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Isoenzyme genotyping and phylogenetic analysis of oxacillin-resistance *Staphylococcus aureus* isolates

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Aim: The propagation of *S. aureus* in hospital and dental environments is considered an important public health problem since resistant strains can cause serious infections in humans. The genetic variability of 99 oxacillin-resistant *S. aureus* isolates (ORSA) from the dental patients (oral cavity) and environments (air) was studied by isoenzyme genotyping. **Methods:** *S. aureus* isolates were studied using isoenzyme markers (alcohol dehydrogenase, sorbitol dehydrogenase, mannitol-1-phosphate dehydrogenase, malate dehydrogenase, glucose dehydrogenase, D-galactose dehydrogenase, glucose-6-phosphate dehydrogenase, catalase and α/β-esterase) and genetic (Nei's statistics) and cluster analysis (UPGMA algorithm). **Results:** A highly frequent polyclonal pattern was observed in this population of ORSA isolates, suggesting various sources of contamination or microbial dispersion. Genetic relationship analysis showed a high degree of polymorphism between the strains, and it revealed three *taxa* (A, B and C) distantly genetically related ($0.653 \leq d_{ij} \leq 1.432$) and fifteen *clusters* (I to XV) moderately related ($0.282 \leq d_{ij} < 0.653$). These *clusters* harbored two or more highly related strains ($0 \leq d_{ij} < 0.282$), and the existence of micro-evolutionary processes in the population of ORSA. **Conclusion:** This research reinforces the hypothesis of the existence of several sources of contamination and/or dispersal of ORSA of clinical and epidemiologically importance, which could be associated with carriers (patients) and dental environmental (air).

Keywords: Oxacillin-resistant *Staphylococcus aureus*. Genetic variability. Propagation dynamics. MLEE. Clustering analysis.

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Introduction

The dissemination of *S. aureus* is considered an important public health problem because resistant strains can cause serious infections, especially in children and hospital patients[1–3]. Dentists treat a wide variety of patients, a fact that exposes these health professionals to people colonized or infected with resistant microorganisms[2,4]. High rates of resistance to antibiotics used during odontological prophylaxis have been detected in pathogens associated with bacterial endocarditis, for example, *S. aureus*[5–8]. Strains of *S. aureus* can be disseminated during dental treatment and occasionally lead to the contamination and infection of patients and dentists. Certain aspects of odontological practice can contribute to the dissemination of microorganisms[9,10]. The skin, environment and instruments can be contaminated with saliva, blood or debris during routine odontological treatment[10,11]. Several researchers have noted an increase in the amount of microorganisms present during clinical procedures in odontological environments, suggesting contamination from aerosols, especially when high-speed devices or ultrasonic scalers are used[12,13]. Among the species identified in microbiological studies, *streptococci* of the group *viridans* and *Staphylococcus* spp. are the most prevalent microorganisms found on surfaces of odontological equipment[12–15], including methicillin-resistant *S. aureus*, which has been detected on odontological operatory surfaces, air-water syringes and recliner chairs[16]. Additionally, bacteria and fungi were significantly more frequent in dentist's hand with rings than those without rings, being *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* highly prevalent among the isolated potentially pathogenic microorganisms[10].

Phenotypic methods (biotyping, serotyping, bacteriophage or bacteriocin typing and antimicrobial susceptibility profiles) and genotypic [pulsed-field gel electrophoresis (PFGE) and other methods based on the restriction of genomes, analysis of plasmids, typing methods based on polymerase chain reaction (PCR)] of microbiological characterization have elucidated the relationship and the distribution of human pathogens, which is considered essentially important for the epidemiology and control of hospital infections[17]. Isoenzymatic typing [multilocus enzyme electrophoresis (MLEE)] has been used for several decades as a "*gold standard*" in population genetics studies of eukaryotes[18–20] and systematic studies[21], as well as in large-scale studies for determining the genetic diversity and structure of natural populations of a variety of bacteria species[22–24] and fungi[25–27]. This method represents an invaluable complement to the more recently developed molecular typing methods, particularly for large-scale epidemiological studies[28]. In addition, MLEE possesses excellent typability (i.e., the percentage of different strains obtained) and reproducibility (i.e., the percentage of strains that display the same results in repetitive tests) and is associated with great discriminatory power (i.e., the ability to differentiate unrelated strains)[23–33].

Epidemiological studies are necessary for the implementation of effective prevention measures. Genotyping of strains from patients in odontological clinical treatment and their environments can provide information that can potentially help control and prevent the spread of *S. aureus* involved in the processes of colonization and human infection. This scientific research evaluated the genetic diversity of natural populations of oxacillin-resistance *S. aureus* dental isolates (dental patients and environments). The

frequency of strains and operational taxonomic groups (*taxon* and *cluster*) and possible epidemiological correlations were investigated by using isoenzymatic markers (MLEE) and genetic and grouping analysis.

