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Detection of resistance determinants among clinical and community isolate of *Staphylococcus* species in Ibadan South-West, Nigeria

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Abstract

Methicillin-resistant *Staphylococcus* species are of global concern in healthcare institutions and community settings due to significant morbidity and mortality associated with its infections. This study was designed to evaluate resistance determinants (methicillin resistant, *mecA*; vancomycin, *vanA* and beta-lactamase, *blaZ* genes) in clinical and community isolates of *Staphylococcus* species. Phenotypic and molecular methods were used to determine the presence of these determinants as well as Panton-Valentine Leukocidin (PVL) status of isolates with *mecA* gene. One hundred and eight *Staphylococcus* species from clinical (55) and community (53) samples which were previously isolated in University of Ibadan and her teaching hospital and identified as *S. epidermidis* (92.6%), *S. aureus* (6.5%) and *S. xylosus* (0.9%) were used. Phenotypic resistance to methicillin was 72.7 and 62.3% while vancomycin was 32.7 and 3.8% in clinical and community isolates respectively. However, PCR results indicated that only clinical isolates (3.6%) were *mecA* positive and no *vanA* in all the isolates. The *blaZ* was found in 16.4% of clinical and 1.8% of community isolates. There was no PVL gene in the isolates with *mecA*. The results showed that methicillin-resistance *Staphylococcus* species were absent in the community isolates studied and its incidence was low in clinical isolates.

Keywords: Methicillin resistance; *Staphylococcus* species; vancomycin resistance

1.0 Introduction

Methicillin-resistant *Staphylococcus* species (MRS) are of global concern in healthcare institutions and

community settings because its infections have been associated with significant morbidity and mortality (Whitby et al, 2003; Farley et al., 2015; Nillius et al,

2016). In the early history of staphylococcal infections, the mortality rate associated with septicemia has been previously documented (Smith and Vickers, 1960). Originally, Methicillin-resistant *Staphylococcus aureus* (MRSA) infections were confined mostly in hospitals, long-term care facilities or their likes (Thompson *et al.*, 1982). However, reports in recent past revealed that they are also emerging in the community settings and colonizing persons with neither history of hospitalization nor risk factors (Layton *et al.*, 1995; Otto, 2007). The gradual emergence of MRSA in community setting and persons without risk factors indicates a paradigm shift in its epidemiology. Methicillin resistance is caused by an encoded gene (*mecA*) which has low affinity for beta-lactam antibiotics and is located on a mobile genetic element known as Staphylococcal cassette chromosome (*SCCmec*).

Resistance of *Staphylococcus* species to beta-lactam antibiotics is mediated by beta-lactamases (produced by some Gram-negative and Gram-positive bacteria) which act by hydrolyzing the β -lactam ring of the antibiotics (Thenmozhi *et al.*, 2014). There are several groups and classes of beta-lactamases that result in antibiotic resistance. Penicillin resistance in *Staphylococcus* species especially *S. aureus* is mediated by encoded *blaZ* gene which expresses beta-lactamase. Four prototypes of this enzyme (A, B, C, and D) have been identified (Richmond, 1965; Zygmunt *et al.*, 1992). For example, several class C beta-lactamases result in cephalosporin resistance and some class A beta-lactamases confer resistance to oxyimino-cephalosporins, cephamycins and carbapenems (Bush *et al.*, 1995; Bush and Jacoby, 2010). The actions of these enzymes together with

emergent of methicillin resistance have compounded the problems of microbial chemotherapy.

MRSA infections are given serious attention due to increased hospital costs and have only few therapeutic options for infected patients. The antibiotic vancomycin has been the last weapon against strains that show resistant to all other antibiotics, but cases of vancomycin resistance exhibiting two different mechanisms (vancomycin-resistant *S. aureus* and vancomycin-intermediate *S. aureus*) were reported (Hiramatsu *et al.*, 1997; Bozdogan *et al.*, 2003; Thati *et al.*, 2011). The eventual detection of vancomycin-resistant *S. aureus* and vancomycin-intermediate *S. aureus* isolates from other countries including Brazil, France, United Kingdom, Germany, India and Belgium is an indication that the emergence of these strains may be a global issue (Thati *et al.*, 2011). Vancomycin resistance is caused by acquired gene (*vanA*) and is characterized by acquired inducible resistance to both vancomycin and teicoplanin (Leclercq *et al.*, 1988; Phukan *et al.*, 2016).

In Nigeria, there are limited information on the co-existence of these resistance determinants in clinical and community isolates of *Staphylococcus* species. This study was designed to evaluate three resistance determinants (methicillin, vancomycin and beta-lactamase genes) in clinical and community isolates of *Staphylococcus* species. In this study, community isolates was defined as isolates outside clinical settings (including environmental isolates).

