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Research article

Bacterial DNA and serum IgG antibody titer assays for assessing infection of human-pathogenic and dog-pathogenic *Porphyromonas* species in dogs

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ABSTRACT

Periodontal disease is highly prevalent in both humans and dogs. Although there have been reports of cross-infection of periodontopathic bacteria, methods for assessing it have yet to be established. The actual status of cross-infection remains to be seen. The purpose of this study was to evaluate the utility of bacterial DNA and serum immunoglobulin G (IgG) antibody titer assays to assess infection of human-pathogenic and dog-pathogenic *Porphyromonas* species in dogs. Four experimental beagles were used for establishing methods. Sixty-six companion dogs at veterinary clinics visiting for treatment and prophylaxis of periodontal disease were used and divided into healthy, gingivitis, and periodontitis groups. Periodontal pathogens such as *Porphyromonas gingivalis* and *Porphyromonas gulae* were investigated as target bacteria. DNA levels of both bacteria were measured using species-specific primers designed for real-time polymerase chain reaction (PCR). Serum IgG titers of both bacteria were measured by enzyme-linked immunosorbent assay (ELISA).

PCR primers were confirmed to have high sensitivity and specificity. However, there was no relationship between the amount of bacterial DNA and the severity of the periodontal disease. In addition, dogs with periodontitis had higher IgG titers against both bacteria compared to dogs in the healthy and gingivitis groups; there was cross-reactivity between the two bacteria. Receiver operating characteristic (ROC) analysis of IgG titers against both bacteria showed high sensitivity

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(>90 %) and specificity (>75 %). Since both bacteria were distinguished by DNA assays, the combination of these assays may be useful in the evaluation of cross-infection.

1. Introduction

Zoonoses are infections transmitted from animals to humans [1]. Companion animals, due to their increased numbers and variety, have been a concern in recent years for causing zoonotic diseases [2]. While companion animals, especially dogs, provide significant physical and mental health benefits to their owners [3], it is important to recognize that they can also transmit zoonoses, which can be particularly severe for older adults with weakened immune systems.

Periodontitis is a disease that affects both humans and dogs, and there have been reports of cross-infection of periodontal pathogens only at the gene level between the two [4], affecting not only the oral cavity but also general health [5,6]. The prevalence of periodontitis is 60 % of humans aged 65 [7] and 89 % of dogs aged 12–14 [8], and there is a risk of cross-infection in community life. However, the actual status of cross-infection remains unclear because of the lack of simple assays to investigate periodontal infections in humans and dogs. We investigated the potential of a bacterial DNA assay and serum IgG antibody titer assay, which have been used for screening of human periodontitis [9,10], to detect periodontopathic bacteria in dogs also. Specifically, we focused on *P. gingivalis*, a common periodontopathic bacterium in humans [11], and *P. gulae*, frequently found in periodontal lesions in dogs [12]. These bacteria share genetic similarities [13,14], and have same virulence factor [15]. We designed new primers for real-time PCR with short amplicon lengths. In addition, we adapted an ELISA developed for humans to measure serum IgG antibody titers [10] in dogs. Our aim was to assess the feasibility of these methods for screening infection of human-pathogenic and dog-pathogenic *Porphyromonas* species in dogs.

2. Materials and methods

2.1. Clinical sampling

In this study, the Animal Care and Use Committee of Okayama University approved the research (OKU-2015396, OKU-2017598). A total of 66 dogs, undergoing anesthesia at a veterinary clinic, were enrolled, and categorized into three groups based on the severity of periodontal disease [16]: healthy, gingivitis, and periodontitis. Samples with bacterial counts below the detection limit of real-time PCR were excluded from further analysis, resulting in 49 dogs included in the final analysis (Fig. 1). Bacterial samples were obtained by swabbing the gingival margin biofilm using a cotton swab. These swabs were preserved in phosphate-buffered saline (PBS) and stored at −20 °C. Blood samples of 3 mL were also collected from the dogs, centrifuged at 12,000×*g* for 3 min, and stored at −20 °C. For general anesthesia, atropine (0.05 mg/kg), midazolam (0.1 mg/kg), and butorphanol (0.2 mg/kg) were administered

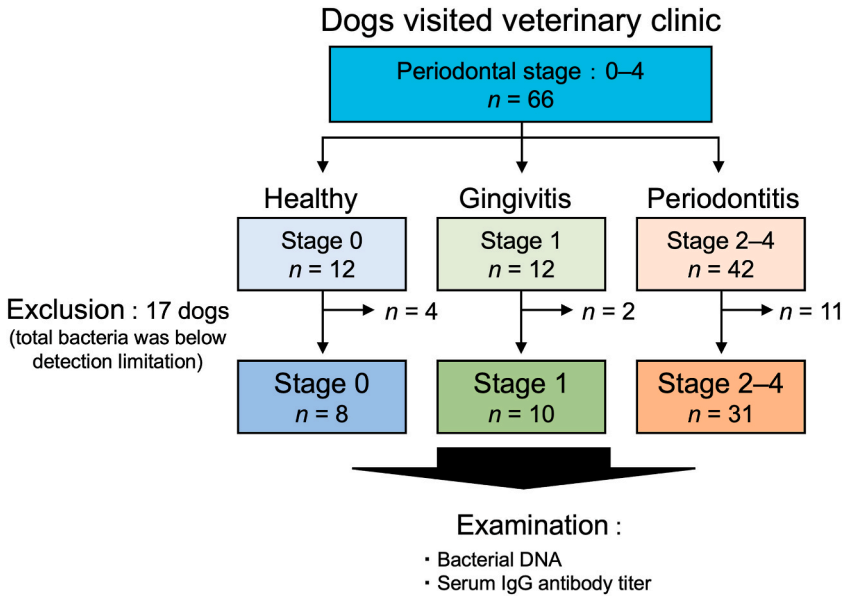


Fig. 1. Flowchart of extraction of samples from companion dogs
Sixty-six companion dogs were evaluated for periodontal diseases. Bacterial samples were collected from the gingival margin and bacterial DNA assay were performed using universal primers, and 17 animals below the detection limit were excluded. The 49 animals used for the final analysis were categorized into a healthy group (n = 8), 10 gingivitis group (n = 10), and 10 periodontitis group (n = 31).

