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VIRULENCE PROFILES OF MICROBIAL STRAINS ISOLATED FROM PATIENTS WITH CHRONIC APICAL LESIONS

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Aim: characterization of virulence patterns of microbial strains isolated from samples taken from 29 adult patients with chronic apical lesions. Materials and methods: the samples were transported in thioglycolate broth, seeded onto 5% sheep blood Columbia agar and incubated at 37°C in 5% CO_2 atmosphere. The colonies were identified based on their morphology and pigmentation, Gram staining and biochemical tests.Virulence factors (VF) were assessed by inoculation of microbial strains on HeLa cells and on specific media. Results: the majority of microbial strains were identified as *Actinomyces* sp. (46.66%), Staphylococcus sp. (33.33%), *Streptococcus* sp. (13.32%) and *Gemellamorbillorum* sp. (6.66%). They exhibited the ability to colonize HeLa cells and produced haemolysins (73.91%), esculin hydrolysis (30.43%), amylases (30.43%), microbial proteases (caseinase and gelatinase) (26.08%), lecithinase and lipase (8.69%), and DNA-ases (4.34%). Conclusion: the microbial strains isolated from adult patients with chronic apical lesions exhibited a wide range of cell-associated and soluble VF contributing to tissue destruction, supporting their potential to initiate and maintain periapical inflammation.

Key words: chronic apical lesions, biofilms, enzymes, virulence.

INTRODUCTION

The species in the endodontic flora are usually of low virulence, but their intraradicular survival and pathogenic properties are influenced by a combination of factors, including: (i) interactions with other micro-organisms in the root canal, to develop synergistically beneficial partners; (ii) the ability to interfere with and evade host defenses; (iii) the release of lipopolysaccharides (LPS) and other bacterial modulins; and (iv) the synthesis of enzymes that damage host tissues (Nair, 2004).

Biofilm can be defined as a sessile multicellular microbial community characterized by cells that are firmly attached to a surface and enmeshed in a self-produced matrix of extracellular polymeric substance (Siqueira, Rocas 2008). Oral bacteria have the capacity to form biofilms on distinct surfaces ranging from hard to soft tissues. Microbial biofilms play an essential role in several infectious diseases such as pulp and periradicular pathosis (Mohammadi *et al*., 2013). A significant number of microorganisms were embedded in the extracellular polymeric substances in apical periodontitis lesions (Fukushima *et al*., 1990; Nair 2006 , Rocha *et al.,* 2008). The ability of oral bacteria to produce exopolysaccharides (EPSs) could contribute to survival and the development of persistent infections in the oral cavity (Costerton *et al*. 1999, Yamane *et al*. 2009, 2010, Yamanaka *et al*. 2010).

Microorganisms endure physiological and morphological modifications in response to environmental changes. In biofilms, different gradients of chemicals, nutrients, and oxygen establish microenvironments to which the microorganisms must adjust to survive. To adapt to a biofilm lifestyle, a large set of genes must be regulated; bacteria are thus able to optimize phenotypic properties for the particular environment.

Consequently, biofilm microorganisms differ phenotypically from their planktonic counterparts (Li YH *et al*., 2000).

Chronic apical periodontitis is included in the group of biofilm-induced diseases. Biofilm morphology varies from case to case and no unique pattern for endodontic infections was observed. Biofilms are more frequently met in association with longstanding pathologic processes, without or incorrect endodontic treatment, and this explains why apical periodontitis is so difficult to treat. The pathogens within a biofilm environment behave very differently from free-floating bacteria. The protective extracellular slime matrix makes bacteria extremely resistant to antibiotics, antimicrobial agents, and host defense mechanisms.

The main etiologic factors of chronic periapical pathosis are bacteria, their metabolic products, and debris of infected pulp tissue remaining in lateral canals, dentinal tubules, or gaps between the root canal wall and root filling materials. If bacteria in infected root canals can invade the extraradicular area via the apical foramen and the dental tubules and can form bacterial biofilm, they and their components may play etiologic roles in refractory and chronic periodontitis. (Noiri *et al*. 2002)

Evidence has been found regarding the presence of bacteria within extraradicular areas and periapical lesions. An extraradicular area is used clinically as a term that contrasts to a root canal, which ends at the apical foramen of the root apex. The solid parts connected with the root canal surface are called the extraradicular area; this is located in the periapical lesion, but is expressed as distinguished from the lesion (Noguchi *et al.*, 2005).

