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Short communication

Adhesion and invasion to epithelial cells by *fimA* genotypes of *Porphyromonas gingivalis*

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Adhesion to and invasion of epithelial cells by the periodontopathogen *Porphyromonas gingivalis* is promoted by the major fimbriae, encoded by *fimA*. The microorganism can be classified in six genotypes, based on *fimA* sequence, and genotype II strains are more prevalent than others in periodontitis patients. This study aimed to determine the adhesive and invasive abilities on KB cells of different *fimA* allelic variants of *P. gingivalis* isolates. Twenty-two isolates and six reference strains representing the six *fimA* genotypes and non-typeable strains were screened for their adhesion and invasion abilities on KB cells, using standard methods. All strains were able to adhere and, except for one, to invade KB cells. However, these properties were not homogeneous among strains belonging to the same genotype. There was no correlation between adhesion and invasion efficiencies. Isolate KdII 865 (*fimA* genotype II) was the most invasive and the second most adhesive strain, whereas reference strain ATCC 33277 (*fimA* I) showed a low adhesion ability but was highly invasive. These data indicated that *fimA* genotypes of *P. gingivalis* are not related to the adhesion and invasion abilities on KB cells, suggesting that the increased prevalence and proportion of certain genotypes may be attributed to other characteristics besides FimA variation.

Key words: *adhesion, fimA*; genotyping; invasion; *Porphyromonas gingivalis*

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Porphyromonas gingivalis is strongly associated with the etiology of periodontitis and has also been detected in diseased atherosclerotic tissue. It has the ability to invade and replicate in epithelial and endothelial cells (7) and expresses two distinct fimbriae (2). The major fimbriae of *P. gingivalis* are involved in both adhesion to epithelial cells and subsequent signaling events associated with invasion, although other mechanisms of entry into gingival epithelial cells, which are less efficient than fimbriae-dependent pathways, have also been reported (24). Intracellular bacteria may be protected from the host immune response and impinge upon aspects of epithelial cell function, including the synthesis of cytokines and the suppression of apoptosis (8, 24). The *fimA* gene, encoding the fimbrillin (FimA) protein of the major fimbriae, has been classified into six variants (types I to V and Ib) based on the nucleotide sequences (2, 14). Most periodontitis patients from a homogeneous population in Japan (13), from Caucasians in Europe (4) and from a multiracial population in Brazil (10) harbor *P. gingivalis fimA* genotype II strains in subgingival dental plaque. Beyond differences in prevalence, the genotypes seem to differ also in their ability to reach high cell densities (18).

The efficiency of *P. gingivalis* invasion differs among strains. The genetic basis associated with these phenotypes is still not fully elucidated (7, 20). Most studies concerning invasion mechanisms have been performed with the highly invasive strain ATCC 33277, which belongs to the *fimA* genotype I (9). However, a recombinant FimA type II exhibited more adhesive properties to HEp-2 cells than other rFimA types, and this type was the only one to markedly invade the epithelial cells and accumulate around the nuclei (13).

This study was designed to test the hypothesis that adhesion and invasion efficiencies are homogeneous traits in each *fimA* genotype. To accomplish this we assayed the adhesive and invasive abilities on KB cells of a collection of *P. gingivalis* clinical isolates and correlated these data with *fimA* allelic variation.

Twenty-two strains of *P. gingivalis* (15 isolates from Brazilian subjects with periodontitis and one with gingivitis, and six isolates from Swedish periodontitis subjects) were analysed. Reference strains ATCC 33277 (*fimA* I), HW 24D-1 (*fimA* II), 6/26 (*fimA* III), HG564 and W83 (*fimA* IV) and HNA99 (*fimA* V) were used as positive controls (13). One of our own clinical isolates of *P. gingivalis* [strain 315B(A)] from a Brazilian periodontitis subject was used as the type Ib positive control (10). All strains of *P. gingivalis* were genotyped by polymerase chain reaction (PCR) using *fimA* homologous primers and evaluated for adherence and invasion abilities.

Frozen stocks *of P. gingivalis* strains were grown on TSHM blood agar plates [tryptic soy agar (Oxoid LTDA, Basingstoke, UK) with 5% defibrinated sheep blood (Soerensen, Marília, Brazil), 0.5 mg/ml hemin and 1 mg/ml menadione (Sigma Chemical Co, St Louis, MO)] for 5–7 days in an anaerobic chamber (Plas Labs, Lansing, MI). After growth, a 3µl bacteriological loop of cells was suspended in sterile water and lysed by immersing the tubes in boiling water for 10 min (22). After centrifugation, the supernatants containing DNA were kept below −20°C until use.

