S. aureus* isolates from Northwestern region of Nigeria by polymerase chain reaction (PCR) so as to determine the diagnostic value of using this technique for detection or confirmation of MRSA.

2. Materials and Methods

2.1. Bacterial Isolates

A total of 100 consecutive non – duplicated *Staphylococcus aureus* isolates were obtained from clinical samples in 8 health institutions (Microbiology department) across Northwestern Nigeria. The isolates were collected for duration of two years from February 2008 to April 2010. The quality control and rejection criteria of specimen were followed (Isenberg, 1998). All plates were examined for Staphylococcus by colonial morphology on nutrient agar (Cheesbrough, 2000). Catalase, coagulase, DNAase tests and test for heamolysin were performed on all the isolates. *Staphylococcus aureus* ATCC 25923 was used as a reference control organism. All confirmed *Staphylococcus aureus* isolates were stored in 16% v/v glycerol broth at -80oC.

2.2. Storage of the Isolates

Using sterile swab, the entire growth of an overnight pure culture was sub-cultured in 5ml of 16% v/v sterile glycerol broth and immediately stored in freezer [Micro bank (Diagnostic pro-lab)] at -80oC. Viability of the organism was checked by thawing the suspension at 35oC and inoculated on blood agar plates after 24 hours.

2.3. Antibiotics Susceptibility Testing

The susceptibility testing of isolates to 19 antibiotics was carried out by the disk diffusion method according to the National Committee for Clinical Laboratory Standards (now Clinical Laboratory Standards institute) guidelines (NCCLS, 2006). The antibiotics (Mast Diagnostics, UK) tested includes penicillin (10µg), fusidic acid (10µg), ciprofloxacin (5µg), gentamicin (10µg), erythromycin (15µg), chloramphenicol (30µg), tetracycline (30µg), trimethoprin (2.5µg), augumentin (30µg), ofloxacin (10µg), peflacine (5µg), streptomycin (30µg), cefuroxime (30µg), cotrimozaxole (25µg) and clindamycin (2µg). *Staphylococcus aureus* (ATCC25923) was the control strain in every test run. Zone diameters for resistance to fusidic acid and streptomycin are as follows: ≤ 18mm – fusidic acid (Skov *et al.,* 2001) and ≤ 14mm –

streptomycin (Kin *et al.,* 2004) using the current guidelines of the Clinical Laboratory Standard Institute (NCCLS, 2006) as described by Ghebremedhin *et al*., (2007). Growth to the edge of the 5µgvancomycin disk indicated high-level resistance. The resistance rate to each antibiotic was calculated as the number of resistance isolates divided by the total number of isolates.

2.4. Beta-Lactamase Test

Beta-lactamase production by the isolates was determined using the modified method of acidimetric test (Bouchillon *et al.,* 2004). A strip of WhatmanNo.1 filter paper was placed in the bottom of a petridish. A few drops of buffered crystalline penicillin bromocresol purple solution were poured until the paper was almost saturated. Using a sterile wire loop, the growth 10-20 colonies of the test organism was spread on the filter paper, covering an area approximately 5mm in diameter. The lid of the petridish was replaced and the plate was incubated at room temperature for 30 minutes. A change of colour from purple to yellow was regarded as the positive for the production of ß-lactamase.

2.5. Oligonucleotide Primers

The oligonucleotide primers used in this study have been previously described (Martineau *et al.,* 1998; Meshref *et al.,* 2011) and were obtained from a commercial source (Inqaba Biotechnical Industries (Pty) Ltd., South Africa). The 3-end region of the *S. aureus* specific gene (chromosomal DNA) was amplified using A 30nucleotide forward primer 5'- AATCTTTGTCGGTACACG ATA TTCTTCACG -3' and A30 nucleotide reverse primer, 5'-CGTAAT GAG ATT TCAGTA GAT AAT ACA ACA-3' (which hybridize to 5-34 and (112-83), respectively, (Martineau *et al.,*1998). While The 3-end region of the *mec*A gene was amplified using A 22nucleotide forward primer 5'- AAAATC GAT GGTAAAGGTTGG C - 3' and A22 nucleotide reverse primer, 5'- AGTTCTGCAGTACCG GAT TTG C-3' (which hybridize to sites 1282-1301 and 1814-1793) (Robert Koch institute, 2003) (Table 1). *Staphylococcus aureus* specific gene and *mec*Agene have the amplicon size of 107 and 532bp using primers described by (Meshref*et al.,* 2011).

