Original Article

# Molecular Characterization of Methicillin-Resistant *Staphylococcus aureus* Isolates from Rural Community Settings in Trinidad and Tobago

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**Abstract**

**Aims:** To determine the virulence and antimicrobial resistant genes in methicillin resistant *Staphylococcus aureus* isolates recovered from patients attending two rural health centers in Trinidad and Tobago. **Settings and Design:** Cross-sectional observational analysis of patients from two local health centers located in communities in northern region of the country. **Materials and Methods:** Nasal and wound swabs from 300 patients were analyzed using standard and molecular techniques. Multiplex polymerase chain reaction was used to detect 16S rRNA, *mec*A, Staphylococcal chromosomal cassette SCC *mec* types, *pvl,* alpha hemolysin (*hla*), and Toxic Shock Syndrome Toxin 1 (*tst*1) genes. *S. aureus* ATCC 33591 and *Staphylococcus epidermidis* ATCC 12228 were used for quality control, respectively. **Results:** Over a quarter (26.7%, 80/300) of the surveyed patient's samples grew bacterial isolates of which 45% (36/80) were *S. aureus* and 44.4% (16/36) were *mecA*-positive. Majority (62.5%, 10/16) possessed the *pvl* gene, whereas 25% (4/16) possessed the alpha hemolysin *(hla)* gene. None of the methicillin-resistant *Staphylococcus aureus* (MRSA) isolates possessed the *tst*1 gene. Also, 18.8% (3/16) isolates possessed both virulence genes, *pvl* and *hla*. Although the SCC*mec* types IV and V were detected, but none of the SCC*mec* I, II, and III were harbored by the isolates. **Conclusions:** SCC*mec* type IV and the *pvl* genes were common among the MRSA isolates from the community. The *hla* gene was found infrequently, but none of the isolates possessed the *tst*1 gene. Knowledge of this is important for robust surveillance of such cases from the community in the country.

**Keywords:** *Community-associated methicillin-resistant Staphylococcus aureus, hya gene, pvl gene, SCCmec types, Trinidad and Tobago, tst1*

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## Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) produces a variety of virulence factors that cause diseases of varying severity in patients treated in hospitals or in community settings.[1,2] Community-associated MRSA (CA-MRSA) strains are replacing hospital-associated (HA-MRSA) strains and eventually, due to competition, may replace specific HA-MRSA strains in the long term.[3-7] This is of concern to public health as it may result in failure of standard hospital treatment protocols for MRSA-infected patients.[8]

Studies on MRSA isolates have detected *pvl* toxin genes in HA-MRSA strains, but in CA-MRSA isolates, they have rarely been identified in Trinidad and Tobago.[9,8] A *pvl*-positive MRSA isolate was identified in 2011/2012 belonging to the ST8 CC8 MRSA known as "USA300"

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in the country.[10] This strain is endemic in the USA and is CA-MRSA, but in Trinidad and Tobago, large number of MRSA isolates identified as ST 239 MRSA-3 is hospital associated.[11]

Toxin genes *hla* and *tst*1 have not been detected in MRSA isolates, but they have been detected in five methicillin-sensitive *S aureus* isolates (MSSA) in the country.[8] The DNA microarray techniques have identified antibiotic resistance genes -*erm*A and *erm*C from HA-MRSA isolates, but *erm*B and *van*A have not been detected.[12] Previous studies on HA-MRSA isolates from the country have focused extensively on phenotypic and molecular resistance testing.[13,14] Also, a study has described SCC*mec* types among HA-MRSA in the country, but none or very few have reported the SCC*mec* types in CA-MRSA isolates.[11]

The aim of this study was to delineate the SCC*mec* type and toxin genes, such as *pvl, hla,* and *tst*1, including genes, such as *erm*A, *erm*C, and *van*A, mediating antibiotic resistance in MRSA isolates from rural or community setting individuals in the country.

## Materials and Methods

### Study sites, population, and sample collection

Study was carried out at two community health centers located in the northern region in Trinidad and Tobago, during a 3-month period – May to July 2016. These health centers are in the rural communities of the country and offer only outpatient care services. Subjects were enrolled in the study after obtaining their written consent. Anterior nares and wound swabs were taken from all participants, if there was no history of hospital visit or admission in the past 12 months. A standardized questionnaire was used to collect clinical and demographic data including age, gender, and occupation. Risk factors for CA-MRSA acquisition such as playing contact sports, living in crowded or long-term unsanitary conditions, prolonged antibiotic usage, being an injecting drug user and surgical intervention were also obtained.