Material and Methods

Microbiological Sampling

A total of ninety-nine bacterial samples of oxacillin-resistant *S. aureus* (ORSA), from the bacteria collection of the *Laboratório de Farmacogenética e Biologia Molecular*, *Faculdade de Ciências Médicas* and *Centro de Pesquisa e Pós-graduação* (UNIFENAS), Alfenas, MG, Brazil, were kindly provided and used for the present research. These samples were previously isolated from dental patients and clinical environment (air) (*Faculdade de Odontologia*, UNIFENAS) (approved by Committee of Ethics in Human Research, protocol no. 174/2009) and characterized using microbiological methods of identification [i.e., stain of Gram, growth in chromogenic medium CHROMagar *Staphylococcus aureus*®, catalase test, coagulase test (Coagu-Plasma, Laborclin Produtos para Laboratórios Ltda.), clumping factor A test (Staphy Test, Probac do Brasil Produtos Bacteriológicos Ltda.), fermentation of mannitol test and DNAse test][34] and antimicrobial susceptibility testing (i.e., diffusion disk and confirmatory triage for resistance to oxacillin)[35].

Multilocus Enzyme Electrophoresis (MLEE)

Preparation of cell extracts, electrophoresis procedures, enzyme staining and genetic interpretation of MLEE patterns were performed according to methods previously reported[23,25,26,31]. To ensure reproducibility of the results, the cellular enzymes of the *S. aureus* ATCC® 25.923™ reference strain were systematically used. A total of nine metabolic enzymes (Table 1) was investigated using systems and solutions previously established for the MLEE analyses[23,25,26,31]. The discriminatory power of the MLEE method was determined using the numeric index of discrimination (*D*), in accordance with the probability that two unrelated isolates sampled from a test population are classified into different types (i.e., strains or ETs)[25,26].

Grouping Analysis

The statistic of Nei (1972)[36] was used to estimate the genetic distance (d_{ij}) among the isolates/strains (ETs) of oxacillin-resistant *S. aureus*. The interpretation in terms of enzyme loci infers that, on average, from zero to an infinite number of allele substitutions are detected (for electrophoresis) for every 100 existing loci from a common ancestral strain. A tree with two-dimensional classification (dendrogram), based on the matrix d_{ij}, was generated by the grouping SAHN method (*Sequential, Agglomerative, Hierarchic, Nonoverlapping Clustering Methods*) and the UPGMA algorithm (*Unweighted Pair-Group Method Using an Arithmetic Average*). Once MLEE provided all levels of relationship that must be solved by DNA fingerprinting methods (i.e., identification of the same strain between independent isolates, identification of microevolutionary changes in the same strain, identification of *clusters* of moderately related isolates and identification of completely unrelated isolates), a threshold (average value: $\overline{d_{ij}}$) in the dendrogram was established to identify identical isolates and highly related isolates, *clusters*

Table 1. Systems and solutions utilized for the MLEE analyses of the *S. aureus* metabolic enzymes.

Enzyme			Compound for staining				
EC number	**Name**	**Symbol**	**Substrate**	**Buffer**	**Salt**	**Coenzyme**	**Dye and Catalyser**
1.1.1.1.	alcohol dehydrogenase	ADH	Ethanol (3mL) Isopropanol (2mL)	200mM Tris-HCl pH 8.0 (q.s.p. 50mL) [a]		NAD 1% (2mL)	PMS 1% (500μL) MTT 1.25% (1mL)
1.1.1.17	mannitol-1-phosphate dehydrogenase	M1P	Mannitol 1-phosphate (5mg)	200mM Tris-HCl pH 8.0 (q.s.p. 50mL) [a]		NAD 1% (2mL)	PMS 1% (500μL) MTT 1.25% (1mL)
1.1.1.37.	malate dehydrogenase	MDH	2M Malic acid (6 mL) [b]	200mM Tris-HCl pH 8.0 (q.s.p. 50mL) [a]		NAD 1% (2mL)	PMS 1% (500μL) MTT 1.25% (1mL)
1.1.1.47	glucose dehydrogenase	GDH	D-glucose (500 mg)	200mM Tris-HCl pH 8.0 (q.s.p. 50mL) [a]		NAD 1% (2mL)	PMS 1% (500μL) MTT 1.25% (1mL)
1.1.1.48	D-galactose dehydrogenase	GLDH	Galactose (450mL)	Tris-HCl 100mM pH 8.4 (q.s.p. 50mL) [c]		NAD 1% (1mL)	PMS 1% (500μL) MTT 1.25% (1mL)
1.1.1.49	glucose-6-phosphate dehydrogenase	G6PDH	Glicose-6-phosphate disodium salt (100 mg)	200mM Tris-HCl pH 8.0 (q.s.p. 50mL) [a]	100mM $MgCl_2$ (1 mL) [d]	NADP 1% (1mL)	PMS 1% (500μL) MTT 1.25% (1mL)
1.11.1.6	catalase	CAT [e]					
3.1.1.1.	α- and β-esterase	EST	α- and β- Naphthyl acetate (1% solution in acetone) (1.5ml)	50mM Sodium phosphate pH 7.0 (q.s.p. 50mL)[f]			Fast Blue RR salt (25 mg)