intravenously as premedication, and anesthesia was introduced with intravenous alfaxalone (2.0–3.0 mg/kg) and maintained with isoflurane inhalation. Intraoperatively, robenacoxib (2.0 mg/kg) and amipicillin sodium (20 mg/kg) were injected subcutaneously. Postoperatively, robenacoxib (1.0 mg/kg, once daily for 2 days) and amoxicillin (11–22 mg/kg, twice daily for 6 days) were administered orally.

2.2. Bacterial strains and growth conditions

P. gingivalis W83 and FDC381 were cultured in modified GAM broth (Nippon Suisan Kaisha, Ltd.) for 12 h, while *P. gulae* ATCC 51700 was cultured on CDC anaerobic blood agar (Becton, Dickinson and Company) for four days and then in tryptic soy broth medium supplemented with 5 mg/mL yeast extract, 0.5 mg/mL L-cysteine, 5 μg/mL hemin, and 1 μg/mL menadione at 37 °C for 24 h.

2.3. DNA primer

The primers used in this study are listed in Table 1. Universal primers targeting the 16S rRNA gene were used to assess the total number of bacteria [9], while species-specific primers for *P. gingivalis* W83 and *P. gulae* ATCC 51700 were designed using tools from the National Center for Biotechnology Information Basic Local Alignment Search Tool and Molecular Evolutionary Genetics Analysis [17]. Due to the similarity of the 16S rRNA genes between *P. gingivalis* and *P. gulae*, the 16S–23S rRNA gene internal transcription region (ITS) [18] was chosen as the target for amplification to distinguish them. AmplifX software (Institute of NeuroPhysiopathology) was employed to validate the specificity of the amplification.

2.4. PCR

DNA from *P. gingivalis* W83 and *P. gulae* ATCC 51700 was amplified by PCR as described previously [9] using the designed primers (Table 1). The PCR reaction mixture contained 0.2 μM of forward and reverse primers, 2 × AmpliTaq Gold® 360 Master Mix (Thermo Fisher Scientific), and 1 μL of template DNA in a total volume of 25 μL. The reaction conditions were 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 7 min. NFW was used as the negative control. The PCR products were examined using 5 % agarose gel electrophoresis and ethidium bromide staining.

2.5. DNA sequence analysis

After electrophoresis, the DNA bands were extracted and purified. The DNA sequences were analyzed using a 3130 × l Genetic Analyzer (Thermo Fisher Scientific). The amplified DNA sequence was determined by aligning the sequences obtained by analyzing both sense and antisense strands. *P. gingivalis* W83 was manually compared with the DNA sequence of *P. gingivalis* ATCC 33277 (GenBank: AY546475) because there was no available data on the ITS DNA sequence of W83. *P. gulae* ATCC 51700 was compared with the DNA sequence of the same strain (GenBank: AY546476).

2.6. Real-time PCR

Real-time PCR was performed as previously described [9] using the listed primers (Table 1). The reaction mixture contained 0.2 μM of forward and reverse primers, 2 × Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific), and 2.5 μL of template DNA in a total volume of 25 μL. The reaction conditions were 34 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. DNA from cultured bacteria (from 1.0×10^5 to 1.0×10^1 cells/μL) and DNA isolated from dog bacterial samples were used as templates. CFX Manager™ software (version 3.1) (Bio-Rad, Hercules, CA, USA) was used for analysis.

2.7. Immunization of experimental dogs with ultrasonically disrupted extracts

This study was approved by the Animal Care and Use Committee of Okayama University (approval no. OKU-2015396). Seven dogs (Toyo Beagle, Kitayama Labes; 11–24 months old, body weight 9.8–12.2 kg, male and female) were used. The dogs were divided into three groups: *P. gingivalis* immunization group, *P. gulae* immunization group, and normal saline group. The ultrasonically disrupted

Table 1
Primers for real-time PCR.

Primer	Sequence (5′-3′)	Target species	Target region	Product size (bp)
Universal-F	GTGSTGCAYGGYTGTCGTCA	Total bacteria	16S	148
Universal-R	ACGTCRTCCMCACCTTCCTC			
P. gingivalis-F	CACGGCGAAAAGCCATATTTG	*P. gingivalis*	ITS	99
P. gingivalis-R	AGAGAACACCCTCCCGGACAT			
P. gulae-F	GCAAGGATGGTTCTTGAGAAACG	*P. gulae*	ITS	87
P. gulae-R	CGAAACCGAAGGCTGATACC			

F: Forward, R: Reverse, 16S: 16S rRNA gene, ITS: 16S–23S rRNA gene internal transcribed spacer.

extracts of *P. gingivalis* FDC381 and *P. gulae* ATCC 51700 were prepared as described previously [10] and adjusted to 1.0 mg/mL for *P. gingivalis* and 0.1 mg/mL for *P. gulae* in normal saline. Ten milliliters of blood were collected from all dogs before immunization and used as the standard serum. The bacterial extracts were administered intravenously at 1.0 mL/kg body weight once a week for six weeks. Blood was collected weekly from the second to the sixth week and every other week until the twelfth week. The collected serum was centrifuged at 1710×*g* at 4 °C for 5 min and stored at −20 °C. Extracts administration and blood sampling were performed without anesthesia.

2.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

SDS-PAGE was performed as described previously [19]. Bacterial extracts of *P. gingivalis* FDC381 and *P. gulae* ATCC 51700 were solubilized in PBS containing 0.05 % Tween-20 (PBS-T) and separated on 4–20 % Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) using reductive conditions with 2-mercaptoethanol.