### Laboratory analysis

The clinical specimens were placed in 5 mL Tryptone soya broth upon collection and streaked on Mannitol Salt Agar (MSA) plates within 6 hours and incubated overnight. Microbiological analysis of the specimens was performed at the Microbiology Laboratory of the Paraclinical Sciences Department, Faculty of Medical Sciences and the Microbiology Laboratory of the Faculty of Science and Technology, the University of the West Indies, St. Augustine Campus.

*S. aureus* bacterial strains were presumptuously identified by morphological (Gram-positive cocci in clusters by Gram staining from small yellow colonies on Mannitol Salt Agar) and biochemical tests such as a positive catalase test, slide, and tube coagulase tests. Isolates were also further identified using latex agglutination test (Oxoid, UK) specific for *S. aureus*.

### Identification of methicillin-resistant *Staphylococcus aureus*

#### *Cefoxitin disk diffusion*

The direct colony suspension of *S. aureus* organisms adjusted to a 0.5 McFarland standard (1–2 × $10^8$ cfu/mL) was inoculated on a Mueller–Hinton Agar (MHA) plate. A 30 μg cefoxitin disc (Oxoid, UK) was then placed onto the plates and incubated for 24 hours at 35°C ± 2°C. Isolates showing inhibition zone sizes ≤21 mm were considered as resistant according to the CLSI guidelines.[15]

#### *Minimum inhibitory concentration*

The oxacillin MIC and cefoxitin screen well tests performed by the MicroScan WalkAway 96 SI system, which identified methicillin resistance in the *S. aureus* isolates. Results for oxacillin MIC of ≥4 μg/mL were considered resistant to oxacillin and hence positive for MRSA. For cefoxitin screening, isolates showing results of ≥4 μg/mL were considered resistant and ≤4 μg/mL were considered sensitive and hence negative for MRSA. Results of cefoxitin screen well test and oxacillin MIC were read when completed by MicroScan automated machine to determine, if *S. aureus* isolates were MRSA. *S. aureus* isolates that had a MIC value of ≥8 μg/mL for erythromycin were considered resistant. Likewise MIC values that were ≥4 and ≥16 μg/mL were considered resistant for clindamycin and vancomycin, respectively.[15]

The MRSA isolates were stored in brain heart infusion broth supplemented with 20% glycerol at −70°C until further testing.

### Inclusion and exclusion criteria

No duplicate patient samples were included in this study. All recovered *S. aureus* isolates and specimens were de-identified and anonymously analyzed but isolates recovered from all individuals who had the risk factors listed above (a long history of hospitalization, indwelling catheters at the time of enquiry or recently, on antibiotic usage over the long term) were excluded from the study analysis.

### DNA extraction process

Before DNA extraction, the MRSA samples were re-inoculated on blood agar plates at 35°C for 18–24 hours. On the following day, a few colonies were taken from the plates and were inoculated into Tryptone Soya Broth, where they were placed in a 37°C shaking incubator for 18–24 hours. DNA

extraction was performed using the Promega DNA extraction Wizard Kit according to the manufacturer's instructions. The eluted DNA was placed at −70°C for later use in molecular testing. The lysing reagents used were lysozyme and lysostaphin, respectively, both at a working concentration of 10 mg/mL.

### 16S rRNA and *mec*A gene detection for identification of methicillin-resistant *Staphylococcus aureus* isolates

Polymerase chain reaction (PCR) as the gold standard for the detection of MRSA was employed in this study. The multiplex PCR (MPCR) was performed according to the protocol of Strommenger *et al*.[16] DNA banding at 420 bp was taken as positive for the 16S rRNA gene, whereas banding at 532 bp as positive for the *mecA* gene, respectively. *S. aureus* ATCC 33591 and *Staphylococcus epidermidis* ATCC 12228 were used as the positive and negative controls, respectively. Amplitaq Gold Mastermix and GC enhancer (Life Technologies, Applied Biosystems) were used for all multiplex PCRs.

