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Identification and molecular characterization of *Porphyromonas gulae fimA* types among cat isolates

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ABSTRACT

Porphyromonas gulae, a Gram-negative black-pigmented anaerobe, is one of several major periodontal pathogens of animals. *P. gulae* isolates from dogs have been classified into three genotypes based on a 41-kDa filamentous appendage (FimA) on the cell surface, which is closely related to virulence in periodontal disease. However, other specific bacterial virulence factors contributing to the aggravation of periodontal disease in cats remain elusive. In the present study, we assessed FimA diversity in *P. gulae* isolates from cats and examined whether this diversity influenced periodontal condition. The putative amino acid sequences of FimA from 15 *P. gulae* isolates from 13 cats were classified into three genotypes (types A, B, and C), which showed 95–100% identity and similarity to the *fimA* types in dogs. The type C isolate showed greater adhesion and invasion properties in periodontal ligament fibroblasts as well as stronger inhibition of scratch closure of the cells compared with type A and B isolates. Next, a PCR-based method for identification of *fimA* genotype was developed and used to analyze 99 oral swab specimens from cats. High *fimA* type A detection rates were observed regardless of the periodontal condition, whereas types B and C were frequently detected from subjects with moderate and severe periodontitis, respectively. These results suggest that *P. gulae* isolates from cats can be classified into three types based on *fimA* genotype, which may be closely related to virulence in periodontitis.

1. Introduction

Periodontal disease is generally defined as a chronic inflammatory disease of the periodontal tissue (Loesche, 1976). Although it is initiated by endogenous bacteria in dental plaque (Loesche, 1976), with the development of the oral microbiome, virulent bacteria associated with periodontal disease can become established in the maturing biofilm (Theilade, 1986). Even at low abundance, the presence of these specific pathogens can change the periodontal tissue environment and induce an abnormal host immune response, resulting in the development of periodontal disease (Hajishengallis and Lambris, 2012).

Periodontal disease is the most common infectious disease of small companion animals such as dogs and cats (Niemiec, 2008). Gingivitis is the early stage of periodontal disease and is characterized by gingival inflammation without loss of periodontal tissues such as periodontal ligament and alveolar bone (Philstrom et al., 2005). In cases of gingivitis, gingiva can be returned to a healthy state following careful tooth brushing or professional dental cleaning (Theilade et al., 1966). In comparison, periodontitis, an advanced form of periodontal disease, is characterized by increased periodontal pocket depth resulting from destruction of periodontal tissues and is generally regarded as irreversible, potentially leading to tooth loss (Philstrom et al., 2005).

Periodontitis-related bacterial species have been detected in periodontal specimens collected from dogs and cats (Kato et al., 2011;

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Booij-Vrieling et al., 2010). *Porphyromonas gulae* (formerly *P. gingivalis*-like or an animal biotype of *P. gingivalis*) is a Gram-negative black-pigmented anaerobic bacterium that, along with several other pathogens, is associated with periodontal disease in many animal species (Fournier et al., 2001). To date, *P. gulae* has been isolated from the oral cavities of both dogs (Kato et al., 2011) and cats (Khazandi et al., 2014).

A 41-kDa cell-surface fimbrillin protein, FimA, a subunit of bacterial fimbriae, was first identified and cloned in a human *P. gingivalis* isolate (Yoshimura et al., 1984; Dickinson et al., 1988). Amongst small animals, FimA has been identified in *P. gulae* strains isolated from dogs (Hamada et al., 2008; Yamasaki et al., 2012) and shows a high degree of homology to the original FimA from *P. gingivalis* (Nomura et al., 2012; Yamasaki et al., 2012). FimA proteins from human *P. gingivalis* strains and *P. gulae* strains from dogs have been classified into six (types I–V and Ib) and three (types A, B, and C) genotypes, respectively, with genotypic variation closely related to the severity of periodontal disease (Kuboniwa et al., 2010; Yamasaki et al., 2012). *P. gulae* exhibits a range of virulence attributes, including cytotoxicity in gingival epithelial cells and systemic inflammation in mice (Lenzo et al., 2016). Among the different genotypes, *P. gulae* strains with FimA type C are generally the most virulent, and bacterial DNA from type C strains is frequently detected in oral swab specimens taken from dogs with severe periodontitis (Nomura et al., 2012; Yamasaki et al., 2012).

It has been reported that approximately 80% of cats at 4 years of age suffer from periodontal disease (Harvey et al., 1995), which also effects overall health and wellbeing (Cave et al., 2012). Several studies have focused on periodontitis-related bacterial species detected from cats (Booij-Vrieling et al., 2010; Pérez-Salcedo et al., 2011; Pérez-Salcedo et al., 2013; Khazandi et al., 2014; Pérez-Salcedo et al., 2015). However, specific bacterial genes or proteins related to the development of periodontitis remain unexplored. It is important to study the characteristics of virulence factors in major periodontal bacteria to identify cats at a higher risk of periodontal disease and to implement proper treatment protocols as early in the periodontal disease process as possible. In the present study, the *fimA* genotypes of *P. gulae* isolates, one of the possible virulence factors associated with periodontal disease in cats, were determined using sequence-based analysis of *fimA* genes from clinical *P. gulae* isolates from cats. We also compared the virulence of *P. gulae* isolates belonging to different *fimA* genotypes using cultured cells obtained from periodontal ligament tissue. Furthermore, the distribution of *fimA* genotypes in oral swab specimens taken from cats with different periodontal conditions was investigated.

2. Materials and methods

2.1. Strains and culture conditions

P. gulae isolates were obtained as previously described (Kato et al., 2011) shown in Table 1. Briefly, oral specimens were collected from the gingival margin of the right fourth maxillary premolar using swabs (Seed-Swab^{R} γ-1 or 2; Eiken Chemical Co., Tokyo, Japan). The specimens were stored at 4 °C and then transported on ice to the laboratory within a few days of sampling. Samples were streaked onto trypticase soy (TS) agar (Becton, Dickinson & Co., Franklin Lakes, NJ, USA) supplemented with 5% defibrinated horse blood, hemin (50 mg/ml), and menadione (5 mg/ml) and then cultured under anaerobic conditions at 37 °C for 7–10 days. At least four colonies were randomly selected from each plate and stored in TS broth supplemented with 10% glycerol at −80 °C until use. In preparation for the assays, the isolates were streaked onto Centers for Disease Control (CDC) anaerobe blood agar plates (Becton, Dickinson & Co) and cultured under anaerobic conditions for 4–10 days. Resulting colonies were picked and inoculated into Todd Hewitt broth containing hemin (50 mg/ml) and menadione (5 mg/ml) and then incubated under anaerobic conditions for 1–2 days for use in subsequent studies.

