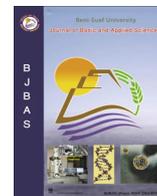


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Detection of methicillin resistant and toxin-associated genes in *Staphylococcus aureus*



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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* is a problem in both healthcare institutions and community settings. This is due to its multi-drug resistant challenges. Hence, this study assessed the prevalence of methicillin resistant gene (*mecA*), exfoliative toxin (*eta* and *etb*) and toxic shock syndrome (*tsst-1*) genes in *S. aureus* isolated from clinical samples. A total of 120 clinical samples of patients (urine, high vagina swab (HVS), semen, wound swab, sputum and urethral swab) from a hospital laboratory were obtained. *S. aureus* was isolated and then identified with API-staph kit. Antibiotic susceptibility of the isolates was determined by agar diffusion while PCR was used to detect the presence of *mecA* and toxin-associated genes. Fifty *S. aureus* isolates were obtained at frequencies of 26(52%), 12(24%), 4(8%), 3(6%), 3(6%) and 2(4%) from the HVS, urine, semen, wound, sputum and urethral swab samples respectively. All the isolates of *S. aureus* were resistant to the antibiotics used in this study. *MecA*, *tsst-1*, *eta* and *etb* were detected in 19(38%), 7(14%), 3(6%) and 2(4%) of the isolates respectively. The prevalence of MRSA and its resistance pattern observed in this study was a signal that the health-care workers and the general public are at risk.

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1. Introduction

Staphylococcus aureus is a normal flora of the mucosa membrane, and skin of both humans and animals (Hanselman et al., 2009). An estimated carriage rate in healthy human population stands between 20% and 30% (Kluytmans et al., 1997). *S. aureus* infection is usually asymptomatic, (Tong et al., 2015) though the bacterium is responsible for several diseases in humans such as abscesses, respiratory infections, bacteremia, endocarditis, osteomyelitis, and acute food poisoning (Kaźmierczak et al., 2014). *S. aureus* continues to be one of the most commonly implicated bacteria causing human diseases throughout the world (I.W. G-S. C. C., 2009).

The pathogenicity of *S. aureus* has become a problem in both health institutions and community settings especially with the emergence of methicillin-resistant to *S. aureus*, MRSA (Panlilio et al., 1992; Layton et al., 1995; Kaźmierczak et al., 2014). The methicillin resistant element, *mecA*, is an inducible 76-kDa penicillin

binding protein (PBP) located on staphylococcal chromosomal cassette which codes for penicillin binding protein (PBP2a) with altered affinity to beta-lactam antibiotics (Cohen, 1992; Neu, 1992). Acquisition of this gene promotes staphylococcal resistance to all β -lactam antibiotics, thereby hampering their efficacy for clinical use during MRSA infections. Since the discovery of MRSA in the early 60s, its prevalence has been on the increase; surveillance in the United States showed a steady rise in the prevalence of infections due to MRSA from 2.4, 5.0, 29.0% in 1974, 1981, 1991 respectively to 43.0% in 1997 (Haley et al., 1982; Panlilio et al., 1992; Gardam, 2000; Saleh et al., 2013).

With the help of potent protein toxins, Virulent strains of *S. aureus* initiate infections as well as express cell-surface proteins that mediate antibody inactivation (Patel et al., 1987; Schneewind et al., 1995; Dinges et al., 2000). *S. aureus* can produce various enzymes such as coagulase, hyaluronidase, deoxyribonuclease and lipase which aid its virulence and spread within the host (Eyre and Stanley, 1987; Balaban and Rasooly, 2000; Lin et al., 2010; Hennekinne et al., 2012; Kong et al., 2016). In addition, other variety of extracellular protein toxins, including enterotoxins, toxic shock syndrome toxin 1 (TSST-1), exfoliative toxins

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(ETs), hemolysins, and coagulase which increase pathogenicity of the organism as well as its ability to invade host cells have been reported (Mehrotra et al., 2000; Kong et al., 2016). These protein toxins were found more in MRSA than non-MRSA cases (Shimaoka et al., 1996; Liu et al., 2010). Of these proteins, toxic shock syndrome (TSST-1) and exfoliative (ETA and ETB) toxins have been documented as the cause of toxic shock syndrome and scalded skin syndrome in human host respectively (Johnson et al., 1991). The former is a pyrogenic toxins of molecular weight 22 kDa which are expressed in 25% of *S. aureus* and produces its toxic effect by stimulating the release of inflammatory markers; interleukin-1, interleukin-2 and tumour necrosis factor (Dinges et al., 2000; Kong et al., 2016).

