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Distribution and molecular characterization of *Porphyromonas gulae* carrying a new *fimA* genotype

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ABSTRACT

Porphyromonas gulae is a gram-negative black-pigmented anaerobe which is known to be a pathogen for periodontitis in dogs. Approximately 41 kDa filamentous appendages on the cell surface (FimA) encoded by the *fimA* gene are regarded as important factors associated with periodontitis. The *fimA* genotype was classified into two major types and strains in type B were shown to be more virulent than those in type A. In the present study, we characterized a strain with a novel *fimA* genotype and designated it as type C. The putative amino acid sequence was shown to be similar to the genotype IV *fimA* of *Porphyromonas gingivalis*, a major pathogen of human periodontitis. Analyses using an oral squamous cell carcinoma cell line derived from tongue primary lesions revealed that the type C strain inhibited proliferation and scratch closure more than genotype A and B strains. In addition, experiments using a mouse abscess model demonstrated that the type C strain could induce much higher systemic inflammation when compared with strains of the other genotypes. Furthermore, molecular analyses of oral swab specimens collected from dogs demonstrated that the detection frequencies of *P. gulae* and the genotype C in the periodontitis group were significantly higher than those in the periodontally healthy group. These results suggest that FimA of *P. gulae* is diverse with the virulence of genotype C strains the highest and that molecular identification of genotype C *P. gulae* could be a possible useful marker for identifying dogs at high risk of developing periodontitis.

1. Introduction

Periodontal diseases are initiated following gingivitis with localized inflammation without the destruction of the periodontal tissues (cementum, periodontal ligament and alveolar bone) (Pihlstrom et al., 2005). Without any intervention, this localized inflammation generally progresses to periodontitis, especially in elder subjects and

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those with systemic disorders. The symptoms of periodontitis are generally irreversible and include severe bleeding, pus discharge and mobility of the affected tooth and ultimately leads to tooth loss. The distribution of the periodontitis-related bacterial species has been investigated in the oral swab specimens taken from Japanese dogs and it was observed that *Porphyromonas gulae*, a gram-negative black-pigmented anaerobe, was one of the species frequently detected (Kato et al., 2011).

The cell surface 41 kDa fimbrillin (FimA), a subunit of fimbriae, was characterized in *P. gulae* and is known to be one of the major factors for periodontitis in dogs (Hamada et al., 2008). Furthermore, *P. gulae* invasion into gingival epithelial cells has also been demonstrated. In our recent study, FimA of *P. gulae* could be classified into two major genotypes, and analyses of the putative amino acid sequences of FimA in many clinical strains revealed that the type A FimA is specific for *P. gulae*, and the amino acid sequence of type B FimA is more closely related to that of genotype III FimA of *Porphyromonas gingivalis*, a major pathogen of human periodontitis (Nomura et al., 2012). On the other hand, there are several *P. gulae*-positive specimens which are negative for both types A and B, indicating the presence of *P. gulae* strains without FimA or those with additional *fimA* genotypes (Nomura et al., 2012).

In the present study, we successfully characterized a new genotype for *fimA* genes in *P. gulae* encoding a novel FimA and designated it as genotype C. The purpose of the present study was to compare the properties of each FimA genotype relative to its virulence potential in periodontitis. In addition, the distribution of the each group was analyzed focusing on the clinical conditions of the dogs sampled.

2. Materials and methods

2.1. Bacterial and cell culture conditions

Table 1 lists the *P. gulae* strains analyzed in the present study, among which all except for one strain (D049) were previously reported (Kato et al., 2011; Nomura et al., 2012). Strain D049 was isolated from an oral swab specimen from a dog and confirmed to be *P. gulae* by a molecular biological method described previously (Kato et al., 2011). In addition, 11 *P. gingivalis* strains listed in Table 1 were also used (Amano et al., 1999; Nakagawa et al., 2000, 2002). *P. gingivalis* and *P. gulae* strains were cultured in mhTS broth [Tripticase soy broth (Becton, Dickinson & Co, Franklin Lakes, NJ, USA) with hemin (50 mg/ml) and menadione (5 mg/ml)] under anaerobic conditions.

The SAS cells, an oral squamous cell carcinoma cell line, were obtained from Japanese Collection of Research Biosources (Tokyo, Japan). Cells were cultured in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2.

Table 1
P. gulae and *P. gingivalis* strains used in the present study.

Species	Name	*fimA* types	Length of *fimA* gene (bp)	Accession numbers of *fimA* gene	References
P. gulae	ATCC 51700[a]	A	1152	AB297918	Hamada et al. (2008)
	D024	A	1152	AB663087	Nomura et al. (2012)
	D025	A	1152	AB663088	Nomura et al. (2012)
	D028	A	1152	AB663089	Nomura et al. (2012)
	D034	A	1152	AB663090	Nomura et al. (2012)
	D035	A	1152	AB663091	Nomura et al. (2012)
	D036	A	1152	AB663092	Nomura et al. (2012)
	D042	A	1152	AB663093	Kato et al. (2011)
	D043	A	1152	AB663094	Kato et al. (2011)
	D060	A	1152	AB663095	Kato et al. (2011)
	D066	A	1152	AB663096	Kato et al. (2011)
	D067	A	1152	AB663097	Kato et al. (2011)
	D068	A	1152	AB663098	Kato et al. (2011)
	B43	B	972[b]	CS228034	Dreier et al. (2005)
	D040[a]	B	1161	AB663099	Kato et al. (2011)
	D044	B	1161	AB663100	Kato et al. (2011)
	D052	B	1161	AB663101	Kato et al. (2011)
	D053	B	1161	AB663102	Kato et al. (2011)
	D077	B	1161	AB663103	Nomura et al. (2012)
	D049[a]	C	1167	AB679295	This study
P. gingivalis	381	I	1044	D17794	Amano et al. (1999)
	ATCC 33277	I	1044	D17795	Amano et al. (1999)
	BH18/10	I	1044	D17796	Amano et al. (1999)
	HW24D1	II	1047	D17797	Amano et al. (1999)
	OMZ314[a]	II	1044	D17798	Amano et al. (1999)
	OMZ409	II	1047	D17799	Amano et al. (1999)
	ATCC 49417	II	1053	D17800	Amano et al. (1999)
	6/26	III	1062	D17801	Amano et al. (1999)
	HG564	IV	1083	D17802	Amano et al. (1999)
	HNA99	V	1104	AB027294	Nakagawa et al. (2000)
	HG1691	Ib	1044	AB058848	Nakagawa et al. (2002)