P. gulae isolates were identified from amongst the selected bacteria based on sequence analysis of the 16S rRNA gene, followed by PCR targeting *P. gulae* as described previously (Kato et al., 2011). Briefly, genomic DNA was extracted from each isolate using a Gentra Puregene Yeast/Bact. Kit B (Qiagen, Hilden, Germany) and used as template for a broad-range PCR targeting the 16S rRNA gene using primers 8UA and 1540R, and for a targeted PCR using *P. gulae*-specific primers (Table 2). Reactions were performed in a total volume of 20 µl containing 2 µl of template DNA (20 µg/ml), Ex *Taq* DNA Polymerase (Takara Bio. Inc., Otsu, Japan), and primers according to the manufacturer's protocol. Amplification reactions were performed in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Thermal cycler parameters for the 16S rRNA gene amplification were: initial denaturation at 95 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.5 min, with a final extension at 72 °C for 7 min. Using the *P. gulae*-specific primers, the assay conditions were: initial denaturation at 95 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min. The resulting amplicons were separated by electrophoresis on a 0.7% agarose gel for the 16S rRNA gene and a 1.5% agarose gel for the *P. gulae*-specific assay. Gels were stained with ethidium bromide (0.5 µg/ml) in distilled water and photographed under UV illumination.

The 16S rRNA gene target fragments were extracted from the gel using a QIAEX Gel Extraction Kit (Qiagen) as per the manufacturer's instructions and then directly cloned into pGEM-T Easy (Promega, Madison, WI, USA). The inserts were then sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA), BigDye Xterminator (Thermo Fisher Scientific), and a 3130xl Genetic Analyzer (Thermo Fisher Scientific) by Fasmac Co. (Kanagawa, Japan). The obtained sequences were compared with those available from the GenBank, EMBL, and DDBJ databases using the gapped BLASTN 2.0.5 program on the National Center for Biotechnology Information server (http:/www.ncbi.nlm.nih.gov/

Table 1
Information regarding cats from which *P. gulae* isolates were recovered in the present study.

Breeds	*P. gulae* strains	Age (years)	Sex	Periodontal conditions
Japanese bobtail	C03Db8, C03Db9	3	Male	Normal
Japanese bobtail	C04Db3	15	Female	Normal
Japanese bobtail	C05Db10	11	Female	Periodontitis
Mix	C20Db1	6	Female	No information
Scottish fold	C28Db2	9	Male	Gingivitis
Japanese bobtail	C29Db1	10	Female	Normal
Munchkin	YC9b	4	Female	No information
Japanese bobtail	YC18a	8	Female	Normal
Japanese bobtail	YC21a	0	Female	Normal
Mix	YC35p3, YC35a	15	Female	No information
Unknown	C13Db2	12	Female	No information
Mix	YC34p1	11	Female	No information
Japanese bobtail	C26Db4	7	Female	Gingivitis

Table 2
PCR primers used in the present study.

Specific primer set	Sequence (5'-3')	References
Confirmation of bacterial species		
8UA	AGA GTT TGA TCC TGG CTC AG	Nomura et al. (2006)
1540R	AAG GAG GTG ATC CAG CC	
Detection of *P. gulae*	TTG CTT GGT TGC ATG ATC GG GCT TAT TCT TAC GGT ACA TTC ACA	Kato et al. (2011)
Determination of *fimA* alignment		
33277-F	TTC ATA CGT CGA CGA CTG CG	Nomura et al. (2012)
33277-R	TTG AGG GTT GAT TAC CAA GT	
6/26-F	AAC TAC GAC GCT ATA TGC AA	Nomura et al. (2012)
6/26-R	TAG ACA AAC TAT GAA AGT T	
HG564-F	GAT TTG CTG CTC TTG CTA TGA CAG CTT GTA	Yamasaki et al. (2012)
HG564-R	TTT AGT CGT TTG ACG GGT CGA AGT	
Specification of *fimA* type		
Type A *fimA*		
Pgfim-AF +	TTG TAG AAG GTA ACG CTA CCA TTA GCG TAG	This study
Pgfim-AR[a]	CTT GCC TGC CTT CAA AAC GAT TGC TTT TGG	
Type B *fimA*		
Pgfim-BF	TAA GAT TGA AGT GAA GAT GAG GGA TTC TTA TGT	Nomura et al. (2012)
Pgfim-BR	ATT TCC TCA GAA CTC AAA GGA GTA CCA TCA	
Type C *fimA*		
Pgfim-CF	CGA TTA TGA CCT TGT CGG TAA GAG CTT GGA	Yamasaki et al. (2012)
Pgfim-CR	TGT GGC TTC GTT GTC GCA GAA TCC GGC ATG	

[a] The reverse primer was designed in our previous study (Nomura et al. (2012).

BLAST/). Identification at the species level was defined as showing > 99% sequence identity to the 16S rRNA gene sequence of *P. gulae* type strain ATCC 51,700. Following PCR and sequencing-based bacterial species confirmation, 15 *P. gulae* isolates were used in the following studies.

2.2. Sequencing and analysis of fimA genes

PCR amplification of the *fimA* region was performed using genomic DNA extracted from the *P. gulae* isolates and primer sets 33277-F/R, 6/26-F/R, and HG564-F/R, which were designed based on the complete *fimA* sequences of *Porphyromonas gingivalis* strains isolated from humans (Table 2). Amplification reactions were performed using TaKaRa Ex *Taq* with the following cycling parameters: initial denaturation at 95 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.5 min, with a final extension at 72 °C for 7 min. The resulting products were separated by electrophoresis on a 0.7% agarose gel and then prepared for sequencing as described above. An alignment of the complete nucleotide sequences of the *fimA* genes was carried out as described previously (Yamasaki et al., 2012). The putative FimA amino acid sequences from the type A, B, and C *P. gulae* strains were used to construct a phylogenetic tree using the neighbor-joining method in CLUSTAL W (DNA Databank of Japan) and Tree View software (http://taxonomy.zoology.gla.uk/rod/treeview.html).