The burdens associated with MRSA infections are not only because of the painful trauma inflicted on the patients, but also as a result of cost incurred due to prolong hospitalization as most of the drug of choice like beta-lactam antibiotics could no longer effectively produce bactericidal effects on this organism (Gould, 2006; Kong et al., 2016). The reason for this development is attributable to production of beta-lactamase which destroys the functional integrity of the beta-lactam antibiotics by cleaving the β -lactam ring of the penicillin molecule. Additionally, the ability of *S. aureus* to produce various antibiotic deactivating enzymes has compounded the problems of antimicrobial therapy resulting in multiple resistances to these agents (CME, 2008).

Knowledge of MRSA and its toxigenic status are important for right antimicrobial intervention and epidemiological purposes. In Nigeria, only scanty studies with regard to methicillin and toxins encoded genes in *S. aureus* have been investigated (Kolawole et al., 2013; Alli et al., 2015). Hence, this study was designed to investigate the resistance pattern, prevalence of methicillin and three associated staphylococcal toxigenic genes of *S. aureus* isolated from clinical samples.

2. Materials and methods

2.1. Isolation procedure and ethical consideration

Clinical samples (HVS, urine, semen, wound swab, urethral swab and sputum) from patients who were on hospital admission due diseases as urinary tract, skin, pulmonary infections to mention a few were obtained. These samples were sent to microbiology laboratory department of the hospital, for routine analysis. Samples were streaked on prepared plates of Mannitol Salt Agar (Oxoid, UK) and incubated using incubator (Gallenkomp, England) at 37 °C for 18–24 h. Colonies showing yellow coloration were picked. Morphologically distinct colonies were sub-cultured on fresh tryptone soy agar (Oxoid, UK) petri plates. Pure colonies obtained were stored in 40% glycerol at 0 °C for subsequent studies. Ethical clearance was obtained from Babcock University/Research Ethics Committee (BUHREC).

2.2. Identification

All the suspected strains of *S. aureus* were identified using both preliminary tests such as Gram staining, catalase, coagulase as previously described (Akobi et al., 2012) and API-Staph identification kit (BioMerieux, France) following the manufacturer's instructions. Briefly, discrete colonies of *S. aureus* strains were removed from an overnight culture inoculated on Trypticase soy agar plates using a sterile wire loop into 6 ml of staph medium. The suspension was ensured to match 0.5 McFarland turbidity standard. Each microcupule on the strip was filled with the cell suspension of the test organism. An anaerobiosis was created in ADH and URE test by filling the cupules with mineral oil. After inoculation, strips were

incubated for 18–24 h at 37 °C. Test results were developed with staph-reagents, read and interpreted following the manufacturer's instructions.

2.3. Antibiotic susceptibility test

Antibiotic susceptibility was determined by agar diffusion method (Bauer et al., 1966). A single pure colony of each isolate was inoculated into a test tube containing 2 ml nutrient broth (Oxoid, UK) and incubated overnight. The young physiological overnight culture of each isolate was diluted with sterile distilled water until the turbidity matched 0.5 McFarland standard. A sterile swab stick was dipped into the adjusted suspension and streaked over the surface of an already prepared Mueller-Hinton Agar (Oxoid, UK) plates. The antibiotic discs: ceftazidime (30 μ g), cefuroxime (30 μ g), cloxacillin (5 μ g), erythromycin (5 μ g), ofloxacin (5 μ g), gentamicin (10 μ g), amoxicillin/clavulanate (30 μ g), ceftriaxone (30 μ g), (Abtek[®]) were applied onto the inoculated plates maintaining a distance of 30 mm edge to edge. The plates were incubated for 18–24 h at 37 °C. The diameter of the inhibition zones was measured using ruler and interpreted according to the criteria recommended by the CLSI (2015).

2.4. Extraction of DNA by boiling method

DNA from isolates was prepared by boiling following previous method (Pe'rez-roth et al., 2001) but with some modifications. A single colony of each isolate was inoculated into nutrient broth and incubated overnight at 37 °C for 18–24 h. The samples were centrifuged at 5000 \times g for 10 min. The supernatant was discarded, and the pellet was re-suspended in 40 μ l of sterilized distilled water. The suspension was subjected to boiling at 100 °C in water bath (Uniscop, Surgifriend, England) for 10 min, and centrifuged at 5000 \times g for 30 s. The supernatant was then transferred into a fresh Eppendorf tubes before it was stored at 4 °C. Aliquots of 1 μ l of template DNA were used for PCR.

