

Exploring difference in subgingival microbial communities in dog and human periodontal diseases using DGGE technique

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Abstract

Periodontal diseases are the most common infectious diseases of dogs. Up to now, the knowledge about periodontopathogenic bacteria in dogs remains sparse. The objectives of this study were to apply the Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) technique, cloning and 16S rRNA gene sequencing to the investigation into subgingival plaque bacteria from healthy and periodontitis dogs; and compare DGGE patterns from the subgingival plaque of dogs and humans. Sixty-eight bacterial species were detected from subgingival plaque of 12 Poodles in Thailand and the predominant genus was *Porphyromonas* (n=11). *Porphyromonas gulae* and *Treponema denticola* were the most common microflora in the periodontitis dog group. In addition, DGGE band patterns from the dogs' subgingival plaque were compared with those of humans' subgingival plaque. The DGGE results showed different bacterial community structures between the two species, which may influence pathogenesis. Therefore, because the pathogenesis of periodontal disease in dogs may not be the same as in human, employing dog as an animal model for studying this disease in human should be carefully analyzed.

Keywords: DGGE, dogs, periodontal diseases, periodontitis, subgingival plaque

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Introduction

Periodontal diseases are the most prevalent oral diseases in dogs (Harvey et al., 1995). They are multi-factorial diseases that can cause various types of discomfort such as anorexia due to pain, teeth and bone loss, and more importantly maxillary or mandibular bone fracture (Carvalho et al., 2015). Data about the pathogenesis of periodontal diseases in dogs are rather limited and knowledge is mainly obtained from extrapolation research of human studies. Bacteria play a major role in the etiology of periodontal diseases and previous studies have revealed various anaerobic bacteria found in the subgingival plaque of dogs (Dahlén et al., 2012). Previous studies also showed some similarities among the general characteristics of subgingival bacteria in dogs and humans (Hardham et al., 2005; Yamasaki et al., 2012). However, there are distinct differences in the oral microflora between animal species (Elliott et al., 2005; Oh et al., 2015; Rober et al., 2008) and information about the bacterial pathogens of periodontal diseases among dogs is still limited. Molecular techniques have been developed to detect unculturable bacteria and several studies have reported the use of denaturing gradient gel electrophoresis (DGGE) for monitoring and identifying microbial communities in complex environments (Muyzer et al., 1993; Petersen et al., 2007). DGGE can be used not only to evaluate bacterial diversity, but also to monitor changes in the community structure (Zijngje et al., 2003). It may also be useful for detecting unidentified, unculturable or hard-to-culture bacteria in the subgingival plaque. The results of this technique show electrophoretic band patterns, in which the number of bands suggests the number of predominant species, with each band representing a different species (Muyzer and Smalla, 1998). Since information on the periodontal diseases of dogs remains unclear, the purposes of this study were to apply the PCR-DGGE technique to the investigation into bacterial communities in the subgingival plaque of dogs with and without periodontal diseases; and to compare DGGE patterns between the subgingival plaque of dogs and humans, with and without periodontal diseases.

Materials and Methods

Sample collection: Plaque samples were obtained from 12 poodles (3-8 years old) at the Veterinary Teaching Hospital of Mahidol University according to the guidelines approved by the Faculty of Veterinary Science-Animal Care and Use Committee (FVS-ACUC) (Protocol No. MUVS-2015-19). All dogs were presented to the veterinary hospital for dental care and neutering. None of the dogs had received antibiotics 3 months before sample collection and suffered from systemic diseases. Physical examination, complete blood count and blood chemistry (creatinine, blood urea nitrogen, alanine aminotransferase and aspartate aminotransferase) were performed in each dog. Dogs were excluded from the study if any disorder was present. Subgingival plaque samples were collected from 6 healthy (clinically healthy, probing depth < 3 mm and no signs of gingival inflammation) and 6 periodontitis (probing depth > 5 mm and attachment

loss) dogs. For samples from human, subgingival samples were taken from 10 volunteers from the Dental Hospital, Faculty of Dentistry, Mahidol University. Ages of the subjects ranged from 20 to 65 years. Five patients had periodontitis (probing depth > 3 mm and attachment loss) and the remainder showed no signs of periodontitis (probing depth < 3 mm and no attachment loss). The study protocol was approved by the Ethics Committee of the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University (COE.No.MU-DT/PY-IRB 2015/039.2812). Plaque was taken with a sterile curette introduced below the gingival margin from gingival sites or periodontal pockets. All samples were placed into individual sterile Eppendorf tubes containing 300 µL nuclease-free water and kept at -80°C until used.