Electrode buffer: Tris–citrate pH 8.0 [83.2 g of $C_4H_{11}NO_3$ (Tris), 33.09 g of $C_6H_8O_7.H_2O$ (Citric acid), 1 liter of H_2O]; Gel buffer: Electrode buffer diluted 1:29.
[a] 24.2 g of $C_4H_{11}NO_3$ (Tris), 1 liter of H_2O (pH adjusted with HCl);
[b] 26.8 g of $C_4H_6O_5$ (DL-malic acid) and 16g of NaOH in 100 ml of H_2O (caution: potentially explosive reaction);
[c] 12.1 g of $C_4H_{11}NO_3$ (Tris), 1 liter of H_2O (pH adjusted with HCl);
[d] 2.03 g of $MgCl_2.6HCl$ (Magnesium chloride) in 100 ml of H_2O;
[e] Incubate gel slice for 30 min at 0 °C in 50 ml of 0.1 M sodium phosphate pH 7.0 buffer, then pour off solution, and immerse it in 50 ml of 1.5% potassium iodide solution (KI) for 2 min. Therefore, rinse gel slice with water, and immerse it in 50 ml of 0.03% hydrogen peroxide (H_2O_2) solution. Mix gently and remove stain solution when white zones appear on dark-blue background;
[f] Sodium phosphate buffer pH 7.0: mix equal parts of 27.6 g of $NaH_2PO_4.H_2O$ (monobasic) in 1 liter of water and 53.6 g of $Na_2HPO_4.7H_2O$ in 1 liter of water, then dilute the mixture 1:25 with water.

($0 \leq d_{ij} < \overline{d_{ij}}$) and *taxa* (singular *taxon*, i.e., taxonomic group of any nature or *rank*) ($d_{ij} \geq \overline{d_{ij}}$). Correlation coefficients based on the Pearson product-moment was used as a measure of agreement between the genetic distance values implicit in the UPGMA dendrogram and the original explicit values in the matrix of genetic distance d_{ij}. All these analyses were performed using the NTSYS-pc program version 2.1[25,26,32].

Results

The electrophoretic isoenzyme patterns of oxacillin-resistant *S. aureus* isolates were reproducible in different gels after three repetitions of each electrophoretic run. According to haploid nature of *S. aureus*, these patterns displayed the following characteristics (Table 2): all 30 enzymatic loci were polymorphic for one, two, three, four, five and six alleles (one allele: Cat-2, β-Est-3, Gdh-2, G6pdh-3, Sdh-1; two alleles: Adh-1, Cat-1, α-Est-1, β-Est-2, M1p-2, M1p-3, Mdh-3 and Sdh-2; three alleles: Gdh-3, Mdh-1, Sdh-3 and Mdh-4; four alleles: Adh-2, Adh-3, α-Est-3, β-Est-1, G6pdh-2 and Mdh-2; five alleles: M1p-1, Gdh-1, Gldh-2 and G6pdh-4; six alleles: α-Est-2, G6pdh-1 and Gldh-1). The average number of alleles per polymorphic locus was equal to 3.16 ±1.62. The existing combination in 30 enzymatic loci revealed 79 strains (ETs) [79% of the isolates, including the reference strain of *S. aureus* ATCC® 25.923, that is, identical isolates that match the same strain ^{ET} (d_{ij} = 0.000)]. Based on the genetic interpretation of electrophoretic patterns, the discriminatory power of the MLEE genotyping method was equal to 0.99051, that is, there was a 99% probability that two non-related isolates of *S. aureus*, from the test population, would be classified as distinct strains ^{ETs}.

The genetic diversity among the strains of oxacillin-resistant *S. aureus* was evaluated using the matrix d_{ij} and the UPGMA dendrogram (Figure 1). Considering the threshold obtained ($0 \leq d_{ij} < 0.282$: isolates identical or highly related; $0.282 \leq d_{ij} < 0.653$: isolates moderately related; $d_{ij} \geq 0.653$: isolates genetically distantly related), the results indicated three main groups or *taxa*, designated A, B and C. *Taxon* A comprised nine isolates/strains (ET2 $^{G22.5}$, ET6 $^{G11.66}$, ET8 $^{G11.86}$, ET9 $^{G11.129}$, ET22 $^{G18.66}$, ET24 $^{G18.8}$, ET32 $^{G18.46}$, ET68 $^{G11.36}$ and ET76 $^{G18.137}$) and eight moderately related *clusters* (from I to VIII; a total of 60 isolates $^{60\%}$ or 43 ETs $^{54.4\%}$):

- *Cluster* I: Thirteen identical and/or highly related isolates, including the reference strain; 11 highly related strains (ET1 $^{ATCC\ 25.923\ and\ G13.172}$, ET41 $^{G18.100\ and\ G20.44}$, ET43 $^{G20.12}$, ET44 $^{G18.155}$, ET45 $^{G22.55}$, ET46 $^{G22.22}$, ET47 $^{G18.51}$, ET48 $^{G15.100}$, ET49 $^{G16.140}$, ET50 $^{G13.165}$ and ET51 $^{G15.40}$).
- *Cluster* II: Five identical and/or highly related isolates; two highly related strains (ET36 $^{G18.20}$ and ET39 $^{G18.110,\ G18.111,\ G18.166\ and\ G18.156}$).
- *Cluster* III: Three highly related isolates; two highly related strains (ET13 $^{G11.135}$, ET15 $^{G13.47}$ and ET21 $^{G18.95}$).
- *Cluster* IV: Three highly related isolates; three highly related strains (ET3 $^{G6.15}$, ET5 $^{G5.38}$ and ET4 $^{G6.12}$).
- *Cluster* V: Four identical and/or highly related isolates; three highly related strains (ET10 $^{G11.139}$, ET11 $^{G11.58\ andG11.19}$ and ET12 $^{G11.39}$).

Table 2. Allele profiles of oxacillin-resistant *S. aureus* isolates (79 strains/ETs), sourced from odontological clinical and environment samples, and obtained for the genetic interpretation of MLEE patterns. Discriminatory power (*D*) = 0.99051. (-) allele null.

