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Original article

Evaluation of changes in periodontal bacteria in healthy dogs over 6 months using quantitative real-time PCR

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Abstract

Porphyromonas gulae, Tannerella forsythia and *Campylobacter rectus* are considered dominant periodontal pathogens in dogs. Recently, quantitative real-time PCR (qRT-PCR) methods have been used for absolute quantitative determination of oral bacterial counts. The purpose of the present study was to establish a standardized qRT-PCR procedure to quantify bacterial counts of the three target periodontal bacteria (*P. gulae, T. forsythia* and *C. rectus*). Copy numbers of the three target periodontal bacteria were evaluated in 26 healthy dogs. Then, changes in bacterial counts of the three target periodontal bacteria were evaluated for 24 weeks in 7 healthy dogs after periodontal scaling. Analytical evaluation of each self-designed primer indicated acceptable analytical imprecision. All 26 healthy dogs were found to be positive for *P. gulae*, *T. forsythia* and *C. rectus.* Median total bacterial counts (copies/ng) of each target genes were 385.612 for *P. gulae*, 25.109 for *T. forsythia* and 5.771 for *C. rectus*. Significant differences were observed between the copy numbers of the three target periodontal bacteria. Periodontal scaling reduced median copy numbers of the three target periodontal bacteria in 7 healthy dogs. However, after periodontal scaling, copy numbers of all three periodontal bacteria significantly increased over time ($p<0.05$, Kruskal-Wallis test) (24 weeks). In conclusion, our results demonstrated that qRT-PCR can accurately measure periodontal bacteria in dogs. Furthermore, the present study has revealed that qRT-PCR method can be considered as a new objective evaluation system for canine periodontal disease.

Key words: dog, periodontal bacteria, periodontal scaling, primers

Introduction

Periodontal diseases, such as gingivitis and periodontitis, are chronic and multifactorial diseases caused by infection with anaerobic bacteria in periodontal pockets (Pavlica et al. 2008). It has been reported that Gram-negative bacteria *Porphyromonas gulae, Tannerella forsythia* and *Campylobacter rectus* are dominant periodontal pathogens in dogs (Kato et al. 2011). The cell wall of these Gram-negative bacterial include lipopolysaccharide (LPS). After bacterial death or lysis, LPS is released and enhances bacterial biofilm formation. Bacterial biofilms such as plaque absorb calcium and phosphate from saliva, leading to

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Table 1. Primers used for quantitative real-time PCR assay.

Target	Expected size of PCR product (bp)	Primer type	Sequence (5'-3')	GenBank Accession No.
Porphyromonas gulae	116	Forward Reverse	TTGCTTGGTTGCATGATCG TCAGTTCCCCTACCCATCGT	JN713221.1
Tannerella forsythia	160	Forward Reverse	AGCGAGGGTAGCAATACCTG CATCCGCAACCGATAAATC	JN713185.1
Campylobacter rectus	80	Forward Reverse	TGTGCTTTAGGTGGGTCAAGG CAAATACGGACTTCGCAGATAGG	JN713167.1

All primers were self designed.

supragingival calculus formation (Jin and Yip 2002, Van der Weijden et al. 2015). Supragingival calculus alters the gingival pocket environment to an anaerobic condition and leads to further growth of anaerobic bacteria (Jin and Yip 2002, Van der Weijden et al. 2015). Calculus removal requires an ultrasonic scaler since dental calculus cannot be removed using a toothbrush.

To evaluate periodontal disease, gingival hyperemia, gingival tumentia and dental calculus buildup are generally assessed by a veterinarian. However, these methods are subjective and thus an objective evaluation system is needed to evaluate canine periodontal disease. Culture methods have been considered the gold standard for identifying periodontal microorganisms in dental biofilms (Kirakodu et al. 2008, Sasaki et al. 2015). However, limitations of this method include difficulty of detecting specific types of microorganisms, such as anaerobes, and inaccuracy of bacterial counting (Kirakodu et al. 2008, Sasaki et al. 2015). Molecular methods are currently available for detecting periodontal pathogens from subgingival microbiota using polymerase chain reaction (PCR) (Ashimoto et al. 1996, Kirakodu et al. 2008). Furthermore, quantitative real-time PCR (qRT-PCR) methods provide an absolute quantitative determination of oral bacterial counts.

