

Phenotypic Detection of Virulence Traits and Antibiotic Susceptibility of Endodontic *Enterococcus faecalis* Isolates

Rakesh Kumar Patidar¹, Mithilesh Kumar Gupta², Vinod Singh^{1,*}

¹Department of Microbiology, Barkatullah University, Bhopal, India

²Department of Oral and Maxillofacial Surgery, People's Dental Academy, Bhopal, India

*Corresponding author: vsingh3@rediffmail.com

Received December 14, 2012; Revised February 03, 2013; Accepted February 28, 2013

Abstract *Enterococcus faecalis* is a Gram-positive member of human gastrointestinal flora that is in recent years emerging as an important cause of endodontic infections. In this study, we have investigated the occurrence of virulence determinants and antibiotic susceptibility pattern of *E. faecalis* isolates, originating from root canals of apical periodontitis. Among 52 *E. faecalis* isolates, 32 (61.5%) isolates produced hemolysin on blood agar while all (100%) isolates showed hemolysin production on BHI-GA ((BHI medium supplemented with 1% glucose and 0.03% L-arginine), 18 (34.6%) produced gelatinase, 38 (73%) produced caseinase, no hemagglutination was observed in *E. faecalis* isolates, whereas all 52 (100%) resistant to serum and formed biofilm. Antibiotic susceptibility results showed that all (100%) *E. faecalis* isolates were susceptible to amoxicillin, amoxicillin/clavulanate, and vancomycin. Whereas, 32 (61.5%) *E. faecalis* isolates were resistant to chloramphenicol, 30 (57.6%) isolates were resistant to ciprofloxacin, 39 (75%) isolates were resistant to erythromycin, and 34 (65.3%) isolates were resistant to tetracycline. Multi-drug resistance was observed in 16 (30.7%) isolates of *E. faecalis* to chloramphenicol, ciprofloxacin, erythromycin and tetracycline antibiotics. These findings demonstrate the presence of putative virulence determinants in *E. faecalis* isolates originating from root canal and suggest amoxicillin, amoxicillin/clavulanate, and vancomycin as more effective than other antibiotics tested.

Keywords: *E. faecalis*, root canal, apical periodontitis, virulence determinants, antibiotics.

1. Introduction

Enterococcus faecalis is the most common enterococci species, and it is responsible for 80-90% of human enterococcal infections [1]. *E. faecalis* is frequently isolated from the patients suffering from dental infections like periodontitis, gingivitis, teeth with failed endodontics and infected root canals [2,3,4].

E. faecalis has some special characteristics that allow them to survive in conditions that are commonly lethal for many other microorganisms. These properties include an ability to grow in high salt concentrations, a wide temperature range, tolerance a broad pH range, as well as persist in the presence of intracanal medicaments. *E. faecalis* has some virulence determinants which are already identified and could be the reason for survival of this microorganism in a very harsh environment of the root canal system [5]. *E. faecalis* possesses certain virulence factors including lytic enzymes, cytolysin, aggregation substance, pheromones, and lipoteichoic acid [6,7]. It has been shown to adhere to host cells, express proteins that allow it to compete with other bacterial cells, and alter host responses [6,8]. *E. faecalis* is able to suppress the action of lymphocytes, potentially

contributing to endodontic failure [9]. *E. faecalis* is not limited to its possession of various virulence factors. It is also able to share these virulence traits among species, further contributing to its survival and ability to cause disease [1]. These factors may or may not contribute to the innate characteristics of *E. faecalis* to cause disease. Because *E. faecalis* is less dependent upon virulence factors, it relies more upon its ability to survive and persist as a pathogen in the root canals of teeth [6].

Another important factor which is started to be evident during the last years is that microbes in the root canals can grow not only as planktonic cells or in aggregates, co-aggregates, but they can also form biofilms consisting of a complex network of different microorganisms [10]. Biofilm formation in root canals is probably initiated some time after the first invasion of the pulp chamber by planktonic oral microorganisms after some tissue breakdown [11]. Biofilms are composed of microcolonies of bacterial cells that are distributed in a matrix which consists of exopolysaccharides, proteins, salts and cell material in an aqueous solution. The matrix takes about 85% of the volume of a biofilm. Bacterial biofilms are reported to be the most common cause of persistent inflammation [12].