ET	Alleles in 30 enzyme *loci*																														
	Adh			*Sdh*			*M1p*			*Mdh*				*Gdh*			*Gldh*		*G6pdh*				*Cat*		α-*Est*			β-*Est*		
	1	2	3	1	2	3	1	2	3	1	2	3	4	1	2	3	1	2	1	2	3	4	1	2	1	2	3	1	2	3
ET1	-	-	2	-	2	-	-	2	1	-	-	1	-	-	1	-	-	1	-	-	1	-	2	-	-	5	-	-	1	-	
ET2	-	-	2	-	2	-	-	2	-	-	-	-	-	-	1	-	6	-	-	-	1	-	2	-	-	5	2	-	-	-	
ET3	-	-	2	-	2	1	-	-	1	-	2	-	-	-	1	2	-	3	-	2	-	-	2	-	-	5	-	-	1	-	
ET4	2	-	-	-	2	1	-	-	1	-	2	-	-	-	1	2	-	3	-	2	-	-	2	-	-	6	4	-	2	-	
ET5	-	-	2	-	-	1	-	-	1	-	4	-	-	-	1	2	-	3	-	2	-	-	2	-	-	5	-	-	1	-	
ET6	-	2	1	-	2	1	-	2	1	-	-	1	2	-	-	-	6	2	-	4	-	-	2	-	-	-	-	-	-	-	
ET7	-	-	-	-	2	1	-	2	1	-	-	-	-	-	-	-	-	-	-	4	-	-	2	-	-	-	-	4	-	-	
ET8	-	-	-	-	2	1	-	2	1	-	-	-	1	-	-	-	-	-	-	-	1	-	2	-	-	1	-	-	-	-	
ET9	-	-	-	-	2	1	-	2	1	-	-	-	-	-	-	-	-	4	-	-	1	-	2	-	-	-	1	-	-	-	
ET10	-	4	2	-	2	-	5	2	-	-	4	1	-	5	1	-	5	1	-	-	1	-	2	-	-	5	-	-	1	-	
ET11	-	4	-	-	2	-	4	-	-	-	4	-	-	5	-	-	5	-	-	-	-	3	2	-	-	3	-	-	1	-	
ET12	-	4	-	-	2	-	4	-	-	-	4	-	-	3	-	-	5	-	-	-	1	3	2	-	-	3	-	-	1	-	
ET13	-	1	-	-	-	-	2	-	-	3	-	-	-	-	-	-	2	-	-	-	-	-	2	-	-	5	-	-	-	-	
ET14	-	-	-	-	-	-	-	-	-	-	3	-	-	5	-	-	-	-	-	-	1	4	2	-	-	5	-	-	-	-	
ET15	-	-	-	-	-	-	-	-	1	3	-	-	-	-	-	-	-	-	-	2	-	-	2	-	-	5	-	-	1	1	
ET16	-	-	-	-	-	-	-	-	1	-	-	-	-	5	-	-	3	-	-	-	1	-	2	-	-	2	-	-	-	-	
ET17	-	-	-	-	2	-	-	-	1	-	-	-	-	-	-	-	-	-	-	4	-	-	2	-	-	-	-	-	-	-	
ET18	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	4	-	-	-	2	-	-	2	-	-	-	-	
ET19	-	-	2	-	-	3	-	1	-	3	-	-	-	-	-	-	-	-	6	-	-	-	2	-	-	-	-	-	-	-	
ET20	-	2	-	-	2	-	-	-	1	3	-	2	-	5	-	-	-	-	-	-	1	-	2	-	-	-	-	-	-	-	
ET21	-	-	2	-	-	3	-	1	-	3	-	-	-	-	-	-	-	1	-	-	-	-	2	-	-	5	-	-	-	-	
ET22	-	-	-	-	-	-	3	-	-	-	3	-	-	-	-	-	4	-	-	-	1	-	2	-	-	5	-	-	-	-	
ET23	-	-	-	-	-	-	-	-	1	-	3	-	-	-	-	-	-	-	-	2	-	-	2	-	-	-	1	2	-	-	
ET24	-	-	-	-	-	-	-	-	1	-	3	-	2	-	-	-	-	-	-	2	-	-	2	-	-	5	-	-	-	-	
ET25	-	-	2	-	-	3	-	-	1	-	4	-	-	5	-	-	-	-	6	-	-	-	2	-	-	-	-	-	1	-	
ET26	-	-	-	-	-	-	-	-	1	-	4	-	-	-	-	-	-	1	-	-	1	-	2	-	-	2	-	2	-	-	
ET27	-	-	2	-	2	-	-	2	-	-	-	-	3	-	1	-	-	1	-	-	1	-	2	-	1	-	-	-	1	-	
ET28	-	-	2	-	2	-	5	2	-	-	-	-	3	-	1	-	-	1	-	-	1	-	2	-	1	-	-	-	1	-	
ET29	-	-	2	-	2	-	-	2	-	-	-	1	3	-	1	-	-	1	-	-	1	-	2	-	2	-	-	-	1	-	
ET30	-	-	2	-	2	-	-	2	-	-	-	1	-	-	-	-	-	1	-	-	1	-	2	-	2	-	-	-	1	-	
ET31	-	-	2	-	2	-	-	2	-	-	-	-	-	1	-	-	-	1	-	-	1	-	2	-	2	-	-	-	1	-	
ET32	-	3	-	-	2	-	-	2	-	-	-	1	-	2	-	-	-	1	-	-	1	-	2	-	2	-	-	-	1	-	
ET33	-	-	2	-	2	-	-	2	-	-	-	1	-	-	1	-	-	1	-	-	1	-	2	-	2	-	-	-	1	-	
ET34	-	-	2	-	2	-	-	2	-	-	-	1	-	-	1	-	-	1	-	-	1	-	2	-	-	-	-	-	1	-	
ET35	-	-	2	-	2	-	-	2	-	-	-	1	-	3	1	-	-	1	-	-	1	-	2	-	-	-	-	3	-	-	
ET36	-	-	2	-	2	-	5	2	-	-	-	1	-	-	1	-	-	1	-	-	1	-	2	-	2	6	-	3	-	-	
ET37	-	-	2	-	2	-	-	2	-	-	-	1	-	3	-	-	-	1	-	-	1	-	2	-	2	-	-	3	-	-	
ET38	-	-	2	-	2	-	5	2	-	-	-	1	-	-	1	-	-	1	-	-	1	-	2	-	2	-	-	3	-	-	
ET39	-	-	2	-	2	-	5	2	-	-	3	-	-	-	1	-	-	1	-	-	1	-	2	-	-	6	-	3	-	-	
ET40	-	-	2	-	2	-	5	2	-	-	-	1	-	-	1	-	-	1	-	-	1	-	2	-	1	-	-	3	-	-	
ET41	-	-	2	-	2	-	-	2	-	-	-	1	-	-	1	-	-	1	-	-	1	-	2	-	-	6	-	3	-	-	