2. Materials and methods

2.1 The organisms and identification:

One hundred and eight (108) *Staphylococcus* species previously collected between 2007 and 2011 from various clinical samples (Table 3) and community

samples (Table 4) which were stored in 40% glycerol at -80°C freezer were used for the study. Combination of preliminary microbiological procedures (Gram staining catalase and coagulase tests), API-Staph identification kit (Biomérieux, France) were used to confirm the isolates as *Staphylococcus* species as these were identified previously with PCR-RFLP and species specific primers (manuscript in preparation).

2.2 Sensitivity test: Single colony of each organism was inoculated in Tryptone Soya broth (Oxoid®) and incubated overnight at 37°C. The overnight suspension of each isolate was diluted with sterile distilled water until the turbidity equivalent of 0.5 McFarland standards was attained. Following the CLSI guidelines, antibiotic discs cefoxitin 10 µg and vancomycin 30 µg (Oxoid®) were applied onto prepared Mueller Hinton agar plates and incubated 37°C. The diameter of the inhibition zones was measured using ruler and interpreted according to the criteria recommended by the CLSI (2011).

2.3 DNA extraction: Bacterial cells harvested from overnight culture were transferred into a 1.5 ml micro centrifuge tube and centrifuged for 5 minutes at 13,000 rpm. The cells were washed twice in normal saline and re-centrifuged at 13,000 rpm at 10°C for 15 min. This was followed by addition of 200 µl of lysis buffer (10mM Tris-HCL, pH 8) and vortexed. This was incubated for 37°C for 2 h. After incubation, cell lysis was carried out by addition of lysis buffer (400 µl) containing 50mM Tris-HCL, 100mM EDTA, 1% SDS, pH 8 and 1 mg/ml of proteinase-K. This was mixed by inversion of the tubes and then incubated at 50°C in water bath for 1 h. Digestion with proteinase-K was followed by addition of 500 µl of a mixture of phenol and chloroforms (1:1 v/v) and emulsified by vigorous shaking for 30 second. The phases were

separated by centrifugation at 13000 rpm at 4°C for 15 min. The aqueous (top) phase was removed and placed in another tube. The high-molecular-weight DNA in the aqueous phase was precipitated by addition chloroform: chloroform:isoamyl alcohol, 24:1 v/v and mixed by inversion. The precipitated DNA was washed twice in 70% ethanol. The DNA was air-dried and dissolved in 100 µl of distilled water. This was placed in 56°C water-bath for at least 30 min for DNA to dissolve. The concentration of the DNA was measured spectrophotometrically with Nanodrop, 3300 (Thermo Scientific).

2.4 Detection of resistance genes (*mecA*, *blaZ* and *vanA* genes) by multiplex PCR:

Multiplex PCR was used for the detection of target genes as described elsewhere (Perez-Roth *et al.*, 2001). Briefly, an aliquot of 1.0 µl of DNA suspension was added up to make 25 µl of PCR mixture consisting of 2.5 µl reaction 10X Taq standard buffer (BioLabs), 0.5 µl of 10 mM deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 0.5 µl of 3 mM MgCl₂, 0.5 µl of 10 µM each *blaZ*, *vanA* and *mecA* primers (Sigma-Aldrich, Germany), 0.125 U of *Taq* DNA polymerase (Invitrogen) and 17.375 µl of sterile water. 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 GeneAmp PCR system 9700 thermocycler with the following cycling parameters for 30 cycles; initial denaturation at 95°C for 3 min, denaturation, 94°C for 30 s, annealing, 45°C for 30 s, Extension, 72°C for 30 s and final extension, 72°C for 7 mins.

2.5 Detection of Pantone-Valentine leukocidin (PVL): The PCR protocol described above was also carried out using only PVL primers in simplex format with annealing temperature of 64°C following a

published protocol (Kader *et al.*, 2011). After PCR amplification, 10 µl of each PCR product was removed and subjected to agarose gel electrophoresis (1.5% agarose, 1x TAE, 100 V, 1 h) to estimate the sizes and quality of the amplicons by comparison with a 100-bp

molecular size standard ladder (BioLabs). Following electrophoresis, gels was examined for bands using a photo documentation system (TranUV, illuminator Biorad).