[a] Strains analyzed in the mouse abscess model.
[b] Only partial sequences corresponding to *fimA* of *P. gulae* are available.

2.2. Sequence of the fimA genes

The nucleotide alignment of the entire length of the *fimA* gene was determined by the method described previously with some modifications as follows (Nomura et al., 2012). PCR was performed with genomic DNA extracted from *P. gulae* D049 using six types of *P. gingivalis fimA* specific sets of primers listed in Table 2 (Amano et al., 1999; Nakagawa et al., 2000, 2002). Since the tested *P. gulae* strains showed positive reaction for *P. gingivalis* type IV *fimA*, we designed the primer sets (HG564-F and HG564-R) based on the adjacent sequence of HG564 (type IV). Next, PCR was performed using these primers to amplify the entire length of the *fimA* gene of *P. gulae*, which was separated by electrophoresis on a 0.7% agarose gel and the amplified DNA fragments were extracted from the gel using a QIAEX gel extraction kit (Qiagen, Düsseldorf, Germany). The DNA was then directly cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA). The nucleotide sequence was determined by the dye-terminator reaction with a DNA sequencing system (373-18 DNA sequencer; Applied Biosystems, Foster City, CA, USA) and an ABI PRISM kit. Data analyses were performed using Gene Works software (IntelliGenetics, Mountain View, CA, USA). The putative amino acid sequences of the FimA of types A and B *P. gulae* strains and those of *P. gingivalis* strains with *fimA* genotypes I through V and Ib listed in Table 1 were compared using the neighbor-joining method to construct a phylogenetic tree using CLUSTAL W (DNA Databank of Japan) and Tree View software (http://taxonomy.zoologygla.ac.uk/rod/treeview.html). Since the sequence from D049 was distinct from those of other strains, we designated it as a novel genotype (type C).

2.3. Construction of a PCR method to classify fimA groups

We previously developed a molecular biological method to classify type A and type B *P. gulae* strains (Nomura et al., 2012). In the present study, the entire *fimA* sequence of D049 was compared to those of genotypes A and B to find specific regions for Type C. A primer set specific for genotype C (Pgufim-CF and Pgufim-CR; Table 2) was then constructed. The amplified fragment was estimated to be 631 bp. The specificities of these primers were tested by the program Amplify (Engels, 1993), based on information on the DNA sequences of *P. gulae* strains obtained in the present study as well as that of *P. gingivalis* in the GenBank database (http://www.ddbj.nig.ac.jp/). The specificity of the primers was also confirmed using the GenBank database. PCR amplification was performed in a total volume of 20 μl with 1 μl of template solution and Ex Taq polymerase (Takara Bio. Inc., Otsu, Japan) according to the supplier's instructions. The PCR amplification reaction was performed in a thermal cycler (iCycler; Bio-Rad, Hercules, CA, USA) with the following cycling parameters: an initial denaturation at 95 °C for 4 min and then 30 cycles consisting 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 PCR products were subjected to electrophoresis in a 1.5% agarose

Table 2
PCR primers used in the present study.

Specific primer set	Sequence (5′–3′)	References
Detection of *P. gulae*		
P. gulae 16S rRNA	TTG CTT GGT TGC ATG ATC GG GCT TAT TCT TAC GGT ACA TTC ACA	Kato et al. (2011)
Determination of *fimA* alignment		
HG564-F[a]	GAT TTG CTG CTC TTG CTA TGA CAG CTT GTA	This study
HG564-R[a]	TTT AGT CGT TTG ACG GGT CGA TTA CCA AGT	
Specification of *fimA* type		
Type A *fimA*		
Pgufim-AF[a]	TGA GAA TAT CAA ATG TGG TGC AGG CTC ACG	Nomura et al. (2012)
Pgufim-AR[a]	CTT GCC TGC CTT CAA AAC GAT TGC TTT TGG	
Type B *fimA*		
Pgufim-BF[a]	TAA GAT TGA AGT GAA GAT GAG CGA TTC TTA TGT	Nomura et al. (2012)
Pgufim-BR[a]	ATT TCC TCA GAA CTC AAA GGA GTA CCA TCA	
Type C *fimA*		
Pgufim-CF[a]	CGA TTA TGA CCT TGT CGG TAA GAG CTT GGA	This study
Pgufim-CR[a]	TGT GGC TTC GTT GTC GCA GAA TCC GGC ATG	
Type I *fimA*	CTG TGT GTT TAT GGC AAA CTT C AAC CCC GCT CCC TGT ATT CCG A	Amano et al. (1999)
Type II *fimA*	ACA ACT ATA CTT ATG ACA ATG G AAC CCC GCT CCC TGT ATT CCG A	Amano et al. (1999)
Type III *fimA*	ATT ACA CCT ACA CAG GTG AGG C AAC CCC GCT CCC TGT ATT CCG A	Amano et al. (1999)
Type IV *fimA*	CTA TTC AGG TGC TAT TAC CCA A AAC CCC GCT CCC TGT ATT CCG A	Amano et al. (1999)
Type V *fimA*	AAC AAC AGT CTC CTT GAC AGT G TAT TGG GGG TCG AAC GTT ACT GTC	Nakagawa et al. (2000)
Type Ib *fimA*	CAG CAG AGC CAA AAA CAA TCG TGT CAG ATA ATT AGC GTC TGC	Nakagawa et al. (2002)