ET and [-] correspond to electrophoretic type (bacterial strain) and allele null, respectively. **Continue**

Table 2. Allele profiles of oxacillin-resistant *S. aureus* isolates (79 strains/ETs), sourced from odontological clinical and environment samples, and obtained for the genetic interpretation of MLEE patterns. Discriminatory power (*D*) = 0.99051. (-) allele null. Continuation

ET	Alleles in 30 enzyme *loci*																													
	Adh			*Sdh*			*M1p*			*Mdh*				*Gdh*			*Gldh*		*G6pdh*				*Cat*		α-*Est*			β-*Est*		
	1	2	3	1	2	3	1	2	3	1	2	3	4	1	2	3	1	2	1	2	3	4	1	2	1	2	3	1	2	3
ET42	-	-	2	-	2	-	-	2	-	-	-	-	-	-	1	-	-	1	-	-	1	-	2	-	1	-	-	3	-	-
ET43	-	-	2	-	2	-	-	2	-	-	3	-	-	-	1	-	-	1	-	-	1	-	2	-	-	5	-	-	1	-
ET44	-	-	2	-	2	-	-	2	-	-	-	-	-	-	1	-	-	1	-	3	-	-	2	-	-	5	-	-	1	-
ET45	-	-	2	-	2	-	-	2	-	-	-	-	-	-	1	-	-	1	-	-	1	-	2	-	-	5	-	-	1	-
ET46	-	-	2	-	2	-	-	2	-	-	-	1	-	-	1	-	-	1	-	-	1	-	2	-	-	5	-	1	-	-
ET47	-	-	2	-	2	-	-	2	-	-	-	-	-	-	1	-	-	1	-	3	1	-	2	-	-	5	-	-	1	-
ET48	-	-	2	-	2	-	-	2	-	-	-	-	-	-	1	-	-	1	-	-	1	-	2	-	-	5	-	3	1	-
ET49	-	-	2	-	2	-	-	2	-	2	-	-	-	-	1	-	-	1	-	-	1	-	2	-	-	4	-	3	-	-
ET50	-	-	2	-	2	-	-	2	-	2	-	-	-	-	1	-	-	1	-	-	1	-	2	-	-	5	-	-	1	-
ET51	-	-	2	-	2	-	-	2	-	2	-	-	-	-	1	-	-	1	-	-	1	-	2	-	-	4	-	-	1	-
ET52	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	1	1	-	2	-	-	-	-	-	-	-
ET53	-	-	4	-	-	2	3	-	2	2	-	-	-	-	-	3	1	5	5	-	-	5	2	1	-	-	-	-	-	-
ET54	-	-	-	-	-	-	-	-	-	2	1	-	-	-	-	-	-	-	-	1	-	2	2	-	-	-	-	-	-	-
ET55	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	3	-	-	-	2	-	-	-	-	-	-	-
ET56	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1	-	-	-	2	-	-	-	2	-	-	-
ET57	1	3	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	2	2	-	-	-	-	-	-	-
ET58	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	2	-	-	-	-	-	-	-
ET59	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	3	-	-	-	2	-	-	-	-	-	-	-
ET60	-	-	3	1	-	-	-	-	-	-	1	-	-	5	-	-	-	-	-	-	-	5	2	-	-	-	-	-	-	-
ET61	-	-	-	1	1	-	-	-	-	2	-	-	-	4	-	1	-	-	1	-	-	-	2	-	-	-	-	-	-	-
ET62	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-
ET63	1	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	1	-	-	2	-	-	-	-	4	-	-
ET64	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	2	-	-	-	-	-	-	-
ET65	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	5	-	-	-	2	-	-	-	-	-	-	-
ET66	-	-	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	1	-	2	-	-	-	-	-	-	-
ET67	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	1	-	-	-	-	-
ET68	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	5	-	-	1	-	-	2	-	2	-	-	-	-	-
ET69	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-
ET70	-	-	-	-	-	-	-	-	-	1	-	-	-	4	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-
ET71	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	2	1	-	-	2	-	-	-	-	-	-	-
ET72	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	2	-	-	-	-	-	-	-
ET73	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	1	-	-	2	-	-	-	-	-	-	-
ET74	-	-	-	-	-	-	1	-	-	-	-	1	-	-	-	-	1	-	5	-	-	-	2	-	-	-	-	-	-	-
ET75	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	-	-	-	-	3	-	-	1	-	-	-	-	-	-	-
ET76	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	-	-	-	-	3	-	-	1	-	-	5	3	-	-	-
ET77	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	2	-	1	1	-	-	-	-	-	1	-
ET78	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	3	-	-	1	-	-	-	-	-	-	-
ET79	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-	-

