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ORIGINAL ARTICLE

Detection of disinfectant and antibiotic resistance genes in *Staphylococcus aureus* isolated from the oral cavity of Tunisian children

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Abstract *Staphylococcus aureus* is an important pathogen associated to dental infection. Many antiseptic agents are used in hygienic handwash to prevent nosocomial infections by methicillin-resistant staphylococci. In this study, 22 *Staphylococcus aureus* strains isolated from the oral cavity of Tunisian children were investigated for their susceptibility to benzalkonium chloride (0.5–512 μg/ml) and antibiotics. The β-lactams resistance gene *blaZ,* the erythromycin resistance methylase genes (*ermA*, *ermB* and *ermC*), the macrolide efflux gene (*msrA*) and the disinfectant resistance genes (*qacH*, *qacA*, *qacB* and *qacC*) were also investigated. Determination of the minimal inhibitory concentration values revealed that 54.5, 54.5, 68, and 82% of isolates were resistant to benzalkonium chloride, oxacillin, tetracycline and erythromycin respectively. The frequency of strains positive for the antibiotic resistance genes tested was 100 (*blaZ*), 50 (*ermA*), 36.4 (*ermB*), 22.7 (*ermC*) and 13.6% (*mrsA*). The *qacH* and the *qacA* genes were found in 22.7% of isolates and *qacB* and *qacC* in 13.6%. Two strains harboured three *qac* (*qacH*, *qacA* and *qacB* or *qacC*) genes. These data highlight the importance of determining the susceptibility to antibiotics and disinfectants of strains isolated in dental medicine in order to monitor the epidemiology and spread of multi-drug resistant staphylococci.

Keywords *Staphylococcus aureus* · MIC · Benzalkonium chloride · Multidrug resistance · *qac* · PCR

Introduction

The human oral cavity is habited by more than 500 cultivable and non-cultivable bacterial species (Paster et al. 2001). The majority of these species are commensals, while a subset comprises opportunistic pathogens. Their key role in the etiology of periodontitis and dental caries in the world is well established (Meyer and Fives-Taylor 1998), and they has been also implicated in the etiology of a number of systemic diseases, such as infective endocarditis (Barrau et al. 2004), respiratory infections (Mojon and Bourbeau 2003) and cardiovascular diseases (Meyer and Fives-Taylor 1998). Interestingly, in some studies, microorganisms such as staphylococci, coliforms and *Candida* spp. have been commonly isolated from peri-implant lesions (Leonhardt et al. 1999; Salvi et al. 2008). More recently, *Staphylococcus aureus* has been demonstrated to have the ability to adhere to titanium surfaces (Gristina 1987), which may be significant in terms of the colonisation of dental implants and subsequent infections (Harris and Richards 2004).

Oral bacteria are exceptionally adept at acquiring resistance to pencillins, tetracycline, macrolides and antiseptic agents (Sweeney et al. 2004). Methicillin-resistant *S. aureus* (MRSA) is considered to be one of the most difficult bacteria to treat in patients, and successful treatment is compounded by the fact that many strains also possess efflux pumps, which export certain tetracyclines, macrolides and

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qacA genes that confer resistance to antibiotics and antiseptics (Marshall and Piddock 1997).

Resistance to macrolides in staphylococci may be based on the active efflux mechanism encoded by the *msrA* gene or on 23S rRNA methylation, which is encoded by erythromycin resistance genes [*erm*(*A*), *erm*(*B*), *erm*(*C*)] (Fluit et al. 2001). It is well-established that in MRSA the *tet(M)* and *tet(K)* genes confer tetracycline resistance through ribosomal protection and an active efflux mechanism, respectively (Trzcinski et al. 2000; Fluit et al. 2001).

Benzalkonium chloride (BC), is frequently used in hospitals to disinfect and prevent the spread of pathogens. The widespread use of BC may impose a selective pressure and contribute to the emergence of disinfectant-resistant microorganisms in these environments (Reverdy et al. 1993; Russell 2000). In clinical cases of staphylococci, *qacA*, *qacB* and *qacC/smr* are generally plasmid borne and are widely distributed in the environment (Leelaporn et al. 1995; Noguchi et al. 1999; Mayer et al. 2001).

In the study reported here, we examined the antibiotic and BC susceptibility of 22 *S. aureus* strains isolated from the oral cavity of Tunisian children. We also investigated the prevalence of *blaZ*, *ermA*, *ermB*, *ermC* and *msrA* genes, which are responsible for antibiotic resistance, as well as the *qac* genes, which are responsible for disinfectant resistance, in *S. aureus* strains.

