

Prevalence and Risk Factors for Nasal and Oropharyngeal Carriage of Staphylococcus Aureus in Insulin-Dependent Diabetics Individuals

Nathalia Bibiana Teixeira (✉ na_tx0402@yahoo.com.br)

Universidade Estadual Paulista Julio de Mesquita Filho Instituto de Biociencias Campus de Botucatu
<https://orcid.org/0000-0002-6185-6086>

Carlos Magno Castelo Branco Fortaleza

Universidade Estadual Paulista Julio de Mesquita Filho Faculdade de Medicina Campus de Botucatu

Matheus Cristovam de Souza

Universidade Estadual Paulista Julio de Mesquita Filho Instituto de Biociencias Campus de Botucatu

Thais Aline Monteiro Pereira

Universidade Estadual Paulista Julio de Mesquita Filho Instituto de Biociencias Campus de Botucatu

Bibiana Prada de Camargo Colenci

Universidade Estadual Paulista Julio de Mesquita Filho Faculdade de Medicina Campus de Botucatu

Maria de Lourdes Ribeiro de Souza da Cunha

Universidade Estadual Paulista Julio de Mesquita Filho Faculdade de Medicina Campus de Botucatu

Research

Keywords: methicillin-susceptible Staphylococcus aureus (MSSA), methicillin-resistant Staphylococcus aureus (MRSA), diabetes mellitus, insulin, resistance, molecular typing

DOI: <https://doi.org/10.21203/rs.3.rs-67652/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Individuals with insulin-dependent diabetes are a risk group for infections caused by *Staphylococcus aureus*. The objective of this study was to determine the prevalence and risk factors for nasal and oropharyngeal carriage of *S. aureus* and MRSA in insulin-dependent diabetic individuals from Botucatu, São Paulo, Brazil. Additionally, susceptibility profiling, detection of the *mecA* gene, SCC*mec* typing and molecular typing by PFGE and MLST were performed in nasal and oropharyngeal swabs of 312 subjects. The prevalence of *S. aureus* and MRSA was 30.4% and 4.8%, respectively. SCC*mec* type IV was the predominant type among isolates, although some carried SCC*mec* types I and II. MRSA and MSSA clones were detected among the isolates of this population. In addition, an important clonal lineage (ST398) was identified among resistant and susceptible isolates. Age and lung disease were negatively associated with *S. aureus* carriage, while lower-extremity ulcers were a risk factor for *S. aureus* carriage. For MRSA, only male gender was a risk factor. These data suggest widespread dissemination of MRSA in the insulin-dependent diabetic population studied, as well as the emergence of important lineages among these individuals.

1. Background

Diabetes mellitus is a progressive chronic disease characterized by high blood glucose levels, which is one of the most prevalent diseases in modern societies. Its treatment is often inadequate or absent [1]. It is estimated that more than 370 million people worldwide have diabetes and more than 5 million deaths were attributed to the disease and its complications in 2017 [2, 3].

Diabetic individuals are known to be more susceptible to infections because of their elevated blood glucose levels and suppression of the immune response. In addition, neuropathy and reduced blood flow to the extremities are common in these individuals. Consequently, wounds tend to heal more slowly, increasing the risk of amputations and death [4, 5].

Staphylococcus aureus is one of the leading causes of infections in diabetic individuals. Infections caused by methicillin-resistant *S. aureus* (MRSA) are associated with a higher mortality rate compared to infections caused by methicillin-susceptible *S. aureus* (MSSA) [2, 6]. Studies suggest that nasal colonization with *S. aureus* and MRSA increases the risk of developing infections and that insulin use is a risk factor for colonization with MRSA in diabetic individuals [7]. However, little is known about the role of throat colonization in this population.

Considering their impact on the health of diabetic individuals, a better understanding of the epidemiology and risk factors attributed to colonization with *S. aureus* and MRSA is necessary. The objective of this study was to determine the prevalence and risk factors for nasal and oropharyngeal colonization with *S. aureus* and MRSA in insulin-dependent diabetic individuals. Additionally, antimicrobial susceptibility testing of the isolates, detection of the *mecA* gene, SCC*mec* typing, clonal profiling, and molecular typing by MLST were performed.

2. Materials And Methods

2.1 Study design

This was a cross-sectional study conducted in the city of Botucatu, São Paulo, Brazil, whose estimated population is 146.497 inhabitants [8]. The size of the sample was calculated based on the number of insulin-dependent diabetic individuals registered at the municipal health department for receiving a glucose meter, which was 1,631 individuals. Using the calculation for proportions, we obtained a suggested n of 312 subjects. The subjects were selected randomly from the database of the health department. If possible, the subjects were recruited during home visits; however, many samples were collected during events promoted at Basic Health Units and at the headquarter of the Botucatu Association for Diabetes Support (ABAD in the Portuguese acronym). A questionnaire including the following data was applied to subjects who agreed to participate in the study: demographic data (gender and age); type of diabetes (1 or 2); time since diagnosis (years); time of insulin use (years); clinical data (comorbidities); presence of ulcers or amputations; tattoos; hospitalizations or medical procedures in the last year; infections and use of antimicrobials in the last year. All data were obtained by interview with the patient and/or legal representative following ethical standards.

2.2 Collection of microbiological specimens

Nasal and oral mucosa samples were collected from 312 insulin-dependent diabetic individuals living in the city of Botucatu, São Paulo, Brazil, using sterile *swabs* in transport medium. Samples from the anterior nares and oropharynx were obtained using one *swab* for each site. For the collection of nasal samples, the *swab* was immersed in 0.9% sterile saline and inserted into both nares, rotating it and gently pressing its end against the mucosa. The technique for oropharyngeal sampling consisted of immersion of the *swab* and passing it gently over the surface of the throat, avoiding contact of the examiner with the tongue.

The *swabs* were transported in Stuart medium to the Laboratory of Bacteriology, Department of Microbiology and Immunology, Institute of Biosciences, UNESP, and seeded onto plates containing Baird-Parker agar, a selective medium for *Staphylococcus*. After incubation for 48 h at 37 °C, the isolated microorganisms were identified.

2.3 Identification of *Staphylococcus aureus*

The microorganisms were submitted to Gram staining for observation of their morphology and specific staining. After confirmation of these features, catalase and coagulase tube tests and biochemical tests (maltose, trehalose, and mannitol) were carried out for differentiating *S. aureus* from other coagulase-positive *Staphylococci* [9, 10].

After DNA extraction with the Illustra Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), the *S. aureus* isolates were confirmed genotypically by detection of the 16S rRNA gene [11] and the DNA SA442 fragment specific for *S. aureus* [12].

2.4 Antimicrobial susceptibility testing

All isolates obtained were subjected to antimicrobial susceptibility testing by the disc diffusion method using impregnated discs according to the criteria of the Clinical Laboratory Standards Institute (CLSI) [13]. The drugs used were: oxacillin (1 µg), ceftiofur (30 µg), linezolid (30 µg), quinupristin/dalfopristin (15 µg), and sulfamethoxazole/trimethoprim (25 µg). Antimicrobial activity was evaluated by determining the diameter of the inhibition zone, which was interpreted according to the CLSI [13].

