
Antimicrobial susceptibility of *Enterococcus faecalis* isolated from canals of root filled teeth with periapical lesions

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Abstract

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Aim To test, *in vitro*, the susceptibility to different antibiotics of *Enterococcus faecalis* isolates from canals of root filled teeth with periapical lesions.

Methodology Twenty-one *E. faecalis* isolates, from canals of root filled teeth with persisting periapical lesions, were tested for their antibiotic susceptibilities. The following antibiotics were used: benzylpenicillin, amoxicillin, amoxicillin-clavulanic acid, erythromycin, azithromycin, vancomycin, chloramphenicol, tetracycline, doxycycline, ciprofloxacin and moxifloxacin. Minimal inhibitory concentrations (MICs) for the antimicrobial agents were determined using the E-test System (AB BIODISK, Solna, Sweden), and the *E. faecalis* strains classified as susceptible or resistant according to the guidelines of National Committee for Clinical Laboratory Standards (NCCLS). The strains were also tested for β -lactamase production with nitrocefin (Oxoid, Basingstoke, UK).

Results All strains were susceptible to penicillins *in vitro*, however, the MICs of amoxicillin and amoxicillin-clavulanic acid ($\text{MIC}_{90} = 0.75 \mu\text{g mL}^{-1}$) were lower than for benzylpenicillin ($\text{MIC}_{90} = 3.0 \mu\text{g mL}^{-1}$). All strains studied were also susceptible to vancomycin and moxifloxacin, whilst 95.2% were susceptible to chloramphenicol. Amongst the isolates, 85.7% were susceptible to tetracycline and doxycycline and 80.9% to ciprofloxacin. The MIC of erythromycin ranged from 0.38 to $>256 \mu\text{g mL}^{-1}$; only 28.5% of the strains were susceptible ($\text{MIC} \leq 0.5 \mu\text{g mL}^{-1}$). Limited susceptibility was also observed with azithromycin which was active against only 14.2% of isolates. No strains produced β -lactamase.

Conclusion *Enterococcus faecalis* isolates were completely susceptible, *in vitro*, to amoxicillin, amoxicillin-clavulanic acid, vancomycin and moxifloxacin. Most isolates were susceptible to chloramphenicol, tetracycline, doxycycline or ciprofloxacin. Erythromycin and azithromycin were least effective.

Keywords: antimicrobial susceptibility, endodontic failure, *Enterococcus faecalis*.

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Introduction

Enterococci are common inhabitants of the human gastrointestinal and genitourinary tracts (Murray

1990, Morrison *et al.* 1997). They have long been known to cause infections, such as enterococcal bacteraemia (Murdoch *et al.* 2002), infective endocarditis (Graham & Gould 2002) and urinary tract infections (Murray 1990, Morrison *et al.* 1997). Over the last two decades, enterococci have been recognized as the leading cause of hospital-acquired infection, paralleling their increased antimicrobial resistance to most currently approved agents (Mundy *et al.* 2000,

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Malani et al. 2002, Udo et al. 2002). Of the enterococcal species associated with colonization and infection in humans, *Enterococcus faecalis* is the most common species (Murray 1990, Mundy et al. 2000, Shepard & Gilmore 2002).

Enterococci are also able to colonize a variety of other sites, including the oral cavity (Smyth et al. 1987). These microorganisms have been associated with oral mucosal lesions in immunocompromised patients (Wahlin & Holm 1988), periodontitis (Rams et al. 1992) and root canal infections (Molander et al. 1998, Sundqvist et al. 1998, Noda et al. 2000, Peciulienė et al. 2000, 2001, Pinheiro et al. 2003a,b). Enterococci constitute a small percentage of the microbial species isolated from root canals of teeth with necrotic dental pulps (Sundqvist 1992, 1994). However, they are the most commonly isolated species from root canals of teeth with failed endodontic treatment. Enterococci are found in approximately 50% of the canals with refractory infection (Molander et al. 1998, Pinheiro et al. 2003a,b). Peciulienė et al. (2000, 2001) have reported an isolation frequency of enterococci as high as 70% when root filled teeth are associated with chronic apical periodontitis. *Enterococcus faecalis* is also the most common *Enterococcus* sp. isolated from root canals; other species are rarely found (Sundqvist et al. 1998, Peciulienė et al. 2000, 2001, Pinheiro et al. 2003a,b). *Enterococcus faecalis* is usually isolated in pure culture or as a major component of the flora of previously root filled teeth with chronic apical periodontitis (Peciulienė et al. 2000).

