Brief communication (Original)

Genotypic characterization of *Enterococcus* species isolated from the oral cavity and their pattern of antibiotic susceptibility

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Background: Enterococci comprise a small share of oral flora and occur as contaminants in food such as meat and cheese. They are commonly encountered in patients with infections of oral tissues such as necrotic pulp, root canals and periodontitis.

Objectives: To characterize the genotypes of *Enterococcus* isolated from patients with oral infection and to determine their antibiotic susceptibility.

Methods: We genotypically characterized a number of isolates of *Enterococcus* species derived from patients with various oral infections. All the isolates were tested for the presence of putative virulence genes; *efaA* (gene for endocarditis), *gelE* (gene for gelatinase), *ace* (gene for collagen binding antigen), *asa* (gene for aggregation substance), *cylA* (gene for cytolysin activator), and *esp* (gene for surface adhesin) of *E. faecalis* and *E. faecium* as described in previous studies.

Results: *E. faecalis* dominated in oral infections compared with *E. faecium*. Isolates were susceptible to most antibiotics (only four were resistant to vancomycin). To our knowledge, the first report from this region.

Conclusions: Treatment of enterococcal infections of the oral mucosal and deep oral areas necessitate microbiological diagnosis and identification of resistance strains.

Keywords: Antibiotic susceptibility pattern, *Enterococcus* species, genotypic characterization, oral infections

Enterococci are commonly encountered and predominate oral infections; especially those associated with necrotic pulp, root canal infections, and periodontitis [1, 2]. They comprise only a minimal proportion of the oral flora, but frequently occur as contaminants of food, such as meat and cheese [2]. Because *Enterococcus* are potential nosocomial oral pathogens, the emergence of multiresistant strains has increased interest in their pathogenicity and potential drug resistance [3].

Many studies have demonstrated a role of *Enterococcus* in causing gingivitis, periodontitis, and carious lesions, other infections of root canals, and aerobic and anaerobic endodontitis [4]. Moreover, these bacteria form biofilms in the root canals so as to survive with increased virulence and cause deep oral infections [5-7]. However, prevalence in oral infections has been relatively low, ranging from 3.7% to 35% in periodontitis. Previous reports have demonstrated the presence of enterococci in periodontitis of immune-compromised individuals [2].

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Recent studies have demonstrated *E. faecalis* and *E. faecium* as two common species recovered from human oral infections with corresponding virulence factors such as gelatinase production, hemolysis, and biofilm formation. *E. faecalis* has been shown to destroy dentinal tubules in vitro [1, 2]. In addition, enterococcal virulence correlates with colonization of host tissue, competition with other bacteria, modulation of host defense mechanisms, and invasion and abscess formation from toxins or inflammatory processes. From the endodontic point of view, virulence factors govern enterococcal action, aggregation, surface adhesion, extracellular superoxide, gelatinase, hyaluronidase and cytolysin (hemolysin) [1]. Aggregation substances mediate binding to the extracellular matrix proteins, especially collagen type 1, a major component of dentin. Virulence factors are associated with biofilm formation and bacterial primary attachment. Gelatinase, an extracellular zinc-containing metalloprotease, hydrolyzes gelatin, collagen, fibrinogen, casein, hemoglobin, insulin, certain *E. faecalis* sex-pheromone-related peptides, and some other bioactive peptides. Cytolysin supports the growth of enterococci, changing oxygen conditions, and increased amounts are produced in anaerobic conditions [1, 4].

The significance of *Enterococcus* in oral infections is not often considered and reported. Even less attention has been paid to phenotypic and genotypic virulent characters of these microbes from dental infections. Because data on virulence factors are necessary to describe the pathogenic cycle of enterococci, we investigated the presence of diverse virulence factors in *E. faecalis* and *E. faecium* isolates from oral infections and their antibiotic patterns of sensitivity and resistance during a 4-year study period.

Materials and methods
Bacterial strains

During 2011 to 2014, at the Dental Health Clinic, College of Applied Medical Sciences (CAMS), King Saud University (KSA), Riyadh, a total of 126 enterococcal cultures from various oral mucosal and deep site infections were isolated for routine diagnosis. These isolates were speciated and categorized as 91 *E. faecalis* and 35 *E. faecium* strains by typical colony morphology on bile esculin agar and other biochemical tests. The Research Ethics Committee, CAMS Research Center, KSA, waived patient consent and formal IRB consideration of this study in 2011 in compliance with principles of the contemporary Declaration of Helsinki, because only bacterial isolates were used as part of the study and the investigators did not use the original routine clinical human tissue samples. The patients providing the samples from which isolates were derived for routine diagnosis were not identified by the investigators; patient anonymity was completely protected by unlinked coded isolates. All experimental protocols were conducted in accordance with the approved guidelines for work with bacterial isolates at Riyadh, KSA.

Phenotypic characterization

Gelatinase activity of the strains was assessed as described previously [4] by inoculating the strains in broth containing 3% gelatin and evaluating liquefaction of the gelatin. Hemolytic activity was measured as a clear zone around colonies on a blood agar plates [8]. Biofilm formation was determined as described previously by growing microorganisms in polystyrene microplates and washing and staining with crystal violet. Absorbance was measured by determining the optical density of the wells and the cells were classified based on the adherence [9].

