

Electrophoresis of Whole-cell Soluble Proteins of *Enterococcus faecalis* and Yeast Isolated in the Root Canals of Previously Root-filled Teeth

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SUMMARY

Microbiological samples were taken from the root canals of 40 previously root filled teeth with apical periodontitis requiring retreatment with special emphasis on the occurrence of *E. faecalis* and *C. albicans*. Microbes were isolated from 33 out of the 40 teeth. Preliminary characterisation of microbial species found in the root canals was based on aerotolerance, gram staining and production of catalase and indole. Identification to species level was based on motility test and carbohydrate fermentation patterns. The detection of glycosidase and aminopeptidase enzymes was used for identification of facultative gram negative enteric rods. Streptococcal isolates growing on bile-esculin agar plates were further identified to species level by a commercial test panel. Identification of Yeasts was based on cellular appearance in gram stained smears and on production of glycosidase enzymes. Results obtained from the primary tests may give inconsistently expressed phenotypic traits.

In order to confirm the results of primary identification tests, electrophoresis of whole-cell soluble proteins as valuable method for rapid and precise characterisation of oral bacteria was used. This test have showed that the protein patterns of reference strain of *E. faecalis* and *C. albicans* exhibited identical or similar characteristics with protein patterns of clinical strains of *E. faecalis* and *C. albicans* isolated from the root canals and it confirmed primary tests results.

E. faecalis was found in 21 out of the 33 culture positive root canals. *C. albicans* was found in 6 out of 33 culture positive root canals.

Key words: *E. faecalis*, *C. albicans*, electrophoresis of whole-cell soluble proteins, phenotypic traits

INTRODUCTION

The microbiota associated with failed cases differs markedly from that reported in untreated teeth (primary root canal infection). Whereas the latter is typically a mixed infection, in which gram-negative anaerobic rods are dominant, the former is usually composed of one or few bacterial species, generally gram-positive bacteria, with no apparent predominance of facultatives or anaerobes [1,2,3,4]. In retreatment cases of apical periodontitis anaerobic bacteria constitute the minority 40-55% of root canal microflora. Increased numbers of some microbial species such as yeasts and gram negative enteric rods which are usually not present in primary apical periodontitis are reported in the retreatment cases [2,4]. *E. faecalis* is not only frequently isolated from secondary root canal infections, but also is the predominant one [2,4].

For identification of microorganisms from microbiological samples molecular typing methods are used. These methods are based on biochemical reactions and antimicrobial resistance patterns which suggests the microorganisms dependence. However, typing methods based solely on these tests have limitations since phenotypic traits of microorganisms may be inconsistently expressed and this could influence the interpretation of the preliminary results.

Electrophoretic separation of cellular proteins has been used for over 20 years for classification, identification and typing of diverse bacterial taxa. This is a highly sensitive analytical technique that provides distinctive phenotypic evidence of the similarity of strains within species [5,6,7].

The present study, using electrophoresis of bacterial whole-cell soluble proteins, was performed in an effort to verify our preliminary findings and to further study the microorganisms involved in endodontic infection.

MATERIAL AND METHODS

Clinical isolates

Microbiological samples were taken from the canals of fourty teeth immediately following the coronal access preparation and removal of old root canal filling under aseptic conditions described by Moller [8]. Microorganisms were isolated from the root canal sample after the preliminary root canal inspection to

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the full working length. Three reference strains from Culture Collection, University of Goteborg (CCUG) or American type Culture Collection (ATCC) were used in the experiment as controls (1 table).

Cultivation of bacteria

The disinfection of the sampling area was done according to the procedures described by Moller [8]. The samples were taken with sterile forceps using sterile paper points inserted into the canal to the approximate apex region. After sampling, the paper points were immediately placed into 1 ml of the transport medium, VMGA III gel [Moller 1966], prepared at the oral microbiological service of the University of Helsinki, Finland. All samples were cultivated within 24-48h after sampling.

The transport media were pre-incubated for 30 min at 37°C, and shaken thoroughly in a mixer for 60 seconds (*Vortex, Scientific Industries Inc., Springfield, MA, USA*). The transport medium contained glass beads with a diameter of the 3 mm to facilitate mixing and homogenisation of the sample prior to cultivation. Serial ten-fold dilutions were made up to 1:10 in 1% sterile peptone water (*Bacto peptone, Difco, MI, USA*). One aliquot of 0,3 ml of the undiluted medium, several aliquots of 0,1 ml of the undiluted medium and the serial dilutions were plated onto several media using sterile plastic spreaders. Aliquots were distributed on the plates with various media for bacterial cultivation:

- Brucella agar plates (*BBL Microbiology Systems, Cockeysville, MD, USA*) enriched with 5% defibrinated horse blood, 5 mg/l of haemin and 10 mg/l of vitamin K for the cultivation of the anaerobic and facultative bacteria;
- TSBV agar plates (*Tryptic-soy-agar, Difco*) enriched with 10% horse serum and supplemented with 75 mg/l of bacitracin (*Sigma Chemical Co., St. Louis, MO*) and 5 mg/l of vancomycin (*Sigma*) for yeasts and gram-negative facultative rods;
- Sabouraud plates (*Difco*) for yeasts;
- Chocolate agar plates (*Tryptic-soy-agar, Difco*) with 10% defibrinated horse blood for facultative bacteria.