Antibiotics are not generally used to treat chronic infections, such as apical periodontitis, in root filled teeth. Chronic alveolar infections are associated with pulpless teeth which have no blood supply reaching the pulp space. Following the systemic administration of an antibiotic, the concentration reaching the root canal is negligible and unlikely to inhibit bacterial growth. Therefore, systemic antibiotic therapy is neither indicated nor likely to be beneficial (Abbott et al. 1990). Prophylactic use of antibiotics is, of course, another matter. Prophylactic use can be indicated if patients are considered at risk of infective endocarditis during endodontic treatment (Abbott et al. 1990, Debelian et al. 1995). In such cases, therapy should be directed primarily against the most important pathogens present.

Furthermore, periapical abscesses can originate from root filled teeth whose apical periodontitis continues following treatment. Some of them need antibiotic therapy prior to surgical treatment (Sousa et al. 2003).

However, it is important to emphasize that, because of ecological changes in an acute situation, the microbiota will change. Polymicrobial infections and obligate anaerobes are frequently found in canals of symptomatic root filled teeth (Pinheiro et al. 2003a). Therefore, bacteria other than enterococci will often be the main target of the antibiotics in the acute infection.

Enterococci possess a vast array of mechanisms that confer antibiotic resistance to a range of antibiotics including penicillin, the drug of choice (Hoellman et al. 1998, Shepard & Gilmore 2002). These microorganisms show intrinsic resistance to certain antibiotics such as cephalosporins, clindamycin and aminoglycosides (Murray 1990, Morrison et al. 1997). In addition to these intrinsic resistances, enterococci have acquired genetic determinants that confer resistance to many classes of antimicrobials, including tetracycline, erythromycin, chloramphenicol, and, most recently, vancomycin (Murray 1990, Morrison et al. 1997, Mundy et al. 2000, Shepard & Gilmore 2002).

Clinical isolates of *E. faecalis* recovered from root canal infections can demonstrate antimicrobial resistance to conventional treatment regimens recommended for dental procedures. Dahlén et al. (2000) have described enterococcal isolates resistant to benzylpenicillin, ampicillin, clindamycin, metronidazole and tetracycline; whilst Noda et al. (2000) have discovered strains that are resistant to cephalosporins. Previous studies (Pinheiro et al. 2003b) have found *E. faecalis* strains which show resistance to azithromycin and erythromycin. Thus, many antibiotics, traditionally used in odontogenic infection, may prove ineffective against *E. faecalis* so that information on alternative agents is required.

In the case of endodontic infections associated with enterococci, very limited antibiotic sensitivity data are available. The present study aimed to test, *in vitro*, the susceptibility to different antibiotics of *E. faecalis* isolated from canals of root filled teeth with periapical lesions.

Materials and methods

Clinical material

The *E. faecalis* strains were isolated from canals of root filled teeth with persisting periapical lesions as described by Pinheiro et al. (2003a) and Gomes et al. (2004). Patients were selected from those who attended the Piracicaba Dental School, SP, Brazil, with a need for

nonsurgical root canal retreatment. Patients who had received antibiotic treatment during the last 3 months or had a general disease were excluded from the study.