Western blotting was performed as described previously [20]. Bacterial extracts were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The serum sample with the highest antibody titer to *P. gingivalis* and *P. gulae* obtained from each dog was used as the primary antibody. Peroxidase rabbit anti-dog IgG (Jackson ImmunoResearch, Philadelphia) was used as the secondary antibody. Protein bands were detected using enhanced chemiluminescence and a ChemiDoc™ MP Imaging System

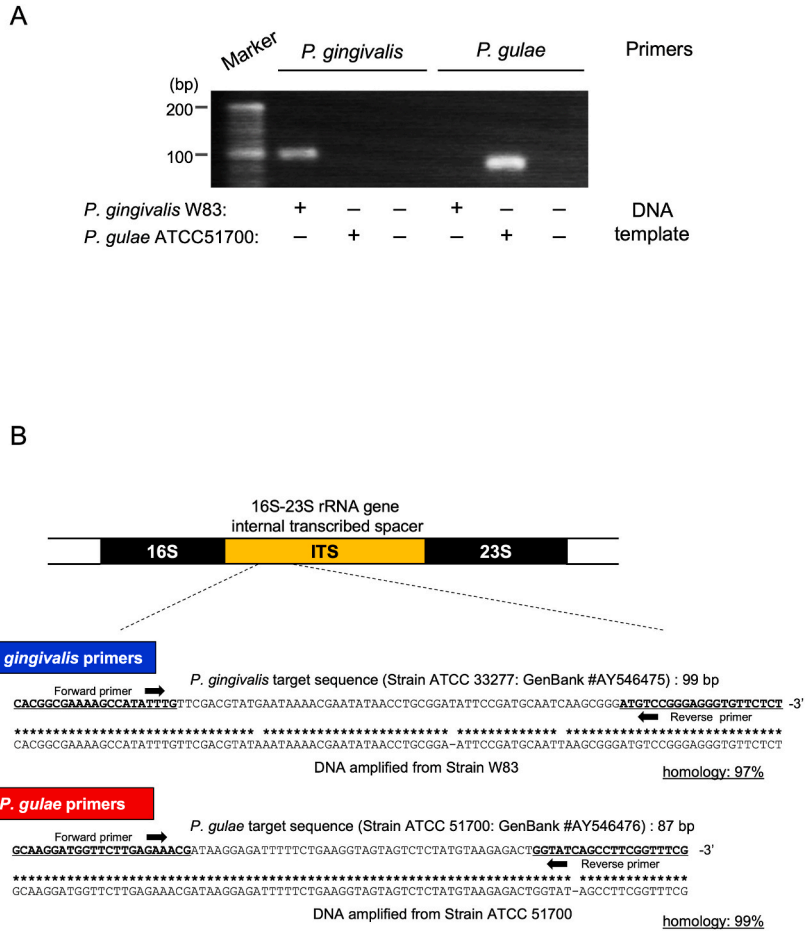


Fig. 2. Specificity of polymerase chain reaction (PCR) primers
PCR primers were designed to detect P. gingivalis species-specifically and *P. gulae* and bacteria associated with periodontal disease in humans and dogs, respectively.
(A) Specificity of *P. gingivalis* and *P. gulae* primer DNA (10^5 bacterial cells/reaction) extracted from *P. gingivalis* W83 and *P. gulae* ATCC 51700 was amplified by PCR (using 0.2 μM *P. gingivalis* species-specific primers (product size, 99 bp) and *P. gulae* species-specific primers (product size, 87 bp). The PCR products were detected by agarose gel electrophoresis (Refer to the uncropped image; Fig. S1).
(B) Homology between the DNA sequence of the target DNA region and the PCR products of *P. gingivalis*- and *P. gulae*-specific primers. The homology between the target DNA sequence of each bacterium in the database (upper panel; *P. gingivalis* ATCC 33277 was substituted for W83 because ITS sequence of W83 was not available) and the DNA sequence of the PCR products (lower panel) was 97 % and 99 % for *P. gingivalis*- and *P. gulae*-specific primers, respectively. * Indicates matching nucleotide sequences.

(Bio-Rad).

2.9. ELISA for IgG titer

ELISA was performed as described previously [10]. Bacterial extracts (*P. gingivalis* FDC381 and *P. gulae* ATCC 51700) dissolved in PBS-T were immobilized on a 96-well ELISA microplate. Sera collected from dogs before immunization were mixed in equal volumes and used as a standard serum. Sera obtained from immunized dogs were used as the test sera. Alkaline phosphatase rabbit anti-dog IgG