2.6. DNA Extraction

In order to accelerate the procedure of identification in clinical microbiology laboratories, a simple and rapid method for DNA extraction is desirable. After overnight culture on brain heart infusion (Difco Laboratories) agar plates, one colony of each sample was resuspended in 25 µl of sterile distilled water and the suspension was then placed in a 100°C heat block for 15 min. From this suspension, a 5-µl volume was directly used as a template for PCR amplification. (Bignardi *et al.,* 1996; Cavassini *et al.,* 1999; Perez *et al.,* 2001; Anna-Kaarina *et al.,*2009).

2.7. PCR Amplification

PCR assays were all directly performed from the bacterial suspension obtained after the rapid DNA extraction method described above. An aliquot of 5 µl of this suspension was added to 95 µl of PCR mixture consisting of 1× reaction buffer [16 mM (NH4)2SO4, 67 mMTris-HCl (pH 8.8)], a 0.5 mM concentration of each of the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP) (Inqaba Biotechnical Industries (Pty) Ltd., South Africa), 1.0µM of each primer, and *mec*A primer, and 1.25 U of The Dream Taq™ Green PCR Master Mix (2x) (Fermentas Life Sciences, supplied by Inqaba Biotechnical Industries (Pty) Ltd., South Africa) is a ready-to-use solution containing Dream Taq™ DNA polymerase, optimized Dream Taq™ Green buffer and 4mMMgCl2. For each sample, one reaction was performed with the pair of primers to identify *S. aureus* specific sequence gene and with the mecA pairs of primers to detect resistance gene (mecA). In order to reduce the formation of nonspecific extension products, a hot-start PCR protocol was used; the tubes were placed in the thermal cycler when the denaturing temperature was reached. All PCR assays were carried out with a negative control containing all of the reagents without DNA template. DNA amplification was carried out in a TechnePCR system TC-5000 thermocycler (Bibby Scientific Ltd.) with the following thermal cycling profile: an initial denaturation step at 94°C for 5 min was followed by 30 cycles of amplification. Each cycle consisted of the following steps; denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s ending with a final extension step at 72°C for 5 min. After PCR amplification, 5 µl was removed and subjected to agarose gel electrophoresis (1.5% agarose, 1× Tris-borate-EDTA, 100 V, 40 min) to estimate the sizes of the amplification products by comparison with a 100-bp O' GeneRuler™ 100 bp molecular size standard DNA Ladder, ready-to-use designed by Fermentas Life sciences (supplied by Inqaba Biotechnical Industries (Pty) Ltd., South Africa) The gel was stained with ethidium bromide, and the amplicons were visualized using a UV light box. This protocol, including the rapid DNA extraction method from a single colony and electrophoretic analysis of the amplified products on an agarose gel, was performed in less than 4 h.

2.8. Statistical Analysis

The Statistical Package for Social Sciences (SPSS) for windows version 11.0 was used for statistical analysis and data interpretation. The statistical analysis was done using median, averages, ranges, ±standard deviation, chi square.

3. Results

The isolates of *Staphylococcus aureus* that carry the mecA gene were reported as methicillin resistant while the isolates that did not carry *mec*A gene were reported as methicillin susceptible. Only 5 of the 100 *Staphylococcus aureus* isolates were found to have the PCR amplification of mecA gene demonstrating the expected 532 bp product. Therefore the prevalence rate of MRSA in current study was 5.0 %. The distribution of *mec*A-positive *Staphylococcus aureus* according to type of specimen is shown in Table 3. The statistical analysis of the result shows significant difference ($p< 0.05$) between mecA positive *Staphylococcus aureus* and *mec*A negative *Staphylococcus aureus* isolates.

Table 1. Oligonucleotide Primers used in the PCR assay.

Oligonucleotide Nucleotide	Sequence position	Target Gene
A30fwd5-34	AATCTTTGTCGGTACACGATATTCTTCACG	Sa
A30 rev112-83	CGTAATGAGATTTCAGTAGATAATACAACA	Sa
A22fwd1282-1301	AAAATCGATGGTAAAGGTTGGC	*mec*A
A22 rev 1814-1793	AGTTCTGCAGTACCGGATTTGC	*mec*A

Table 2. Distribution of *Staphylococcus aureus* isolates used in the PCR assay according to the study area.

	***Staphylococcus aureus* isolates**		
Study Area	**No. of isolates (%)**	**No. of mecA+ (%)**	**No. of mecA-(%)**
AKTH	17 (17.0)	0 (0.0)	17 (100)
ABUTH	12 (12.0)	1 (8.3)	11 (91.7)
MMSH	26 (2.0)	1 (3.8)	25 (96.2)
FMCB	13 (13.0)	1 (7.7)	12 (92.3)
FMCG	8 (8.0)	1 (12.5)	7 (87.5)
FMCK	6 (6.0)	0 (0.0)	6 (100)
IDH	14 (14.0)	1 (7.1)	13 (92.9)
SHS	4 (4.0)	0 (0.0)	4 (100)
Total	**100 (100)**	**5 (5.0)**	**95 (95.0)**

Mean = 0.714286, SE = 0.184428, SD = 0.48795, CL (95.0%) =0.451279.