Sampling procedure

All coronal restorations, posts and carious defects were removed. After access cavity preparation, the teeth were individually isolated from the oral cavity with a rubber dam, and disinfection was carried out using 5.25% sodium hypochlorite. The root filling was removed using Gates Glidden drills (Dentsply Maillefer, Ballaigues, Switzerland) and endodontic files without the use of chemical solvents. Irrigation with sterile saline solution was performed in order to remove any remaining materials and to moisten the canal prior to sample collection. For microbial sampling, a sterile paper point was introduced into the full length of the canal (as determined with a preoperative radiograph), and kept in place for 60 s. The paper point samples from the root canal were transferred to a transport medium-VMGA III (Möller 1966, Dahlén *et al.* 1993) and taken to the microbiology laboratory for processing within 4 h.

Microbial identification

The samples were inoculated onto nonselective blood agar plates and incubated in aerobic and anaerobic conditions. The enterococcal identification was performed using colonial morphology, oxygen tolerance, Gram staining characteristics, and Rapid ID 32 Strep (Bio Merieux, Marcy-l'Etoile, France). In most of the cases, enterococcal strains, bile resistant, facultatively anaerobic Gram-positive cocci, were identified as *E. faecalis*.

Antimicrobial susceptibility tests

The susceptibility/resistance of 21 *E. faecalis* strains to 11 antibiotics was measured. The following antimicrobials were tested: benzylpenicillin, amoxicillin, amoxicillin-clavulanic acid, erythromycin, azithromycin, vancomycin, chloramphenicol, tetracycline, doxycycline, ciprofloxacin and moxifloxacin.

The antimicrobial susceptibility of isolates was investigated by means of the E-test System (AB Biodisk, Solna, Sweden). The E-test uses plastic strips; one side of the strip contains a concentration gradient of the antimicrobial agent; the other contains a numeric scale

that indicates the drug concentration in $\mu\text{g mL}^{-1}$ (Bolmström 1993).

Mueller-Hinton agar plates (Oxoid, Basingstoke, UK) 4 mm thick were inoculated using a swab that had been submerged in a bacterial suspension standardized to match the turbidity of the 0.5 McFarland standard. The surface of the plate was swabbed in three directions to ensure a complete distribution of the inoculum over the entire plate. Within 20 min of inoculation, the antimicrobial agents' strips were applied and the plates were inverted for incubation at 35 °C in air for 16–18, 24 h for vancomycin. After incubation, the plate was examined and an elliptical zone of growth inhibition was seen around the strip. The minimal inhibitory concentration (MIC) was read from the scale on the strip at the intersection of the growth with the E-strip. Once the MICs for the antimicrobial agents had been recorded, they were translated into interpretative categories of susceptible or resistant according to the guidelines of National Committee for Clinical Laboratory Standards (NCCLS) (2002). All the tests were completed in duplicate.

Beta-lactamase production

Enterococcus faecalis isolates were tested for β -lactamase production with nitrocefin (Oxoid) according to the manufacturer's instructions. Nitrocefin solution (5 μL) was dropped onto a single colony of an overnight culture. Development of a red colour within 60 s indicated a positive result.

Results

MIC range, MIC₅₀ and MIC₉₀ values obtained by the E-test method are shown in Table 1. Susceptibility rates are also shown. All isolates proved susceptible to benzylpenicillin, amoxicillin and amoxicillin-clavulanic acid. No strains produced β -lactamase. The strains studied were also completely susceptible to vancomycin and moxifloxacin. The latter was the most active antibiotic, *in vitro*, against *E. faecalis* with the lowest MIC values: all isolates were inhibited by $\leq 0.5 \mu\text{g mL}^{-1}$. Eight strains were found to be resistant to azithromycin, and two of them were also resistant to erythromycin. Three strains were resistant to both tetracycline and doxycycline. One strain was resistant to multiple drugs, viz. erythromycin, azithromycin, tetracycline, doxycycline and chloramphenicol.