[a] Names of primers are indicated.

gel–Tris–acetate–EDTA buffer. The gel was stained with 0.5 μg ethidium bromide per ml and photographed under UV illumination. A 100 bp DNA ladder (New England BioLabs, Beverly, MA, USA) was used as the molecular size standard. The sensitivity of the PCR assay was determined by using titrated cultures of D049.

2.4. In vitro assays using cultured cells

Infected or control SAS cells were incubated with the TetraColor ONE Cell Proliferation Assay System (SEIKAGAKU, Tokyo, Japan) according to the instructions provided by the manufacturer. Data were calculated as the relative ratio of infected/uninfected (no infection) animals and expressed as the means ± SD from three independent experiments. As for the scratch assays, confluent cell monolayers, grown on gelatin-coated 6 well dishes, were scraped using a plastic tip to form 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. The SAS cells were mixed with bacterial strains at a MOI of 10 and 100 for 2 h and then incubated with metronidazole (200 mg/ml) and gentamicin (300 mg/ml) for 12 h. The rate of scratch closure was determined using ImageJ software as described previously (Rincon et al., 2003).

2.5. Mouse abscess model

All animal procedures and protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry prior to the experiments. The virulence of *P. gulae* D049 was evaluated using a mouse abscess model developed for evaluation of *P. gingivalis* strains with some modifications as follows (Nakano et al., 2004; Nomura et al., 2012). As a reference, *P. gulae* ATCC 51700 (type A), D040 (type B) and *P. gingivalis* OMZ314 (type II) were also analyzed. Fifty female BALB/c mice (5 weeks old), which were randomly divided into 5 groups, were maintained in horizontal-flow cabinets and provided with sterile food and water *ad libitum*. At 40 days of age, a single site approximately 1 cm lateral from the midline on the dorsal surface was depilated, and 0.1 ml of bacterial suspension, 1×10^9 colony-forming units (CFU) of a test strain or PBS (control group), was injected subcutaneously. For quantitative evaluation of the infectious inflammatory change, serum C-reactive protein (CRP) was measured. However, 90% of all mice in the group infected with D049 died by day 4. Therefore, the present experiment was terminated on that day although the original model evaluated inflammatory changes for 2 weeks. Blood specimens (0.1 ml) were collected from an orbital vein at 0, 1, 2 and 4 days after bacterial infection and then centrifuged at 3000 rpm for 10 min to separate the serum. CRP concentrations in sera were calorimetrically quantified using a commercial kit (MOUSE C-REACTIVE PROTEIN (CRP) ELISA TEST KIT; Life Diagnostics, Inc., West Chester, PA, USA) according to the supplier's instructions. The mice were also monitored for signs and symptoms of infection, *i.e.* ruffled hair, abscess formation and emergence of erosion, as described previously.

2.6. Distribution of *P. gulae fimA* genotypes in oral swab specimens

All study protocols were approved by the Animal Research Committee of Azabu University, the Ethics Committees of Osaka University Graduate School of Dentistry and Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. Prior to collection of the specimens, the owners of all dogs were informed of the protocols of the present study and gave approval for their participation. Oral swab specimens were collected from one specific location, the gingival margin of the mandibular left canine in the oral cavity of 139 dogs using swabs (Seed-Swab® γ-1, Eiken Chemical Co. Ltd., Tokyo, Japan, or 2), as described previously (Kato et al., 2011). The ages of the dogs ranged from 0 to 17 years and mean age was 7.72. These dogs came to the clinic for health examinations, vaccinations, coxarthropathy, external otitis, external disc disease, trauma, or diarrhea. All of the dogs were reported to be kept indoors.

The periodontal condition of each dog was evaluated by measuring several parameters of a representative tooth (mandibular left canine) as follows. The periodontal pocket depth was measured to the nearest millimeter around the circumference of each tooth from the gingival margin to the deepest probing point, using a round-ended probe tip 0.4 mm in diameter. The maximum values were recorded as the values for periodontal pocket depth of each tooth. The periodontal pocket depths of 3 mm or less for large dogs, 2 mm or less for medium and small dogs were regarded as healthy. In addition, other criteria, such as no bleeding on probing, no pus discharge and no tooth mobility were also observed in healthy subjects. When observing the deeper periodontal pockets and bleeding on probing, the dogs were diagnosed for gingivitis or periodontitis. Periodontitis was diagnosed by pathological mobility due to obvious destruction of periodontal tissues, such as alveolar bone. The bacterial DNA of each specimen was extracted and PCR was performed using *P. gulae*-specific sets of primers, as described above. The *fimA* genotype was determined for *P. gulae*-positive specimens using specific primer sets for types A, B and C (Table 2).