2.5 Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of vancomycin was determined by the E-test. This quantitative test consists of inert and transparent plastic strips (60 mm long x 5.5 mm wide) with a predefined gradient of concentrations of the antimicrobial to be tested. The MIC results were classified as susceptible, intermediate, or resistant according to the definitions of the CLSI [13].

2.6 Detection of the *mecA* gene of methicillin resistance

The parameters described by Murakami et al. [14] was used for detection of the *mecA* gene by PCR. International reference strains were included as positive (*S. aureus* ATCC 33591) and negative (*S. aureus* ATCC 25923) controls in all reactions.

2.7 Determination of staphylococcal cassette chromosome *mec* (SCC*mec*) type

SCC*mec* typing was performed by multiplex PCR as described by Oliveira and de Lencastre [15] and updated by Milheiriço et al. [16] The following strains were used as controls: COL for SCC*mec* type I; N315 for SCC*mec* type IA; PER34 for SCC*mec* type II; AN546 for SCC*mec* type III; HU25 for SCC*mec* type IIIA, and MW2 for SCC*mec* type IV.

2.8 Visualization of amplified products

The efficiency of the amplifications was confirmed by electrophoresis on 2% agarose gel prepared in 0.5 M Tris-borate-EDTA (TBE) buffer. A 100-bp marker was used as molecular weight standard. The gel was stained with SYBR® Safe and photographed under UV transillumination.

2.9 Pulsed field gel electrophoresis (PFGE)

The modified protocol of McDougal et al. [17] was used to type all *S. aureus* isolates by PFGE. The isolates were grown in BHI broth for 24 h. Next, 400 µL of the sample was added to a microtube and centrifuged at 12,000 rpm for 50 seconds. The supernatant was discarded and 300 µL TE solution (10 mM Tris, 1 mM EDTA, pH 8.0) was added. The samples were kept in a water bath for 10 min at 37 °C. After vortexing, 5 µL lysostaphin (1 mg/mL in 20 mM sodium acetate, pH 4.5) and 300 µL low-melting agarose were added.

The samples were transferred to plug molds. The plugs were allowed to solidify and then placed in a 24-well plate with 2 mL EC solution (6 mM Tris-HCl, 1M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosine) and incubated at 37 °C for at least 4 h. The EC solution was removed and the plugs were washed four times with 2 mL TE at room temperature at intervals of 30 min.

The *Sma*I enzyme (Fast Digest *Sma*I, Life Science, Canada) was used for restriction of genomic DNA. Electrophoresis was carried out in a CHEF-DR III System (BioRad Laboratories, USA) on 1% agarose gel prepared with 0.5 M TBE (Pulsed Field Certified Agarose, BioRad Laboratories, USA) under the following conditions: pulse switch time of 5 to 40 s for 21 h using a linear ramp; 6 V/cm; angle of 120°; 14 °C; 0.5M TBE as running buffer. The Lambda PFG Ladder (New England BioLabs) was used as molecular marker. The gel was stained with GelRed (10,000X in water; Biotium, USA) for 1 h and photographed under UV transillumination.

Similarity was analyzed with the BioNumerics 7.6 software (Applied Maths, Belgium). The dendrogram was constructed by the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean), with band position tolerance and optimization adjusted to 1.2% and 1%, respectively.

Forty-five isolates, including one MRSA, could not be typed with the *Sma*I enzyme and were therefore digested with the *Apa*I restriction enzyme.

2.10 Multilocus sequence typing (MLST)

MLST was performed according to the protocol of Enright et al. [18] by amplification and sequencing of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqjL*). The PCR products were purified using the HiYield™ Gel/PCR Fragments Extraction Kit and the sequencing reaction is described below. The sequences were analyzed and compared to an online database (<http://www.mlst.net>) (2004). The sequencing reactions were performed in an ABI3500 8-capillary (50 cm) sequencer using POP7 as polymer (Applied Biosystems). The sequences were visualized (electropherogram) using the BioNumerics 7.6 program (Applied Maths, Belgium).

2.11 Statistical analysis

The Epi-Info for Windows software (version 7.26; ©Centers for Disease Control and Prevention, Atlanta, USA) was used for univariate analysis. Dichotomous variables were compared using nonparametric tests of proportion, χ^2 test, and Fisher's exact test. Numerical variables were compared by the Mann-Whitney U test.

Multivariate analysis was performed with the SPSS 20.0 software (©SPSS, Inc.) using a logistic regression model. The outcomes of interest were the overall presence of *S. aureus* or the presence of MRSA irrespective of sampling site. The variables were selected using a backward stepwise strategy. The criterion for entry and permanence of the variables in the models was $p < 0.1$. Final statistical significance was set at $p < 0.05$.

3. Results

3.1 Prevalence of *Staphylococcus aureus* and MRSA carriage

Ninety-five of the 312 subjects included in the study were colonized with *S. aureus*; 15 were colonized with MRSA either in the nose or oropharynx. The overall prevalence of *S. aureus* was 30.4% (95%CI = 25.6%-35.8%) and that of MRSA was 4.8% (95%CI = 2.9%-7.8%). Regarding the sampling site, *S. aureus* was isolated exclusively from the nose in 44 (14.1%) individuals, exclusively from the oropharynx in 34 (10.9%), and from both sites in 17 (5.4%). Analysis of MRSA demonstrated that seven of the 15 colonized individuals carried the microorganism exclusively in the nose and six exclusively in the oropharynx. Interestingly, one subject carried MSSA in the oral mucosa and MRSA in the nasal mucosa (Fig. 1).

Note

Flow chart of the total number of individuals colonized with *Staphylococcus aureus* and of individuals colonized with MSSA and MRSA according to sampling site. MSSA = methicillin-susceptible *S. aureus*; MRSA = methicillin-resistant *S. aureus*. * Overall prevalence of *S. aureus*. ** Prevalence of MRSA.

Figure 1. Flow chart of the total number of individuals colonized with *Staphylococcus aureus* and number of individuals colonized with MSSA and MRSA according to sampling site.

3.2 Determination of *in vitro* antimicrobial susceptibility

All 112 isolates identified as *S. aureus* were submitted to *in vitro* antimicrobial susceptibility testing as previously described. The disc diffusion method revealed 11 strains that were resistant to oxacillin and cefoxitin, including seven resistants to both drugs and four that were resistant only to cefoxitin (inhibition zone ≤ 21). It should be noted that four of the isolates that were susceptible to both drugs exhibited resistance by the genotypic method.

Among all isolates, only one was resistant to sulfamethoxazole/trimethoprim and there was no case of resistance to quinupristin/dalfopristin or linezolid. In addition, the MIC₅₀ and MIC₉₀ for vancomycin were 0.50 and 1.0 $\mu\text{g/mL}$, respectively, and all isolates were susceptible (Supplementary Material Table S1).

3.3 Detection of the *mecA* gene and characterization of SCC*mec*

Fifteen of the 112 *S. aureus* isolates carried the *mecA* gene of methicillin resistance. There was a predominance of the SCC*mec* type IV among isolates ($n = 10$), but three isolates harboring SCC*mec* type I and two harboring SCC*mec* type II were also identified. Most individuals harboring MRSA isolates reported no contact with the hospital environment in the last year, except for one subject with SCC*mec* type II and one with SCC*mec* type IV.