Among the MRSA strains, three MRSA isolate was detected in wound samples; two from in-patients at the Murtala Muhammad Specialist Hospital (MMSH) and Federal Medical Centre Gusau (FMCG) while the remaining 1 MRSA was isolated from out-patient at Ahmadu Bello University Teaching Hospital (ABUTH). The remaining 2 MRSA isolates were detected in urine samples from out-patients at the Federal Medical Centre Birnin-kudu and Infectious Diseases Hospital (IDH). The overall distribution of *Staphylococcus aureus* used in the PCR assay according to the study area is given in Table 2.

Table 3. Distribution of *Staphylococcus aureus* isolates according to the type of specimen.

Specimen type	***Staphylococcus aureus* isolates**		
	No. of mecA$^+$(%)	**No. of mecA(%)**	**Total(%)**
Wounds wabs	3(9.7)	28(90.3)	31(31.0)
Earswabs	0(0.0)	9(100)	9(9.0)
Bloodculture	0(0.0)	8(100)	8(8.0)
Urine	2(7.7)	24(92.3)	26(26.0)
Highvaginalswabs	0(0.0)	12(100)	12(12.0)
Sputum	0 (0.0)	11(100)	11(11.0)
Semen	0(0.0)	1(100)	1(100)
Urethralswabs	0(0.0)	2(100)	2(100)
Total	5(5.0)	95(95.0)	100(100)

Mean = 0.285714, SE = 0.285714, SD = 0.755929, CL (95.0%) =0.699118.

All the mecA gene positive isolates identified by PCR were fully resistant to tetracycline and ciprofloxacin but susceptible to gentamicin, rifampicin and fusidic acid. Three of the isolates were susceptible to chloramphenicol and four isolates to ofloxacin. The inducible MLSB phenotype was detected in 3 MRSA isolates and only 3 were multi-resistant MRSA (mMRSA). Table 4 shows the antibiotic susceptibility of the MRSA isolates identified by PCR.

4. Discussion

This result indicates the acquisition of mecA gene is responsible for methicillin resistance; concurring with the previous studies on the role of mecA gene in developing high methicillin resistance (Hartman and Tomasz, 1984). In addition to presence of mecA gene in Methicillin-resistant

Staphylococcus aureus, PBP2a and ica gene cluster can also encode resistant in MRSA (Cramton *et al., 1999*, Memmi *et al.,* 2008). In this study, out of the 100 isolates, 5 (5.0%) of the isolates were confirmed as MRSA by the detection of mecA gene. In addition to our finding, Martin-Lopez *et al*., (2002) recorded 96.5% of MRSA by detection of mecA gene, which is slightly lower than the 100% detection of mecA gene in MRSA from South Western, Nigeria. A recent multi-centre study in South Western Nigeria carried out by Adesida *et al.,* (2005) confirmed resistance to methicillin by the detection of the mecA gene using PCR and reported a prevalence of 1.4 %.The prevalence of MRSA in this study (5.0%) was lower than previous study of Nwankwo *et al.,* (2010) in Northwestern Nigeria where they reported 28.6%. That figure (5.0%) was also lower than previous studies in Southwestern Nigeria, which ranged from 9% to 50% (Rotimi *et al.,* 1987; Ako-Nai *et al.,* 1990; Okesola *et al.,* 1999; Martin-Lopez *et al.,* 2002; Kesah *et al.,* 2003; Taiwo *et al.,* 2004; Adesida *et al.,* 2005). Though mecA gene is responsible for phenotypic behaviour of methicillin resistance in this part of the world, it s noteworthy that low level resistance to methicillin has been reported to be conferred by penicillin binding protein 2a (PBP2a) (Memmi *et al.,* 2008). It is also interesting to note that the MRSA nasal carriage rate was very low among the hospital workers with the non-detection of *mec*A gene in the entire *S. aureus* isolates from the hospital workers suggesting lesser role of hospital workers in dissemination of MRSA in hospitals. The use molecular epidemiological techniques (Taiwo *et al.,* 2005, Alli *et al.,* 2007, Okon *et al.,* 2009) in order to monitor the epidemiology of MRSAis highly desirable in view of the disparity in prevalence of mecA in Nigeria (Rotimi*et al.,*1987; Ako-Nai *et al.,* 1990; Okesola *et al.,*1999; Martin-Lopez *et al.,*2002; Kesah *et al.,*2003; Taiwo*et al.,*2004; Adesida *et al.,*2005). This would provide a better understanding of the pattern of resistance in Nigeria as well as putting measures in place to curtail the prevalence from taking epidemic dimension. The prevalence of MRSA (5.0%) in this study is low compared to similar work in people living with HIV in Nigeria and South Africa where 34.7% and 77% were reported respectively (Taiwo *et al.,* 2005 and Cotton *et al.,* 2008). Beta lactamase production by *S. aureus* identified as a risk factor for the prevalence of MRSA in South Western, Nigeria. Olowe *et al*., (2007) has also been confirmed in our study where all the isolates of *S. aureus* produced the enzyme. The link between MRSA and beta lactamase production in *S. aureus* is very clear, since the use of methicillin (beta lactamase resistant penicillin) is to combat the beta lactamase producing *S. aureus* infections. Though majority of beta lactamase producers were methicillin resistant (Alli, 1988), yet still few isolates don't produce the enzyme. Therefore, because of the variations in results between tests and since several resistance mechanisms are known to mediate methicillin resistance in *S. aureus*, the reliable detection of MRSA cannot be solely based on one technique. Like present study, several other studies (Barski *et al.,* 1996; Kuzucu *et al.,* 2002; Fridkin *et al.,* 2005) have highlighted the problem of detecting heterogeneous MRSA strains using phenotype methods and usefulness of direct detection of mecA gene as gold standard for the detection of methicillin resistance, mainly because phenotype method may be difficult to interpret and some isolates do not express their *mec*A gene unless selective pressure via antibiotic treatment is applied. The presence of the *mec*A gene is considered the hallmark for identification of MRSA strains can be difficult to distinguish using phenotypic methods since heterogenous phenomena may compound the possibility of missing some resistant strains using standard phenotypic susceptibility testing methods due to. This phenomenon was recognized soon after the discovery of MRSA where cultures of few strains were highly resistant to methicillin while majority expressed resistant level at or near those of susceptible strains such as heterogenecity of resistance is a characteristic of MRSA in contrast to almost all other bacteria (Hiramatsu, 1995; Chambers, 1997).

