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# Antimicrobial susceptibility and molecular analysis of *Enterococcus faecalis* originating from endodontic infections in Finland and Lithuania

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**Background/aims:** *Enterococcus faecalis* strains with multiple antibiotic resistances can cause infections that are difficult to treat. The microbial flora in treatment-resistant apical periodontitis is dominated by *E. faecalis*, and is a potential source of infections at other sites.

**Material and methods:** Sensitivities to a range of antibiotics were determined for 59 endodontic *E. faecalis* isolates from Finland and Lithuania. The DNA sequence of the gene responsible for the species' intrinsic quinupristin-dalfopristin resistance, *lsa*, was determined from two isolates with diminished resistance. Four pairs of isolates from the same root canal were typed by pulsed-field gel electrophoresis.

**Results:** A high prevalence of resistance to rifampicin was found, whereas all isolates were susceptible or showed intermediate susceptibility to penicillin and ampicillin and four isolates were unusually susceptible to cefotaxime. No vancomycin or high-level gentamicin resistance was detected. Nine of 59 isolates were susceptible to quinupristin-dalfopristin. A fully quinupristin-dalfopristin-susceptible isolate also susceptible to clindamycin produced a truncated Lsa polypeptide, and an isolate with borderline quinupristin-dalfopristin-susceptibility had mutations proximal to the predicted ribosomal binding site. Pulsed-field gel electrophoresis showed that the same root canal could harbor two different strains of *E. faecalis* during the course of the same infection.

**Conclusion:** Despite the differing antibiotic usage in Finland and Lithuania, *E. faecalis* from endodontic infections in these countries showed similar susceptibility patterns with levels of resistance considered typical for the species, and decreased resistance to clindamycin and quinupristin-dalfopristin as well as lesions in the *lsa* gene which were similar to those described in other clinical isolates.

Key words: apical periodontitis; enterococcal sensitivity; Lsa-mutation

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Bacteria may be retained in the dental root canal system for many years (16). Several authors have reported *Enterococcus faecalis* as the most frequently isolated species in treatment-resistant dental root canal infections (8, 12, 20, 22, 25). The necrotic root canal is a secluded cavity inaccessible to the local immune system (35), and during an endodontic infection microorganisms may enter the periapical area by extrusion due to over-instrumentation or as a result of exacerbation (34, 36), possibly allowing spread to the general circulation and other body sites under altered local or systemic immunity, and potentially causing such bacteremias such as those which occur frequently during endodontic treatment (13). Thus far, the main focus in enterococcal research has been on the emergence of antibiotic-resistant strains in nosocomial infections, with only a

handful of studies published on the antimicrobial susceptibility of oral enterococcal isolates (5, 21, 22, 24, 28).

Multiply resistant enterococci isolated from hospital infections are of increasing concern as treatment is difficult. Closer attention has been focused on enterococcal susceptibility to different antibiotics since the first report of vancomycin-resistant enterococci in the UK in 1988 (15, 38, 39), as it limits treatment options, and because plasmid-borne resistance genes may spread by horizontal transfer to other strains. Quinupristin-dalfopristin is one treatment option for vancomycin-resistant *Enterococcus faecium* infections (26), but is not active against most *E. faecalis* due to the presence of the *lsa* gene, thought to encode a type II ABC protein (30). Lsa confers intrinsic resistance to streptogramin A and lincosamides (e.g. clindamycin) in *E. faecalis*, most likely by active efflux of the drugs (31). However, susceptibility to quinupristin-dalfopristin can occur in *E. faecalis*, as a result of mutations in the *lsa* gene (6).

The prevalence of antibiotic-resistant strains is associated with selective pressure by antibiotics (10). We aimed to study the antibiotic susceptibility of isolates of *E. faecalis* originating from endodontic infections in two different countries, Finland and Lithuania, as these differ both in their antibiotic guidelines and in their treatment of endodontic infections, calcium hydroxide being commonly used as an endodontic dressing in Finland, unlike in Lithuania (4, 9, 11, 19, 29, 37). We further aimed to compare their relatedness by pulsed-field gel-electrophoresis (PFGE) and to investigate the observed sensitivity to quinupristin-dalfopristin of some oral isolates of *E. faecalis*.

