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**MICROFLORA OF ROOT FILLED
TEETH WITH APICAL PERIODONTITIS
AND ITS SENSITIVITY TO
ANTIBACTERIAL SUBSTANCES**

Doctoral Thesis Summary for Doctor's degree in
Medical Sciences
Speciality – Endodontics

Rīga, 2012

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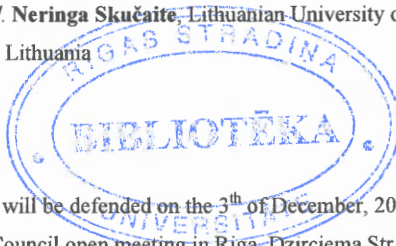
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ANNOTATION

This doctoral thesis “Microflora of root filled teeth with apical periodontitis and its sensitivity to antibacterial substances” focuses on a study of root canal infection which is a primary aetiological factor of apical periodontitis.

Apical periodontitis (AP) is a periodontal inflammation caused by infection in the root canal system and may appear as a chronic asymptomatic process that is frequently detected during a dental radiographic investigation. Epidemiological studies show a relationship between endodontically treated teeth and chronic AP in 25-40 % of cases, thus indicating a potentially high need for endodontic retreatment in different populations. In a study carried out in Latvia, it was showed that chronic AP is present in more than 30% of teeth which have undergone root canal treatment. Microbial flora of filled root canals is different from primary infection of the root canal and is more resistant to antibacterial agents. The microbial flora of endodontically treated root canals has not been studied in Latvia.

The aim of the study was to investigate the microbial flora of endodontically treated root canals with chronic apical periodontitis and to determine their sensitivity to antibacterial substances used in root canals, and as to determine the prevalence of β -lactamase producing bacterial strains in Latvian patients. Thirty-five patients scheduled for root canal retreatment in Endodontic Department at the Institute of Stomatology of Riga Stradins University and an extensive private dental clinic in Riga, Latvia were selected for the study. During retreatment, microbiological samples were collected and transported to the microbiological laboratory. Identification of isolated bacterial strains, determination of β -lactamase producing strains and evaluation of sensitivity of bacterial strains to 2.0% sodium hypochlorite solution, 0.2 % chlorhexidine digluconate solution and calcium hydroxide paste using the agar diffusion test were carried out.

The data was entered in the Microsoft Office Excel database. Statistical analysis of the data was performed by SPSS and Microsoft Office Excel softwares. Standard descriptive statistical methods were used to characterize microbial species isolated from endodontically treated root canals. Chi square statistical test was performed to detect possible correlation between bacterial aerotolerance, Gram staining and nitrocefine test results. Sensitivity of strains of the isolated microorganisms to

antibacterial substances used in root canals was evaluated using analysis of dispersion (ANOVA).

The analysis of data showed that

- Gram-positive microorganisms prevail in the microbial flora of endodontically treated root canals with chronic apical periodontitis in Latvian patients.
- 1 to 6 species of microorganisms were isolated and identified with a method of cultivation from endodontically treated root canal with chronic apical periodontitis.
- Bacterial species most frequently isolated from endodontically treated teeth with chronic apical periodontitis belong to the genera of *Actinomyces*, *Streptococcus*, *Staphylococcus*, *Lactobacillus* and *Enterococcus*.
- β -lactamase producing microorganisms add up to almost one-fifth of bacterial strains isolated from retreated root canals.
- The most frequently found β -lactamase producing bacterial species belong to the genera of *Actinomyces* and *Staphylococcus*.
- β -lactamase producing bacterial strains were found in about one-third of patients included in the study.
- Bacterial strains isolated from the retreated root canals are sensitive to sodium hypochlorite and chlorhexidine gluconate solutions and are weakly sensitive to calcium hydroxide paste when tested in vitro.
- Different strains of the same microbial species may have different sensitivity to antibacterial substances used in root canals.

Based on the present results and scientific literature, practical recommendations are elaborated for irrigation and temporary dressing of retreated root canals with chronic apical periodontitis.

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ABBREVIATIONS

ANOVA	statistical analysis of variance
AP	apical periodontitis
CHX	chlorhexidine digluconate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
MTAD	mixture of tetracycline, citric acid, and detergent
NaOCL	sodium hypochlorite
PAI	Periapical Index
RNA	ribonucleic acid

INTRODUCTION

1.1. Topicality of the Study

Apical periodontitis (AP) is an inflammation of the periodontal ligament surrounding the root apex of a tooth, usually as a consequence of pulpal inflammation or necrosis (Ørstavik, 2007). Apical periodontitis is a sequel to endodontic infection and manifests itself as the host-defence response to microbial challenge emanating from the root canal system. As clinical symptoms may be absent or scarce, periapical lesions are often detected by radiographic investigations.

The ultimate biological aim of endodontic treatment is either to prevent or to cure apical periodontitis (Ørstavik, 2007). Clinical studies have demonstrated that successful endodontic treatment may be achieved in over 90% of cases (Chevigny et al., 2008; Ng et al., 2007) while results from epidemiological studies represent success-rates from 60 to 75%, indicating the potentially high need for endodontic retreatment in different populations (Eriksen, 2007). The prevalence of endodontically treated teeth and teeth with AP gradually increases with age, half of individuals aged over 50 may have teeth with radiographic signs of AP (Kirkevang et al., 2001; Jimenez-Pinzon et al., 2004). The investigation based on samples from 35- 44 year old patients from Riga, Latvia, indicates that 87% of patients have at least one endodontically treated tooth. Thirty one percent of the endodontically treated teeth presented with a radiographically detectable periapical radiolucency (Jerša et al., 2010). Nonsurgical retreatment is an option to save the patient's natural tooth in such cases. Meta-analysis of outcomes of secondary root-canal treatment shows the estimated success rate 77% (Ng Y et al., 2008). It is important to use evidence-based knowledge of root canal microflora and apical periodontitis to develop treatment principles, and to improve the outcome of endodontic retreatment.

The presence of microorganisms within the root canal system is considered to be the major cause of periapical pathology of endodontically treated teeth. Studies indicate differences in the composition of the flora in retreatment cases compared to primary necrotic cases. The microflora associated with persistent secondary infections is usually composed of a low number of species, with predominance of Gram-positive bacteria (Peciuliene et al., 2000; Hancock et al., 2001; Pinheiro et al., 2003). Lower prognosis in root canal retreatment cases may be associated with difficulties in the elimination of the specific microbial flora. Microorganisms may persist in the apical

part of a root canal, in isthmuses and lateral canals. In addition, the environmental conditions and nutritive conditions in treated root canals differ from those in untreated cases (Lazizzera et al., 2000). Such factors as dentine buffer capacity, bacterial invasion into dentine and biofilm also contribute to difficulties of infection elimination (Gilbert et al., 1997 Haapasalo et al., 2000).

Debridement of a root canal by instrumentation and irrigation is considered as the most important factor in eradication of endodontic infection. Studies demonstrate that mechanical preparation leaves 35% or more of the canals' surface area unchanged (Paque et al., 2009; Peters et al., 2001). The use of irrigating solutions (e.g. sodium hypochlorite, chlorhexidine digluconate) is an important part of effective chemomechanical preparation. It enhances bacterial elimination and facilitates removal of necrotic tissue and dentine remnants from the root canal. It is traditionally recommended that the root canal should be filled with an antibacterial dressing, e.g. calcium hydroxide, between appointments to secure the disinfection of the canal space, until it is filled at the next appointment. Microbial flora of root filled teeth with apical periodontitis possesses greater resistance to antimicrobial agents used in endodontic treatment than microorganisms of primary infected teeth (Waltimo et al., 1999; Estrela et al., 2003; Baker et al., 2004).

Antibiotics are typically prescribed in dental practice for treatment of acute odontogenic infections. Dentists should acknowledge that it is essential to use antimicrobials in an appropriate and responsible manner, both to treat an infection effectively and to minimize the likelihood that bacteria will develop resistance to antimicrobials in the general population. Bacterial resistance to the antibacterial agents has been a clinically significant problem for over 40 years (Kunin et al., 1993). β -lactam antibiotics are the antimicrobial agents most commonly used in treatment of many infectious diseases (Matagne et al., 1998). The major mechanism of β -lactam resistance is bacterial production of β -lactamases, which are a group of enzymes that catalyze the hydrolysis of the beta-lactam ring of the antibiotics yielding inactive products (Maddux, 1991). Evidence show that antibiotic resistance and prevalence of β -lactamase-producing microorganisms has increased in the oral microflora over the last 10-20 years (van Winkelhoff et al., 1997; Fosse et al., 1999; Lewis et al., 1995; Brook & Frazier 1995; Kuriyama et al., 2000). Until now, the microbial flora of endodontically treated root canals, its sensitivity to antibacterial substances and the prevalence of β -lactamase-producing bacterial strains has not been studied in Latvia.

1.2. Objective of the Study

The aim of the present study was to investigate the microbial flora of root filled teeth with apical periodontitis, to determine the antimicrobial efficacy of antibacterial substances on isolated microorganisms, and to determine the prevalence of β -lactamase producing strains in isolated bacteria in Latvian patients.

1.3. Tasks of the Study

1. To identify microbial strains isolated from root filled teeth with apical periodontitis and to detect their aerotolerance and Gram staining.
2. To detect prevalence of bacterial species isolated from retreatment cases.
3. To detect β -lactamase producing strains in isolated bacteria.
4. To detect prevalence of β -lactamase producing strains in isolated bacteria.
5. To detect the antimicrobial efficacy of 2. 5% sodium hypochlorite on selected bacterial strains.
6. To detect the antimicrobial efficacy of 0. 2% chlorhexidine digluconate on selected bacterial strains.
7. To detect the antimicrobial efficacy of calcium hydroxide paste on selected bacterial strains.

