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Distribution of genes encoding aminoglycoside modifying enzymes amongst methicillin resistant and methicillin sensitive *Staphylococcus aureus* isolates from Nigerian hospitals

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Staphylococcus aureus **has long been recognized as one of the major pathogenic organism of human which is responsible for a variety of infections including life threatening infections such as pneumonia. Aminoglycosides antibiotics play an important role in the therapy of staphylococcal infections despite the increase resistant to these drugs. This study aimed at determining the prevalence of genes encoding aminoglycoside modifying enzymes (AMEs) in clinical isolates of *S. aureus* from teaching hospitals in Nigeria. In this study, 86 culture collections of *S. aureus* obtained from 4 teaching hospitals in Nigeria were screened for the presence of genes encoding aminoglycoside resistant genes: (*aac*(6')/*aph*(2"), *aph*(3')-IIIa, ant(4')-Ia) and *mecA* gene by polymerase chain reaction (PCR). Prior to this, antibiotic susceptibility testing was carried out on all the *S. aureus* strains against several antibiotics including gentamicin and cefoxitin. The prevalence of *mecA* gene was 44.2% (38 out of 86 *S. aureus*). Forty eight (55.8%) of the 86 *S. aureus* identified as gentamicin resistant phenotypically contained at least one of the 2 gentamicin resistant genes: aac(6')/aph(2") or *aph(3')-IIIa* while *ant(4')-Ia* gene was not detected in any of the isolates. The MIC$_{50}$ and MIC$_{90}$ for gentamicin resistant strains were 32 and >256 μg/ml, respectively while the MIC$_{50}$ and MIC$_{90}$ for gentamicin sensitive strains were 1 and 8 μg/ml, respectively. The prevalence of gentamicin resistant genes was 29.3% for *aac* and 19.0% for *aph*. No *ant* gene was detected among the gentamicin resistant strains. SCC*mec* typing for all the gentamicin resistant methicillin resistant *S. aureus* strains showed diversity of the isolates with 4 (17.4%) out of the 23 were SCC*mec* II; 4 (17.4%) out of 23 were SCC*mec* III and 9 (39.1) out of 23 were SCC*mec* V while the remaining 8 (34.8%) were non-typeable. Using this scheme gentamicin-methicillin resistant *S. aureus* strains were found to be widely distributed in all the four teaching hospitals studied. The study found an association between genes encoding AMEs and *mecA* on the genome of *S. aureus* isolates from Nigerian hospitals especially the gentamicin-methicillin resistant *S. aureus*, hence the need for establishment of effective infection control measures and antibiotic policies that will reduce the emergence of gentamicin methicillin resistant strain.**

Key words: *aac*(6')/*aph*(2"), *aph*(3')-IIIa, *ant*(4')-Ia genes, gentamicin methicillin resistant *Staphylococcus aureus*, Nigeria.

