

Research Report

Hemolysin activities as virulence factor of *Enterococcus faecalis* isolated from saliva and periapical abscess (gene detection by PCR)

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ABSTRACT

Background: *Enterococcus faecalis* is a normal flora of the oral cavity, commonly detected in saliva and persistence in endodontic infections. These bacteria have diverse survival and virulence factors. Hemolysin is one of the factor and still had unclear role as a virulence factor of the *Enterococcus faecalis* to survive in the root canal. **Purpose:** The purpose of this research was to analyze the presence and activity of hemolysin gene and its activity as a virulence factor isolated from saliva and root canals with periapical abscess. Yet by understanding one of the phenotypes characters which is hemolysin, it is expected a successful endodontic treatment can be provided with the persistent of *Enterococcus faecalis* bacteria. **Methods:** Method of the research starting with the identification of *Enterococcus faecalis* bacteria in isolated saliva and periapical abscess was done in the first part of the study. Then the phenotypes character of *Enterococcus faecalis* such as gene detection and expression of hemolysin in blood agar cultures of the 60 colonies samples were performed in the later part. **Results:** Not all of the colonies cultured were identified as *Enterococcus faecalis*. All positive detection on hemolysin gene showed hemolysin expression in both isolated samples. However, there were samples with hemolysin expression eventough no hemolysin gene detected. Hemolysin expression detection in saliva was higher due to different activation phase of hemolysin in saliva. The study with just one primer could lead to the possibility of undetected hemolysin gene, eventough there were samples that did not have hemolysin gene. The proportion of hemolysin expression in root canals were less than saliva, this could be influenced by environmental factors. However, Hemolysin was considered as important virulence factor, particularly for disease therapy. **Conclusion:** The conclusion of this research was hemolysin gene discovered in clinical isolated saliva and root canals samples as virulence factor of the *Enterococcus faecalis*, and hemolysin expression occured from both sources.

Key words: Hemolysin, virulence, *Enterococcus faecalis*, saliva, root canal, primer

ABSTRAK

Latar belakang: Bakteri *Enterococcus faecalis* adalah flora normal rongga mulut dan merupakan mikroorganisme yang umum dideteksi dalam saliva dan infeksi endodontik persistensi. Bakteri ini memiliki berbagai faktor survival dan virulensi. Hemolysin adalah salah satunya tetapi masih merupakan faktor virulensi yang belum terlalu jelas mekanismenya. **Tujuan:** Tujuan riset ini adalah untuk menganalisis keberadaan gen hemolysin dan aktifitas hemolysin *Enterococcus faecalis* sebagai faktor virulen yang diisolasi dari saliva dan saluran akar gigi dengan abses periapikal. Mengetahui salah satu karakter fenotip *Enterococcus faecalis* yaitu hemolysin diharapkan berguna untuk kesuksesan perawatan endodontik akibat persistensinya bakteri tersebut. **Metode:** Penelitian diawali pada bagian pertama penelitian adalah identifikasi bakteri *Enterococcus faecalis* isolat saliva dan abses periapikal. Bagian kedua melihat karakter fenotip *Enterococcus faecalis* berupa deteksi gen hemolysin serta ekspresi hemolysin di kultur agar darah dari 60 sampel koloni. **Hasil:** Tidak semua kuman yang tumbuh dikultur teridentifikasi sebagai *Enterococcus faecalis*. Pada deteksi gen hemolysin positif menunjukkan seluruhnya terekspresi hemolysin di kedua sumber isolat klinik. Namun, terdapat sampel yang menunjukkan terekspresi hemolysin meskipun gen hemolysin tidak ada dan itu lebih banyak di saliva, walaupun tidak bermakna. Hal ini dapat disebabkan perbedaan tahap aktivasi hemolysin di saliva. Pengujian hanya dengan satu primer dapat menyebabkan kemungkinan

ada gen hemolysin tetapi tidak terdeteksi. Walaupun memang ada sampel yang tidak memiliki gen hemolysin. Proporsi keberadaan ekspresi hemolysin pada saluran akar lebih sedikit dari saliva karena ekspresi hemolysin dipengaruhi faktor lingkungan. Namun demikian, Hemolysin adalah faktor virulensi yang penting khususnya untuk terapi penyakit. **Kesimpulan:** Kesimpulan penelitian ini adalah ditemukan gen hemolysin pada sampel isolat klinik saliva dan saluran akar sebagai faktor virulen bakteri *Enterococcus faecalis* serta terjadi ekspresi hemolysin dari kedua sumber tersebut.