The purpose of the present study was standardization of qRT-PCR procedure for evaluation of changes in periodontal bacteria in healthy dogs.

Materials and Methods

Twenty-six healthy beagles (5 males, 7 castrated males and 14 spayed females; 6.2-13.0 kg body weight; 1-9 years old), maintained in our laboratory were used. All dogs were fed a commercial diet (Select Protein, Royal Canin Japon, Tokyo, Japan) twice a day (8 am and 6 pm). The caloric intake was set at $0.5 \times 1.4\text{-}1.6 \times$ RER (BW0.75×70) for each feeding, where RER indicates the resting energy requirement and BW indicates body weight for dogs. Approval for this work was given by the Animal Research Committee of the Nippon Veterinary and Life Science University.

Oral bacteria were collected once per each dog using a commercial sterilized toothbrush (Lion Corporation, Tokyo, Japan) by 20 s of brushing the gingival margin on the buccal side of the maxillary bilateral molar region. After tooth brushing, the toothbrush was soaked in 400 µl of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4) as oral bacterial solution. Total DNA was extracted from the oral bacterial solution using the QIAmp DNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Extracted DNA samples were stored at -80°C until further use.

First, qRT-PCR was performed using extracted DNA from 26 healthy beagles with various periodontitis statuses to identify three target bacteria: *P. gulae, T. forsythia* and *C. rectus*. The primers were self-designed from GenBank information (Table 1). All qRT-PCR reactions for target bacteria were performed in triplicate. The reactions were carried out with a commercial kit (SYBR Premix Ex Taq II, TAKARA BIO Inc., Shiga, Japan) using the Applied Biosystems 7500 Real Time PCR Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The protocol was as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Following amplification, melting curve analysis was performed to verify the authenticity of the amplified products by their specific melting temperatures (Applied Biosystems). The resulting products were subjected to nucleotide sequencing to confirm their specificity. PCR amplification was carried out in 20-µl reactions containing 1 µl of template DNA, 0.8 µl of each specific primer (10 µM), 10 µl of SYBR Premix Ex Taq, 0.4 µl ROX Reference Dye II and 7.0 µl of distilled water. After qRT-PCR amplification, absolute quantification

Table 2. Intra- and inter-assay coefficients of variation for each primer.

		Intra-assay (n=10)	Inter-assay (n=5)
Porphyromonas gulae	Mean (SD) CV (%)	15.9*(0.03) 0.2	16.8*(0.3) 1.7
Tannerella forsythia	Mean (SD) CV (%)	17.9*(0.03) 0.2	20.7*(0.5) 2.3
Campylobacter rectus	Mean (SD) CV (%)	21.8*(0.1) 0.6	30.1*(0.5) 1.6

SD – standard deviation; CV – coefficient of variation.
* Theshold cycle.

was performed, as described previously (Whelan et al. 2003), by establishing a linear amplification curve from 10-fold serial dilutions of cloned and sequenced plasmid DNA containing target PCR products. Furthermore, intra-assay reproducibility of the qRT-PCR assay with each primer was estimated using pooled extracted oral bacterial DNA with 10 replicates. Another group of pooled DNA was also used to evaluate the inter-assay imprecision, with 5 independent runs over 4 weeks.