Bacterial DNA extraction: Bacterial DNA was extracted from reference strains and the subgingival plaque samples by QIAamp® DNA Mini Kit (Qiagen, USA) according to the manufacturer's instructions. This DNA was used as template DNA in PCR.

The reference bacteria namely *Aggregatibacter actinomycetemcomitans* (ATCC 43717), *Fusobacterium nucleatum* (ATCC 25586) and *Porphyromonas gingivalis* (ATCC W50) were obtained from the American Type Culture Collection (ATCC). *F. nucleatum* was kindly provided by Dr. Ratchapin Srisatjaluk, of the Faculty of Dentistry, Mahidol University. *Treponema denticola* and *Tannerella forsythia* DNA were kindly provided by Dr. Oranart Matangkasombut, of the Faculty of Dentistry, Chulalongkorn University. *Porphyromonas gulae* DNA was kindly provided by Dr. Kazuhiko Nakano, of the Graduate School of Dentistry, Osaka University, Japan.

Polymerase chain reaction (PCR): The 16S rDNA was amplified in the PCR by using the universal bacterial primers 27F containing a 40-base pair GC-clamp at the 5' end (5'-CGCCCGCCGCGCGCGGGC GGGGC GGGGGCACGGGGG + AGAGTTTGATCMTGGCT CAG, where M is C or A) and 342R (5'-CTGCTGCSYC CCGTAG, where S is G or C and Y is C or T) as needed for DGGE analysis. The PCR reaction consisted of 50 ng of template DNA, 10 µL of EmeraldAmp® GT PCR Master Mix (Takara Bio Inc., Japan), 0.25 µM of each primer and 0.5 mM of magnesium chloride in a total volume of 100 µL. PCR amplification was performed under the following conditions: initial denaturation at 95°C for 3 min, followed by denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 30 sec (30 cycles), and final extension at 72°C for 3 min. PCR products were determined using 1.5% (W/V) agarose gel electrophoresis and then stained with ethidium bromide.

Denaturing gradient gel electrophoresis (DGGE): DGGE was performed using the Bio-Rad DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). The amplified 16S rDNA from the samples and reference strains were loaded on 6% (W/V) polyacrylamide gel containing a 30% to 70% urea/formamide denaturing gradient. Electrophoresis was performed in 0.5X TAE buffer at a constant voltage of 145 V and a temperature of 60°C for 12 h. After electrophoresis, the gel was stained with ethidium

bromide. Images were obtained by gel doc system (G:Box, Syngene). DGGE band patterns of the healthy and periodontitis groups were compared. Finally, distinct bands were excised. DNA fragments were eluted from the polyacrylamide gel by adding 30 µL deionized water to DGGE gel and incubating at 4°C overnight. The DNA fragments were then re-amplified again using the primers 27F and 342R. PCR products were purified and cloned using InsTAclone PCR cloning kit (Thermo Scientific), and the inserted DNA was sequenced and analyzed by using databases from the National Center for Biotechnology Information (NCBI) server.

Statistical Analysis: Distribution of bacterial species found in the healthy and periodontitis dogs was presented using descriptive statistics.

Results

The DGGE profiles of the amplified 16S rDNA from the subgingival bacteria are shown in Fig. 1. The three marker lanes (M1, M2, M3) contained PCR products of *A. actinomycetemcomitans*, *F. nucleatum*, *T. denticola*, *T. forsythia*, *P. gingivalis*, and *P. gulae* types A, B, and C. In the healthy group, similar electrophoretic band patterns were found, but distinct differences were also detectable. A total of 97 bands were excised from the DGGE gel for identification purposes. The sequencing results from these bands were analyzed

using databases from the National Center for Biotechnology Information (NCBI). The bacterial species present are summarized in Table 1.