2.7. Statistical analyses

Statistical analyses were performed using the computational software package StatView 5.0 (SAS Institute Inc., Cary, NC, USA) and Prism 4 (GraphPad Software Inc., La Jolla, CA, USA). Intergroup differences in *in vitro* assays and animal experiments were estimated using Bonferroni's method after an analysis of variance (ANOVA). Fisher's protected least-significant difference test was utilized to compare the detection frequencies of each *fimA* genotype in each gingival condition. Odds ratio (OR) and 95% confidence interval (CI_{95}) values were calculated to determine any significant association regarding each *fimA* genotype in dogs with each periodontal condition. A *P* value of less than 0.05 was considered statistically significant.

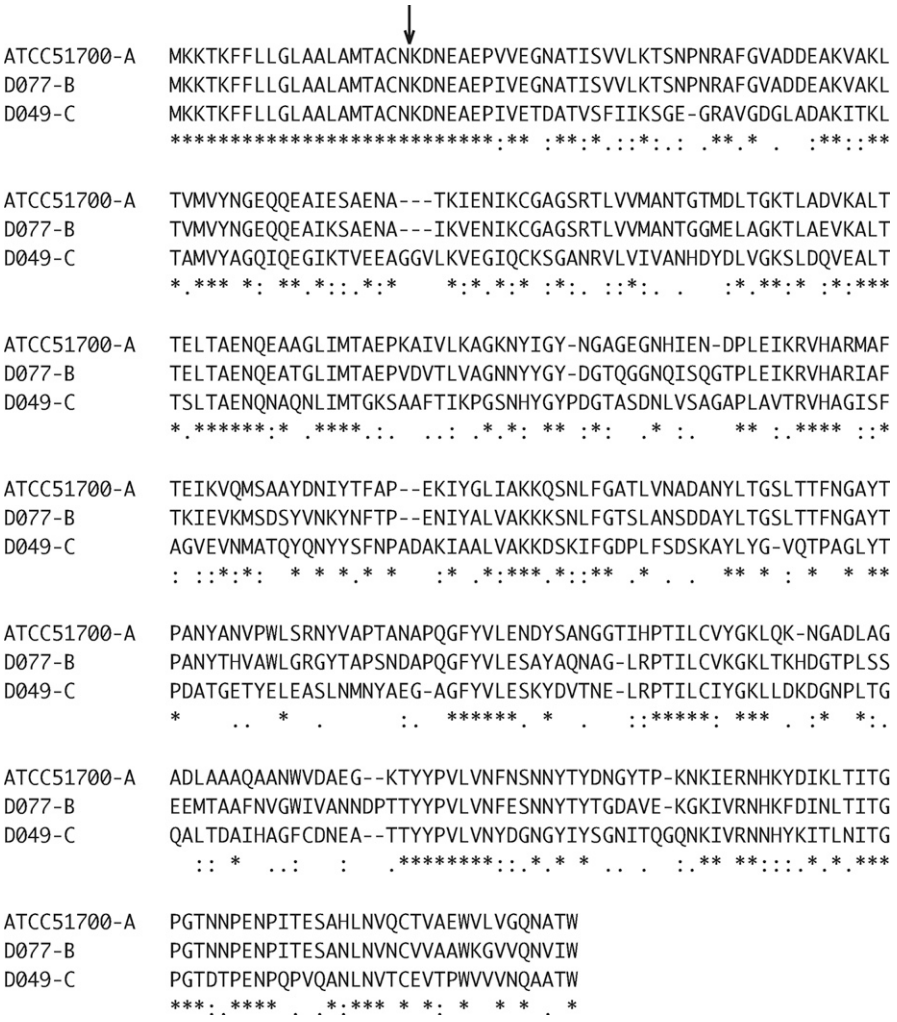

Fig. 1. Comparison of the deduced amino acid sequences of FimAs encoded by type A, B and C *fimA* genes of *P. gulae*. Single and double dots indicate similar and highly similar amino acid residues, respectively. Dashes indicate the gaps when multiple alignments were performed. The arrow indicates the cleavage site estimated by the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The alignment was made using the CLUSTAL W program of the DNA Data Bank of Japan.

3. Results

3.1. Molecular biological properties of type C FimA

The entire length of the *fimA* gene of *P. gulae* strain D049 is 1167 bp, which was deposited in the DNA Database of Japan under accession no. AB679295. Fig. 1 shows the deduced amino acid sequence of strains ATCC 51700 (type A) and D077 (type B) as compared to that of D049 (type C). The FimA in D049 and that in *P. gulae* types A and B strains ranged from 49 to 50% identical, which was almost similar to those of types I, II, III, and Ib FimA in *P. gingivalis*. On the other hand, the identity of type C FimA with that in *P. gingivalis* HNA99 was 59%. In contrast, the FimA of D049 and that of *P. gingivalis* HG564 (type IV) showed 92% identity. Fig. 2 shows a phylogenetic tree comprised of the FimA of *P. gingivalis* types I through V and Ib as well as *P. gulae* types A, B and C. Type C FimA was shown to be the most closely related to genotype IV *P. gingivalis*.

3.2. Effects of P. gulae infection in SAS cells

P. gulae ATCC 51700 (type A) and D049 (type C) caused the cells to change into a contracted and rounded morphology, which was similar to that produced by *P. gingivalis* OMZ314 at a MOI 100 (Fig. 3A). In a proliferation assay, D049 (type C), but not types A and B strains, inhibited cell proliferation at a MOI 10 (Fig. 3B). In addition, D049 showed the maximum inhibition of cell proliferation and the other strains, except for D066 (type A), slowed proliferation at a MOI 100 (Fig. 3C). As for scratch closure assays, the migration rate of D049 was 42.4%, which was the lowest rate among the strains tested at a MOI 10 (Fig. 3D). The closure rate of D049-infected cells decreased to 31.4% at a MOI 100, although the rates for the other strains were shown to be similar to D049 or slightly decreased (data not shown).