2.3. Evaluation of adhesion to and invasion of human periodontal ligament fibroblasts (HPdLF)

To evaluate the virulence of *P. gulae* isolates, HPdLF cells (Lonza, Walkersville, MD, USA) were cultured in stromal cell basal medium (Lonza) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. The adhesion and invasion properties of the *P. gulae* isolates were then evaluated using a previously described method (Abranches et al., 2011; Nomura et al., 2013), with some modifications. Briefly, confluent HPdLF cells grown in stromal medium were adjusted to a density of 1×10^5 cells/ml in antibiotic-free medium before being plated into 24-well trays (1 ml/well). Following incubation at 37 °C for 24 h, the medium was removed and 300 µl of *P. gulae* culture adjusted to approximately 1×10^7 colony-forming units (CFU) in antibiotic-free stromal medium were inoculated into each well. For adhesion assays, the medium was removed following 1.5 h of anaerobic incubation at 37 °C and infected cells were washed three times with 1 ml of phosphate-buffered saline (PBS), followed by addition of 1 ml of sterile distilled water to disrupt the cells. For invasion assays, the medium was removed after 2 h of anaerobic incubation at 37 °C and the cells were washed three times with 1 ml of PBS. A 1 ml aliquot of medium containing gentamycin (300 µg/ml) and metronidazole (200 µg/ml) was then added to each well and plates were incubated at 37 °C for a further 3 h to kill any *P. gulae* adhering to the cell surfaces. After the cells were washed three times with 1 ml of PBS, 1 ml of sterile distilled water was added to disrupt the cells. For both adhesion and invasion assays, a dilution series of the cell lysates was plated onto CDC anaerobe blood agar plates and incubated at 37 °C for 48 h under anaerobic conditions. The numbers of adherent and invasive bacteria were determined by calculating the number of recovered bacteria from the wells with HPdLF cells minus the number of bacterial cells recovered from the wells without HPdLF cells.

Adhesion of *P. gulae* to HPdLF was also observed by scanning electron microscopy as described previously (Nomura et al., 2014). Briefly, HPdLF cells with adhered bacteria were fixed with 3% paraformaldehyde, dehydrated with ethanol, and then freeze-dried with *t*-butyl alcohol. The dried samples were mounted on the stage and coated with osmium for conductive processing and then observed using a scanning electron microscope (Hitachi S-4800; Hitachi High Technologies Corporation, Tokyo, Japan).

2.4. Cell proliferation assays

Cell proliferation assays using methyl tetrazolium (MTT) were performed as described previously (Nomura et al., 2016), with some modifications. Three-hundred microliter aliquots of each of the tested *P. gulae* isolates (1×10^7 CFU) were used to infect 1×10^5 HPdLF cells that had been precultured in 24-well culture plates in antibiotic-free medium at 37 °C for 24 h, as described in the adhesion and invasion assays. The infected cells were incubated for 6 h and then washed three times with 1 ml of PBS. A 1 ml volume of medium containing gentamycin (300 µg/ml) and metronidazole (200 µg/ml) was then added to each well to kill extracellular bacteria and plates were incubated at

37 °C for a further 18 h. The cells were then washed three times with PBS before the addition of 100 µl of MTT (Sigma-Aldrich) solution (5 mg/ml) and 1 ml of medium to each well. After 4 h of incubation, 1 ml of sodium dodecyl sulfate dissolved in 0.01 N HCl was added to each well and the plates were allowed to stand at room temperature for 5 h. A 96-well microplate reader (Thermo Fisher Scientific) was then used to measure the absorbance at 595 nm. The cell proliferation rates were calculated as the relative ratio of infected/uninfected cells and were expressed as means ± SD from three independent experiments.

2.5. In vitro wound healing assay using HPdLF cells

For *in vitro* wound healing assays, confluent cell monolayers (2×10^5 HPdLF cells) grown in gelatin-coated 6-well dishes (2 ml/well) were scraped using a plastic tip to produce a scratch wound, as described previously (Inaba et al., 2004). The exposed surfaces were then re-coated with gelatin dissolved in RPMI 1640 at 37 °C for 1 h. Aliquots (600 µl) of bacteria at a concentration of 2×10^7 CFU were then added to the cell monolayers and incubated for 2 h before the addition of 1 ml of medium containing metronidazole (200 mg/ml) and gentamicin (300 mg/ml). The plates were incubated for a further 18 h and then cell migration was determined using ImageJ software.

2.6. Development of a PCR-based method to classify fimA groups

We previously developed a PCR-based method to classify the *fimA* genotypes of *P. gulae* strains isolated from dogs (Nomura et al., 2012; Yamasaki et al., 2012). Primers used for the detection of type B and C *fimA* genes from dog isolates were used for the sequence analysis of type B and C *P. gulae* isolates, respectively, from cats. However, 4 of the 10 type A isolates from cats did not contain sequences that were consistent with the target region of the type A forward primer for dog isolates, despite the presence of homologous sequences at the site of the canine-isolate type A reverse primer. Thus, we compared the complete *fimA* sequences from all type A *P. gulae* isolates with those from all type B and type C isolates to find a region that was unique to type A isolates and successfully constructed a type A-specific forward primer named Pgufim-AF+ (Table 2, Supplementary Fig. 1 Fig. 1). PCR amplification was performed using TaKaRa Ex *Taq* with the following cycling parameters: an initial denaturation at 95 °C for 4 min followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min. The resulting PCR products were subjected to electrophoresis on a 1.5% agarose gel.

2.7. Distribution of P. Gulae fimA types in oral swab specimens

All study protocols were approved by the Animal Research Committee of Azabu University. Samples were collected from 99 cats visiting the veterinary clinics for health examinations, vaccinations, and medical check-ups. Mean age of the cats was 6.4 years, with a range of 0.3–20.5 years. Prior to the collection of specimens, all cat owners were informed of the content of the study and gave approval for their pets' participation. Oral swab specimens were collected from the gingival margin of the left fourth maxillary premolar as described previously (Kato et al., 2011).

The periodontal condition of each cat was evaluated at the left fourth maxillary premolar based on previously described methods (Finch et al., 2016). Dental calculus accumulation was scored visually, as follows: (0) no calculus, (1) minimal layer of calculus visible on teeth at the gingival margin, (2) moderate amount of calculus visible at the gingival margin, (3) large amount of calculus covering a significant surface area of the tooth and extending into the interdental space. In addition, gingival scores were evaluated visually as follows: (0) no gingivitis, (1) thin area of mild inflammation at the gingival margin, (2) larger area of moderate inflammation affecting gingiva ± bleeding, (3) severe inflammation of gingiva ± bleeding and stomatitis. Severity of the periodontal condition was assessed based on the total of the dental calculus score plus the gingival score, as follows: (0) no dental disease, (1–2) mild, (3–4) moderate, (5–6) severe. Bacterial DNA was extracted from each specimen and PCR analysis was performed using *P. gulae*-specific primer sets, as described above. The *fimA* genotype was then determined for *P. gulae*-positive specimens using specific primer sets for types A, B, and C (Table 2).