Next, 7 healthy dogs out of 26 dogs underwent overnight fasting. Each dog was sedated by intravenous injection of 7 mg/kg BW of propofol (Propofol Intravenous Injection 1%; Fresenius Kabi Japan Corporation, Tokyo, Japan), and then anesthetized with isoflurane. Dental scaling was subsequently performed using an ultrasonic scaler (Oral scaler type OVC-S1, J. Morita Mfg. Corp., Kyoto, Japan). Dental scaling for each dog was performed by the same veterinarian. After dental scaling was completed, polishing was performed using a tooth polishing instrument (VOLVERE Vmax, Nakanishi Inc., Tochigi, Japan), paste (Scaling cream adonest coarse and fine, Neo Dental Chemical Products Co., Ltd., Tokyo, Japan) and a polishing brush and a rubber cup. The day after dental scaling was defined as week 0. Oral bacterial DNA extraction was performed at 0, 4, 8, 12, 16, 20 and 24 weeks after dental scaling with the same protocol as described above.

Data are presented as median and 95% non-parametric interfractile interval. To assess significance between groups, the Kruskal-Wallis test and Dunn's multiple comparison test were employed. All tests were conducted using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). P values <0.05 were considered statistically significant.

Results

Analytical evaluation of each primer indicated acceptable analytical imprecision, with intra- and inter-assay coefficients of variation not exceeding the commonly accepted 5% limit for each primer (Table 2).

All 26 healthy dogs were found to be positive for *P. gulae*, *T. forsythia* and *C. rectus*. Median total bacterial counts (copies/ng) of each target gene were 385.612 (minimum-maximum range, 46.361-1.177.774) for *P. gulae*, 25.109 (minimum-maximum range, 8.664-128.153) for *T. forsythia* and 5.771 (minimum-maximum range, 805-22.764) for *C. rectus* (Fig. 1A-C). Significant differences were observed between the copy numbers of the three target periodontal bacteria ($p<0.05$, Kruskal-Wallis test) (Fig. 1D). *P. gulae* copy numbers were significantly greater than those for *T. forsythia* ($p<0.05$, Dunn's multiple comparison test). Furthermore, *C. rectus* copy numbers were significantly lower than those for *T. forsythia* ($p<0.05$, Dunn's multiple comparison test).

Next, changes in oral bacterial copy numbers of *P. gulae*, *T. forsythia* and *C. rectus* after periodontal scaling (0, 4, 8, 12, 16, 20 and 24 weeks) were evaluated in 7 healthy dogs (Fig. 2). A significant increase was observed in all three periodontal bacteria over time after periodontal scaling ($p<0.05$, Kruskal-Wallis test). Median oral *P. gulae* copy numbers at 0, 4, 8, 12, 16, 20 and 24 weeks were 7.751, 186.387, 409.216, 642.333, 678.430, 995.133 and 608.424, respectively. Median oral *T. forsythia* copy numbers at 0, 4, 8, 12, 16, 20 and 24 weeks were 979, 17.099, 36.392, 30.029, 29.920, 26.521 and 24.861, respectively. Median oral *C. rectus* copy numbers at 0, 4, 8, 12, 16, 20 and 24 weeks were 200, 1.642, 2.513, 2.895, 2.456, 4.274 and 4.035, respectively. Significant differences were observed between week 0 vs. weeks 16, 20 and 24 and week 4 vs. weeks 20 and 24 for *P. gulae*; week 0 vs. weeks 8, 12, 16 and 20 for *T. forsythia*; and week 0 vs. week 16 for *C. rectus* ($p<0.05$ for all, Dunn's multiple comparison test).