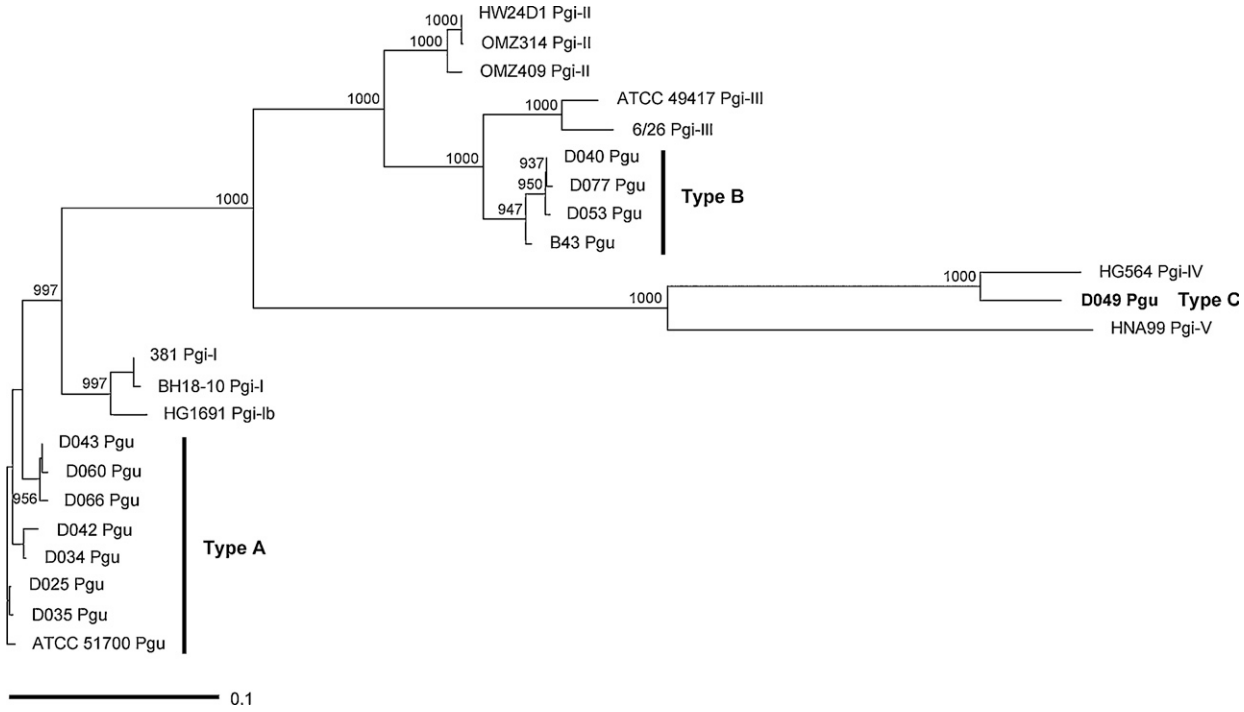

Fig. 2. Evolutionary relationship based on the synonymous site variation in the putative amino acid sequences of FimA encoded by *fimA* genes of *P. gulae* and *P. gingivalis*. The neighbor-joining method was used to construct the phylogenetic tree using CLUSTAL W (DNA Databank of Japan) and Tree View software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Pgu and Pgi indicate *P. gulae* and *P. gingivalis*, respectively. The roman numerals following Pgi indicate the *fimA* genotypes of *P. gingivalis*. The numbers shown in the tree are bootstrap values.

3.3. Mouse abscess model

Subcutaneous injections of D049 (type C) caused drastic weakened systemic conditions in the mice on Day 1 and the formation of erosions was observed on Day 2 although similar lesions were not observed in the mice injected with types A and B strains (Fig. 4). The serum CRP values in mice infected with D049 on Days 1 and 2 were significantly higher than those injected with the other strains (data not shown). On Day 4, 90% of all mice injected with D049 died.

3.4. Clinical specimens

139 specimens were divided into periodontally healthy ($n = 22$), gingivitis ($n = 94$) and periodontitis ($n = 23$) groups based on the clinical conditions of the dogs from which the specimens were isolated. The ages of the healthy group (mean 5.05) were significantly lower than those for the gingivitis (7.97) and periodontitis (10.57) groups ($P < 0.01$ and $P < 0.001$, respectively). Comparing the periodontitis and gingivitis groups, ages of the periodontitis group were significantly higher ($P < 0.05$).

The PCR system for identification of type C *fimA* was developed based on the specific nucleotide alignment of type C *fimA* as compared to those of types A and B *fimA* (Fig. 5A). The specificity of this PCR system was confirmed using the strains listed in Table 1, which demonstrated that primers designed for type C *fimA* genes of *P. gulae* did not generate positive bands from the *P. gingivalis* strains tested. In addition, the sensitivity was shown to be approximately 10–100 CFU per reaction (data not shown).

Fig. 5B shows representative results for the analyses of clinical specimens. *P. gulae* was detected in 110 out of 139 (79.1%) oral swab specimens (Table 3). The detection rate for *P. gulae* in the periodontitis group was 100%, which was significantly higher than that in healthy (63.6%) and gingivitis (77.7%) groups ($P < 0.001$ and $P < 0.05$, respectively). Single and multiples genotypes were identified in *P. gulae*-positive specimens and there were a few untypeable specimens in each group. The numbers of the specimens containing multiple genotypes were higher in the gingivitis and periodontitis groups than that of the periodontally healthy group ($P < 0.05$ and $P < 0.01$, respectively). The numbers of subjects with multiple genotypes or type B in the gingivitis and periodontitis groups and those of multiple types or type C in the periodontitis group were higher than those of the healthy group ($P < 0.01$ each).