Kata kunci: Hemolysin, virulen, *Enterococcus faecalis*, saliva, saluran akar, primer

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INTRODUCTION

Enterococcus faecalis is a normal commensal flora, normally found in gastrointestinal and oral cavity.¹⁻³ This bacteria could become pathogenic and caused of many infections including root canal infection.⁴⁻¹³ The characteristics of these microbes allow it to survive in conditions that are not common to other microbes, because it has some virulence factors which hold important role in the pathogenesis such as lipoteichoic acid, aggregation substance (AS), hemolysin, gelatinase (gel E) and extracellular surface protein (ESP).^{7-10,14}

Hemolysin has been reported as an important virulence factor especially for disease therapy. This virulence factor can be used for guard antibiotic and corticosteroid combinations of treatment in hemolysin or non-hemolysin strain cases.¹⁵ On the other hand, even though hemolysin has been researched in many aspects but still had unclear role as a virulence factor of the *Enterococcus faecalis* to survive in the root canal, and induce inflammation in the periapical tissues.¹⁶

Enterococcus faecalis bacteria is a normal flora of the oral cavity, but often found in cases of periapical abscess and endodontic treatment failure cases, therefore it is assumed that there are differences in phenotype activity of hemolysin in saliva and root canals with periapical abscess.⁶ The involvement of *Enterococcus faecalis* in periapical tissue infections was not fully understood from literatures. Thus, more detailed data is needed to explain the behavior of the bacterial population in the root canal of the tooth, whether as the pathogen causing the infection of periapical tissues or opportunistic species that become pathogenic because of the conducive micro-environment to their survival. A question needs to be answered through this research because these bacteria are normal flora of the mouth and digestive tract.

The purpose of this research was to analyze the presence and activity of hemolysin gene and its activity as a virulence factor isolated from saliva and root canals with periapical abscess. Yet by understanding one of the phenotypes characters which is hemolysin, it is expected a successful endodontic treatment can be provided with the persistent of *Enterococcus faecalis* bacteria.

MATERIALS AND METHODS

The first part of the study was the identification of *Enterococcus faecalis* bacteria in isolated clinical saliva and periapical abscess. Then the phenotypes character of *Enterococcus faecalis* such as gene detection and expression of hemolysin in blood agar cultures were performed in the later part. Samples were taken from six patients with periapical abscess. The samples taken from saliva and root canals were then cultured in chromatogenic agar. Sixty greenish blue color colonies were taken. Extraction of DNA proceeded for PCR preparation. DNA was extracted using the Real Genomic Hi-Yield DNA mini kit. Sequence primers use for 16sRNA, forward: TGGC ATAA GAGT GAAA GGCGC, revers: GGGG ACGT TCAG TTAC TAAC GT. Sequence primers use for hemolysin gene forward: GACT CGGG GATT GATA GGC, revers: GCTG CTAAGCT GCGC TTAC.¹⁷⁻¹⁹

Two PCR reactions were performed. The first one was using 16sRNA to ensure *Enterococcus faecalis* DNA analyzed, while the second reaction was to confirm the extracted DNA contains hemolysin genes. PCR reaction performed in a total volume of 25 µl containing PCR mix dream Tag Fermentation (Real Biotech Co. USA), 3 µl Primer 16sRNA forward 10 µM, 3 µl Primer 16sRNA revers 10 µM, 3 µl Nuclease free water. A total of 3 µl of extracted DNA was added to the reaction mixture. PCR was also performed using a positive control (DNA extracted from the American Type Culture Collection [ATCC] species). For the first amplification, samples were subjected to 22 denaturation cycles at 95° C for 15 min, heating at 94° C for 20 seconds. For the second amplification, PCR reaction conditions were 40 cycles of 67° C for 45 seconds, 72° C for 15 seconds, 72° C for 7 minutes and then 4° C.

Hemolysin gene PCR reaction was performed in the same way. The first temperature was 95° C during 15 minutes as the stage of activation of the DNA denaturation enzyme. Followed with 35 cycles at a temperature of 94° C for 20 seconds, 56° C for 45 seconds and 72° C for 60 seconds.

PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with Gelred Nucleic Acid Gel Stain (Biotium Inc. USA) in TAE electrophoresis buffer II,

and viewed under ultraviolet transillumination. Positive or negative identification was done based on the presence of clear bands of the expected molecular size using a 100bp DNA Ladder (GeneRuler, CA).

Then the *Enterococcus faecalis* phenotypic characters test was done to confirm the hemolysin expression of isolated saliva and root canal samples in blood agar plates. Blood agar medium was made by mixing 40 ml fresh lamb blood into 1 L of blood agar solution. Colony dilution was done (10^{-5}) in order to distribute colonies on blood agar. Hemolysin expression activity will be seen in the form of clear circular nodes (halo) around the bacterial colonies.

RESULTS

About 15% of 70 colonies samples were not *Enterococcus faecalis* colonies. This suggests the possibility of other types of bacteria isolated in the sample and were not studied further. A total of 60 *Enterococcus faecalis* colony samples were taken for the next stage in this research (Figure 1).

The proportion of hemolysin gene in the isolated saliva samples were higher compared to root canal but no significant differences (Table 1). The proportion of hemolysin expression in root canal sample is less than in saliva samples, but no significant differences (Table 2 and Figure 2). Table 1 and 2 showed proportion of hemolysin gene and hemolysin expression not compare each other.

On positive hemolysin gene detection showed that all of them had hemolysin expression in both clinical isolated samples. However, there were samples with hemolysin expression eventough no hemolysin gene detected (Table 3). The proportion of hemolysin expression without hemolysin gene was 23.1% in root canal samples and 18.2% in saliva samples.

Table 1. Hemolysin gene distribution of clinical isolated samples

Clinical isolated samples	Hemolysin gene				Total n	p*
	Yes		No			
	n	%	n	%		
Root canal	17	56.7	13	43.3	30	0.792
Saliva	19	63.3	11	36.7	30	
Total	36	60.0	24	40.0	60	

* Chi Square Test

Table 2. Hemolysin expression of clinical isolated samples

Clinical isolated samples	Hemolysin expression				Total n	p*
	Yes		No			
	n	%	n	%		
Root canal	20	66.7	10	33.3	30	1.000
Saliva	21	69.0	9	31.0	30	
Total	41	67.8	19	32.2	60	

* Chi Square Test

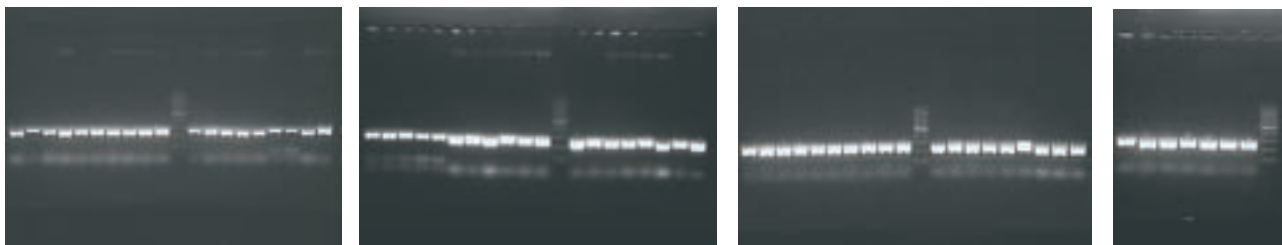


Figure 1. PCR 16sRN Result from 60 samples using a 100 bp DNA Ladder (GeneRuler, CA).

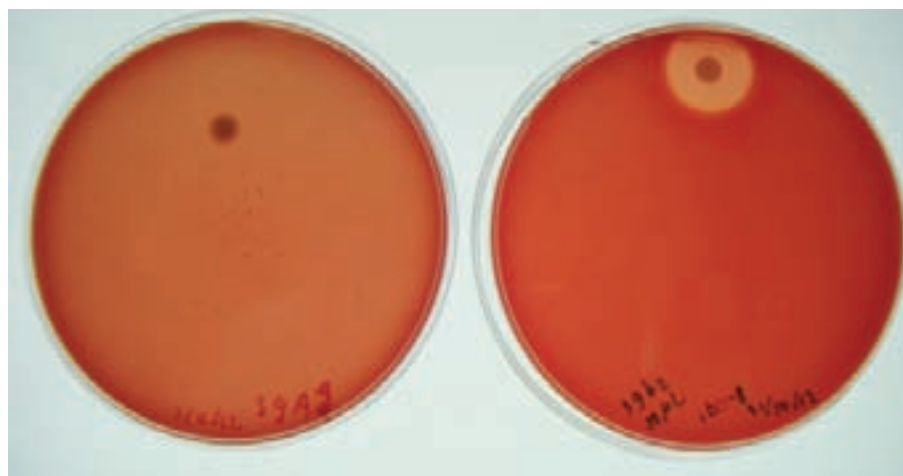


Figure 2. Bacterial colonies showed no halo (left) and positive halo (right).