1.4. Hypotheses of the Study

1. Gram-positive facultative anaerobic microorganisms are prevalent in microbial flora isolated from root filled teeth with apical periodontitis.
2. Sodium hypochlorite and chlorhexidine digluconate have an antimicrobial efficacy on microorganisms isolated from endodontic retreatment cases. Calcium hydroxide has a weak antimicrobial effect *in vitro*.

1.5. Scientific Novelty and Practical Implication of the Study

The study of the microbial flora of root filled teeth was carried out for the first time in Latvia, involving determination of efficacy of antibacterial substances on isolated microorganisms and determination of the prevalence of β -lactamase producing strains in isolated bacteria.

The outcome of the study supported the proposed hypotheses and provides information about microbial flora in root filled teeth with apical periodontitis in Latvian patients. Practical recommendations for antibacterial rinse and temporary

medication in retreatment cases are developed based on information obtained in the study and scientific literature.

1.6. Structure of the Doctoral Thesis

The Doctoral Thesis consists of introduction, objective of the study, tasks and hypotheses, literature review, methodology, results, conclusions, discussion, and publications. Practical recommendations are presented in task 3. Reading list comprises 235 references. The total volume of the Doctoral Thesis is 97 A4 format pages; symbols size 12 and 1.5 rows spaces, including 13 pictures and 5 tables.

2. MATERIALS AND METHODS

2.1. Clinical material

The consultations of 112 patients were creating the study sample. Thirty-five patients requiring non-surgical endodontic retreatment were selected from those who attended the Endodontic Department at the Institute of Stomatology of Riga Stradins University and an extensive private dental clinic in Riga, Latvia. Patients' age varied from 18 to 64 years. Nineteen female and 16 male patients were included in the study. Patients' medical and dental history was obtained, and a clinical and radiographic investigation of oral cavity was performed.

The clinical investigation of root filled teeth included evaluation of restoration (permanent/ temporary) and dental hard tissue, evaluation of periodontal status (probing, palpation, percussion), and evaluation of soft tissue. Only asymptomatic teeth were included in the study and were defined as follows:

- 1) Negative tests of palpation and percussion,
- 2) Absence of sinus tract,
- 3) Absence of intraoral and extraoral swelling,
- 4) Absence of signs of vertical fracture.

Radiographic investigation of root filled teeth using digital imaging was performed. The quality of previous root canal treatment was assessed (distance from the filling depth to radiological apex and presence of voids or pores). Periapical status of root filled teeth was assessed using PAI score system (Ørstavik et al. 1986) (Fig. 2.1.).

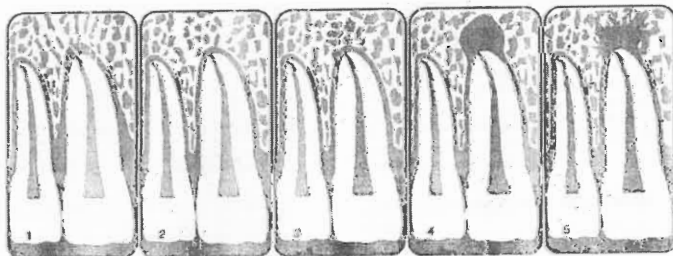


Fig. 2.1. The PAI score system (Ørstavik et al. 1986)

Criteria of inclusion:

- 1) Radiolucency seen on radiographs (PAI = 4 and 5) at the apex of endodontically treated teeth,
- 2) Improper quality of root canal filling (voids, pores), distance from the filling depth to the radiological apex equals 0-5 mm,
- 3) Time from previous endodontic treatment more than 4 years,
- 4) Teeth having permanent restoration.

Criteria of exclusion:

- 1) Antibiotic treatment during last 3 months,
- 2) General diseases,
- 3) Signs of acute periapical pathology,
- 4) Teeth having sinus tracts,
- 5) Teeth with temporary filling or with missing restoration,
- 6) Non-restorable teeth,
- 7) Teeth with marginal periodontitis.

2.2. Endodontic Retreatment

The root canal retreatment was performed at the Endodontic Department of the Institute of Stomatology of Riga Stradins University and a private dental clinic in Riga, Latvia. The retreatment was carried out by one specialist – the author of the present study. The endodontic treatment was performed under aseptic conditions. After preparation of access cavities, the teeth were isolated with rubber dam and disinfected with 5.25% Na hypochlorite. Na hypochlorite solution was inactivated with Na thiosulphate. After opening of the root canal entrance the type of the filling material was evaluated and appropriate method of removal was chosen. Following partial removal of the filling material and preparation of access to the apical part of the canal, microbiological samples were taken (see Microbiological Procedures). Prior the collection of samples, the apical part of the root canal was treated with endodontic files to make dentin shavings. After sampling, removal of the filling material was completed using a solvent, an irrigation solution (2.5% sodium hypochlorite solution, 0.2% chlorhexidine digluconate solution) and a lubricant. Working length was determined by apex locator (Root ZX, Morita, USA) and radiographically. In all cases root canal preparation was performed in one session.

Gutta-percha filling was removed using endodontic files, Gates-Glidden drills and rotary instruments (Retreatment Pro Taper, Dentsply Maillefer, Switzerland), and the canals were rinsed with sterile physiological saline solution. After the sampling, filling removal was completed using a solvent (Guttasolv, Septodont, France). Root canal obturation material containing zinc-oxide-eugenol base was removed using endodontic files, Gates-Glidden drills and rotary instruments and rinsing canals with physiological saline solution. Following the sampling, filling removal was completed using a solvent (Endosolv R, Septodont, France), irrigation solutions and a lubricant. Removal of resin-based material was performed using endodontic files and Gates-Glidden drills. Removal of metal objects was performed using ultrasound equipment and endodontic tips (EMS, Switzerland). Rotary instruments (Pro Taper, Dentsply Maillefer, Switzerland) were used for preparation of root canals.

2.3. Sampling and Identification of Microbiological Samples

After partial removal of the filling material and preparation of access to the apical part of the canal, microbiological sampling was carried out. The canal was rinsed with sterile physiologic saline solution to moisten it prior collection of a sample. Sterile paper points were introduced into canals and left for 1 minute. The paper points were immediately placed in transportable tubes (Port-A-Cul-tube, Becton Dickinson, USA). Processing of samples in the laboratory was performed within 4 hours.

R2A (LAB M, UK) and sheep blood agar (National Diagnostic Centre, Riga) culture media were used for cultivation of microorganisms. Samples were incubated in aerobic as well under anaerobic conditions in CO₂ atmosphere (Gas Pak, Becton Dickinson, USA) and 37°C for up to 14 days. Examination of the morphology of grown microorganisms was studied under a microscope “Leica”, magnification of 600x. To identify the pure culture of microorganisms, test systems “BBL Crystal™” (Becton Dickinson, USA) with Gram-positive ID, Enteric/Nonfermenter ID and Anaerobe ID kits were used. In addition, Gram-staining as well as oxidase and indole reactions for detection of Gram-negative bacteria and catalase and indole reactions for detection of anaerobic bacteria were carried out. Yeasts were identified according to micro-morphological signs using Fungiscreen 4H (Sanofi Diagnostics, Pasteur, France). All isolated strains of microorganisms are registered and stored in the Microbial Strain Collection of Latvia.

2.4. Sensitivity of Microbial Strains to Antibacterial Substances

In order to determine efficacy of antibacterial substances, 31 microbial strains belonging to 27 species were used. They were selected randomly from ninety-three microbial strains isolated from retreated root canals. Using agar diffusion method the sensitivity of microorganisms to sodium hypochlorite (2.5% Dzirciema aptieka, Riga), chlorhexidine digluconate (0.2%, Dzirciema aptieka, Riga), calcium hydroxide paste (UltraCal XS, Ultradent Products Inc., USA) was evaluated. Antibacterial efficacy was tested on monocultures of bacteria.

Microbial suspension was prepared in screw-capped glass test tubes and poured into sterile Petri dishes with the agar cultural medium and levelled evenly over the entire surface. Excess fluid was carefully drained with a sterile pipette. After the surface of the culture medium was drained (after 20–30 minutes), a metal cylinder (diameter 5 mm) was used to obtain 3 round beds in agar. Beds were filled with antibacterial substance. Samples were incubated at 37°C for 1–3 days; anaerobes were cultivated in anaerobic camera. Liquids diffused in agar and suppressed the growth of microorganisms. The inhibition zones were measured (mm) (Figure 2.2.).