The purpose of this study was to characterize the virulence potential of microbial strains isolated from samples taken from adult patients with chronic apical lesions, using phenotypic assays to evidence cell-associated and soluble, enzymatic factors implicated in the pathogenicity of oral bacteria.

MATERIALS AND METHODS

Isolation and identification of the microbial strains

The apical lesion samples were taken from twenty-nine patients (15 men and 14 women), 21 of them being between 20 and 31 years old, and 8 of them between 42 and 68 years old. They presented to a dentistry clinic in Bucharest for current dental evaluation and treatment, and discussed their personal treatment plan with the dentists, taking into account the therapeutical indications. Patients signed an informed consent about the medical procedures.

From these teeth, a number of 10 had inaccurate endodontic treatments, and 19 of them showed no endodontic treatment. All the teeths presented fibrous chronic apical lesions that could be detected on individual radiographs, and the endodontic retreatment was not possible. There were 14 mandibular molars, 2 mandibular premolars, 3 upper premolars, 7 maxillary molars, 3 incisors. All the patients had radiographic evidence of apical periodontitis lesions.

The extractions were made using aseptic and antiseptic techniques, under local anesthesia.

After drawing out, and by using aseptic techniques and sterile instruments, bacterial samples were taken and placed into a 2-mL centrifuge tube containing 1.5 mL thioglicolat medium. The samples were immediately processed.The inoculated tubes were vortexed for a few seconds and then cultured on 10% sheep blood Brucella agar. Plates were incubated aerobically and anaerobically for 48 to 7 day sat 37°C. The microbial isolates obtained were presumptively identified based on colony morphology, Gram staining and conventional biochemical tests (catalase and oxidase). Colonies were then identified by using the commercial biochemical kits API (bioMérieux, France): API Staph, API Strep, API 20E, API NE, API 20A.

Microtiter plate biofilm production assay

The study of microbial isolates ability to develop a biofilm on inert substratum was assessed by the microtiter plate method. The isolates were grown in 10 ml of rich undefined medium, TSB (tryptoy soy broth) at 37°C overnight. Biofilm production assays were performed with TSB. Overnight cultures in TSB were transferred (0.1 mL) to 1 mL of fresh TSB and vortexed. After vortexing, 100-µL volumes were transferred into microtiter plate wells. Plates were made in duplicate, incubated, and covered at 37°C for 24 h. Each plate included eight wells of TSB without microbial strain as control wells. After incubation period, the culture medium was removed from the wells and microtiter plate wells were washed 3 times with sterile distilled water to remove loosely associated bacteria. Wells were fixed with methanol for 5 minutes and stained with 200 µl of 1% crystal violet solution in water for 20 min. After staining, plates were washed with sterile distilled water five times. The quantitative analysis of biofilm production was performed by adding 200 µl of acetic acid 33% to destain the wells. The level (OD) of the crystal violet present in the destaining solution was measured at 492 nm.

Evaluation of microbial adherence to HeLa cells

For the adherence assay, Cravioto's adapted method was used.In this purpose, HeLa cells monolayers cultivated in 6 multi-well plastic plates at 80–100% confluence were washed 3 times with PBS; 1 mL of fresh medium without antibiotics was added to each well (Cravioto *et al.*, 1979). Microbial suspensions from mid-logarithmic phase cultures grown in TSB was adjusted at 10^7CFU/mL, and 1 mL was used for the inoculation of each well.The inoculated plates were incubated for 2 hours at 37ºC. After incubation, the monolayers were washed 3 times in PBS, briefly fixed in cold methanol (3 min), Giemsa stained and incubated for 30 min. The plates were washed, dried at room temperature, examined

microscopically (magnification, ×100) and photographed with a Sony camera adapted to Zeiss microscope.Three distinct patterns of adherence have been investigated during this study: localized adherence (LA), in which the bacteria attach to and form microcolonies in distinct regions of the surface; diffuse adherence (DA), in which bacteria adhere evenly to the whole cell surface, and aggregative adherence (AggA), in which aggregated bacteria attach to the cell in a stacked-brick arrangement . The adherence index was expressed as the ratio between the number of the eukaryotic cells with adhered bacteria: 100 eukaryotic cells counted on the microscopic field.

