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Molecular characterization of antimicrobial resistance genes against *Staphylococcus aureus* isolates from Trinidad and Tobago

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Summary *Staphylococcus aureus* continues to pose major public health challenges in many areas because of antibiotic resistance problems. In the Caribbean, especially Trinidad and Tobago, the challenge is not different. This study was performed to evaluate the antimicrobial resistance gene prevalence among *S. aureus* isolates in Trinidad and Tobago.

Standard and molecular microbiological methods, including the Microscan automated system, DNA microarray and multi locus sequence typing (MLST) analysis, were performed on 309 clinical *S. aureus* isolates recovered from patients who were treated at three of the country's main health institutions.

S. aureus exhibited susceptibilities ≥80% to eleven of the 19 antimicrobials tested against it, and these belong to the most commonly used and available antibiotics in the country. While the antibiotic to which it was most susceptible of the commonly used antibiotics was trimethoprim/sulfamethoxazole, the antibiotics to which it was least susceptible or most resistant to were ampicillin and penicillin. *S. aureus* isolates from the pediatric ward produced the greatest rate of susceptibility among the isolates recovered from patients admitted into hospitals, while isolates from Accident and Emergency rooms displayed the greatest susceptibilities among patients from the community.

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S. aureus isolates from the country did not harbor acquired resistant genes targeting clindamycin/macrolides (*erm*B), linezolid (*cfr*) or vancomycin (*van*A). The *bla*Z gene, which is the most common beta lactam (Penicillinase) resistance mechanism for *S. aureus*, was observed in 88.7% of the methicillin susceptible *S. aureus*, while methicillin resistance mediated by the *mec* gene was present in 13.6%. Most of the resistance markers found in MRSA isolates were significantly associated with the ST239-MRSA-III strain in this study, and all isolates that belonged to the USA300 strain, which additionally encoded both the *PVL* gene and ACME cluster, belonged to CC8.

Several resistant genes, such as *van*A, *cfr* and *erm*B, mediating resistance in *S. aureus*, are currently non-existent in Trinidad and Tobago. However, the majority of SCC*mec* genes were observed, suggesting that there is ongoing nosocomial transmission with minimal community transmission. This calls for stringent antibiotic stewardship and policies in the country.

Introduction

Severe *S. aureus* infections require combative treatment, including incision and drainage for abscesses and systematic antibiotics [1]. Systematic antibiotics are necessary for deep-seated and systematic infections [2]. In the 1940s, penicillin, a widely used antibiotic derived from *Penicillium* fungi, was introduced for use [3]. Penicillin proved to be an effective antibiotic as it decreased the incidence and spread of infections as well as deaths caused by *S. aureus*. Penicillin acts by preventing cell wall formation as it competes for protein binding sites on the bacterium. However, resistance to penicillin was observed in the late 1940s with the emergence of the enzyme penicillinase, which inactivates penicillin [3]. Treating penicillin-resistant *S. aureus* led to the synthesis and further introduction of methicillin in 1959 [4]. However, in 1961, there were reports from the United Kingdom of methicillin-resistant *S. aureus* (MRSA). This trend was similarly seen in other European countries, Japan, the United States of America and Australia [4]. In the subsequent years, resistance developed to a range of antibiotics, including macrolides, fluoroquinolones, aminoglycosides, glycopeptides and tetracyclines.

Phenotypic methods have been used to identify and susceptibility test *S. aureus* isolates in many studies, and data on the characterization of *S. aureus* using molecular methods are limited in most undeveloped countries [5,6]. Knowledge of *S. aureus* susceptibility patterns and molecular characterization of genes mediating resistance are very important for developing effective infection control measures and treating or combating staphylococcal infections [5,6].

Many staphylococcal strains are known to resist multiple antibiotics and exhibit reduced susceptibility to glycopeptides, such as vancomycin. However, with the rise of glycopeptide intermediate susceptible *S. aureus* (GISA) and resistant genes, such as *van*A, there is a great concern in administering treatment [7—9]. Alternatively, other drugs have been used, such as linezolid, whose resistance is uncommon and encoded for by the *cfr* gene [8,9]. Several other genes are responsible for antibiotic resistance in *S. aureus*. For example, macrolide/clindamycin resistance is encoded by the *erm* gene, while the *aphA*3 and *sat* genes confer resistance to neo-/kanamycin and streptomycin, respectively. Gentamycin and tobramycin resistance are encoded for by the *accA-aph*D genes and tetracycline resistance is carried on the *tet* genes [8,9]. In an era of rapid antimicrobial resistance gene spread on multi resistance plasmids, our main objective was to evaluate and document the genes encoding antibiotic resistance in *S. aureus* isolates recovered from several major hospitals in Trinidad and Tobago.