INTRODUCTION

Aminoglycoside antibiotics have been the cornerstone of effective therapy for bacterial infections including life threatening infections such as pneumonia caused by *Staphylococcus aureus*. These antibiotics are often combined with a beta lactam antibiotic with synergistic effect (Chadwick et al., 1986; Eliopoulos and Moellering, 1996). Bacterial resistance against aminoglycosides has constituted a major threat to successful antimicrobial treatment of patients. The emergence of methicillin resistant *S. aureus* (MRSA) in the early 1960s in United Kingdom (Barber, 1961) has made the use of penicillinase resistant penicillin such as methicillin, flucloxacillin, oxacillin, cloxacillin and even cephalosporins in the treatment caused by this strain very difficult. This strain of *S. aureus* has been reported virtually in all the countries of the world with varying frequencies (Dufour et al., 2002; Kesah et al., 2003; Crum et al., 2006). Nigeria has not been exempted from the global epidemic strain (Ghebremedhin et al., 2009; Okon et al., 2009; Alli et al., 2012). The gene encoding the MRSA phenotype has been linked to *mecA* gene on the chromosome that encodes a modified penicillin binding protein 2 (PBP2). This *mecA* gene has been found to be located within a SCC*mec* cassette within the chromosome and this particular cassette has been found useful in typing the MRSA strains (Zhang et al., 2005). The degree of success that has been achieved in the treatment of staphylococcal infections can be short-lived because of the emergence of aminoglycosides resistant *S. aureus* complicating treatment especially if the strain is MRSA. Aminoglycoside resistance is occasionally caused by intrinsic failure of bacteria to take up drug. However, in most cases, resistance is due to the production by bacteria of an aminoglycoside modifying enzyme (AME), the genetic information for which is acquired by plasmid transfer or another means of gene exchange. About 20 different AMEs are known; and they can be categorized generally into three main groups: acetyltransferases (AACs), phosphotransferases (APHs) and adenylyl transferases (ANTs) (Shaw et al., 1993). Systematic analysis of resistance mechanisms in clinical isolates is indispensable for the design of effective antibiotic policies and could also provide insight into an impending epidemic of antibiotic resistance in *S. aureus*. There is dearth of information on the prevalence of genes encoding AMEs in *S. aureus* from Nigeria apart from the phenotypic description of aminoglycoside resistant *S. aureus* usually described as gentamicin resistant *S. aureus* - being the most commonly available aminoglycoside used for the treatment of staphylococcal infections. It was in view of this that we embarked on the study to examine the clinical isolates of *S. aureus* in Nigeria for the presence of genes encoding AMEs.

Therefore, the aim of the study was to determine the distribution of genes encoding AMEs and *mecA* gene in clinical isolates of *S. aureus* from Nigerian hospitals including the diversity of SCC*mec* types within the MRSA with gentamicin resistant phenotype.

MATERIALS AND METHODS

Bacterial isolates

A culture collection of eighty six (86) *S. aureus* isolates obtained from non-duplicate 500 clinical specimens from sterile and non-sterile sites including aspirates, ear swab, wound swab, blood, and endocervical swab submitted to four diagnostic laboratories of tertiary health care institutions in Nigeria between July and August 2013 were investigated. The four teaching hospitals were Ladoke Akintola University Teaching Hospital (LTH), Osogbo, Obafemi Awolowo University Teaching Hospital Complex (OAUTHC), Ile-Ife, University College Hospital (UCH), Ibadan and University of Ilorin Teaching Hospital (UITH), Ilorin, Nigeria. *S. aureus* identification was based on API 20 Staph kit (Biomerieux, France) and tube coagulase test. All the isolates were stored at 4°C on Mueller Hinton (MH) agar slope until ready for use.

Antibiotic susceptibility testing

The antimicrobial disc susceptibility testing was performed on Mueller Hinton agar. The following antibiotics: penicillin G (10 Units), cefoxitin (30 µg), clindamycin (2 µg), erythromycin (15 µg), tetracycline (30 µg), gentamicin (10 µg), fusidic acid (10 µg) and trimethoprim-sulphamethaxazole (1.25/23.75 µg) were used to determine the susceptibility pattern of the isolates according to the guidelines of CLSI (2012). *S. aureus* ATCC 25923 was used as a control strain for the assay.

Susceptibility of S. *aureus* to vancomycin was determined by using macro dilution technique for minimum inhibitory concentration as described previously with cation adjusted Mueller Hinton broth (CAMHB) (CLSI, 2012). Briefly, about 12 dilutions were made using concentration covering 0.128 to 256 µg/ml of vancomycin. Suspension of the isolates was made to obtain 0.5 MacFarland standard of the organism and 0.05 ml of the suspension was added to each dilution. Three controls were set up; positive control (containing CAMHB and ATCC 25913 strain of *S. aureus*), negative control (containing CAMHB and the antibiotic), and sterility control (containing only CAMHB). MIC breakpoint of ≤ 4 µg/ml was used in defining vancomycin susceptible.

