Association between *Bifidobacterium, Fusobacterium nucleatum* and Type of Root Canal Infections in Primary Teeth and Clinical Symptom: A Quantitative Real-Time PCR Analysis

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Abstract

Objective: To quantify levels of *Bifidobacterium* and *Fusobacterium nucleatum* in two types of primary teeth root canal which are irreversible pulpitis and pulp necrosis and to analyze the association between these bacteria, clinical symptoms and radiographic findings.

Materials and Methods: Subjects were chosen from patients aged 2-10 years old who came to the Pediatric Dental Clinic, Faculty of Dentistry, Mahidol University and needed pulpectomy treatment for this cross-sectional study. Pulpal diagnosis based on the American Academy of Pediatric Dentistry. Recorded clinical signs and symptoms including pre-operative radiographs before treatment. Collected fluid inside root canals using paper points by aseptic technique. Performed DNA extraction and quantitative real-time PCR using fluorescent dye (SYBR green) using specific primers to identified *Bifidobacterium* and *F. nucleatum.*

Results: Total of 134 primary teeth was selected. Subjects consisted of 70 males (52%) and 64 females (48%). Mean (± standard deviation) age was 5.25 ± 1.37 years old. Sixty eight samples were diagnosed with irreversible pulpitis (51%) and 66 with pulp necrosis (49%). Amounts of total bacteria ($p \leq 0.001$), *F. nucleatum* ($p = 0.025$) and *Bifidobacterium* ($p = 0.183$) in the pulp necrosis group were higher than in the irreversible pulpitis group. The ratio of *Bifidobacterium* to total bacteria was higher in irreversible pulpitis group ($p = 0.016$). There was a correlation between levels of *F. nucleatum* and swelling at gingiva area present clinically.

Conclusion: Levels of total bacteria and *F. nucleatum* were significantly higher in pulp necrosis group. *F. nucleatum* was correlated with swelling at gingiva area present clinically.

Keywords: Root canal infection, Pulpectomy, Primary teeth, Real-Time PCR, *F. nucleatum, Bifidobacterium*

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Introduction

Early childhood caries (ECC) is an advanced progressive demineralization of the tooth in children younger than 6 years old due to the imbalance or homeostasis of dental plaque or biofilm is disrupted and pathological microorganisms start prevailing and eventually overgrowth the healthy microorganisms resulting in demineralization to develop (1,2). It is one of the most prevalent biofilm-dependent diseases in childhood which cause pain and infection in preschool children, result in extensive carious lesions and destruction of primary teeth and reduce quality of life (1).

Dental biofilm on occlusal surfaces of primary teeth is associated with progression of carious lesions (3). When dental caries progresses deeper, bacteria which located in deep carious lesion of biofilms are directly involved in inducing damage and consequential infect dental pulp tissue. Eventually, the microorganisms that initially occupy the pulp chamber and root canal lumen invade the entire root canal system (3). Root canal infection is a common consequence of dental caries. Without proper treatment, pulpitis becomes irreversible and finally turn into pulp necrosis (4). Studies have identified bacteria isolated from advanced carious lesions and dental pulp after caries exposure in primary teeth. Their results showed that the microbiota of the carious exposed pulp and irreversible pulpitis were similar to those of deep carious lesions (5-8). The dominant bacteria detected in pulpitis were *S. mutans* and *Bifidobacterium, Fusobacterium nucleatum*, and *Veillonella* (5, 8).

Bifidobacterium is anaerobic, gram-positive, rod-shaped and one of the probiotic bacteria detected in the gastrointestinal tracts of humans and animals (9). It is acidogenic and able to produce a final pH below 4.2 that causes extensive demineralization of tooth surfaces (10). Recent finding showed that *Bifidobacterium* are present in human oral cavities and associated with early childhood caries (ECC) (11-14). A previous study in Thai children reported that *Bifidobacterium* levels were significantly higher in the supra gingival plaque of ECC children when compared with caries-free children (11). Interestingly, a study previously found that *Bifidobacterium* was detected higher in dental pulp than in the carious lesions of severe early childhood caries (S-ECC) children (5).