Materials and methods

This was an observational cross-sectional study to investigate the susceptibility profiles of several antimicrobial agents on *S. aureus* isolates encountered in several infection types for patients admitted into the hospitals and those coming directly from communities in Trinidad and Tobago over a 10-month period in 2011—2012. The study was conducted at three of the five major regional hospitals across the country. Ethical approval for

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this study was granted by the Ethics Committee, the University of the West Indies, St. Augustine and written permissions were obtained from the health care facilities where samples were collected.

Bacterial isolates and susceptibility tests

Staphylococcus aureus isolates used for the analysis were previously reported [10—12]. Antibiotic susceptibility testing was performed using conventional, automated (Microscan Walkaway, Siemens USA) and molecular methods (DNA Microarray assay and Multi Locus Sequence Type — MLST). The minimum inhibitory concentrations (MIC) of the antimicrobial agents were measured using the Walkaway system. The conventional disk diffusion method with antibiotic disks (Oxoid) was used to determine the susceptibility profiles of all isolates to ampicillin, amoxicillin, ciprofloxacin, erythromycin, gentamicin, levofloxacin, oxacillin, rifampin, trimethoprim-sulfamethoxazole (TMP-SMX), tetracycline and vancomycin in accordance with the Clinical and Laboratory Standard Institute (CLSI) guidelines [13]. The minimum inhibitory concentrations (MIC) determined using the Microscan system (Panel for Gram positive organisms) were analyzed for all of the above agents, including linezolid, nitrofurantoin, clindamycin, chloramphenicol, penicillin, rifampin, quinupristin/dalfopristin, tetracycline, trimethoprim/sulfamethaoxazole and vancomycin. The zone sizes of the disc diffusion method and MIC were interpreted according to CLSI breakpoints [13]. American Type Culture Collection *S. aureus* strains ATCC 29213; 25923 and *S. epidermidis* ATCC 12228 were used as quality controls.

Molecular analysis

Further *S. aureus* identification and molecular analysis were performed using DNA Microarray at Institute for Medical Microbiology and Hygiene, Medizinische Fakultaet Carl Gustav Carus, Fiedlerstr Dresden, Germany. The *S. aureus* Genotyping kit (Alere Technologies GmbH, Germany) was utilized, which allows for DNA-based detection of resistance genes, pathogenicity markers of *S. aureus* and assignment of unknown isolates to known strains. The principle of the procedure is that 40-fold amplification of clonal *S. aureus*, which is labeled with biotin-dUTP, has similarities to the linear PCR protocol. Multi-Locus Sequence Typing for *S. aureus* developed by Mark Enright [14] and the corresponding database of allelic profiles for over 1000 isolates with approximately 3000 sequence types from invasive diseases in several areas were utilized. The allelic profile of a *S. aureus* strain is attained by internal fragments of seven housekeeping genes that are sequenced. Statistical Package for the Social Sciences (SPSS) version 20 was used to analyze the data. The chi-squared and Fisher's exact tests were used, as appropriate, to compare data from different groups. The data were descriptive and were reported as comparisons of the frequency distributions. *P* values <0.05 were considered statistically significant.

Results

The *S. aureus* isolates ($n = 309$) included in this analysis were from a previously reported study [12]. The antimicrobial patterns were analyzed and the genes were detected by DNA hybridization. Two hundred and sixty-seven isolates (267/309, 86.4%) were methicillin sensitive *S. aureus*, while forty-two (42/309; 13.6%) were MRSA and the difference between these were statistically significant ($P < 0.05$).

A high incidence of susceptibility was observed among most of the isolates from each infection to the 19 antibiotic drugs tested, as shown in Table 1 where *S. aureus* isolates were 80% susceptible to eleven of the antibiotics commonly available and used in the country.

Staphylococcus aureus were 92.9% susceptibility to trimethoprim/sulfamethoxazole, which is one of the antibiotics available and used in the country, with an MIC value of <2/38 μg/ml. The isolates were least susceptible to ampicillin (10.7%) and penicillin (11.7%) despite having very low MIC with values of <0.25 μg/ml and <0.12 μg/ml, respectively. Among the methicillin susceptible *S. aureus* isolates, the susceptibility rates were high to imipenem (85%), trimethoprim/sulfamethoxazole (84%) and cephalothin (84%). One hundred percent (100%) of MRSA isolates were resistant to ampicillin and penicillin, while the isolates were 1—2% susceptible to oxacillin, amoxicillin/clavulanate and erythromycin and produced low susceptibility rates that were below 10% to several other antibiotics, such as gentamicin and clindamycin.

