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Zaki Mubarak
Faculty of Dentistry, Syiah Kuala University, Banda Aceh 23111, Indonesia, zakimubarak54@yahoo.com

Widya Asmara
Department of Microbiology, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta 55281, Indonesia

Tri Wibawa
Department of Microbiology, Faculty of Medicine, Gadjah Mada University, Yogyakarta 55281, Indonesia

Boy Bachtiar
Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta 10430, Indonesia

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ORGINAL ARTICLE

Phenotype and Genotype of *Enterococcus faecalis* Isolated from Root Canal and Saliva of Primary Endodontic Patients

Zaki Mubarak[1], Widya Asmara[2], Tri Wibawa[3], Boy Bachtiar[4]

[1]Faculty of Dentistry, Syiah Kuala University, Banda Aceh 23111, Indonesia
[2]Department of Microbiology, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta 55281, Indonesia
[3]Department of Microbiology, Faculty of Medicine, Gadjah Mada University, Yogyakarta 55281, Indonesia
[4]Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta 10430, Indonesia
Correspondence e-mail to: zakimubarak54@yahoo.com

ABSTRACT

Objective: To investigate the phenotype and genotype of *E. faecalis* isolated from the root canal and saliva of primary endodontic patients with periapical lesions. **Methods:** Eighteen adult male and female individuals suffering from primary endodontic infection, either with or without periapical lesions, were involved in this study. Root canal scraping and saliva were collected from each subject and used for bacterial quantitation using a real-time polymerase chain reaction (RT-PCR). Enterococci were isolated using ChromAgar medium and then identified using both biochemical (Gram staining and catalase tests) and molecular biology (conventional PCR) methods. Gelatinase activity, polysaccharide capsul profile and mRNA *ace* expression level were determined using microbiological, biochemical and molecular biology approach, respectively. Genotype of *E. faecalis* was determined based on nucleotide sequence of *ace* and *gelE* genes analyzed using web-based 3730xl DNA Analyze software. **Results:** The results showed that except for its proportion, no significant difference was found in phenotypes (gelatinase activity and mRNA *ace* expression levels) and genotypes (polymorphism of Cps operon and variation of *ace* and *gelE* nucleotide sequences) of *E. faecalis* isolated from the root canal and saliva of primary endodontic patients had or had no periapical lesions. **Conclusion:** It can be concluded that *E. faecalis* proportion had a role in the occurrence of periapical lesions in the primary endodontic patients, but not gelatinase activity, mRNA *ace* expression level, Cps operon polymorphism or *ace* and *gelE* nucleotide sequence variations.

Key words: Ace, Cps, Enterococcus, GelE, sequence

INTRODUCTION

The commensal bacterium *Enterococcus faecalis* is a non-oral microbiota that may inhabit in mouth cavity.[1,2] Once occur in oral cavity, infected pulp or root canal *E. faecalis* may enter the entire system of root canal and survive to induce a number of dental problems although chemomechanic treatments have been applied.[3] As consequences, *E. faecalis* becomes the most common bacteria isolated from the mouth cavity of patients suffering from a number of primary or secondary (post-treatment) endodontic infections.[4-11] Here, *E. faecalis* may occur as a single infection agent or a mix infection with *E. faecalis* as the most dominant species.[12]

High infectivity of *E. faecalis* in human and animal tissues or organs and its remarkable endogenous resistance to extreme environmental conditions and antimicrobials may relate to this bacterium's ability to produce some virulence factors such as hemolysin (Hln), aggregation substance (AS) and gelatinase (GelE) as well as *adhesin for collagen from E. faecalis* (Ace) and polysaccharide capsule (Cps).[13-16] Evidences for the role of Hln, AS and GelE in the pathogenesis of *E. faecalis* infection, so far, come from *in vivo* inflammation studies in non-endodontic animal models and from *in vitro* adherence test using certain cell lines.[17-20] Additionally, the avail-

able data explaining the relationship between the polymorphism of *E. faecalis* Cps and root canal infections is still controversial.[21-23] Ace is the solely virulence factor of *E. faecalis* that has been known its role in facilitating the adherence of this bacterium in the collagen of human dentin.[20] The data suggest that, except for that of Ace, the actual roles of virulence factors produced by *E. faecalis* in the pathogenesis of enterococcal infections in the root canal or dental tubuli are not clearly understood or experimentally proved.