Materials and methods

Patients and Bacterial strains

Samples were taken with a sterile swab from the oral cavity of Tunisian children (n = 90). The mean age of the children was approximately 4–12 years. Prior to study commencement, all clinical procedures were approved by the Ethical Committee of the Faculty of Medicine, University of Monastir, Tunisia, and written informed consent was obtained from the parents of all participants. The criteria for inclusion were: no antibiotic treatment during the 4 weeks preceding sampling and no systemic disease.

Species identification was performed using API 20 Staph strips (BioMerieux, Marcy l'Etoile, France) according to the manufacturer's recommendation, and the results were read using an automated microbiological mini-API (Bio-Merieux).

Molecular identification of *S. aureus* strains was performed using specific primers (Sa442-1 and Sa442-2), as previously described (Martineau et al. 1998).

Minimal inhibitory concentration determination

The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of BC (Acros Organics, Geel, Belgium), oxacillin (OX), tetracycline (TET) and erythromycin (ERY) (0–512 μg/ml) according to the Comité de l'antibiogramme de la société française de microbiologie (CASFM 2010). After incubation, bacterial growth was evaluated by the presence of turbidity and a pellet on the well bottom. The MIC was defined as the lowest concentration of compound that resulted in no macroscopically visible growth.

Detection of antibiotic resistance gene by PCR

Genomic DNA was extracted using a Wizard Genomic Purification kit (Promega, Madison, WI). The presence of *blaZ* DNA was detected by PCR using forward and reverse primers as described previously (Martineau et al. 2000). Multiplex PCR for the detection of macrolide resistance genes *ermA*, *ermB*, *ermC* and *msrA* was performed as described elsewhere (Martineau et al. 2000; Lim et al. 2002).

Detection of *qacH*, *qacA*, *qacB* and *qacC* genes by PCR–restriction fragment length polymorphism

The presence of the *qacH* gene was detected by PCR using forward and reverse primers as described previously (Sidhu et al. 2002).

Multiplex PCR–restriction fragment length polymorphism (RFLP) analysis of the *qacA*/*B* and *qacC* genes was performed as described by Sekiguchi et al. (2004).

Following PCR, the product was digested with 5 U of *Alu*I (Promega) at 37°C for 90 min. The treated product (10 μl) was analysed by gel electrophoresis in 3% agarose gel in 1× Tris-borate-EDTA buffer (TBE, pH 8.3). The amplification products were photographed and their size determined using a 25-bp molecular size marker (Promega). The *qacB* gene was expected to be digested into two fragments of 176 and 44 bp, respectively. Genes *qacA* and *qacC* were not expected to be digested (Sekiguchi et al. 2004).

Results

Molecular identification of *S. aureus* strains

Molecular identification using specific primers showed the presence of 22 *S. aureus* among 59 Gram-positive strains isolated from the oral cavity of Tunisian children with dental caries (n = 60) and controls (n = 30) as presented in Table 1 .

Antimicrobial susceptibility

The isolates were screened for their susceptibility to BC (Table 1) according to the MIC values. Among the 22

Table 1 Relationship between the minimal inhibitory concentration of benzalchonium chloride and the presence of disinfectant resistance genes *qacH*, *qacA*, *qacB* and *qacC*

Strains	State	MIC benzalchonium chloride (μg/ml)	Presence of genes[a]			
B240	Caries-active	0.50	*qacH*+	*qacA*−	*qacB*−	*qacC*−
B226	Caries-active	2	*qacH*−	*qacA*−	*qacB*−	*qacC*−
B40	Caries-active	1	*qacH*−	*qacA*−	*qacB*−	*qacC*−
B290	Caries-active	32	*qacH*−	*qacA*−	*qacB*−	*qacC*+
B374	Caries-active	128	*qacH*−	*qacA*−	*qacB*−	*qacC*+
B285	Caries-active	8	*qacH*−	*qacA*+	*qacB*+	*qacC*−
B295	Caries-active	4	*qacH*−	*qacA*−	*qacB*−	*qacC*−
B230	Caries-active	2	*qacH*−	*qacA*	*qacB*−	*qacC*−
B621	Caries-active	2	*qacH*−−	*qacA*−	*qacB*−	*qacC*−
B136	Caries-active	8	*qacH*−	*qacA*−	*qacB*−	*qacC*−
B364	Caries-active	128	*qacH*+	*qacA*+	*qacB*+	*qacC*−
B73	Caries-active	16	*qacH*−	*qacA*+	*qacB*+	*qacC*−
B456	Caries-active	4	*qacH*−	*qacA*−	*qacB*−	*qacC*−
B712	Caries-active	8	*qacH*−	*qacA*−	*qacB*−	*qacC*−
B398	Caries-active	4	*qacH*+	*qacA*−	*qacB*−	*qacC*−
B401	Caries-active	1	*qacH*−	*qacA*−	*qacB*−	*qacC*−
B244	Caries-active	2	*qacH*−	*qacA*−	*qacB*−	*qacC*−
B147	Caries-active	2	*qacH*+	*qacA*−	*qacB*−	*qacC*−
B193	Caries-active	2	*qacH*−	*qacA*−	*qacB*−	*qacC*−
B302	Caries-free	2	*qacH*−	*qacA*−	*qacB*−	*qacC*−
B289	Caries-free	8	*qacH*−	*qacA*+	*qacB*−	*qacC*−
B291	Caries-active	16	*qacH*+	*qacA*+	*qacB*−	*qacC*+

