Original article

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Virulence factors and antibiotic susceptibility in enterococci isolated from oral mucosal and deep infections

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Objective: This study evaluates the presence of virulence factors and antibiotic susceptibility among enterococcal isolates from oral mucosal and deep infections.

Methods: Forty-three enterococcal strains from oral mucosal lesions and 18 from deep infections were isolated from 830 samples that were sent during 2 years to Oral Microbiology, University of Gothenburg, for analysis. The 61 strains were identified by 16S rDNA, and characterized by the presence of the virulence genes *efa A* (endocarditis gene), *gel E* (gelatinase gene), *ace* (collagen binding antigen gene), *asa* (aggregation substance gene), *cyl A* (cytolysin activator gene) and *esp* (surface adhesin gene), tested for the production of bacteriocins and presence of plasmids. MIC determination was performed using the E-test method against the most commonly used antibiotics in dentistry, for example, penicillin V, amoxicillin and clindamycin. Vancomycin was included in order to detect vancomycin-resistant enterococci (VRE) strains.

Results: Sixty strains were identified as *Enterococcus faecalis* and one as *Enterococcus faecium*. All the virulence genes were detected in more than 93.3% (*efa A and esp*) of the *E. faecalis* strains, while the presence of phenotypic characteristics was much lower (gelatinase 10% and hemolysin 16.7%). Forty-six strains produced bacteriocins and one to six plasmids were detected in half of the isolates.

Conclusions: Enterococcal strains from oral infections had a high virulence capacity, showed bacteriocin production and had numerous plasmids. They were generally susceptible to ampicillins but were resistant to clindamycin, commonly used in dentistry, and no VRE-strain was found.

Keywords: *Enterococci*; *oral mucosal infections*; *opportunistic infections*; *antibiotic susceptibility*; *virulence*

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Enterococci are common inhabitants of the human intestinal microflora and the genitourinary tract of men and women (1). Enterococci are also frequently present in most animals and common contaminants in food or used as starters in meat and cheese processing (2). Enterococci are potential pathogens in many body sites and enterococcal infections are often opportunistic and more prevalent in hospitalized patients (3). The general interest for enterococci and treatment of enterococcal infections has increased due to the appearance of antibiotic multiresistant strains and especially to the occurrence of vancomycin-resistant strains (vancomycin-resistant enterococci VRE).

Enterococci sometimes occur in the oral cavity, although in low amounts in healthy individuals (4). They do sometimes occur and predominate in oral infections. In persistent chronic endodontic failures, they often are major participants (5–9). Enterococci show prevalence from 3.7 to 35% in periodontitis (10–12), while they are more rarely found in peri-implantitis (13, 14). There are few reports on enterococci in oral mucosal lesions (15). Studies on compromised patients (16–18) have reported higher levels of enterococci than in healthy subjects. In the above-cited studies, enterococci are rarely specified and characterized with respect to phenotype, virulence and antibiotic susceptibility.

This study was conducted to evaluate some virulence and phenotype characteristics and the susceptibility against antibiotics commonly used in dentistry, of enterococcal isolates from oral mucosal and deep

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infection samples, collected in the department's microbiological diagnostic service during 2 years.

Materials and methods

Bacterial strains

During the period of 2006–2007 the oral microbiological diagnostic service at the Institute of Odontology at University of Gothenburg received 820 samples from mucosal and deep infections from dentists. The majority were from oral medicine and/or surgical clinics in the western area of Sweden. Altogether 61 enterococcal strains were collected from 43 patients with oral mucosal infection and from 18 patients with deep infections. The inclusion criteria were that the bacteria should be present in predominant numbers (e.g. moderately to heavy growth, see below) in a sample to reduce the risk that their presence was due to temporary colonization or contamination. In addition, one reference strain each of *Enterococcus faecalis* (OMGS 3199/ATCC 47077), and *Enterococcus faecium* (OMGS 3386/CCUG 542^T) from the laboratory collection were used as positive controls in the identification procedures.