2.8. Statistical analyses

Statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Intergroup differences in *in vitro* assays and animal experiments were estimated using Bonferoni's method following the analysis of variance. Fisher's protected least-significant difference test was used to compare the detection frequencies of each *fimA* type in gingivitis- and periodontitis-positive cats compared with those in periodontally-healthy cats. Results were considered significantly different at $P < 0.05$.

3. Results

3.1. Molecular biological properties of P. gulae strains and FimA

Of the 394 colonies selected from the 90 oral specimens, *P. gulae*, *Bacteroides pyogenes*, *Pasteurella multocida*, *Porphyromonas macacae*, *and Porphyromonas circumdentaria* were frequently identified by sequence analysis targeting the 16S rRNA gene. Among these isolates, 15 colonies were confirmed as *P. gulae* based on 16S rRNA gene sequence analysis and PCR-based analysis using *P. gulae*-specific primers. *fimA* was amplified from each of the 15 *P. gulae* isolates and sequence analysis showed that the genes could be classified into three genotypes, designated types A, B, and C, on the basis of sequence differences (Table 3). Among the *P. gulae* isolates, type A was the most frequently observed ($n = 10$), followed by type B ($n = 4$) and type C ($n = 1$). The putative amino acid sequences of the type A (C04Db3), type B (C13Db2), and type C (C26Db4) gene products were considerably different (Fig. 1). Interestingly, both type A and B *P. gulae* isolates were recovered from two cats (Table 1, Supplementary Fig. 2). The putative FimA amino acid sequences from type A, B, and C isolates showed 99–100% identity and similarity to type A, B, and C isolates obtained from dogs, respectively (Table 4), although four type A *P. gulae* isolates (C04Db3, C05Db10, Yc9b, and Yc21a) showed lower sequence identities (95–96%). Fig. 2 shows a phylogenetic tree generated from the FimA sequences of *P.*

Table 3
P. gulae and *P. gingivalis* isolates examined in the present study.

Species	Name	FimA types	Length of *fimA* (bp)	Accession number of *fimA*	Reference
P. gulae	C03Db8[a]	A	1152	LC372924	This study
	C04Db3	A	1152	LC372925	This study
	C05Db10	A	1152	LC372926	This study
	C20Db1	A	1152	LC372927	This study
	C28Db2	A	1152	LC372928	This study
	C29Db1	A	1152	LC372929	This study
	YC9b	A	1152	LC372930	This study
	YC18a	A	1152	LC372931	This study
	YC21a	A	1152	LC372932	This study
	YC35p3[b]	A	1152	LC372933	This study
	C03Db9[a]	B	1161	LC372934	This study
	C13Db2	B	1161	LC372935	This study
	YC34p1	B	1161	LC372936	This study
	YC35a[b]	B	1161	LC372937	This study
	C26Db4	C	1167	LC372938	This study
P. gingivalis	OMZ314	II	1044	D17798	Amano et al. (1999)

[a] *P. gulae* isolates were recovered from the same swab specimen.
[b] *P. gulae* isolates were recovered from the same swab specimen.

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C04Db3-A        MKKTKFFLLGLAALAMTACNKDNEAEPVVEGNATISVVLKTSNPNRVFGVADDEAKVAKL 60
C13Db2-B        MKKTKFFLLGLAALAMTACNKDNEAEPIVEGNATISVVLKTSNPNRAFGVADDEAKVAKL 60
C26Db4-C        MKKTKFFLLGLAALAMTACNKDNEAEPIVETDAT-VSFIIKSGEGRAVGDGLADAKITKL 59
                ************************** **  **       *   *  *     **  **

C04Db3-A        TVMVYKGEQQEAIKSAENAIKVENI-----KCGAGQRTLVVMANTGGMELAGKTLAEVKA 115
C13Db2-B        TVMVYNGEQQEAIKSAENAIKVENI-----KCGAGSRTLVVMANTGGMELAGKTLAEVKA 115
C26Db4-C        TAMVYAGQIQEGIKTVEEAGGVLKVEGIQCKSGANRVL--VIVANHDYDLVGKSLDQVEA 117
                * *** *  ** **  * *  *        * **      *        * ** *  * *

C04Db3-A        LTTELTKENQEAAGLIMTAEPKAIVLKAGKNYIGY-NGAGEGNHIE-NDPLEIKRVHARM 173
C13Db2-B        LTTELTAENQEATGLIMTAEPVDVTLVAGNNYYGY-DGTQGGNQISQGTPLEIKRVHARI 174
C26Db4-C        LTTSLTAENQNAQNLIMTGKSAAFTIKPGSNHYGYPDGTASDNLVSAGAPLAVTRVHAGI 177
                *** ** *** *  ****          * *  **  *    *      **   ****

C04Db3-A        AFTEIKVQMSA-AYDNIYTFTPEKIY-------GLIAKKQSNLFGATLVNADANYLTGSL 225
C13Db2-B        AFTKIEVKMSD-SYVNKYNFTPENIY-------ALVAKKKSNLFGTSLANSDDAYLTGSL 226
C26Db4-C        ------SFAGVEVNMATQYQNYYSFNPADAKIAALVAKKDSKIFGDPLFSDSKAYLYGVQ 231
                                              * *** *  **  *      ** *

C04Db3-A        TTFNGAYTPANYANVPWLS--RNYVAPTA-NAPQGFYVLENDYSANGGTIHPTILCVYGK 282
C13Db2-B        TTFNGAYTPANYTHVAWLG--RGYTAPSN-DAPQGFYVLESAY-AQNAGLRPTILCVKGK 282
C26Db4-C        TP-AGLYTPDA--AGETYELEASLNMNYAEGA--GFYVLESKY-DVTNELRPTILCIYGK 285
                *   * ***                       *  ******  *        *****  **

C04Db3-A        LQKNGADLAG---ADLAAAQAANWVDAEGKTYYPVLVNFNSNNYTYDNGYTPK-NKIERN 338
C13Db2-B        LTKHDGTPLSSEEMTAAFNAGWIVANNDPTTYYPVLVNFESNNYTYTGDAVEK-GKIVRN 341
C26Db4-C        LLDKDGNPLTGQALTDAINAGFCDNEA--TTYYPVLVNYDGNGYIYSGNITQGQNKIVRN 343
                *               *             ********   * * *         ** **