## Material and methods

### Enterococcal isolates

Fifty-nine *E. faecalis* isolates, 23 originating from root filled teeth with apical periodontitis, were collected by an endodontist at the Dental Faculty in Vilnius, Lithuania (20), and 36 root canal samples from apical periodontitis were collected by general practitioners in Finland and referred for identification at the Oral Microbiological Service Laboratory at the Institute of Dentistry in Helsinki. Among the Lithuanian isolates, a second sample was taken after instrumentation and irrigation with 10 ml NaOCl (2.5%) and 5 ml disodium EDTA (17%). In four cases, *E. faecalis* was reisolated from the same root canal, giving rise to four pairs of isolates. The isolates were identified as described (19, 20, 33).

### Susceptibility testing

The minimum inhibitory concentrations (MIC) of ampicillin, penicillin, cefotaxime, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, rifampicin, streptomycin, teicoplanin, tetracycline, vancomycin, clindamycin, quinupristin-dalfopristin, and linezolid were determined by agar dilution on Diagnostic Sensitivity Test (DST) agar (Oxoid, Basingstoke, UK), containing 5% saponin-lysed horse blood (TCS Microbiology, Buckingham, UK). Plates were inoculated with $10^4$–$10^5$ colony forming units (cfu)/spot and incubated at 37°C for 18 h. Isolates were considered susceptible or resistant according to breakpoint values recommended by the British Society for Antimicrobial Chemotherapy (BSAC). Isolates requiring cefotaxime MIC = 2 mg/l, vancomycin MIC ≥8 mg/l, clindamycin MIC = 4 mg/l or quinupristin-dalfopristin MIC = 2 mg/l were confirmed by Etest® (AB Biodisk, Solna, Sweden) or by agar dilution.

### Polymerase chain reaction (PCR) and DNA sequencing of the *lsa* gene in quinupristin-dalfopristin-susceptible strains

The *lsa* genes from one fully quinupristin-dalfopristin susceptible isolate (F10) and one borderline susceptible isolate (VP3-197) were PCR amplified as *ca*. 1 kb and 1.3 kb portions using the primer pairs abc2F (GGCAATCGCTTGTGTTTTAGCG) /*lsa*r1179 (TCAAGCGATTGACTTCTTTTTTC), and *lsa*f960 (CAAGTGGCTGAATATTTGAAG)/ abc2R (GTGAATCCCATGATGTTGATACC). PCR amplicons were cloned into pCR2.1 (Invitrogen, Groningen, the Netherlands) according to the manufacturer's instructions. The plasmid inserts were sequenced using M13 forward (GTAAAACGACGGCCAG) and reverse (CAGGAAACAGCTATGAC) primers, on a Beckman CEQ8000 DNA sequence analyser (Beckman Coulter, High Wycombe, UK).

### PFGE

Eight isolates were analyzed by PFGE, representing four pairs of isolates from the same tooth in four patients, before and after instrumentation and irrigation as previously described (20). PFGE was carried out essentially as described by Kaufmann (14). DNA was digested with *Sma*I and the macrorestriction fragments were separated on a CHEF DRII apparatus (Bio-Rad Laboratories, Hemel Hempstead, UK). Gel images were analyzed with BIONUMERICS software (Applied Maths, Sint-Martens-Latem, Belgium), and the percentage of relatedness was calculated by use of the Dice coefficient. The unweighted pair group method with arithmetic averages was used for clustering to produce a dendrogram with a band position tolerance of 0.6%.

### Statistical analysis of differences between sensitivity patterns of Lithuanian and Finnish isolates

The null hypothesis was tested on susceptible, intermediate or resistant strains in the Lithuanian vs. the Finnish material using the Chi-squared test with significance defined as $P < 0.05$.