Antibiotic susceptibility

Antibiotic susceptibility testing was determined on blood agar plates by disc diffusion using the following antibiotics: penicillin, amoxicillin, clindamycin, erythromycin, tetracycline, ciprofloxacin, gentamycin, vancomycin, and teicoplanin. Minimal inhibitory concentration (MIC) was determined by E-test against penicillin, amoxicillin, vancomycin, and teicoplanin.

Genotypic characterization

Species identification was determined by performing specific gene uniplex polymerase chain reaction (PCR) for *E. faecalis* and *E. faecium* as previously described [3]. Identification of putative virulence genes; *efaA* (gene for endocarditis), *gelE* (gene for gelatinase), *ace* (gene for collagen binding antigen), *asa* (gene for aggregation substance), *cylA* (gene for cytolysin activator), and *esp* (gene for surface adhesin) of *E. faecalis* and *E. faecium* were performed as described previously (**Table 1**).

Table 1. Primers used to identify species and to detect the virulence genes

Gene	**Primers Sequence 5′–3′**	**Product**	**References**
E. faecalis	5′-ATCAAGTACAGTTAGTCT-3′ 5′-ACGATTCAAAGCTAACTG-3′	941	[3]
E. faecium	5′-TTGAGGCAGACCAGATTGACG-3′ 5′-TATGACAGCGACTCCGATTCC-3′	685	[3]
gelE	5′-ACCCCGTATCATTGGTTT-3′ 5′-ACGCATTGCTTTTCCATC-3′	419	[9]
esp	5′-TTGCTAATGCTAGTCCACGACC-3′ 5′-GCGTCAACACTTGCATTGCCGA-3′	932	[8]
cylA	5′-GACTCGGGGATTGATAGGC-3′ 5′-GCTGCTAAAGCTGCGCTTAC-3′	688	[8]
ace	5′-GGAATGACCGAGAACGATGGC-3′ 5′-GCTTGATGTTGGCCTGCTTCCG-3′	616	[8]
efaA	5′-GCCAATTGGGACAGACCCTC-3′ 5′-CGCCTTCTGTTCCTTCTTTGGC-3′	688	[8]

Results

In all, 126 enterococcal isolates were obtained from 2011 to 2014 of which 91 (72%) were identified as *E. faecalis* and 35 (28%) as *E. faecium*. Species-specific PCR detected a 941 and 685 bp sequence in all of the 91 *E. faecalis* and 35 *E. faecium* strains respectively. Sixty-four (70%) *E. faecalis* and 23 (66%) *E. faecium* were from oral mucosal infections, while 27 (30%) *E. faecalis* and 12 (34%) *E. faecium* were from deep oral infections. Gelatinase activity was detected in 82 (90%) *E. faecalis* and 18 (51%) *E. faecium* strains. A clear halo zone of hemolysis was found around 66 (73%) *E. faecalis* and 15 (43%) *E. faecium* strains respectively.

Antibiotic susceptibility testing showed a good effect for most of the antibiotics tested on the enterococci, but with only 95% efficiency. By E-test, the estimation of MIC revealed 100% susceptibility to teicoplanin, 96% to vancomycin, and 94% to penicillin and amoxicillin. Teicoplanin and vancomycin showed a maximum effect on enterococcal strains, whereas penicillin and amoxicillin showed comparatively lower effect on these strains. Four of the *E. faecalis* strains tested were resistant to vancomycin with an MIC ≥ 256 µg/ml. No vancomycin-resistant enterococci was seen among the *E. faecium* strains.

Overall, the distribution of virulence factors showed 86/91 (95%) *E. faecalis* and 33/35 (94%) *E. faecium* carrying *esp*, and 82/91 (90%) *E. faecalis* and 30/35 (86%) *E. faecium* strains carrying *efaA*. Furthermore, all (100%) the enterococcal strains isolated carried *gelA*, *ace*, and *asa*. *CylA* was seen in 71/91 (78%) *E. faecalis* and 15/35 (43%) *E. faecium* strains and *asa1* was seen in 89/91 (98%) *E. faecalis* and 34/35 (97%) *E. faecium* strains. Biofilm production was found in 48/91 (58%) *E. faecalis* strains, whereas only 15 (43%) *E. faecium* strains showed biofilm production. Most of the biofilm formed by *E. faecalis* was strongly adherent compared with that from *E. faecium* strains, which formed only weakly adherent biofilms.

Considering the vancomycin resistance in vitro in the 4/91 *E. faecalis* isolates, we evaluated the gene responsible for the vancomycin resistance in these stains and detected the presence of *VanA* (4%) in all the four strains.

Discussion

Enterococci are commensals of the human gastrointestinal tract and are a transient flora of the oral cavity. [3]. Even though they occur in low numbers in the oral cavity as resident flora, they are important in nosocomial infections. Enterococci have gained more importance as an endodontic pathogen because of their common occurrence in root canal infections, gingivitis, periodontitis, and deep oral abscesses, and because of the frequency of vancomycin-resistant *Enterococcus* strains [1, 2].