2.5. Detection of toxin genes (*eta*, *etb* and *tsst-1*) by multiplex PCR

Multiplex PCR was carried out as described previously (Mehrotra et al., 2000) with modifications. An aliquot of 1.0 μ l of DNA suspension was added to PCR mixture consisting of 12.5 μ l OneTaq Quick-Load 2X Master Mix with Standard Buffer (New England Biolabs), 0.5 μ l of 10 μ M each *eta*, *etb* and *tsst-1* primers produced by Sigma-Aldrich, Germany (Table 1). The reaction mixture was rounded up to 25 μ l with sterile distilled water. Multiplex Polymerase Chain Reaction assays were carried out with a negative control containing all of the reagents without DNA template. DNA amplification was carried out using 9700 thermocycler with the following thermal cycling profile: Initial denaturation at 95 °C for 3 min, denaturation 94 °C for 30 s, annealing 40 °C for 30 s, extension 72 °C for 30 s and final extension 72 °C for 7 min with a programmable period of 30 cycles. After PCR amplification, 10 μ l of PCR product was resolved by agarose gel electrophoresis.

3. Results

3.1. API-identification system and the distribution of isolates

One hundred and twenty clinical samples were obtained of which 50 *S. aureus* strains were isolated from 40 female and 10 male subjects. Of the 50 *S. aureus* isolates, 26(52%), 12(24%), 4 (8%), 3(6%) and 2(4%) were isolated from HVS, urine, semen, wound and sputum and urethra, respectively. All the isolates were correctly identified with the API-identification kit.

Table 1
The sequence of primers used in this study.

Primers	Sequences 5'–3'	Genes	Size (bp)	Genes	References
TSST F R	ATGGCAGCATCAGCTTGATA TTTCCAATAACCACCCGTTT	<i>Tsst-1</i>	350	<i>Tsst-1</i>	Johnson et al., 1991
ETA F R	CTAGTGCATTGTATTCAA TGCATTGACACCATAGTACT	<i>eta</i>	119	<i>eta</i>	Johnson et al., 1991
ETB F R	ACGGCTATATACATTCAATT TCCATCGATAATATACCTAA	<i>etb</i>	200	<i>etb</i>	Johnson et al., 1991
MecA F R	GGG ATC ATA GCG TCA TTA TTC AAC GAT TGT GAC ACG ATA GCC	<i>mecA</i>	527	<i>mecA</i>	Hanssen et al., 2004

3.2. Status of methicillin resistant, exfoliative genes and antibiogram

Methicillin resistant determinant was detected in 10(20%), 5 (10%), 2(4%), 1(2%) and 1(2%) *S. aureus* isolated from HVS, urine, semen, sputum and urethral samples, respectively (Table 2). In general, *mecA* (Fig. 1) was detected in 19 isolates (38%). The distribution of *mecA* positive isolates indicated that young females have highest colonization followed by older females (Fig. 2). This distribution fell into three age categories; 18–26, 33–36 and 34–50 years. The *tsst-1* 7(14%), *eta* 3(6%) and *etb* 2(4%) were detected in these isolates (Fig. 3) but in a relatively low degree. Only one isolate has three genes (*mecA*, *etb* and *tsst-1*). The *tsst-1* gene was detected in three isolates of *S. aureus* obtained from HVS samples while only one isolate of *S. aureus* isolated from urine, sputum, urethral swab and semen samples harbor this gene. All the strains of *S. aureus* were resistant to the antibiotics used in this study.

4. Discussion

The isolation of *S. aureus* from various clinical samples (semen, HVS, urethral, wound swab, sputum and urine) indicative that it is widely distributed. This distribution could be an evidence of *S. aureus* versatility and cause of most nosocomial associated infections. The frequency of isolation of MRSA in HVS and urine over other samples is unclear, but it is possible that sexual behaviors of the patients under study might have been responsible for this outcome. Certain lifestyle behaviors such as illicit drug use and high-risk sexual behaviors increase the risk of MRSA colonization (Shadyab and Crum-Cianflone, 2012). A cohort study (Farley et al., 2015) reported that illicit drug and public gym use were predisposing factors associated with prevalence of *S. aureus* colonization and these result in spread of *S. aureus* through person-to-person contact or fomites. Other risk factors for hospital acquisition of MRSA include prolonged hospitalization, stay in an intensive care unit, chronic diseases, prior exposure to antibiotics, surgery and contact with a patient known to be colonized or infected with MRSA (Lucet et al., 2003). The above risk factors might somehow contributed to MRSA colonization of patients in this study.

Table 2
Percentage distribution of target genes per source in *S. aureus* isolated from various specimens.