Table 1 *In vitro* susceptibility of 21 *E. faecalis* isolates from canals of root filled teeth with periapical lesions

Antibiotic	MIC ($\mu\text{g mL}^{-1}$)			% Susceptible ^a
	MIC ₅₀	MIC ₉₀	Range	
Benzylpenicillin	2.0	3.0	1.0–4.0	100
Amoxicillin	0.5	0.75	0.25–0.75	100
Amoxicillin-clavulanic acid	0.5	0.75	0.25–0.75	100
Erythromycin	1.0	2.0	0.38–>256	28.5
Azithromycin	4.0	24.0	2.0–>256	14.2
Vancomycin	3.0	3.0	1.0–4.0	100
Chloramphenicol	4.0	6.0	3.0–>256	95.2
Tetracycline	0.5	32	0.19–>256	85.7
Doxycycline	0.38	12	0.12–>256	85.7
Ciprofloxacin	1.0	1.5	0.38–2.0	80.9
Moxifloxacin	0.38	0.5	0.19–0.5	100

MIC₅₀, minimal inhibitory concentration including 50% of the strains; MIC₉₀, minimal inhibitory concentration including 90% of the strains.

^aSusceptibility and resistance MIC breakpoints ($\mu\text{g mL}^{-1}$) recommended by NCCLS (2002): benzylpenicillin, amoxicillin and amoxicillin-clavulanic acid (≤ 8 S, ≥ 16 R); erythromycin (≤ 0.5 S, ≥ 8 R); vancomycin (≤ 4 S, ≥ 32 R); chloramphenicol (≤ 8 S, ≥ 32 R); tetracycline and doxycycline (≤ 4 S, ≥ 16 R); ciprofloxacin (≤ 1 S, ≥ 4 R). The breakpoints used for azithromycin were ≤ 2 S and ≥ 8 R (Fass 1993); and for moxifloxacin were ≤ 2 S, ≥ 8 R (Mather et al. 2002).

Discussion

Penicillins are the most frequently used antimicrobial agents. Due to their historical effectiveness, minimal toxicity and relatively low cost, penicillins constitute the first-choice antibiotics for odontogenic infections. Important classes of penicillins include penicillins G and V, which are highly active against susceptible Gram-positive cocci, and amoxicillin with an improved Gram-negative spectrum. β -Lactamase inhibitors such as clavulanate are used to extend the spectrum of penicillins against β -lactamase producing organisms (Petri 2001).

Bacterial resistance to penicillins has become a problem of great clinical significance because of its widespread use for many years (Appelbaum et al. 1990). The development of enterococcal resistance to β -lactams can be mediated by alterations in the expression or binding affinities of penicillin-binding proteins. Additionally, resistance has been associated with the production of β -lactamase, occasionally (Morrison et al. 1997). However, in this study, all isolates were negative for β -lactamase production, which agrees with the findings of Udo et al. (2002). β -Lactamase production occurs only rarely in *E. faecalis*

(Murray 2000, Murdoch et al. 2002, Shepard & Gilmore 2002).

All strains studied were susceptible to penicillins *in vitro*, however, the MICs of amoxicillin and amoxicillin-clavulanic acid were lower than for benzylpenicillin. These findings are in agreement with previous studies (Rams et al. 1992, Pinheiro et al. 2003b) which have found that enterococci are more sensitive to amoxicillin than to benzylpenicillin, bearing in mind that the latter can be given i.m. or i.v. not orally. Phenoxymethyl penicillin, which can be given orally, is less active against enterococci than benzylpenicillin is (Nord & Wadström 1973). The results indicated that *E. faecalis* strains isolated from canals of root filled teeth with periapical lesions remain susceptible, *in vitro*, to amoxicillin. Nevertheless, the lack of enterococcal resistance to penicillins in this study may be due to the limited number of strains investigated and/or geographical differences. The presence of enterococcal strains resistant to penicillin and ampicillin has been reported in endodontic infections in the USA (Matusow 1981) and Sweden (Dahlén et al. 2000) which underlines the need to perform susceptibility tests of these isolates. However, those authors did not provide information about the nature of the endodontic infections, i.e. primary or secondary infections. There most likely is a difference in resistance pattern between enterococci from primary infections and from root filled teeth with continuing apical periodontitis. Further investigation involving enterococcal strains isolated from both situations would improve knowledge about resistance pattern of enterococci in endodontic infections.