When the bacteria from healthy and periodontitis dogs were compared, forty-five bacteria genera, comprising 68 species, were found. Of these, 47 species were identified from the healthy group and 36 species from the periodontitis group. The most abundant bacteria identified in both groups belonged to the genus *Porphyromonas* (*P. cangingivalis*, *P. canoris*, *P. catoniae*, *P. crevioricanis*, *P. gingivicanis*, and *P. gulae*), *Fusobacterium* (*F. canifelinum*, *F. nucleatum*, and *F. periodonticum*) and *Treponema* (*T. denticola*, *T. maltophilum*, *T. medium*, and *T. socranskii*). In addition, three *Porphyromonas* strains were more prevalent in the healthy group than the periodontitis group (*P. cangingivalis*, *P. catoniae*, and *P. gingivicanis*), while 2 strains were more prevalent in the periodontitis group than the healthy group (*P. crevioricanis* and *P. gulae*).

For the healthy group, *P. cangingivalis* and *P. catoniae* were commonly found in the subgingival plaque samples while *P. gulae* and *T. denticola* were commonly found in the periodontitis group. Moreover, as shown in Table 1, *P. catoniae* was identified in 5 of 6 healthy dogs but not in the periodontitis dogs, while *Parvimonas micra* and *Peptostreptococcus canis* were likely to be found in the periodontitis dogs but not in the healthy dogs. *Frederiksenia canicola*, *Enhydrobacter aerosaccus*, and *F. nucleatum* were more prevalent in the healthy dogs than periodontitis dogs.

Table 1 Bacterial species detected in healthy and periodontitis dogs

Bacterial species	Healthy	Periodontitis
<i>Acholeplasma axanthum</i>	+	+
<i>Acinetobacter calcoaceticus</i>	+	-
<i>Acinetobacter johnsonii</i>	+	-
<i>Acinetobacter junii</i>	+++	-
<i>Actinobacillus minor</i>	+	-
<i>Actinomyces coleocanis</i>	+	-
<i>Actinomyces viscosus</i>	+	-
<i>Anaerorhabdus furcosa</i>	+	-
<i>Anaerovorax odorimutans</i>	+	+
<i>Bacteroides coprophilus</i>	+	-
<i>Bacteroides oleiciplenus</i>	+	-
<i>Bacteroides plebeius</i>	+	-
<i>Bacteroides pyogenes</i>	-	+
<i>Bergeyella zoohelcum</i>	+	-
<i>Frederiksenia canicola</i>	++++	-
<i>Brachymonas chironomi</i>	+	-
<i>Campylobacter rectus</i>	+	+
<i>Capnocytophaga canimorsus</i>	+	-
<i>Catabacter hongkongensis</i>	-	+
<i>Christensenella minuta</i>	-	+
<i>Clostridium fimetarium</i>	-	+
<i>Desulfomicrobium baculatum</i>	-	+
<i>Dielma fastidiosa</i>	+	-
<i>Enhydrobacter aerosaccus</i>	++++	+
<i>Eubacterium sulci</i>	+	-
<i>Eubacterium yurii</i>	++	-
<i>Filifactor villosus</i>	-	+
<i>Fretibacterium fastidiosum</i>	+	++
<i>Fusobacterium canifelinum</i>	+	-
<i>Fusobacterium nucleatum</i>	++++	++
<i>Fusobacterium periodonticum</i>	++	++
<i>Gemella palaticanis</i>	+	-
<i>Granulicatella elegans</i>	-	+
<i>Lautropia mirabilis</i>	+	-
<i>Luteococcus sanguinis</i>	+	-
<i>Moraxella osloensis</i>	+	+

<i>Mycoplasma feliminutum</i>	-	+
<i>Neisseria shayeganii</i>	+	-
<i>Neisseria wadsworthii</i>	-	+
<i>Oribacterium asaccharolyticum</i>	+	-
<i>Oribacterium parvum</i>	-	+
<i>Parabacteroides johnsonii</i>	-	+
<i>Paraprevotella xylaniphila</i>	-	+
<i>Parvimonas micra</i>	-	++++
<i>Pasteurella stomatis</i>	-	+
<i>Peptostreptococcus canis</i>	-	++++
<i>Porphyromonas cangingivalis</i>	+++++	++
<i>Porphyromonas canoris</i>	++	++
<i>Porphyromonas catoniae</i>	+++++	-
<i>Porphyromonas crevioricanis</i>	-	+++
<i>Porphyromonas gingivicanis</i>	++	-
<i>Porphyromonas gulae</i>	+++	+++++
<i>Prevotella amnii</i>	+	-
<i>Prevotella bryantii</i>	++	-
<i>Prevotella dentasini</i>	-	+
<i>Prevotella intermedia</i>	-	++
<i>Pseudobutyriovibrio ruminis</i>	+	-
<i>Robinsoniella peoriensis</i>	+	-
<i>Roseburia inulinivorans</i>	++	-
<i>Ruminococcus gnavus</i>	+	-
<i>Sphingomonas jinjuensis</i>	-	+
<i>Streptobacillus moniliformis</i>	+	-
<i>Streptobacillus hongkongensis</i>	+	+++
<i>Tannerella forsythia</i>	+++	+++
<i>Treponema denticola</i>	++	+++++
<i>Treponema maltophilum</i>	+	++
<i>Treponema medium</i>	-	+
<i>Treponema socranskii</i>	-	+