Discussion

Gingivitis and periodontitis in dogs are the most common diseases in veterinary medicine

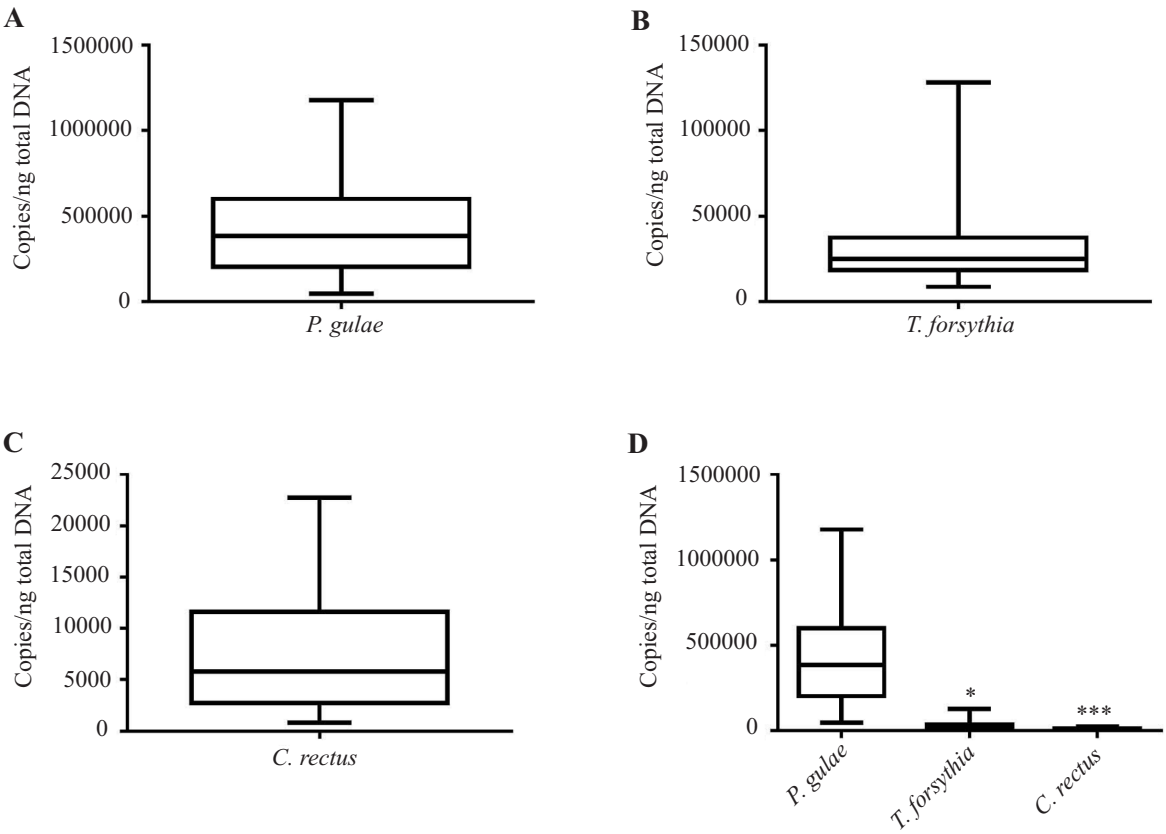

Fig. 1. Total DNA counts (copies/ng) of *Porphyromonas gulae* (A), *Tannerella forsythia* (B) and *Campylobacter rectus* (C) and comparison of copy numbers of the three target periodontal bacteria (D) in 26 healthy dogs. Values are expressed as box plots. The box represents the interquartile range (i.e., 25-75% range), horizontal line in the box represents the median and error bars represent the 2.5th-97.5th percentile. *p<0.05, compared with *P. gulae* (Dunn's multiple comparison test). **p<0.05, compared with *T. forsythia* (Dunn's multiple comparison test).

(Kato et al. 2011). To establish an objective evaluation system for periodontitis, we designed qRT-PCR primers targeting *P. gulae*, *T. forsythia* and *C. rectus*. If these primers could accurately quantify bacterial counts in dogs, then periodontal status could be determined objectively (by mathematical values). Our self-designed primers for the three target periodontal bacteria demonstrated acceptable analytical imprecision, and results were reproducible.

