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In Vitro Alkaline pH Resistance of *Enterococcus faecalis*

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Enterococcus faecalis is a bacterial species often found in root canals with failed endodontic treatment. Alkaline pastes are widely used in Endodontics because of their biocompatibility and antimicrobial activity, but this microorganism can resist alkalinity. The purpose of this study was to evaluate *in vitro* the alkaline pH resistance of *E. faecalis* for different periods up to 14 days. Samples were obtained from the oral cavity of 150 patients from the Endodontic clinic. The pH of the experimental tubes (n=84) was first adjusted with 6M NaOH to pH values of 9.5, 10.5, 11.5 and 12.5 (21 tubes *per* pH). Twenty clinical isolates and the ATCC 29212 strain were tested. The 5 positive controls and experimental tubes of each pH were inoculated with 10 µL of bacterial suspension and incubated at 36 °C for 24, 48 and 72 h, 7 and 14 days. For each period, the turbidity of the medium was visually compared with a 0.5 McFarland standard. The presence of the microorganism was confirmed by seeding on M-Enterococcus agar. Four tubes containing BHI broth adjusted to the tested pHs were incubated for 14 days to verify if pH changes occurred. The pH of inoculated BHI broth was also measured on day 14 to determine if the microorganism acidified the medium. The growth of all *E. faecalis* strains occurred at pH 9.5 to 11.5 in all periods. Although turbidity was not observed at pH 12.5, there was growth of 13 and 2 strains at 24 and 48 h, respectively, on M-Enterococcus agar. No tube showed growth at pH 12.5 after 72 h. It was concluded that *E. faecalis* can survive in highly alkaline pH, and some clinical isolates require 72 h at pH 12.5 to be killed.

Key Words: *Enterococcus faecalis*, alkaline pH, microbial resistance.

Introduction

Bacterial species belonging to the genus *Enterococcus* have their usual ecological niche in the gastrointestinal tract of humans and other animals, but they may be found free-living in plants and soil. These microorganisms may also colonize the genitourinary tract and the oral cavity (1). *Enterococci* are facultative, fermentative, Gram-positive cocci with oval shape and 0.5-1.0 µm diameter, which can live isolated or arranged in small chains (1). They are opportunistic pathogens associated with cardiovascular problems, urinary tract, and wound infections (2).

The most commonly isolated species is *Enterococcus faecalis*, which can be found in the oral cavity and infections, such as marginal periodontitis, endodontic infections, periradicular abscesses and failed root canal therapy (3,4,5). A distinguishing characteristic of *E. faecalis* is its ability to grow in an alkaline pH that normally inhibits other bacteria (6,7,8). The mechanism of *E. faecalis* resistance to alkaline pH, and consequently to calcium hydroxide pastes, regularly used in Endodontics (9,10), may be related to the existence of a working active proton pump (11).

It would be interesting to evaluate whether *E. faecalis* field strains isolated from oral samples have the same resistance profile to high pH as that of a reference *E. faecalis* ATCC strain. This could help establishing the risks of leaving a tooth exposed to an oral cavity contaminated with a highly resistant microorganism to alkaline environments.

This study evaluated *in vitro* the alkaline pH resistance of *E. faecalis* for different periods up to 14 days.

Material and Methods

One hundred and fifty patients treated at the service of Endodontics at the University of Sagrado Coração were enrolled in the study after approval of the research protocol by institutional Ethics Committee (Process #069/2005).

For collection of clinical samples from the patients' oral cavity, a sterile cotton swab was rubbed over the lingual surface of teeth and placed in tubes containing 5 mL of thioglycollate medium (Oxoid Brasil Ltda., São Paulo, SP, Brazil), which were immediately processed by conventional bacteriological culture techniques (12).

Thioglycollate medium containing the sample was vortexed for 1 min and seeded onto the surface of M-Enterococcus Agar plates (Difco Laboratories Inc., Detroit, MI, USA), which were incubated at 36 °C for 24 h and analyzed for presence of *E. faecalis* colonies. Bile-esculin agar (Difco Laboratories Inc.) was used to confirm the biochemical features. The appearance of diffuse blackish color colonies indicated positive proof to the test. Colonies positive for the bile-esculin test were subjected to the tolerance to 6.5% sodium chloride (11).

pH Tolerance Test

The *E. faecalis* clinical isolates positive in the bile-esculin and sodium chloride tests were used. An American Type culture collection (ATCC) 29212 reference strain was

used as control. All bacterial strains were cultivated and maintained at -20 °C until use. The strains were reactivated on M-Enterococcus agar (Difco Laboratories Inc.) and incubated at 36 °C for 24 h. Colonies formed after this period were transferred to tubes (one for each strain) containing brain heart infusion (BHI) broth (Oxoid Brazil Ltda.) and incubated at 37 °C until complete turbidity of the medium was observed.