C04Db3-A        HKYDIKLTITGPGTNNPENPITESAHLNVQCTVAEWVLVGQNATW 383
C13Db2-B        HKFDINLTITGPGTNNPENPITESANLNVNCVVAAWKGVVQNVIW 386
C26Db4-C        NHYKITLNITGPGTDTPENPQPVQANLNVTCEVTPWVVVNQAATW 388
                    * * ******  ****    * *** * *  *  * * *   *
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Fig. 1. Alignment of the putative amino acid sequences of the type A, B, and C *fimA* genes of the *P. gulae* isolates. Asterisks indicate amino acid residues that are conserved among all three sequences. Dashes indicate gaps when multiple alignments were performed. Alignment was carried out using CLUSTAL W, available from the DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp).

gulae isolates from dogs and cats. In general, the FimA sequences from both dog and cat isolates were distributed throughout the tree; however, a close relationship was observed between four type A FimA sequences obtained from cats (C04Db3, C05Db10, Yc9b, and Yc21a) that showed lower identities to those obtained from dogs.

3.2. Effect of P. gulae genotype on infection of HPdLF cells

The numbers of adherent bacterial cells from assays using type A and B isolates ranged from 2×10^3 to 6×10^4 CFU, with the numbers of invasive cells calculated as 1–3 $\times$ 10^3 CFU (Fig. 3A, B). In comparison, the number of adherent and invasive C26Db4 cells (type C) was 2×10^5 CFU and 5×10^4 CFU, respectively. The adhesion and invasion rates of the type C isolate (C26Db4) and *P. gingivalis* OMZ314, a highly virulent (genotype II) human isolate, were significantly higher than those of the type A and B isolates ($P < 0.001$). Scanning electron microscopy images also showed that a larger number of C26Db4 cells adhered to HPdLF compared with the type A and B isolates (Fig. 3C). In addition, the type B and C isolates demonstrated strong inhibition of cellular proliferation compared with the type A isolates and no-infection controls (Fig. 3D, E). Although most of the *P. gulae* isolates significantly inhibited the scratch closure of HPdLF, C26Db4 showed the most prominent inhibition (Fig. 3F, G).

3.3. Distribution of fimA genotypes in oral specimens

Most of the oral swab specimens taken from cats showed a positive result from the PCR screening using *P. gulae*-specific primer sets. The

Table 4
BLAST-based identification of proteins with high homology to the putative amino acid sequences of *fimA* genes from cat isolates obtained in the current study.

	P. gulae strains isolated from dogs with highest similarities			BLAST results	
P. gulae strains (FimA types)	Strain Name (FimA types)	Accession number	References	Identity (%)	Similarity (%)
C03Db8 (A)	D024 (A)	BAL46681	Nomura et al. (2012)	382/383 (99)	382/383 (99)
C04Db3 (A)	D060 (A)	BAL46689	Nomura et al. (2012)	368/383 (96)	372/383 (97)
C05Db10 (A)	D060 (A)	BAL46689	Nomura et al. (2012)	368/383 (96)	372/383 (97)
C20Db1 (A)	D034 (A)	BAL46684	Nomura et al. (2012)	383/383 (100)	383/383 (100)
C28Db2 (A)	D066 (A)	BAL46690	Nomura et al. (2012)	383/383 (100)	383/383 (100)
C29Db1 (A)	D034 (A)	BAL46684	Nomura et al. (2012)	379/383 (99)	381/383 (99)
YC9b (A)	D066 (A)	BAL46690	Nomura et al. (2012)	365/383 (95)	371/383 (96)
YC18a (A)	D034 (A)	BAL46684	Nomura et al. (2012)	382/383 (99)	382/383 (99)
YC21a (A)	D066 (A)	BAL46690	Nomura et al. (2012)	365/383 (95)	371/383 (96)
YC35p3 (A)	D024 (A)	BAL46681	Nomura et al. (2012)	382/383 (99)	383/383 (100)
C03Db9 (B)	D077 (B)	BAL46697	Nomura et al. (2012)	385/386 (99)	385/386 (99)
C13Db2 (B)	D053 (B)	BAL46696	Nomura et al. (2012)	385/386 (99)	386/386 (100)
YC34p1 (B)	D053 (B)	BAL46696	Nomura et al. (2012)	385/386 (99)	386/386 (100)
YC35a (B)	D053 (B)	BAL46696	Nomura et al. (2012)	385/386 (99)	386/386 (100)
C26Db4 (C)	D049 (C)	BAM14715	Yamasaki et al. (2012)	386/388 (99)	387/388 (99)