3.4 Risk factors for *S. aureus* and MRSA carriage

The results of univariate and multivariate (logistic regression model) analysis to identify risk factors for *S. aureus* and MRSA carriage are shown in Table 1 and 2. With respect to the study of risk factors for *S. aureus* carriage, univariate analysis showed a negative association with age (median 61years, $p = 0.04$), heart disease ($p = 0.03$) and lung disease ($p = 0.02$), and a positive association only with the presence of lower-extremity ulcers ($p = 0.02$). However, the only factors that remained independently associated with *S. aureus* carriage in insulin-dependent diabetic individuals were age (OR = 0.98, 95% CI = 0.93–0.99, $p = 0.02$) and lung disease (OR = 0.31, 95% CI = 0.10–0.92, $p = 0.03$), which were negatively associated. A positive association was only observed with lower-extremity ulcers (OR = 2.44, 95% CI = 1.11–5.34, $p = 0.03$).

Table 1

Uni- and multivariate (logistic regression) analysis of predictors of *Staphylococcus aureus* carriage in diabetic individuals.

Factor	<i>S. aureus</i> (n = 95)	Negative (n = 217)	Univariate analysis		Logistic regression (multivariate)	
			OR (95%CI)	p	OR (95%CI)	p
Male gender	42 (44.2)	98 (45.2)	0.96 (0.59–1.56)	0.88		
Age (median, quartiles)	61 (49–70)	65 (56–71)	-	0.04	0.98 (0.93–0.99)	0.02
Time since diagnosis, years (median, quartiles)	14 (9–20)	16.5 (8–25)	-	0.84		
Use of insulin, years (median, quartiles)	8 (4–14)	7 (3–11)	-	0.22		
Diabetes type 2	78 (82.1)	184 (84.8)	0.82 (0.43–1.56)	0.35		
Heart disease	16 (17.4)	61 (29.0)	0.51 (0.28–0.95)	0.03		
Lung disease	4 (4.3)	29 (13.8)	0.28 (0.10–0.83)	0.02	0.31 (0.10–0.92)	0.03
Kidney disease	23 (25.0)	59 (28.1)	0.85 (0.49–1.49)	0.58		
Liver disease	9 (9.8)	18 (8.6)	1.16 (0.50–2.68)	0.73		
CNS disease	19 (20.7)	31 (14.8)	1.50 (0.80–2.82)	0.20		
Cancer	11 (11.8)	28 (13.1)	0.89 (0.42–1.87)	0.75		
Trauma	8 (8.7)	23 (11.0)	0.77 (0.33–1.80)	0.55		

Note. All values are reported as number (%) unless otherwise specified. Significant associations are indicated in bold. OR = odds ratio; CI = confidence interval; CNS = central nervous system.

Factor	<i>S. aureus</i> (n = 95)	Negative (n = 217)	Univariate analysis		Logistic regression (multivariate)	
			OR (95%CI)	p	OR (95%CI)	p
Tattoo	4 (4.3)	12 (5.7)	0.75 (0.23–2.39)	0.78		
Lower-extremity ulcers	15 (16.3)	16 (7.6)	2.36 (1.11–5.01)	0.02	2.44 (1.11–5.34)	0.03
Amputation	2 (2.2)	8 (3.8)	0.56 (0.18–2.70)	0.73		
Charlson comorbidity index \geq 1	3 (2–4)	3 (2–4)	-	0.84		
Hospitalization in the last year	11 (12.0)	37 (17.6)	0.63 (0.31–1.31)	0.21		
Surgery in the last year	10 (10.9)	22 (10.5)	1.04 (0.47–2.30)	0.91		
Antimicrobial use in the last year	21 (22.8)	57 (27.3)	0.79 (0.44–1.40)	0.42		
Note. All values are reported as number (%) unless otherwise specified. Significant associations are indicated in bold. OR = odds ratio; CI = confidence interval; CNS = central nervous system.						

The study of risk factors for MRSA carriage revealed a positive association only with male gender in uni- and multivariate analysis (OR = 3.64, 95% CI = 1.12–11.78, $p = 0.03$).

Table 2

Uni- and multivariate (logistic regression) analysis of predictors of MRSA carriage in diabetic individuals.

Factor	MRSA (n = 15)	Negative (n = 287)	Univariate analysis		Logistic regression (multivariate)	
			OR (95%CI)	p	OR (95%CI)	p
Male gender	11 (73.3)	129 (43.3)	3.58 (1.11– 11.51)	0.02	3.64 (1.12– 11.78)	0,03
Age (median, quartiles)	58 (46– 73)	63 (54– 71)	-	0.27		
Time since diagnosis, years (median, quartiles)	13 (10– 19)	15 (9– 24)	-	0.56		
Use of insulin, years (median, quartiles)	8 (3– 15)	7 (3–12)	-	0.94		
Diabetes type 2	11 (73.3)	251 (84.5)	0.50 (0.15– 1.65)	0.27		
Heart disease	3 (20.0)	74 (25.8)	0.72 (0.20– 2.62)	0.77		
Lung disease	0	33 (11.5)	-	0.39		
Kidney disease	4 (26.7)	78 (27.2)	0.97 (0.30– 3.15)	1.00		
Liver disease	1 (6.7)	26 (9.1)	0.72 (0.09– 5.67)	1.00		
CNS disease	1 (6.7)	49 (17.1)	0.35 (0.04– 2.70)	0.48		
Cancer	2 (13.3)	37 (12.7)	1.06 (0.30– 4.87)	1.00		
Trauma	0	31 (10.8)	-	0.38		

Note. All values are reported as number (%) unless otherwise specified. Significant associations are indicated in bold. OR = odds ratio; CI = confidence interval; CNS = central nervous system.

Factor	MRSA (n = 15)	Negative (n = 287)	Univariate analysis		Logistic regression (multivariate)	
			OR (95%CI)	p	OR (95%CI)	p
Tattoo	1 (6.7)	15 (5.2)	1.29 (0.16– 10.52)	0.57		
Lower-extremity ulcers	3 (20.0)	28 (9.8)	2.31 (0.61– 8.69)	0.19		
Amputation	1 (6.7)	9 (3.1)	2.21 (0.26– 18.65)	0.40		
Charlson comorbidity index ≥ 1	3 (2– 4)	3 (2–4)	-	0.41		
Hospitalization in the last year	2 (13.3)	46 (16.0)	0.81 (0.18– 3.69)	1.00		
Surgery in the last year	3 (20.0)	29 (10.1)	2.22 (0.59– 8.34)	0.20		
Antimicrobial use in the last year	4 (26.7)	74 (25.9)	1.04 (0.32– 3.37)	1.00		

Note. All values are reported as number (%) unless otherwise specified. Significant associations are indicated in bold. OR = odds ratio; CI = confidence interval; CNS = central nervous system.

3.5 Determination of the clonal profile of *S. aureus* and MRSA by pulsed field gel electrophoresis (PFGE)

A total of 112 *S. aureus* isolates were analyzed by PFGE. Forty-five of them could not be typed repeatedly with *SmaI*, including one MRSA isolate. However, molecular typing of all 45 isolates was possible using the *ApaI* enzyme.