The presence of high prevalence of *E. faecalis* in treatment-resistant root canal infections is widely

recognized [13,14,15]. Enterococci have an acquired resistance to several classes of antibiotics either by mutation or by receipt of foreign genetic material through the transfer of plasmids and transposons [16].

The increasing incidence of *E. faecalis* infections in recent years little is clearly understood about enterococcal virulence determinants.

Data on oral prevalence of *E. faecalis* and its virulence determinants vary from one study to another and still not clearly understood [2,17,18]. Therefore, more investigation on potential virulence determinants and susceptibility pattern of different antibiotics would be useful in understanding their role in dental infection.

The aim of the study was to assess the prevalence of the virulence determinants and antibiotic susceptibility in root canal isolates of *E. faecalis*.

2. Materials and Methods

2.1. Bacterial Strains

Fifty-two *E. faecalis* isolates from root canal samples of apical periodontitis were obtained from 20 patients of People's Dental Academy and for control ATCC 14506 *E. faecalis* strain was used in this study.

2.2. Sampling Procedure

Sterile paper point was introduced into the full length of the canal and kept it place for 60s. The paper point samples from the root canal were transferred immediately to test tubes containing Amies transport medium (Himedia Laboratories, Mumbai) and taken to the laboratory in cold condition within 4h for microbiological analysis.

2.3. Bacterial Isolation and Identification

The samples were inoculated onto the blood agar (Himedia Laboratories, Mumbai) plates and incubated in microaerobic condition. Every growth showing gram-positive cocci, positive bile-esculin, positive 6.5% NaCl tests, catalase negative. All tentative Enterococcus isolates were subcultured on brain heart infusion agar (Himedia laboratories, Mumbai) and processed for further biochemical identification. Fifty two isolates were identified as *E. faecalis*.

2.4. Hemolytic Assay

E. faecalis isolates were assessed for hemolytic activity on blood base agar supplemented with 5% (v/v) human blood. A single colony was cultured onto blood agar plates and its hemolytic activity was determined by presence of clear zone around the colonies. Extracellular hemolysin was assayed on microplates using culture supernatant from *E. faecalis* grown on brain heart infusion broth (Himedia Laboratories, Mumbai) supplemented with 1% glucose and 0.03% L-arginine (BHI-GA) [19]. The supernatants were serially diluted with 5mM phosphate buffer saline (PBS), pH 7.4, mixed with equal volume of a 1% sheep erythrocytes suspension and incubated at 37 °C for 1h, followed by a further overnight incubation at 4 °C.

2.5. Protease Production

Gelatinase production was detected by inoculating *E. faecalis* isolates onto trypticase soya agar (TSA) containing 3% gelatin (Himedia laboratories, Mumbai). The appearance of turbid halos or zones around the colonies after incubation at 37 °C for 24h was considered to be a positive indication of gelatinase production [20]. Casein hydrolysis was tested on mueller hinton agar containing 3% (w/v) skimmed milk, by streaking with 10µL of each suspension followed by incubation at 37 °C for 1h [21]. The presence of transparent zone around the colonies indicated caseinase activity [22].

2.6. Hemagglutination Assay

After centrifugation, the *E. faecalis* bacterial pellet was suspended in 5mM PBS, pH 7.4, to a final concentration of 10⁹cfu/mL 50µL of this suspension was added to an equal volume of 2% (v/v) suspension of sheep erythrocytes, and the plates were incubated at 37 °C for 1h.