The plates were incubated at 37°C in 5% CO₂ and anaerobically in anaerobic jars. Bacterial and yeast colony types were enumerated, isolated, coded, preliminary identified and preserved in glycerine milk at -70°C to wait for further identification.

Preliminary characterisation of microbial species was based on: aerotolerance, gram staining, production of catalase and indole. Identification to species level was based on motility test and carbohydrate fermentation patterns. The detection of glycosidase and aminopeptidase enzymes (*Roscozym-4-hour ent., Rosco, Taastrup, Denmark*) was used for identification of facultative gram negative enteric rods.

Table 1. Bacterial type strains.

Bacterial strains	Source	Reference
<i>Candida albicans</i>	CCUG	19915
<i>Candida glabrata</i>	CCUG	32725
<i>Enterococcus faecalis</i>	ATCC	29212

Streptococcal isolates growing on bile-esculin agar plates (*Difco*) were further identified to species level by a commercial test panel Rapid ID 32 (*Strep BioMerieux, Marcy/Etoile, France*).

Yeasts were identified based on cellular appearance in gram stained smears, production of glycosidase enzymes (*Chromogenic substrate tablets; Rosco*).

Comparison of silver stained (*Silver Stain Plus, Biorad Laboratories, Richmond, CA, USA*) whole cell protein profiles of the isolates in sodium dodecyl sulphate polyacryl amide gel electrophoresis (*SDS-PAGE*) (reducing sample buffer, 12% gels, Mini Protean II, Biorad) with reference strains was made [9]. One per cent SDS was used for the extraction of the cell proteins for half an hour at 20°C with repeated mixing (*Vortex, Scientific Industries Inc.*).

40 µl of the sample suspension were mixed with 40 µl of sample buffer supplemented with 20g/l of sodium dodecyl sulphate (SDS) (*Pfarmacia, Uppsala, Sweden*), 10 mM of dithiothreitol (*Sigma*) and 0,2 g/l of bromophenol blue (*Pharmacia*). Supernatants obtained after centrifugation (12000 x g, 5 min, 4°C). 10 µl of supernatant from each tube was applied directly onto the surface of an 0,5mm thin precast polyacrylamide gradient gel and were used for the SDS-PAGE.

The proteins were separated using Mini protean II electrophoresis apparatus following the running conditions given by the manufacturer (200V, 50mA) at constant temperature. Duration of electrophoresis procedure was 80 min.

After electrophoresis, the gels were placed in a fixing solution for at least for 30 min in 30°C and rinsed with deionized distilled water. The gels were then photographed.

RESULTS

The bacteriology of 40 symptom-free teeth with apical periodontitis and previously filled root canals was studied with special emphasis focused on the presence of *E. faecalis* and *C. albicans* in the root canals. Bacteria were isolated from the 33 (82,5%) root canals out of the 40 teeth studied. According to the preliminary tests results *E. faecalis* was found in 21 (63,6%) out of the 33 culture positive root canals. *C. albicans* according to preliminary tests was found in 6 (18,2%) out of 33 culture positive root canals.

Results obtained from the primary tests may give inconsistently expressed phenotypic traits. Electrophoretic separation of cellular proteins provides distinctive phenotypic evidence of the similarity of strains within species and was used in this study to prove preliminary test results. This is a highly sensitive analytical technique. The electrophoretic bands of cellular proteins of the microbial cells of *E. faecalis* and *C. albicans* on the gels after staining were distinct. The protein patterns of *E. faecalis* and *C. albicans* in the root canals obtained by electrophoresis are shown in figures 1,2.

The clinical strains from the root canals of *C. albicans* gave identical or similar protein patterns (1 fig.). The reference strain of *C. albicans* CCUG 19915 (A) exhibited characteristics identical, to those of the clinical strains of *C. albicans* (B, C, D, E, F, H,

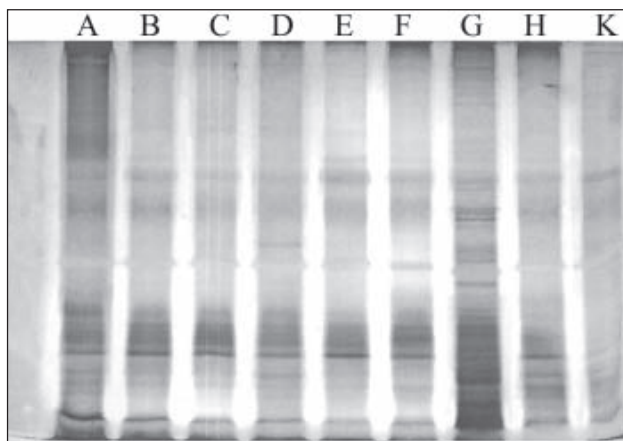


Figure 1. The clinical strains from the root canals of *C. albicans* (B, C, D, E, F, G, H, K) gave identical protein patterns exhibited by the reference strain of *C. albicans* CCUG 19915 (A).