Aliquots of 150 mL of pure BHI broth were placed in Erlenmeyer flasks and had their pH adjusted with NaOH 6M to values of 9.5, 10.5, 11.5 and 12.5 using a pH meter (Orion Research, Boston, MA, USA). After alkalinization, the BHI broth was cold sterilized using a Millipore filter with 0.22 µm pore size with a vacuum pump (Millipore Corporate, Billerica, MA, USA). Aliquots of 4 mL of each broth at pH 9.5-12.5 were distributed in sterile test tubes (n=21 tubes for each pH). Ten tubes containing only BHI broth (pH 7.3±0.1 at 25 °C) served as controls. All tubes were incubated overnight at 37 °C to ensure sterility.

The 5 positive controls and the experimental tubes of each corresponding pH were inoculated with 10 µL of standardized bacterial suspension (1.0×10^8 UFC mL^{-1}) and incubated at 37 °C for 24 h, 48 h, 72 h, 7 days, and 14 days. The 5 negative controls were not inoculated. At the end of each period, the tubes with and without turbidity of the medium were visually compared to a 0.5 McFarland standard, and plated onto M-Enterococcus agar to confirm the presence or absence of the microorganism.

Four tubes containing BHI broth adjusted at pH 9.5, 10.5, 11.5 and 12.5 (without inoculum) were also incubated for 14 days to verify if pH changes occurred. The pH of inoculated BHI broth was also measured at the end of the experiment (day 14) to determine if the microorganisms had acidified the medium. All procedures in this study were performed under aseptic conditions. The results obtained in each period for each pH were expressed in percentage of bacterial growth. pH values and periods were compared using the chi-square test (α=0.05).

Results

E. faecalis was identified in the oral cavity of 13.3% of the patients (n=20). The controls confirmed the validity of the experimental protocol, as bacterial growth occurred in the positive controls and no bacterial growth was observed in the negative controls. Growth of *E. faecalis* strains at pH 9.5 to 11.5 was observed in all periods. Although turbidity was not observed at pH 12.5, growth of 13 (61.9%) and 2 (9.52%) strains out of 21 samples, occurred at 24 and 48 h, respectively on M-Enterococcus agar. No tube showed growth at pH 12.5 after 72 h. The pH x period interaction showed a significant ($p<0.05$) inhibition of bacterial growth over time at pH 12.5. No changes were observed in the pH of non-inoculated BHI broth throughout the experiment. The initial pH values of 9.5, 10.5, 11.5 and 12.5 decreased to 7.6, 8.3, 8.6 and 10.1, respectively, at day 14.

Discussion

E. faecalis was present in oral samples of 20 out of 150 patients, using conventional bacteriological culture techniques. This value is higher than those of other studies (4,13) that used the same methodology to isolate *E. faecalis* from the oral cavity, which could be explained by differences in the populations and their oral hygiene habits.

E. faecalis is the most important microorganism associated with root canal therapy failure (3,5) because of its resistance to the endodontic procedures and intracanal medications (10), even in alkaline environments (11,14).

The mechanisms involved in the survival of *E. faecalis* in high alkaline pH are related to the existence of a working proton pump (11). When negatively charged hydroxyl ions penetrate the bacterial cytoplasm, elevating the pH, the proton pump drives positively charged potassium ions into the cell to acidify the cytoplasm, impeding (blocking) the occurrence of enzymatic inhibition (11,15,16).

An *in vitro* study (20) investigated the pH required to kill *E. faecalis* and found that pH 10.5 to 11.0 only delayed the growth of this microorganism, whereas at pH 11.5 or greater the microorganism was killed. The present study used a similar methodology to evaluate the resistance of a reference ATCC strain and clinical isolates of *E. faecalis* to high alkaline pH. Contrary to a previous finding (20), in the present study growth of all bacterial strains occurred above pH 11.5. A possible explanation for this difference may be attributed to the methodology. MacHugh et al. (14) assessed the bacterial growth spectrophotometrically by turbidity and confirmed the presence of the original organism by Gram stain morphology. The present study used subcultures in M-Enterococcus agar, a selective medium for this bacterium, to verify its growth. The methodology employed in this study presents greater sensitivity relative to the turbidity method because only the viable cells can grow on the surface of the M-Enterococcus agar.