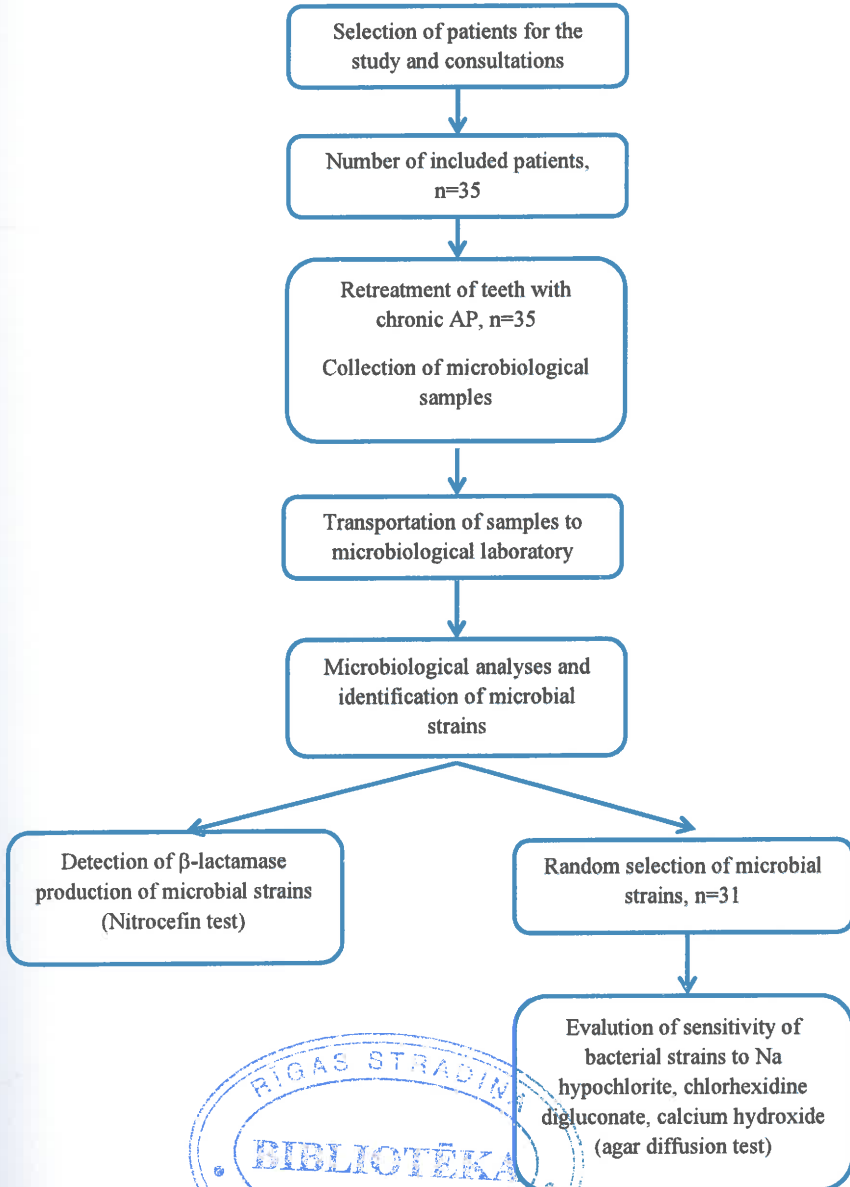


Fig. 2.2. The agar diffusion method. Plate with inhibition zones

2.5. Detection of β -lactamase Producing Microbial strains

β -lactamase test was conducted on 85 microbial strains isolated from 32 endodontically treated root canals. β -lactamase determination was carried out using a nitrocefin kit. The bacterial pure culture was placed onto a Nitrocefin slide (Dry Slide™ nitrocefin, Becton Dickinson, USA). In the presence of β -lactamase producing microorganism, a change of colour from yellow to red was observed on slides.

2.6. The study design



2.7. Data Processing

The data were entered in the Microsoft Office Excel database and statistical analysis was carried out using SPSS Statistics 17.0 and Microsoft Office Excel.

Standard descriptive statistical methods were used to characterize the microbial species isolated from endodontically treated root canals. Chi square statistical test was performed to detect correlation between bacterial aerotolerance, Gram staining and β -lactamase (nitrocefine test) results. The statistical analysis of variance (ANOVA) was used to detect the sensitivity of isolated microbial strains to antibacterial substances used in root canals,

Significance of differences was set at a significance level of 5% ($p=0.05$).

3. RESULTS

3.1. The number of Microbial Isolates, Gram staining, aerotolerance

Microorganisms were cultured in 34 cases (97.1%) from 35 teeth involved in the study. Ninety-three microbial strains were isolated. From 1 to 6 species were isolated from each sample (mean 2.7 species). In one sample microorganisms were not detected, in 6 cases single species were found (17.7%), 2 species were isolated in 13 cases (38.2%) and 3 species were isolated in 3 cases (8.8%). Four species were isolated in 9 cases (26.5%), 5 species were isolated in 2 cases (5.9%), 6 species were found in 1 case (2.9%). Strains of the *Streptococcus intermedius*, *Bacteroides caccae*, *Streptococcus constellatus*, *Escherichia coli* and *Actinomyces viscosus* were isolated as monoinfection.

Ninety-three isolates pertained to 29 genera of microorganisms. The majority of identified microorganisms (77.4%) were Gram-positive. Fifty (53.8%) isolates were facultative anaerobes, 30 isolates (32.2%) were obligate anaerobes and 13 isolates (14.0%) were obligate aerobes and microaerophiles. Chi square statistical analysis was performed to detect correlation between microbial aerotolerance and Gram staining indicators (Fig. 3.1). No statistically significant difference ($p=0.22$) between these parameters was proven. Gram positive bacteria were isolated 4.2 times more frequent than Gram negative. Gram positive facultative anaerobes were found 6.3 times more frequent than Gram negative facultative anaerobes.

Four yeast strains were isolated from three (9.1%) teeth involved in the study. Two yeast species were isolated from one sample. *Candida albicans* was isolated in 2 cases. Other isolated yeast strains belonged to the genera of *Saccharomyces* and *Cryptococcus*.

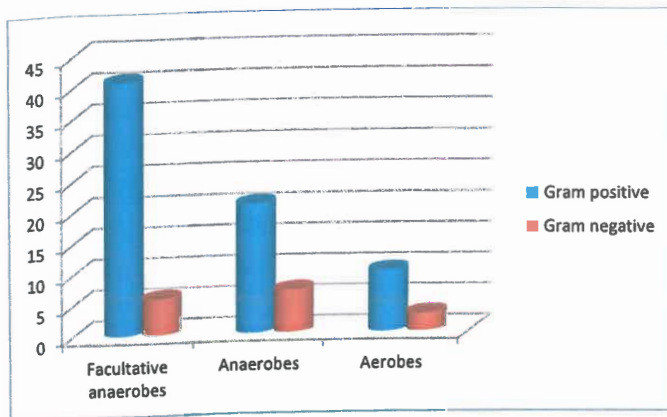


Fig 3.1. Aerotolerance and Gram staining indicators of isolated microbial species

3.2. The Prevalence of Microbial Species and Genera.

Most frequently isolated microorganism species (Table 3.1.) pertained to the genera of *Actinomyces* (29.4%), *Streptococcus* (27.3%), *Staphylococcus* (21.2%), *Lactobacillus* (18.2%) and *Enterococcus* (18.2%).

Table 3.1.

Identity and prevalence of isolated microorganisms in root filled teeth with apical periodontitis

Microbial genus	Number of isolates	Number of teeth	Prevalence
<i>Actinomyces</i>	13	10	29,4%
<i>Streptococcus</i>	11	9	27,3%
<i>Staphylococcus</i>	9	7	21,2%
<i>Enterococcus</i>	6	6	18,2%
<i>Lactobacillus</i>	6	6	18,2%
<i>Bacillus</i>	7	5	15,2%
<i>Lactococcus</i>	3	3	9,1%
<i>Peptostreptococcus</i>	3	3	9,1%
<i>Fusobacterium</i>	3	3	9,1%
<i>Bacteroides</i>	3	3	9,1%
<i>Enterobacter</i>	3	3	9,1%
<i>Escherichia</i>	3	3	9,1%
<i>Arcanobacterium</i>	2	2	6,1%
<i>Micrococcus</i>	2	2	6,1%
<i>Prevotella</i>	2	2	6,1%

Table 3.1. continued

Gemella	2	2	6,1%
Candida	2	2	6,1%
Eubacterium	2	2	6,1%
Corynebacterium	1	1	3,0%
Campylobacter	1	1	3,0%
Rhodococcus	1	1	3,0%
Veilonella	1	1	3,0%
Stenotrophomonas	1	1	3,0%
Propionibacterium	1	1	3,0%
Clostridium	1	1	3,0%
Cryptococcus	1	1	3,0%
Sacharomyces	1	1	3,0%
Pseudomonas	1	1	3,0%
Aerococcus	1	1	3,0%

Thirteen strains of species belonging to the *Actinomyces* were isolated. *Actinomyces israelii* was isolated in 2 cases (6.1%), *Actinomyces viscosus* was isolated in 2 cases (6.1%). *Actinomyces naeslundii* was found in 3 cases (9.1%), *Actinomyces odontolyticus* was found in 3 cases (9.1%) and *Actinomyces pyogenes* was also found in 3 cases (9.1%).

Eleven strains pertained to the genus *Streptococcus* were isolated from 9 retreated root canals. *Streptococcus mitis*, *Streptococcus porcinus*, *Streptococcus salivarius*, *Streptococcus sanguinis*, *Streptococcus uberis* were isolated in 1 case each (3%), *Streptococcus constellatus* – in 2 cases (6.1%), *Streptococcus intermedius* was isolated in 4 cases (12.2%).

Nine species belonging to the genus *Staphylococcus* were isolated from 7 retreated dental root canals. *Staphylococcus cohnii*, *Staphylococcus kloisii*, *Staphylococcus lentus*, *Staphylococcus saccharolyticus*, *Staphylococcus saprophyticus* were isolated in 1 case each (3%). *Staphylococcus capitis*, *Staphylococcus epidermidis* were isolated from 2 samples each (6.1%).

Six species belonging to the genus *Lactobacillus* were found. Strains of the *Lactobacillus acidophilus* were found in 3 cases (9.1%) and strains of the *Lactobacillus johnsonii* were also found in 3 cases (9.1%).

The genus *Enterococcus* was represented by 6 strains. *Enterococcus faecium* was found in case, and *Enterococcus faecalis* was isolated in 5 cases (15.2%). *E.faecalis* was not isolated as a mono-infection in any cases.

3.3. Prevalence of β -lactamase-producing Bacterial Strains

β -lactamase production test (nitrocefine test) was carried out on 85 bacterial strains that were isolated from 32 endodontically treated teeth. β -lactamase producing microorganisms were found in 12 (37.5%) of the 32 patients with cultivable microbial flora. Four patients (12.5%) had all isolated microorganisms as β -lactamase producing ones. Sixteen β -lactamase producing bacterial strains belonging to 13 species were found in the root canals (Table 3.2). β -lactamase producers were 18.5% of the 85 microbial strains isolated from the root canals of the retreated teeth. The most common β -lactamase producing microbial strains pertained to the genera of *Actinomyces* and *Staphylococcus*.

Table 3.2.