For clonal profile analysis, one dendrogram was constructed for susceptible *S. aureus* isolates (MSSA) and one for resistant isolates (MRSA) using *SmaI* and *ApaI*, which permitted to identify clusters with similarity $\geq 80\%$ in both groups.

Figure 2 shows the dendrogram of the PFGE-*SmaI* and PFGE-*ApaI* profiles of MRSA isolates, including the *in vitro* susceptibility profile to oxacillin and ceftazidime, presence of the *mecA* gene, and SCC*mec* type. Analysis of PFGE-*SmaI* isolates revealed the presence of two clusters (A and B). Cluster A contained five isolates, four of them showing 100% similarity (554O, 555N, 659N, and 665N). All of them were isolated from different individuals and harbored SCC*mec* type IV. Cluster B contained three strains, two of them showing 100% similarity (72O and 615N). The MRSA isolate typed with *ApaI* is shown in Fig. 2 (b). None of the isolates grouped with international clones.

Note

a. Isolates showing > 80% similarity (clusters A and B) after digestion with *SmaI*. b. Band pattern of strain 735N obtained by digestion with *ApaI*. N = nasal mucosa; O = oropharyngeal mucosa; S = susceptible; R = resistant. * International clones used as controls.

Figure 2. Dendrogram generated by Dice analysis/UPGMA (BioNumerics, Applied Maths) of the PFGE *SmaI* and PFGE *ApaI* profiles of MRSA isolated from insulin-dependent diabetic individuals.

Two clusters (A and C) containing four strains and two clusters (B and D) containing strains were obtained for the MSSA isolates (Supplementary Material Figure S1). Cluster A contained two isolates from the nasal and oropharyngeal mucosa of the same subject, demonstrating colonization of different sites with the same isolate. Similarly, in cluster B, two isolates from the nasal and oropharyngeal mucosa of the same subject were grouped with one nasal isolate of another subject. On the other hand, the four isolates of cluster C were obtained from unrelated individuals and in cluster D all isolates were from the oral mucosa of different subjects. These findings suggest widespread dissemination of *S. aureus* in the community.

Four (26.7%) of the 17 subjects colonized at both sampling sites (nasal/oropharyngeal) carried the same isolate in the nasal and oral mucosa, while five (33.4%) carried different *S. aureus* strains in the nose and throat. Interestingly, two patients concomitantly colonized with *S. aureus* in the nose and throat had their strains (nasal and oropharyngeal) typed after digestion with different restriction enzymes. Isolates 691O and 747N were typed with *SmaI*, while 691N and 747O could only be typed with *ApaI*, confirming that they are different *S. aureus* strains.

Analysis of typable isolates with *ApaI* revealed three major clusters (A, C, and D) and three minor clusters of 3 isolates (B, E, and F). In cluster A which contained 14 isolates with 85.6% similarity, two isolates were from the nasal and oropharyngeal mucosa of the same patient and the remaining from different subjects. The same was observed for clusters C and D. In cluster D, the isolates were grouped with a strain previously identified in another study as ST398 (strain 76N). These findings are shown in Supplementary Material Figure S2.

3.6 Molecular typing of *S. aureus* and MRSA by multilocus sequence typing (MLST)

Based on the clusters obtained by PFGE, nine *S. aureus* isolates (four MSSA and five MRSA) were selected for molecular typing by the MLST technique. Typing of the MRSA isolates revealed a predominance of sequence type ST5 in three of the four isolates analyzed and one isolate with ST8. In addition, the strain typed with *ApaI* by PFGE was characterized as ST398 (Fig. 3).

Note

a. Resistant isolates showing > 80% similarity (clusters A and B) by digestion with *SmaI*. b. Band pattern of strain 735N typable with *ApaI*. N = nasal mucosa; O = oropharyngeal mucosa; * International clones used as control. *arcC* = carbamate kinase; *aroE* = shikimate dehydrogenase; *glpF* = glycerol kinase; *gmk* =

guanylate kinase; *pta* = phosphate acetyltransferase; *tpi* = triosephosphate isomerase; *yqjL* = acetyl coenzyme A; ST = sequence type.

Figure 3. Dendrogram generated by Dice analysis/UPGMA (BioNumerics, Applied Maths) of the PFGE-*SmaI* and PFGE-*ApaI* profiles of MRSA isolates and sequence types obtained by MLST.

Among the clusters obtained by PFGE of the MSSA isolates that could not be typed with *SmaI* and were digested with *ApaI*, four lineages were selected for MLST. There was a predominance of ST398 (n = 3). However, one isolate exhibited divergence in the allele of the *arcC* gene and was sent to the curator of the MLST database (<http://www.mlst.net>) for identification of the ST. This isolate was identified with ST 6133 (Fig. 4).

Note

Clustering of isolates digested with *ApaI* that were analyzed by MLST. All isolates except for 735N were susceptible to methicillin (MSSA). N = nasal mucosa; O = oropharyngeal mucosa; *arcC* = carbamate kinase; *aroE* = shikimate dehydrogenase; *glpF* = glycerol kinase; *gmk* = guanylate kinase; *pta* = phosphate acetyltransferase; *tpi* = triosephosphate isomerase; *yqjL* = acetyl coenzyme A; ST = sequence type. Isolate 76N was identified as ST398 in a previous study from our group. Isolate 7000 was sent to the curator of the MLST database (<http://www.mlst.net>) for identification of the new ST. This isolate was identified with ST 6133.

Figure 4. Dendrogram generated by Dice analysis/UPGMA (BioNumerics, Applied Maths) of the PFGE *ApaI* profiles of MSSA isolates and sequence types obtained by MLST.

4. Discussion

Bacterial resistance has become a great global threat. *Staphylococcus aureus* plays a particularly important role in this scenario by causing infections that range from superficial to severe and potentially fatal systemic infections, in addition to its ability to acquire resistance to multiple drugs. In the past, the pathogen was mainly found in hospital environments, but we are now witnessing an increase particularly in resistant isolates (MRSA) acquired in the community that are genetically different from traditional nosocomial strains [19–21]. Furthermore, studies suggest that staphylococcal infections are preceded by the process of colonization [2, 22–26] and at least one third of colonized healthy adults are at risk of developing subsequent invasive infections [19, 20].

Diabetic individuals are considered a risk group for skin infections such as those caused by *S. aureus* and are also more prone to developing severe systemic infections [26]. Studies have shown that the prevalence of nasal colonization with *S. aureus* (27%-56.6%) and MRSA (1%-7.3%) varies according to geographic location and that the use of insulin is a risk factor for nasal MRSA carriage in diabetic individuals [7].

The prevalence of colonization of *S. aureus* and MRSA in insulin-dependent diabetic individuals was 30.4% and 4.8%, respectively. Our results differ from those reported by Kutlu et al. [27], who analyzed 304 patients and found a prevalence of 41.9% for *S. aureus* and of 9.9% for MRSA among diabetic individuals. Alizargar et al. [7] also obtained a higher prevalence of *S. aureus* (42.5%) and MRSA (24.7%). In a population-based survey conducted in the same city, Pires et al. [29] found a similar prevalence among community-dwelling individuals, with an overall prevalence of *S. aureus* of 32.7%. However, the prevalence of MRSA was higher in our study (4.6% vs 0.9%). In a recent study, Lin et al. [2] found a lower prevalence of *S. aureus* and MRSA than that obtained in the present study (16.4% of *S. aureus* and 2.8% MRSA). The authors suggested that the presence of different microorganisms in the microbiota of these individuals causes competition for the same site, which could explain the variation found in prevalence studies. None of the studies included oral mucosa as a potential site of colonization.