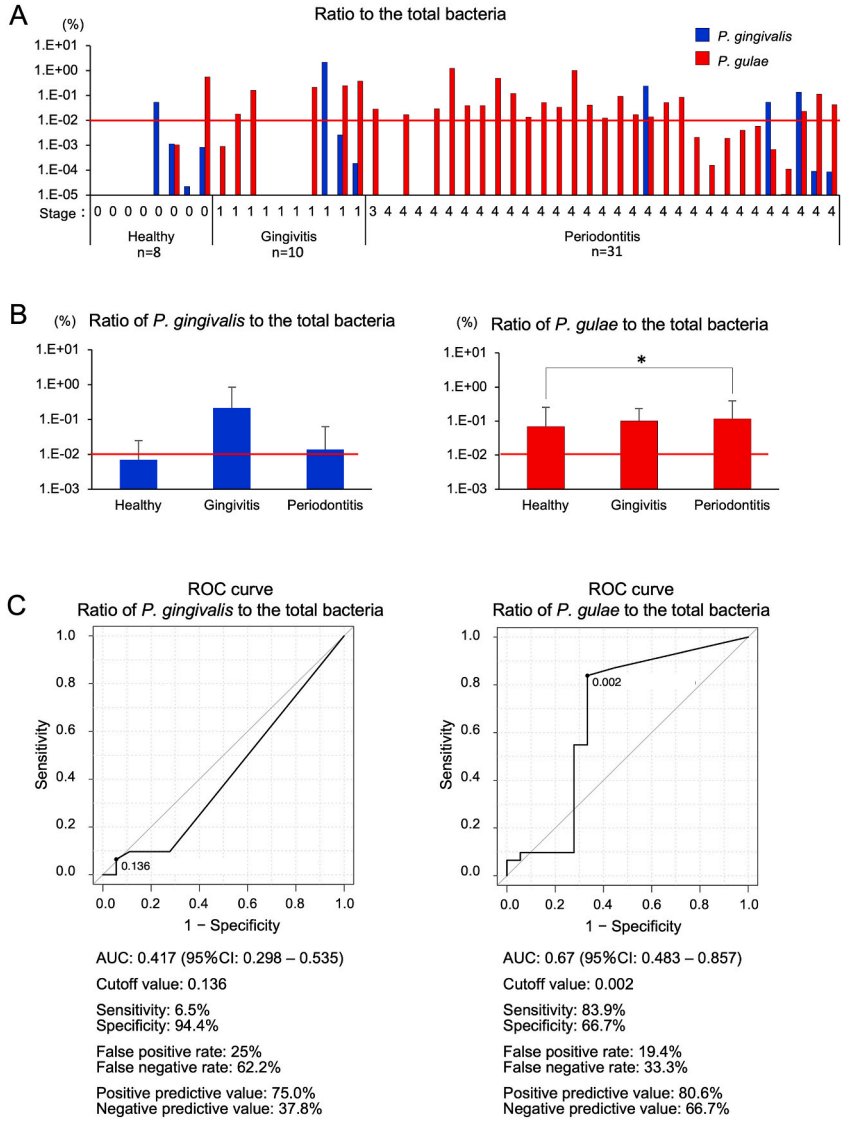


Fig. 3. Detection of *P. gingivalis* and *P. gulae* at the DNA level
(A) Ratio (%) of the number of *P. gingivalis* and *P. gulae* bacteria to the total number of bacteria for dogs in each group.
Forty-nine dogs were classified into healthy (n = 8), gingivitis (n = 10), and periodontitis (n = 31) groups based on the severity of periodontal disease (stages 0–4). Reactions were performed in duplicate on three PCR plates. The mean value of the six measurements was used to calculate the ratio. Solid bars and hollow bars indicate the ratio of the number of *P. gingivalis* and *P. gulae* bacteria, respectively, to the total number of bacteria.
(B) Comparison of the ratio of each bacterium among the groups.
The mean value of the ratio (%) of the number of *P. gingivalis* and *P. gulae* bacteria to the total number of bacteria was compared between the healthy (n = 8), gingivitis (n = 10), and periodontitis (n = 31) groups. Error bars indicate the standard deviation (SD). *, $P < 0.05$; Kruskal-Wallis and Mann-Whitney U tests.
(C) Receiver operating characteristic (ROC) curve for real-time PCR
The periodontitis group was disease-positive (n = 31), and the healthy and gingivitis groups were disease-negative (n = 18). Area under the curve (AUC) for *P. gingivalis* and *P. gulae* was 0.417 and 0.67, respectively.