2.7. Sensitivity to the Bactericidal Effect of Human Normal Serum

The sensitivity of *E. faecalis* to the bactericidal effect of human normal serum was tested as described by Pelkonen and Finne [23]. Bacteria grown in BHI for 18h were diluted in PBS (10⁹bacteria/mL) and 175µL of the bacterial suspension and the same quantity of PBS were pipetted into the wells of microtitre plates, 100µL of serum (final concentration 36%) were added and incubated at 37 °C. The absorbance at 630nm was measured at 0, 30, 60, 120 and 180 min. the strains were classified as resistant, intermediate or sensitive as suggested by Taylor [24].

2.8. Biofilm Formation by Microtiter Plate and Coverslip Assay

In microtiter assay, all *E. faecalis* strains (10⁹cfu/30µL) were cultured in BHI in 96-well microtiter plates at 37 °C for 48 h. After incubation broth was aspirated and wells were washed with PBS. 0.5% crystal violet stain was added for 5min. The plates were then washed with tap water and 200µL of 95% ethanol was added. The biofilm formation was considered positive when an optical density at 570nm was equal or more than 0.2 [25].

In coverslip assay, biofilms of *E. faecalis* were grown as follows, individual sterile culture dishes were filled with 2.5mL of BHI broth and sterile 18mm diameter glass microscope coverslip was added to each dish, and culture dish was covered. Each sample was inoculated with defined volume of overnight culture. The dishes were incubated microaerobically at 37 °C for 48h. Glass coverslips containing attached biofilm were removed from dishes and rinsed briefly with PBS and stained with 0.5% crystal violet for 5min. Stained biofilms were observed at 40X using compound microscope (Olympus, Magnus-MLX-Series, India)

2.9. Antibiotic Susceptibility Testing

The antibiotic susceptibility of the test strains to different antibiotics (amoxicillin, amoxicillin/clavulanate, chloramphenicol, ciprofloxacin, erythromycin, tetracycline and vancomycin) was purchased from

Himedia Laboratories, Mumbai and determined by disc diffusion method according to the clinical and Laboratory Standards Institute (CLSI) guidelines.

3. Results

Among 52 *E. faecalis* isolates, 32 (61.5%) isolates showed hemolytic activity on blood agar, determined by the appearance of clear zone around the colonies (β -hemolysis). All (100%) culture of the supernatants from *E. faecalis* isolates, grown in BHI-GA showed hemolysed sheep RBC (Table 1).

Gelatinase production is a major virulence determinant of *E. faecalis*, whereas only 18 (34.6%) isolates showed gelatinase production. Casein hydrolysis was observed in 38 (73%) *E. faecalis* isolates (Table 1).

The hemagglutinating activity of all (100%) *E. faecalis* isolates with sheep erythrocytes was negative. All (100%) *E. faecalis* isolates were resistant to the bactericidal action of normal serum (Table 1).

All (100%) isolates of *E. faecalis* formed biofilms on microtitre polystyrene plates and coverslips (Table 1). Figure 1 showed biofilm formation by coverslip assay.

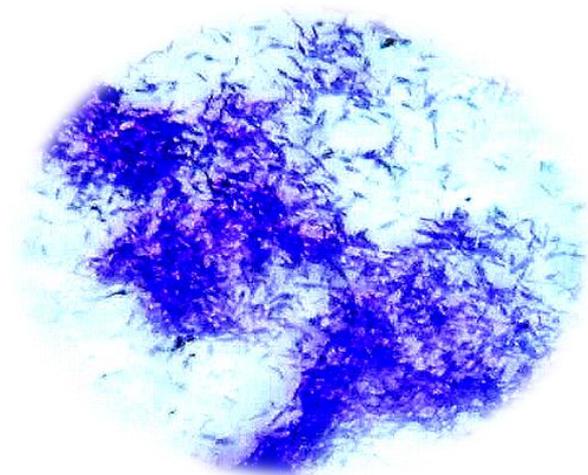


Figure 1. Microscopic view: *E. faecalis* biofilm development by coverslip assay

Antibiotic susceptibility results showed that all (100%) *E. faecalis* isolates were susceptible to amoxicillin, amoxicillin/clavulanate, and vancomycin. Whereas, 32 (61.5%) *E. faecalis* isolates were resistant to chloramphenicol, 30 (57.6%) isolates were resistant to ciprofloxacin, 39 (75%) isolates were resistant to erythromycin, and 34 (65.3%) isolates were resistant to tetracycline. Multi-drug resistance was observed in 16 (30.7%) isolates of *E. faecalis* to chloramphenicol, ciprofloxacin, erythromycin and tetracycline antibiotics (Figure 2, Table 2).