Identity and prevalence of β -lactamase-producing bacterial strains

Bacterial species	Number of isolates	Prevalence
<i>Actinomyces odontolyticus</i>	1	3,1%
<i>Actinomyces israelii</i>	1	3,1%
<i>Actinomyces viscosus</i>	2	6,3%
<i>Bacillus licheniformis</i>	1	3,1%
<i>Bacteroides caccae</i>	1	3,1%
<i>Corynebacterium aquaticum</i>	1	3,1%
<i>Enterobacter cloacae</i>	2	6,3%
<i>Esherichia coli</i>	2	6,3%
<i>Propionibacterium avidum</i>	1	3,1%
<i>Rhodococcus equi</i>	1	3,1%
<i>Staphylococcus capitis</i>	1	3,1%
<i>Staphylococcus epidermidis</i>	1	3,1%
<i>Staphylococcus lentus</i>	1	3,1%

3.4. Gram Staining, Aerotolerance of the β -lactamase-producing Microorganisms

Five strains (31.2%) of the 16 β -lactamase producing microbial strains were Gram-negative and 11 strains (68.8%) were Gram-positive.

Five (31.3%) β -lactamase producing microbial strains were anaerobes, one strain (6.2%) was aerobe and 10 strains (62.5%) were facultative anaerobes. Chi square statistical analysis was performed to detect correlation between bacterial nitrocefine test and Gram staining indicators (Fig. 3.2) and statistically significant difference ($p=0.05$) was found. Gram-positive nitrocefine negative microorganisms are more than nitrocefine negative, respectively, the majority of the isolated Gram-positive bacteria do not produce β -lactamase.

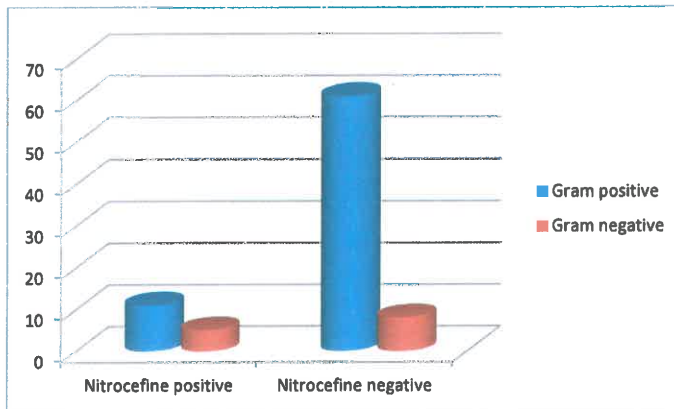


Fig.3.2. Gram staining and nitrocefine test indicators of microbial isolates

3.5. Sensitivity of Microbial Strains to Antibacterial Substances Used in Root Canals

To determine the sensitivity of the microbial flora to antibacterial substances for root canals, 31 microbial strains were used. Measurements microbial inhibition zones formed by antibacterial substances are described in the Table 3.3.

Table 3.3.

Zones of bacterial inhibition

Bacterial species	Inhibition zones (mm)		
	Antibacterial substance		
	Chlorhexidine digluconate	Sodium hypochlorite	Calcium hydroxide
<i>Actinomyces israelii</i>	16	26	0
<i>A. haemolyticum</i>	26	42	0
<i>A. naeslundii</i> (6) *	19	35	0
<i>A. naeslundii</i> (13) *	0	0	0
<i>A. odontolyticus</i>	18	38	1
<i>Arcanobacterium pyogenes</i>	20	32	2
<i>Bacteroides caccae</i>	8	30	0
<i>Campylobacter gracilis</i>	21	38	0
<i>Corynebacterium aquaticum</i>	2	9	6
<i>Enterobacter cloacae</i> (2) *	2	9	2
<i>E. cloacae</i> (17) *	4	1	3
<i>Enterococcus faecalis</i>	5	1	2
<i>E. faecium</i>	3	8	3
<i>Escherichia coli</i> (16) *	2	8	3
<i>E. coli</i> (4) *	3	1	1
<i>Fusobacterium nucleatum</i>	22	39	1
<i>Gemella morbillorum</i>	5	14	1
<i>Lactococcus garvieae</i>	5	25	4
<i>Peptostreptococcus anaerobius</i>	9	25	1
<i>P. tetradius</i>	0	0	3
<i>Prevotella disiens</i>	19	42	2
<i>Propionibacterium avidum</i>	21	35	1
<i>Rhodococcus</i> sp.	4	14	3
<i>Staphylococcus capitis</i> (23) *	7	15	1
<i>S. capitis</i> (26) *	4	11	2
<i>S. epidermidis</i>	4	7	2
<i>S. lentus</i>	2	1	2
<i>S. kloosii</i>	4	14	2
<i>S. saccharolyticus</i>	1	25	1
<i>S. saprophyticus</i>	5	2	3
<i>Streptococcus intermedius</i>	2	8	4

*Strains from different samples

Inhibition zones created by sodium hypochlorite solution (Figure 3.3.) ranged from 0 to 42 mm (mean 17.9 ± 14.4 mm). Inhibition zones formed by chlorhexidine solution (Figure.3.4.) ranged from 0 to 26 mm (mean 8.5 ± 8.0 mm) and calcium hydroxide paste created inhibition zones (Figure. 3.5.) from 0 to 6 mm (mean 1.8 ± 1.4 mm). Results of inhibitory zones created by the above antibacterial substances were submitted to analysis of variance (ANOVA) and significant difference ($p = 0.001$) was found. Sodium hypochlorite was the most effective antibacterial substance.

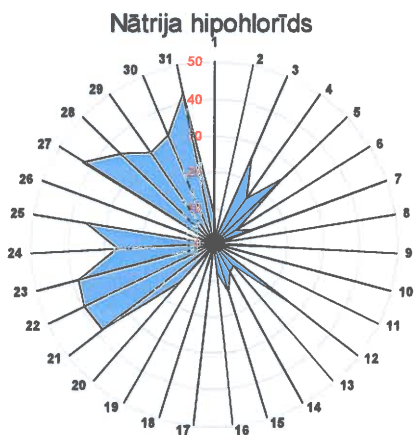


Fig.3.3. The susceptibility of microorganisms to sodium hypochlorite
(1-31- number of strain; 0-50- zones of bacterial inhibition, mm)

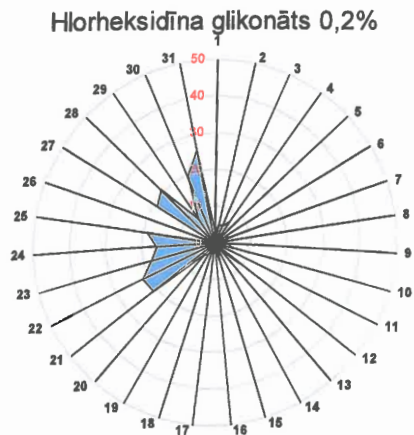


Fig.3.4. The susceptibility of microorganisms to chlorhexidine digluconate
(1-31- number of strain; 0-50- zones of bacterial inhibition, mm)

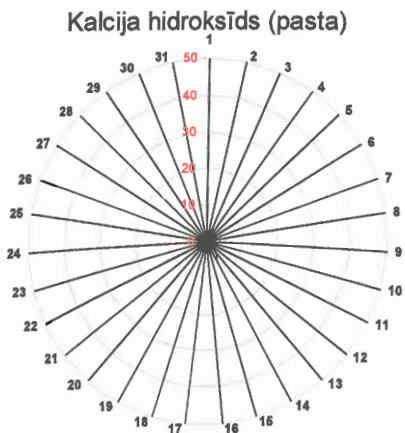


Fig. 3.6. The susceptibility of microorganisms to calcium hydroxide
(1-31- number of strain; 0-50- radiuses of zones of bacterial inhibition, mm)

Most of the microorganisms (87.1%) were more susceptible to the root canal irrigants (sodium hypochlorite, chlorhexidine digluconate) than to calcium hydroxide paste. Three microbial isolates (9.7%) were more susceptible to calcium hydroxide than to chlorhexidine digluconate, but the susceptibility was no higher than their susceptibility to sodium hypochlorite. One microbial strain (*Peptostreptococcus tetradius*) was resistant to root canal irrigation solutions however it was susceptible to calcium hydroxide paste.

Sodium hypochlorite showed strong inhibitory effects on the genera *Actinomyces*, *Arcanobacterium*, and on the species *Fusobacterium nucleatum*, *Prevotella disiens*, *Propionibacterium Avidum*, *Bacteroides caccae*, *Lactococcus garvieae*, *Streptococcus saccharolyticus*, *Peptostreptococcus anaerobius*.

Chlorhexidine gluconate solution most explicitly inhibited such bacterial genera as *Actinomyces*, *Arcanobacterium* and *Campylobacter gracilis*, *Fusobacterium nucleatum*, *Prevotella disiens*, *Propionibacterium avidum*.

Calcium hydroxide paste showed the least inhibiting effect, most bacteria continued to grow. It was slightly effective only on *Corynebacterium Aquatic*, *Streptococcus intermedius* and *Lactococcus garvieae*.

Different strains of microbial species isolated from retreated root canals had different susceptibility to antibacterial substances. Strains of the *Escherichia coli*, *Enterobacter cloacae*, and *Actinomyces naeslundii* bacteria species were obtained from different samples. Inhibitory zones of the *Escherichia coli* isolated from the sample No 16 were larger than the ones from the sample No 4. A similar situation was shown by the results of inhibition zone of the *Enterobacter cloacae*, the strain isolated from the 2nd sample was more sensitive to sodium hypochlorite than the strain from the 17th sample. Strain of the *Actinomyces naeslundii* isolated from the 13th sample showed no sensitivity to the tested antibacterial substances.