Fusobacterium nucleatum is gram-negative, rod-shaped, non-spore-forming, non-motile, obligate anaerobic bacteria that colonise in the oral cavity (15). It has been isolated from primary endodontic infections in permanent teeth from adults and it is the most prevalent species found in root canal infections (16-18). Previous studies reported that *F. nucleatum* is associated with the clinical condition and reflects the persistent instance of endodontic infection in primary teeth (19,20). It is predominant in teeth with apical abscesses and related to the degree of patient pain (20). Studies showed that *F. nucleatum* was found at a high prevalence (97%) in pulp necrosis of the primary teeth (21,22). However, there is still limited knowledge of bacteria involved in pulp infections in primary teeth especially in Thai children. Further study to quantitatively identify root canal infection related bacteria would be beneficial and help to more understanding the role of bacteria in pulp infections in the root canals of primary teeth.

Quantitative real-time PCR provides an accurate result and is a sensitive method for the detection and quantification of bacterial species (23). This study aimed to quantitatively identify *Bifidobacterium* and *F. nucleatum* in two groups of infected root canals of primary teeth between teeth diagnosed as irreversible pulpitis and pulp necrosis in Thai children using real-time PCR, and analyze the relationship between these bacteria, clinical signs and symptoms and radiographic findings. The hypothesis is that the levels of *Bifidobacterium* and *F. nucleatum* in irreversible pulpitis and pulp necrosis groups should be different.

Materials and Methods

This cross-sectional study protocol was approved by the Ethical Institutional Review Board, Faculty of Dentistry and the Faculty of Pharmacy, Mahidol University (COA. 2018/ 009.2301).

Subject selection

Based on previous studies with $\alpha = 0.05$ and power of 80%, using the software package Primer of Biostatistics (McGraw-Hill, NY, USA) (9). Sample size calculations determined that a minimum of 69 children in each group was enough to achieve statistical difference (9). A Total of 134 primary molar teeth from 138 Thai children aged 2 to 10 years old were selected in the study. All subjects were chosen from patients who came to the pediatric dental clinic, Faculty of Dentistry, Mahidol University, Bangkok, Thailand and needed pulpectomy treatment. Consent forms were signed. Sixty eight samples were diagnosed with irreversible pulpitis and 66 with pulp necrosis.

Clinical examination, inclusion and exclusion criteria

All subjects had normal physical growth, no systemic disease and cooperated during dental treatment. A clinical examination was performed by 2 Pediatric dental residents. They were calibrated for clinical examination (kappa co-efficiency = 0.80). Oral examination was performed following the American Academy of Pediatric Dentistry (AAPD) guideline. The diagnosis of a pulpal and periapical condition was based on the AAPD guideline (24). Clinical signs and symptoms of infected primary teeth included pain history, swelling and pathologic mobility (grade I, II). Recorded the presence of abscess or sinus tract, the presence or absence of tenderness to percussion and tooth mobility. The roots should exhibit minimal or no resorption. For the diagnosis, tooth that has; 1. History of pain; Intense, lingering pain to temperature changes, spontaneous pain, diffuse or referred pain 2. Clinical examination; deep caries, response to thermal stimuli, hypersensitive to cold, excessive hemorrhage that is not controlled with a damp cotton pellet applied for several minutes 3. Radiographic examination; no evidence found of osseous changes was diagnosed as Irreversible pulpitis. For tooth that has; 1. History of pain; a few months ago, or no history of pain 2. Clinical examination; deep caries that can be found on pulpal exposure, no response to thermal stimuli, pain on percussion if PDL (periodontal ligament) around apical region is inflamed 3. Radiographic examination; radiographic change and periapical lesions can be found was diagnosed as Pulp necrosis. Pre-operative radiographs were taken before pulpectomy treatment in order to assess furcation involvement or periapical radiolucency,

pathologic external root resorption and internal root resorption. If the tooth was unrestorable, or root resorption was more than 2/3 of root length, or the degree of tooth mobility was more than grade II, or showed a significant gingival recession or periodontal pockets deeper than 4 mm, they were excluded. Subjects who had any systemic disease(s), taking any kind of antibiotics, had professional fluoride application or any dental treatment within 2 months prior to the sample collection period were excluded.