Expression of soluble enzymatic factors

The expression of soluble enzymatic factors was studied by cultivating the isolated microbial strains on media containing specific substrate: haemolysins, other pore forming toxins (lecithinases, lipases), proteases (caseinases, gelatinasse), DNA-ses and amylase. An enriched agar base medium was used for preparing the media for soluble virulence factors, in order to enable the growth of streptococci. Haemolysins production was assessed by spotting the strains on 5% sheep blood agar medium. After incubation at 37°C for 24 hours, a transparent area around the colony reveal a full hemolysis or a greenish area that shows incomplete hemolysis (characteristic for alpha-haemolitic strains). Investigation of lipase production was perfomed by cultivation on 1% Tween 80 for 7 days. An opaque (precipitation) zone around the spot was registered as positive reaction; for the lecithinase production, the cultures were spotted into 2.5% yolk agar and incubated at 37°C for 7 days. An opaque (precipitation) zone around the spot indicated the lecithinase production; the DNA-ase production was studied on DNA supplemented agar with blue toluidin. A zone colored in pink around the culture was interpreted as positive reaction; the caseinase activity was determined using 15% soluble casein agar as substrate; for caseinase activity, the strains were inoculated on casein agar as substratum, and after incubation at 37°C for 24 hours, a clearing zone surrounding the growth indicated casein proteolysis. For the production of gelatinase the strains were spotted in the solid medium containing gelatin for 7 days at 37°C. The presence of a precipitation zone around the growth area indicated gelatine proteolysis.The amylase production was tested on 10% starch supplemented agar medium, the presence of a clear area around the culture after 7 days of incubation at 37°C being registred as positive reaction.

RESULTS

The root canal flora of teeth with clinically intact crowns, but having necrotic pulps and periapical disease, is dominated (> 90%) by obligate anaerobes (Sundqvist 1976; Byström and Sundqvist 1981; Haapasalo 1989; Sundqvist *et al.* 1989), usually belonging to the genera *Fusobacterium, Porphyromonas* (formerly *Bacteroides*), *Prevotella* (formerly *Bacteroides*) (Shah and Collins, 1988), *Eubacterium,* and *Peptostreptococcus.* In contrast, the microbial composition – even in the apical third of the root canal of periapically affected teeth with pulp canals exposed to the oral cavity – is not only different from but also less dominated (< 70%) by strict anaerobes (Baumgartner and Falkler, 1991). Using culture techniques (Hampp 1957; Kantz and Henry, 1974; Dahle *et al.* 1996), dark-field (Brown and Rudolph, 1957; Thilo *et al.* 1986; Dahle *et al.* 1993), and transmission electron microscopy (Nair 1987), investigators have found spirochetes in necrotic root canals.

The microbiological study of the twenty-nine periapical samples taken from adult patients with chronic apical lesions led to the isolation of 59 microbial strains belonging to 28 bacterial species. The majority of the recovered strains were facultative anaerobes. Gram positive bacteria constituted 90,74% of the isolated microoganisms, the most prevalent genus being *Streptococcus (mitis, salivarius, oralis, constellatus, intermedius, acidominimus)*, followed by *Actimomyces (naeslundii, israelii, viscosus), Gemella (morbillorum, haemolysans), Staphylococcus (aureus, xylosus, epidermidis), Enterococcus (faecalis, faecium), Lactococcus (lactis), Eubacterium (lentum), Aerococcus (viridans), Bifidobacterium Propionibacterium*. Gram negative bacteria genera (9,26%) isolated was represented by *Prevotella (intermedia, oralis), Pantoea and Veionella.* The isolation frequencies of microorganisms retrieved from patients with chronic periapical lesions are shown in Figure 1.