+ : 1 sample, ++ : 2 samples, +++ : 3 samples, ++++ : 4 samples, +++++ : 5 samples

In general, DGGE banding patterns differ greatly between dogs and humans, especially in healthy samples. However, similarity in the banding patterns among the same species in healthy conditions was found in this study (Fig. 2). Variations in

individual DGGE profiles, however, increased in the human periodontitis group as well as in the dog periodontitis group compared with the healthy group from both species, as shown in Fig. 3.

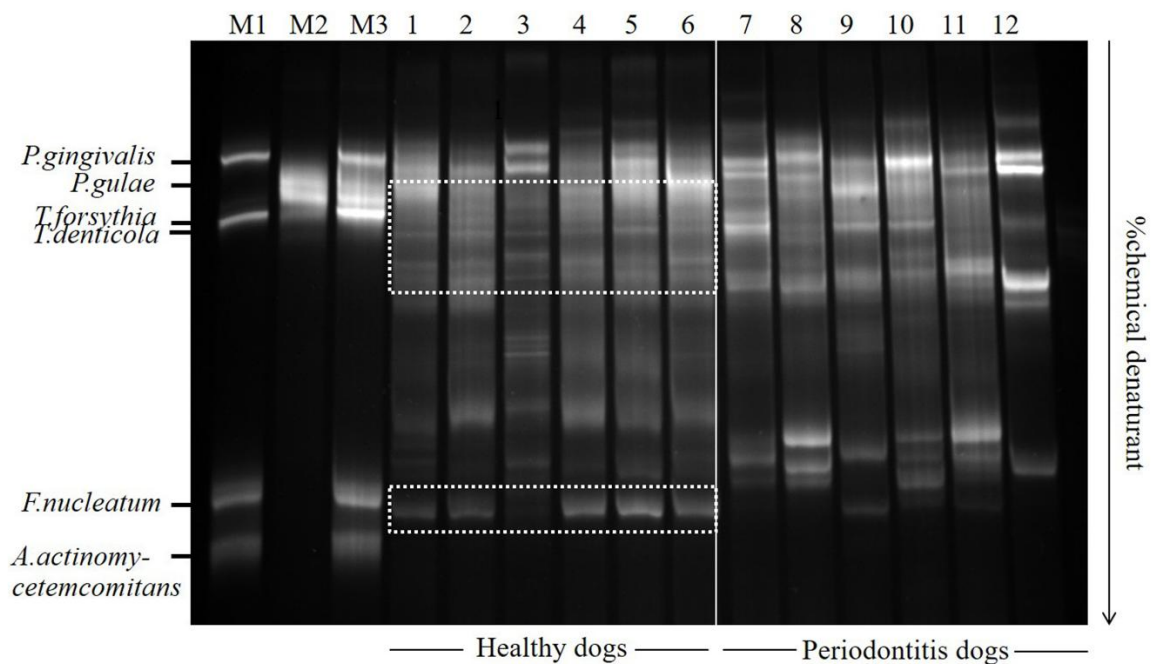


Figure 1 DGGE profile representing bacterial diversity in the subgingival plaque of the dogs. Lane M1: *P. gingivalis*, *T. forsythia*, *F. nucleatum*, *A. actinomycetemcomitans*. Lane M2: *P. gulae*, *T. denticola*. Lane M3: Mixture of reference bacterial species (*A. actinomycetemcomitans*, *F. nucleatum*, *T. denticola*, *T. forsythia*, *P. gingivalis* and *P. gulae*). Lanes 1-6: Healthy dog samples. Lanes 7-12: Periodontitis dog samples. Areas in the dotted rectangles represent the similar band patterns in healthy group.