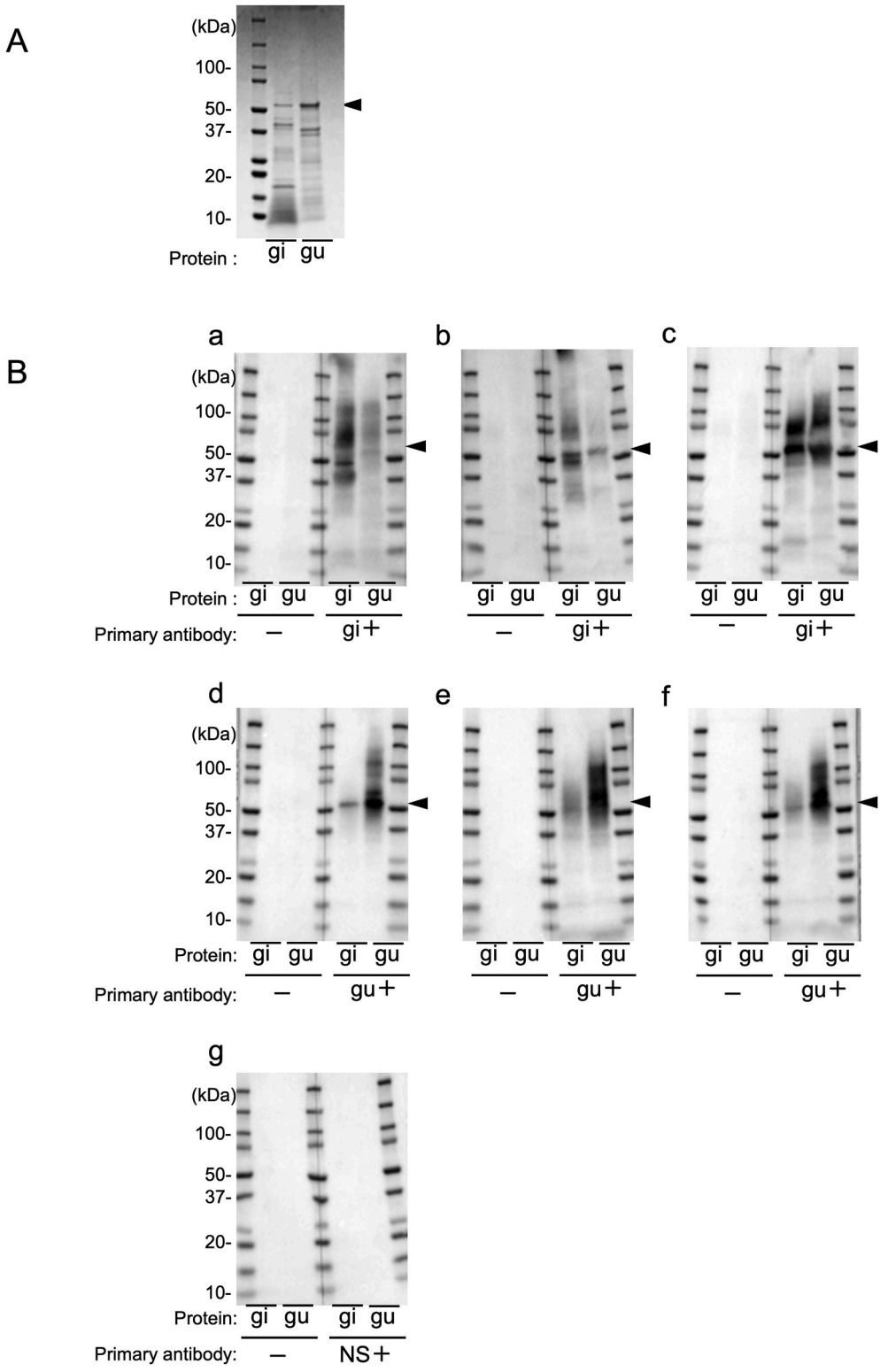


Fig. 4. Characters of bacterial extracts and serum IgG titers in dogs after immunization with bacterial extracts

(A) SDS-PAGE

Proteins (10 μg/lane) in extracts of *P. gingivalis* FDC381 (gi) and *P. gulae* ATCC 51700 (gu) were separated by SDS-PAGE. The arrow indicates the band between 50 and 55 kDa detected in both extracts (Refer to the uncropped image; Fig. S3).

(B) Western blotting

Dogs a, b, and c were administered *P. gingivalis* extracts, whereas dogs d, e, and f were administered *P. gulae* extracts. As a negative control, dog g was administered normal saline solution. Bacterial extracts were electrophoresed and transferred onto polyvinylidene difluoride membranes (Refer

to the uncropped image; Fig. S4). The membrane to the left side of the central marker was probed with pre-immune serum (−). The membrane on the right side was probed with serum collected from dogs immunized with bacterial extracts (gi+ and gu+) or normal saline (NS+). The arrowhead indicates the band between 50 and 55 kDa detected for both bacterial extracts.

(C) Serum IgG titers in dogs after immunization with bacterial extracts

Dogs a, b, and c were immunized with *P. gingivalis* extracts (n = 3), dogs d, e, and f were immunized with *P. gulae* extract (n = 3), and dog g was administered normal saline solution (n = 1) as a negative control. Each ELISA was performed in duplicate in three independent experiments. Data are presented as mean and standard deviation (error bars). The horizontal line at EU 100 indicates the threshold value. Closed and open circles indicate IgG titers against *P. gingivalis* and *P. gulae*.

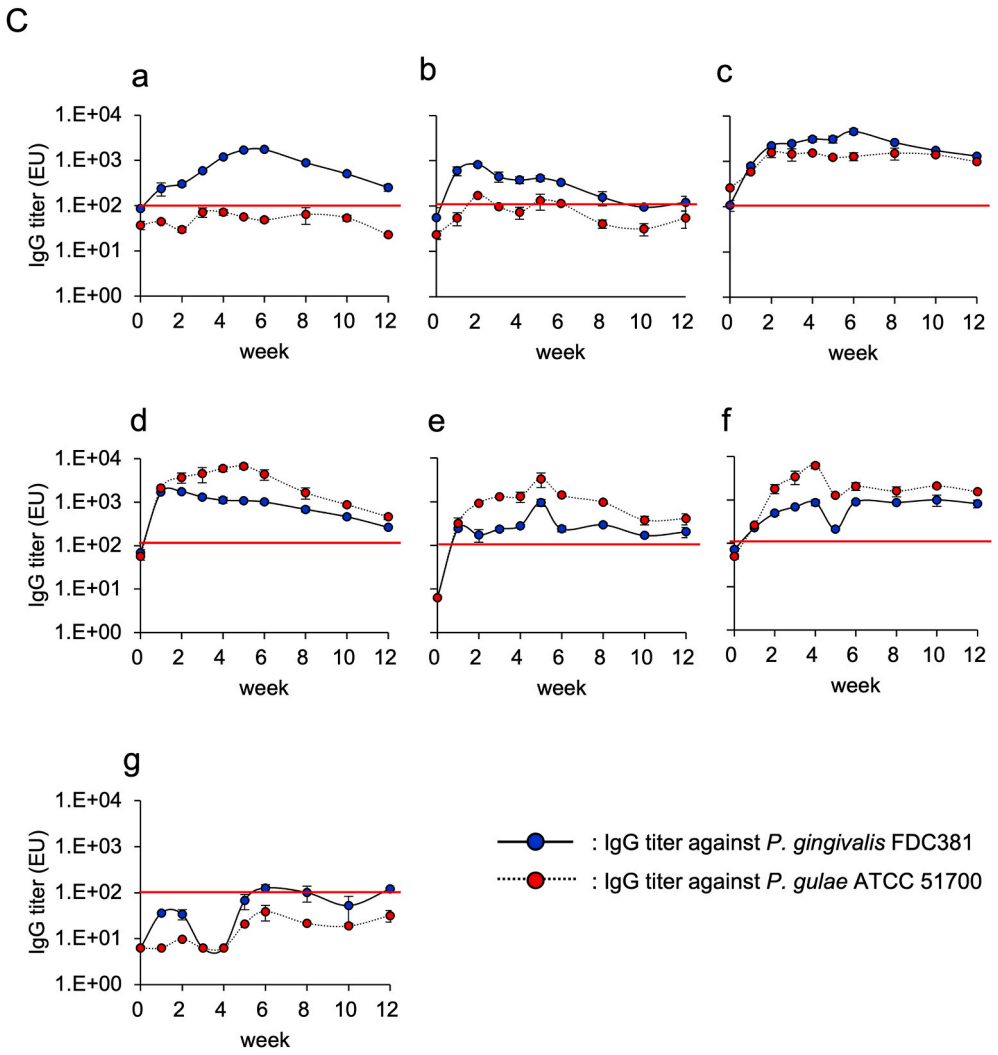
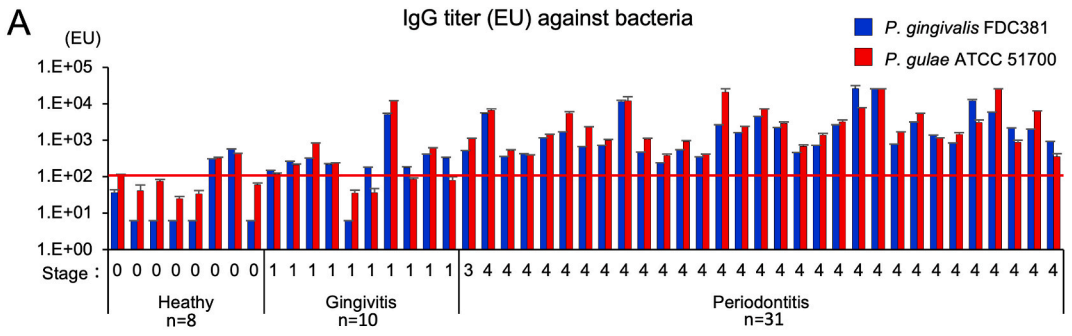