It should be noted that 10.9% (n = 34) of the subjects were colonized exclusively in the oral mucosa, six of them with MRSA. This finding reinforces what was reported of Partida et al. [29] that colonization of the oral mucosa can compromise control measures of pathogen dissemination since the throat is not part of routine screening.

The MRSA isolates were identified by phenotypic methods (disc diffusion) and by PCR for detection of the *mecA* gene. The latter is considered the gold standard for detecting methicillin-resistant isolates. The CLSI [13] recommends the use of the ceftioxin disc for the detection of methicillin resistance because of its greater sensitivity in identifying isolates carrying the *mecA* gene. The results of the present study confirm the greater sensitivity of the ceftioxin disc compared to oxacillin, with only seven of the 15 isolates being identified as resistant when the oxacillin disc was used, while 11 were resistant to ceftioxin. However, we found four isolates carrying the *mecA* gene that did not exhibit phenotypic resistance to ceftioxin or oxacillin.

The most common agent isolated from infections in diabetic individuals is *S. aureus*, with a high prevalence of MRSA [30]. In a recent study, Chaudhry et al. [6] investigated clinical isolates and observed that 84% of the *S. aureus* isolates were MRSA and 20% were resistant to vancomycin. In our study, the prevalence of MRSA was lower and none of the isolates was resistant to vancomycin, although one MRSA isolate had a MIC of 1.5 µg/mL, indicating a potential therapeutic risk [31–33]. Two other MSSA isolates had a vancomycin MIC of 1.5 µg/mL.

In the present study, the analysis of risk factors revealed a positive association only with lower-extremity ulcers, which is consistent with literature findings showing that the same isolate colonizing the nares was found in foot ulcers and wounds [34, 35]. Age was negatively associated with *S. aureus* colonization, with an odds ratio of 0.98, indicating a reduction of 2% in the risk of colonization for each additional year of age. Similar data have been reported by Pereira-Franchi et al. [36] Lung disease was also negatively associated with *S. aureus* colonization, a fact that might be related to colonization of the respiratory tract by other microorganisms that are competing with *S. aureus*. Mueller et al. [37] also found a negative

association with age but, in contrast to our findings, lung disease was a risk factor for *S. aureus* colonization.

Multivariate analysis identified male gender as a risk factor for colonization with MRSA. These results are in contrast with those reported by Pereira-Franchi et al. [36] who found no risk factors for MRSA colonization.

With respect to clonality of the isolates, one important MRSA clone was detected in the community insulin-dependent diabetic individuals studied. Five of the 15 diabetic individuals with MRSA carried isolates that belonged to the same cluster (A), all of them harboring SCC*mec* type IV. It is important to emphasize that isolates 6390 and 659N present in this cluster harbor ST5, possibly reflecting a common origin. There was also a smaller cluster (B) with three isolates obtained from three different subjects, one of nasal origin (615N-ST5-SCC*mec* I) and two of oral origin. One of them (720) harbored ST8 and SCC*mec* type IV. This fact reinforces the importance of throat colonization, which could be a route of transmission within the population studied. Other studies involving individuals from the same city and region also found MRSA ST5-SCC*mec* type IV and ST8-SCC*mec* type IV, suggesting that these strains are prevalent in the region [28, 38–43].

Studies suggest a high clonal diversity among *S. aureus* isolates, particularly among MSSA [44]. We observed the formation of only four clusters among the MSSA isolates, but several isolates from the same individual were grouped together, with the identification of five individuals with different *S. aureus* in the nose and throat. There was a large number of isolates that showed similarity with the ST398 clonal lineage. Isolates of this lineage are not digested with *Sma*I because of a yet unknown restriction/DNA methylation system in the genus *Staphylococcus* spp. However, these strains can be digested with the enzyme *Apa*I [45, 46]. Forty-five isolates that could not be typed by conventional PFGE (restriction with *Sma*I) were identified, which formed three major clusters and four clusters with a smaller number of strains after digestion with the restriction enzyme *Apa*I. Among the 45 isolates, four were typed by MLST and were characterized as ST398. This fact was also observed by Souza et al [42].

The ST398 clonal lineage has been associated with infection and colonization of humans and domestic animals, such as dogs, horses and pigs, in many countries around the world [21]. This lineage is called livestock-associated *S. aureus* and was described for the first time among both MSSA and MRSA on pig farms in France [45, 46]. Since then, ST398 has spread rapidly to other animals and has been increasingly related to infections not only in rural workers but also in people and animals without risk factors [13, 47]. Although susceptible to oxacillin, this *S. aureus* lineage is associated with severe infections, as reported by Bonesso et al. [48] in patients with ventilator-associated pneumonia in whom the infection was fatal in most cases.

Our finding demonstrated a predominance of SCC*mec* type IV among isolates, in agreement with the findings of other prevalence studies on non-diabetic individuals conducted in the State of São Paulo [38, 42]. However, SCC*mec* types I and II were also detected, which are commonly found circulating in health services. The presence of SCC*mec* type II has also been reported by Pereira-Franchi et al. [36].

The present study provides important data about the epidemiology of *S. aureus* and MRSA in a population of insulin-dependent diabetic individuals. The prevalence found is lower than that reported in the international literature, probably because the individuals were living in the community, most of them rarely visiting the hospital, and were generally in good health. A higher prevalence of MRSA colonization was observed in the population studied. Within this context, screening for oral colonization is extremely important since some individuals were colonized only at this body site.

The isolates analyzed had a low rate of resistance to the tested drugs, with only one isolate being resistant to sulfamethoxazole-trimethoprim; however, the prevalence of MRSA was higher than that found in a population-based study conducted in the same city on healthy individuals [16]. In the population studied here, clones were detected among the MSSA and MRSA isolates and an important clonal lineage (ST398) was identified. These data suggest widespread dissemination of MRSA in the insulin-dependent diabetic population studied, as well as the emergence of important *S. aureus* lineages in these individuals.

5. Declarations

5.1 Ethical approval and consent to participate

The study was approved by the Ethics Committee of the Botucatu Medical School, Brazil (Approval No. 1.107.685). All data were obtained by interview with the patient and/or legal representative following ethical standards, by signing the consent form.

5.2 Consent for publication

Not Applicable.

5.3 Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

5.4 Competing interests

The author(s) declare no competing interests.

5.5 Acknowledgements and Funding

This work was funded by the State funding agency São Paulo Research Foundation (FAPESP - Grant 2017/21396-0), Coordination for Improvement of Higher Education Personnel (CAPES) through the Master/Doctorate Grant program and the National Council for Scientific and Technological Development (CNPq; grant 304051/2017-9).

5.6 Contributions

NBT and M.L.R.S.C designed the study and wrote the manuscript. N.B.T. and M.C.S. performed the experiments. N.B.T., T.A.M.P and B.P.C.C. collected the samples. C.M.C.B.F contributed the study design and performed the statistical analysis. All authors wrote and agreed the final version.