Phenotype and genotype of *E. faecalis* could be characterized using a combination of microbiological and biochemical approaches supported by information obtained from molecular biology techniques such as conventional and real time polymerase chain reaction (RT-PCR) and DNA sequencing analysis.[19,24] Since unique microenvironment may exist in certain regions of the mouth cavity, in particular between the root canal and saliva, it is assumed that there are some differences in the phenotype and genotype characteristic of virulence factors produced by *E. faecalis* lives in these two environments.[25]

Unfortunately, there is no literature, so far, comprehensively describes the properties of virulence factors produced by *E. faecalis* lives in the root canal and saliva or explains the relationships between *E. faecalis* virulence factor genotype and phenotype diversities with the occurrence of periapical lesions in the primary endodontic patients. The objective of this study, therefore, was to investigate the relationship between phenotype (GelE activity and mRNA *ace* expression level) and genotype (polymorphism of Cps operons and ace and GelE nucleotide sequences) of *E. faecalis* isolated from the root canal and saliva with the incidence of periapical lesions in the patients suffered from primary endodontic infection.

METHODS

This study was approved by the Ethics Committee of Dentistry Faculty of Syiah Kuala University (Document no. 020/KG/FKG/2014). Subjects were 18 male and female individuals, aged 18-50 years old, who suffered from primary endodontic infection (4 with periapical lesions and 14 without periapical lesions) and voluntarily participated in the study after completing the informed consent. The status of necrotic pulp and periapical lesions was determined by thermal and radiographic examinations, respectively.

Root canal sample scrapings and saliva were aseptically collected from each subject using reamer and sterile cotton bud, respectively. Samples were placed in sterile plastic containers, diluted in NaCl 0.9% (E-Merck), and used for quantitation of bacterial proportion using RT-PCR technique.[24] For isolation, samples were cultured in the ChromAgar medium (Brilliance VRE, Oxoid, Basingstoke, UK) for 18-24 hours at 37°C and observed by binocular Olympus microscopy using 100x Phase contrast.[26] The pink colonies isolates were then identified using Gram staining and catalase test. The colonies were also regrowth in the brain heart infussion (BHI) medium (Acumedia) containing 10% fetal bovine serum (FBS) (Acumedia) before finally being identified by conventional PCR using *E. faecalis* 16S rRNA primers.[24,27]

Phenotypes of *E. faecalis* examined were: (1) GelE activity that was determined based on the diameter of halo produced after this bacterium growth in the tryptic soy agar (TSA) medium (BD Diagnostic Systems) containing 1.5% skim milk (Himedia Laboratories), and (2) and mRNA *ace* expression level that was quantitated by RT-PCR using *E. faecalis ace* forward and reverse primers.[24] Genotype of the *E. faecalis* isolated was determined based on nucleotide sequences of *ace* and *gelE* genes analyzed using web-based 3730xl DNA Analyze software. Data obtained was tabulated and then analyzed descriptively.

RESULTS

Isolation and identification of *E. faecalis*

Culturing root canal sample scrapings and saliva from primary endodontic patients with or without periapical lesions in the ChromAgar medium successfully recovered 38 *Enterococcus* isolates. *Enterococcus spp*., including *E. faecalis* and *E. faecium*, resulted in pink colonies in ChromAgar because they produced β-glucosidase that was able to change chromogenic substances in the medium.[26]

To differentiate *E. faecalis* (Gram positive and catalase negative) from *E. faecium* (Gram positive and catalase positive), the pink colonies obtained were screened further using Gram staining and catalase test, two biochemical methods commonly used to identify bacteria isolated from oral samples.[9] Results of these biochemical tests showed that there were 19 *E. faecalis* from 38 *Enterococcus* colonies isolated using ChromAgar medium.

Proportion of *E faecalis* based on the source of isolate

The detection of *E. faecalis* isolates' DNA with specific primers that were subsequently amplified by quantitated PCR technique may explain the differences in the number and proportion of these bacteria according to the source of clinical samples used in this study (Figure 1).