DNA extraction and PCR for detection of *mecA* gene

S. aureus suspension was lysed to release DNA from a centrifuged deposit of 5 ml overnight Mueller Hinton broth culture of the organism in 0.5 ml of TES (Tris HCl pH 8.0, 1 mM EDTA and 0.1 M NaCl) containing lysostaphin (1 mg/ml) (Sigma, UK) incubated at 37°C for 15 min. After which the DNA was extracted with phenol-chloroform, purified and precipitated with ethanol as previously described (Alli et al., 2007). PCR for the *mecA* gene was carried out on all the strains as previously described (Murakami and Minamide, 1993). Briefly, the DNA template from each isolate was amplified

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Table 1. Antimicrobial susceptibility pattern of *S. aureus* isolates from teaching hospitals in Nigeria

Antibiotics	Number of *S. aureu*s sensitive isolates (%)	Number of *S. aureus* resistant isolates (%)	Number of MSSA sensitive isolates (%)	Number of MSSA resistant isolates (%)	Number of MRSA sensitive isolates (%)	Number of MRSA resistant isolates (%)
Clindamycin	8 (9.30)	78 (90.7)	5 (10.4)	43 (89.6)	3 (7.9)	35 (92.1)
Erythromycin	42 (48.8)	44 (51.2)	22 (45.8)	26 (54.2)	18 (47.4)	20 (52.6)
Gentamicin	37 (43.0)	49 (57.0)	25 (52.1)	23 (47.9)	13 (34.2)	25 (65.8)
Trimethoprim-sulphamethaxazole	36 (41.9)	50 (58.1)	22 (45.8)	26 (54.2)	14 (36.8)	24 (63.2)
Tetracycline	23 (26.7)	63 (73.3)	18 (37.5)	30 (62.5)	5 (13.2)	33 (86.8)
Penicillin	0 (0.0)	86 (100)	0 (0.0)	48 (100)	0 (0.0)	38 (100)
Vancomycin	86 (100)	0 (0.0)	48 (100)	0 (0.0)	38 (100)	0 (0.0)
Cefoxitin	48 (55.8)	38 (44.2)	48 (100)	0 (0.0)	0 (0.0)	38 (100)
Fusidic acid	17 (19.8%)	69 (80.2)	8 (16.7)	40 (83.3)	9 (23.7)	29 (76.3)

%, percentage; MRSA, methicillin resistant *S. aureus*; MSSA, methicillin sensitive *S. aureus* Gm resistant, Chi square = 5.90; p value = 0.015 between MRSA and MSSA.

using forward primer designated mecA_F1 (AGTTCTGCAGTACCGGATTG) and backward primer designated mecA_B1 (AAAATCGATGGTAAGGTTCGC) in a 30 µl reaction containing 50 ng of DNA, *Taq* polymerase and dNTP using the cycling parameter - denaturation temperature at 94°C for 30 s, annealing temperature at 55°C for 30 s, followed by extension at 72°C for 1 min for 40 cycles. Positive control (MRSA DNA) and negative control DNA from NCTC 6571 (Oxford *S. aureus*) were included in each batch of PCR run.

PCR Amplication of *aac(6')/aph(2"), aph(3')-IIIa* and *ant(4')-Ia* genes

Simplex PCR amplication of *aac(6')/aph(2"), aph(3')-IIIa* and *ant(4')-Ia* genes was carried out as previously described by Choi et al. (2003) using the following forward and backward primers, 5'-GAAGTACGCAGAAGAGA-3' and 5'-ACATGGCAAGCTCTAGAA-3'; 5'-AAATACCGCCTGCGTA-3' and 5'-CATACTTCTTCCGAGCAA -3'; 5'-AATCGGTAGAAGCCCAA-3' and 5'-GCACCTGCCATTGCTA, respectively. The PCR mix was in a 30 µl reaction volume as described for *mecA* amplification with the following cycling parameters: denaturation at 95°C for 2 min; annealing at 54°C for 1 min; extension at 72°C for 1 min; for 30 cycles using

GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, UK). The expected product sizes of 491, 242 and 135 bp, respectively were used to determine successful amplification of the genes.