## Results

### Antibiotic susceptibilities

The antibiotic susceptibilities of the 23 Lithuanian and 36 Finnish isolates are shown in Table 1. All isolates were sensitive to penicillin and ampicillin, glycopeptides, and linezolid. The borderline antienterococcal activity of ciprofloxacin (18) was apparent, with seven Lithuanian and nine Finnish ciprofloxacin-resistant isolates. Overall, the differences between the Finnish and Lithuanian isolates were not statistically significant. Resistance to erythromycin, which was most likely acquired, was observed in one Lithuanian (MIC = 8 mg/l) and five Finnish isolates (MIC ≥ 32 mg/l), tetracycline resistance in seven Lithuanian and 10 Finnish isolates (MIC = 16 mg/l), and high-level streptomycin resistance (MIC = 4096 mg/l) in one Lithuanian and three Finnish isolates. More unusually, all of the Lithuanian and 34 of 36 (94%) Finnish isolates required rifampicin MIC = 2 mg/l. Unusual cefotaxime susceptibility was also observed in four of the 23 Lithuanian isolates (17%) (MIC = 2 mg/l).

### Streptogramin A and lincosamide susceptibilities and *lsa* analysis

Despite the intrinsic resistance of *E. faecalis* to quinupristin-dalfopristin and clindamycin (1, 26, 27, 31), six of 23 (26%) Lithuanian and three of 36 (8%) Finnish isolates were susceptible to quinupristin-dalfopristin according to BSAC criteria (MIC = 2 mg/l) (2); two of these did not display a classic $LS_A$ phenotype (3) of the

*Table 1.* Susceptibility of 59 *Enterococcus faecalis* isolates from treatment-resistant endodontic infections

| | Minimum inhibitory concentration (MIC) (mg/l) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 2048 | 4096 |
| AMP | | | 5 | 46 | 8 | | | | | | |
| CHL | | | | | | 52 | 7* | | | | |
| CIP | | | 5 | 38 | 11 | 5 | | | | | |
| CTX | | 1 | 1 | 2 | 1 | 1 | 53* | | | | |
| ERY | 4** | 15 | 34 | | | 1 | | 5 | | | |
| GEN | | | | | | 1 | 25 | 31 | 2 | | |
| LIN | | | | 3 | 56 | | | | | | |
| PEN | | | 3 | 52 | 4 | | | | | | |
| RIF | | | 2 | 57* | | | | | | | |
| STR | | | | | | | | | | 54** | 5* |
| TEI | | 9** | 43 | 7 | | | | | | | |
| TET | | | | 16 | 25 | 1 | 17* | | | | |
| VAN | | | 3 | 30 | 25 | | 1 | | | | |

* The highest concentration tested. Isolates indicated had MICs ≥ this concentration.
**The lowest concentration tested. Isolates indicated had MICs ≤ this concentration.
AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; ERY, erythromycin; GEN, gentamicin; LIN, linezolid; PEN, penicillin; RIF, rifampicin; STR, streptomycin; TEI, teicoplanin; TET, tetracycline; VAN, vancomycin.

species, and required an MIC of quinupristin-dalfopristin of only 0.5 mg/l (Table 2), an MIC of clindamycin of 0.5 mg/l, and an MIC of erythromycin of 0.5 mg/l, thus showing no evidence of *ermB*-mediated resistance (MIC = 1 mg/l). The remaining seven quinupristin-dalfopristin-susceptible isolates were erythromycin susceptible (MIC 0.5–1 mg/l), and were among a larger group of 13 isolates with borderline quinupristin-dalfopristin susceptibility (MIC = 2–4 mg/l), three of which had reduced resistance to clindamycin (MIC = 2–4 mg/l). Isolates with normal resistance to quinupristin-dalfopristin (modal MIC = 16 mg/l) were consistently highly resistant to clindamycin (MIC > 8 mg/l) (Table 2).