4. Discussion

Enterococci are able to colonize the oral cavity, particularly in patients with periodontitis or root canal infections associated with oral mucosal lesions and in immunocompromised patients [26,27]. *E. faecalis* is the most commonly isolated species from root canal samples

with treatment failure, periapical lesions or chronic apical periodontitis [14,28]. It represents a potential threat to general health is not yet known because endodontic infecting microorganisms are localized in a restricted area within the root canal system and little is known on the extraradicular presence of *E. faecalis* [29].

Table 1. Virulence characteristics of *Enterococcus faecalis* isolates

Isolates	Hemolysin(a)		Protease (b)	
	Agar	SN	Gel	CH
EF01	+	+	-	-
EF02	-	+	+	+
EF03	-	+	-	+
EF04	-	+	+	-
EF05	-	+	+	+
EF07	+	+	-	+
EF08	+	+	-	+
EF09	+	+	-	-
EF10	+	+	-	+
EF11	+	+	-	+
EF12	-	+	-	+
EF13	-	+	-	-
EF14	+	+	-	+
EF15	+	+	+	-
EF16	+	+	+	+
EF17	-	+	+	+
EF18	+	+	-	-
EF19	+	+	-	-
EF20	+	+	-	+
EF21	+	+	+	+
EF22	-	+	-	+
EF23	+	+	+	+
EF24	+	+	-	+
EF25	+	+	+	+
EF26	-	+	+	+
EF27	-	+	-	+
EF28	+	+	-	+
EF29	+	+	-	+
EF30	-	+	+	+
EF31	-	+	+	+
EF32	+	+	+	+
EF33	+	+	-	+
EF34	+	+	-	+
EF35	-	+	-	+
EF36	+	+	-	+
EF37	-	+	-	+
EF38	+	+	-	+
EF39	-	+	-	-
EF40	+	+	+	+
EF41	+	+	-	-
EF42	-	+	+	+
EF43	-	+	-	+
EF44	-	+	-	-
EF45	+	+	+	+
EF46	+	+	-	-
EF47	-	+	+	+
EF48	-	+	-	+
EF49	+	+	-	-
EF50	+	+	-	+
EF51	+	+	-	-
EF52	+	+	-	+

(a) Hemolytic activities on agar plates and of culture supernatants (SN)

(b) Protease activity; casein hydrolysis (CH) and gelatinase (Gel)

Hemolysin producing strains of *E. faecalis* are virulent in animal models and human infections and are associated with increasing infection severity. In the present study, hemolysin production was expressed by 61.5% of *E. faecalis* isolates on blood agar, whereas all (100%) isolates of *E. faecalis* exhibited extracellular hemolytic activity towards sheep erythrocytes with BHI-GA.

Previous study [30] indicated that glucose and arginine are essential elements for heat-stable hemolysin production.

Table 2. Antibiotic susceptibility testing of *Enterococcus faecalis*

Antibiotics	No. (%) of resistant strains of <i>E. faecalis</i> (n=52)
Amoxicillin	0 (0)
Amoxicillin/Clavulanate	0 (0)
Chloramphenicol	32 (61.5)
Ciprofloxacin	30 (57.6)
Erythromycin	39 (75)
Tetracycline	34 (65.3)
Vancomycin	0 (0)
Chloramphenicol, Ciprofloxacin Erythromycin and Tetracycline	16 (30.7)