Biofilm development

Previous studies suggested that EPSs-producing bacteria in the periapical lesion play an important role in the recurrence of acute apical periodontitis or of abscess lesions from asymptomatic periapical pathosis. Some strains of *Prevotella intermedia* and *P. nigrescens* isolated from chronic periodontitis lesions produce EPSs in a sucrose-independent manner and form biofilms that contribute to their virulence (Fukushima *et al*. 1992, Yamane *et al*. 2005, Yamanaka *et al*. 2009).

Clinical strains of *Bacillus subtilis*, *Rothia mucilaginosa* and *Escherichia hermannii* isolated from persistent periapical lesions have an ability to produce self-synthesized EPSs, suggesting that these organisms can cause a persistent biofilm infection in the apical region of root canal (Yamane *et al*., 2009, 2010, Yamanaka *et al*., 2010).

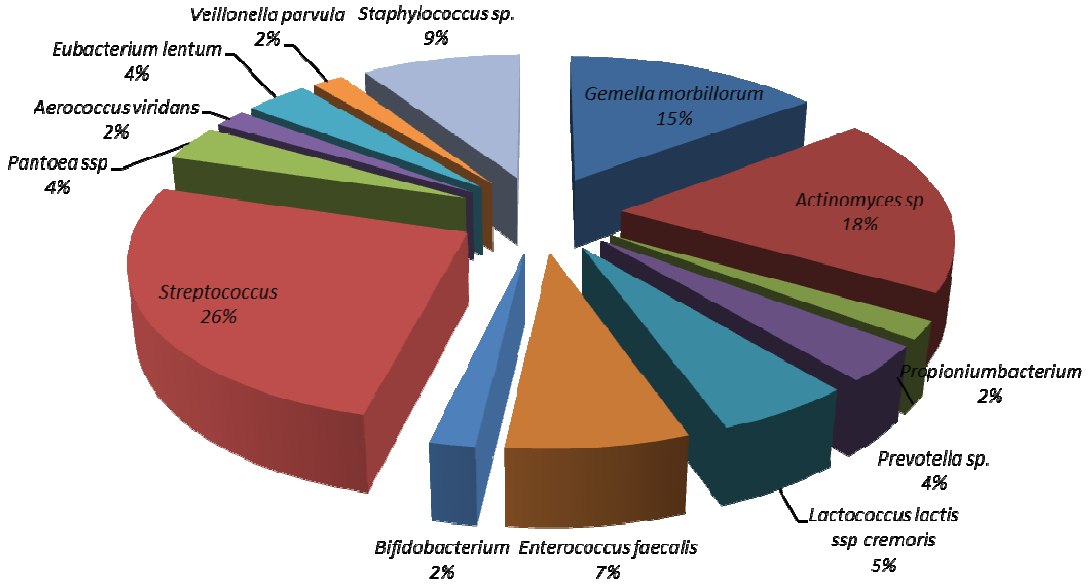

Figure 1. Graphic representation of the isolation frequencies of the microorganisms species isolated from periapical samples.