Sources	<i>S. aureus</i>		<i>mecA</i>		<i>eta</i>		<i>etb</i>		<i>Tsst-1</i>	
	no	%	no	%	no	%	no	%	no	%
Urine	12	24	5	10	2	4	x	0	1	2
Sputum	3	9	1	2	x	0	x	0	1	2
HVS	26	52	10	20	1	2	2	4	3	6
Urethral swab	2	4	1	2	x	0	x	0	1	2
Semen	4	8	2	4	x	0	x	0	1	2
Wound	3	9	x	0	x	0	x	0	x	0
Total	50	100	19	38	3	6	2	4	7	14

X: Absence of target gene, no: number of detected target gene per source.



Fig. 1. Electrophoregram of methicillin resistant gene in studied isolates. M: Molecular ladder (100 bp), *mecA* positive: 1–3, 5, 7–10, 13–14; *mecA* negative: 4, 6, 11, 15; control: 12.

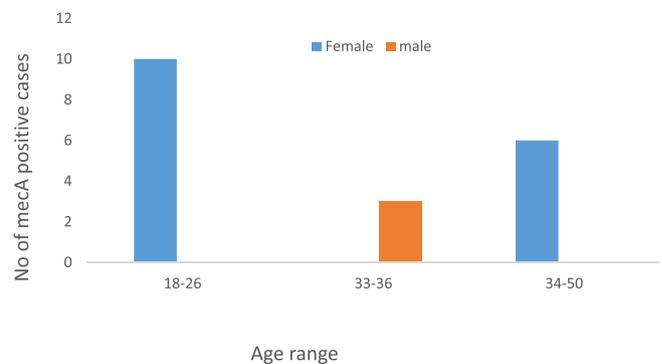


Fig. 2. The distribution of *mecA* positive cases with age amongst human subjects.

In this study, the prevalence of MRSA was 38% and this is comparable to 0.3–34% in European countries (de Neeling et al., 1998; Gbaguidi-Haore et al., 2009; Bocher et al., 2010), 41.6% in the USA (Yu et al., 2012), but less than 72% in Lebanon, (Tokajian et al., 2011), 77.65–80% in China (Peng et al., 2010; Tokajian et al., 2011) and 87.6% in Iran (Koosha et al., 2016). The distribution of *mecA* positive strains seems to be sex and age dependent. In this study, strains with *mecA* genes were more frequent in females than

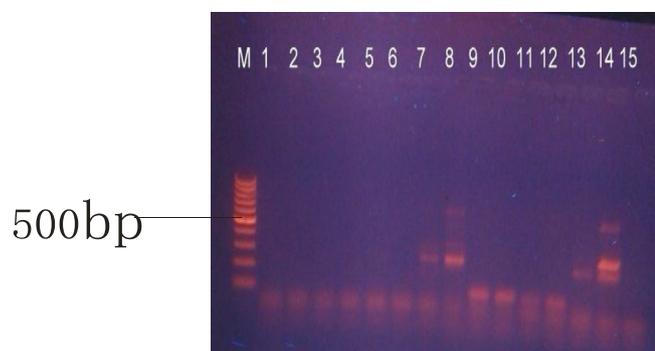


Fig. 3. Electrophoregram of staphylococcal toxin genes in studied isolates. M: Molecular ladder (100 bp), 7, 8 & 14 = *tsst-1* positive, 9 & 10 = *eta* positive, 13 & 14 = *etb* positive.

males. One study reported that men are likely to be less colonized with MRSA than women (Farley et al., 2015). The limited number of male participants in this study hampered the overall scientific significance. However, among females, this study showed that it was more in young girls between 18–26 years of age. This may be explained in terms of sexual activities which occur mostly amongst these age groups in recent times. It is also possible that the use of protective devices such as condoms, may likely be disregarded amongst these groups.

All the antibiotics used in this study have no bactericidal effect on the *S. aureus* strains including strains without methicillin resistant gene. This observation was contrary to a study of (Nwankwo and Nasiru, 2011) where nearly the same set of antibiotics (gentamicin 7.6%; ofloxacin, 23.4%; ceftriaxone, 28.6% amoxicillin/clavulanate, 37.0%; cloxacillin, 64.2%; erythromycin, 67.6%) were used. Another study observed resistance level of 56.71% to gentamicin and 65.67% to erythromycin in MRSA cases (Shahkarami et al., 2014). This study observed no significant difference in resistance pattern between MRSA and non-MRSA strains. Regulatory genes such as transport and signal transduction may influence the magnitude of resistance expressed. It is probably that non-MRSA strains acquired resistance genes that are plasmid regulated and or chromosomally encoded order methicillin resistant gene. It has been reported that some strains of *S. aureus* over-express β -lactamase while some strains possess a modification of existing penicillin-binding proteins rather than acquisition of a PBP2a (Gunawardena et al., 2012). Recent works have also shown that the susceptibility to antibiotics is controlled by bacterial metabolism which dictated this phenotype (Corona and Martinez, 2013). This regulation makes bacteria either more resistant or more susceptible to antibiotics. However, a time bacteria become momentarily resistant to antibiotics even in the absence of a genetic change (Levin and Rozen, 2006).