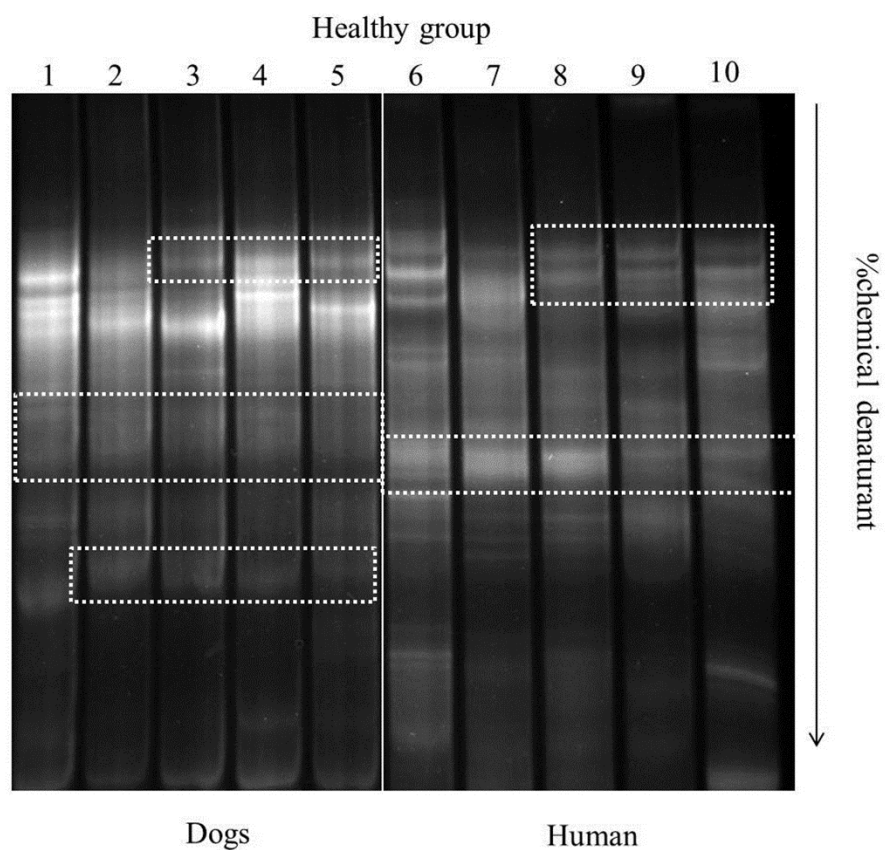


Figure 2 DGGE banding pattern of subgingival plaque taken from healthy dogs and humans. Lanes 1-5: Dog samples. Lanes 6-10: Human samples. Areas in the dotted rectangles represent the similar band patterns.