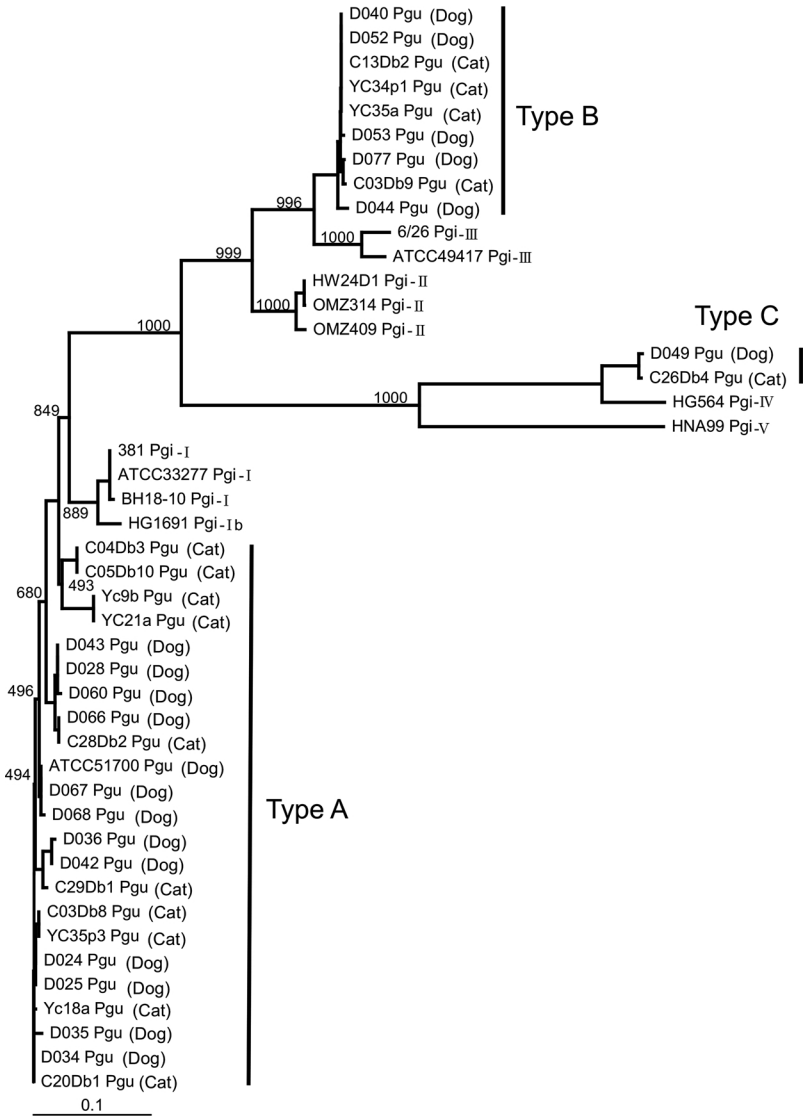

Fig. 2. Evolutionary relationships among *P. gulae* isolates from dogs and cats and *P. gingivalis* isolates from humans based on the putative FimA amino acid sequences. The neighbor-joining method was used to construct the phylogenetic tree using CLUSTAL W (DNA Databank of Japan) and Tree View software (http://taxonomy.zoology.gla.uk/rod/treeview.html). Pgu and Pgi indicate *P. gulae* and *P. gingivalis* strains, respectively. The numbers shown on the tree are bootstrap values.

fimA genotype was then determined for the *P. gulae*-positive specimens using the *fimA*-specific primer sets (Fig. 4A). A total of 99 specimens taken from the 99 cats were analyzed and were classified into healthy ($n = 20$), mild ($n = 41$), moderate ($n = 24$), and severe ($n = 14$) periodontal condition groups (Table 5). Age was positively correlated with the severity of periodontal condition. Representative results from the analyses of clinical specimens are shown in Fig. 4B. Among all specimens, *fimA* type A was significantly more frequently detected than types B and C ($P < 0.01$) (Fig. 4C). In addition, the number type B-positive specimens in the moderate group was significantly higher than that in the healthy group ($P < 0.01$) (Table 5), while type C-positive specimens were significantly more prevalent in the severe group than in the healthy group ($P < 0.05$).

4. Discussion

Periodontal disease is the most common oral concern in small companion animals (Niemiec, 2008). Recently, several studies focusing on the presence of periodontopathic bacterial species in cats and their relationship to periodontal disease have been published (Booij-Vrieling et al., 2010; Pérez-Salcedo et al., 2011; Khazandi et al., 2014; Pérez-Salcedo et al., 2015). Among the bacterial species examined, *P. gulae* was strongly associated with periodontal disease in cats (Pérez-Salcedo et al., 2013). Although most cats at 4 years of age suffer from periodontal disease (Harvey et al., 1995), the relationship between *P. gulae* virulence factors and periodontal condition has not been defined, making it difficult to identify cats at high risk of periodontal disease. In the present study, we hypothesized that *P. gulae* isolates from cats would contain the FimA-encoding gene *fimA*, one of the most important virulence factors of *P. gingivalis* isolates from humans and *P. gulae* isolates from dogs, which has been associated with periodontal condition. We therefore carried out molecular biological analyses to identify and characterize *fimA* in *P. gulae* isolates from cats. Our analyses revealed that all 15 *P. gulae* isolates examined were *fimA*-positive and that the putative amino acid sequences of FimA could be classified into three genotypes.

Among the 15 *P. gulae* isolates, 10 were classified as *fimA* genotype type A and four as type B, with only one isolate classified as type C. The putative amino acid sequences of each of the *fimA* genotypes from cats showed high levels of identity and similarity to those from dogs, and the frequencies of each of the *fimA* genotypes within the two species showed equivalent distribution patterns. However, the *fimA* sequences