Sample collection

Samples were collected using a strictly asepsis technique (25). The tooth was cleaned with pumice and isolated with a rubber dam and the surrounding field was sterilized with iodine solution (Italmar (Thailand) Co., Ltd). A sterile bur with sterile normal saline for the coolant was used to access the root canal. A #15 K-file (Maillefer, Ballaigues, Switzerland) was inserted to a level approximately 1 mm short of the tooth apex, and a discrete filing motion was applied. Afterward, two sequential paper points (Denjoy Dental Co., Ltd, P.R. China) were placed at the same level and left in the wet canal for 60 seconds in order to soak up the fluid in the canal. Then, the paper points were transferred to tubes containing 1.0 ml of TE buffer. All samples were immediately transported to the Oral Biology Laboratory (Department of Oral Microbiology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand) on ice and stored at -20°C until the DNA extraction process.

DNA extraction

DNA was extracted based on enzymatic lysis using a commercial kit (Flavogen, Pingtung, Taiwan) as previously described (11). The extracted DNA concentration and purity was measured using a spectrophotometer at 260 nm/280 nm (Nanodrop 2000C Thermo Scientific, Delaware, USA).

Culture condition and standard strains

Two bacterial strains were used as standard strains. *Bifidobacterium longum* (subspecies 51139) was purchased from BIOTEC (National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand) and cultured on BL agar. *F. nucleatum* (ATCC 25586) was cultured on Brain Heart Infusion agar. Both strains were incubated at 37°C for 24-48 hours in anaerobic conditions (5% CO2). Genomic DNA was extracted from the overnight culture as described above. A ten-fold serial dilution, starting from 10^8-10^2 CFU/ml, was performed.

Conventional PCR

All extracted DNA samples were confirmed with 16srRNA universal primers (Table 1). Conventional PCR was performed as previously described (11). Thermocycle (GeneAmp PCR System 9600 PCR machine, PerkinElmer, CA, USA) was set at 45 cycles. The procedure started with preheating at 95°C for 10 minutes. Each cycle consisted of a denaturing step at 95°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 30 seconds, and incubation for an additional extension at 72°C for 10 minutes.

Quantitative Real-time PCR

Using specific primers (Table 1), the reaction mixture (total volume of 20μl) contained 8.2μl of water, 10μl of 2X KAPA SYBR® FAST qPCR Master Mix, 0.4μl of 10 μM forward and reverse primer, and 1μl of standard bacteria DNA. The thermocycler (C1000™ Thermal cycler and CFX 96 Real-time System) was set for 40 cycles. Each cycle consisted of enzyme activation at 95°C for 3 minutes, denaturing at 95°C for 3 seconds, annealing for 20 seconds for *Bifidobacterium* and *F.nucleatum,* respectively. Melting curves were generated from 60°C to 95°C and read every 0.5°C for 5 seconds (11).

Agarose gel electrophoresis

Stained 2% agarose gel (UltraPure Agarose, ThermoFisher Scientific, USA) with ethidium bromide and direct visualized PCR products.

Statistical Analysis

All data were recorded and analyzed using SPSS 23.0 software (Microsoft Corporation, USA). Data distribution was tested using Kolmogorov-Smirnov ($p < 0.001$). The different amounts of two bacteria between two groups using a Mann-Whitney U test for non-parametric data ($p < 0.05$) were analyzed. Analysis for the correlation between the amount of each bacterium, clinical signs and symptoms and radiographic finding using Spearman's correlation test ($p < 0.05$) was carried out.

Results

A total of 134 primary teeth were included in this study. Subjects consisted of 70 males (52%) and 64 females (48%). Mean (± standard deviation) age of the children was 5.25 ± 1.37 years old. Sixty eight samples were diagnosed with irreversible pulpitis (51%) and 66 with pulp necrosis (49%).