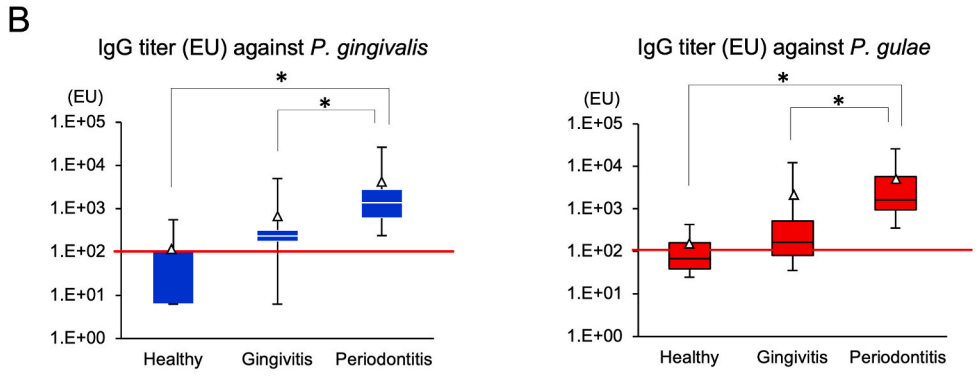
Fig. 4. (*continued*).

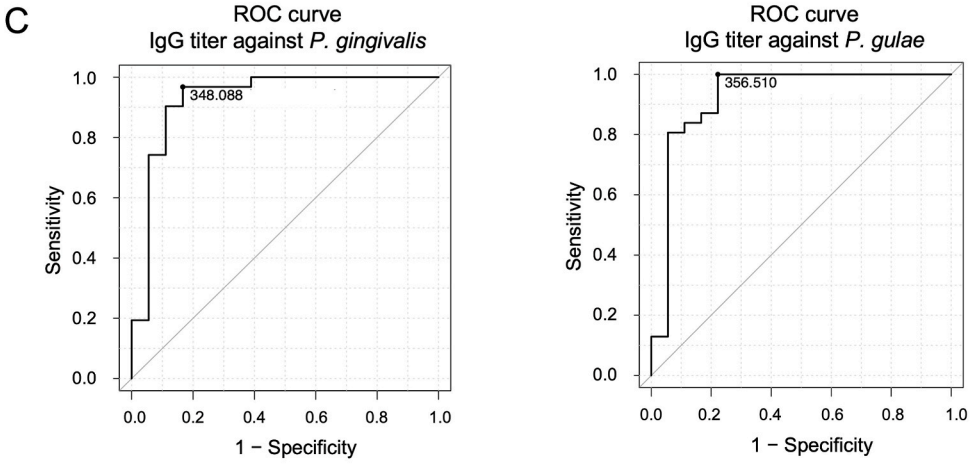
(Jackson ImmunoResearch) was used as the secondary antibody and disodium *p*-nitrophenyl phosphate hexahydrate was used as the chromogenic antibody. Absorbance was measured using a microplate reader and Microplate Manager® Software 5.2.1 (Bio-Rad). Then, the absorbance of the standard serum diluted 3200 times was set to an ELISA unit (EU) of 100.

2.10. Statistical analysis

The differences between the groups were tested using Kruskal-Wallis test and Mann-Whitney *U* test. The accuracy of real-time PCR and ELISA was evaluated using receiver operating characteristic (ROC) curves. EZR software [21] was used for statistical analyses, and a significance level of $P < 0.05$ was used.