Dentists in clinics situated in the western region of Sweden took the clinical samples and the majority came from dentists working in or close to hospitals. The indication for taking a sample was the patient's complaint or the dentist's clinical diagnosis of a general stomatitis; abnormal appearance of the mucosa or localized white or red lesions of the mucosa. In addition, samples taken in surgical departments from acute deep infections (abscesses) were included in the survey. The samples were transported and cultured as previously described (15). The plates were examined for typical colony morphology and were semi-quantified according to the scale previously published (15). Very sparse growth was used for colonies <10, sparse growth for 10–100, moderate growth for 100–1,000, heavy growth for 1,000–10,000 and very heavy growth for $>10{,}000$ colonies.

Phenotype characterization

All isolates were checked for growth on bile-esculine (Enterococcosel agar plate, BBL, Becton, Dickinson and Company, Sparks, MD) and tested for gelatinase activity and hemolysis. Gelatinase activity was assessed by inoculation of the strains in a broth containing 3% gelatine, which was then incubated in 37°C for 1–2 days in an aerobic atmosphere. After 1 h cooling of the tubes in a refrigerator, positive gelatinase activity was recorded as degradation of the gelatine to liquid. Hemolysin activity was recorded as a clear halo around each colony after growth on a blood agar plate with 4% horse blood.

Genotype characterization

All strains were genotypically tested with 16S rRNA gene sequence PCR as previously described in detail by Sedgley et al. (9) using primers to the virulence genes: *efa A* (endocarditis gene), *gel E* (gelatinase gene), *ace* (collagen binding antigen gene), *asa* (aggregation substance gene), *cyl A* (cytolysin activator gene) and *esp* (surface adhesin gene).

Antibiotic susceptibility

Routine screening for antibiotic susceptibility was performed using blood agar plates and the disc diffusion method (AB Biodisk, Solna, Sweden) against: penicillin G, ampicillin, amoxicillin, clindamycin, erythromycin, tetracycline, doxycycline, ciprofloxacin, gentamycin and vancomycin. After incubation, the diameter of the inhibition zone of each strain was measured and the strains were graded as sensitive (S), intermediate (I) and resistant (R). Minimal inhibitory concentration (MIC) was determined using the E-test method (AB Biodisk) against penicillin V, amoxicillin and clindamycin. Vancomycin was also included in order to confirm presence of tentative VRE strains. The MICs were read from the intercept where the elipse inhibition zone intersected with the scale. The MICs including 90 and 50% of the strains were calculated.

Bacteriocin testing

Bacteriocin production was tested according to Sedgley et al. (4) against the following bacterial target strains: *Enterococcus faecium* strain OMGS 3386, *Enterococcus faecalis* strain OMGS 3382 (bacteriocin positive strain termed GS31 in Sedgley et al. (9)) and *E. faecalis* strain OMGS 3199 (bacteriocin negative control termed GS3 in Sedgley et al. (9)), *Streptococcus mutans* (OMGS 2482), *Streptococcus mitis* (OMGS 1770), *Streptococcus oralis* (OMGS 2470), *Lactobacillus fermentum* (OMGS 3182) and *Lactobacillus rhamnosus* (OMGS 3179). The target (indicator) strains were grown overnight in BHI broth (BBL) and then 0.5 ml (OD_{600} 0.8–1.0) was added to 10 ml of liquefied soft agar (0.75%) and poured on a BHI agar plate. After solidification, samples from single colonies of the producer strains (60 *E. faecalis* strains) were placed on the agar. After aerobic incubation overnight at 37°C, clear zones were visible around the bacteriocin producing strains. The zones were graded as strong, moderate, weak and negative with reference to their size. Some producer strains seemed to interact with other enterococcal target (indicator) strains giving a turbid zone around the colonies instead of a clear one.