Table 1: Primer sequences, expected weight and their sources

Primers	Sequence 5'-3'	Expected weight	Sources
mecA F	GTA GAA ATG ACT GAA CGT CCG ATA A	310	Perez-Roth <i>et al.</i> , 2001
R	CCA ATT CCA CAT TGT TTC GGT CTA A		
blaZ F	ACT TCA ACA CCT GCT GCT TTC	173	Lee <i>et al.</i> , 2001
R	TGA CCA CTT TTA TCA GCA ACC		
vanA F	CAT GAA TAG AAT AAA AGT TGC AAT A	3010	Clark <i>et al.</i> , 1993
R	CCC CTT TAA CGC TAA TAC GAT CAA		
PVL F	ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A	433	Kader <i>et al.</i> , 2011
R	GCA TCA AGT GTA TTG GAT AGC AAA AGC		

3.1 Results

3.2 Distribution of *Staphylococcus* species

A total of 55 clinical *Staphylococcus* species were obtained (49 *Staphylococcus epidermidis*, 5 *S. aureus* and 1 *S. xyloso*). *Staphylococcus epidermidis* obtained from wound swab, eye swab, semen and ear swab accounted for 36.4% (20/55), 20.0% (11/55), 14.5% (8/55) and 10.9% (6/55) respectively of all the clinical isolates (Table 2). One *Staphylococcus epidermidis* each was isolated from Sputum, throat, soft tissue and high vagina swab (HVS). Only one *S. xyloso* was isolated from urethral swab. In wound swab, 3 *S. aureus* (5.5%) were obtained while one *S.*

aureus each was recovered from eye and ear specimens. In community isolates (Table 3), only two species were isolated (51 *S. epidermidis* and 2 *S. aureus*). Again, *S. epidermidis* constituted the largest percentage, 96.2% (51/53). Thirty eight *S. epidermidis* (71.70%) and 9(17.0%) recovered from human nostril and waste water respectively. One *S. epidermidis* each was obtained from air and skin sample while and 2(3.8%) was recovered from private suite surfaces. One *Staphylococcus aureus* each was isolated from both nostril and private suite surfaces.

Table 2: Distribution of clinical isolates according to their sources

Sources	<i>S. epidermidis</i>	<i>S. xyloso</i>	<i>S. aureus</i>
HVS	1	0	0
Semen	8	0	0
Ear	6	0	1
Eye	11	0	1
Soft tissue	1	0	0
Sputum	1	0	0
Throat	1	0	0
Urethra	0	1	0
Wound	20	0	3
Total	49	1	5

Table 3: Distributions of community isolates according to their sources

Sources	<i>S. epidermidis</i>	<i>S. xylosus</i>	<i>S. aureus</i>
Air	1	0	0
Waste water	9	0	0
Nasal swab	38	0	1
Skin swab	1	0	0
Private suite surface swab	2	0	1
Total	51	0	2

3.3 Status of resistance determinants (*mecA*, *blaZ* and *PVL*)

Phenotypic detection of methicillin resistance using both methicillin and cefoxitin disks put prevalence at 62.3% in community and 72.3% in clinical isolates. However, only two clinical isolates had visible bands on gel electrophoresis when *mecA* specific primers were used in polymerase chain reaction (Fig 1). However, no clinical and community isolates studied had *vanA* gene when its specific primers were used. Among the clinical isolates investigated, 9(16.4%) had detectable β -lactamase gene while in community, only 1 (1.8%) of the isolates possessed this gene. The PVL status of three *mecA* positive isolates were determined using its specific primers. However, no amplification was found after repeated trials.

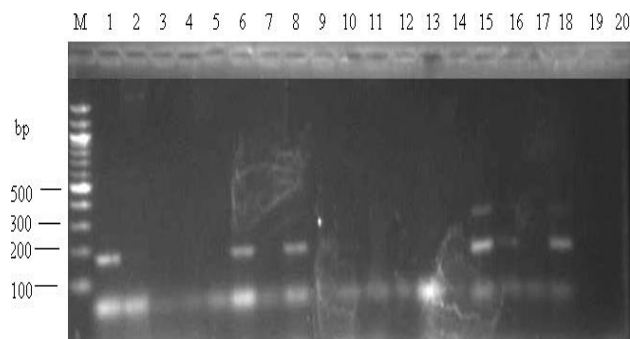


Fig 1: Multiplex PCR for the identification of *mecA*, *vanA* and *blaZ* genes.

Lane M=Molecular marker (100bp), 1, 6 & 8 & 16=*blaZ* positive isolates and *mecA* negative, 15 & 18=*mecA* & *blaZ* positive isolates, negative control= 20.