Next, *P. gulae*, *T. forsythia* and *C. rectus* copy numbers were measured in 26 healthy dogs with various periodontal statuses. Surprisingly, all three periodontal bacteria were detected in all 26 dogs in the current study. Yamasaki et al. (2012) demonstrated that the detection rates of *P. gulae*, *T. forsythia* and *C. rectus* were 71.2%, 77.3% and 66.7% in 66 canine oral cavities, respectively, using PCR and agarose gel electrophoresis. Kato et al. (2011) observed detection rates >88% for *P. gulae*, *T. forsythia* and *C. rectus* in the oral cavities of 26 dogs. Moreover, Senhorinho et al. (2011) also showed the presence of *P. gulae* in 92% and 56% of dogs with and without periodontitis, respectively. These reports used bacterial culture before PCR as detection methods. As such, differences in detection methods have a significant effect on the detection rate of periodontal pathogens. In this study, we used qRT-PCR methods to evaluate periodontal pathogens. Therefore, this highly sensitive measurement system might explain the 100% bacterial detection rate observed in the current study.

Median copy numbers of the three target periodontal bacteria indicated that *P. gulae* is detected at 15.4-fold and 66.8-fold higher rates than *T. forsythia* and *C. rectus*, respectively. *P. gulae* is reported to be closely related to *Porphyromonas gingivalis*, which is considered a major pathogen in human periodontitis (Fournier et al. 2001). *P. gingivalis* produces several important virulence factors such as fimbriae, LPS, capsule and protease, which are associated with adhesion to and invasion of gingival cells (Amano et al. 2004). Since *P. gulae* also has fimbrial proteins, it might readily exist as indigenous oral bacteria in dogs (Hamada et al. 2008, Nomura et al. 2012). *T. forsythia*, as well as *P. gingivalis*, is a member of red complex bacteria and a major cause of periodontal disease in humans (Holt and Ebersole 2000). *C. rectus* is also considered