There was a 100% (134/134) detection rate using the 16srRNA universal primers. Both *Bifidobacterium* and *F. nucleatum* were detected at 99% (133/134). Mean levels (± standard deviation) of total bacteria, *Bifidobacterium* and *F. nucleatum* in irreversible pulpitis group were $(3.47x10^{5)}\pm(14.20x10^{5)}$,$(1.11x10^{4)}\pm(5.26x10^{4)}$, $(0.54x10^{4)}\pm(22.23x10^{4)}$,respectively. Mean levels (±standard deviation) of total bacteria, *Bifidobacterium* and *F. nucleatum* in pulp necrosis group were $(2.06x10^{6)}\pm(3.68x10^{6)}$,$(9.47x10^{4)}\pm(50.20x10^{4)}$, $(2.03x10^{4)}\pm (4.74x10^{4)}$, respectively. There were a significant difference of levels of total bacteria ($p = 0.001$) and *F. nucleatum* ($p = 0.025$) between two groups (Table 2). The ratio of *Bifidobacterium* to total bacteria in irreversible pulpitis and Pulp necrosis groups were $(1.11x10^{4)}\pm(5.26x10^{4)}/(3.47x10^{5)}\pm(14.20x10^{5}$and $(9.47x10^{4)}\pm(50.2x10^{4)}/(2.06x10^{6)}\pm(3.68x10^{6)}$, respectively. The ratio of *F. nucleatum* to total bacteria in irreversible pulpitis and Pulp necrosis groups were $(0.54x10^{4})\pm(22.23x10^{4})/(3.47x10^{5)}\pm(14.20x10^{5})$and $(2.03x10^{4})\pm(4.74x10^{4})/(2.06x10^{6})\pm(3.68x10^{6})$, respectively. The ratio of *Bifidobacterium* to total bacteria in the irreversible pulpitis group was significantly higher than in the pulp necrosis group ($p = 0.016$) (Table 3). There was no correlation between the levels of total bacteria and any clinical signs and symptoms (Table 4). Likewise,

there was no correlation between the levels of *Bifidobacterium* and any clinical signs and symptoms (Table 5). However, there was a correlation between level of *F. nucleatum* and swelling clinically ($p = 0.04$) (Table 6). Radiographic evaluation in both groups included discontinuity of lamina dura, widening periodontal ligament (PDL) space, periapical radiolucency, furcation involvement, root resorption and involvement of permanent tooth bud. There was no correlation between levels of total bacteria, *Bifidobacterium* and *F. nucleatum,* nor any radiographic signs.

Table 1. Primers used in this study.

Primers		Nucleotide sequence 5' to 3'	Expected amplicon (bp)	Annealing Temp (°c)	References
Universal BAC16S	Forward primer	5'-TGG AGC ATG TGG TTT AAT TCG A-3′	160	52	Sinsimer et al., 2005 (39)
	Reverse primer	5'-TGC GGG ACT TAA CCC AAC A-3′			
Fusobacterium nucleatum	Forward primer	F 5'-CGC CCG TCA CAC CAC GAG A-3'	75	60	Amman et al., 2013 (40)
	Reverse primer	5′-ACA CCC TCG GAA CAT CCC TCC TTA C-3′			
Bifidobacterium	Forward primer	5'-CTC CTG GAA ACG GGT GG-3'	550	55	Matsuki et al., 2004 (41)
	Reverse primer	5'-GGT GTT CTT CCC GAT ATC TAC A-3'			

Table 2. Bacteria levels between 2 groups.