All strains were identified and genotyped by PCR using primers from Invitrogen (São Paulo, Brazil) and the thermocycler Gene amp PCR system 2400 (Applied Biosystems, Foster City, CA). The PCR products were resolved by electrophoresis in 2% agarose gels in 1X Tris-acetate-EDTA (TAE) buffer. Digital images of the ethidium bromide stained gels were obtained with the Photo PC 3100Z (Epson, Hemel Hempstead, UK). Primer pairs homologous to 16S rRNA of *P. gingivalis* (5′-TGT AGA TGA CTG ATG GTG AAA ACC-3′, 5′-ACG TCA TCC CCA CCT TCC TC-3′) (1) was used for identification, yielding a product of 197 bp for *P. gingivalis*. A negative and a positive control were used. PCR was performed in a total volume of 25 µl consisting of 25 pmol of each primer, 5 µl template DNA, 2.5 µl of 10X PCR buffer, 1.5 mmol $MgCl_2$, 0.5 µl 0.2 mmol dNTP (Invitrogen); 25 pmol of each primer and 2.5 U *Taq* polymerase (Invitrogen). The initial denaturation step occurred at 94°C for 5 min, and was followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 7 min.

PCR primer pairs homologous to each *fimA* variant (*fimA* I: 5′-CTG TGT GTT TAT GGC AAA CTT C-3′ and 5′-AAC CCC GCT CCC TGT ATT CCG A-3′; *fimA* II: 5′-ACA ACT ATA CTT ATG ACA ATG G-3′ and 5′-AAC CCC GCT CCC TGT ATT CCG A-3′; *fimA* III: 5′-ATT ACA CCT ACA CAG GTG AGG C-3′ and 5′-AAC CCC GCT CCC TGT ATT CCG A-3′; *fimA* IV: 5′-CTA TTC AGG TGC TAT TAC CCA A-3′ and 5′-AAC CCC GCT CCC TGT ATT CCG A-3′; *fimA* V: 5′-AAC AAC AGT CTC CTT GAC AGT G-3′ and 5′-TAT TGG GGG TCG AAC GTT ACT GTC-3′) were used for genotyping (1, 12). The composition and conditions for DNA amplification and electrophoresis were the same as described above. Samples in which types I and II amplicons were detected simultaneously were submitted to another amplification reaction using a type Ib primer pair (*fimA* Ib: 5′-CAG CAG AGC CAA AAA CAA TCG-3′ and 5′-TGT CAG ATA ATT AGC GTC TGC-3′) (14). The 271-bp amplicons were digested with *Rsa*I and electrophoresed.

Adhesion and invasion abilities were assessed in KB cells, kindly provided by Dr P. Fives Taylor (University of Vermont, VT). The cells were cultivated in DMEM-AS [Dulbeccos modified Eagle's medium (DMEM; Cultilab, Campinas, Brazil), supplemented with 10% fetal calf serum (Cultilab), penicillin (1664 U/ml) (ICN Biomedicals, Aurora, OH)/streptomycin (745 U/ml) (Calbiochem, Darmstadt, Germany) solution (PEST) 100 mg/ml and 2.20 g/l sodium bicarbonate (Mallinckrodt Baker, Paris, France)]. About 2×10^5 KB cells were inoculated into each well in 24-well tissue culture plates (Corning Inc., Corning, NY) and incubated at 37°C for 24 h to reach a confluent monolayer (5×10^5 KB cells) in DMEM-AS. The wells were washed three times with phosphate-buffered saline (PBS) (pH 7.5, 0.8% NaCl). The *P. gingivalis* strains were grown on tryptic soy broth (Oxoid) supplemented with 0.1% yeast extract (Difco, Le Pont de Claire, France), 0.5 mg/ml hemin and 1 mg/ml menadione (Sigma Chemical Co.) under anaerobic conditions to an OD at $A_{660\ nm} = 1.2$. The bacterial suspensions were centrifuged, resuspended in DMEM without antibiotics and serum at 10^8colony-forming units (CFU)/ml, added to confluent KB monolayers at a multiplicity of infection of 1:200 and incubated at 37°C in 5% CO_2 for 90 min.