The production of hemolysin and gelatinase as putative virulence determinants were not always expressed by *E. faecalis* isolates in association with dental diseases. Sedgley et al [28,31] reported different results with hemolysin production; first study has proved that 36% of the *E. faecalis* isolates recovered from endodontic patients produced hemolysin, while the second has not detected production of hemolysin in any enterococcal isolates from endodontic cases. Gelatinase has been implicated as a

virulence determinant in animal models. The ability of this enzyme to hydrolyse gelatin, collagen and certain bioactive peptides suggests its participation in the initiation and propagation of inflammatory processes involving *E. faecalis* [32]. In our study, gelatinase activity was observed in 18 (34.6%) of *E. faecalis* isolates only. Casein hydrolysis was observed in 38 (73%) isolates of *E. faecalis*. Data does suggest that the gelatin hydrolyzing activity is different from caseinase and also supported previous findings (30). Sedgley et al [33] found that gelatinase gene (*gelE*) was detected in all endodontic isolates of *E. faecalis* while expressed gelatinase activity was observed in two thirds of the isolates. These studies concluded that evidence of potential virulence determinants were identified in endodontic *Enterococcus* spp., specifically production of gelatinase and response to pheromones. Other studies indicated that expression of gelatinase of gelatinase gene contributed to the increased dissemination of *E. faecalis* in high density environments and was associated with increased adhesion of *E. faecalis* to dentine in vitro [32,34].

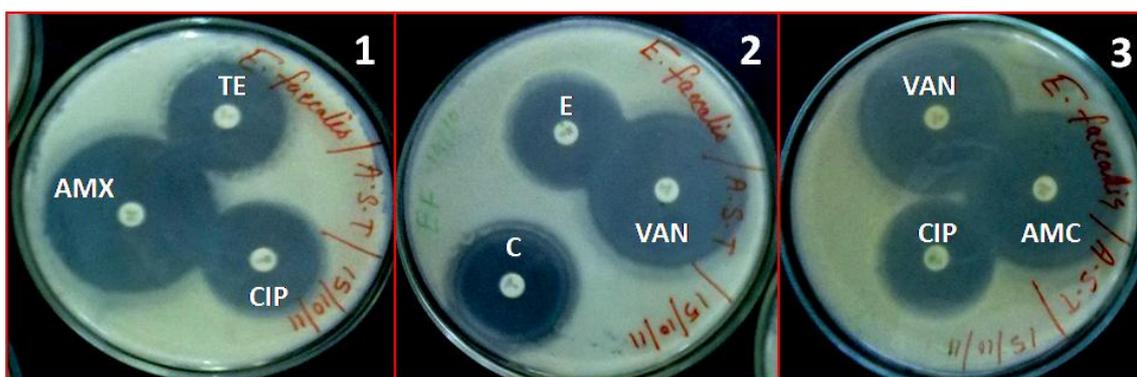


Figure 2. Antimicrobial activity of different drugs against *Enterococcus faecalis*. In Figure 2 (1) shows amoxicillin (AMX); sensitive, Tetracycline (TE); resistant, Ciprofloxacin (CIP); resistant, in Figure 2 (2) shows Erythromycin (E); resistant, Chloramphenicol (C); resistant, Vancomycin (VAN); sensitive, in Figure 2 (3) shows Vancomycin (VAN); sensitive, Ciprofloxacin (CIP); resistant, Amoxicillalve (AMC); sensitive pattern against *E. faecalis*.

Hemagglutinating activity was negative in all (100%) *E. faecalis* isolates with sheep erythrocytes. Our results substantiate the findings of Furumura et al [30].

The growth of bacteria in host tissue is limited, not only by host defense mechanisms, but also by the availability of iron, an essential and important factor for bacterial growth that functions mainly as a redox catalyst for proteins participating in oxygen and electron transport [35]. All (100%) isolates of *E. faecalis* examined in this investigation were resistant to serum.

Biofilm is a population of cells attached irreversibly on various biotic and abiotic surfaces and encased in a hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids [36]. Adherence and production of a biofilm by *E. faecalis* on different biomaterials and medical devices has been documented. Among the factors involved in this type of adherence are physic-chemical forces, such as polarity, van der Waals forces and hydrophobic interactions [37] but factors involved in enterococcal adherence to biomaterials have not been well defined [38].