AUC: 0.928 (95%CI: 0.835 - 1)

Cutoff value: 348

Sensitivity: 93.5%
Specificity: 83.3%

False positive rate: 9.4%
False negative rate: 11.8%

Positive predictive value: 90.6%
Negative predictive value: 88.2%

AUC: 0.925 (95%CI: 0.826 - 1)

Cutoff value: 356

Sensitivity: 100%
Specificity: 77.8%

False positive rate: 11.4%
False negative rate: 0%

Positive predictive value: 88.6%
Negative predictive value: 100%

(caption on next page)

Fig. 5. Detection of *P. gingivalis* and *P. gulae* infections using ELISA
(A) Serum IgG titers against *P. gingivalis* FDC381 and *P. gulae* ATCC 51700 for dogs in each group.
Dogs were classified into three groups based on the severity of the periodontal disease. Each reaction was performed in duplicate in three independent experiments. Data are presented as the mean and SD. The horizontal line at EU 100 indicates the threshold values. Solid and hollow bars indicate IgG titers to *P. gingivalis* and *P. gulae*.
(B) Comparison of serum IgG titers among the groups.
The IgG titers against *P. gingivalis* and *P. gulae* were compared between the healthy (n = 8), gingivitis (n = 10), and periodontitis (n = 31) groups. The box plot was shown with the median (bar) and mean (triangle). *, $P < 0.05$; Kruskal-Wallis and Mann-Whitney U tests.
(C) ROC curve for ELISA
The Periodontitis group was disease-positive (n = 31), and the health and gingivitis groups were disease-negative (n = 18). AUC for *P. gingivalis* and *P. gulae* were 0.928 and 0.925, respectively.

3. Results

3.1. Primer specificity, nucleotide sequence analysis, and quantification of DNA

Sequence analysis of the PCR products of *P. gingivalis*- and *P. gulae*-specific primers was performed to confirm that the target DNA was amplified using each primer pair (Table 1). The specificity of *P. gingivalis* and *P. gulae* primers for each bacterium was confirmed by agarose electrophoresis and real-time PCR. Agarose electrophoresis revealed species-specific amplicon bands for both primer pairs (*P. gingivalis*: 99 bp; *P. gulae*: 87 bp), whereas no bands were detected in other bacteria or the negative control (Fig. 2A, Fig. S1).

The homology between the DNA base sequence of the PCR product and the target DNA base sequence was 97 % and 99 % for *P. gingivalis* and *P. gulae* primers, respectively (Fig. 2B). In real-time PCR, both primers specifically and efficiently amplified the target regions of both bacteria. No primer dimers were generated, and the detection limit for the DNA copy number was 10^1 (Fig. S2).

3.2. Association of bacterial DNA with periodontal disease severity

Bacterial DNA assay for clinical samples was performed using real-time PCR to determine the total number of bacteria in gingival swab samples using universal primers and the number of *P. gingivalis* and *P. gulae* using species-specific primers. *P. gingivalis* was detected in 12 cases/49 dogs (24.5 %), whereas *P. gulae* was more frequently detected in 37 cases/49 dogs (75.5 %), and 29 cases/31 dogs (93.5 %) particularly in the periodontitis group (Fig. 3A). Furthermore, the number of *P. gingivalis* was not related to the severity of the periodontal disease. However, the number of *P. gulae* was significantly higher in the periodontitis group than in the healthy group (Fig. 3B).

ROC curves were analyzed for the ratio of the number of *P. gingivalis* and *P. gulae* to the total number of bacteria (healthy + gingivitis vs. periodontitis). The area under the curve (AUC) for *P. gingivalis* was 0.417 (95 % confidence interval [CI]:0.298–0.535), the cutoff value was 0.136, the sensitivity was 6.5 %, and the specificity was 94.4 %. The AUC for *P. gulae* was 0.67 (95 % CI:0.483–0.857), the cutoff value was 0.002, the sensitivity was 83.9 %, and the specificity was 66.7 % (Fig. 3C–Table S1).

3.3. Effectiveness of ultrasonically disrupted extracts for serum IgG antibody production

A clear broad band between 50 and 55 kDa was detected for both *P. gingivalis* and *P. gulae* extracts using SDS-PAGE; many other bands were also detected specific to each bacterium (Fig. 4A, Fig. S3). In addition, sera collected from dogs immunized with the bacterial extracts were subjected to western blotting. Multiple bands with long-range smears, especially between 50 and 55 kDa, were detected in both extracts upon reaction with sera collected from immunized dogs (Fig. 4B, Fig. S4; dog a–f); however, no bands were detected in the reaction with sera collected before immunization (Fig. 4B, Fig. S4; dog g). Furthermore, sera immunized with *P. gingivalis* showed strong bands with *P. gingivalis* extract or both extracts (Fig. 4B, Fig. S4; dog a–c). On the other hand, sera immunized with *P. gulae* showed strong bands with *P. gulae* extract (Fig. 4B, Fig. S4; dogs d–f).

Serum IgG antibody titers in serum against both extracts were quantified by ELISA (Fig. 4C). When the threshold value was set at EU100, sera immunized with *P. gingivalis* showed high antibody titers against the *P. gingivalis* extract or both extracts (Fig. 4C, dog a–c). Sera from dogs immunized with *P. gulae* also showed high antibody titers to both extracts (Fig. 4C, dog d–f).

3.4. Association of serum IgG titer with periodontal disease severity

Serum IgG antibody titers in clinical samples were quantified using ELISA against *P. gingivalis* and *P. gulae* extracts. The periodontitis group detected both *P. gingivalis* and *P. gulae* over EU 100 IgG titers (Fig. 5A). In addition, IgG titers against both *P. gingivalis* and *P. gulae* were significantly higher in the periodontitis group than in the healthy and gingivitis groups (Fig. 5B).

ROC curves for antibody titers against *P. gingivalis* and *P. gulae* were also analyzed (healthy + gingivitis vs. periodontitis). The AUC for *P. gingivalis* was 0.928 (95 % CI:0.835–1), the cutoff value was 348, the sensitivity was 93.5 %, and the specificity was 83.3 %. The AUC for *P. gulae* was 0.925 (95 % CI:0.826–1), the cutoff value was 356, the sensitivity was 100 %, and the specificity was 77.8 % (Fig. 5C–Table S2).

4. Discussion

The purpose of this study was to evaluate the utility of bacterial DNA and serum IgG antibody titer assays for assessing cross-infection between humans and dogs.