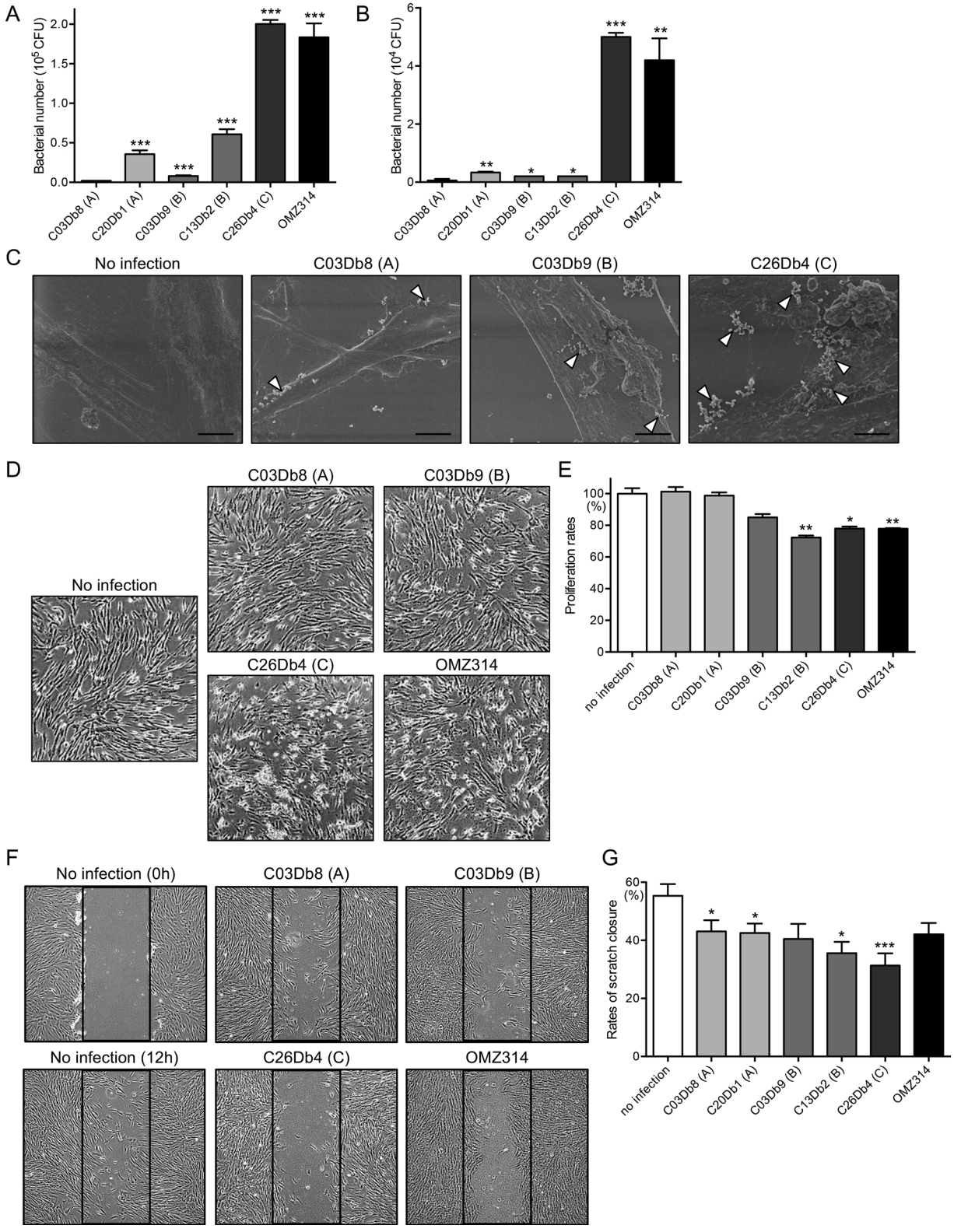

Fig. 3. Effects of *P. gulae* infection on HPdLF cells. Adhesion (A) and invasion properties (B) of *P. gulae* isolates in HPdLF cells (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *versus* C03Db8 infection). (C) Representative scanning electron microscopy images showing adhesion of *P. gulae* to HPdLF cells. Bar = 10 μm. (D) Light microscopy images showing morphology of the HPdLF cells infected with *P. gulae* isolates during the cell proliferation assay. (E) Cell proliferation as measured by MMT assay following infection with *P. gulae* isolates. Data are expressed as the relative ratio of infected/uninfected (no infection) cells and are means ± SD from three independent experiments (*$P < 0.05$, **$P < 0.01$). (F) *In vitro* wound healing assay using HPdLF cells infected with *P. gulae* isolates. Bars indicate the scratched wound regions at 0 h. (G) Rates of wound closure determined from wound healing assays. Data are expressed as means ± SD from three independent experiments.

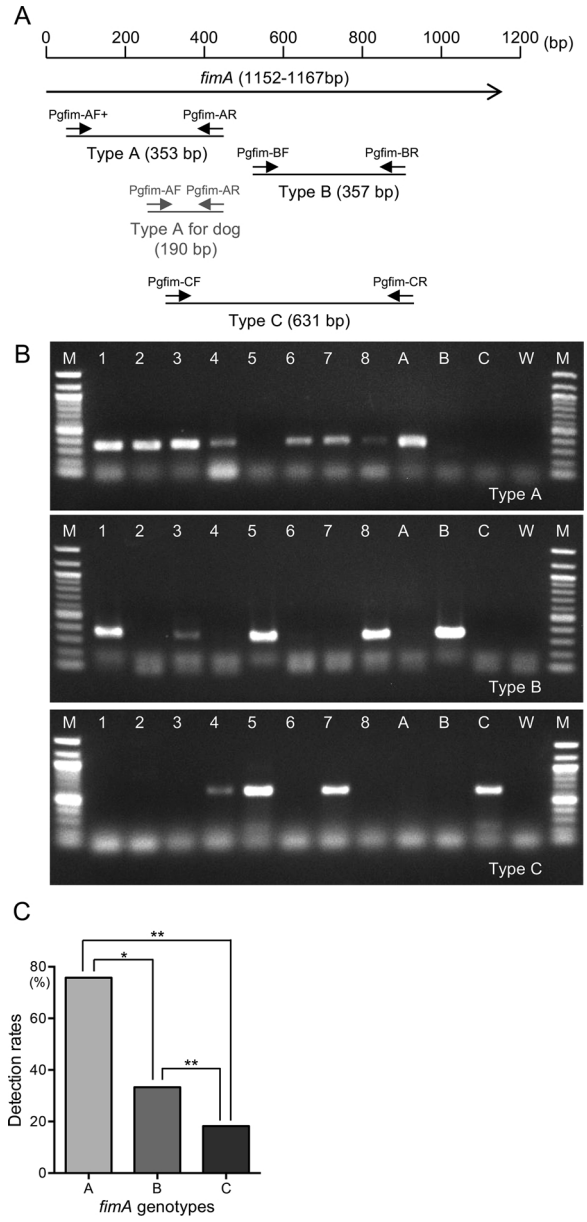

Fig. 4. Primer sets for identification of *fimA* from swab specimens. (A) The locations of the primer sets for detection of *fimA* genotypes. Long arrow indicates the entire length of *fimA*. Short arrows indicate the primer positions. Primers were designed based on the nucleotide alignment of the *fimA* genes. (B) Representative results using swab specimens. Samples in lanes 1–8 are specimens collected from eight different cats. Lanes: M, molecular size marker (100-bp DNA ladder); A, C04Db3; B, C13Db2; C, C26Db4; W, sterile water. (C) Detection rates of *fimA* genes from swab specimens. Significant differences indicated by $^{*}P < 0.05$, $^{**}P < 0.01$.

of four of the type A isolates from cats clustered in a separate location on the phylogenetic tree, indicating that some of the type A *P. gulae* isolates may be specific to cats.

Although we successfully isolated 15 *P. gulae*, isolation of *Porphyromonas* species is difficult, which may hinder research focusing on the virulence of *P. gulae* in periodontal disease. Therefore, we developed a PCR-based method for the direct detection of *fimA* genotypes from oral swab specimens collected from cats without isolation of the bacteria. Such a tool may be useful for analyzing the relationship between different *P. gulae fimA* genotypes and clinical conditions.

We initially tried a PCR-based method that we previously used for the successful detection of *fimA* genotypes in dog oral specimens (Nomura et al., 2012; Yamasaki et al., 2012). However, sequence analyses revealed that the type A primers sometimes produced false-positive results from the cat samples (data not shown). In addition, four of the 10 type A *P. gulae* isolates from cats did not contain the homologous binding site for the type A forward primer, which was designed based on the consensus sequence of type A *P. gulae* strains from dogs. Therefore, we designed a new type A forward primer based on the consensus sequence of all type A *P. gulae* isolates from cats, which was successfully used to amplify type A *fimA* gene sequences from cat oral swab specimens. Interestingly, the sequence of the new type A forward primer was also identified in all canine type A strains present in the GenBank database (http://www.ddbj.nig.ac.jp/); thus, the type A, B, and C primer sets used in the current study could be applied to both cat and dog samples.