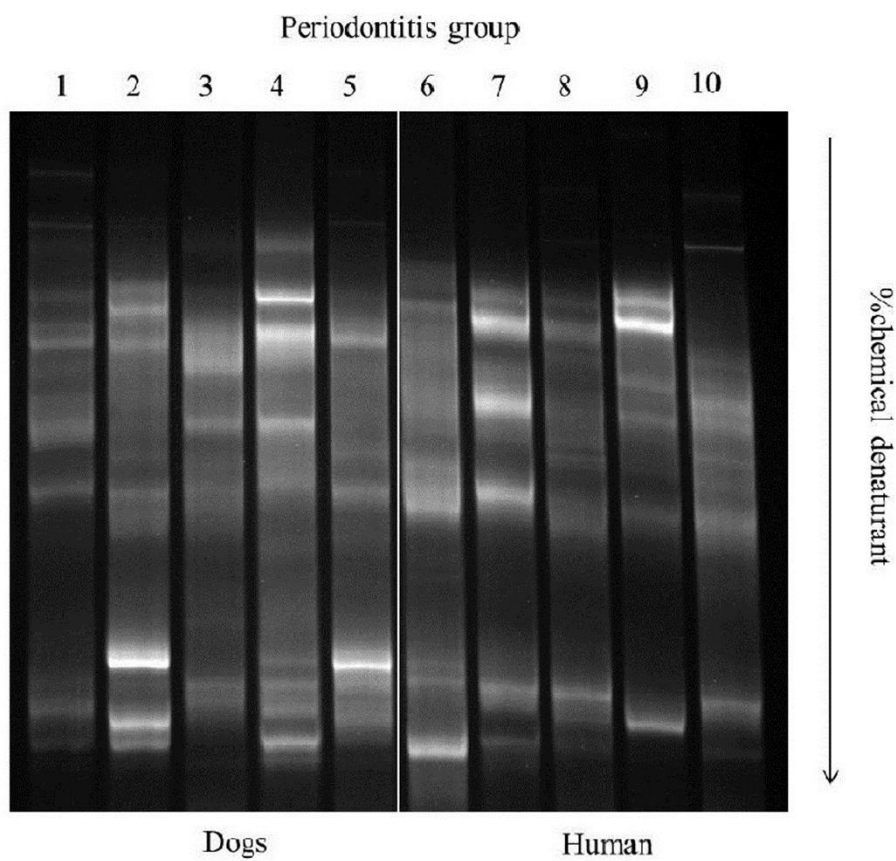


Figure 3 DGGE banding pattern of subgingival plaque taken from periodontitis dogs and humans. Lanes 1-5: Dog samples. Lanes 6-10: Human samples.

Discussion

The advantages of the DGGE technique are its ability to provide the overall image of the microbial community, detect the presence of any amplifiable bacterial sequence above the detection threshold, and identify multiple bacteria in a single sample (Fujimoto et al., 2003; Muyzer and Smalla, 1998). The present study used the DGGE technique to separate bacterial community in subgingival plaque, followed by molecular cloning, and sequencing of bacterial 16S rRNA genes to identify the bacteria associated with dog periodontal diseases. Sixty-eight bacterial species were identified from the subgingival plaque of 12 dogs with and without periodontitis. Of these, 52 species were gram negative and some species had not been previously reported in dog subgingival plaque. By comparing DGGE profiles, different band patterns were observed in the healthy and periodontitis dogs, which may imply that dog periodontal diseases are associated with change in the balance of multiple microbial species rather than a single pathogen. A similar pattern was found among the samples in the healthy group. In contrast, the pattern varied in the periodontitis group. The varied patterns among the samples in the diseased group may result from the different pathogenic factors in the individuals. This study tried to use this technique to evaluate the difference between healthy and periodontitis dogs and it clearly showed that this technique could be useful for screening subgingival microorganism.

As mention earlier that many scientists extrapolated the knowledge of periodontal pathogen and pathogenesis in dogs from human, it is of interest to compare the microorganism in subgingival dental plaque between human and dog. There are three main species of periodontal pathogens in human, namely *P. gingivalis*, *T. denticola*, and *T. forsythia*, which are called the red complex bacteria (Buonavoglia et al., 2013; Socransky et al., 1998). In this study, *T. denticola* and *T. forsythia* were found in the dog samples, but not *P. gingivalis*, a well-known periodontopathic bacteria in human chronic periodontitis. *Porphyromonas* spp. have been reported as pathogens in dog periodontal diseases (Isogai et al., 1999); however, these bacteria were also found in the healthy group (Senhorinho et al., 2011). Therefore, the *Porphyromonas* species represents the predominant bacteria in the subgingival plaque of both healthy and diseased dogs, even though it is claimed to be a pathogen in canine periodontal diseases. The periodontal diseases in dog might result from the interaction and imbalance of multi-bacteria apart from *Porphyromonas* species; moreover, other factors may be involved in disease etiology. In this study, six *Porphyromonas* species were detected. *P. gulae* was the predominant species in the periodontitis dog samples. Normally it is not present in human subgingival plaque (Yamasaki et al., 2012). *P. gulae* is related to periodontitis in dogs (Hamada et al., 2008; Kato et al., 2010). Previous studies showed that *P. gingivalis* and *P. gulae* were genetically closely related, but *P. gulae* was catalase-positive when compared with *P. gingivalis* (Fournier et al., 2001; Hamada et al., 2008). *T. denticola* is known as the major bacterium related to disease in human (Simonson et al., 1988) while *P. gulae*

is mainly found in dogs (Yamasaki et al., 2012). In this study, *P. gulae* and *T. denticola* were found in almost all periodontitis dogs, which differs from the predominant species in human periodontitis. The present study suggests the possible role of *P. gulae* and *T. denticola* in the etiology of periodontal diseases in dogs. Therefore, virulent factors in these dominant pathogens, as well as bacterial interactions, should be further studied.