In the present study, we observed that all (100%) isolates of *E. faecalis* showed ability to form biofilm on polystyrene plates and glass coverslips.

In recent years, enterococci have received increasing attention because of the development of multi-drug resistance and its common prevalence in nosocomial and endodontic infections. Vancomycin-resistant enterococci (VRE) probably represent currently most serious challenge among many microbes with antibiotic resistance causing human infections.

In the present study of antibiotic susceptibility, the strains were seen to be highly sensitive to the more commonly used antibiotics (amoxicillin, amoxicillin/clavulanate and vancomycin). Whereas resistance was observed in *E. faecalis* isolates against chloramphenicol, ciprofloxacin, erythromycin and tetracycline. This is a serious problem, as it reduces the number of possible antimicrobial therapies for dental infections associated to enterococci.

Enterococci resistance to currently available antibiotics pose real therapeutic difficulties and can lead to the endodontic treatment failures result [39]. The acquisition of antibiotic resistance occurs either through the acquisition of resistance genes on plasmids from other organisms. Enterococci can secrete pheromones [40] which are stimulating the synthesis of the surface aggregation substance [41]. This facilitates the contact between the cells and the formation of the mating aggregate, which

finally will lead to the exchange of plasmids carrying resistance.

All the findings of the present study demonstrate the presence of putative virulence determinants in *E. faecalis* isolates originating from root canal and suggest amoxicillin, amoxicillin/clavulanate, and vancomycin as more effective than other antibiotics tested. The emergence of resistance against multiple antibiotics and the increasing frequency of *E. faecalis* isolates from infected root canal of endodontic patients underscore the necessity for a better understanding of the virulence determinants, mechanisms of this pathogen, and the development of alternatives to current antibiotic treatments. The genetic plasticity of *E. faecalis* and its ability to rapidly acquire and/or develop resistance against many clinically important antibiotics and to transfer these resistance determinants to other more pathogenic microorganisms makes the search for alternative treatment and preventive options even more important.

Acknowledgements

This study was supported by Barkatullah University Bhopal (M.P.) India and University Grant Commission (New Delhi, India). We would like to extend our thanks to staff members of Department of Endodontics, People's Dental Academy, Bhopal (M.P.) India for providing clinical samples.

Conflict of Interest

There is no conflict of interest in this study.