### SCC*mec* typing of methicillin-resistant *Staphylococcus aureus* isolates

A multiplex PCR was performed for the detection of SCC*mec* types I (415 bp), II (937 bp), III (518 bp), IV (937 and 415 bp), and V (518 and 359 bp) following the protocol of Boye *et al*.[17] The following PCR conditions were used in the amplification of the SCC*mec* genes being investigated in this study: 4 min at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 60 seconds at 72°C, with a final extension for 4 min at 72°C. The initial 4 minutes at 94°C serves as a "hot start," which is required to activate DNA polymerase required for enzyme activation before the start of the PCR amplification cycles (denaturation, annealing, and extension).

### Detection of toxin genes

The primers used in this study for the amplification of *pvl, hla,* and *tst*1 genes are as listed by Havaei *et al*.[18] The presence of *pvl*, *tst*1, and *hla* genes were considered positive at 502, 398, and 744 bp DNA banding, respectively.

### Detection of antibiotic resistant genes

The primers used for the amplification of the *erm*A, *erm*C were as previously reported by Strommenger *et al*.[16] and for *van*A genes as described by Havaei *et al*.[18] The protocol was performed with the following settings: initial denaturation at 94°C for 3 minutes, followed by 30 cycles of amplification (denaturation at 94°C for 30 seconds; annealing at 55°C for 30 seconds; extension at 72°C for 30 seconds), and final extension at 72°C for 4 minutes. The presence of *erm*A, *erm*C, and *van*A genes were considered positive at 190, 299, and 1,032 bp DNA banding, respectively.

### Gel electrophoresis confirmation

About 8 μL of amplified PCR product was loaded on a 2.0% agarose gel containing ethidium bromide at a concentration of 1 μg/mL at 120 V for 40–60 minutes. The resulting band patterns were analyzed by visual inspection for the respective sizes according to the published literature for the respective primers listed above. A 100-bp molecular ladder was used in all experiments.

### Ethical approval

Ethical approval and permission for this study was given by the Campus Ethics Committee of the University of the West Indies, St. Augustine, and the North Central Regional Health Authority (NCRHA).

## Results

During the study period, 300 clinical specimens (nasal swabs, $n$ = 294; wound swabs, $n$ = 6) from the subjects were deidentified and anonymously analyzed. Just about 26.7% (80/300) of the patients' samples had positive bacteria growth of which 36 of the isolates were *S. aureus* (45%, 36/80). Phenotypic tests indicated that 50% (18/36) were MRSA positive; however, molecular tests confirmed only 44.4% (16/36) of the *S. aureus* isolates as MRSA with the presence of the *mec*A gene [Figure 1] and were included in the final molecular analysis. Also, worth noting was the fact that all isolates from patients ($n$ = 36) included in the analysis had no current infective process. A majority of them were diabetic (25%, 4/16), hypertensive (18.8%, 3/16), asthmatic (18.8%, 3/16), or healthy (37.5%, 6/16); and more than half of the analyzed isolates 56% (9/16) were from females. A majority of patients identified as having MRSA were students 31% (5/16), and half of the MRSA isolates (50%, 8/16) were from those who participate in sports for which there were physical contacts.

As depicted in Figure 2, the SCC*mec* type IV was the most predominant with 75% (12/16) and the remaining 25% (4/16) were SCC*mec* type V. The SCC*mec* types I, II, and III were not detected among this community MRSA isolates. The toxin gene screening of the CA-MRSA isolates [Figure 3] shows that the majority of isolates, 62.5% (10/16), possessed the *pvl* gene, whereas 25% (4/16) had the alpha hemolysin *(hla)* gene. None of these CA-MRSA isolates possessed the *tst* 1 gene. In addition, it is important to note that 12.5% (2/16) isolates possessed the virulence genes, *pvl* and *hla*. The remaining *pvl* toxin genes are associated with SCC*mec* type IV and 2 *hla* toxin genes belong to SCC*mec* type V.