SCC*mec* typing

SCC*mec* typing of all the *mecA*+ isolates of *S. aureus* was carried out as previously described (Zhang et al., 2005) using PCR technology. The PCR products were analysed on agarose gel containing 0.5 µg/ml ethidium bromide following electrophoresis and the image captured using Sygene Gel documentation system (Sygene, UK).

Statistical analysis

Data were analysed using statistical package within the Microsoft Excel and Epi-info software for Centre for Disease Control and Prevention, USA. The p value < 0.05 was considered to be significant.

RESULTS

The result of the antibiotic susceptibility pattern of the culture collections from 4 tertiary hospitals in

Nigeria mainly from South-Western region of the country is shown in Table 1. Varying degrees of susceptibility to antibiotics were observed with highest degree of antibiotic resistance observed for penicillin where all the isolates examined were resistant to penicillin. The highest degree of antibiotic sensitivity was observed in vancomycin where all the clinical isolates (100%) of *S. aureus* were susceptible using MIC breakpoint of ≤4 µg/ml according to CLSI (2012) (Table 1). All the isolates were screened for *mecA* gene and the result show that 38 (44.2%) of the 86 isolates possessed *mecA* gene indicating there were 38 methicillin resistant *S. aureus* (MRSA) and 48 methicillin sensitive *S. aureus* (MSSA) isolates. This result conformed with the result of the cefoxitin (30 µg) disc susceptibility testing. On the basis of these, the isolates were divided into two: MRSA and MSSA. The MRSA strains appeared to be more resistant to antibiotics than MSSA (Table 1) but there was no significant difference in the susceptibility patterns of the isolates (Chi square = 5.61; P = 0.469). However, there was a significant difference in the resistance of MRSA and MSSA

Table 2. Distribution of *aac(6')/aph(2")* and *aph(3')-IIIa* genes amongst *S. aureus* isolates.

|	mecA+	%mecA+	mecA-	%mecA-	Gm res	%Gm res	Gm sens	%Gm sens	mecA+ Gm res	%mecA+ Gm res
aph(3')-IIIa										
Negative	21	55.3	32	66.7	12	24.5	22	59.5	1	50.0
Positive	17	44.7	16	33.3	37	75.5	15	40.5	1	50.0
aac(6')/aph(2")										
Negative	9	23.7	30	62.5	19	38.8	22	59.5	1	50.0
Positive	29	76.3	18	37.5	30	61.2	15	40.5	1	50.0
aph(3')-IIIa* and *aac(6')/aph(2")										
Negative	2	8.0	10	62.5	4	23.5	8	57.1	2	8.0
Positive	23	92.0	6	37.5	13	76.5	6	42.9	23	92.0

Test of association between having *aac(6')/aph(2")* and *aph(3')-IIIa* genes and methicillin resistant gentamicin resistant *S. aureus* (Chi square = 6.197; P = 0.0127). Gm res – gentamicin resistant; Gm sens – gentamicin sensitive.

to gentamicin (Chi square = 5.90; P = 0.015). Overall, methicillin and gentamicin resistant *S. aureus* represented 29.1% of the 86 clinical isolates of *S. aureus*. The MIC_{50} and MIC_{90} to gentamicin for gentamicin resistance isolates were 32 and >256 µg/ml, respectively indicating high level of resistance to gentamicin while that of gentamicin sensitive isolates MIC_{50} and MIC_{90} were 1 and 8 µg/ml, respectively. Twenty one (26.6%) of the 79 clindamycin resistant *S. aureus* were inducible clindamycin resistance as detected by the double disc diffusion method giving 24.4% of the total *S. aureus* examined. It was found that 11 (52.4%) of 21 inducible clindamycin resistance were MRSA while the remaining 10 (47.6%) were MSSA.