Plasmid determination

The presence of plasmids in each strain was estimated according to Engbrecht et al. ((19), basic protocol 1:

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Miniprep by alkaline lysis). Bacterial cells grown overnight in 1.5 ml Brain heart infusion broth (BBL) were centrifuged during 1 min and the supernatant was removed. The pellet was re-suspended in glucose/tris/EDTA (GTE) solution, with 2 mg/ml lysozyme (Roche, Stockholm, Sweden) and kept at room temperature for 30 min before 200 µl NaOH (0.2N in 1% SDS) solution was added. After mixing, 150 µl of potassium acetate solution was added for neutralization, vortexed and placed on ice for 5 min. The cell debris and chromosomal DNA were spun down and the supernatant was transferred to a new tube with 0.8 ml of 95% ethanol and kept at room temperature for precipitation of nucleic acids (plasmid DNA and RNA). The supernatant was removed and the pellet washed with 1 ml of 70% ethanol and dried. After the pellet was re-suspended in 30 µl TE buffer/0.1 mg/ml RNase, a volume of 3–5 µl was used as a restriction digest. Plasmid DNA restriction fragments were separated on 0.7% agarose gels in TBE buffer. The gels were stained with ethinium bromide for 30 min and the bands were made visible by fluorescence under UV light.

Results

Altogether 61 enterococcal strains were isolated during the period of 2006–2007. Sixty of the 61 isolates were identified as *E. faecalis* and 1 as *E. faecium*. Samples from 39 females and 32 males were included and the age ranged from 3 to 99 years (mean 63.2, median 67). Forty-three strains, including the *E. faecium* strain, were isolated from oral mucosal lesions and 18 from deep oral infections. While 27 (62.8%) of the 43 isolates from mucosal lesions came from patients with various forms of general diseases (Table 1), the majority (77.8%) of the deep infections isolates came from patients with acute infections with local and specified symptoms. Most of the mucosal infection isolates came from the tongue and from pus in case of a deep infection.

Table 1. Patient characteristics in relation to sampling from mucosal or deep oral infections and the frequency of enterococci (60 *E. faecalis*, 1 *E. faecium*) in high numbers in each category

Patient characteristics	No. of patients (%)	
	Oral mucosal infection	Oral deep infection
General disease[a]	27 (62.8)	4 (22.2)
Local symptoms only (specified)[b]	8 (18.6)	14 (77.8)
Local symptoms only (uncertain)	8 (18.6)	0
Total	43	18

[a]General diseases included: immune compromised (leukemia, transplantation, radiation, and cancer) 13; cardiovascular diseases: 3 (two sepsis); rheumatoid arthritis: 2; bone disease: 1; dislabeled, demens: 1; brain disease: 1; B12 anemia: 1; renal disease: 2; liver disease: 1; lung disease: 1; Parkinson's disease: 1

[b]Local symptoms all included burning sensations and clinically visible inflammation

Among the patients with oral mucosal infections, four were on antibiotics (penicillin, isoxapenicillin or amoxicillin), two were on antiviral medication, six were on antifungal medication, nine had no antimicrobial medication and for 22 the data were missing. For the patients with oral deep infections, all 18 patients were on antimicrobial medication, 10 used clindamycin (3 in combination with ciprofloxacin), 1 penicillin, 1 isoxapenicillin, 1 cephalosporin + vancomycin. For five subjects, data on type of antibiotics used were lacking.

α-Hemolytic streptococci were the most common co-isolates in samples from the oral mucosa. Also *Prevotella* spp. and *Fusobacterium* spp. were quite common in the predominant flora on the tongue as well as *Haemophilus parainfluenzae* on the buccal mucosa. Notably many of the mucosal samples had other opportunists in significant quantities. Twenty-eight had *Candida*, 21 enteric rods and 5 *Staphylococcus aureus* in heavy growth.