4. DISCUSSION

4.1. Aim and Design of the Study

Microbial flora of root canals has not been studied in Latvia before. Therefore methods of treatment and choices of antibacterial substances for retreatment of root canals with apical periodontitis were based on the assumption that the resistant Gram-positive bacteria prevail in the microbial flora of these teeth. The aim of the present study was to investigate microflora of endodontically treated root canals with chronic apical periodontitis, its sensitivity to the most commonly used antibacterial root canal substances and to determine the prevalence of β -lactamase producing strains in Latvian patients. The hypothesis of the study was based on similar studies conducted in other countries (Molander et al., 1998; Sundqvist et al., 1998; Peciuliene et al., 2000; Hancock et al., 2001; Pinheiro et al., 2003).

Strict inclusion criteria were used for selection of patients- teeth with root filling and chronic apical periodontitis endodontically treated more than 4 years previously (Molander et al., 1998). In compliance with the guidelines of the European Society of Endontology, an infection is considered to be persistent if apical periodontitis fail to diminish radiographically within 4 years following the treatment or retreatment of root canals (ESE, 2006). The sample size was restricted by study budget. To yield microbial flora from root canals with persistent chronic infection, teeth with the filling depth of 0-5 mm from the radiological apex were selected for the study. Patients with teeth with chronic periodontitis and fistula, or those with signs of exacerbation of chronic periodontitis were not included in the study. The study did not include patients with general medical conditions or those who had used antibiotics within last 3 month, as well as it did not include those teeth with temporary fillings and teeth with no restorations. Such a strict inclusion and exclusion criteria for the study made it possible to compare the results obtained with qualitative studies conducted previously and to present the data at scientific conferences and journals.

4.2. Number of Teeth with Cultivable Microbial Flora and Number of Isolated Microorganisms

Microorganisms were found in 97% of cases from 35 teeth involved in the study. In similar studies microorganisms were isolated in a smaller frequency of cases – 44.4% (Sundqvist et al., 1998), 73.4% (Molander et al., 1998), 82.5% (Peciuliene

et al., 2001) and in 61.1% of cases (Hancock et al., 2001). Similar inclusion criteria were mentioned in those studies (asymptomatic endodontically treated teeth with apical periodontitis, time from the previous endodontic treatment exceeded 2 years), microbial cultivation and similar methods of microbial identification. The high recovery rate of intracanal bacteria might be explained by the sampling technique used. Following the guidelines described in studies (Molander et al., 1998; Schiimeister et al., 2007), dentin chips were made from the apical part of the root canals and were not rinsed out before sampling. This method enables yielding of not only planktonic microorganisms in a sample but also fragments of biofilm formed on root canal walls. Literature also refers to the impact of geographic locations of the studies, that may lead to differences both in the amount and content of microorganisms (Siquiera, 2009). In the present study, microorganisms were not found in one case. This might be explained by the fact that microorganisms could be lost in the process of sampling and laboratory operations, particularly it could result from their small quantities or they might be located in inaccessible parts of the root canal system. Method used for bacterial identification is not sufficiently sensitive for such cases. It could also be an infection of non-cultivable bacteria (Munson et al., 2002; Olsen et al., 2009). Bacterial cultivation techniques make it possible to multiply and identify the living and the cultivable microorganisms. Time-consumption and possible loss of anaerobic microorganisms in the process of sampling and laboratory operations is a disadvantage of cultivation techniques. Despite its disadvantages, cultivation techniques remain essential in studies of bacterial physiology and pathogenicity studies as well as in determining the susceptibility to antibiotics (Gomes et al., 2011).

Higher number of microbial species isolated from the root canals than in similar studies was also found. Results show that only 18% of cases isolated from 34 root canals with cultivable microflora were monoinfection, 2 species were isolated in 38 % of cases, polymicrobial infection with 3 or more species - in 44 % of cases. In Scandinavian studies, monoinfection was found in 79% from positively cultivated teeth (Sundqvist et al., 1998), presence of 1 to 2 species in 85% of teeth and polymicrobial infection in 15% of teeth (Molander et al., 1998). In the USA, 1 or 2 species were isolated from a canal in 84.8% of cases, when paper points were used for sampling and in 89.2% of cases when endodontic files were used for sampling (Hancock et al., 2001). Studies show relationship between polymicrobial infection and inadequate root canal filling (Pinheiro et al., 2003) and inadequate restoration

(Hommez et al., 2004). In unfilled parts of a canal, the microflora is similar to the microflora of a tooth with pulpal necrosis (Pinheiro et al., 2003). In present study, samples from multi-rooted teeth were taken from the canal in which the filling was closer to radiological apex. Teeth without restorations or with temporary restorations were not included in the study. In a UK study, that included only endodontically treated teeth with signs of leakage of permanent restorations, micro-organisms were identified in different parts of the root canal system and tooth crown, and up to 41 microbial species were isolated from a tooth. The authors found no statistically significant relationship between the isolated strains and their location sites (Adib et al., 2004). Higher polymicrobial infection rate in the present study compared to similar studies might be due to the technique of sampling from the apical part of the canal.

Higher number of isolates is found in studies employing molecular microbial identification methods: DNA-DNA hybridization and polymerase chain reaction. The main advantage of the molecular microbial identification methods is a possibility to identify uncultivable microorganisms and the technique is less time-consuming (Siqueira et al., 2003). Disadvantages of the molecular microbial identification methods are high costs and possibility to identify the DNA of dead microorganisms (Josephson et al., 1993; Keer & Birch, 2003).

Amplification of genes 16S or 23S rRNA of bacteria is used in polymerase chain reaction. Thus, microorganisms previously undiagnosed in endodontic infection were identified such as *Bacteroides forsythus* and *Treponema denticola*, as well as bacteria belonging to the *Olsenella* genus (Fouad et al., 2002). When using DNA-DNA hybridization method, a high number of micro-species (6-10) was found also in samples of periapical tissues of asymptomatic endodontically treated teeth. Bacterial DNA was found in all cases (Gatti et al., 2000). In another study, bacterial DNA was found in 85% of periapical tissue samples (Handal et al., 2009). The use of molecular identification methods in studies and the new evidence have changed the knowledge about the amount of microbial species in the infected root canals and the bacterial presence in apical granuloma tissues. This finding has changed the previous opinion that apical granuloma is not contaminated.

4.3. Bacterial Gram Staining, Aerotolerance

In the present study, 53.8% of the isolated bacteria were anaerobes and 77.4% of isolates were Gram-positive species. Rates of aerotolerance and Gram staining are similar to the results of previous researches, e.g., in Scandinavian studies by Sundqvist (Sundqvist et al. 1998) and by Molander *et al.* (Molander et al., 1998), 58% and 69% of bacteria were facultative anaerobes and 87% and 74.3% were Gram-positive bacteria. In a USA study 80.4% were Gram-positive bacteria (Hancock et al. 2001), in Brazil - 57.4% were facultative anaerobes and 83.3% were Gram-positive species (Pinheiro et al., 2003). In the present study, the most frequently isolated facultative anaerobic species pertain to the genera *Streptococcus*, *Actinomyces*, *Enterococcus*, *Lactobacillus* and *Staphylococcus*. In similar studies, *Streptococcus*, *Actinomyces* and *Enterococcus* were the most frequently isolated species (Sundqvist et al., 1998; Pinheiro et al., 2003).

4.4. Species of the Most Frequently Isolated Microorganisms

Species pertaining to the genus *Actinomyces* were isolated in 29.4% of the cases. In similar Scandinavian studies *Actinomyces* were isolated in 2.9% and 12.0% of the teeth with cultivable microflora (Molander et al., 1998; Sundqvist et al., 1998). In other researches the *Actinomyces* were found in 23.5%, 19.6% and 24.0% of the cases with cultivable microflora (Hancock et al., 2001; Pinheiro et al., 2003; Cheung et al., 2001). *Actinomyces* is an opportunistic Gram-positive facultative anaerobic microorganism that is frequently found in the retreated root canals and is also involved in extra-radicular infection.

Species pertaining to the genus *Streptococcus* were isolated in 27.3% of the cases. In similar studies with teeth with cultivable microbial flora, the *Streptococcus* was isolated more rarely - in 8.8%, 17.6% and 20.6% of the cases (Sundqvist et al., 1998; Hancock et al., 2001; Molander et al., 1998) or in higher number of cases in one study - 33.3% (Pinheiro et al., 2003). *Streptococci* are also opportunistic Gram-positive facultative anaerobic bacteria that are frequently found in the retreated root canals and they facilitate co-invasion of other microbial species (Love, 2002).

Gram-positive facultative anaerobic bacteria pertaining to the genus *Staphylococcus* was isolated relatively frequently (21.2%). In a study conducted in Sweden, *Staphylococcus* was found in no cases (Sundqvist et al. 1998). In similar studies, the occurrence of *Staphylococcus* was lower. In another study by

Scandinavians, bacteria of this genus were found in 10.3% of the cases (Molander et al., 1998). In studies in the USA and Brazil, *Staphylococcus* was found in 11.8% and 3.9% of the teeth with cultivable microbial flora (Hancock et al. 2001, Pinheiro et al. 2003).

Lactobacillus was isolated more frequently (18.2%) than in other studies. In Scandinavian studies *Lactobacillus* was found in 4.2% and 16.2% of the cases (Sundqvist et al., 1998; Molander et al., 1998). In other similar studies *Lactobacillus* was isolated in 5.9% and 3.9% of the cases (Hancock et al., 2001; Pinheiro et al., 2003).

Higher isolation rate of *Staphylococcus* and *Lactobacillus* in comparison to other studies cannot be associated with different geographic location. It must be pointed out that the above similar studies show higher rate of teeth with uncultivable microbial flora - 15% to 56% of the cases. *Staphylococcus* and *Lactobacillus* are Gram-positive facultative anaerobes. *Lactobacillus* often is detected in a primary root canal infection (Sjögren et al., 1997; Siren et al., 1993) and, possibly, in retreated root canals they are persistent microorganisms that have survived treatment and disinfection procedures. The different prevalence of bacterial species might be due to sampling and cultivation techniques. It must be noted that the overall prevalence of Gram-positive facultative anaerobic microorganisms isolated in the present study is similar to the figures obtained in other studies.