The OR of *P. gulae* detection for periodontitis was 27.55 (CI_{95}; 1.48–514.10). In addition, those with multiple genotypes, multiple genotypes or single genotypes B and C were 8.23 (CI_{95}; 1.89–35.83), 8.44 (CI_{95}; 2.12–33.61) and 12.75 (CI_{95}; 3.06–53.19), respectively. Furthermore, the OR of multiple types or single type B detection for gingivitis was 4.50 (CI_{95}; 1.42–14.30).

4. Discussion

The present study characterizes a novel *fimA* genotype of *P. gulae*. We previously isolated many *P. gulae* strains

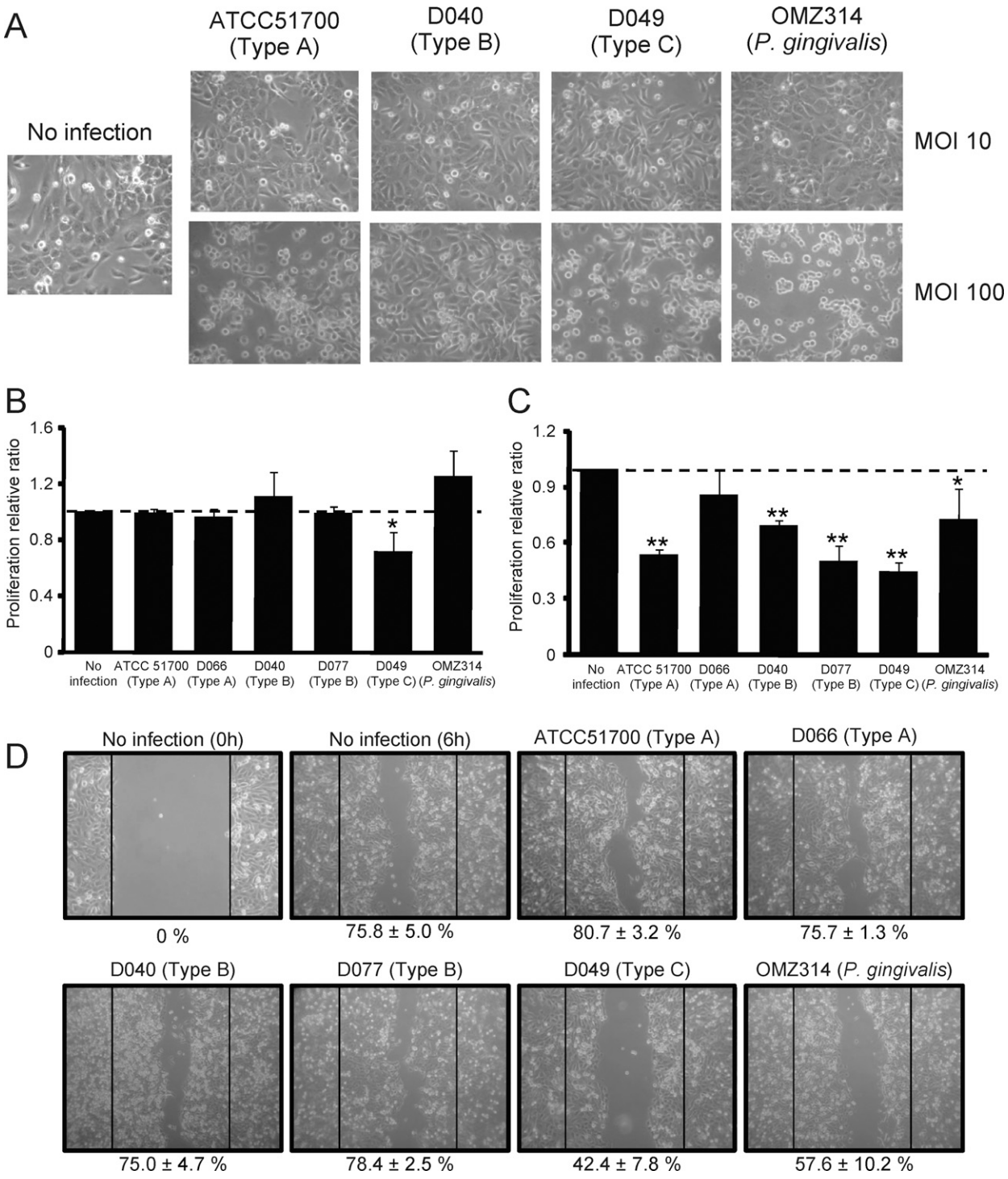

Fig. 3. Effects of *P. gulae* strains infection in cultured cells. (A) Light microscopy images showing morphology of the cells infected with *P. gulae* at a MOI of 10 and 100 for 24 h. Control cells were uninfected. (B and C) Cell proliferation assay measured by tetrazolium following infection with *P. gulae* strains at a MOI of 10 (B) and 100 (C). Data are expressed as relative ratio infected/uninfected (no infection) and are means ± SD from three independent experiments analyzed with a *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$). (D) *In vitro* scratch assay in the cells infected with *P. gulae* at a MOI of 10. Bars show the scratched wound regions at 0 h. The rates of wound closure are determined from assays and are means ± SD from three independent experiments.