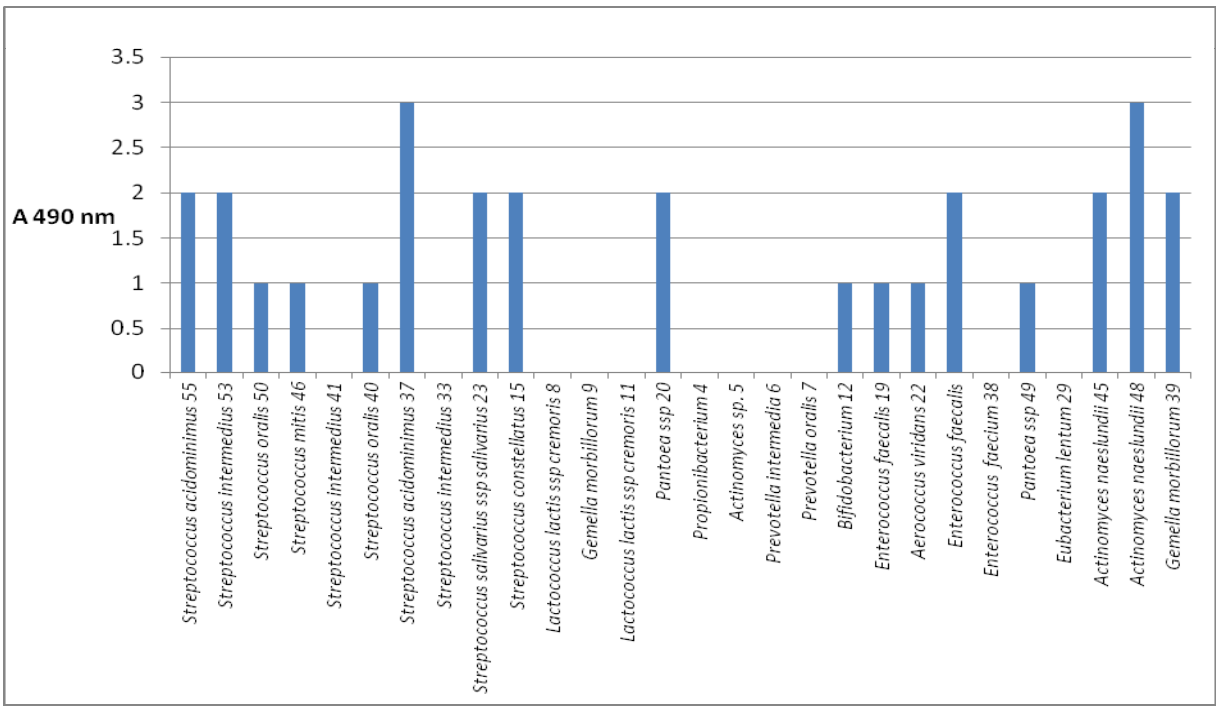

Figure 2. Graphic representation of the intensity of adherence to the inert substratum, expressed by measuring the A 490 nm of the adhered and colored biofilms.

In our study the microbiota of persistent apical periodontitis lesions, composed by diverse types of microorganisms exhibited biofilm-forming capacity. A total of 79.62 % of the bacterial strains were shown to adhere to inert substratum represented by the plastic wells, with different intensities (Figure 2). The presence of an apical bacterial biofilm is clinically important, and it may cause failures of endodontic treatment as a consequence of persistent infection.

Regarding the ability of the strains to adhere to cellular substratum represented by eukaryotic cells belonging to HeLa cell line, the results showed that 96,42% of the analyzed strains were able to adhere, with an adherence index ranging from 10 to 100% (Figure 3). The observed adherence patterns were very diverse, the predominant ones being the diffuse and aggregative ones (Table 1, Figure 4).