Bacteria	Group	Subject (n)	Bacteria levels (cells/ml) Mean	p-value[1]
Total bacteria	Irreversible pulpitis	68	$3.47 \times 10^5 \pm 14.20 \times 10^5$	0.001*
	Pulp necrosis	66	$2.06 \times 10^6 \pm 3.68 \times 10^6$	
Bifidobacterium	Irreversible pulpitis	68	$1.11 \times 10^4 \pm 5.26 \times 10^4$	0.183
	Pulp necrosis	66	$9.47 \times 10^4 \pm 50.20 \times 10^4$	
Fusobacterium nucleatum	Irreversible pulpitis	68	$0.54 \times 10^4 \pm 22.23 \times 10^4$	0.025*
	Pulp necrosis	66	$2.03 \times 10^4 \pm 4.74 \times 10^4$	

[1]Mann-Whitney U test. * p-value < 0.05

Table 3. The ratio of *Bifidobacterium* and *Fusobacterium nucleatum* to total bacteria between 2 groups.

The ratio of bacteria		Bacteria levels (cells/ml)	Subject (n) (%)	p-value[1]
Bacteria	**Group**	**Mean**		
Bifidobacterium to total bacteria	Irreversible pulpitis	$1.11 \times 10^4 \pm 5.26 \times 10^4$/ $3.47 \times 10^5 \pm 14.20 \times 10^5$	68 (16%)	0.016*
	Pulp necrosis	$9.47 \times 10^4 \pm 50.2 \times 10^4$/ $2.06 \times 10^6 \pm 3.68 \times 10^6$	66 (11%)	
Fusobacterium nucleatum to total bacteria	Irreversible pulpitis	$0.54 \times 10^4 \pm 22.23 \times 10^4$/ $3.47 \times 10^5 \pm 14.20 \times 10^5$	68 (6%)	0.689
	Pulp necrosis	$2.03 \times 10^4 \pm 4.74 \times 10^4$/ $2.06 \times 10^6 \pm 3.68 \times 10^6$	66 (5%)	

[1]Mann-Whitney U test. * p-value < 0.05

Table 4. Association between level of total bacteria and clinical signs and symptoms.

Clinical signs and symptoms	N	Median (P_{25}, P_{75})	p-value[1]
History of pain			
Yes	115	5.84×10^4(0.93×10^4, 120.23×10^4)	0.175
No	19	2.08×10^4 (0.62×10^4, 9.03×10^4)	
Pain from mastication			
Yes	84	4.9×10^4 (0.86×10^4, 104.8×10^4)	0.872
No	50	3.42×10^4 (0.98×10^4, 76.53×10^4)	
Pain from percussion			
Yes	46	5.80×10^4 (0.94×10^4, 56.3×104^4)	0.732
No	88	4.11×10^4 (0.82×10^4, 100.04×10^4)	
Pain from palpation			
Yes	41	3.41×10^4 (0.64×10^4, 81.82×10^4)	0.628
No	93	5.84×10^4 (0.86×10^4, 79.87×10^4)	
Sensitivity			
Yes	9	0.94×10^4 (0.30×10^4, 5.95×10^4)	0.075
No	125	5.35×10^4 (0.97×10^4, 106.56×10^4)	
Swelling			
Yes	15	9.47×10^4 (2.02×10^4, 515.23×10^4)	0.094
No	119	3.5×10^4 (0.64×10^4, 58.49×10^4)	

Clinical signs and symptoms	N	Median (P_{25}, P_{75})	p-value[1]
Sinus tract			
Yes	4	17.01 X 10^4 (2.7 X 10^4, 392.56 X 10^4)	0.456
No	130	4.11 X 10^4 (0.91 X 10^4, 76.53 X 10^4)	
Tooth mobility			
Yes	21	17.57 X 10^4 (1.50 X 10^4, 153.89 X 10^4)	0.264
No	113	3.79 X 10^4 (0.83 X 10^4, 46.16 X 10^4)	

[1]Mann-Whitney U test. * p-value < 0.05

Table 5. Association between level of *Bifidobacterium* and clinical signs and symptoms.