Enterococcus faecalis was isolated from 15.2% of teeth with cultivable microbial flora. This rate is lower than in other studies. In Scandinavian studies *E.faecalis* was isolated in 47% and 38% of cases (Molander et al., 1998; Sundqvist et al., 1998), in Lithuania in 64% of cases (Peciuliene et al., 2001). In present study *E. faecalis* was not isolated as monoinfection in any case. In another study, *E. faecalis* as a single species was found in 18 of the 27 cases (Pinheiro et al., 2003). In Lithuanian study *E.faecalis* as monoinfection was found in 11 of the 21 cases (Peciuliene et al., 2001). In a study done by Zoletti et al., similar prevalence of *Enterococcus faecalis* (13%) was found in retreated root canals using cultivation technique, however higher prevalence (78%) was revealed using polymerase chain reaction (PCR) method (Zoletti et al., 2006). In another study *Enterococcus faecalis* was isolated in 22% of cases from retreated root canals using PCR identification technique (Fouad et al., 2005). In untreated root canals, *E. faecalis* was found in less than 11% of cases (Sedgley et al., 200; Siqueira et al., 2002).

Mechanisms of enterococci penetration in root canal system is not quite clear. One of the explanations could be that the *Enterococcus faecalis* occur in the root canal system during the process of treatment or between appointments. More often *E. faecalis* is isolated from poorly isolated root canals as well as from those canals that has undergone 10 or more endodontic treatments (Svensäter et al., 2004). Low rate of *E. faecalis* prevalence might be associated with the patient inclusion criteria and identification techniques. In present study, teeth with temporary restorations and teeth without restorations were excluded, and good isolation with rubber dam and the light curable resin was provided.

High prevalence of *Enterococcus faecalis* in the retreated root canals and the isolation in a form of monoinfection has drawn researcher's attention to this bacterium. Enterococci are elements of a normal human oral microflora, but in healthy individuals they are found in relatively small quantities and cannot be identified if standard sampling and cultivation techniques are used (Sedgley et al., 2004; Bergman et al., 1991). However, the high prevalence of *E. faecalis* in the infected retreated root canals enhances possibility that enterococci in the oral microflora are at greater quantities than it was thought previously. Studies show that different phenotypes and genotypes of *E. faecalis* are found in the root canals. Pinheiro et al. has also found that some patients have genotypically similar *E. faecalis*, and in one patient genotypically different strains were isolated from different dental root canals (Pinheiro et al., 2006). The role of *E. faecalis* in the pathogenesis of apical periodontitis is not entirely clear. *E. faecalis* is isolated more frequently from asymptomatic root canals (Siqueira et al., 2002; Pirani et al., 2008). *E. faecalis* has an ability to form a structure of biofilm, to invade dentinal tubules and to facilitate invasion of other organisms into the dentine. It is possible that those bacteria promote the participation of other microorganisms in formation and maintenance of apical periodontitis.

Yeasts were isolated in 9.1% of cases. This rate is higher than in most similar studies. In Scandinavian studies yeasts were found in 8.3% and 4.4% of the cases (Sundqvist et al., 1998; Molander et al., 1998). In other similar studies, yeasts were isolated in 2.4% and 3.9% of the teeth with cultivable microbial flora (Hancock et al., 2001; Pinheiro et al., 2003). In a Lithuanian study, the prevalence of yeasts was similar - 9% (Peciuliene et al., 2001). In the present study, half of isolated yeast species were *Candida albicans*. Other isolated yeast strains pertained to the genera

Saccharomyces and *Cryptococcus*. In similar studies *C. albicans* was the only yeast species (Sundqvist et al., 1998; Molander et al., 1998; Peciuliene et al., 2001; Hancock et al., 2001). In one study the prevalence of microorganisms of the genus *Candida* was not indicated (Pinheiro et al., 2003). Yeasts are opportunistic pathogens and it is possible that yeast species in retreated root canals are mostly members of secondary infections.

4.5. Prevalence of β -lactamase Producing Microorganisms

β -lactamase producing microorganisms were found in 37.5% of the patients with cultivable microbial flora. Similar prevalence of these bacteria (38.5%) was found in the samples of acute purulent infection in a study in the U.K. (Lewis et al., 1995). In another study, the prevalence of β -lactamase producing strains and the resistance to antibiotics of microorganisms isolated from different locations of the oral cavity – from marginal periodontium, buccal mucosa, tongue and saliva – was analysed and enzyme producing microorganisms were found in 38.5% of the patients (Villagran Valdes et al., 1982). β -lactamase producing microorganisms were found more often (in 55-73% of patients) in the microbial flora of marginal periodontitis (Herrera et al., 2000; Handal et al., 2004). In the present study, the prevalence of β -lactamase producing microorganisms in patients was interpreted as moderately high.

β -lactamase producing bacteria were in less than one fifth (18.5%) of the 85 microbial strains isolated from root canals of the retreated teeth. Similar prevalence of β -lactamase producing strains – 18.2% were found in samples of primary symptomatic and asymptomatic infections of the dental root canals (Gaetti-Jardim et al., 2007). These microorganisms might be a source of antibiotic resistance genes for other microorganisms involved in the root canal infection. Studies have shown that the resistance genes can be passed over both vertically – from mother cells to daughter cells – and horizontally – from one microbial species or strain to another microbial species or strain. Resistance genes can be retrieved both from living cells and released from dead microorganisms (Ferry et al., 2005; Tenover et al., 2006). The microbial resistance to antibiotics is clinically significant in cases of acute abscess, since it requires oral antibiotic treatment. It is not clear which gene mechanisms are involved in the resistance transfer to bacteria of the abscess. The resistance of root canal microflora to antibiotics tends to increase within several years in certain

populations (Gomes et al., 2011). Antibiotics of the penicillin group in combination with clavulanic acid are the most efficient on the microbial flora of acute apical abscesses (Baumgartner & Xia, 2003). In a study conducted in Lithuania, it was found that all microbial isolates of acute dental abscess were sensitive to penicillin, 74% of the strains were sensitive to clindamycin and 55% were sensitive to erythromycin (Skucaite et al., 2010).

Although oral antibiotics are not used for treatment of chronic apical periodontitis, this diagnosis is frequently used in epidemiological and clinical studies, since it is an asymptomatic pathology easily diagnosed on dental radiographs. The study design was based on the fact that β -lactamase producing organisms are potentially resistant to antibacterial agents and that the resistance of oral microbial flora to antibiotics has not been studied in Latvia. The study was carried out to collect information about the prevalence of β -lactamase producing bacterial strains in the root canal microbial flora. It was discovered in the present study, that the majority of the isolated Gram-positive bacteria do not produce β -lactamase.

4.6. Bacterial Strain Sensitivity to Antibacterial Root Canal Substances

To determine sensitivity of the microbial flora to antibacterial root canal substances, a large number of microbial strains were used (31 strain), that were isolated from the treated root canals. It was found that the most explicit inhibitor of microorganisms was sodium hypochlorite solution (NaOCL). Chlorhexidine digluconate solution (CHX) showed lower antibacterial efficacy, and calcium hydroxide paste was ineffective to root canal microorganisms under the conditions of the study. In the present study a large number of microorganisms isolated from the retreated root canals were used for evaluation of efficiency of the root canal medications. Those studies in which sensitivity of certain bacterial species occurring in retreated root canals to Na hypochlorite, chlorhexidine and calcium hydroxide paste was determined using the agar diffusion test may be considered similar. To determine the effectiveness of antibacterial root canal substances, one or few microbial species, not always isolated from root canals, are often used in studies. The methodology in such studies is very different and it is difficult to compare result of such studies. Some studies analyse the concentration of irrigants and the duration of their exposure for achievement of a negative cultivation (Gomes et al., 2001; Radcliffe et al., 2004;

Vianna et al., 2004). Other studies examine the decrease of bacterial quantity after root canal preparation and / or rinsing (Siqueira et al., 2007). Some laboratory studies determine the effects of antibacterial substances on selected microbial species analysing inhibition caused by these substances. For a wider picture, the present results were interpreted in comparison with other types of laboratory studies, clinical trials and data of systematic analysis.

The finding that sodium hypochlorite is the most effective root canal irrigation solution is confirming the data described in the literature (Zehnder, 2006). In a study conducted in 2003, growth of germs was analysed *in vitro* in the presence of antibacterial root canal substances and similar results were obtained. In a direct contact, sodium hypochlorite was the most effective antibacterial substance against the 5 microbial species tested. Chlorhexidine solution was effective against some germs, while calcium hydroxide and detergent solution showed low efficacy (Estrela et al., 2003). Other studies have shown that the antibacterial efficacy of sodium hypochlorite depends on concentration of the solution. 5.25% NaOCl solution produced the greatest inhibition zone, while 0.5% solution resulted in statistically significant lower antibacterial activity on 6 species of microorganisms (Ayhan et al., 1999). In a study carried out in 2006, efficacy of various concentrations of NaOCl solution (5.25%, 2.5%, and 0.5%) against one *E. faecalis* strain was determined. It was found that a solution with higher concentration (5.25% and 2.5%) is effective for elimination of *E. faecalis* from experimentally infected root canal dentinal tubules regardless of the method selected for root canal preparation (Berber et al., 2006). In our study, sensitivity against antibacterial root canal substances was determined using one *E. faecalis* strain and the largest inhibition zone was created by a chlorhexidine gluconate solution. Contrary to what was expected, sodium hypochlorite solution created smaller *E. faecalis* inhibition zone than calcium hydroxide paste. As previously mentioned, different phenotypes and genotypes of *E. faecalis* are found in the retreated root canals (Pinheiro et al., 2006). *E. faecalis* resistance against sodium hypochlorite solution in our study might be explained by a bacterial strain that is phenotypically or genotypically different from other studies.