Besides differences in geographical areas and origins of infections, changes in resistance pattern of bacteria may occur over time. Earlier studies (Zeldore & Ingle 1962, Engström 1964) of enterococci isolated from root canals had shown that 100% of isolates were susceptible to erythromycin. Heintz et al. (1975) found more than 90% of isolates were susceptible, whilst Stern et al. (1990) have found 61.9% of enterococcal isolates susceptible to this drug. The present findings support the finding of a decrease in the enterococcal susceptibility to erythromycin over time. In this study, the MIC of erythromycin varied between 0.5 and $>256 \mu\text{g mL}^{-1}$. Two isolates were classified as resistant (MIC $\geq 8 \mu\text{g mL}^{-1}$) and 6 (28.5%) as susceptible (MIC $\leq 0.5 \mu\text{g mL}^{-1}$) according to the susceptibility breakpoints determined by the NCCLS protocol; most of the isolates (65.4%) showed an intermediate pattern. Similar results have been reported by Sedgley et al.

(2004) who have found, amongst 12 oral enterococci, two strains resistant to erythromycin, two (16.6%) susceptible and eight (66.6%) with an intermediate pattern. Those studies have shown that the MIC of erythromycin, when tested against enterococcal strains, has increased over time; which suggests that oral enterococci have become less susceptible to this drug.

Azythromycin is able to achieve higher and more sustained blood levels than erythromycin, without the gastrointestinal side effects (Grad 1997, Andrade 2000). Azythromycin was tested as a substitute for erythromycin and was found to be less effective against enterococci than erythromycin, with only 14.2% of isolates being susceptible. This finding is in accordance with those of Fass (1993). Furthermore, the latter study has also reported that there is cross-resistance between azithromycin and erythromycin.

In this study, erythromycin and azythromycin resistance was found amongst *E. faecalis* isolates. Furthermore, *E. faecalis* has intrinsic resistance to clindamycin (Murray 1990, Morrison et al. 1997). Thus, this drug is not clinically effective for *Enterococcus* spp. Therefore, when patients are allergic to penicillins, the alternative prophylactic regimens recommended for dental procedures seems to be of limited value against enterococci. Due to the predominance of *E. faecalis* in root filled teeth with periapical lesions, alternative drugs should be considered for prophylaxis in individuals at risk for endocarditis during endodontic retreatment. Amongst the alternative drugs investigated in this study, *E. faecalis* strains were found to be resistant to tetracycline, doxycycline, ciprofloxacin and chloramphenicol. Owing to geographical differences as well as differences over time, previously discussed in this paper, the findings of this study are not general but rather only applicable to the microbes tested.

Tetracyclines are broad-spectrum antibiotics with activity against aerobic and anaerobic Gram-positive and Gram-negative organisms. Doxycycline is one of the most active derivative of tetracycline. However, bacterial resistance to any member of the class usually results in cross-resistance to other tetracyclines (Chambers 2001), which was observed in the present study. The strains resistant to tetracycline were also resistant to doxycycline, the latter showing lower MICs against *E. faecalis*. Tetracycline resistance observed in 14.3% of strains in this study agrees with resistance in 13.8% of isolates reported by Dahlén et al. (2000). In contrast, some studies have shown even higher percentages of *E. faecalis* to be resistant to this antibiotic, i.e. 58% (Rams et al. 1992), 65.1% (Udo et al. 2002) and 68.5%

(Cotter & Adley 2001). Resistance to tetracyclines has reduced their clinical usefulness.