ET and [-] correspond to electrophoretic type (bacterial strain) and allele null, respectively.

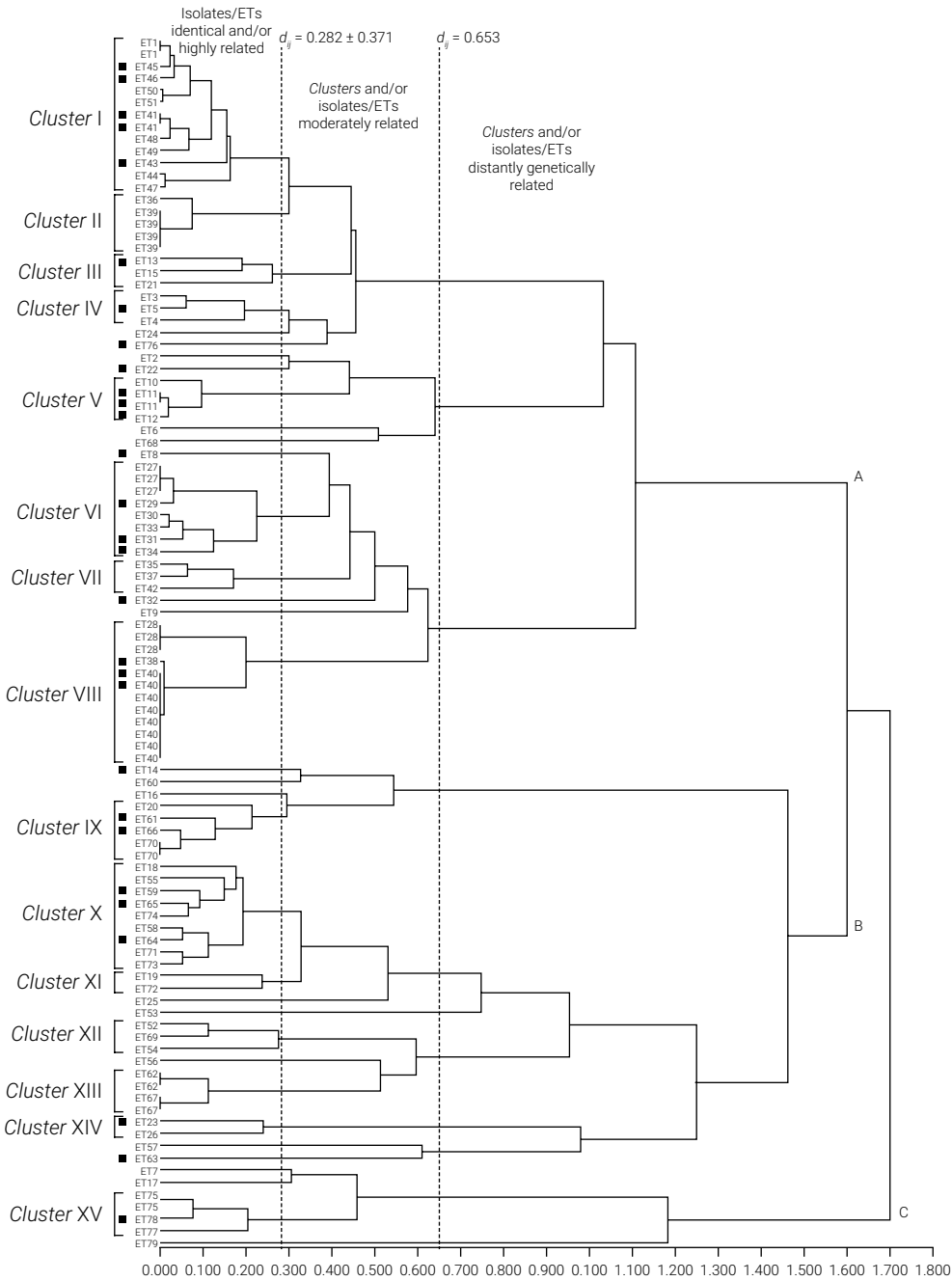

Figure 1. Genetic diversity of 99 oxacillin-resistant *S. aureus* isolates sourced from a population of odontological clinical and environment samples. The UPGMA dendrogram (r_{jk} = 0.79908) was generated from a matrix of genetic distance d_{ij} (Nei, 1972).