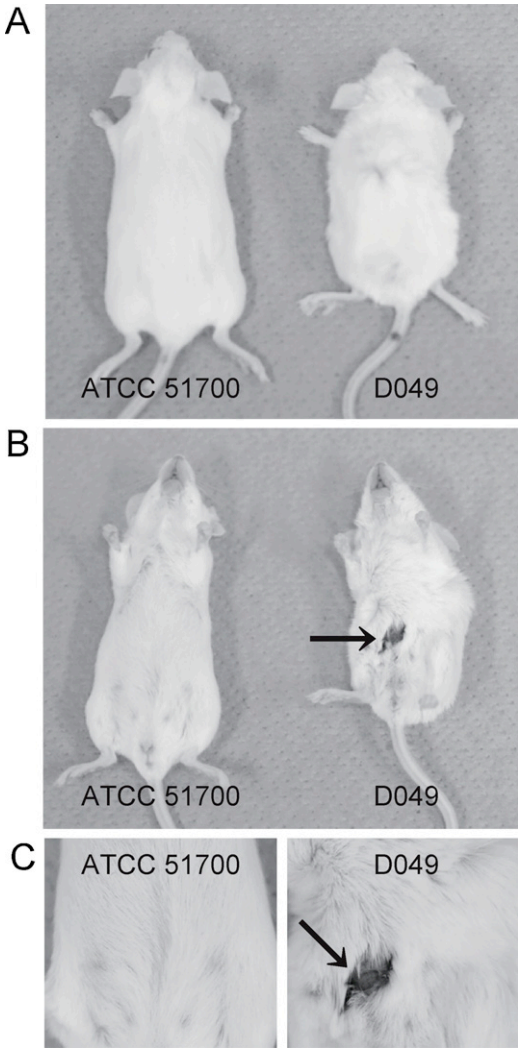

Fig. 4. Macroscopic observation of a representative mouse in the abscess model experiment. (A) Dorsal view, (B) ventral view and (C) magnification of the erosion area. Arrows indicate the areas of erosion.

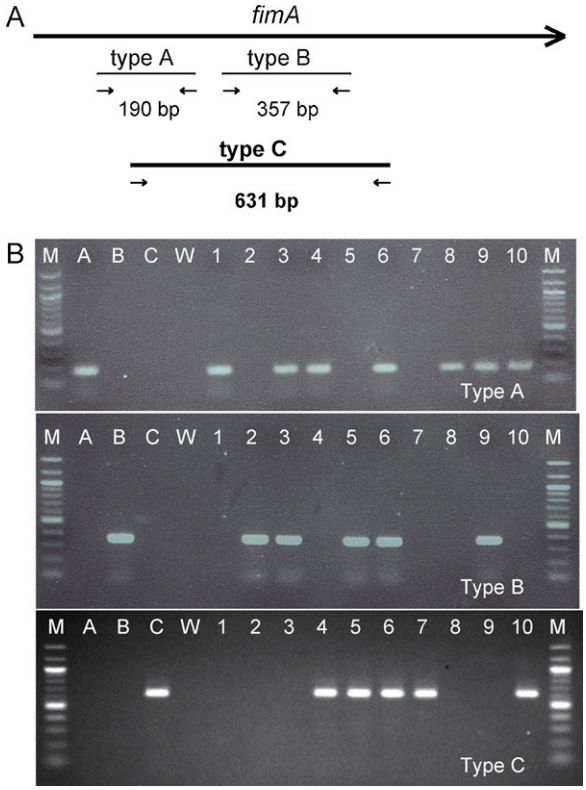

Fig. 5. Construction of the primers specific for type C of *P. gulae*. (A) The position of the primers for *fimA* genotypes A, B and C are illustrated, (B) analyses of clinical specimens for determination of *fimA* types. Lanes: M; molecular size marker (100-bp DNA ladder), A; ATCC 51700, B; D077, C; D049, W, sterile water. 1–10; clinical specimens.

from Japanese dogs, almost all of which were classified into types A or B *fimA* (Kato et al., 2011). Only a single strain (D049) was shown to be of a non-A and non-B FimA genotype, which is designated as type C in the present study. Type A FimA was shown to be *P. gulae*-specific and shares 73% amino acid homology with *P. gulae* type B and *P. gingivalis* type III FimA (Nomura et al., 2012). On the other hand, the present study revealed that the similarity of type C FimA and type IV FimA of *P. gingivalis* was very high. Relative to the virulence of *P. gingivalis*, types II/IV/Ib was shown to be more virulent than types I/III/V (Kuboniwa et al., 2010). Thus, we speculated that the virulence of type C would be the highest among all *fimA* types of *P. gulae*.

Initially, we used cultured cells to perform *in vitro* analyses. To begin the process of characterizing the response of SAS cells to infection with *P. gulae* strains, the infected cells were observed by microscopy and found to be altered in the morphology. Next, we analyzed the inhibition of cell proliferation, which demonstrated that the genotype C strain D049 caused the maximum inhibition of proliferation. It was reported that oral bacteria can inhibit cell migration (Inaba et al., 2004, 2005) and the *in vitro* scratch assay is effective in characterizing the properties of cell proliferation and migration (Liang et al., 2007). Thus, using the cell migration assay, we also demonstrated that D049 inhibited cell migration the most. These results support the proposal that type C fimbriae might be one of the important factors for the impairment of cellular function caused by *P. gulae*. However, further analyses, such as evaluation of multiple type C strains or strains with replacement of type C *fimA* with other genotype *fimA* genes and *vice versa* should be carried out to confirm this hypothesis.