References

1. WHO guideline. Global report on diabetes. Geneva: WHO.2016. 88 p.
2. Lin SY, Lin NY, Huan YY, Hsieh CC, Huang YC. Methicillin-resistant *Staphylococcus aureus* nasal carriage and infection among patients with diabetic foot ulcer. J Microbiol Immun Inf. 2018;xx:1–8.
3. Cho NH, et al. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. Diab Resear Clin Prat. 2018;138:271–81.
4. Trivedi U, et al. Prevalence of Multiple Antibiotic Resistant Infections in Diabetic versus Nondiabetic Wounds. J Pathog. 2014; 1–6.
5. Enoch S, et al. Randomized clinical trial and economic analysis of four-layer compression bandaging for venous ulcers. Brit J of Surg. 2003;90(7):794–8.
6. Chaudhry WN, et al. Clinico-microbiological study and antibiotic resistance profile of *mecA* and ESBL gene prevalence in patients with diabetic foot infections. Experim Therap Med. 2016;11:1031–8.
7. Alizargar J, Sharif M, Sharif A. Risk factors of methicillin-resistant *Staphylococcus aureus* colonization in diabetic outpatients. A prospective cohort study. Int J Microbiol Res. 2013;4:147–51.
8. Instituto Brasileiro de Geografia e Estatística. Panorama do município
Instituto Brasileiro de Geografia e Estatística. Panorama do município. de Botucatu SP. 2019.
<https://www.ibge.gov.br/cidades-e-estados/sp/botucatu.html>. Accessed July 6, 2019.
9. Konemann EW, Allen SD, Dowell VR, Sommer HM. Introdução à microbiologia médica. Diagnóstico microbiológico: texto e Atlas colorido. 5th ed. Rio de Janeiro: Medsi; 2001.
10. Cunha MLRS, Sinzato YK, Silveira LV. Comparison of methods for the identification of coagulase-negative *Staphylococci*. Mem Inst Oswaldo Cruz. 2004;99(8):855–60.
11. Mason WJ, et al. Multiplex PCR protocol for the diagnosis of staphylococcal infection. J Clin Microbiol. 2001;39(9):3332–8.
12. Martineau F, Picard FJ, Roy PH, Ouellette M, Bergeron MG. Species-specific and ubiquitous-DNA-based assays for rapid identification of *Staphylococcus aureus*. J Clin Microbiol. 1998;36(3):618–23.
13. CLSI. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard— 26th Edition (M100S). CLSI, 2016.
14. Murakami K, Minamide W, Wada K, Nakamura E, Teraoka H, Watanabe S. Identification of methicillin-resistant strains of *Staphylococci* by polymerase chain reaction. J Clin Microbiol. 1991;29(10):2240–4.
15. Oliveira DC, de Lencastre H. Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. Antimicrob Ag Chem.

- 2002;46(7):2155–61.
16. Milheiriço C, Oliveira DC, de Lencastre H. Update to the multiplex PCR strategy for assignment of *mec* element types in *Staphylococcus aureus*. *Antimicrob Ag Chem*. 2007;51(9):3374–7.
 17. McDougal LK, et al. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. *J Clin Microbiol*. 2003;41(11):5113–20.
 18. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol*. 2000;38(3):1008–15.
 19. Hewagama S, et al. The Epidemiology of *Staphylococcus aureus* and Panton-Valentine Leucocidin (*pvf*) in Central Australia, 2006–2010. *BMC Infect Dis*. <http://dx.doi.org/10.1186/s12879-016-1698-5>. Accessed December 15, 2016.
 20. Park SY, et al. Sequence type 72 community-associated methicillin-resistant *Staphylococcus aureus* emerged as a predominant clone of nasal colonization in newly admitted patients. *J Hosp Infect*. 2016;93:386–9.
 21. Camoez M, et al. Prevalence and Molecular Characterization of methicillin-resistant *Staphylococcus aureus* ST398 resistant to tetracycline at a Spanish hospital over 12 years. *Plos One*. 2013;8(9):1–6.
 22. Kalmeijer MD, et al. Surgical site infections in orthopedic surgery: the effect of mupirocin nasal ointment in a double-blind, randomized, placebo-controlled study. *Clin Infect Dis*. 2002;35:353–8.
 23. Eiff C, Becker K, Machka K, Stammer H, Peters G. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. Study group. *N Engl J Med*. 2001;344:11–6.
 24. Stanaway S, Johnson D, Moulik P, Gill G. Methicillin-resistant *Staphylococcus aureus* (MRSA) isolation from diabetic foot ulcers correlates with nasal MRSA carriage. *Diabetes Res Clin Pract*. 2007;75:47–50.
 25. Haleem A, et al. Concordance of nasal and diabetic foot ulcer staphylococcal colonization. *Diagn Microbiol Infect Dis*. 2014;79:85–9.
 26. Shah BR, Hux JE. Quantifying the risk of infectious diseases for people with diabetes. *Diabetes Care*. 2003;26:510–3.
 27. Kutlu SS, et al. Prevalence and risk factors for methicillin-resistant *Staphylococcus aureus* colonization in a diabetic outpatient population: a prospective cohort study. *Am J Infect Control*. 2012;40:365–8.
 28. Pires FV, Cunha MLRS, Abraão LM, Martins PY, Camargo CH, Fortaleza CMCB. Nasal carriage of *Staphylococcus aureus* in Botucatu, Brazil: a population-based survey. *PLoS One*. 2014;9:925–37.
 29. Partida AH, Espunes TS, Martínez JB. Characterization and persistence of *Staphylococcus aureus* strains isolated from the anterior nares and throats of healthy carriers in a Mexican community. *J Clin Microbiol*. 2010;48(5):1701–5.