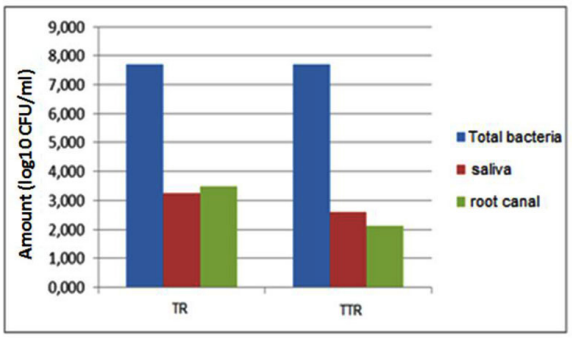

Figure 1. The number of *E. faecalis* based on the sources of sample (root canal and saliva) of primary endodontic patients had (TR) or had no (TTR) periapical lesions. Total DNA of *E. faecalis* and total bacteria was amplified using *E. faecalis* 16S rRNA primers and universal primers, respectively.[24,27]

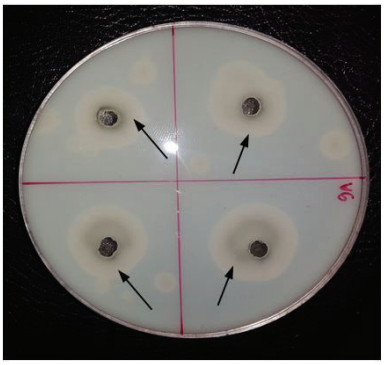

Figure 2. Gelatinase activity showed by *E. faecalis* isolates. Black arrows represent the halo of wells containing isolates cultures for 24 hour in the TSA medium.

The results quantitated PCR analysis suggested that the higher numbers of *E. faecalis* in root canal (3.489 CFU/mL) than that in saliva (3.236CFU/mL) of primary endodontic patients with periapical lesions.

Gelatinase activity

Using microbiology method described previously, GelE activity of *E. faecalis* isolated from the root canal and saliva of primary endodontic patients with or without periapical lesion was successfully determined as illustrated in Figure 2.[24,26] Based on GelE activity showed by the control *E. faecalis* strain ATCC 29212, GelE activity was categorized into negative, weak (0.1-0.2cm), medium (0.3-0.8cm) or strong (1.0-1.9cm).

The majority (84.2%) of *E. faecalis* had GelE activity ranged from weak to strong, and only small amount (15.8%) showed no GelE activity. In primary endodontic patients with periapical lesions there was no significant difference ($p>0.05$) in the numbers of *E. faecalis* isolates live in root canal and saliva showed either weak or strong GelE activity. In primary endodontic patients without periapical lesions, *E. faecalis* isolates had a weak GelE activity were more prevalent in saliva (36.8%) than in root canals, whereas strong GelE activity was produced by *E. faecalis* mainly isolated from root canals. However, there was no significant difference ($p>0.05$) found in the activity of GelE *E. faecalis* isolates between the two groups of primary endodontic patients evaluated. This study also observed the incongruent GelE activity showed by *E. faecalis* isolated from root canal and saliva of one primary endodontic patient without periapical lesions.

Level of mRNA *ace* expression

Phenotype of Ace of *E. faecalis* isolated in this study was determined based on the level of mRNA *ace* expression quantitated using qPCR technique after the bacterium was cultured in the BHI solution containing 10% FBS. The results indicated that 8 out of 19 *E. faecalis* isolated from root canals and saliva of primary endodontic patients without periapical lesions expressed mRNA *ace*. According to the ratio, mRNA *ace* expression levels belonged into 3 categories: weak (-0.87 – -2.63), moderate (0.19 – 0.30), and strong (3.58 – 8.27). Here, weak mRNA *ace* expression level showed by the control *E. faecalis* ATCC 29212 was used a reference.

Isolates had moderate and strong mRNA *ace* expression levels showed higher ($p<0.05$) mRNA *ace* expression compared to *E. faecalis* ATCC 29212. *E. faecalis* isolates had strong (4.47) mRNA *ace* expression were mainly found in primary endodontic patients had no periapical lesions whereas those having weak (0.08) mRNA *ace* expression were prevalent in primary endodontic patients with periapical lesions.