For the adhesion assay (19), unattached bacteria were removed after incubation with KB cells by washing the wells three times with PBS. KB cells were detached by adding 1 ml trypsin/EDTA solution (250 mg%, 1:250) (Cultilab) to each well and the CFU of adherent *P. gingivalis* cells were estimated by viable counts.

The method described by Lamont et al. (9) with slight modifications was used for the invasion assay. After a 90-min incubation of bacteria with the KB cells, unattached bacteria were removed by washing the wells three times with PBS, and extracellular bacteria were killed by incubation with metronidazole (0.1 mg/ml; Acros Organics, Geel, Belgium) and gentamicin (0.5 mg/ml; Cultilab) for 60 min. Monolayers were washed, and KB cells were lysed by adding 2 ml of sterile water per well followed by incubation for 20 min. The CFU of invasive organisms were estimated by viable counting. Adhesion and invasion efficiencies were expressed as the percentage of viable bacterial cells in relation to the initial inoculum (21). All assays were performed in triplicate.

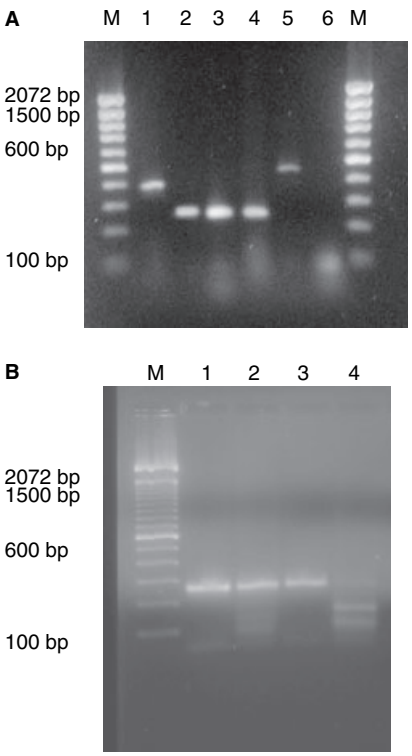

Fig. 1. (A) Amplicons after electrophoresis on a 2% agarose gel. Lanes 1–5, PCR product using primers homologous to gene *fimA* I, II, III, IV and V and DNA from *Porphyromonas gingivalis* strains – lane 1, ATCC 33277 (392 bp); lane 2, HW 24D-1 (257 bp); lane 3, 6/26 (247 bp); lane 4, HG 564 (251 bp); lane 5, HNA 99 (462 bp), lane 6, negative control. (B) Detection of type Ib *fimA* by PCR amplification and *Rsa*I digestion. Lanes 1 and 2, *fimA* amplicons from *P. gingivalis* ATCC 33277 (type I *fimA*) and 315B(A) (type Ib *fimA*) using *fimA* type Ib primers. In lanes 3 to 4, amplicons of lanes 1 and 2 digested with *Rsa*I. Lane M, molecular weight marker 100 bp (Invitrogen, São Paulo, Brazil).