There was a statistically significant difference between susceptibility of MSSA and MRSA isolates to all antibiotics ($P < 0.05$).

Of the 309 *S. aureus* tested, 124 (40.1%) were resistant to erythromycin, while 111(36%) were resistant to clindamycin. The MIC values for both erythromycin and clindamycin among the MSSA strains were ≤0.5 μg/ml. The MRSA strains resistant to macrolides or macrolides/lincosamides harbored the *erm*A ($n = 20$), *erm*C ($n = 2$) and *msr*A ($n = 17$)

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Table 1 The MIC values and susceptibility percentages of MSSA and MRSA isolates (*n*).

Antibiotics	Resistant	Sensitive	Sensitive isolates		
			Mic μg/ml	MSSA	MRSA
Amox/Clavu	21 (65)	79 (244)	≤4/2	77	2
Ampicillin	89.3 (276)	10.7 (33)	≤0.25	11	0
Cephalothin	11.3 (35)	88.7 (274)	≤8	84	5
Chloramphenicol	43 (133)	57 (176)	≤8	49	8
Ciprofloxacin	16.8 (52)	83.2 (257)	≤1	80	3
Clindamycin	36 (111)	4 (198)	≤0.5	58	6
Erythromycin	40.1 (124)	59.9 (185)	≤0.5	67	2
Gentamicin	25.2 (78)	74.8 (231)	≤4	68	7
Imipenem	10.4 (32)	89.6 (277)	≤4	85	5
Levofloxacin	19.4 (60)	80.6 (249)	≤1	78	3
Linezolid	19.1 (59)	80.9 (250)	≤4	69	12
Nitrofurantoin	0.3 (1)	99.7 (308)	≤32	86	14
Oxacillin	15.2 (47)	84.8 (262)	≤2	84	1
Penicillin	88.3 (273)	11.7 (36)	≤0.12	11	0
Rifampin	6.5 (20)	93.5 (289)	≤1	81	13
Quinu/Dalfo	28.8 (89)	71.2 (220)	≤1	61	10
Tetracycline	13.9 (43)	86 (266)	≤4	80	6
Trime/Sulfa	7.1 (22)	92.9 (287)	≤2/38	84	8
Vancomycin	19.1 (59)	0.9 (250)	≤2	70	11

MIC = minimum inhibitory concentration values; MSSA = methicillin sensitive *S. aureus*; MRSA = methicillin resistant *S. aureus*; Resist = resistant isolates; Amox/Clavul = amoxicillin/clavulanate; Quinu/Dalfo = quinupristin/dalfopristin; Trime/Sulfa = trimethoprim/sulfamethaoxazole.

genes. The genes associated with antibiotic resistance in both MSSA and MRSA isolates in this study are outlined in Table 2. In this analysis, Staphylococcal cassette chromosome *mec* (SCC*mec*), one of the MRSA resistance islands, was encountered. Types I and IV only occurred in 2 isolates each, while Types II and III were encountered in 17 and 21 isolates, respectively. The *blaZ* gene, which is the most common resistance mechanism of *S. aureus* to beta lactams (Penicillinase), was observed in 88.7% (274/309) of the isolates (MRSA 42/309 and MSSA 233/309). Of interest is that, in Trinidad and Tobago, the acquired resistance genes were normally located on the plasmid or chromosome targeting macrolides/clindamycin (*erm*(B)), linezolid (*cfr*), vancomycin (*van*A) in *S. aureus* and none of the MRSA and MSSA harbored *erm*(B) or *mef*(A) genes.

However, among MRSA isolates, the following genes were observed: *mec*A, *SCCmec* II, *SCCmec* III — 0% in MSSA; 42%, 17%, and 21% in MRSA, targeting beta lactam antibiotics; *erm*(A) — 1% in MSSA and 48% MRSA, generally targeting macrolides and lincosamides; *aac*A-*aph*D — 0% in MSSA and 20% in MRSA, targeting aminoglycosides like gentamicin; *tet*(K) — 2% in MSSA and 45% in MRSA; and *tet*(M) — 2% in MSSA and 48% in MRSA, targeting tetracycline. All differed significantly when compared with the MSSA isolates (p-value <0.05). The resistance markers commonly found in the MRSA isolates were, in the current study, significantly associated with the ST239-MRSA-III strain. Common resistance genes, *msr* (2% in MSSA and 40% in MRSA) and *mph* (1% in MSSA; and 40% in MRSA) were also notably associated with USA300. The 2% SCC*mec* IV observed in this analysis occurred in the MRSA isolates recovered from patients who were from the community areas in the country.