Polymorphism of polysaccharide capsule

Using the method described previously, this study successfully identified three types of Cps operons in *E. faecalis* lived in root canals and saliva of primary endodontic patients without periapical lesions, namely Cps-1 (73.7%), Cps-2 (10.5%) and Cps 5 (10.5%). Only 5.3% (1/19) were Cps negative.[40] The number of isolates had Cps 1, 2 and 5 found in the root canals or saliva of these primary endodontic patients slightly varied (Figure 3). The differences, however, were not statistically analyzed due to the limited number of samples and *E. faecalis* had Cps 2 and 5 operons.

Enterococcus faecalis isolates had Cps-1 operon, those did not produce polysaccharide capsule, were mainly isolated from root canals and saliva of primary endodontic patients without periapical lesions (47.3%)

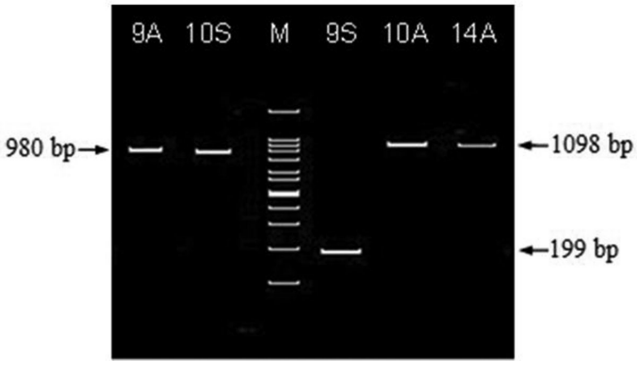

Figure 3. Electrogram showing the band of Cps1 (980 bp), Cps2 (1098 bp) and Cps 5 (199 bp) operons. Number stands for sample number. M = Marker, A = root canal, S= Saliva

whereas isolates had Cps-2 or Cps-5 operons were more prevalent in primary endodontic patients with periapical lesions. The dominance of non-Cps *E. faecalis* in the saliva of primary endodontic patients with periapical lesions indicated that saliva was a convenient environment for these strains compared to root canals. Further study is needed to do to explain this assumption.

DISCUSSION

Isolation and identification of *E. faecalis*

Results of the biochemical tests showed that there were 19 *E. faecalis* from 38 *Enterococcus* colonies isolated using ChromAgar medium. Since detection of *E. faecalis* from oral samples required more specific and sensitive approaches such as a PCR that was able to amplify DNA encoding 16S rRNA, bacterial identification was also performed by PCR method using *E. faecalis* 16S RNA primers.[24,27-29] Similar to those of biochemical methods, this molecular biological approach confirmed the occurrence of 19 *E. faecalis* isolates from 38 Enterococci colonies recovered in the ChromAgar medium. It can be concluded that the occurrence of Enterococcus bacteria in ChromAgar medium cultured with root canal sample scrapings and saliva collected from primary endodontic patients did not necessarily indicate the presence of *E. faecalis* in the clinical samples analyzed.

Results of this study suggested that in the primary endodontic patients without periapical lesions, *E. faecalis* was more frequently found in saliva rather than in root canals. In the primary endodontic patients with periapical lesions, in contrast, equal numbers of *E. faecalis* were found in both root canal and saliva. These data indicated that saliva became the main source of *E. faecalis* in the mouth of patients from where clinical samples for this study were obtained. This finding was in agreement with results of previous studies reported that *E. faecalis* was consistently isolated from both root canal and saliva of patients suffered from primary and secondary endodontic infections.[10,12,30-33] Data of this study, however, could not explain whether the isolated *E. faecalis* was part of mouth flora or just a transient bacterium in this organ as this organism could be present in the mouth cavity from dietary sources.[34-35]