Bacterial adhesion to and invasion of host cells are important virulence factors and are essential for establishing infection (Pizarro-Cerdá and Cossart, 2006). *P. gingivalis* adheres to and invades cells obtained from periodontal tissue, and different *fimA* genotypes are associated with differing degrees of virulence (Nakagawa et al., 2006). However, there have been no *in vitro* studies focusing on the adhesion and invasion properties of *P. gulae* in host cells. In the present study, the *fimA* type C *P. gulae* isolate and a type II *P. gingivalis* positive control strain, which is one of the most virulent periodontopathic bacteria, showed significantly greater adhesion to and invasion of periodontal ligament fibroblasts than type A and B isolates. While we selected periodontal ligament fibroblasts, originally cultured from periodontal tissue, to evaluate the virulence of *P. gulae* isolates, further analyses should be performed using other cell lines obtained from periodontal tissue, such as gingival epithelial cells or periodontal ligament fibroblasts from cats or dogs, to verify the results of the current study in other tissue types.

We also analyzed the responses of HPdLF cells to infection with *P. gulae* strains using cell proliferation and *in vitro* wound healing assays. In the cell proliferation assays, both type B and type C *P. gulae* isolates inhibited the growth of HPdLF. *In vitro* wound healing assays were performed to evaluate whether *P. gulae* infection inhibited cell migration. Results showed that all *P. gulae* isolates inhibited scratch closure, although type C strain C26Db4 showed the greatest degree of inhibition. Importantly, the inhibition resulting from infection with C26Db4 was significantly greater than that of OMZ314, a highly virulent clinical *P. gingivalis* strain. Based on these results, we suspect that *P. gulae* infection, regardless of genotype, may induce damage of periodontal tissues, but that cats infected with type C *P. gulae* might have a greater risk of impairment of periodontal cellular function.

The severity of the periodontal condition was positively correlated with age in cats, which is in accordance with results obtained from dogs (Yamasaki et al., 2012). In dogs, *P. gulae* detection rates were also positively correlated with the severity of periodontal condition. In addition, the rates of *P. gulae* recovery in periodontally-healthy and gingivitis-positive dogs were not high, at 63.6% and 77.7%, respectively, whereas 100% of periodontitis subjects were positive for *P. gulae* (Yamasaki et al., 2012). However, the detection rates of *P. gulae* in cats were approximately 90% regardless of the periodontal condition. Based on these results, it seems likely that *P. gulae* may more readily colonize the oral cavities of cats compared with dogs.

Although we assessed dental calculus accumulation and gingival condition using a previously described method (Finch et al., 2016), we could not evaluate bone loss using dental X-ray, a useful method of differentiating between gingivitis and periodontitis, because very few owners gave permission for radiographic determination under general anesthesia. However, we did identify a correlation between the distribution of *P. gulae fimA* types and oral condition; thus, a more detailed analysis including evaluation of bone loss should be performed in future studies.

The distribution frequencies of the type A, B, and C *P. gulae fimA* genotypes in the oral specimens collected from cats were approximately

Table 5
Distribution frequency of *fimA* genotypes among *P. gulae*-positive isolates recovered from swab specimens obtained from cats.

	Healthy (n = 20)	Mild (n = 41)	Moderate (n = 24)	Severe (n = 14)
Age [mean ± standard deviation]	3.7 ± 0.6	4.7 ± 0.7	7.9 ± 1.1*	12.9 ± 1.4*
P. gulae-positive	18 (90.0 %)	37 (90.2 %)	23 (95.8 %)	12 (85.7 %)
A positive	15 (75.0%)	34 (82.9%)	16 (66.7%)	10 (71.4%)
B positive	4 (20.0%)	11 (26.8%)	15 (62.5%)**	3 (21.4%)
C positive	1 (5.0 %)	8 (19.5 %)	5 (20.8 %)	4 (28.6 %)*
Untypeable	2 (10.0 %)	2 (4.9 %)	0 (0 %)	1 (7.1 %)
Single type	12 (60.0 %)	20 (48.8 %)	12 (50.0 %)	7 (50.0 %)
A	11 (55.0 %)	19 (46.3 %)	6 (25.0 %)	6 (42.9 %)
B	1 (5.0 %)	1 (5.0 %)	5 (20.8 %)	1 (7.1 %)
C	0 (0 %)	0 (0 %)	1 (4.2 %)	0 (0 %)
Multiple types	4 (20.0 %)	15 (36.6 %)	11 (45.8 %)	4 (28.6 %)
A and B	3 (15.0 %)	7 (17.1 %)	7 (29.2 %)	0 (0 %)
A and C	1 (5.0 %)	5 (12.2 %)	1 (4.2 %)	2 (14.3 %)
B and C	0 (0 %)	0 (0 %)	1 (4.2 %)	0 (0 %)
A, B and C	0 (0 %)	3 (7.3 %)	2 (8.3 %)	2 (14.3 %)

*$P < 0.05$ and **$P < 0.01$ *versus* the healthy control group.

75%, 30%, and 20%, respectively. The type A genotype was frequently associated with all periodontal conditions, whereas the detection rates of types B and C were higher in the moderate and severe periodontal condition animals, respectively. Although these results imply that *P. gulae fimA* types B and C may be major periodontal pathogens in cats, the detection rate of type C isolates in the severe periodontal condition group was less than 30%. A recent study reported that *P. gulae* contained genes encoding gingipains such as RgpA, RgpB and Kgp, which induced alveolar bone resorption in a mouse periodontitis model (Lenzo et al., 2016). Thus, further studies should be performed to compare additional virulence factors other than FimA among *P. gulae* strains, or to identify co-infection with other highly virulent bacterial species, which may be associated with severe periodontitis.

In summary, we identified and classified the *fimA* genotypes of *P. gulae* isolates from cats. The *fimA* genes were classified into three genotypes, among which, type C showed the highest association with virulence in periodontal cells. However, the prevalence of type C *P. gulae* isolates was lower than that of type A and B isolates. The molecular method developed in this study to classify *fimA* genotypes directly from oral specimens will be a useful tool to determine the risk of periodontitis in cats.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.12.018.

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