- *Cluster* VI: Eight identical and/or highly related isolates; six highly related strains (ET27 [G18.33, G18.104 and G20.45], ET29 [G18.14], ET30 [G19.43], ET31 [G22.48], ET33 [G18.135] and ET34 [G20.14]).
- *Cluster* VII: Three highly related isolates; three highly related strains (ET35 [G18.45], ET37 [G19.10] and ET42 [G18.126]).

- *Cluster* VIII: Twelve identical and/or highly related isolates; three highly related strains (ET28 G18.94, G18.9 and G21.1, ET38 G18.55, ET40 G15.52, G16.40, G18.142, G18.26, G18.50, G18.74, G22.63 and G19.21).

Taxon B comprised eight isolates/strains (ET14 G13.112, ET60 G14.71, ET16 G12.14, ET25 G18.89, ET53 G14.126, ET56 G15.159, ET57 G13.41 and ET63 G16.258) and six moderately related *clusters* (from IX to XIV; a total of 33 isolates 33% or 30 ETs 37.9%):

- *Cluster* IX: Thirteen identical and/or highly related isolates; four highly related strains (ET20 G18.15, ET61 G13.120, ET66 G5.31, ET70 G13.174 and G14.199).
- *Cluster* X: Nine highly related isolates; nine highly related strains (ET18 G13.51, ET55 G17.68, ET58 G17.86, ET59 G16.269, ET64 G15.131, ET65 G17.13, ET71 G15.64, ET73 G17.128 and ET74 G17.62).
- *Cluster* XI: Two highly related isolates; two highly related strains (ET19 G11.13 and ET72 G17.63).
- *Cluster* XII: Three highly related isolates; three highly related strains (ET52 G16.88, ET54 G16.49 and ET69 G13.142).
- *Cluster* XIII: Four identical and/or highly related isolates; two highly related strains (ET62 G17.115 and G16.167 and ET67 G11.131 and G11.32).
- *Cluster* XIV: Two highly related isolates; two highly related strains (ET23 G18.91 and ET26 G18.10).

Taxon C comprised three isolates/strains (ET7 G11.96, ET17 G12.13 and ET79 G22.64) and only one moderately related *cluster* (XV; a total of six isolates 6% or five ETs 6.3%):

- *Cluster* XV: Four identical and/or highly related isolates; three highly related strains (ET75 G17.42 andG18.124, ET78 G20.48 and ET77 G19.44).

Discussion

In this study, the enzyme electrophoretic profiles of oxacillin-resistant *S. aureus* isolates on different gels were reproducible after three repetitions of each electrophoretic run. The discriminatory capacity (i.e., 99% probability that two unrelated isolates sampled from a population test are classified in different strains ETs) of the MLEE method, based on genetic interpretation of electrophoretic enzyme patterns, was also observed (i.e., the combination of existing alleles on 30 enzyme loci revealed 79 ETs). Once again, MLEE proved to be a powerful tool for the typing of *S. aureus* in epidemiological studies. These results are in agreement with previously reported data on the discriminatory power and reproducibility of the MLEE method as applied to bacteria and yeasts of medical importance[23–27,31], but the discriminatory power was higher than the values reported for *S. aureus* by other groups of researchers[29,30].

Genetic polymorphism has been found in almost all natural populations and at all levels of genetic organization, from genotype characteristics to phenotypic traces. The possible reasons of its existence have been the subject of a long debate in the population genetics and molecular evolution fields[37,38]. *S. aureus* is a heterogeneous species (polymorphic)[39] that has been observed to have a clonal population structure[40]. Therefore, it is believed that *S. aureus* does not suffer extensive recombination, diversifies extensively by nucleotide mutations and displays a high degree of linkage disequilibrium (non-random asso-

ciations between gene loci). A particular structural gene locus is defined as polymorphic when the frequency of its more common allele presents a value below 0.99 (99%). Some of the measures used to quantify this variability in populations of organisms are the allele and gene frequencies, the percentage of polymorphic loci, the average number of alleles per locus and heterozygosity[41]. In this study, quantitative and qualitative variations of polymorphic loci (30 $^{100\%}$ polymorphic enzyme loci to one, two, three, four, five and six alleles) and the average number of alleles per polymorphic locus (3.16 ±1.62) were observed in the population of oxacillin-resistant *S. aureus*. These variations have been observed in several studies of genetic diversity of populations of *S. aureus* obtained from human and bovine sources[29,30,42,43]. In addition, the genetic polymorphism observed in the population of oxacillin-resistant *S. aureus* isolates revealed a highly frequent polyclonal pattern and infrequent monoclonal pattern, suggesting various sources of contamination or microbial dispersion from an epidemiological point of view.