K) found in the root canals of retreated cases. The reference strain of *C. albicans* CCUG 19915 (A) exhibited characteristics similar to the strain of *C. albicans* (G) found in root canal (1 fig.).

In the protein patterns no similarity between reference strain of *C. glabrata* and clinical strains of yeasts isolated from the root canals of retreated cases was found.

The clinical strains of *E. faecalis* from the root canals were compared with the reference strain of *E. faecalis* ATCC 29212 (A) (2 fig.). The clinical strains of *E. faecalis* (B, C) isolated from the root canals exhibited characteristics identical to the reference strain of *E. faecalis* ATCC 29212 (A) (2 fig.). The clinical strains of *E. faecalis* (D, E) isolated from the root canals exhibited characteristics similar to the reference strain of *E. faecalis* ATCC 29212 (A) (2 fig.).

The results obtained after electrophoresis procedure confirmed the results of the primary tests used for the detection of bacteria.

DISCUSSION

Failure of endodontic treatment is a result of microorganisms persisting in the apical portion of the root canal system, even in a well treated teeth.

E. faecalis is frequently isolated from secondary or persistent root canal infections often as mono-infection. The results of the present study demonstrated and proved the results of previous studies where they have stated that *E. faecalis* is the dominant microorganism in the retreatment cases [2,3]. Strains of this microorganism have demonstrated to be extremely resistant to the several medicaments used during endodontic treatment procedures [10]. This proves that eradication of *E. faecalis* by conventional means maybe extremely difficult.

Yeasts is the group of microorganisms which are not found during primary endodontic treatment, but are

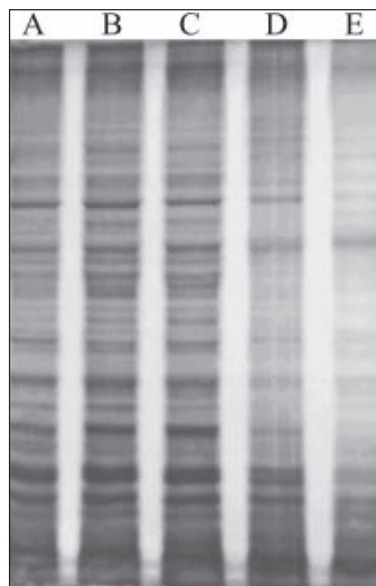


Figure 2. The clinical strains *E. faecalis* (B, C, D, E) isolated from the root canals exhibited characteristics identical the reference strain of *E. faecalis* ATCC 29212 (A).

isolated from the root canals in retreatment cases only more rare than *E. faecalis* [11]. Studies have shown that yeast – like microorganisms may exhibited resistance to the conventional endodontic treatment procedures like *E. faecalis* [11,12].

The teeth which were included in this study had been previously filled with a variety of different root filling materials. Results have proved that *E. faecalis* and *C. albicans* are ecologically strong microorganisms and can successfully survive in the environment of incompletely filled root canals where the availability of nutrients is limited in comparison with cases of primary apical periodontitis [1,2,3,4].

Early described microbiological methods for primary identification of enteric bacteria and yeasts which are based on biochemical reactions and antimicrobial resistance patterns may not give the full picture of specificity of microorganisms found in the root canals since phenotypic traits of isolated microorganisms may be inconsistently expressed and by these means can influence the interpretation of the final results.

For this purpose electrophoresis of whole-cell soluble proteins was used. This method proved to be a valuable method for rapid and precise characterisation of oral bacteria. This method provides important information and verification on the similarity of the strains within the same species and subspecies [13, 14].

This method enabled us to verify and confirm primary test results of the identification of microorganisms found in the root canal system. This allowed us to draw precise conclusions.

The results of our study contributed to the understanding of the etiology of the enteric and yeast infection in the root canal system in retreatment cases. Further studies are necessary in order to develop effective measures for the prophylaxis and treatment of root canal infections caused by the resistant species of microorganisms.

CONCLUSIONS

1. Typing methods based solely on the molecular typing tests have limitations since phenotypic traits may be inconsistently expressed and this could influence the interpretation of the preliminary results.

2. Electrophoretic separation of cellular is a highly sensitive analytical technique that provides distinctive phenotypic evidence of the similarity of strains within species.

3. The results obtained after electrophoresis procedure confirmed the results of the primary tests used for the detection of bacteria.

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Received: 20 02 2003

Accepted for publishing: 25 03 2003