Proportion of *E faecalis* based on the source of isolate

The results quantitated PCR analysis suggested that the higher numbers of *E. faecalis* in root canal (3.489 CFU/mL) than that in saliva (3.236 CFU/mL) of primary endodontic patients with periapical lesions. This is contrast to condition found in the primary endodontic patients without periapical lesions where the numbers of *E. faecalis* in root canal (2.117 CFU/mL) were lower than that in saliva (2.608 CFU/mL). Overall, the numbers of *E. faecalis* in the root canal and saliva of primary endodontic patients had periapical lesions (58.7%) were higher than that of primary endodontic patients without periapical lesions (41.3%). The qPCR technique was also able to show dominance of *E. faecalis* population over other bacteria species in both root canal and saliva of these patients. These results support the notion that the qPCR was a sensitive technique for detection and quantitation of bacterial number and proportion in clinical oral samples.[24]

It was revealed from this study that periapical lesions were mainly found in primary endodontic patients with high number and proportion of *E. faecalis* in both root canal and saliva. Periapical lesions, on the other hand, were absent in primary endodontic patients had lower number and proportion of *E. faecalis* in root canal or saliva. These facts suggested that the high presence of *E. faecalis* in root canal and saliva was strongly related to the case of periapical lesions in primary endodontic patients.

Phenotypes of *E. faecalis*

Gelatinase activity

This study also observed the incongruent GelE activity showed by *E. faecalis* isolated from root canal and saliva of one primary endodontic patient without periapical lesions. Here saliva isolate was GelE negative, but root canal isolate showed weak GelE activity. This was not uncommon phenomenon because previous showed that although *gelE* gene existed in the chromosome of all *E. faecalis* cells, this gene was not regularly expressed.[36,37] Previous studies also found that *E. faecalis* isolated from oral cavity generally expressed GelE, but activity of the enzyme *in vitro* was heterogenic.[24,38]

Level of mRNA *ace* expression

Results obtained in this study were different from previous reports suggesting that *E. faecalis* expressed higher levels of mRNA *ace* when cultured in the

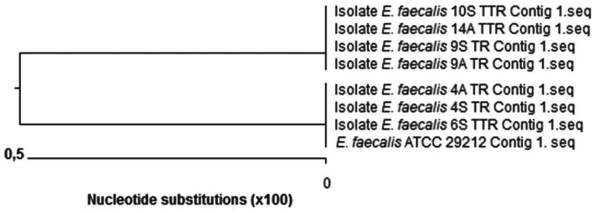

Figure 4. Phylogenic tree of *E. faecalis* isolates constructed based on gelE sequence analysis showing the difference and similarity of the isolates with overseas strains. S = saliva, A = root canal, TR = patients had periapical lesions; TTR = patients had no periapical lesions

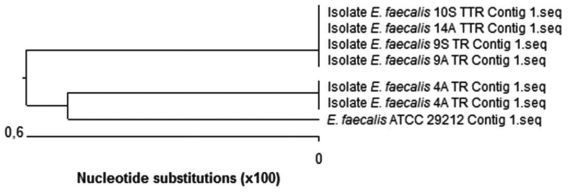

Figure 5. Phylogenic tree of *E. faecalis* isolates constructed based on ace sequence analysis showing the difference and similarity of the isolates with overseas strains. S = saliva, A = root canal, TR = patients had periapical lesions; TTR = patients had no periapical lesions

serum *E. faecalis* or growth medium supplemented by serum.[15,39] The mRNA *ace* expressions reported by these authors were not categorized as weak, moderate or strong as done in this study. Moreover, study did not address the relationship between mRNA *ace* expression level and the occurrence of periapical lesions.[39]

From this study could be assumed that level of mRNA *ace* expression in the *E. faecalis* live in the root canal was not directly related to the incidence of periapical lesions in primary endodontic patients. This was contrast to results showing that there was a positive correlation between the levels of mRNA *ace* expressed and *E. faecalis* infections in experimental endocarditis. More detailed research is, therefore, required to explain this assumption.[15]