Chloramphenicol is effective against most aerobes and anaerobes, but its potential side-effect of aplastic anaemia usually makes selection of another effective and safer antibiotic a better choice (Moenning et al. 1989). It was effective against 95.23% of the strains in this study. However, other studies have reported that 20% (Cotter & Adley 2001) to 26% (Udo et al. 2002) of enterococci are chloramphenicol resistant.

Amongst the drugs tested, vancomycin and moxifloxacin were active against all *E. faecalis* isolates *in vitro*. Vancomycin is a drug primarily active against Gram-positive bacteria. However, it should be employed only to treat serious infections (Chambers 2001). Administration of vancomycin is an effective alternative, in patients who are allergic to penicillin, for the treatment of endocarditis caused by viridans streptococci as well as enterococci. In the latter case, penicillin or vancomycin is given in combination with an aminoglycoside (Murray 1990, Graham & Gould 2002). All *E. faecalis* strains examined in this study were susceptible to vancomycin. Previous studies of the susceptibility of oral enterococci have also shown high susceptibility to vancomycin (Rams et al. 1992, Dahlén et al. 2000). However, studies have highlighted the emergence of vancomycin-resistant enterococci, especially amongst *E. faecium* and in lower frequency amongst *E. faecalis* (Murray 2000, Malani et al. 2002). These vancomycin-resistant enterococci have emerged as major nosocomial pathogens in hospitals, and frequently possess determinants conferring multiple drug resistance so that few therapeutic options remain for treating these infections (Morrison et al. 1997, Rice 2001, Shepard & Gilmore 2002).

Moxifloxacin and ciprofloxacin are members of the quinolones. Ciprofloxacin has antimicrobial activity against most Gram-negative bacilli and cocci, but limited activity against most Gram-positive organisms. Moxifloxacin is a new fluoroquinolone with expended spectrum of activity, including anaerobes and Gram-positive organisms, especially the multi-resistant ones (Fass 1997, Oliphant & Green 2002, Speciale et al. 2002, Andersson & MacGowan 2003). In the present study, moxifloxacin was one of the most active antibiotics against *E. faecalis* with the lowest MIC₅₀ and MIC₉₀, and proved more active than ciprofloxacin, which agrees with data that have been reported by several authors (Fass 1997, Mather et al. 2002, Speciale et al. 2002). In addition to antimicrobial activity studies, the pharmacokinetic and pharmacodynamic properties of moxifloxacin have been studied, showing

excellent bioavailability, long half-life and good tissue penetration of this drug. Furthermore, it has an excellent tolerability (Krasemann *et al.* 2001).

Recent studies have shown that moxifloxacin has good antibacterial activity against periodontal pathogens (Milazzo *et al.* 2002) and bacteria isolated from dentoalveolar abscesses (Sobottka *et al.* 2002). The latter have suggested the potential use of moxifloxacin in the treatment of odontogenic infections. This study revealed that moxifloxacin had good *in vitro* activity against *E. faecalis* isolates from the root canal and seems to be a reasonable alternative for patients who are allergic to penicillin or show resistance to the antibiotics usually prescribed. However, further investigation involving a larger number of bacterial isolates from root canal as well as clinical studies would be necessary to test the use of moxifloxacin as an alternative drug when antibiotic therapy is indicated during endodontic treatment.

Conclusion

In conclusion, the results have shown that amoxicillin, amoxicillin-clavulanic acid, vancomycin and moxifloxacin were the most active antibiotics, *in vitro*, against *E. faecalis*, with all the isolates being susceptible. Less effective were chloramphenicol, tetracycline, doxycycline and ciprofloxacin, which were effective against most strains. Azithromycin and erythromycin were least effective, with low percentages of isolates being susceptible, during laboratory testing. Owing to geographical differences as well as differences over time, the findings of this study are not general but rather only applicable to the microbes tested.

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