The *lsa* DNA sequences of one fully susceptible isolate (F10) (clindamycin MIC = 0.25 mg/l and quinupristin-dalfopristin MIC = 0.25–0.5 mg/l) and one borderline-resistant isolate (VP3-197) (clindamycin MIC = 8 mg/l and quinupristin-dalfopristin MIC = 4 mg/l) were determined and compared with sequences from two quinupristin-dalfopristin and clindamycin-resistant strains (AY225127 and AE016955) (Fig. 1). VP3-197 possessed an AG insertion at positions − 7 and − 8 relative to the ATG codon, within the predicted ribosome binding site (Fig. 1) and an A-G mutation at position − 83, relative to the ATG codon, as observed previously for the borderline quinupristin-dalfopristin-susceptible isolate TX0263 (30). The Lsa coding sequence from VP3-197 was predicted to produce a full length polypeptide of 498 amino acids, but that of the fully quinupristin-dalfopristin and clindamycin-susceptible isolate, F10, did not. A premature TAG stop codon at nt +207 in the *lsa* gene resulted in a likely nonfunctional polypeptide of only 68 amino acids. In addition, F10 also had the A-G mutation at position − 83 relative to the *lsa* ATG codon. Notably the MIC (0.5 mg/l) to antibiotics of F10 resembled those described previously for an Lsa null-mutant (31) and nosocomial isolates producing truncated Lsa proteins (6, 31).

*Table 2.* Minimum inhibitory concentrations (MIC) of quinupristin-dalfopristin vs. clindamycin of the 59 endodontic *Enterococcus faecalis* isolates. The central cross indicates BSAC breakpoints

| | Quinupristin-dalfopristin MIC (mg/l) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | > 16 |
| Clindamycin MIC (mg/l) | | | | | | | | |
| 0.25 | | 2 | | | | | | |
| 0.5 | | | | | | | | |
| 1 | | | | | | | | |
| 2 | | | | 1 | 1 | | | |
| 4 | | | | | 1 | | | |
| 8 | | | | | | 1 | | |
| > 8 | | | | 6 | 4 | 8 | 32 | 3 |
| Total | | 2 | | 7 | 6 | 9 | 32 | 3 |

### PFGE

Two of the four pairs of isolates taken from the same teeth before and during therapy had identical PFGE profiles, indicating that the same strain was reisolated after instrumentation and irrigation. This may have resulted from treatment tolerance by the strain. Two other pairs of isolates originating from the same tooth showed different PFGE profiles, suggesting the coexistence of more than one strain within the same root canal (Fig. 2).

## Discussion

The lack of circulation in the root canal makes the use of systemic antibiotics inefficient in the treatment of endodontic infections, which necessitates the use of topical antimicrobial therapies and mechanical instrumentation. Nevertheless, antimicrobial susceptibilities of endodontic isolates are important as they may provide a reservoir for infection at other body sites (13, 32). In Finland, a Program for Antimicrobial Treatment Strategies has existed since 1998, whereas in Lithuania the use of antibiotics is subject to no national guidelines, nor do Lithuanian hospitals have common guidelines (4, 37). Despite a presumed different antibiotic usage in Finland and Lithuania, and the fact that *E. faecalis* is a commensal in the oral cavity (17), the study isolates had similar antibiotic resistance profiles. This could be attributed to the size of this sample set or could be a result of the ecologic pressure in the root canal. To our knowledge, this study is the first report of more than one *E. faecalis* strain in the same root canal, as indicated by PFGE, which further emphasizes the complexity of the endodontic microflora.

Overall, antibiotic susceptibilities among the *E. faecalis* study isolates were typical for the species, with infrequent mutational resistance and expected levels of acquired resistance. An exception was that 97% of these endodontic *E. faecalis* required a rifampicin MIC > 1 mg/l, contrasting with other studies where 64–74% of *E. faecalis* isolates required an MIC > 1 mg/l (23, 40). Notably, no vancomycin-resistant enterococci were found among the Lithuanian endodontic isolates. A vancomycin-resistant enterococci prevalence of 4–10%, depending on isolation site, among nosocomial enterococci (60% were *E. faecalis*) has been reported in Vilnius University Hospital (Lithuania) (11), but direct comparison with this data set is not possible due to the size of the