Genotypes of *E. faecalis*

Polymorphism of polysaccharide capsule

Results obtained in this study were different from those reported.[22] In their studies Bachtiar and coworkers reported that *E. faecalis* species had Cps-2 were more prevalent in primary and secondary endodontic patients.[22] This difference might be caused by individual variation or unique environment triggering endodontic infections among populations.[41,42] Similar to this study, Bachtiar and coworkers did not explain whether the *E. faecalis* had Cps 2 reported were isolates with positive or negative Cps phenotypes. According to Gaspar *et al*., *E. faecalis* had Cps 2 genotype consisted of 2 phenotypes, namely Cps positive and Cps negative.[22,23] The two strains could be identified based on the occurrence of 6770 bp insertion that could be detected using qPCR method.[20] This study focused on Cps operons of *E. faecalis* in primary endodontic patients with or without periapical lesions, different from other researchers that did not address periapical lesions in the primary endodontic patients investigated.[22,23]

***gelE* and *ace* nucleotide sequences**

The variation observed in the activity of gelatinase and the level of mRNA *ace* expression of *E. faecalis* isolates collected from primary endodontic patients with or without periapical lesions might relate to the polymorphism of *gelE* and *ace*, the encoding genes. To address this assumption, coding regions for both *gelE* and *ace* preliminary were separately amplified by PCR, sequenced and then analyzed using web-based 3730xl DNA Analyze software programs. These unraveled similarity and difference of *gelE* and *ace* nucleotide sequences of *E. faecalis* isolates as shown by phylogenic tree of the isolates created according to the sequence of each gene (Figure 4 and 5).

Results of PCR amplification suggested that from 19 *E. faecalis* isolates, 7 were gene *gelE* positive (Figure 4) and 6 were gene *ace* positive (Figure 5). Comparative analysis on the sequences of the two genes indicated that *E. faecalis* isolates found in the root canals of primary endodontic patients, either with or without periapical lesions, had 100% homology with those found in their saliva. It was predicted, therefore, that periapical lesions occurred in the patients were caused by *E. faecalis* strains in saliva entered root canals as reported by Mozini *et al*. and de Oliviera *et al*.[43,44]

Based on nucleotide sequences of *ace* and *gelE, E. faecalis* strains isolated from primary endodontic patients with or without periapical lesions involved in this study could be grouped into two clusters with 0.5-0.6 differences per 100 nucleotides. These small differences, however, were enough to classify the isolates into two separate branches in the phylogenic trees.

Similarity and difference of these *E. faecalis* isolated in this study with overseas isolates were also analyzed by comparing nucleotide sequences of *gelE* and *ace* of the isolates with those of overseas isolates downloaded from the database (http://www.ncbi.nlm.nih.gov). The results showed that the first cluster occurred in a branch separated from overseas isolates. There were four isolates in this cluster, namely 9A, 9S, 10S and 14A. The first two isolates were obtained from the root canals and saliva of primary endodontic patients with

periapical lesions, respectively; whereas the other two were obtained from saliva and root canals of primary endodontic patients without periapical lesions. These isolates (except for 10S), uniquely, showed very strong gelatinase activity.

Different from those in the first cluster, *E. faecalis* isolates in the second cluster showed closer relationships with a number of overseas isolates such as JH2-2 and *collagen adhesin* from America, 75A from India, TLME3 from China and 494-95 from Portugal as well as local isolates of South Korea, Japan and the control *E. faecalis* ATCC 29212. Two isolates in this cluster (4A and 4S) were collected from primary endodontic patients had periapical lesions, and one (6S) was collected from saliva of primary endodontic patient had no periapical lesions. These all three isolates showed weak gelatinase activity.

Nucleotide differences ranged from 0.5 upto 1.0 between *E. faecalis* strains isolated in this study and overseas *E. faecalis* isolates suggested specificity of these isolates. Contribution of this genetic specificity on the incidence of periapical lesions in primary endodontic patients, however, was not obviously identified. Further studies are, therefore, required to unravel the roles of *gelE* and *ace* polymorphisms on the incidence and pathogenesis of periapical lesions in primary endodontic patients.

CONCLUSION

Based on results of the study and discussion it can be concluded that high number of *E. faecalis* found in both root canal and saliva was related to the incidence of periapical lesions in primary endodontic patients. This is contrast to the insignificant relationship found between gelatinase activity, mRNA *ace* expression, Cps polymorphism, and genetic variation of *gelE* and *ace* with the occurrence of periapical lesions in these patients, respectively.

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