Moreover, (Corona and Martinez, 2013) highlighted best examples as the growth in biofilms, phenomenon of persistence, and drug indifference. They also explained that bacteria can present change in their susceptibility to antibiotics depending on the status of their metabolism.

The high level resistance observed in this study could be due to increasing antibiotic pressure in clinical settings as reported previously in literature (Davies and Davies, 2010). Hence, accumulation of these antibiotics in the clinical environment might have facilitated development of resistance. In addition, antibiotic abuse associated with self-medication which often results in inadequate dosage could have contributed significantly to this resistance profile. Antibiotic abuse emanates from its availability across the counters with or without prescription. Beside the aforementioned

factors, the variability in the performance of disc diffusion may be attributable to the nature of phenotypic expression of resistance in these isolates. Attributable factors like growth conditions (Annear, 1968; Sinsimer et al., 2005), salt concentrations (Yoon et al., 2013), inoculums size (Huang et al., 1993) and medium compositions (Coombs et al., 1996) might have had considerable effects on the susceptibility test.

The expression of variety of virulent factors is key to success of staphylococcal pathogenesis under the influence of accessory gene regulator (*agr*). Factors which activate this include environmental stimulus from either bacterial cell density (quorum sensing), nutritional status, or energy availability (Lyon and Novick, 2004; Bukowski et al., 2010; Roux et al., 2014). In this study, the prevalent rate of exfoliative toxin genes (*eta*, 6% and *etb*, 4%) were relatively similar to earlier cases in Nigeria and Karachi (Kolawole et al., 2013; Taj et al., 2014). The low rate of positive exfoliative toxin-producing isolates in this study is in accordance with previous studies (Sila et al., 2009; Megevand et al., 2010). The exfoliative toxin genes have been found to be present in the clinical isolates of *S. aureus*. Geographical variations in the occurrence of *eta* and *etb* have been reported. The *eta* toxin gene is prevalent in Europe, USA and Africa (De Azavedo and Arbutnott, 1981; Adesiyun et al., 1991). The *etb* has been found to be more frequent in Japan than *eta* toxin gene (Kondo et al., 1975; Yamasaki et al., 2005). According to a review of (Bukowski et al., 2010), there was no significant difference in the prevalence rate between methicillin resistant and methicillin susceptible *S. aureus* to *eta* toxin gene and that 3–4% of the later can carry either gene (*eta* or *etb*). However, only few MRSA strains were positive for toxin genes in this study. What might be responsible for the absence of virulent factors (*eta*, *etb* and *tsst-1*) in MSSA is unknown, but there may be positive correlation between *mecA* gene and virulence factors. Some other researchers have also obtained the same observation where MRSA strains possess *tsst-1* gene than its methicillin susceptible counterpart (Shimaoka et al., 1996; Liu et al., 2010) while others reported the contrary (Tokajian et al., 2011; Yu et al., 2012). Among the sample sources, HVS has the highest cases of *tsst-1* gene. This might not be surprising since there have been reports of an association of *tsst-1* gene with tampon use among women (Durand et al., 2006; Tristan et al., 2007; Parsonnet et al., 2008) with increased morbidity and mortality (Berkley et al., 1987). The severity of *tsst-1* in disease progression involves host receptors as well as release chemokines like IL-8, MIP-3, IL-2 and tissue necrosis factor (Kong et al., 2016). It is very imperative to attribute the high rate of morbidity and mortality associated with MRSA infections to acquisition of resistant and virulent genes. While resistant genes offer protective covering of *S. aureus* from external forces such as antimicrobials, virulent genes on the other hand ensure invasiveness to the host cells. This intra-cooperation results in pathogenicity and the survival strategies of *S. aureus* strains.

5. Conclusion

The prevalence of MRSA and its resistance pattern observed in this study is a signal that the health-care workers and the general public are at risk. Besides, appearance of toxin genes only in *mecA* positive isolates may indicate an interplay and cooperation between resistant and toxin genes within the strains. Hence, effort must be put in place to curtail the spread which include renewed awareness and establishment of drug resistance surveillance system.

Conflicts of interest

The authors declared no conflict of interest.

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