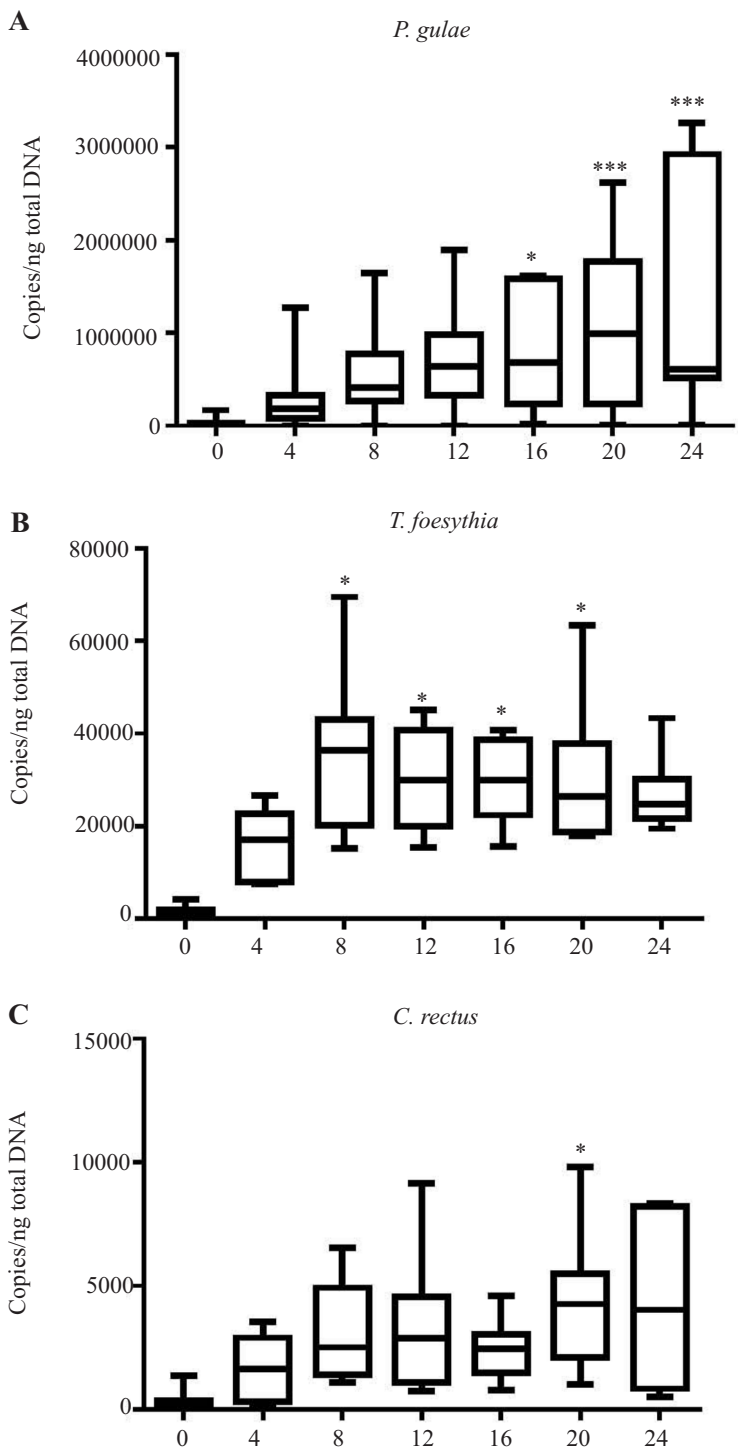

Fig. 2. Changes in total DNA copy numbers of *Porphyromonas gulae* (A), *Tannerella forsythia* (B) and *Campylobacter rectus* (C) after periodontal scaling (0, 4, 8, 12, 16, 20 and 24 weeks) in 7 healthy dogs. Values are expressed as box plots. The box represents the interquartile range (i.e., 25-75% range), horizontal line in the box represents the median and error bars represent the 2.5th-97.5th percentile. *p<0.05, compared with week 0 (Dunn's multiple comparison test). **p<0.05, compared with week 4 (Dunn's multiple comparison test).

a causative bacterium of human periodontal disease and has been detected in periodontal patients (Suda et al. 2004). These three bacteria might exist as oral indigenous bacteria in dogs since they were detected in all 26 dogs.

Finally, changes in the three target periodontal bacteria were evaluated for 24 weeks after periodontal scaling to investigate whether time without toothbrushing affects oral periodontal bacteria in dogs. A significant increase was observed in all three target

periodontal bacteria over time after scaling. Especially, a significant increase was observed from week 16 for *P. gulae*, week 8 for *T. forsythia* and week 20 for *C. rectus* as compared to week 0. Thus, the bacterial proliferation rate differed for each periodontal bacterium in the healthy canine oral cavity. Because a clear increase in *T. forsythia* copy numbers was observed starting at week 8, *T. forsythia* might be an early detection marker of periodontitis. Additionally, *P. gulae* copy numbers sequentially increased over time, thus it might be a marker of periodontitis severity. For *C. rectus*, a significant increase was only observed at week 20. Therefore, *C. rectus* might not be suitable as a marker of periodontitis as compared to *P. gulae* and *T. forsythia*.

qRT-PCR methods are now commonly applied to evaluate periodontal disease in humans (Kirakodu et al. 2008). Our results demonstrated that qRT-PCR methods can similarly measure periodontal bacteria in dogs. Furthermore, the qRT-PCR method established in the current study might be a new objective evaluation system for canine periodontal disease. Periodontal pathogens are associated with disorders including respiratory and cardiovascular disease (Seymour et al. 2007). Therefore, the prevention of periodontitis positively affects periodontal health as well as general health. Periodic toothbrushing following periodontal scaling is one of the most effective methods to prevent periodontitis (Watanabe et al 2015). Further investigations should utilize qRT-PCR methods to assess whether periodic toothbrushing can prevent proliferation of canine periodontal bacteria.

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