Gelatinase was detected in 6 (10%), hemolysin in 10 (16.7%) and plasmids in 30 (50%) of the *E. faecalis* strains (Table 2). None of these were detected in the *E. faecium* strain. It was, however, positive for the six investigated virulence genes for which the detection frequency ranged from 93 to 100% of the *E. faecalis* isolates (Table 2).

Clearly visible zones around enterococcal strains indicating a significant production of bacteriocins were detected for 10 isolates against enterococccal target strains (Table 3). Little effect was noticed against oral streptococci and lactobacilli, except for *S. salivarius* for which six *E. faecalis* strains and the *E. faecium* strain showed growth inhibition.

Ampicillin and amoxicillin showed the strongest effect on the enterococci but only 57.4 and 31.1%, respectively, of the isolates were susceptible, as screened routinely by the disc diffusion method. Using the E-test method for estimation of MIC values, all of the *E. faecalis* strains were susceptible as well as the *E. faecium* isolate for amoxicillin. Of the strains from mucosal and deep infection samples, 90% showed minimal inhibitory concentrations (MIC_{90}) of 256 µg/ml or more against clindamycin (Table 4). All enterococcal strains were sensitive to vancomycin.

Discussion

This study describes phenotypic and genotypic characteristics of 60 *E. faecalis* and 1 *E. faecium* isolates from oral mucosal infections and deep oral infections.

Table 2. Phenotype and genotype characteristics of the 60 *E. faecalis* strains isolated from oral infection

Characteristic	No of strains (%)		
	Mucosal infections ($n = 42$)	Deep infections ($n = 18$)	All infections ($n = 60$)
Gelatinase	4[a] (9.5)	2[b] (11.1)	6 (10.0)
Hemolysin	5 (11.9)	5 (27.8)	10 (16.7)
Plasmids	19[c] (45.2)	11[d] (61.1)	30 (50.0)
efaA positive	39 (92.8)	17 (94.4)	56 (93.3)
gelE positive	42 (100.0)	18 (100.0)	60 (100)
ace positive	41 (97.6)	18 (100.0)	59 (98.3)
asa positive	41 (97.6)	17 (94.4)	58 (96.7)
cylA positive	42 (100.0)	18 (100)	60 (100)
esp positive	40 (95.2)	16 (88.9)	56 (93.3)

[a]2 strains also positive for hemolysin
[b]2 strains also positive for hemolysin
[c]15 strains containing 1 plasmid, 1 containing 2, 1 containing 3, 1 containing 4 and 1 containing 6
[d]6 strains containing 1 plasmid, 3 containing 2, 1 containing 3 and 1 containing 6

No significant differences between the isolates due to their origin were disclosed.

Enterococci are widely distributed in the environment and they are predominant in the upper part of the intestine. They are also considered transient in the oral cavity and may even occur in low numbers in the resident flora of some individuals (4). It is an important micro-organism in foods, either as probiotic, starters or contaminants in meat and cheese handling or processing (2, 20, 21). On the other hand, they are important pathogens, and reported as a major cause of nosocomial infections and are commonly isolated in urinary tract infection, in the blood steam and at surgical sites (3). The predominant species in infections is *E. faecalis* followed by *E. faecium*. *E. faecium* has, however, gained much attention lately since it is reported to be frequently identified among VRE isolates (22).

Studies on oral enterococci have been quite extensive due to their common appearance in root canal infections. Less is known on oral transient/resident strains and strains from oral infections apart from endodontic ones (15). In this study of samples arriving in the laboratory during 2 years, we found enterococci to be part of the predominant flora in 61 cases with acute symptoms from the mucosa or from deeper located abscesses. The samples were rarely monoinfections, but rather accompanied by other oral bacteria or other opportunists. *E. faecalis* was present in amounts (moderate growth or more) that indicated them to be part of the infection process and not only as resident bystanders. The species distribution in oral mucosal or deep oral infections seems to be very similar to infections in other body sites, with the majority being classified as *E. faecalis*. Enteroccocci in oral mucosal infections are classical