The genetic relationship between the oxacillin-resistant *S. aureus* strains was determined by using the statistic d_{ij} of Nei (1972) and the UPGMA dendrogram[25,26,32,36], which displayed a value r_{jk} acceptable ($r_{jk} \sim 0.8$) based on the correlation coefficient of Pearson's product-moment [i.e., good agreement between the elements d_{ij} (matrix of genetic distance) and C_{jk} (correlation matrix derived from UPGMA dendrogram)]. A high degree of genetic polymorphism ($0.000 \leq d_{ij} \leq 1.705$) was observed between the ORSA isolates (i.e., on average, from zero to 170.5 allele substitutions were detected in each 100 loci from a common ancestor strain). These isolates were allotted to three *taxa* (A, B and C), which were distantly genetically related ($0.653 \leq d_{ij} \leq 1.705$). *Taxon* A presented a larger number of isolates, strains or *clusters* of bacteria (60 isolates $^{60\%}$, 43 ETs $^{54.4\%}$ and eight *clusters* $^{I\text{-}VIII}$), followed by *taxon* B (33 isolates $^{33\%}$, 30 ETs $^{37.9\%}$ and six *clusters* $^{IX\text{-}XIV}$) and *taxon* C (six isolates $^{6\%}$, five ETs $^{6.3\%}$ and one *cluster* ^{XV}). Each *taxon* presented one or more *clusters* and/or moderately related isolates ($0.282 \leq d_{ij} < 0.653$). In turn, these *clusters* harbored two or more identical and/or highly related isolates ($0 \leq d_{ij} < 0.282$). Considering that highly related isolates/strains highly come from a common ancestor [i.e., descendants have suffered microevolutions and adaptations as a result of recombination (not extensive), nucleotide mutations and non-random association between gene loci (linkage disequilibrium)[39,40], these results suggest the existence of microevolutionary processes in the population of oxacillin-resistant *S. aureus*, as demonstrated in each *cluster* (i.e., on average, from zero to < 28.2 allele substitutions were detected in each 100 loci from a common ancestor strain). However, these data reinforce the hypothesis of the existence of several sources of contamination and/or dispersal of oxacillin-resistant *S. aureus* of clinical and epidemiologically importance, which could be associated with carriers (patients) and dental environmental (air). These epidemiological investigations have also been a goal of our research group and contribute to (*i*) knowledge about the dynamics of the spread and retention of *S. aureus* strains resistant to antibiotics in hospital and odontological environments (i.e., surgical devices, dental instrumentation, various surfaces, air and other) and (*ii*) the implementation or restructuring of containment barriers, use of personal protective equipment, means of identification and periodic treatment from professionals carriers of microorganisms (nasal cavities, oral and oropharyngeal, perineum and armpits), techniques and devices for air purification, hygiene and more efficient prophylaxis.

Certain aspects of practicing dentistry may contribute to the transmission of microorganisms[9]. The skin, environment, and instruments can be contaminated with saliva, blood or organic debris during routine dental treatment[11]. In the dental environment, investigators have observed an increase in the amount of microorganisms during clinical procedures, suggesting contamination by aerosols, especially when high-speed handpieces or ultrasonic scalers are used[12,13,15]. Among the species identified in microbiological studies, *Streptococcus viridans* and *Staphylococcus* spp. are the most prevalent microorganisms found on the surfaces of dental equipment[12–15]. In addition, the high-speed drills and cavitrons used in dental offices generate aerosols and droplets that are contaminated with blood and bacteria and may be a route for the transmission of diseases such as SARS (severe acute respiratory syndrome), tuberculosis, and Legionnaires' disease[44–46]. Methicillin-resistant *S. aureus* (MRSA) has frequently been detected on surfaces in dental operatories, including the air-water syringe and reclining chair[16]. Nosocomial infections or the colonization of MRSA occurred in eight out of 140 patients who displayed no evidence of MRSA upon admission to a clinic. Antibiogram tests revealed that the isolates from the eight patients were of the same strain as those from the surfaces of the dental operatory, suggesting *S. aureus* transmission between the patients and the dentist via the clinical environment[16]. The frequency of *S. aureus* isolated from the noses, hands, and tongues of students and patients and from the clinical environment of a pediatric dentistry clinic before and after dental treatment was determined[47]. The highest concentration of *S. aureus* was found in the noses and on the tongues of children and among the dental students, and the highest level of contamination was observed on gloved hands, which was followed by the tongue and hands without gloves before clinical care. At the end of dental treatment, *S. aureus* colonies isolated from the gloved hands of students decreased significantly. Considering the clinical environment, *S. aureus* dissemination increased at the end of dental procedures, and the most contaminated areas were the auxiliary table and the storeroom, which was located at the center of the clinic. Such results can be explained by the intense circulation of people in the clinic and the use of high-speed dental handpieces. However, it is still speculated that much of the *S. aureus* contamination detected in the clinical environment came from other sources, such as direct contact, skin exfoliation or the improper handling of plates, and it is concluded that the dental clinic is an appropriate environment for *S. aureus* cross-transmission.

Because molecular-based epidemiological studies are useful in identifying possible sources of the spread of microorganisms in hospitals and dental clinical settings, this study contributes to our knowledge on the dynamics of the spread of *S. aureus* strains resistant to antibiotics and points to the need for containment barriers, use of personal protective equipment, periodic identification and treatment of carriers among clinical staff, and installation of air purifiers. Thus, infection control guidelines and published research pertinent to dental infection control principles and practices must be applied by the dentist as a matter of routine in academic dental offices. This research showed a genetic polymorphism in the population of oxacillin-resistant *S. aureus* isolates (dental patients and air of the clinical environment) and a highly frequent polyclonal pattern of these bacterial strains, supporting the hypothesis of various sources of contamination or microbial dispersion in the dental clinic environment. The isoenzyme typing and genetic relationship analysis revealed also some taxa and

clusters exhibiting different frequencies of strains and possibly microevolutionary changes. In addition to the genetic information of *S. aureus*, the present methodology potentially collaborates with measures of prevention, management, and tracking of *S. aureus*, especially in dental clinics with great workflow.

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