Clinical signs and symptoms	N	Median	p-value[1]
History of pain			
Yes	115	677 (162, 5199)	0.106
No	19	263 (72, 1362)	
Pain from mastication			
Yes	84	440 (120, 4460)	0.370
No	50	754 (224, 5352)	
Pain from percussion			
Yes	46	565 (154, 8949)	0.530
No	88	620 (110, 3744)	
Pain from palpation			
Yes	41	478 (147, 5528)	0.815
No	93	706 (129, 4632)	
Sensitivity			
Yes	9	556 (135, 4796)	0.919
No	125	669 (147, 4837)	
Swelling			
Yes	15	1004 (677, 23564)	0.130
No	119	502 (123, 4276)	
Sinus tract			
Yes	4	4282 (1109, 224108)	0.219
No	130	563 (139, 4763)	
Tooth mobility			
Yes	21	320 (75, 5528)	0.468
No	113	627 (168, 4632)	

[1]Mann-Whitney U test. * p-value < 0.05

Table 6. Association between level of *F. nucleatum* and clinical signs and symptoms.

Clinical signs and symptoms	N	Median	p-value[1]
History of pain			
Yes	115	405 (26, 2766)	0.247
No	19	163 (9, 1328)	
Pain from mastication			
Yes	84	335 (25, 1941)	0.765
No	50	332 (24, 2660)	
Pain from percussion			
Yes	46	507 (27, 2896)	0.613
No	88	238 (22, 1941)	
Pain from palpation			
Yes	41	163 (26, 1071)	0.307
No	93	422 (23, 4308)	
Sensitivity			
Yes	9	297 (0.99, 1888)	0.348
No	125	355 (25, 2031)	
Swelling			
Yes	15	740 (297, 5721)	0.040*
No	119	229 (21, 1876)	
Sinus tract			
Yes	4	5008 (510, 29115)	0.123
No	130	270 (24, 1898)	
Tooth mobility			
Yes	21	405 (109, 1155)	0.495
No	113	242 (21, 2432)	

[1]Mann-Whitney U test. * p-value < 0.05

Discussion

Results from this study demonstrated that the total bacterial level from the pulp necrosis group was significantly higher than the irreversible pulpitis group. Similar to previous study which showed that the number of bacterial cells included anaerobic and facultative microorganisms were higher in the pulp necrosis group than in the irreversible pulpitis group (26). This was the first quantitative analysis of *Bifidobacterium* in infected root canals in primary teeth in Thai children. Most of previous studies were done to analyze the association between this bacteria and advanced dental caries. Previous studies have suggested that bacteria located in advanced dental caries are directly involved in inducing damage and consequent inflammation in the pulp tissue, and *Bifidobacterium* is one of those bacteria that are involved in pulpal inflammation and initiate endodontic infection (5,7,27). Previous study reported the bacteria associated with advanced dental caries in adult permanent teeth were *Lactobacillus, Prevotella, Fusobacterium,* and *Bifidobacterium* (6). Another previous study demonstrated bacteria involving in severe dental caries in primary and permanent teeth in children and young adults and the results reported high level of *Bifidobacterium* in deep dentin caries (8). *Bifidobacterium* was not only detected in deep carious lesions but also in root canals infection in permanent teeth (28,29). In addition, it was found in the primary teeth with necrotic pulps in children aged 4-7 years old together with *Streptococcus intermedius* (30). In this study, a higher level of *Bifidobacterium* was found in the pulp necrosis group than in the irreversible pulpitis group. Even though it was not significantly different, it may imply that *Bifidobacterium* is definitely involved in infected root canals in primary teeth. In addition, in this study, results showed that the ratio of *Bifidobacterium* to total bacteria in the irreversible pulpitis group was significantly higher than in the pulp necrosis group. From previous study that collected samples from pulp diagnosed apical periodontitis from permanent teeth showed that *Actinomyces, Bifidobacterium,* four different *Lactobacillus, Propionibacterium,* and *Streptococcus* were mainly detected (31). *Bifidobacterium* were shown to have similar acidogenicity and aciduricity to *S. mutans* and the ability to produce an acidic environment, to resist low pH and to promote biofilm formation when co-adhered with primary colonizers (10). Haukioja and colleagues reported that *Bifidobacterium* did not bind to saliva-coated hydroxyapatite, but bound well to *F. nucleatum*-coated surfaces, indicating the importance of other oral bacteria in modulating the colonization potential of the strains (32). This might be one of the reasons that the detection of *Bifidobacterium* was in the same direction as *F. nucleatum.* However, this is the first study that quantitatively detected *Bifidobacterium* level in pulp infection of primary teeth, it is difficult to compare results with previous study due to the limitation of this kind of study. Further study is recommended to confirm the role of *Bifidobacterium* in root canal infection of primary teeth.