MIC, Minimal inhibitory concentration

[a] +, Gene present; −, gene absent

S. aureus strains, 12 (54.5 %) were considered to be resistant to BC (MIC 4–128 μg/ml) and ten (45.45 %) to be sensitive (MIC ≤2 μg/ml) according to the guideline of the CASFM (2010).

Several strains were resistant toward all three antibiotics tested (TET, OXA, ERY), 12 (54.5%) were resistant to oxacillin (MIC >2 μg/ml), 15 (68%) were resistant to tetracyline (MIC >2 μg/ml) and 18 (82%) were resistant to erthromycin (MIC >2 μg/ml) according to the CASFM 2010. The incidence of strains resistant to tetracycline and erythromycin was higher among MRSA than among methicillin-resistant *S. aureus* (MSSA) isolates, with 10/12 (83%) MRSA isolates being resistant to tetracycline and 11/12 (91%) of isolates being resistant to erythromycin (Table 2).

Detection of antibiotics resistance genes

All the isolated *S. aureus* were positive for the *blaZ* gene. The incidence of the three erythromycin ribosomal methylase genes was as follows: 11 of 22 (50%) *S. aureus* contained *ermA*, eight strains harboured *ermB* (36.4%) and five (22.7 %) strains were positive for *ermC*. The *msrA* gene was present in three (13.6%) *S. aureus* strains. Four strains (18%) were susceptible to erythromycin and did not contain any erythromycin resistance gene. In contrast, no erythromycin resistance gene was detected in one strain (B136), although this strain was resistant to erythromycin (Table 2).

PCR–RFLP detection of *qacA*, *qacB*, *qacC* and *qacH* genes

Among the 22 *S. aureus* strains tested, 12 were *qac*-negative, while *qacA* and *qacH* were detected in five (22.7%) strains and *qacC* and *qacB* were detected in three (13.6%) strains (Table 1). Notably, two of the ten *qac*-positive strains (B364 and B291) carried three different genes (*qacA*, *qacB*, *qacH* and *qacA*, *qacC*, *qacH*, respectively).

Discussion

The large consumption of antibiotics worldwide has resulted in the development and spread of a large number of antibiotic resistance determinants among bacterial populations. Concerns have arisen regarding the potential emergence of cross-resistance between widely used disinfectants and antibiotics (Paulsen et al. 1998; Russell 2000).

In this study, we report the presence of *S. aureus* in carious children. A large number of the *S. aureus* isolates were resistant toward the three antibiotics tested (OXA,

Table 2 Relationships between MIC of antibiotics, and the presence of resistance genes *blaZ*, *ermA*, *ermB*, *ermC* and *msrA*