Real-time PCR primers were designed to perform bacterial DNA assays targeting *P. gingivalis* and *P. gulae*. In this study, we designed primers suitable for real-time PCR with an amplicon length of less than 150 bp, different from previously reported PCR primers with amplicons of 300 bp [22]. Due to the high homology (97–98 %) of the 16S rRNA gene between *P. gingivalis* and *P. gulae* [13], designing primers with high specificity and sensitivity for bacterial DNA detection was challenging. Therefore, we targeted the ITS region, which has a lower homology between two bacteria than that of 16S rRNA, and designed primers capable of detecting DNA from both bacteria with high specificity and sensitivity.

Using the designed primers, bacterial DNA assay of clinical samples revealed a low detection rate of *P. gingivalis* in dogs, while *P. gulae* was more frequently detected in the periodontitis group (Fig. 3A). Interestingly, the number of *P. gingivalis* was unrelated to the severity of periodontal disease, whereas the number of *P. gulae* was significantly higher in the periodontitis group compared to the healthy group (Fig. 3B). This suggests that bacterial DNA assay for *P. gulae* could be used for screening periodontal disease in dogs. However, the accuracy of the assay may be limited as the AUC was <0.7 (Fig. 3C). Furthermore, approximately 26 % of oral bacterial samples obtained by wiping the gingival margin had bacterial counts below the detection limit and were excluded from the analysis (Fig. 1). These results indicate that the wiping method may recover *Porphyromonas* species for DNA detection because *P. gulae* was detected as other reports [4]. Although *P. gingivalis* may infect dogs less than *P. gulae,* alternative methods such as using a paper point or curette, commonly employed in human dentistry to collect plaques from the gingival sulcus and subgingival root surface, could improve the collection process from deep periodontal pockets where *Porphyromonas* species exist in anaerobe conditions. However, in veterinary medicine, this alternative method is challenging due to the narrow oral cavity of small dogs and the requirement for general anesthesia for the collection process from deep periodontal pockets. Simple and easy-to-use sampling methods should be developed for small dogs under no general anesthesia. In addition, Oh et al. (2015) [23] utilized next-generation sequencing to analyze oral microbiomes and found oral-to-oral infection between dogs and humans. However, this study performed only the gene-level analysis but no host responses.

P. gingivalis and *P. gulae* extracts were used to assess serum IgG antibody titers and their specificity. Experimental dogs were immunized with these extracts, and ELISA was conducted using them as antigens, resulting in increased serum IgG antibody titers (Fig. 4C). Sera from dogs immunized with *P. gingivalis* showed high antibody titers against *P. gingivalis* extracts, and sera immunized with P. *gulae* against *P. gulae* extracts. The reactivity of sera to bacterial extracts was also evaluated by Western blotting (Fig. 4B). Multiple bands, particularly between 50 and 55 kDa range, were detected in both extracts, but not in pre-immunized sera; sera from dogs immunized with *P. gingivalis* showed a strong band against *P. gingivalis* extracts and sera immunized with *P. gulae* against *P. gulae* extracts, consistent with the antibody titer results. These results suggest that the protein in the size range of between 50 and 55 kDa is responsible for the elevated antibody titers and that specific and non-specific antigens are present in these extracts. Proteins in this size range include the 53 kDa outer membrane protein of *P. gingivalis* [19] and the 53 kDa fimbrial protein [24], which are closely associated with periodontal disease. Identification of each specific antigens for of *P. gingivalis* and *P. gulae* would allow specific serum antibody titer assays without cross-reactivity.

Serum IgG antibody assays using *P. gingivalis* and *P. gulae* extracts showed higher antibody titers in the periodontitis group (Fig. 5B). The significant AUC of the ROC curve and high sensitivity and specificity (Fig. 5C) indicate that the serum IgG antibody titer assay is a useful screening method for periodontal disease in dogs. This assay can be conveniently performed during routine veterinary visits using blood samples. However, precise quantification of antibody titers for *P. gingivalis* and *P. gulae* is challenging due to cross-reactivity caused by non-specificity of crude antigens. Identification of the antigens specific to each bacterium is needed to dissolve this problem.

In conclusion, the study demonstrated that a combination of bacterial DNA assay using real-time PCR and serum IgG antibody titer assay using ELISA was effective for screening *Porphyromonas gingivalis* and *Porphyromonas gulae* in dogs, and the serum IgG antibody titer assay was effective for screening periodontal disease in dogs. However, the bacterial DNA assay has limitations due to technique-dependent sampling, and the serum IgG antibody titer assay suffers from non-specific antigens. These limitations can be overcome to enhance test accuracy. By improving these assays, it becomes possible in the future to investigate the cross-infection of periodontopathic bacteria between humans and dogs.

Data availability statement

No data associated with this study has been deposited into a publicly available repository. Data included in article/supp. Material/referenced in the article. Original data will be made available on request.

Additional information

No additional information is available for this paper.

Ethics statement

The Animal Care and Use Committee of Okayama University approved the research (OKU-2015396, OKU-2017598).

CRediT authorship contribution statement

Masako Tai-Tokuzen: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Takashi Ito:** Supervision, Methodology, Investigation, Conceptualization. **Kazuya Tamura:** Supervision, Methodology, Investigation. **Haruko Hirayama:** Supervision, Methodology, Investigation. **Hirohito Ogawa:** Writing – review & editing, Visualization, Validation. **Shin Nakamura:** Writing – review & editing, Visualization, Validation. **Keisuke Okubo:** Writing – review & editing, Visualization, Validation. **Kazuhiro Omori:** Writing – review & editing, Supervision. **Tadashi Yamamoto:** Writing – review & editing, Supervision. **Katsumi Mominoki:** Supervision, Methodology, Investigation. **Shogo Takashiba:** Writing – review & editing, Supervision, Project administration, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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