PCR detection of (*aac(6')/aph(2")* and *aph(3')-IIIa*, genes showed that there were varying distributions of (*aac(6')/aph(2")* and *aph(3')-IIIa* genes amongst the *S. aureus* isolates (Table 2). Highest prevalence of *aac(6')/aph(2")* gene representing 47 (54.7%) of 86 was recorded for clinical isolates of *S. aureus* followed by *aph(3')-*

IIIa gene where 33 (38.4%) out of 86 clinical isolates of *S. aureus* were detected. No *ant(4')-Ia* gene was detected in all the 86 *S. aureus* isolates. The distribution of *aac(6')/aph(2")* and *aph(3')-IIIa* genes in *mecA+* and *mecA- S. aureus* is shown in Table 2. There was no association between *aph(3')-IIIa* gene and *mecA+/mecA-* strains of *S. aureus* (Chi square = 0.73; P = 0.39). However, association was found between *aac(6')/aph(2")* gene and *mecA+/mecA-* strains of *S. aureus* (Chi square = 11.38; P = 0.007). The distribution of genes encoding AMEs showed that more of *aph(3')-IIIa* gene was detected in gentamicin resistant *S. aureus* isolates (75.5%) than gentamicin sensitive isolates (40.5%), that is, association was found between the presence of this gene and gentamicin resistant *S. aureus* isolates (Chi square = 9.37; P = 0.0022) while 30 (61.2%) of 49 gentamicin resistant *S. aureus* were positive for *aac(6')/aph(2")* gene and 15 (40.5%) out of 37 gentamicin sensitive *S. aureus* were positive for *aac(6')/aph(2")* gene. No association was found between *aac(6')/aph(2")* gene and

gentamicin resistant *S. aureus* isolates in this study (Chi square = 2.83; P = 0.09). It is noteworthy that there was strong association between genes encoding AMEs (*aph(3')-IIIa* and *aac(6')/aph(2")* genes*) and *mecA+* gentamicin resistant *S. aureus* isolates (Chi square = 16.85; P = 0.000038). Twenty three (26.7%) of the 86 *S. aureus* isolates examined in this study constituted the *mecA+* gentamicin resistant *S. aureus* isolates. The result of the SCC*mec* typing for all the gentamicin resistant methicillin resistant *S. aureus* strains showed that 4 (17.4%) out of the 23 were SCC*mec* II; 4 (17.4%) out of 23 were SCC*mec* III and 9 (39.1) out of 23 were SCC*mec* V, while the remaining 8 (34.8%) were non-typeable using this scheme. This could be interpreted as 8 of the 23 gentamicin resistant, methicillin resistant *S. aureus* strains were most likely to be hospital acquired strains, that is, SCC*mec* II and III while the SCC*mec* V was considered to be community acquired strains. The interpretation was based on the fact that large SCC*mec* I to III had been found to be associated
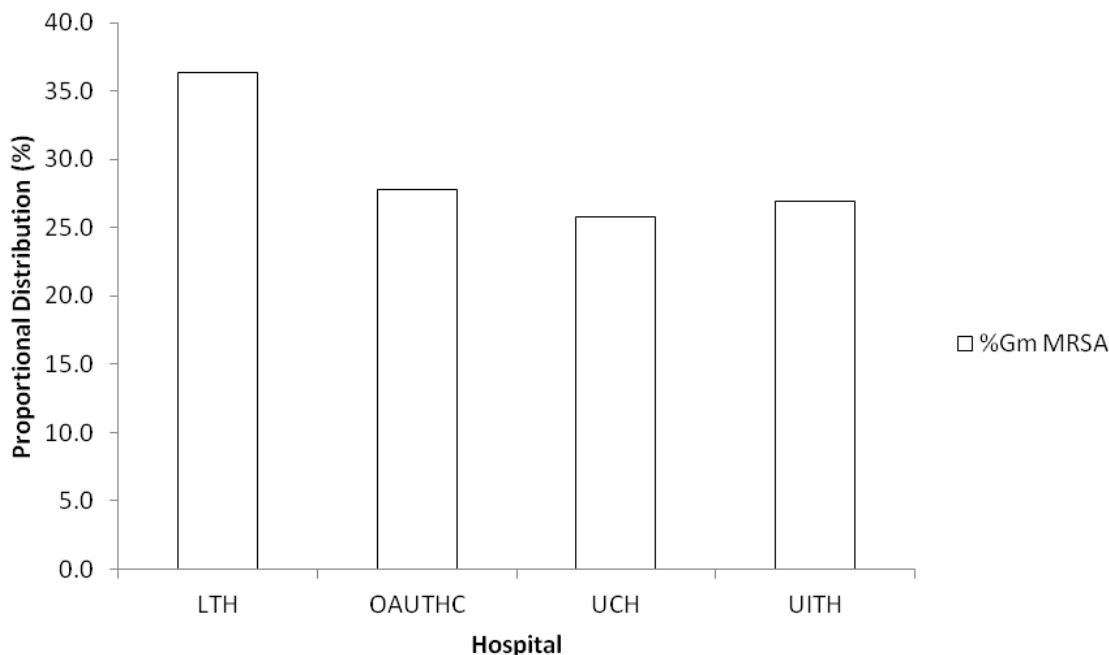
Figure 1. Proportional distribution of gentamicin methicillin resistant *S. aureus* amongst four teaching hospitals in Nigeria.