Table 3. Hemolysin expression presence distribution with hemolysin gene of clinical isolated samples

	Expression				Total n
	Yes		No		
	n	%	n	%	
Root canal					
Positive hemolysin gene	17	100.0	0	0.0	17
Negative hemolysin gene	3	23.1	10	76.9	13
Saliva					
Positive Hemolysin gene	19	100.0	0	0.0	19
Negative Hemolysin gene	2	18.2	9	81.8	11

DISCUSSION

The identification of *Enterococcus faecalis* bacteria was performed using chromatogenic agar as medium. This medium was used for positive Gram bacteria. A more distinctive color display showed bacteria colonies in chromatogenic agar, greenish blue color colonies suspected as *Enterococcus faecalis* or *Enterococcus faecium*. PCR 16sRNA was used to confirm the *Enterococcus faecium* colonies.²⁰ The results showed that 85% of the samples were *Enterococcus faecalis* colonies and the remaining 15% were not. This suggests the possibility of other types of bacteria isolated in the samples and were not studied further.

The proportion of hemolysin gene in isolated root canal was 56.7%, smaller than the proportion of saliva which was 63.3%, there were no significant difference ($p > 0.05$). In this study, cytolysin A primers were used to detect the presence of hemolysin gene, while hemolysin gene can detect using cytolysin A, B or M primers. The production and activation of hemolysin has several stages. Lysis Precursor factors (CylL) synthesized in ribosome, modified and undergo a process of translation by CylM, secreted from the cell by CylB, then activated by CylA.¹⁵ CylA primers selection was based on the final stage. Hemolysin gene was discovered more in saliva than in the root canal, although not significant, due to the activation of extracellular stage of hemolysin in saliva, while in root canal was still in the initial stage where bacteria grow. One primers was used in this test (CylA), with only CylA might cause other types of hemolysin gene undetected.

The results of this study found that not all of the samples had hemolysin gene. A total of 43% of root canal samples and 36.7% saliva samples showed negative hemolysin gene. The literature showed that most of the strains of *Enterococcus faecalis* were non-haemolytic.^{7,9} Several studies supported the finding:¹⁸ Nevertheless, primers of all hemolysin phases should be used to ensure complete detection of all stages in the formation of hemolysin gene.

Hemolysin expression of root canal samples was less than saliva samples, this could be caused by silent Cyl genes; hemolytic activity on blood agar was not likely to

happen because of environmental factors. The infected surrounding environment would be activated during the expression. It found that 6 of the 31 samples had CylA *Enterococcus faecalis* gene but none expressed hemolysin activity.¹⁷ Because of the small percentage, the role of this protein as a virulence factor considered very small. However, recent findings indicated that negative phenotype profile could be activated due to environmental factors to finally express hemolysin.

Researches using animal models demonstrated that Hemolysin as an important virulence factor. Antibiotic and corticosteroid combinations of treatment showed effective results in non-hemolysin strain cases, and has the effect to decrease tissue damage in endophthalmitis cases, while not useful in the case of strains that produce hemolysin.¹⁵

It was found that positive hemolysin gene indicates expression of hemolysin (100%) in both isolated clinical sources (root canal and saliva). However, there were samples that showed hemolysin expression without hemolysin gene existed. In root canal samples, the proportion of hemolysin expression with negative hemolysin gene was 23.1%, and 18.2% in the saliva samples. Only CylA was used in this study, this might showed negative result but perhaps other types of hemolysin gene was involved.

This research showed that hemolysin is one of the virulence factor of *Enterococcus faecalis* isolated from saliva and root canal. All samples detected hemolysin gene indicates expression of hemolysin. On the other hand, there were samples that had hemolysin expression without hemolysin gene. It can occur because the research used only one primers, in fact, there are three stage of formation hemolysin gene. Highly suggestion to use all of the kind of primers to detect all of the hemolysin gene for the further researches.

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