Table 3. Bacteriocin production pattern among 60 *E. faecalis* isolates

Target bacteria	No. of positive strains (graded activity)				No. of negative strains
	Strong	Moderate	Weak	unclear	
S. oralis (OMGS 2470)	1	0	2	0	57
S. mitis (OMGS 1770)	1	0	7	0	52
S. salivarius (OMGS 2473)	7	0	4	0	49
L. fermentum(OMGS 3182)	3	0	4	0	53
L. rhamnosus (OMGS 3179)	3	5	7	0	45
L. casei (OMGS 3184)	1	1	2	0	56
L. acidophilus (OMGS 3185)	9	2	0	6	43
E. faecalis (OMGS 3382)	10	4	15	17	14
E. faecalis (OMGS 3199)	10	3	13	18	16
E. faecium (OMGS 3386)	10	5	12	19	14

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Table 4. Antibiotic susceptibility (MIC_{90} μg/ml, including 90% of the strains and MIC_{50} μg/ml, including 50% of the strains) oral mucosal and deep infection enterococcal isolates using E-test

	Mucosal isolates ($n = 43$)		Isolates from deep infections ($n = 18$)	
Antibiotics	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}
Penicillin V	1.5	0.5	1.5	0.5
Amoxicillin	2	0.5	4	0.5
Clindamycin	256	24	256	16
Vancomycin	4	3	4	3

opportunists and, similar to other opportunists such as *Candida* spp., *S. aureus* and enteric rods (16), they appear commonly in patients who are immunosuppressed for various reasons. This was also the case in this study, where the patients were generally older and where 62.8% had general and systemic diseases. This ecological disharmony may be a consequence of the hard medical treatment, which has reduced the resident streptococci, *Neisseria*, *Haemophilus* and anaerobes (*Prevotella* and *Fusobacterium* spp.). This condition is difficult to treat as long as the medication is ongoing and the treatment will consequently be symptomatic.

This study shows that deep enterococcal infections in the jaws (abscesses, bone sequestration and open surgical wounds) do occur and should be considered in the choice of antibiotics. All 18 cases were on antimicrobial treatment and 10 of them were on clindamycin, a drug that is not suitable for enterococcal infections. Unfortunately, clindamycin prescription by dentists is increasing, probably due to overuse or recommendations to use clindamycin in penicillin allergy cases (23). The frequent occurrence of enterococcal infections in the oral cavity points to the importance for an appropriate microbiological diagnosis and susceptibility test in cases of need for antibiotic treatment.