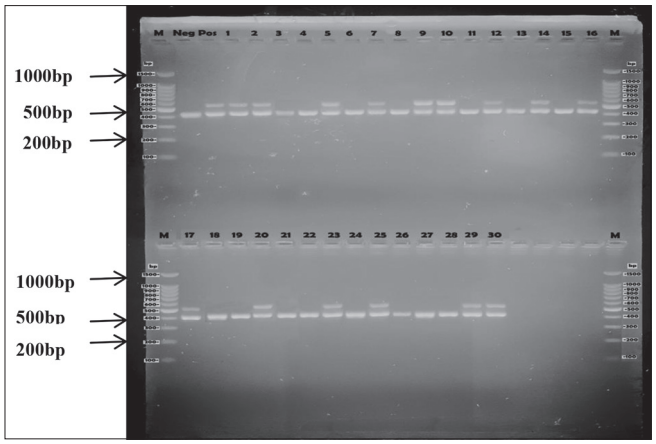


**Figure 1:** 16S rRNA and *mecA* results for community-associated methicillin-resistant *Staphylococcus aureus* isolates

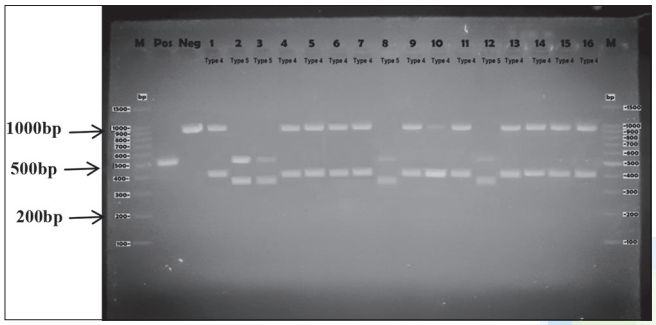


**Figure 2:** SCC*mec* typing of community-associated methicillin-resistant *Staphylococcus aureus* isolates

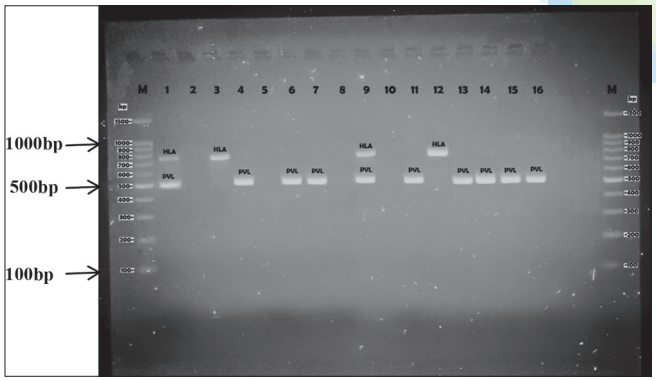


**Figure 3:** Toxin gene results for the community-associated methicillin-resistant *Staphylococcus aureus* isolates

The antibiotic resistance gene screening of the isolates analysis revealed that 31% (5/16) of the isolates tested positive for the *erm*A gene. None of them tested positive for the *erm*C and *van*A genes, respectively.

Figure 1 shows 16S rRNA and *mec*A results of MRSA isolates from patients recruited from the health centers, Trinidad. M lanes indicate the 100 bp DNA ladder. The ATCC strains (*S. epidermidis* and *S. aureus*) used as quality controls are in lanes Neg and Pos, respectively. Lanes 1–16 and 17–30 represents bands of PCR products for the MRSA isolates from the patients studied. Half of the isolates were positive for *mec*A genes, whereas half were not. Lanes 3, 4, 6 8, 11, 13, 15, 18, 19, 21, 22, 24, and 26–28 were all negative for the *mec*A gene bands, whereas the others had the bands for *mec*A gene confirming MRSA strains.

Figure 2 shows the SCC*mec* type of MRSA isolates from patients recruited from the health centers. M lanes indicate the 100 bp DNA ladder. The "Pos" and "Neg" quality control lanes were the ATCC strains isolates of *S. aureus* and *S. epidermidis*. Lanes 1–16 represents bands of PCR products of the MRSA isolates from the patients. Lanes 1, 4–7, 9–11, and 13–16 are SCC*mec* type IV, whereas lanes 2, 3, 8, and 12 are SCC*mec* type V.

Figure 3 shows toxin gene results of MRSA isolates from patients recruited from the health centers, Trinidad. M lanes indicate the 100 bp DNA ladder. Lanes 1–16 represents bands of PCR products of the MRSA isolates from the patients. Lanes 1, 3, 9, and 12 are the isolates that possess the *hla* gene, lanes 1, 4, 6, 7, 9, 11, and 13–16 are the isolates that possess the *pvl* gene, respectively.