The *mec*A positive (13.6%, 42/309) isolates were spread over the following three lineages: CC5, CC8 and CC59 (data not shown). Clonal complex 5 accounted for 2% (1/42) of MRSA isolates, while CC8 contributed seven sequence types to the MRSA group, accounting for 93% (39/42) of the MRSA isolates. The most common SCC*mec* type was SCC*mec* III (21 isolates) for which all isolates belonged to ST239. These isolates also encoded *mer*A/B genes, which are likely to indicate a presence of a composite SCC*mec* III/SCC*mer* element. Seventeen isolates belonged to the CC8 USA300 strain and additionally encoded both the *PVL* genes and ACME cluster.

Discussions

Susceptibility patterns of *S. aureus* strains differ considerably in different regions. Ampicillin and penicillin remain the least efficient antibiotics as

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Table 2 Genes associated with antibiotic resistance among the MSSA and MRSA isolates in Trinidad and Tobago.

Gene	Gene name	*N*	MSSA	MRSA
*mec*A	Alternate penicillin binding protein 2, defining MRSA	42	0	42
SCCmec I	Cassette Chromosome mec I	2	0	2
SCCmec II	Cassette Chromosome mec II	17	0	17
SCCmec III	Cassette Chromosome mec III	21	0	21
SCCmec IV	Cassette Chromosome mec IV	2	0	2
*mer*A/B	Mercury resistance operon	21	0	21
*bla*Z/I/R	Beta-lactamase operon	274	233	41
erm(A)	Methyltransferases, erythromycin, clindamycin resistance	23	3	20
erm(B)	Methyltransferases, erythromycin, clindamycin resistance	0	0	0
erm(C)	Methyltransferases, erythromycin, clindamycin resistance	2	0	2
lnu(A)	Lincosamid-Nucleotidyltransferase	1	1	0
mef(A)	Macrolide efflux protein A	0	0	0
mph(C)	Probable lysyphosphatidylglycerol synthetase	20	3	17
msr(A)	Energy dependent efflux of erythromycin	22	5	17
*aac*A-*aph*D	Bifunctional enzyme Aac/Aph, gentamicin resistance	20	0	20
*aad*D	Aminoglycoside adenyltransferase, tobramycin resistance	12	8	4
*fus*B	Fusidic acid resistance gene	1	1	0
*fus*C	Fusidic acid resistance gene from ''SCCfus'' elements	6	3	3
mupA	Mupirocin resistance protein	15	11	4
tet(K)	Tetracycline resistance	24	5	19
tet(M)	Tetracycline resistance	25	5	20
cat	Chloramphenicol acetyltransferase	1	0	1
*fex*A	Chloramphenicol/florfenicol exporter	0	0	0
cfr	Linezolid resistance	0	0	0
dfrS1	Dihydrofolate reductase type	1	11	0
*qac*A	Quaternary ammonium compound resistance protein A	14	2	12
*qac*C	Quaternary ammonium compound resistance protein C	7	5	2
sat	Streptothricine-acetyltransferase	39	3	36
*van*A	Vancomycin resistance gene	0	0	0

they are older β-lactams and are now ineffective antibiotics in the treatment and control of more than 80% of *S. aureus* infections in the country [15]. Trimethoprim/sulfamethaoxazole, imipenem, cephalothin and oxacillin are among the antibiotics with the highest sensitivity rates in MSSA isolates, but the MRSA isolates were highly resistant to oxacillin as expected. A significant difference between MSSA and MRSA isolates was observed, which further suggests that certain antibiotics are more effective in treating either MSSA or MRSA infections in the country.