Chlorhexidine gluconate solution created lower antibacterial effect on isolates of the retreated root canals than sodium hypochlorite solution. In similar study by Estrela et al., the chlorhexidine solution was effective against some microorganisms (*S. aureus*, *E. faecalis* and *C. albicans*) (Estrela et al., 2003). Oncag et al. studied the

effectiveness of several antibacterial irrigants used for 5 minutes and 48 hours in root canals infected with *E.faecalis*. It was found that 2.0% chlorhexidine and 0.2% cetrimide solutions had higher antibacterial effect on *E.faecalis* in both time periods than 5.25% Na hypochlorite solution (Oncag et al., 2003). In another study, the efficacy of presence of dentine and various organic components on the antibacterial activity of CHX and potassium iodide solution against *E. faecalis* was examined. The authors found that the presence of dentin matrix and the heat-killed microorganisms decreases the antibacterial efficacy of chlorhexidine gluconate solution (Portenier 2002). Systematic analysis of the ability of chlorhexidine and sodium hypochlorite to eliminate *Enterococcus faecalis* from the root canal system showed that the above substances have limited ability to eliminate *E. faecalis* (Estrela et al., 2008). As mentioned, in the present study the sensitivity to antibacterial root canal substances was evaluated using one *E. faecalis* strain, and chlorhexidine gluconate created greater inhibition zone than sodium hypochlorite. This finding cannot be generalised, and it only complies with the results obtained in other *in vitro* studies.

Studies have shown that the efficacy of irrigation solutions depends on concentration of the agent type (solution or gel), and bacterial sensitivity to an irrigant. Studies involving the use of different concentrations and types of chlorhexidine demonstrated that 2.0% CHX gel and solution kill *Staphylococcus aureus* and *Candida albicans* in 15 seconds. Time required for killing microorganisms at the given study was the same both when using 1.0% and 2.0% chlorhexidine and 5.25% sodium hypochlorite solution (Gomes et al., 2001; Vianna et al., 2004). In our study, sensitivity of *Candida* against root canal agents was not tested, since the microorganisms for the test were selected randomly from all root canal isolates. Laboratory and clinical studies have shown that chlorhexidine and sodium hypochlorite solutions reduce the number of cultivated bacteria in root canals (Ercan et al., 2004; Manzur et al., 2007, Siqueira et al., 2007). Studies during the last decade have investigated efficacy of chlorhexidine solution on biofilms in root canals and this efficacy is often compared to the efficacy of sodium hypochlorite. It was stated in a literature review that chlorhexidine targets microorganisms in biofilm, while sodium hypochlorite is the only irrigation solution with the capability of disrupting biofilms (Mohammadi & Abbot, 2009). In the present study, lower antibacterial efficacy of chlorhexidine digluconate can be explained by 0.2% the concentration of the solution used. It is

possible that the higher rates of sensitivity of microorganisms to chlorhexidine solution could be achieved when 2.0% solution is used.

Calcium hydroxide paste produced the least inhibition zone in the microbial strains involved in the study. Calcium hydroxide paste is most often used as interappointment dressing of root canals and was long regarded as an effective antibacterial substance, based on a research conducted in 1980-ies (Haumann & Lowe, 2003). The resistance of *E.faecalis* to calcium hydroxide *in vitro* was detected in a study carried out in 1990 (Ørstavik et al., 1990). In 1999, Waltimo *et al.* studied the sensitivity of *Candida albicans* to potassium iodide, NaOCl, chlorhexidine acetate and calcium hydroxide in an agar diffusion test. It was found that *C. albicans* is highly resistant to calcium hydroxide. Potassium iodide and sodium hypochlorite solutions were the most effective on the bacterium. Yeast cells were destroyed within 30 seconds, while chlorhexidine acetate destroyed them in 5 minutes (Waltimo et al., 1999). These studies led to a notion that the microbial flora in retreated root canals is resistant to calcium hydroxide, and it has been widely quoted in literature reviews (Stuart et al., 2006). In the present study, sensitivity of *Candida* to root canal agents was not tested.

In the study by Estrela et al., a similar finding was obtained, calcium hydroxide solution resulted in less inhibition of four microbial strains than sodium hypochlorite and chlorhexidine solutions. The amount of time required to kill microorganisms *in vitro* was also determined. Calcium hydroxide inhibited growth of *E. faecalis* after 20-minutes contact, but was ineffective to *Bacillus subtilis* and *Candida albicans* (Estrela et al., 2003). The *E. faecalis* strain used in our study was more sensitive to calcium hydroxide paste than to sodium hypochlorite. In another investigation, the amount of time required for the application of calcium hydroxide paste to achieve a negative experimental cultivation in the root canals infected by *E. faecalis* was studied. When the paste was applied for a period of one week, a negative cultivation was found in 70% of the teeth, while when applied for 2 weeks – in 100% of the teeth appeared negative (Lana et al., 2009). It should be noted, that laboratory studies might show contradictory results. Two studies carried out in 2004 show a low antimicrobial activity of calcium hydroxide in the infected root canals. Baker *et al.* found that *E. faecalis* was cultivable 24 hours after application of calcium hydroxide paste into the experimentally infected teeth (Baker et al., 2004). As similar finding was discovered in the study by Siren et al. – calcium hydroxide was unable to

kill microorganisms in dentin, while in the combination with chlorhexidine and potassium iodide it was more effective (Siren et al., 2004). Whereas *Chai et al.* studied the efficacy of antibiotics and calcium hydroxide to the experimentally created biofilm of *E. faecalis* and they found that calcium hydroxide solution kills microorganisms in a biofilm within 1 h (Chai et al., 2007).

In the present study, sensitivity of three microbial isolates (9.7%) to calcium hydroxide exceeded the sensitivity to chlorhexidine digluconate, however it was no higher than the sensitivity against sodium hypochlorite. One microbial strain (*Peptostreptococcus tetradius*) was resistant to root canal irrigation solutions, while it was sensitive to calcium hydroxide paste. These data cannot be generalised. However they show that bacteria in the retreated root canals may have different sensitivities to different root canal substances.

Calcium hydroxide in a water vehicle has antimicrobial qualities that are believed to be due to the very high pH resulting from the dissociation of hydroxyl (OH⁻) ions. In laboratory studies the efficacy of calcium hydroxide can be affected by diffusion of OH⁻ ions in agar. The time required for the diffusion of hydroxyl ions in dentin, buffering capacity of dentin and bacterial biofilm plays a significant role in studies in which the teeth of patients are used *in vivo* and *ex vivo*. The role of dentine buffering capacity has been studied. Haapasalo et al. have found that the antibacterial effect of calcium hydroxide and potassium iodide on *E. faecalis* was completely reduced and the efficacy of sodium hypochlorite and chlorhexidine was lowered in the presence of dentine powder (Haapasalo et al., 2000). The antibacterial activity of calcium hydroxide is also reduced in the presence of hydroxyapatite and serum albumin (Portenier et al., 2001). The buffering capacity of dentine may affect the antimicrobial efficacy of calcium in clinical trials.

Two independent systematic literature reviews on the efficacy of calcium hydroxide in primary infected root canals were found. In the literature review conducted in 2006 by Sathorn et al. eight studies were included, and it was concluded that calcium hydroxide has limited effectiveness in human teeth when using cultivation techniques (Sathorn & Parashos, 2007). In a systematic analysis carried out in 2008, five studies were included and it was concluded that adequate root canal disinfection and an application of mixture of calcium hydroxide and physiological saline reduces the amount of microorganisms in the infected root canals (Estrela et al., 2008). The effectiveness of calcium hydroxide also has not been established in

clinical trials in which the results of single-visit endodontic treatment of teeth with apical periodontitis were compared to the ones of two-visit treatment. The systematic analysis conducted in 2005 reported that a single-visit and two-visit endodontic treatment show similar outcomes. Single-session treatment showed 6.3% higher rates, while the difference was not statistically significant (Sathorn & Parashos, 2005). No systematic analysis of clinical trials on the efficacy of calcium hydroxide on retreated root canals is present in the electronic databases (PubMed, cohrane.org, 1.05.2011.)

When analysing the outcomes of the present study, it must be taken into consideration that the structure of biofilm provides natural habitats for bacteria in the root canal system and that they may have greater resistance to antimicrobials. It should be noted that evaluation of antimicrobial activity of irrigants and medicaments on biofilms in root canals is a difficult task in clinical and laboratory studies. In 2009 a literature analysis was carried out on antibacterial effects of intracanal medications on microbial biofilm. The authors of the article set up strict criteria for inclusion and exclusion of studies. 91 studies were selected and analysed, however most of the studies did not meet the inclusion criteria. It was impossible to draw conclusions about clinical effectiveness of antibacterials, since none of the studies carried out *in vivo* met the criteria, lack of randomized clinical trials was reported. A number of *in vitro* studies showed the efficacy of the above substances, while it should be taken into consideration that it was impossible for these studies to provide conditions identical to natural biofilms in root canals (Estrela et al., 2009).