## Discussion

In this study, the number of isolates screened was very small and the time frame of this study was short; however, the results are not similar to the findings from previous studies conducted in the country.[10,14] The majority of the MRSA isolates were SCC*mec* type IV, whereas the remaining were SCC*mec* type V. This supports international published findings, which state that a large number of CA-MRSA strains found worldwide belong to SCC*mec* type IV.[19,20] No SCC*mec* types I, II, or III were found associated with CA-MRSA in this study; and the ratio of SCC*mec* types III, IV, and V in this study is similar to that conducted by Monecke *et al*. (2014) on HA-MRSA isolates from Trinidad and Tobago.[8]

A review of published literature available shows that "no MRSA isolates possessing SCCmec1 have been identified in the country." A plausible explanation could be that MRSA isolates containing SCC*mec* I, which is found worldwide may not have been introduced into the country, or if they have, they have not persisted due to strict competition with already prevailing hospital strains. The MRSA isolates possessing SCC*mec* type II have previously been detected in the country and typing by PFGE designated such isolates as belonging to clonal complex 5.[13] This is a hospital strain, which is common in Europe and North America.[21] The importation of this specific strain into the country may have been the result of the travel activities of health care personnel and from recovered hospital patients.[22]

Toxin gene analysis of MRSA isolates has proven to be interesting with respect to the first report of the identification of virulence genes, *hla and tst* 1, among MRSA isolates within Trinidad and Tobago. The *tst* 1 gene was found in a previous study conducted in Trinidad and Tobago in MSSA isolates only.[8] It is important to note that the *hla* gene is the most prominent *S. aureus* cytotoxin that can act against a wide range of host cells including erythrocytes, epithelial cells, endothelial cells, T cells, monocytes, and macrophages.[23] The *pvl* gene which is a potent toxin gene was detected in a minority of HA-MRSA isolates and was found to be associated with both the *hla* and *tst*1 genes except for one isolate. Isolates which contain these three virulence genes can be postulated to have increased pathogenicity and therefore cause more serious infections in patients.

In this study, the predominant toxin gene identified among MRSA isolates was the *pvl* gene. This is expected in CA-MRSA isolates as the majority of these strains possess the *pvl* gene as reported in literature[20] but is less than that reported by Borlaug *et al*., 2011[24] who stated almost 100% of CA-MRSA isolates possess the *pvl* gene. It must be noted that the *pvl*-positive CA-MRSA isolates obtained in this study, all belonged to SCC*mec* type IV. It could be postulated that these patients may have initially acquired these infections in the community setting before being admitted and treated in the hospital. Recent studies have reported that CA-MRSA strains are spreading in hospital settings and are replacing traditional HA-MRSA strains.[25] More studies need to be conducted in the country to deduce whether this trend is occurring. Two CA-MRSA isolates that are SCC*mec* type V did not possess any of the virulence genes screened for in this study and we hypothesized that if more virulence genes were screened, this particular SCC*mec* type would have one or more toxin genes.

The molecular antibiotic resistance analysis of CA-MRSA isolates revealed that a minority of CA-MRSA isolates tested positive for the *ermA* gene, whereas no isolates contained the *erm*C or *van*A genes. These findings support the report of previous studies in the country that MRSA isolates are much less resistant to non β-lactam antibiotics.[12,14] An increasing number of studies has shown that CA-MRSA isolates from certain areas have become resistant to multiple drugs, which have enabled them to invade hospitals and affect the distribution of MRSA clones.[26] A more extensive population study focusing on the collection and screening of CA-MRSA isolates for specific antibiotic resistance genes would most likely detect more than one resistance gene in the country. The authors are of the opinion that surveillance system or close monitoring of community acquired MRSA infections especially in healthcare centers in rural communities should be robust, timely, and efficient, so as to report their epidemiology in the country.

## Conclusion

A major limitation of this current study was the limited number of patients and samples investigated which may not have given a true picture of the distribution of *S. aureus* resistant and virulent genes. But despite this, the authors conclude that SCC*mec* type IV was the most predominant and this is in agreement with international published findings. The *pvl* gene was possessed by the majority of CA-MRSA isolates, whereas the *hla* gene was found infrequently among these isolates and the *tst*1 gene was not detected. The antibiotic resistance analysis of the isolates revealed that the *erm*A gene was detected in low frequency, whereas the *erm*C and *vanA* antibiotic resistance genes were not detected.

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### Conflicts of interest

There are no conflicts of interest.

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