References

- [1] Jett B.D., Huycke M.M., Gilmore M.S. "Virulence of enterococci". *Clin Microbiol Rev*, 7. 462-78. 1994.
- [2] Sedgley C., Buck G., Appelbe O. "Prevalence of *Enterococcus faecalis* at multiple oral sites in endodontic patients using culture and PCR". *J Endod*, 32. 104-09. 2006.
- [3] Johnson E.M., Flannagan S.E., Sedgley C.M. "Coaggregation interactions between oral and endodontic *Enterococcus* and bacterial species isolated from persistent apical periodontitis". *J Endod*, 32. 946-50. 2006.
- [4] Pinheiro E.T., Gomes B.P., Ferraz C.C., Sousa E.L., Teixeira F.B., Souza-Filho F.J. "Microorganisms from canals of root-filled teeth with periapical lesions". *Int Endod J*, 36. 1-11. 2003.
- [5] Evans M., Davies J.K., Sundqvist G., Figdor D. "Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide". *Int Endod J*, 35. 221-28. 2002.
- [6] Rocas I.N., Siqueira J.F., Santos K.R.N. "Association of *Enterococcus faecalis* with different forms of periradicular diseases". *J Endod*, 30. 315-20. 2004.
- [7] Patidar R.K., Gupta M.K., Singh V. "Virulence of *Enterococcus faecalis* and impact of genome-wide approaches". *South Asian J Exp Biol*, 1. 16-24. 2011.
- [8] Love R.M. "*Enterococcus faecalis*: a mechanism for its role in endodontic failure". *Int Endod J*, 34. 399-05. 2001.
- [9] Lee W., Lim S., Son H., Bae K. "Sonicated extract of *Enterococcus faecalis* induces irreversible cell cycle arrest in phytohemagglutinin-activated human lymphocytes". *J Endod*, 30. 209-12. 2004.
- [10] Nair P.N.R. "On the causes of persistent apical periodontitis: a review". *Int End J*, 39. 249-81. 2006.
- [11] Svensäter G., Bergenholtz G. "Biofilms in endodontic infections". *Endod Top*, 9. 27-36. 2004.
- [12] Costerton J.W., Stewart P.S. "Biofilms and device-related infections". In: Nataro P.J., Balser M.J., Cunningham-Rundles S., eds. *Persistent Bacterial Infections*. Washington, 2000. 423-39.
- [13] Hancock H.H., Sigurdsson A., Trope M., Moiseiwitsch J. "Bacteria isolated after unsuccessful endodontic treatment in a North American population". *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 91. 579-86. 2001.
- [14] Molander A., Reit C., Dahle'n G., Kvist T. "Microbiological status of root-filled teeth with apical periodontitis". *Int Endod J*, 31. 1-7. 1998.
- [15] Peculiene V., Reynaud A.H., Balciuniene I., Haapasalo M. "Isolation of yeasts and enteric bacteria in root-filled teeth with chronic apical periodontitis". *Int Endod J*, 34. 429-34. 2001.
- [16] Murray B.E. "The life and times of the enterococci". *Clin Microbiol Rev*, 3. 46-65. 1990.
- [17] Gomes B.P.F.A., Pinheiro E.T., Gade-Neto C.R., Sousa E.L., Ferraz C.C., Zaia A.A., Teixeira F.B., Souza-Filho F.J. "Microbiological examination of infected dental root canal". *Oral Microbiol Immunol*, 19. 71-76. 2004.
- [18] Reynaud af Geijersstam A., Culak R., Molenaar L., Chattaway M., Røslie E., Peculiene V., Haapasalo M., Shah H.N. "Comparative analysis of virulence determinants and mass spectral profiles of Finnish and Lithuanian endodontic *Enterococcus faecalis* isolates". *Oral Microbiol Immunol*, 22. 88-94. 2007.
- [19] Booth M.C., Bogie C.P., Sahl H.G., Siezen R.J., Hatter K.L., Gilmore M.S. "Structural analysis and proteolytic activation of *Enterococcus faecalis* cytolysin, a novel lantibiotic". *Mol Microbiol*, 21. 1175-84. 1996.
- [20] Vergis E.N., Shankar N., Chow J.W., Hayden M.K., Snyderman D.R., Zervos M.J., Linden P.K., Wagener M.M., Muder R.R. "Association between the presence of enterococcal virulence factor gelatinase, hemolysin, and enterococcal surface protein and mortality among patients with bacteremia due to *Enterococcus faecalis*". *Clin Infect Dis*, 35. 570-75. 2003.
- [21] Kurl D.N., Haataja S., Finne J. "Hemagglutination activities of group B, C, D and G streptococci: demonstration of novel sugar specific cell-binding activities in *Streptococcus suis*". *Infect Immun*, 57. 384-89. 1989.
- [22] Archimbaud C., Shankar N., Forestier C., Baghdayan A., Gilmore M.S., Charbonne F., Joly B. "In vitro adhesive properties and virulence factors of *Enterococcus faecalis* strains". *Res Microbiol*, 153. 75-80. 2002.
- [23] Pelkonen S., Finne J. "A rapid turbidimetric assay for the study of serum sensitivity of *Escherichia coli*". *FEMS Microbiol Lett*, 42. 55-57. 1987.
- [24] Taylor P.W. "Bactericidal and bacteriolytic activity of serum against gram-negative bacteria". *Microbiol Rev*, 47. 46-83. 1983.
- [25] Wakimoto N., Nishi J., Sheikh J., Nataro J.M., Sarantuya J., Iwashita M., Manago K., Tokuda K., Yoshinaga M., Kawano Y. "Quantitative biofilm assay using microtiter plate to screen for enteroaggregative *Escherichia coli*". *Am J Trop Med Hyg*, 71. 687-90. 2004.
- [26] Pinheiro E.T., Anderson M.J., Gomes B.P.F.A., Drucker D.B. "Phenotypic and genotypic identification of enterococci isolated from canals of root-filled teeth with periapical lesions". *Oral Microbiol Immunol*, 21. 137-44. 2006.
- [27] Guven Kayaoglu, Orstakavik D. "Virulence of *Enterococcus faecalis*: relationship to endodontic disease". *Crit Rev Oral Biol Med*, 15. 308-20. 2004.
- [28] Sedgley C., Nagel A., Dahlen G., Reit C., Molander A. "Real-time quantitative polymerase chain reaction and culture analyses of *Enterococcus faecalis* in root canals". *J Endod*, 32. 173-77. 2003.
- [29] Sunde P.T., Olsen I., Debelian G.J., Tronstad L. "Microbiota of periapical lesions refractory to endodontic therapy". *J Endod*, 28. 304-10. 2002.
- [30] Furumura M.T., Figueiredo P.M.S., Carbonella G.V., da Costa Darini A.L., Yano T. "Virulence-associated characteristics of *Enterococcus faecalis* strains isolated from clinical sources". *Braz J Microbiol*, 37. 230-36. 2006.
- [31] Sedgley C.M., Lennan S.L., Clewell D.B. "Prevalence, phenotype and genotype of oral enterococci". *Oral Microbiol Immunol*, 19. 95-01. 2004.
- [32] Waters C.M., Antiporta M.H., Murray B.E., Dunny G.M. "Role of the *Enterococcus faecalis* GeIE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins". *J Bacteriol*, 185. 3613-23. 2003.