In this study, a high prevalence of *F. nucleatum* was found (99%), which is in agreement with previous studies (18,21,22). Previous study reported that in pulp necrosis with periradicular lesions from primary teeth, the most prevalent bacteria was *F. nucleatum* (100%) (18). Another study investigated the microbial composition of 30 infected root canals in primary

teeth, their results showed that *F. nucleatum* was the most prevalent bacteria (97%) (21). However, some studies showed a lower detection rate of *F. nucleatum.* Fabris and colleagues investigated primary teeth with 103 necrotic pulp (n = 103) and fistula presented at gingiva area (n = 7) using the PCR technique, their results showed that *F. nucleatum* was detected at 25% (22). Another study using Real-Time PCR technique to evaluate microbiota in primary endodontic infections in permanent teeth. Their results showed that *F. nucleatum* was detected at 27% (33). In this study, *F. nucleatum* levels in the irreversible pulpitis group and the pulp necrosis group were $0.54x10^4 \pm 22.23x10^4$ and $2.03 \times 10^4 \pm 4.74 \times 10^4$, respectively. *F. nucleatum* was detected significantly higher in the pulp necrosis group when compared to the irreversible pulpitis group. Our result was different from the previous study that was found mean level of *F. nucleatum* were 15.38×10^5 and 5.59×10^5 in the irreversible pulpitis group and pulp necrosis with sinus tract group in permanent teeth, respectively (34). Their results showed the higher levels of *F. nucleatum* in the irreversible pulpitis group. Another study investigated amount of *F. nucleatum* in pulpitis, they reported the number of 4.2×10^5 (35). A different detection rate among studies might be from the different guideline of pulp diagnosis between permanent and primary teeth and the technique used to identify bacteria. In this study, there was a correlation between levels of *F. nucleatum* and swelling at the gingiva area clinically. Similarly, several studies previously revealed that *F. nucleatum* has been associated with clinical symptoms. In permanent teeth, *F. nucleatum* was reported to relate with a history of pain, tenderness to percussion, gingiva swelling, fistula, purulent exudate, and periapical radiolucency (36,37). In addition, some studies in primary teeth showed that *F. nucleatum* was detected higher in teeth that were tender to percussion and where mobility was present (25,37). Another study obtained samples from 30 teeth in children with both primary and permanent dentitions found a relationship between *F. nucleatum* and hemorrhagic exudate, purulent exudate, and periapical radiolucency (21). *F. nucleatum* is a gram-negative bacteria. Previous studies had reported that gram-negative bacteria cell wall containing endotoxin which can stimulate the release of bradykinin. It is a pain mediator that associates with acute symptoms such as pain (38). The apical part of root canal has low oxygen tension and large availability of proteins and glycoproteins which contributes to anaerobic bacteria establishment. Most of them are strictly anaerobic species, such as *F. nucleatum, Porphyromonas endodontalis, Tannerella forsythia* and *Treponema denticola* (38). This study might not directly useful in the clinical outcome. However, bacterial infection is the cause of caries and pulp infection. Knowledge regarding bacteria involving the pulp infection process especially in primary teeth is very limited. This study might fill some gap that missing in the information regarding role of bacteria in pulp infection in primary teeth.

In conclusion, total bacteria and levels of *F. nucleatum* in the pulp necrosis group were significantly higher than in the irreversible pulpitis group. The ratio of *Bifidobacterium* to total bacteria in the irreversible pulpitis group was significant higher than in the pulp necrosis group. There was a correlation between levels of *F. nucleatum* and gingiva swelling clinically.

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