with HA-MRSA while SCC*mec* IV and V had been found to be associated with CA-MRSA (Ma et al., 2002; David and Daum, 2010). The proportional distribution of gentamicin methicillin resistant *S. aureus* amongst the 4 teaching hospitals in Nigeria is shown on Figure 1. Highest prevalence of this strain was recorded at LTH while UCH recorded the lowest. There was no association between hospital and gentamicin methicillin resistant *S. aureus* (Chi square = 0.25; P = 0.97). The proportional distribution of genes encoding aminoglycosides modifying enzymes and *mecA* gene is shown on Figure 2. LTH recorded highest prevalence (8 (80%) out of 10) of *aac(6')/aph(2")* gene; UITH recorded the lowest prevalence (13 (50%) out of 26) of this gene. No association was found between prevalence of *aac(6')/aph(2")* gene and hospitals (Chi square 3.46; P = 0.326). Similarly, no associations were found with *aph(3')-IIIa* gene (Chi square = 1.40; P = 0.71), *mecA* gene (Chi square = 4.00; P = 0.26) and hospitals.

DISCUSSION

Methicillin resistant *S. aureus* (MRSA) has been reported all over the world in clinical isolates with varying frequency (Crum et al., 2006; Diep et al., 2008; Egyir et al., 2014) including Nigeria where studies have shown general increase in the prevalence of this strain of *S. aureus* (Alli et al., 2012). In this present study, survey of MRSA amongst clinical isolates of *S. aureus* from 4 tertiary hospitals in Nigeria was undertaken along with

antimicrobial susceptibility pattern of the isolates. The prevalence of MRSA was found to be 44.2% in this study as compared to 41.2% obtained about 2 years ago in this area (Alli et al., 2012). The antimicrobial susceptibility patterns of the clinical isolates of *S. aureus* also showed general increase in the prevalence of bacteria resistance to other with the exception of vancomycin that has been shown to be active against these isolates. This study is in contrast with a report at a nearby country – Ghana where the prevalence of antibiotics resistant has been found to be low (Egyir et al., 2014). MRSA has been shown over the years to have propensity for antibiotic resistance. This present study found that there was no significant difference in the antibiotics susceptibility patterns between MRSA and MSSA (P > 0.05); this is a worrying trend in this part of the world.