The results of molecular typing showed that over 90% of MRSA in the country belonged to SCC*mec* types II and III isolates, while types I and IV accounted for less than 10%. Types V, VI and other types reported in the literature [16] were not observed in our analysis. The prevalence of two types of SCC*mec* II and III among the MRSA isolates encountered in Trinidad and Tobago indicates very low clonal diversity. Previous studies have reported that *S. aureus* isolates exhibiting SCC*mec* I, II and III belong to health care-associated clones (HA-MRSA) and harbor multiple resistance determinants, while types IV were associated with community-associated clones [17,18]. This means that with majority of SCC*mec* cases are present in health institutions in the country, which warrants more efforts focusing on antibiotic stewardship and infection control measures in the health care facilities because there is now very low clonal diversity. It has also been reported that SCC*mec* type II is associated with increased MRSA mortality [14,19], which was in agreement with our experience wherein the only two cases of mortality belonged to SCC*mec* type II. As a result, there should be measures to rapidly identify the MRSA strains harboring the SCC*mec* genes. As has been previously reported by this author and others [20], evaluation of the cost for detecting MRSA from clinical specimens should now be reviewed and incorporated into routine laboratory tests to prevent any mortality caused by missing such MRSA infections in the country.

The vancomycin gene (*van*A) was not observed in this study, which is similar to reports from Brazil [21]. The *van*A resistance gene has nonetheless been reported in *S. aureus* isolates in the USA

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[22,23] and other bacterial species in Costa Rica [24]. Detection of the resistance mechanisms and their genetic basis is an important reason to support antibiotic susceptibility surveillance in *S. aureus*.

Among resistance markers, however, only the *bla*Z/I/R was commonly found, while other genes had low frequencies or were not found, such as the *erm*(B), *mef*(A), *fex*A, *cfr* and *van*A genes. It could be hypothesized that resistance of *S. aureus* to these agents could be mediated by other mechanisms and not by these genes. Both clonal spread of resistant strains and horizontal transfer of resistance genes contribute to the rising global prevalence of multi resistant bacteria, and horizontal transfer enables the acquisition of multidrug resistance by previously susceptible bacteria [25]. Non-existence of *fex*A among the *S. aureus* isolates is not surprising considering that this gene mediates resistance towards these agents, chloramphenicol and florfenicol, which are not available or have been withdrawn from local markets in the country for over 20 years. We did not encounter the *cfr* gene among our *S. aureus* isolates despite reports of its existence in several countries, including the USA, Mexico, Ireland, Spain, Italy and Colombia, where the first clinical isolate was documented [26—31]. More good news for the country is that there is no resistance to fourth and fifth generation cephalosporins, such as cefepime and ceftaroline (although we did not include these in our studies), because they have not yet been included in the National drug formulary and are not available in the country.

The most common clonal complex in our study was CC8, accounting for 93% (39/42) of MRSA isolates in our analysis. Similar results have been reported in other countries, such as Japan and the USA, where this complex is the most common clone in skin and soft tissue-associated infections, especially among PVL-positive *S. aureus* [32,33]. More than 50% of these MRSA isolates harbored the SCC*mec* II and III cassettes and were positive for the PVL gene, while six (14.3%) harboring the SCC*mec* III cassette were PVL-negative.

Our multi locus sequence typing analysis revealed unique *S. aureus* strains with similarities to ST2250/2277 that belonged to the newly described species *S. argenteus*. They were not observed to carry the species marker *coA* or accessory gene regulator. They do have these genes, but they had other, unrecognized alleles. Thus, while we did not see them, we cannot say that they are absent. They were *mec*A negative and lacked various resistant genes, but the β-lactamase structural gene (*bla*Z) was present. Known virulence factors, including the PVL gene, were also lacking.

Conclusions

Despite the limitation of not correlating the specific antimicrobial resistance with the outcomes of *S. aureus* infections, this study highlighted several resistance genes of *S. aureus* organisms that are currently non-existent in Trinidad and Tobago, such as *van*A, *cfr* and *erm*(B), suggesting that there is some other method of *S. aureus* resistance to vancomycin, linezolid and clindamycin. The study also reported a high prevalence of SCC*mec* genes among *S. aureus* clones, suggesting an ongoing nosocomial transmission and minimal community transmission. This observation indicates that routine rapid detection methods of MRSA strains, as has been previously delineated, should now be implemented. Additionally, there should be concerted efforts to implement stringent antibiotics stewardship and policy measures in hospitals throughout the country. The authors propose that a more detailed molecular epidemiological population study should be performed to evaluate correlations with antimicrobial resistance, outcomes and *S. aureus* infections in Trinidad and Tobago.

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Competing interests

None declared.

Ethical approval

Ethical approval for this study was granted by the Ethics Committee, the University of the West Indies, St. Augustine and written permissions were obtained from the health care facilities where samples were collected.

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