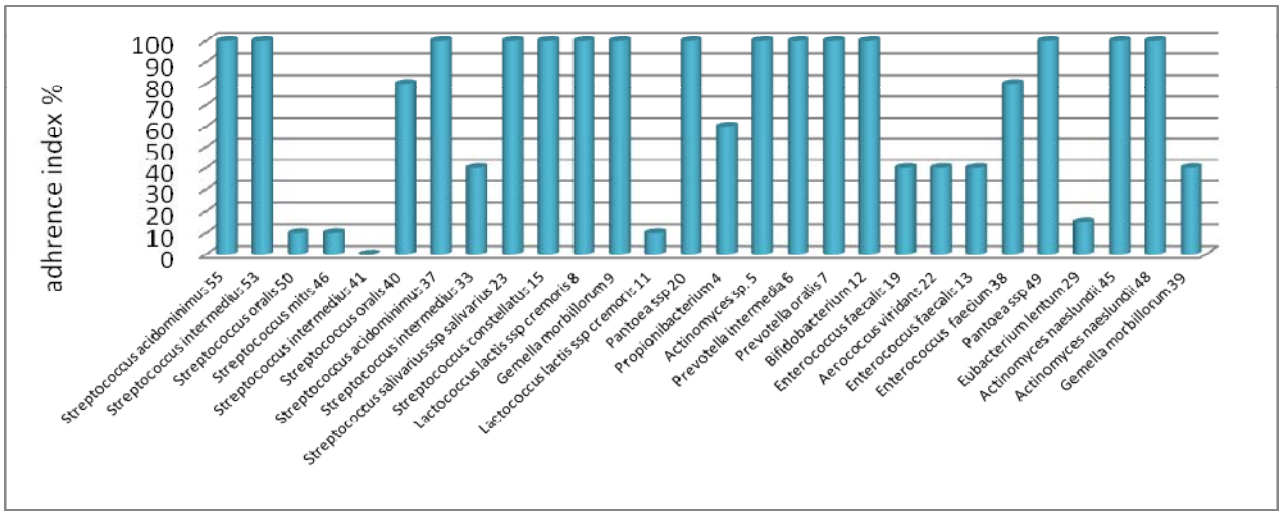

Figure 3. Graphic representation of the ability of the tested strains to adhere to the cellular substratum.

Table 1
The adderence pattern recorded for the analyzed microbial strains

Microbial strain	Adherence pattern
Streptococcus acidominimus 55	**Diffuse-aggregative**
Streptococcus intermedius 53	**Diffuse-aggregative**
Streptococcus oralis 50	**Localized-aggregative**
Streptococcus mitis 46	**Diffuse-aggregative**
Streptococcus oralis 40	**Diffuse-aggregative**
Streptococcus acidominimus 37	**Diffuse-aggregative**
Streptococcus intermedius 33	**Diffuse-aggregative**
Streptococcus salivarius ssp salivarius 23	**Localized-aggregative**
Streptococcus constellatus 15	**Diffuse-aggregative**
Lactococcus lactis* ssp *cremoris 8	**Diffuse-aggregative**
Gemella morbillorum 9	**Diffuse-aggregative**
Lactococcus lactis* ssp *cremoris 24	**Localized-aggregative**
Pantoea* ssp *20	**Diffuse**
Propionibacterium 4	**Localized-aggregative**
Actinomyces sp. 5	**Diffuse-aggregative**
Prevotella intermedia 6	**Diffuse**
Prevotella oralis 7	**Diffuse**
Bifidobacterium 12	**Diffuse**
Enterococcus faecalis 19	**Diffuse**
Aerococcus viridans 22	**Diffuse**
Enterococcus faecalis 13	**Diffuse**
Enterococcus faecium 38	**Diffuse**
Pantoea ssp 49	**Diffuse**
Eubacterium lentum 29	**Localized-aggregative**
Actinomyces naeslundii 45	**Localized-aggregative**
Gemella morbillorum 39	**Diffuse-aggregative**

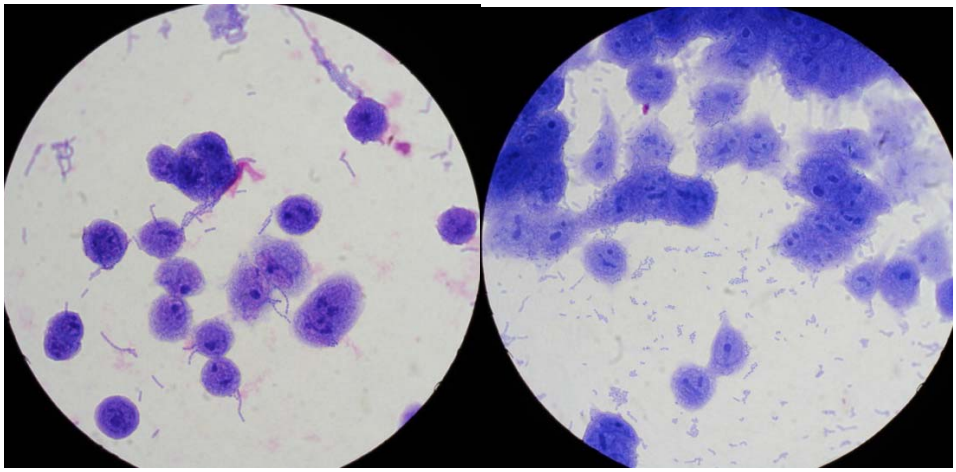

Figure 4. Giemsa stained HeLa cells infected with two bacterial strains
(*i.e.* exhibiting a diffuse-aggregative (left) and diffuse (right) adherence patterns.