Strains	State	MIC (μg/ml of antibiotic)			Presence of genes[a]				
		TET	OXA	ERY					
B'240	Caries-active	0.50	0.50	512	*blaZ*+	*ermA*+	*ermB*+	*ermC*+	*msrA*−
B226	Caries-active	0.50	0.50	32	*blaZ*+	*ermA*−	*ermB*+	*ermC*−	*msrA*−
B40	Caries-active	4	0.50	8	*blaZ*+	*ermA*−	*ermB*−	*ermC*+	*msrA*−
B290	Caries-active	256	512	512	*blaZ*+	*ermA*−	*ermB*−	*ermC*−	*msrA*+
B374	Caries-active	128	32	512	*blaZ*+	*ermA*−	*ermB*−	*ermC*−	*msrA*+
B285	Caries-active	4	512	512	*blaZ*+	*ermA*+	*ermB*+	*ermC*−	*msrA*−
B295	Caries-active	128	0.50	512	*blaZ*+	*ermA*+	*ermB*+	*ermC*−	*msrA*−
B230	Caries-active	0.50	0.50	0.50	*blaZ*+	*ermA*−	*ermB*−	*ermC*−	*msrA*−
B621	Caries-active	0.50	512	512	*blaZ*+	*ermA*+	*ermB*−	*ermC*−	*msrA*−
B136	Caries-active	64	32	4	*blaZ*+	*ermA*−	*ermB*−	*ermC*−	*msrA*−
B364	Caries-active	4	0.50	512	*blaZ*+	*ermA*+	*ermB*−	*ermC*+	*msrA*−
B73	Caries-active	64	512	512	*blaZ*+	*ermA*+	*ermB*−	*ermC*−	*msrA*−
B456	Caries-active	64	0.50	512	*blaZ*+	*ermA*+	*ermB*+	*ermC*−	*msrA*−
B712	Caries-active	0.50	0.50	0.50	*blaZ*+	*ermA*−	*ermB*−	*ermC*−	*msrA*−
B398	Caries-active	16	0.50	64	*blaZ*+	*ermA*+	*ermB*+	*ermC*−	*msrA*−
B401	Caries-active	0.50	512	0.50	*blaZ*+	*ermA*−	*ermB*−	*ermC*−	*msrA*−
B244	Caries-active	32	512	512	*blaZ*+	*ermA*+	*ermB*+	*ermC*−	*msrA*−
B147	Caries-active	0.50	0.50	0.50	*blaZ*+	*ermA*−	*ermB*−	*ermC*−	*msrA*−
B193	Caries-active	8	256	64	*blaZ*+	*ermA*−	*ermB*−	*ermC*+	*msrA*−
B302	Caries-free	256	512	512	*blaZ*+	*ermA*+	*ermB*+	*ermC*−	*msrA*−
B289	Caries-free	4	8	512	*blaZ*+	*ermA*−	*ermB*−	*ermC*+	*msrA*−
B291	Caries-active	128	512	512	*blaZ*+	*ermA*+	*ermB*−	*ermC*−	*msrA*+

TET, Tetracycline; OXA, oxacillin; ERY, erythromycin

[a] +, Gene present; −, gene absent

TET and ERY) and harboured the antibiotic resistance genes. Our data demonstrate that all *S. aureus* (either MSSA and MRSA) strains tested, including the two strains from caries-free children, harboured the *blaZ* gene. The frequency of MRSA estimated in our study is significantly higher than that reported by Petinaki et al. (2001) who found that only 14.8% of strains were resistant to oxacillin. We also found that among the MRSA isolates, 83% of strains were resistant to tetracycline and 91% of strains were resistant to erythromycin. These results are in contrast with those of Petinaki et al. (2001), who showed that 16.21% of MRSA were resistant to tetracycline and 81% were resistant erythromycin.

Erythromycin resistance in staphylococci is predominantly mediated by *erm* genes encoded by methylases (Weisblum 1995). Accordingly, our investigation on the prevalence of erythromycin resistance genes showed that of the 17 strains resistant to erythromycin, 15 contained at least one *erm* gene (among them only two were from caries-free children). Of the remaining two strains, one (B136) did not carry any of the investigated erythromycin resistance genes (Table 2) and showed low-level resistance. Similarly, Sekiguchi et al. (2004) reported discordance between phenotypic susceptibility and the presence of *erm* genes. This result may be explained by the location of resistance genes in small plasmids that could occasionally be lost (Fluit et al. 2001).

Staphylococcal strains resistant to macrolides and type-B streptogramins frequently harbour *msrA*, which encodes an ATP-dependent efflux pump (Eady et al. 1993). In contrast,