Table 4. Antibiotic susceptibility of the MRSA isolates recovered from PCR.

MRSA(n=5)			
Antibiotics	**No. of isolates that were**		**Resistance rate (%)**
	S	**R**	
Gentamicin	5	0	0.0
Ciprofloxacin	0	5	100
Chloramphenicol	3	2	40.0
Ofloxacin	4	1	20.0
Rifampicin	5	0	0.0
Fusidic acid	5	0	0.0
Tetracycline	0	5	100

Key: S, Sensitive; R, Resistant.

The indiscriminate use of antibiotics as a result of obtaining drugs without prescription may have led to increase in resistant strains (Kim *et al.,* 2004, Adebayo *et al.,*2006).Many investigators have reported an increase in the incidence of MRSA. Hospitals' location was not recognized as a risk factor in this study, association between hospitals location and the prevalence of MRSA was not significant. Other variables such as gender and age were also not risk factors in MRSA infections. This is in sharp contrast to a study carried out in Taiwan where age has been found to be a risk factor for *S. aureus* colonization (Lu *et al.,* 2005). Interestingly, site of isolation of MRSA / specimen type has been found to be associated with prevalence of MRSA in this study; hence considered more of a risk factor. High rate of isolation of MRSA had been found in dwelling devices and endocervical swabs, with low rate of isolation of MRSA in blood. This is in agreement with a previous study (Carnicer-

Pont *et al.,* 2006) where MRSA was found to be associated with catheter or indwelling devices.

Antimicrobial disc susceptibility pattern of the methicillin resistant *S. aureus* isolates was determined in order to establish multiple antibiotics resistant nature of methicillin resistant *S. aureus* isolated. Some of the *S. aureus* isolates were susceptible to streptomycin, and pefloxacin. High level of resistance was recorded for penicillin despite the fact that the hospitals antibiotic usage policy in Northwestern, Nigeria has put a stop to the use of penicillin in treatment of infection. Although this cannot rule the possibility of patients obtaining it as over the counter medicine in pharmacy shops, hence, the selective pressure on S. aureus.

5. Conclusion

The prevalence of mecA gene in *S. aureus* in Northwestern Nigeria is 5.0% and mecA gene detection is a good predictor of methicillin resistance in S. aureus. On the basis of this finding; establishment of molecular diagnostic laboratory in secondary and tertiary health units is highly desirable in order to monitor the pattern of resistance and factors responsible for the increase in mecA gene prevalence. The application of the rapid and reliable detection method of methicillin resistance can decrease unnecessary use of toxic and expensive drugs such as vancomycin. It also provides a tool to control the emergence and spread of multi-resistant organism.

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