According to the current results, *P. canis* was the dominant *Peptostreptococcus* in the dogs of the periodontitis group, but not in human. Moreover, *Actinomyces* spp., which is commonly found in humans, was not the predominant bacterium in this study. *A. coleocanis* and *A. viscosus* were detected in one healthy sample. Likewise, *A. actinomycetemcomitans*, one periodontal pathogen in aggressive human periodontitis (Fives-Talor et al., 1996), was not found among the dog samples in this study. This may be due to the different environment in the oral cavity. *F. nucleatum*, another bacterium often identified from human subgingival periodontitis samples (Moore and Moore, 1994), was present in the subgingival samples from both healthy and periodontitis dogs. Interestingly, Streptococcus species, the dominant bacteria in both supragingival and subgingival plaque of humans, were not detected in the dogs, which may be due to the alkaline condition of the dog's oral cavity (Lavy et al., 2012).

In conclusion, the DGGE technique could detect many bacterial species, and may potentially provide a fingerprint of the microbial community in healthy and periodontitis dogs. However, it is important to keep in mind that the periodontal disease in dogs may not be similar to human. Therefore, care should be taken when applying the knowledge about periodontal pathogenesis in human in treatment and care for dogs.

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บทคัดย่อ

การสำรวจความแตกต่างของกลุ่มเชื้อจุลินทรีย์ได้เหงือกในสุนัขและมนุษย์ ที่เป็นโรคปริทันต์อักเสบโดยใช้เทคนิคดีจีจีจี

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โรคปริทันต์อักเสบเป็นโรคติดต่อที่พบได้มากที่สุดที่สุนัข ในปัจจุบันความรู้เกี่ยวกับแบคทีเรียที่เป็นสาเหตุของโรคปริทันต์อักเสบในสุนัขยังมีไม่มากนัก การศึกษานี้มีวัตถุประสงค์เพื่อประยุกต์ใช้เทคนิคปฏิกิริยาลูกโซ่พอลิเมอร์ร่วมกับเทคนิค Denaturing Gradient Gel Electrophoresis หรือ PCR-DGGE รวมถึงเทคนิคการโคลนนิ่งและการหาลำดับนิวคลีโอไทด์ของยีน 16S rRNA เพื่อสำรวจกลุ่มชนิดของเชื้อแบคทีเรียจากคราบจุลินทรีย์ได้เหงือกของสุนัขที่มีสุขภาพช่องปากปกติและเป็นโรคปริทันต์อักเสบ และเปรียบเทียบรูปแบบของแถบดีเอ็นเอที่ได้จากเทคนิค DGGE ของคราบจุลินทรีย์ได้เหงือกของสุนัขกับของมนุษย์ จากการศึกษาเชื้อแบคทีเรียจากคราบจุลินทรีย์ได้เหงือกของสุนัขสายพันธุ์พุดเดิลในประเทศไทยจำนวน 12 ตัว สามารถตรวจพบเชื้อแบคทีเรียเป็นจำนวนทั้งหมด 68 สปีชีส์ โดยแบคทีเรียส่วนใหญ่ที่พบอยู่ในยีนส Porphyromonas ในกลุ่มตัวอย่างสุนัขที่เป็นโรคปริทันต์อักเสบนั้นเชื้อแบคทีเรียที่พบมากที่สุดคือเชื้อ *Porphyromonas gulae* และ *Treponema denticola* การศึกษารูปแบบของแถบดีเอ็นเอที่ได้จากเทคนิค DGGE ของคราบจุลินทรีย์ได้เหงือกของสุนัขเปรียบเทียบกับของมนุษย์พบว่า โครงสร้างของกลุ่มเชื้อแบคทีเรียได้เหงือกของสุนัขและมนุษย์มีความแตกต่างกัน ซึ่งอาจมีความเกี่ยวข้องกับกลไกการก่อโรคจากผลการทดลองอาจกล่าวได้ว่า โรคปริทันต์อักเสบในสุนัขอาจแตกต่างจากในมนุษย์ ดังนั้นการนำสุนัขมาเป็นสัตว์ทดลองเพื่อใช้ศึกษาวิจัยโรคปริทันต์อักเสบในมนุษย์ต้องมีการวิเคราะห์อย่างระมัดระวัง

คำสำคัญ: DGGE สุนัข โรคปริทันต์อักเสบ ปริทันต์อักเสบ คราบจุลินทรีย์ได้เหงือก

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