- [33] Sedgley C.M., Nagel A.C., Shelburne C.E., Clewell D.B., Appelbe O., Molander A. "Quantitative real-time PCR detection of oral *Enterococcus faecalis* in humans". Arch Oral Biol, 50. 575-83. 2005.
- [34] Hubble T.S., Hatton J.F., Nallapareddy S.R., Murray B.E., Gillespie M.J. "Influence of *Enterococcus faecalis* proteases and the collagen binding protein, Ace, on adhesion to dentin". Oral Microbiol Immunol, 18. 121-26. 2003.
- [35] Podschun R., Ullmann U. "Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors". Clin Microbiol Rev, 11. 589-03. 1998.
- [36] Costerton J.W. "Introduction to biofilm". Int J Antimicrobial Agents, 11. 217-21. 1999.
- [37] Di Martino P., Cafferini N., Joly B., Darfeuille-Michaud A. "Klebsiella pneumoniae type 3 pili facilitate adherence and biofilm formation on abiotic surfaces". Res Microbiol, 154. 9-16. 2002.
- [38] Kritish C.J., Li Y.H., Cvithkovitch D.G. Dunny G.M. "Esp independent biofilm formation by *Enterococcus faecalis*". J Bacteriol, 186. 154-63. 2004.
- [39] Coque T.M., Tomayko J.F., Ricke S.C., Okhyusen P.C., Murray B.E. "Vancomycin-resistant enterococci from nosocomial, community, and animal sources in the United States". Antimicrob Agents Chemother, 40. 2605-09. 1996.
- [40] Dunny G.M., Leonard B.A., Hedberg P.J. "Pheromoneinducible conjugation in *Enterococcus faecalis*: interbacterial and host-parasite chemical communication". J Bacteriol, 177. 871-76. 1995.
- [41] Galli D., Lottspeich F., Wirth R. "Sequence analysis of *Enterococcus faecalis* aggregation substance encoded by the sex pheromone plasmid pAD1". Mol Microbiol, 4. 895-04. 1990.