4.0 Discussion

It has been reported that detection of methicillin resistance in staphylococci depends on prompt synthesis of the peptidoglycan precursors as well as production of penicillin binding protein with chromosomal factors regulating the whole process (Sadeghian *et al.*, 2004). Therefore, strains with *mecA* do exhibit varying resistance pattern which is a function of the genetic background that acquire *mecA*. The detection of methicillin-resistant staphylococci in this study (3.6%) in clinical isolates was comparable to finding of Shittu *et al.* (2009) who reported 2% MRSA (1/50) prevalence of clinical isolates in Ife, Nigeria. This also was similar to some European countries, including Denmark, Finland, the Netherlands, Norway, and Sweden (Bocher *et al.*, 2010). It should be noted that if this report was based on phenotypic method of assessing methicillin resistance, a range of 62.3-72.3% prevalence would have been reported as against 3.6% with molecular method, thus highlighting the importance of molecular over phenotypic methods. The variability in the performance of disc diffusion may be attributable to the heterogeneous nature of phenotypic expression of

resistance in these isolates. Notwithstanding to above factor, other attributable factors like growth conditions, salt concentrations, inoculums size, medium compositions (Annear, 1968; Huang *et al.*, 1993; Coombs *et al.*, 1996; Yoon *et al.*, 2013) might have had considerable effects on the susceptibility test. Recent work has also shown that the susceptibility to antibiotics is highly dependent on the bacterial metabolism and that global metabolic regulators can modulate this phenotype (Corona and Martinez, 2013). In addition, occasion where bacteria become constantly resistant to antibiotics without alteration to genetic make-up was previously reported (Levin and Rozen, 2006).

The limited number of *S. aureus* in this study hampered the overall MRSA prevalence. However, the detection of methicillin resistance in both *S. aureus* and *S. epidermidis* is a warning signal that in nearest future, high prevalence rates can be anticipated especially in the hospital settings. To the best of my knowledge, this is the first time *S. epidermidis* with *mecA* positive as confirmed by PCR is reported in Nigeria. The absence of methicillin resistance in community isolates may be due to low resistance recorded in the clinical settings and probably because horizontal gene transfer of *mec* gene is a rare event as compare to genetic events occurring in plasmid associated resistance.

Vancomycin has been drug of choice for over 50 years in the treatment of staphylococcal infections, especially methicillin-resistant *S. aureus*. However, its efficacy is now debatable regarding the emergence of strains of *S. aureus* expressing varying degree of resistance (Holmes *et al.* 2012) i.e. intermediate resistance, heteroresistance and total vancomycin resistance. In this study, the vancomycin resistance determinant (*vanA*) was not detected in any of the

isolates studied. However, vancomycin-intermediate and vancomycin-resistant *S. aureus* clinical isolates in some centers in Nigeria has been reported, although this were based on phenotypic screening methods (Onolitola *et al.*, 2007; Taiwo *et al.*, 2011). The vancomycin resistant outcome in this study was also contrary to some reports from US, Iran, India where vancomycin-resistant *S. aureus* was due to acquisition of the *vanA* (Thati *et al.*, 2011; Holmes *et al.* 2012). The absence of *vanA* in isolates studied could probably be as a result of rare use of vancomycin for treatment of staphylococcal infections in this region. This practically reduces the exposure potential of these strains to the drug. In addition, the presentation of this antibiotic makes its abuse very difficult by unqualified personnel. Hence, the antibiotic pressure for this drug is low. Another possibility accounting for the absence of this resistance determinant could be that the transfer of this resistance determinant from enterococci to *S. aureus* is a slow event because even in the developed countries where vancomycin is frequently used for treatment of patients, its incidence is relatively very low (Tenover *et al.*, 2004).

Beta-lactamase encoded by the *blaZ* gene accounts for the resistance to penicillins and cephalosporins. The assessment of beta-lactamase using molecular method similar to previous work (Fagade *et al.*, 2010) by conventional iodometric method where the clinical isolates was three times higher than the community counterpart. In this study, percentage of beta-lactamase detection in clinical isolates was seven times more compare to the community isolates. This is not surprising since antibiotic selective pressure is more pronounced in clinical environment compare to the community environment. Accumulation of antibiotics in the clinical environment might have facilitated development of beta-lactamase. In

addition, antibiotic abuse resulting from self-medication which is often associated with inadequate dosage could have contributed significantly to this development. This antibiotic abuse is a result of its availability across the counters with or without prescription.

4.1 Conclusion: The results showed that methicillin-resistance *Staphylococcus* species were absent in the community isolates studied and its incidence was low in clinical isolates. Hence, vancomycin may still remain the drug of choice in treatment of multi-drug resistance associated with methicillin-resistant *Staphylococcus* species in this region.

4.2 Limitation of the study: Limited fund and high cost of molecular techniques hindered the repeat of this PCR amplification for clearer gel documentation.

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