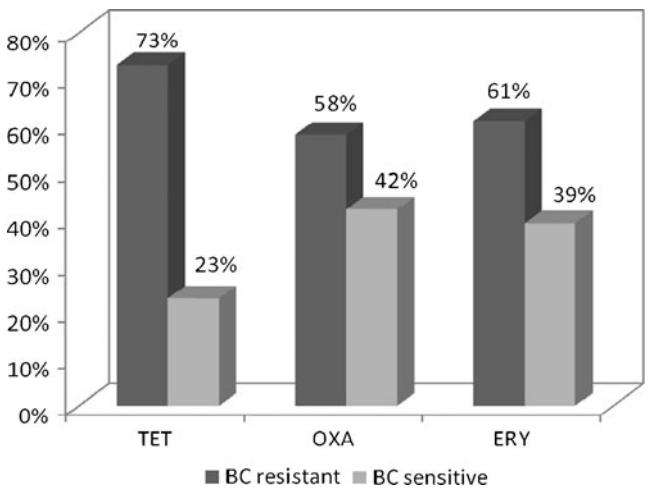

Fig. 1 Percentages of antibiotic-resistant *S. aureus* isolates among benzalkonium chloride (BC)-resistant and BC-sensitive isolates. Antibiotics used for testing were tetracycline (TET), oxacillin (OXA) and erythromycin (ERY)

we detected *msrA* only in three strains. These findings are in disagreement with the study by Eady et al. (1993) conducted with staphylococci in the UK in which an incidence of 5.9% for *ermA*, 7.2% for *ermB* and 33% for *msrA* was reported. Our results also deviate from those of a recent study of Ardic et al. (2005) in which a higher level of *ermA* and *ermC* was found in MRSA isolated from a hospital setting.

Disinfectants using quaternary ammonium compounds (QAC) are frequently used in hospitals to prevent the spread of pathogens. However, the widespread use of these disinfectants in hospitals actually contributes to the emergence of disinfectant-resistant bacteria (Reverdy et al. 1993; Russell 2000). In *S. aureus*, *qacA* and *qacB*, which encode multidrug-transporter proteins (Rouch et al., 1990), have been identified as antiseptic resistance genes that confer resistance to cationic antiseptic agents, including dyes such as ethidium bromide, QACs, such as BC, and biguanides, such as chlorhexidine digluconate (Littlejohn et al. 1992; Putman et al. 2000). Among the bacteria tested in this work, 12 (55%) isolates, 11 from caries-active and one from caries-free children, were considered to be BC resistant (MIC 4–128 μg/ml), and the remaining ten (45%) were considered to be sensitive (MIC ≤2 μg/ml). Similarly, it has been reported that 50% of *S. aureus* isolated from bloodstream infections were resistant to BC (Sidhu et al. 2002). Different genes, including *qacH*, *qacA* and *qacB* or *qacC*, confer effux-mediated resistance to lipophilic cationic compounds, including BC and dyes, in staphylococci (Paulsen et al. 1996).

Our molecular detection analysis of the disinfectant's resistance genes showed that eight strains resistant to BC (7 from caries-active and one from caries-free children) contained at last one of the four *qac* genes. The *QacA* and *qacH* genes were the most frequent (both 22.7%), followed by *qacC* and *qacB* (both 13.6%). Previous investigators have reported a similar distribution of these *qac* resistance genes in clinical *S. aureus* and CoNS (Leelaporn et al. 1995; Mayer et al. 2001).

On the other hand, our data disagree with those of Sekiguchi et al. (2004), who found that 49% of their MRSA isolates were positive for *qacA*, 10% were positive for *qacC* and only one isolate was positive for *qacB*. It should be noted that, as shown in Table 1, two strains (B364 and B291) harboured three *qac* resistance genes (*qacA*, *qacB*, *qacH* and *qacA*, *qacC*, *qacH*) but showed different MIC values for BC (128 and 16 μg/ml, respectively). Little is known about the occurrence and possible genetic linkage of *qac* and antibiotic resistance in staphylococci. Interestingly, we observed that staphylococci resistant to BC were generally more often resistant to antibiotics (Oxa, Ery and Tet) than BC-sensitive isolates (Fig. 1). This result is in agreement with data reported by Sidhu et al. (2002). MRSA isolates resistant to antiseptics and disinfectants have been reported in Australia and in the UK in the last decade (McDonnell and Russell 1999). *QacA*/*B* genes are typically located on a transposon of transmissible multidrug-resistant plasmids, such as pSK1 (Littlejohn et al. 1991). Noguchi et al. (1999) reported that when the antiseptic susceptibility and the distribution of antiseptic resistance genes of MRSA isolated in Japan in 1992 were studied, *qacA*/*B* genes were detected in 10.2% of the strains tested (10/98). However, 7 years later, in a similar study of MRSA isolates in Japan, *qacA*/*B* genes were detected in 47.9% (198/413) of the isolates.

In conclusion, monitoring susceptibility to antibiotics and disinfectants in bacteria isolated during dental procedures can greatly contribute to the epidemiological control of multidrug resistant strains.

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