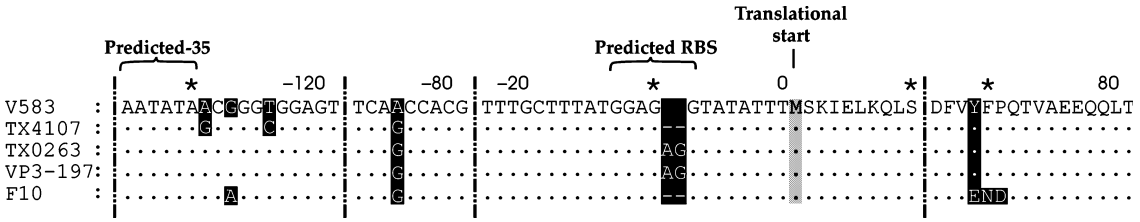


*Fig. 1.* Sequence differences of wild-type quinupristin-dalfopristin-resistant *Enterococcus faecalis* V583, and decreased quinupristin-dalfopristin resistance promoter variants TX4107 [AY737525][6], and TX0263 [AY737526][6]. Predicted −35 promoter[6] and ribosome binding site (RBS)[6] sequences are annotated. Nucleotide differences in the promoter regions of the *lsa* genes are shown as is the premature truncation of the peptide sequence of Lsa from F10.

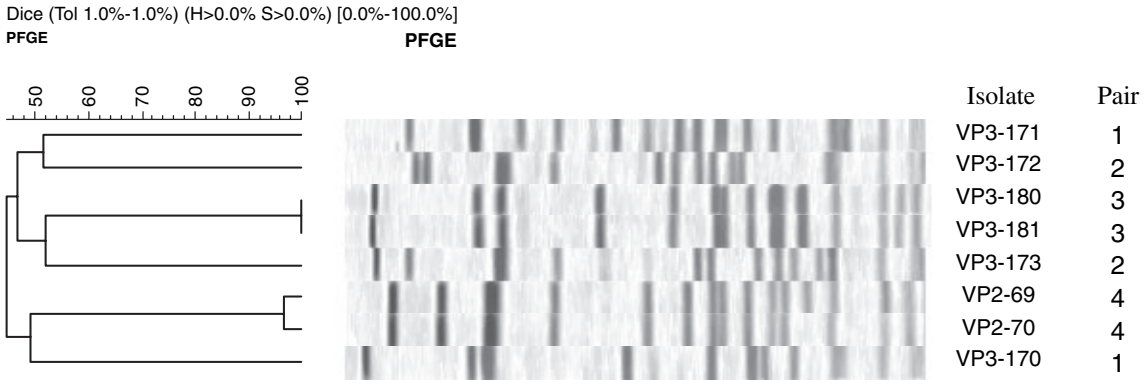


*Fig. 2.* Dendrogram and pulsed-field gel-electrophoresis (PFGE) banding patterns of four pairs of *Enterococcus faecalis,* taken from the same root canal, during the treatment course of the same infection. Pairs were: VP3-170 & VP3-171; VP3-172 & VP3-173; VP3-180 & VP3-181; and VP-69 & VP-70.

study sample, differences in the patient group types, exposure to antibiotics and the possibility that the above study may have included an outbreak.

Despite the intrinsic resistance of *E. faecalis* to quinupristin-dalfopristin and clindamycin (1, 26, 27, 31), we found a higher frequency of isolates sensitive to both of these antibiotics than the frequency observed amongst isolates from healthy subjects (7), but the significance of this cannot be easily estimated with the current data set. We observed a bimodal clindamycin and quinupristin-dalfopristin MIC distribution in *E. faecalis*, with a fully sensitive group which included the isolate predicted to produce a truncated Lsa protein, F10. Isolates with resistance to clindamycin but borderline susceptibility to quinupristin-dalfopristin also occurred and included isolate VP3-197, which had mutations in the predicted *lsa* promoter and ribosome binding site regions, which may decrease Lsa expression (30), although this remains unproven. In contrast to the fully susceptible group, this group appeared within a larger group including the fully resistant types. In addition to *lsa* expression analyses, further work is also required to confirm potential efflux of quinupristin-dalfopristin and clindamycin by Lsa and to identify potential selectors of this unusual susceptibility in *E. faecalis*.

Despite different antibiotic selection pressures in Finland and Lithuania we found little evidence of differing levels of resistance in endodontic isolates of *E. faecalis* from these countries. The typical antibiotic susceptibility profiles of these isolates indicated susceptibility to standard antibiotic therapies in the event of an exacerbation of apical periodontitis caused by *E. faecalis* with a following systemic infection or of endocarditis prophylaxis in compromised individuals.

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