30. Vandendriessche S, et al. Previous healthcare exposure is the main antecedent for methicillin-resistant *Staphylococcus aureus* carriage on hospital admission in Belgium. *Eur J Clin Microbiol Infect Dis*. 2012; 2283–2292.
31. Soriano A, et al. Influence of vancomycin minimum inhibitory concentration on the treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. *Clin Infect Dis*. 2008;46:193–200.
32. Howden BP, et al. Treatment outcomes for serious infections caused by methicillin-resistant *Staphylococcus aureus* with reduced vancomycin susceptibility. *Clin Infect Dis*. 2004;38:521–8.
33. Yeg YC, et al. Impact of vancomycin MIC creep on patients with methicillin-resistant *Staphylococcus aureus* bacteremia. *J Microbiol Immunol Inf*. 2012;45:214–20.
34. Richard JL, Sotto A, Lavigne JP. New insights in diabetic foot infection. *World J Diabetes*. 2011;2:24–32.
35. Taha AB. Relationship and susceptibility profile of *Staphylococcus aureus* infection diabetic foot ulcers with *Staphylococcus aureus* nasal carriage. *Foot (Edinb)*. 2013;23:11–6.
36. Pereira-Franchi EP, Barreira MRN, da Costa NSLM, Fortaleza CMCB, Cunha MLRS. Prevalence of and risk factor associated with the presence of *Staphylococcus aureus* in the chronic wounds of patients treated in primary health care settings in Brazil. *Ver Soc Bras Med Trop*. 2017;50(6):833–8.
37. Muller LMAJ, et al. Increased risk of common infections in patients with type 1 and type 2 diabetes mellitus. *Clin Infect Dis*. 2005;41:281–8.
38. Silveira M. Prevalência e fatores de risco para carreamento de *Staphylococcus aureus* resistente à metilina em idosos institucionalizados na cidade de Bauru-SP. Dissertação (Mestrado). Programa de Pós-Graduação em Doenças Tropicais, 2013.
39. Witzel CL, Fortaleza CM, de Souza CS, Riboli DF, Cunha MLRS. Nasopharyngeal carriage of *Staphylococcus aureus* among imprisoned males from Brazil without exposure to healthcare: risk factors and molecular characterization. *Ann Clin Microbiol Antimicrob*. 2014;13:1–6.
40. Lastoria LC. Colonização por *Staphylococcus aureus* em pessoas vivendo com HIV/AIDS acompanhadas em um serviço ambulatorial de referência em Botucatu (SP): prevalência, resistência à metilina e epidemiologia molecular. Dissertação (Mestrado). Programa de Pós-Graduação em Doenças Tropicais, 2016.
41. Abraão LM. Carreamento nasal/oral de *Staphylococcus aureus* em populações indígenas do norte e sudeste do Brasil: resistência antimicrobiana, virulência, fatores de risco e epidemiologia molecular. Tese (Doutorado). Programa de Pós-Graduação em Doenças Tropicais, 2017.
42. Souza CSM. Determinação da relação clonal e virulência de *Staphylococcus aureus* isolados de pacientes vivendo com HIV/AIDS e seus familiares. Tese (Doutorado). Programa de Pós-Graduação em Doenças Tropicais, 2018.
43. Pereira-Franchi, et al. Molecular epidemiology of MRSA in the Brazilian primary health care system. *Trop Med Intern Heal*. 2018. doi:10.1111/tmi.13192.
44. Robinson DA, Enright MC. Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chem*. 2003;47(12):3926–34.

45. Bens CCPM, Voss A, Klaassen CHW. Presence of a novel DNA methylation enzyme in methicillin-resistant *Staphylococcus aureus* isolates associated with pig farming leads to uninterpretable results in standard pulsed-field gel electrophoresis analysis. J Clin Microbiol. 2006;44(5):1875–6.
46. Witte W, Strommenger B, Stanek C, Cuny C. Methicillin-resistant *Staphylococcus aureus* ST398 in humans and animals, Central Europe. Emerg Infect Dis. 2007;13(2):255–8.
47. Chung M, et al. Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. Microb Drug Resist. 2000;6:189–98.
48. Bonesso MF, et al. Key role of α -toxin in fatal pneumonia caused by sequence type 398. Amer J Respirat Critl Care Med. 2016;193:217–20.

Figures

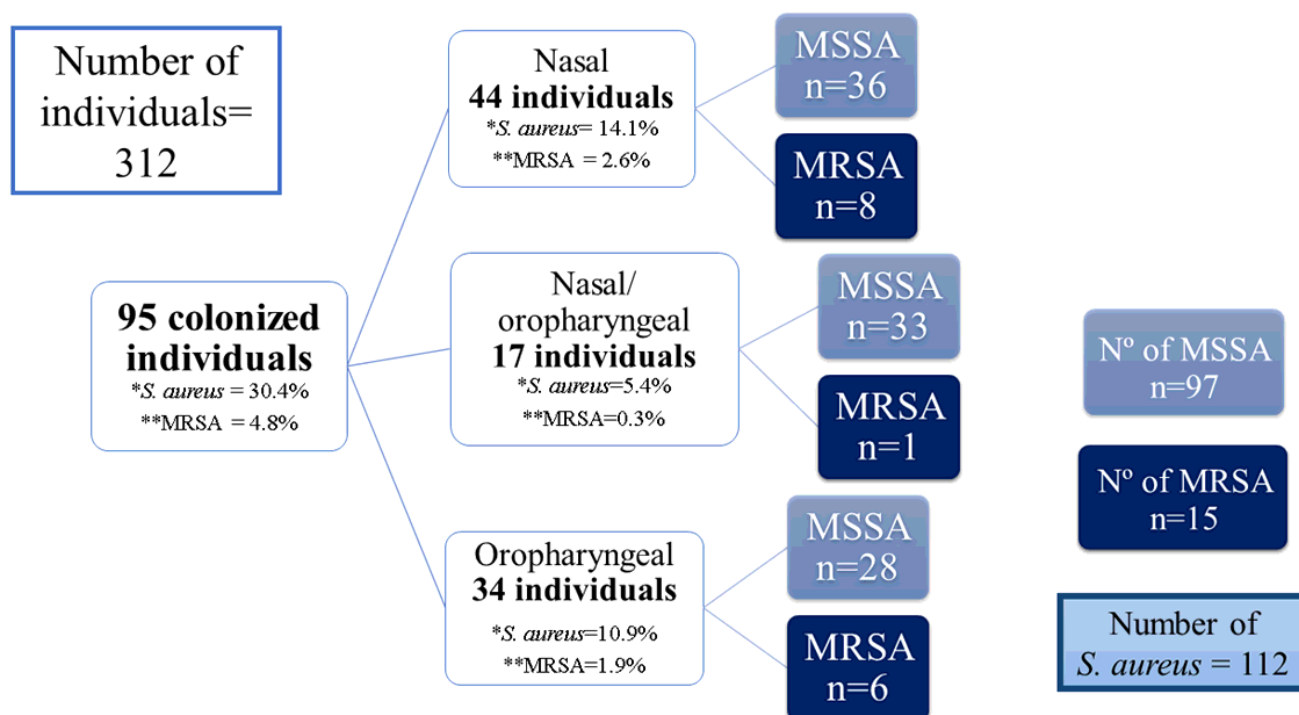


Figure 1

Flow chart of the total number of individuals colonized with *Staphylococcus aureus* and number of individuals colonized with MSSA and MRSA according to sampling site.