Enterococcal species do not display a large panel of virulence factors of the type seen in other Gram-positive cocci, such as *S. aureus* and hemolytic Group A streptococci. Factors that are commonly discussed for enterococci are hemolysin (cytolysin), gelatinase, aggregation factor and surface adhesins (24, 25). The frequency of hemolysin and gelatinase positive phenotypes was low (10 and 16.7%, respectively) although the presence of the genes (*cylA* and *gelE*) was identified in almost all of the *E. faecalis* strains. The frequency of hemolysin and gelatinase positive strains varies greatly among the studies and the clinical conditions from which the enterococci were isolated. Sedgley et al. (9) reported gelatinase in 93% of primary endodontic infections but only 25% in retreatment cases. Gelatinase positive *E. faecalis* has further been isolated in a large proportion of hospitalized patients and patients with endocarditis (26). Interestingly, previous studies reported that none of 35 endodontic isolates was hemolytic (9) while 37% of clinical isolates and 31% of fecal isolates from hospitalized patients were hemolytic (26). Among healthy Norwegian infants, 29% of the *E. faecalis* strains were cytolysin positive and 48% positive for gelatinase, while the genes *cylL* and *gelE* were identified in 52 and 94% of the strains (27). An even greater discrepancy between the genotype and phenotype characteristics was seen in the present study. It seems that enterococci participating in clinical infections express more of the virulence factors than enterococci in chronic persistent endodontic cases where non-expressed ('sleeping') genes are common. This is in line with Creti et al. (28), who reported that gelatinase activity was correlated with sleeping genes of *gelE*. They also concluded that strains from endocarditis and commensals expressed a lower number of virulence factors than isolates from other sites, while strains from urinary tract infections had expressed the most. The presence of the genes in this study shows a similar pattern to that previously reported for endodontic and non-endodontic oral strains, where *efaA*, *gel E*, *ace* and *asa* were found in 100% of the isolates (4). Two other genes *cyl A*, coding for hemolysin and *esp*, coding for surface adhesion, were present in 100 and 93.3% of samples respectively in this study. This is considerably higher than reported for isolates from healthy subjects, where they were present in 18 and 60% of samples respectively (4). Both these genes are present in pathogenicity islands (PAI) that are suggested to be enriched among infection-derived enterococcal isolates (29). Coque et al. (26) found *cylA* in 50% of the enterococcal strains in bacteremia cases, in 11% of endocarditis cases and none from stool samples. Our isolates that were related to acute infections may be another indication of this enrichment, despite the phenotypic expression of, for example, hemolysin was low. Eaton and Gasson (20) also concluded that medical isolates had more virulence genes than *E. faecalis* isolates from food that in turn had more than those used as starters in food processing. Conclusively, almost all isolates from the oral mucosa as well as deep infections seem to have the capacity to produce and express all the common virulence factors. This might also suggest that oral isolates are not primarily obtained in the oral cavity

as contaminants from food, but rather transmitted from human sources.

An important aspect of virulence is how the specific bacteria can compete with the resident flora in the normal ecology of a body site. In healthy individuals, the ecology is to some extent self-limiting and does not allow overgrowth of other microorganisms. Apparently, enterococci are sometimes part of the resident flora, even though their prevalence in healthy individuals (students) is quite low. Enterococci are well-known producers of bacteriocins (4). However, in this study we have seen that the bacteriocins are targeted mostly at other enterococcal strains rather than against other oral species such as α-hemolytic streptococci and lactobacilli. This is in agreement with Sedgley et al. (30), who identified 'siblicides' among the enterococcal strains. Enterococci are believed to be important biofilm participants (31–34). Even if enterococci are not present in high numbers in the dental plaque biofilm, they may have an important role as a reservoir for antibiotic-resistant genes, which can be transferred to other bacteria in the biofilm (12, 35).

Twenty-two of the *E. faecalis* isolates (37%) also contained plasmids, of which nine had several (range 2–6). Sedgley et al. (9), found one to four plasmids in 25 out of the 31 endodontic strains. Plasmids are also commonly found in medical and food isolates and exchange of plasmids between enterococcal strains are potentially likely (21). Plasmids are also commonly found in medical and food isolates and exchange of plasmids between enterococcal strains are potentially likely (21). Thus, conjugation and horizontal spread of genes including resistance genesis are probably quite common in humans, further explained by the common production of pheromones (9). Pheromones are a kind of clumping factor that supports close contact with the bacterial cells, allowing conjugation to take place. The supposedly frequent conjugation between enterococcal cells facilitates the spread of antibiotic resistance.

Conclusions

Enterococci, predominantly *E. faecalis* were detected in both mucosal and deep oral infections. The frequency of hemolysis and gelatinase positive strains was low but almost all isolates had the virulence genes *efaA*, *gelE*, *ace*, *asa*, *cylA* and *esp*. The isolates produced bacteriocins, mostly directed against other enterococcal isolates. Of the isolates, 37% had plasmids. The 61 enterococcal isolates showed a tested antibiotic susceptibility for amoxicillin but were resistant to clindamycin. These are two of the most used antibiotics in dentistry for severe oral mucosal and deep infections. All isolates were susceptible to vancomycin.

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Conflict of interest and funding

There is no conflict of interest in the present study for any of the authors.

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