In this study we focused on the phenotypic and genotypic characterization of 91 *E. faecalis* and 35 *E. faecium* isolates from oral mucosal and deep oral infections. Studies on enterococcal virulence and the essential factors for its pathogenicity are quite complex

and multifactorial [1]. Several genes and factors are involved in the endodontic pathogenesis of enterococci. Moreover, the frequent use of clinical regimens for treating dental infections increase the expression of antibiotic resistance to the conventional drugs.

In the present study, we identified *E. faecalis* (72%) as the predominant strain of enterococci followed by *E. faecium* (28%). Gelatinase activity was characterized by the liquefaction of gelatin encoded by *gelE* and the hemolytic activity was characterized by a zone of hemolysis around the colony encoded by *cylA* [10]. In our study, (100%) of the strains carried the *gelE*; whereas, only 78% strains carried the *cylA*. However, these phenotypic characters could not be correlated with the presence of the *gelE* and *cylA*. We found strains with *gelE* and *cylA* did not express gelatinase or hemolytic activity in vitro. This may be the result of the presence of a mutated gene or the involvement of other genes responsible for expression control [11, 12, 13].

Dahl n et al. observed that the frequency of hemolysis and gelatinase activity was as low as 10% and 16.7% respectively, even though *cylA* was present in all the strains [2]. In the present study, we found a high frequency of hemolysis (64%) and gelatinase (79%) activity among the strains (81/126 and 100/126 respectively), although 68% *cyl A* and 100% *gel E* were identified in 86/126 and 126/126 strains respectively.

Based on our findings, the presence of *esp* and *asa* could not be directly linked with biofilm production. Although 95% and 98% *E. faecalis* and 94% and 97% *E. faecium* were positive for *esp* and *asa1* respectively, only 58% *E. faecalis* and 43% *E. faecium* formed biofilms in vitro. This suggests the influence of other extrinsic and intrinsic factors in the expression of the biofilm phenotype. By contrast with previous studies, we did not find any significant association of *esp*, *gelE*, or *asa* with biofilm formation [14, 15]. This is consistent with findings reported by Dworniczek et al. [16].

Up to 6% (5/86) of the *cylA*-positive strains and 21% (26/126) of the *gelE*-positive strains did not express the corresponding phenotype. This is concordant with a previous study wherein unexpressed sleeping genes were reported [2, 8]. Other genes; *ace*, *efaA*, and *asa1* were found in 100%, 88%, and 98% strains respectively. Sedgley et al. reported 100% of *efaA*, *ace*, and *asa* [17],

whereas in our study the percentage of *efaA* and *asa1* was relatively low.

Like previous investigators, we did not find any significant discrepancies in virulence traits of the isolates from different sources [2]. Very recently, *Enterococcus* strains have attracted increased research interest because of the emergence of multiple resistance to antimicrobial drugs, especially in nosocomial strains. Vancomycin resistant enterococci pose great challenges to clinicians, and have now disseminated worldwide. Pinheiro et al. observed 100% susceptibility of enterococcal isolates from oral specimens to amoxicillin, amoxicillin-clavulanic acid, and vancomycin, but to less extent to erythromycin, moxifloxacin, chloramphenicol, tetracycline, doxycycline, and ciprofloxacin [18]. Similar findings were reported by Sedgley et al. who observed the enterococcal isolates were susceptible to ampicillin, benzylpenicillin, gentamicin, and vancomycin [19]. Moreover a study by Salah et al. showed that all *E. faecalis* they isolated were susceptible to chloramphenicol, ampicillin, vancomycin, ciprofloxacin, and teicoplanin, but the isolates were much less susceptible to erythromycin [9]. We showed that all the *E. faecalis* and *E. faecium* strains were susceptible to teicoplanin, clindamycin, erythromycin, tetracycline, ciprofloxacin, gentamycin, but less susceptible to penicillin (94%), amoxicillin (95%), and vancomycin (97%). By contrast, our present study showed increased rates of resistance to the β-lactam antibiotics, and we also found 4 isolates to be resistant to vancomycin phenotypically and genotypically. Molecular studies showed the presence of *vanA* in all the 4 strains. To our knowledge, this study is the first report on vancomycin-resistant enterococci from oral infections in Saudi Arabia.

We also reported a high prevalence of virulence determinants including *gelE*, *ace*, *asa1*, *esp*, and *efaA*, which is consistent with the virulence process and the complexity of enterococci and supports the findings of Sharifi et al [20]. Further studies need to be performed to determine sequence types and the clonal relationship of the isolates. In the present study, we reported a large number of *Enterococcus* isolates from oral infections in a 4-year-study period. This frequency of enterococcal infections in the oral mucosal and deep oral areas necessitates accurate microbiological diagnosis and identification of resistance strains and also of the mechanism involved.

Acknowledgments

Conflicts of interest statement

The authors have no conflicts of interest to declare.

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