Aminoglycosides group of antibiotics especially gentamicin has been the cornerstone for the treatment of infection caused by *S. aureus* especially when it is used along with β-lactamase resistant penicillin or cephalosporin for the treatment of infective endocarditis. In this study, the prevalence of gentamicin resistant *S. aureus* was found to be 55.8%; higher than what the study reported for MRSA. This study is comparable to what was obtained in this locality in Nigeria where 56.5% was obtained (Alli et al., 2012). Interestingly, recent report from Ghana, a closest neighbour to Nigeria showed 3% (Egyir et al., 2014). *S. aureus* resistance to gentamicin is essentially as a result of acquisition of genes encoding aminoglycosides modifying enzymes
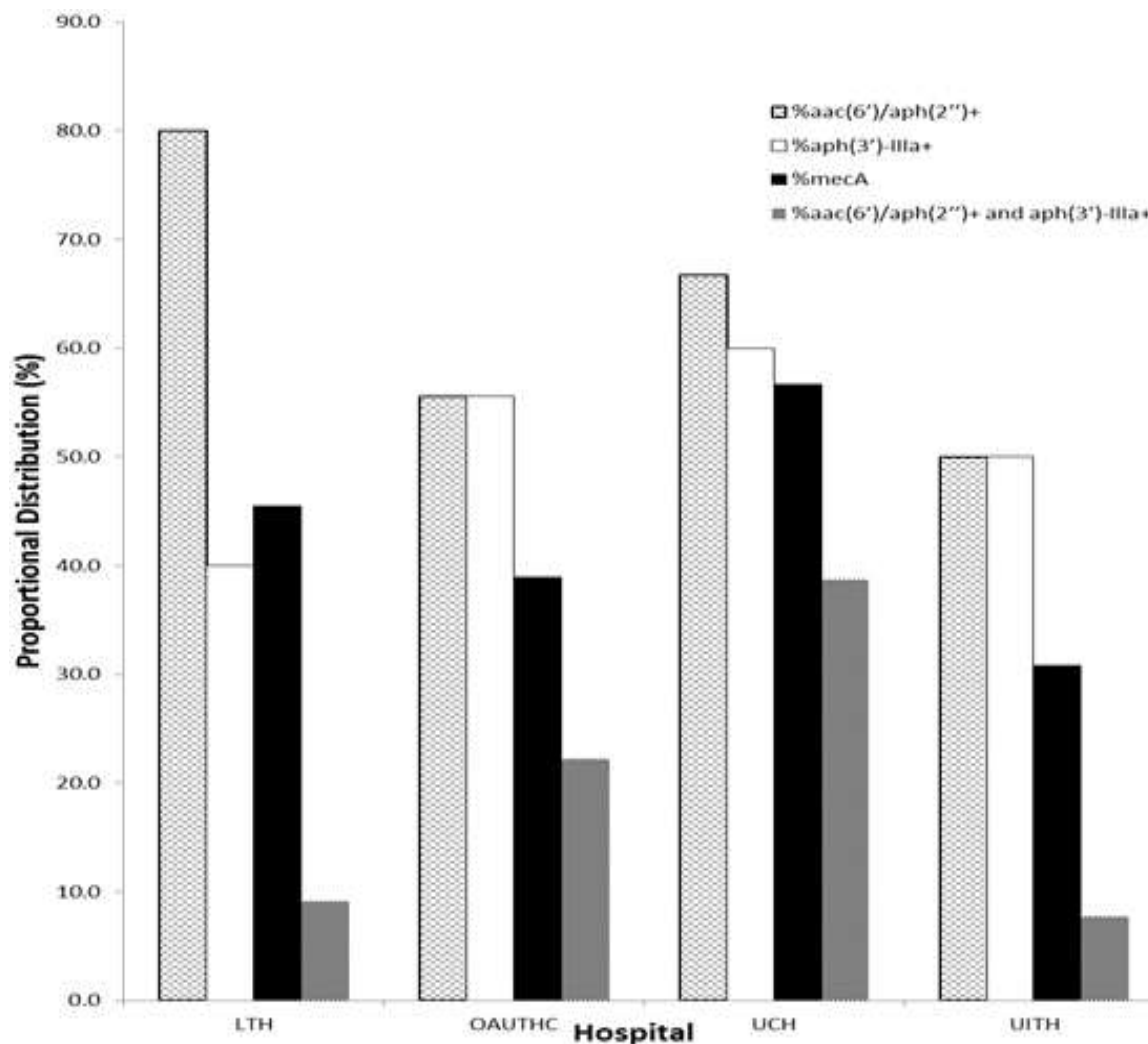
Figure 2. Proportional distribution of genes encoding aminoglycoside modifying enzymes and *mecA* gene in *S. aureus* isolates from four teaching hospitals in Nigeria.