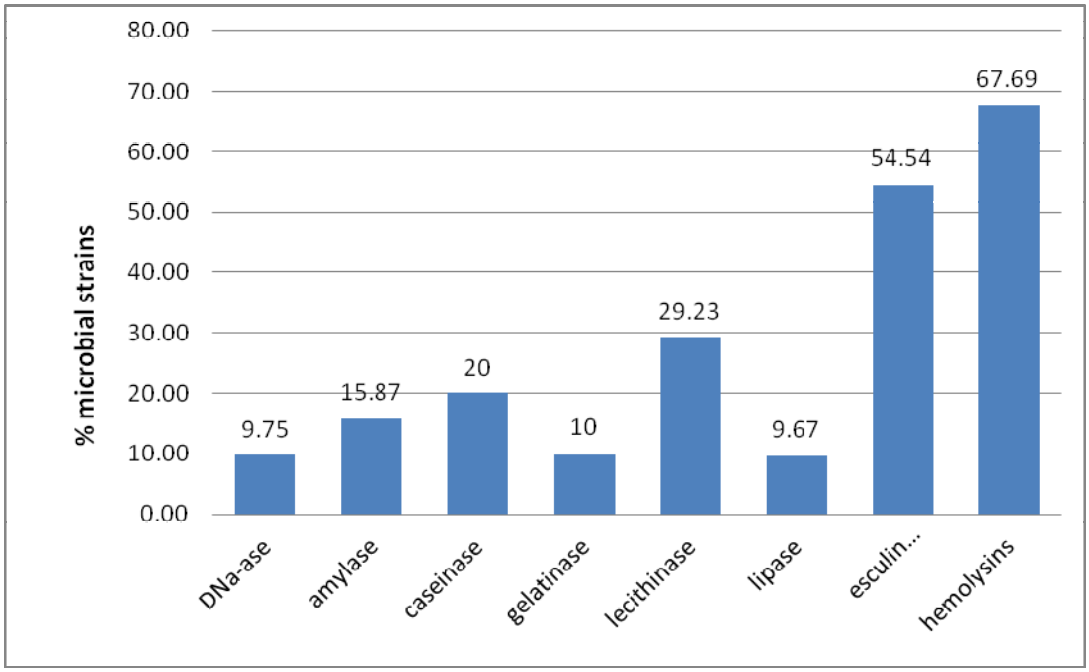

Figure 5. Expression level of different virulence factors in the microbial strains isolated from periapical lesions.

Endodontic microorganisms produce a variety of enzymes that are not directly toxic but may aid the spread of the organisms in host tissues, such as collagenase, hyaluronidase, fibrinolysins, and several proteases. Microbes are also known to produce enzymes that degrade various plasma proteins involved in blood coagulation and other body defenses. The ability of some *Porphyromonas* and *Prevotella* species to break down plasma proteins – particularly IgG, IgM (Killian 1981), and the complement factor C3 (Sundqvist *et al.* 1985) – is of particular significance, since these molecules are opsonins necessary for both humoral and phagocytic host defenses (Nair 2004).

In the present study a high percentage of the analyzed strains produced haemolysins (67.69%) (Figure 5). These extracellular enzymes act in the pathogenesis of bacterial species by production of tissue damage (pore-forming toxins). This action adds to the effect of lecithinases (29.23%) indisruption of host cell membrane by affecting membrane lipids, suggesting the implication of these enzymes in host invasion.

A high percentage of the analyzed strains exhibited the ability to produce iron chelating agents (resulted from the esculin hydrolysis) (54.54%) which are implicated in the regulation of multiplication rate as well as in the virulence factors expression.

CONCLUSIONS

The microbial strains isolated from samples taken from adult patients with chronic apical lesions exhibited a wide range of cell-associated and soluble virulence factor.

Apical lesions may contain toxin and hydrolytic enzyme-producing microorganisms contributing to tissue destruction and spreading, supporting their potential to initiate and maintain periapical inflammation.

Microbiological analysis and antimicrobial susceptibility testing should ideally form the basis for selecting the optimal antimicrobial treatment.

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