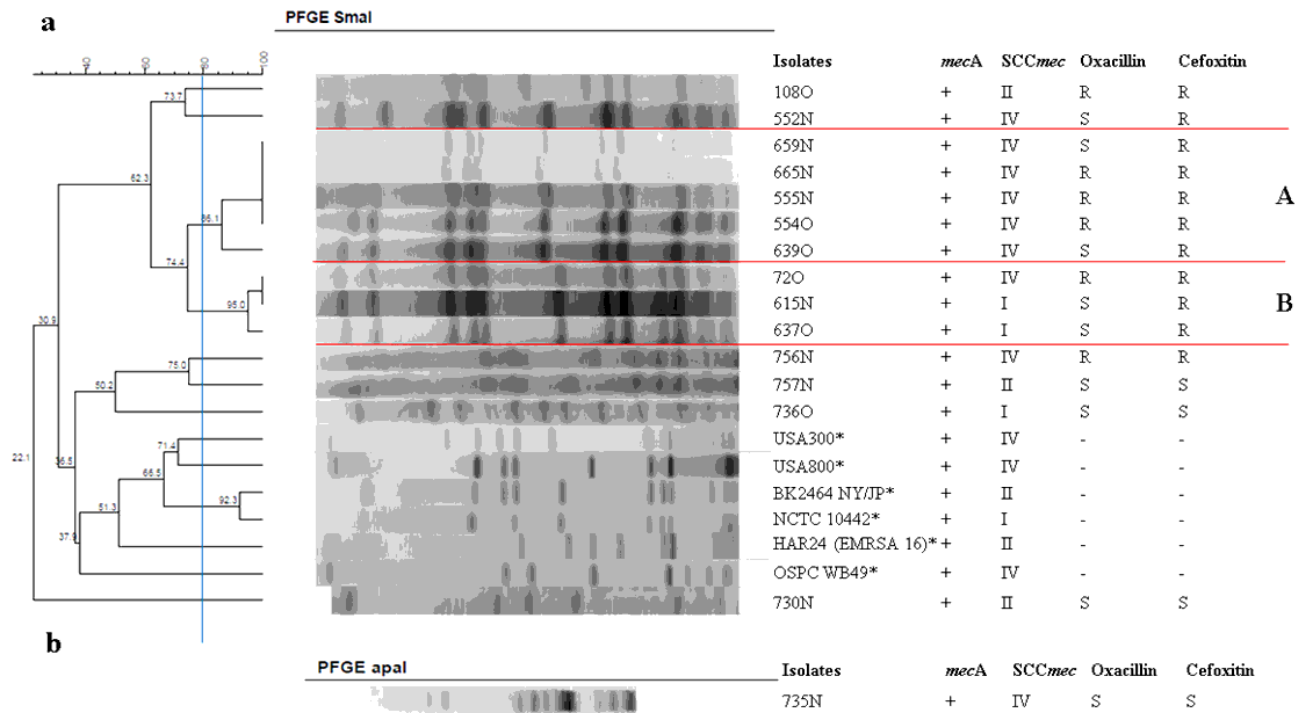


Figure 2

Dendrogram generated by Dice analysis/UPGMA (BioNumerics, Applied Maths) of the PFGE SmaI and PFGE ApaI profiles of MRSA isolated from insulin-dependent diabetic individuals.

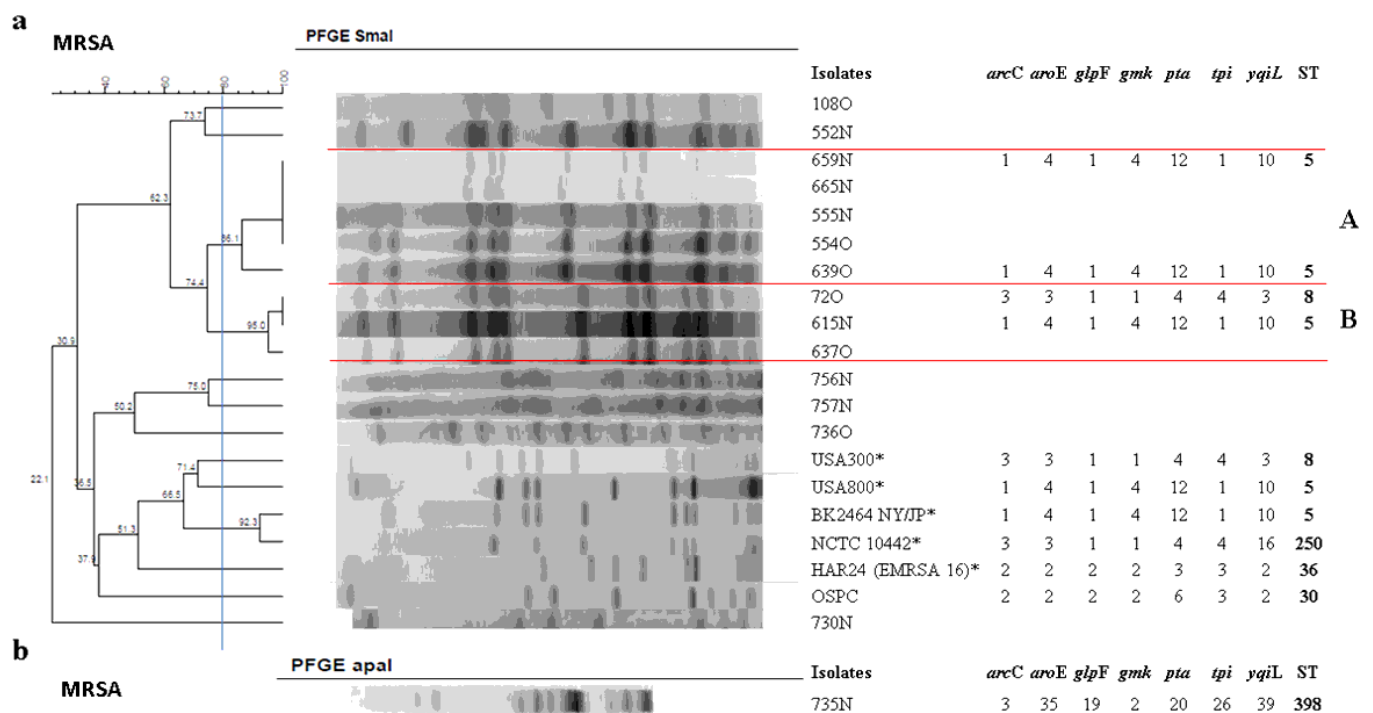


Figure 3

Dendrogram generated by Dice analysis/UPGMA (BioNumerics, Applied Maths) of the PFGE-SmaI and PFGE-ApaI profiles of MRSA isolates and sequence types obtained by MLST.

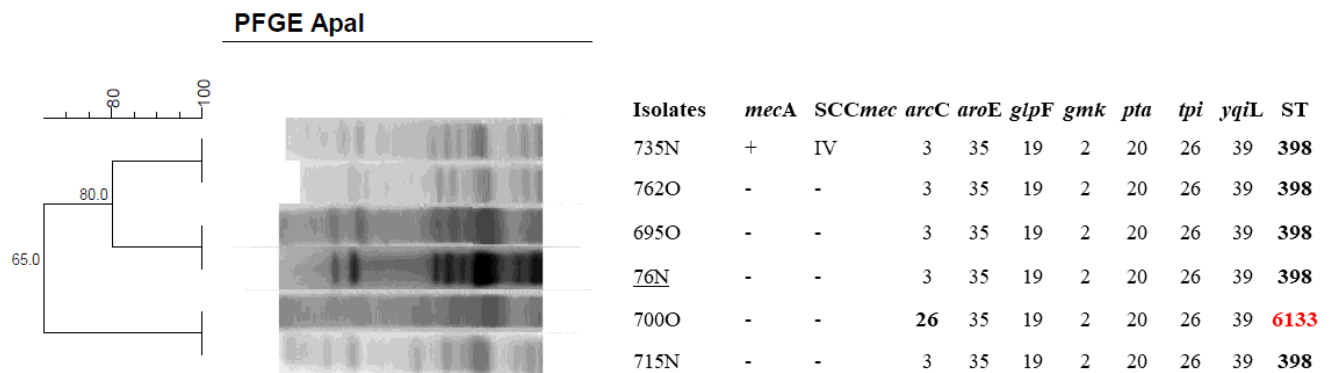


Figure 4

Dendrogram generated by Dice analysis/UPGMA (BioNumerics, Applied Maths) of the PFGE ApaI profiles of MSSA isolates and sequence types obtained by MLST.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterialfigureandtable.docx](#)