(*aac(6')/aph(2")*, *aph(3')-IIIa* and *ant(4')-Ia* genes). The study did not detect the *ant(4')-Ia* gene amongst the gentamicin resistant *S. aureus* nor gentamicin sensitive isolates which is in contrast with a study carried out in South Korea where prevalence of this gene was found to be 41%. The product of *ant(4')-Ia* gene (Ant 4") has activity against neomycin, kanamycin, tobramycin and amikacin in staphylococci. None detection of this gene amongst clinical isolates of *S. aureus* in Nigeria may have something to do with little or no usage of these antibiotics in the treatment of infections or the sample size used in this study. The prevalence of *aac(6')/aph(2")* gene was found to be 54.7% in *S. aureus* isolates examined as compared to 65% obtained by Choi et al. (2003), this suggests the widespread of this gene. In gentamicin resistant isolates, the prevalence of *aac(6')/aph(2")* gene was found to be 61.2% which is in contrast to 100% obtained in a study by Choi et al. (2003).

Conversely, the prevalence of *aph(3')-IIIa* gene was found to be 75% amongst the gentamicin resistant *S. aureus*. This prevalence is very high as compared to what was obtained in Iran where Yadegar et al. (2009) reported 6% prevalence amongst the clinical isolates of *S. aureus* and the prevalence of 9% reported in South Korea by Choi et al. (2003). In gentamicin sensitive *S. aureus* isolates in our study, we found the prevalence of *aph(3')-IIIa* gene to be 40.5% suggesting that this gene does not necessarily confer resistance to gentamicin alone. *aph(3')-IIIa gene* has been shown from previous study to mediate resistance to kanamycin, neomycin, gentamicin and amikacin (Shaw et al., 1993), suggesting cross resistance to other aminoglycosides. Gentamicin is used more regularly in treating infections than other aminoglycosides in Nigeria especially when used in conjunction with penicillin to treat endocarditis. Interestingly, our study showed 92% concordance to gentamicin resistant when we had co-occurrence of two

aminoglycoside modifying enzyme genes (*aac(6')/aph(2"*) *and aph(3')-IIIa*) in *S. aureus* isolates with *mecA* gene in gentamicin-methicillin resistant *S. aureus* strains. The importance of this finding to diagnosis and treatment cannot be overemphasized because there is most likely to be clinical failure if gentamicin is used in treating infection caused by gentamicin-methicillin resistant *S. aureus* as a result of the resistant of these strains to gentamicin that would be brought about by the full expression of the resistance genes. Several studies have linked aminoglycoside resistance to methicillin resistance (Choi et al., 2003; Schmitz et al., 1999; Shaw et al., 1993), this study also observed a similar trend. The result of the SCC*mec* showed that the gentamicin methicillin resistant *S. aureus* is diverse because it is not restricted to one SCC*mec* type and one teaching hospital. Although more is needed to be done in this area in order to determine the clonality of this strain, but this does not rule out the importance of this technique in giving a snapshot of the diversity of this strain in developing countries like ours.

To conclude, this study has shown the importance of genes encoding aminoglycoside modifying enzymes in gentamicin resistant *S. aureus* and also the emergence of gentamicin-methicillin resistant *S. aureus* that can complicate treatment of endocarditis where combination therapy such as gentamicin and beta-lactamase resistant penicillin is employed because of synergistic effect of these antibiotics. Hence, there is need for constant surveillance of gentamicin methicillin resistant *S. aureus* strains in our community with good infection control policy that could curb the spread of this strain. Further work is needed to detect and characterize the plasmid backbones or vectors underpinning these resistance determinants and also determine the effect of fitness cost result from acquisition of antibiotic resistance of these strains. The use of genes encoding AMEs and *mecA* in screening for gentamicin methicillin resistant strain of *S. aureus* should be encouraged as employed by Choi et al. (2003) in multiplex PCR.

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Conflict of interest

No conflict of interest is reported in this study.

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