A previous investigation using the mouse abscess model showed that strains with type B *fimA* caused more severe systemic inflammation than those with type A *fimA* (Nomura et al., 2012). The present study clearly showed that the properties inducing systemic inflammation by D049 (genotype C) were strongest compared to other *P. gulae* strains in the mouse model. When we developed a mouse model for evaluating the properties of *P. gingivalis* involved in inducing systemic inflammation, we found that strain OMZ314 (type II *fimA*) possessed the highest potential (Nakano et al., 2004). In fact, subcutaneous injection of OMZ314 resulted in the death of mice under

Table 3
Distribution frequency of *fimA* genotypes in *P. gulae*-positive specimens in periodontally healthy, as well as dogs with gingivitis and periodontitis.

	Healthy ($n = 22$)	Gingivitis ($n = 94$)	Periodontitis ($n = 23$)
Age [mean ± standard deviation (range)]	5.05 ± 4.29 (0–12)	7.97 ± 3.91 (1–17)**	10.57 ± 3.58 (2–16)***,#
P. gulae-positive	14 (63.6%)	73 (77.7%)	23 (100%)**#
Single type	9 (40.9%)	36 (38.3%)	10 (43.5%)
A	7 (31.8%)	20 (21.3%)	4 (17.4%)
B	1 (4.5%)	13 (13.8%)	2 (8.7%)
C	1 (4.5%)	3 (3.2%)	4 (17.4%)
Multiple types	3 (13.6%)	34 (36.2%)*	13 (56.5%)**
A and B	0 (0%)	4 (4.3%)	4 (17.4%)
A and C	1 (4.5%)	16 (17.0%)	8 (34.8%)
B and C	2 (9.1%)	4 (4.3%)	1 (4.3%)
A, B and C	0 (0%)	10 (10.6%)	0 (0%)
Untypeable	2 (9.1%)	3 (3.2%)	0 (0%)
Single A type or multiple types	10 (45.5%)	54 (57.4%)	17 (73.9%)
Single B type or multiple types	4 (18.2%)	47 (50.0%)**	15 (65.2%)**
Single C type or multiple types	4 (18.2%)	37 (39.4%)	17 (73.9%)**

* $P < 0.05$ against healthy group.
** $P < 0.01$ against healthy group.
*** $P < 0.001$ against healthy group.
\# $P < 0.05$ against gingivitis group.

the conditions tested. Since we attempted to develop a mouse model to evaluate systemic virulence over prolonged periods, we modified the mouse model so that all mice survived even in the group infected with OMZ314. Thus, it was very surprising that erosion was identified in almost all of the mice with subcutaneous injections of D049 even on Day 1, and dramatically weakened systemic conditions were observed in almost all of the mice leading to death by Day 4. Thus, we demonstrated that strain D049 could cause extremely severe systemic inflammation.

We are interested in applying the results obtained in the present study for clinical purposes. Therefore, we plan to develop a system for identifying high risk subjects for periodontitis. As a first step, the periodontal condition of the mandibular left canine was evaluated as a representative tooth for several reasons. First, it is relatively easy to visually inspect this region without general anesthesia. In addition, traction of the frenulum close to the mandibular canine can produce wide views of the area. For clinical evaluation, we can further simplify the criteria for diagnosis based on our current knowledge of periodontitis in humans. Periodontal pocket depth, bleeding on probing and tooth mobility were used as major criteria for diagnosis of gingivitis and periodontitis together with other evaluations such as pus discharge.

The distribution of *P. gulae* in the dogs in the periodontitis group was significantly higher than those of the healthy and gingivitis groups. The detection rates of *P. gulae* in Brazilian dogs with and without periodontitis were reported to be 92% and 56%, respectively (Senhorinho et al., 2011), which was almost similar to those of Japanese dogs analyzed in the present study. As for humans, the distribution rates of *P. gingivalis* in Japanese patients with periodontitis and periodontally healthy subjects were reported to be 87.1%, and 36.8%, respectively (Amano et al., 2000), indicating that the presence of *P. gingivalis* itself could be one possible risk marker for periodontitis. The predominant cultural subgingival flora in dogs shows great similarity to the subgingival bacteria from humans at the genus level, but distinct differences at the species level (Dahlén et al., 2012). In the present study, the statistical analyses indicated that *P. gulae* detection itself could be one possible risk factor for development of periodontitis. However, there are other species possibly associated with periodontitis. Further studies should focus on the analyses of the other species related to periodontitis in dogs.

It should be noted that there exist specimens which are negative for genotypes A, B and C *fimA* in *P. gulae*-positive specimens. This finding indicates the presence of *P. gulae* strains without FimA or those with additional *fimA* genotypes. Further studies are required to elucidate the *fimA* genotypes of these specimens. However, we speculate that the existence of unknown type(s) would not influence the results obtained in the present study since the number of such specimens are extremely low. Thus, we can design a system for identification of dogs at high risk for developing periodontitis without elucidating the details of the untypeable specimens.

Prediction of the risk for developing future periodontitis would be clinically beneficial. However, it was reported that the oral flora in health and periodontitis is highly diverse and that it contains high proportions of uncultured and potentially novel species (Riggio et al., 2011). It should be noted that the present study demonstrated that specific FimA genotypes of *P. gulae* could be one possible risk factor. Specifically, three factors, such as the presence of *P. gulae*, detection of multiple *fimA* genotypes with single type B or type C detection were suggested. The molecular biological analyses of the specimens taken from the dogs enable the identification of high risk dogs, who should be provided with intensive care for periodontitis. In addition, we propose that the dogs designated as risk subjects in healthy

and/or gingivitis groups should be carefully followed up for prevention of the onset of subsequent periodontitis. Further studies should also focus on large-scale analyses to identify the geographic specificity of the presence of the *fimA* genotypes. Such worldwide surveys could elucidate the validity of this method for identification of high risk subjects for periodontitis in dogs.

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