Based on the present research results and scientific literature review, practical recommendations for irrigation and temporary medication of root canals have been developed. When summarizing the data, it can be unequivocally stated that adequate rinsing of the root canal system is essential for elimination of root canal infections in endodontic retreatment. Sodium hypochlorite solution is the most commonly used irrigant. It remains an effective disinfectant and it dissolves organic parts of the root canal contents – pulp remnants, necrotic pulp, and bacterial cells. However, the scientific literature and the guidelines of professional associations recommend an additional use of other irrigants as well. It is suggested to use also an acid solution (ethylenediaminetetraacetic acid or citric acid) restraining dentinal tubules and removing smear layer therefore acting on inorganic parts of root canal content (Zehnder, 2006; AAE, 2011). Chlorhexidine digluconate is the second most widely used antibacterial irrigant for root canals, it has some advantages over sodium

hypochlorite. Chlorhexidine solution has no irritating effect on periapical tissues, no specific odour, does not cause erosion of dentin and it has prolonged antibacterial effect on the root canal walls, however it has no proteolytic effect (Haapasalo et al., 2010). Other root canal irrigants and combinations of various substances, such as MTAD, have also been examined (Singla et al., 2011). Analysis of the scientific literature led to the conclusion that there is no single root canal irrigation protocol, while sodium hypochlorite solution is considered the as most effective substance, 1.0% - 5, 25% solution is used for rinsing. When using solutions of lower concentrations (1.0% - 1.25%) in larger amounts, the antibacterial effects achieved are equal to the ones of higher concentrations (Siqueira et al., 2002). It is recommended to rinse each canal with 2 - 12 ml of a solution (van der Sluis et al., 2006). Heating of the solution increases the efficiency, and therefore it is recommended to heat up the solution with low concentration up to 40 ° C (Sirte et al., 2005). For a complete solution exchange, the tip of the rinsing needle should be placed to within 1 mm from preparation length of the root canal (Boutsiokis et al., 2008). Studies have shown that ultrasonic activation of Na hypochlorite more effectively removes the smear layer from a root canal walls (van der Sluis et al., 2007). At the end of the root canal preparation, the use of 5-10 ml ethylenediaminetetraacetic acid (EDTA) or citric acid for 1 minute is suggested. Acid solution is not suggested for a long use, as it weakens the root dentin (Calt et al., 2002). Acid reduces the effect of NaOCl. In order to avoid mixing of the solutions, rinsing should therefore be performed with distilled water (Zehnder et al., 2005). If interappointment medicines are intended to be used, the canals shall be repeatedly rinsed with 5-10 ml NaOCl solution. Sodium hypochlorite solution can be used for preparation of a calcium hydroxide paste, as such paste dissolves the remaining tissue more efficiently and acts on the root canal microflora (Zehnder et al., 2003). At the second session of root canal therapy, rinsing with sodium hypochlorite and acid should be repeated. The 2.0% chlorhexidine digluconate solution is used as the final irrigant before the canal filling, while during the procedure its mixing with sodium hypochlorite should be avoided. Distinct orange-brown precipitate occurring in the process of mixing may stain the teeth and contain a potentially mutagenic substance parachloroaniline. Before the use of hypochlorite, root canals should therefore be rinsed with distilled water (Marchesan et al., 2007).

Usually irrigation of root canal systems is performed using a disposable plastic syringe and a needle. However alternatives for elimination of infection and irrigation, such as laser, ultrasonic, sonic vibrations and the use of negative pressure have been examined over the past decade (Kimura et al., 2003; van der Sluis et al., 2007; Townsend et al., 2009; Desai et al., 2009, Nielsen et al., 2007). A reflective practitioner in endodontics should regularly follow up the latest scientific literature and should choose treatment methods and root canal irrigants based on scientific evidence.

5. CONCLUSIONS

- In Latvian patients, Gram-positive microorganisms prevail in microbial flora of endodontically treated root canals with chronic apical periodontitis.
- 1 to 6 microbial species can be isolated and identified by cultivation from the endodontically treated root canal with chronic apical periodontitis.
- Microbial species isolated from endodontically treated teeth with chronic apical periodontitis most frequently pertain to the genera *Actinomyces*, *Streptococcus*, *Staphylococcus*, *Lactobacillus* and *Enterococcus*.
- β -lactamase producers account for almost one-fifth of microbial strains isolated from the retreated root canals.
- The most frequently found β -lactamase-producing microbial species pertain to the genera *Actinomyces* and *Staphylococcus*.
- β -lactamase-producing microbial strains are found in almost one-third of patients with endodontically treated teeth.
- Microbial strains isolated from the retreated root canals are sensitive to sodium hypochlorite and chlorhexidine digluconate solution and are weakly sensitive to calcium hydroxide paste when tested *in vitro*.
- Different strains of a single microbial species may have different sensitivity to the root canal antimicrobials.

6. PRACTICAL RECOMMENDATIONS

Based on the research results and scientific literature, following practical recommendations are suggested for irrigation and temporary dressing of retreated root canals with chronic apical periodontitis:

Retreatment in one session:

- Irrigation during root canal preparation - 2-5 ml 2.5% (cold) or 1.25% (heated) sodium hypochlorite solution after each instrument
- If possible, to use ultrasonic activation of Na hypochlorite solution
- At the end of root canal preparation - 5-10 ml NaOCl for each root canal
- Irrigation at the end of root canal preparation - 5 ml 17.0% EDTA or 20.0% citric acid solution, and 5 ml distilled water for each root canal
- Before filling of the root canals - 5-10 ml 2.0% chlorhexidine digluconate solution for each root canal

Retreatment in two sessions:

First session

- Irrigation during root canal preparation - 2-5 ml 2.5% (cold) or 1.25% (heated) sodium hypochlorite solution after each instrument
- If possible, use ultrasonic activation of Na hypochlorite solution
- At the end of root canal preparation - 5-10 ml NaOCl for each root canal
- Interappointment dressing – calcium hydroxide paste prepared with 2.5% Na hypochlorite solution

Second session

- Irrigation during root canal preparation - 2-5 ml 2.5% (cold) or 1.25% (heated) sodium hypochlorite solution after each instrument
- If possible, use ultrasonic activation of Na hypochlorite solution
- Irrigation at the end of root canal preparation - 5 ml 17.0% EDTA solution or 20.0% citric acid, 5 ml distilled water for each root canal
- Before filling of the root canals - 5-10 ml 2.0% chlorhexidine digluconate solution for each root canal

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8. PUBLICATIONS AND APPROBATION

Publications

1. **A. Mindere**, R. Kundzina, V. Nikolajeva, D. Eze, Z. Petrina. Microflora of root filled teeth with apical periodontitis in Latvian patients. *Stomatologija, Baltic Dental and Maxillofacial Journal*, 2010; 12: 116-121.
2. **A. Mindere**, R. Kundziņa, V. Nikolajeva, D. Eze, Z. Petriņa, A. Babarikina. Sakņu kanālu skalojamo līdzekļu un kalcija hidroksīda antibakteriālā iedarbība uz pārārstējamu sakņu kanālu mikrofloru. *RSU Zinātniskie raksti*, 2010, 444-451.
3. **A. Mindere**, V. Nikolajeva, D. Eze, Z. Petriņa, R. Kundziņa. Endodontiski ārstētu sakņu kanālu ar apikālu periodontītu mikroflora. *RSU Zinātniskie raksti*, 2008, 356-363.

Presentations

1. **A. Mindere-Gubele**, V. Nikolajeva, E. Eze, Z. Petrina, A. Babarikina, R. Kundzina. The antimicrobial efficacy of irrigants and calcium hydroxide on persistent endodontic microorganisms. 15 Biennial congress of the European society of Endodontology 2011, Rome, Italy.
2. **A. Mindere**, R. Kundziņa. Mutes dobuma mikrofloras rezistence pret antibiotiskām vielām. The 6. Latvian Congress of Physicians, 2009, Riga, Latvia.
3. **A. Mindere**, V. Nikolajeva, D. Eze, Z. Petriņa, R. Kundziņa. Endodontiski ārstētu sakņu kanālu mikroflora un β -laktamāzi producējošo celmu izplatība. The RSU Scientific conference, 2009, Riga, Latvia.
4. **A. Mindere**, R. Kundzina, V. Nikolajeva, D. Eze, Z. Petrina. Microbiological status of root filled teeth with apical periodontitis. 14 Biennial congress of the European society of Endodontology, 2009, Edinburgh, Great Britain.
5. **A. Mindere**, R. Kundzina, V. Nikolajeva, D. Eze, Z. Petrina. Microflora of root filled teeth with apical periodontitis. The 3rd Baltic scientific conference in dentistry, 2008, Vilnius, Lithuania.
6. A. Babarikina, **A. Mindere**, V. Nikolajeva, D. Eze, Z. Petriņa. Endodontiski ārstētu zobu ar apikālu periodontītu mikroflora un tās jutība pret antibakteriālajiem līdzekļiem. 66 conference of Latvia University, 2008, Riga, Latvia.
7. **A. Mindere**, V. Nikolajeva, D. Eze, Z. Petriņa, R. Kundziņa. Endodontiski ārstētu zobu ar apikālu periodontītu mikrobiālais status Latvijas pacientiem. The RSU Scientific conference, 2008, Riga, Latvia.

8. **A. Mindere**, V. Nikolajeva, D. Eze, Z. Petrina, R. Kundzina. Microbiological status of root filled teeth with apical periodontitis. The 2nd Baltic scientific conference in dentistry, 2007, Riga, Latvia.

Abstracts

1. **A. Mindere-Gubele**, V. Nikolajeva, E. Eze, Z. Petrina, A. Babarikina, R. Kundzina. The antimicrobial efficacy of irrigants and calcium hydroxide on persistent endodontic microorganisms. 15 Biennial congress of the European society of Endodontology. Research abstracts. Int Endod J 2011; 44: 1188 - 1188.
2. **A. Mindere**, V. Nikolajeva, D. Eze, Z. Petriņa, R. Kundziņa. Endodontiski ārstētu sakņu kanālu mikroflora un β-laktamāzi producējošo celmu izplatība. RSU 2009.gada zinātniskā konference. Tēzes, 132. lpp.
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4. **A. Mindere**, R. Kundzina, V. Nikolajeva, D. Eze, Z. Petrina. Microflora of root filled teeth with apical periodontitis. Stomatologija, Baltic dental and maxillofacial journal, 2008; 10, Suppl 5: 23-24.
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6. **A. Mindere**, V. Nikolajeva, D. Eze, Z. Petrina, R. Kundzina. Microbiological status of root filled teeth with apical periodontitis. The 2nd Baltic scientific conference in dentistry, 2007, Abstract book, p 5.