An interesting finding of the present study was that turbidity of the medium was not observed in any tube at pH 12.5. However, after seeding on M-Enterococcus agar, growth of 13 (including the ATCC strain) out of the 21 bacterial strains occurred after 24 h, 2 clinical isolates grew after 48 h, and no colony formation occurred at 72 h or longer periods. This reveals the ability of *E. faecalis* to remain viable, but not detectable. In order words, under stress conditions, this microorganism remains latent and may grow again when the conditions become favorable (17). The variation of alkaline pH resistance may be related to the variable genotypes among *E. faecalis* strains (2).

Another important aspect is that bacteria can produce acid and reduce the pH of the environment, making it favorable for their survival. Although it was not the scope of this study, a progressive decrease in the initial pH values was observed. This finding shows that the microorganism probably acidified the medium since the BHI containing tubes without inoculum, adjusted with the tested pH values, did not undergo changes throughout the experiment.

This study investigated whether *E. faecalis* clinical isolates from the oral cavity of patients with endodontic infections have the same resistance profile to high alkaline pH as those of the reference strain (ATCC 29212). Two clinical isolates survived 48 h at pH 12.5, while the ATCC strain was viable only up to 24 h. The differences between our results and the aforementioned study (14) may be due to the 5-year interval between them and to the fact that the resistance profile of microorganisms can change over time (18). In this time interval, a genetic variation of *E. faecalis* strains may have assigned them a phenotype of greater resistance to alkaline pH.

Although the pH of calcium hydroxide pastes used in Endodontics is 12.3, this value drops to 8.5-9.0 within the root canal (9,19) because of the buffering effect of dentin, making it not high enough to kill the *E. faecalis*. The combination of calcium hydroxide pastes with different vehicles such as chlorhexidine (8,20), paramonochlorophenol (10,21) and ozone (21) has been suggested to produce a more effective intracanal medication, especially against *E. faecalis*. Further studies should be focused on evaluating *E. faecalis* resistance to alkaline conditions in other populations.

References

Enterococcus faecalis é uma espécie bacteriana frequentemente encontrada em canais radiculares com insucesso do tratamento endodôntico. Pastes alcalinas são amplamente utilizada em Endodontia por causa de sua biocompatibilidade e atividade antimicrobiana, porém esse microrganismo pode ser resistente a alcalinidade. Este estudo avaliou in vitro a resistência do *E. faecalis* ao pH alcalino por diferentes períodos até 14 dias. Amostras foram obtidas da cavidade oral de 150 pacientes da Clínica de Endodontia. O pH dos tubes experimentais (n=84) foram inicialmente ajustados com NaOH 6M a valores de pH 9.5, 10.5, 11.5 e 12.5 (21 tubes per pH). Vinte isolados clínicos e a cepa ATCC 29212 foram testados. Os 5 controles positivos e os tubos experimentais de cada pH foram inoculados com 10 µL de suspensão bacteriana e incubados a 36 °C por 24, 48 e 72 h, 7 e 14 dias. Para cada período, turvação do meio foi compara visualmente com padrão 0.5 da escala de McFarland. A presença de microorganism foi confirmada por semeadura no meio ágar M-Enterococcus. Quatro tubos contendo caldo BHI ajustado aos pHs testados foram incubados por 14 dias para verificar a ocorrência de alterações de pH. O pH do caldo BHI inoculado também foi medido no 14º dia para determinar se o microrganismo acidificou o meio. O crescimento de todas as cepas de *E. faecalis* ocorreu com pH entre 9.5 e 11.5 em todos os períodos. Embora não tenha sido observada turvação do meio no pH 12.5, houve crescimento de 13 e 2 cepas às 24 e 48 h, respectivamente, no meio ágar M-Enterococcus. Nenhum tube apresentou crescimento bacteriano no pH 12.5 após 72 h. Concluiu-se que o *E. faecalis* pode sobreviver em pH altamente alcalino, que alguns isolados clínicos requerem 72 h em pH 12.5 para serem eliminados.

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