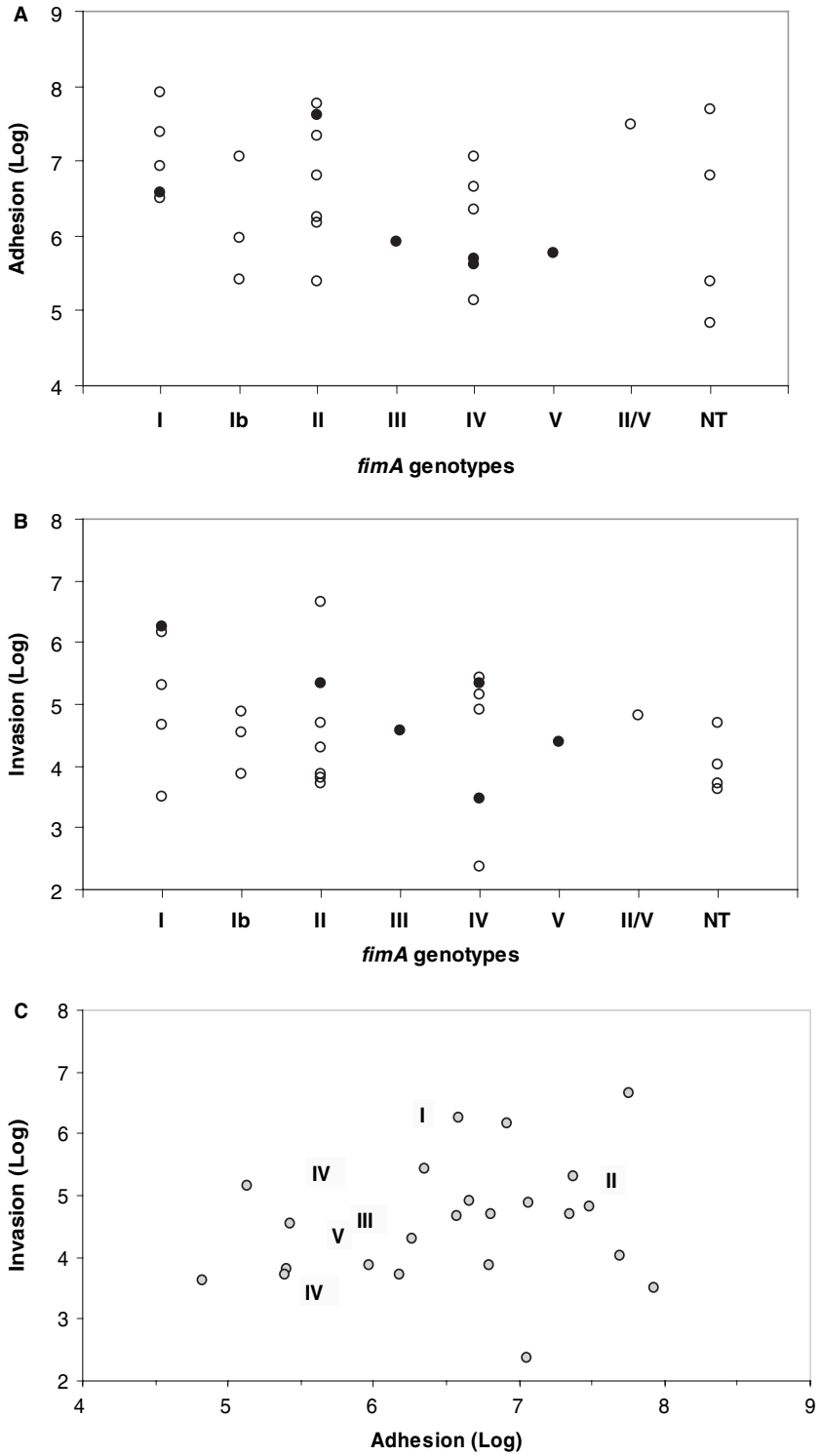

Fig. 2. Distribution of *Porphyromonas gingivalis* strains according to genotype and adherence (A) and to invasion (B) to KB cells (log of CFU/well). Reference strains are marked as black circles. Distribution of *P. gingivalis* strains according to invasion and adhesion in (C) (log of CFU/well). Reference strains are marked as I, ATCC 33277; II, strain HW 24D-1; III, strain 6/26; IV, HG564 (upper) and W83 (lower); and V, HNA99.

Analyses of variance (ANOVAs) were used to test whether there are significant differences among the means of log of CFU of *P. gingivalis* per well after the adherence and invasion assays for the different genotypes.

The *fimA* genotypes of 22 clinical isolates and six reference strains of *P. gingivalis* were determined. The amplicons produced after PCR using specific primer pairs for reference strains are shown in Fig. 1A. Type Ib strains were considered to be those that were amplified with type Ib primers and the amplicons digested with *Rsa*I resulted in 162 bp and 109 bp fragments, as shown in Fig. 1B. Twenty-three of the 28 studied strains were classified in a single known genotype. Among 22 clinical isolates, four belonged to genotype I, three to genotype Ib, six to genotype II and four to genotype IV. PCR with DNA of one strain from Brazil resulted in products with two primers pairs (*fimA* II/V). No amplicons were obtained from the DNA of four isolates, which were classified as 'untypable'.

All strains adhered to KB cells, however a significant strain variability was observed, even when strains from the same genotype were evaluated, as shown in Fig. 2A. All strains were also able to invade KB cells, except for one isolate from a Brazilian periodontitis subject (genotype IV, invasion efficiency ≤0.001%). However, only three strains exhibited invasion efficiency higher than 1% (ATCC 33277, one genotype I clinical isolate from Brazil, and one genotype II clinical isolate from Sweden) and most of the *P. gingivalis* strains studied exhibited moderate (<1% and ≥0.1%) or low (<0.1% and ≥0.001%) invasion efficiency. Among invasive isolates, the number of internalized bacteria varied considerably and ranged from 4.63% (Kd II 865– *fimA* genotype II) to 0.003% (W83– *fimA* genotype IV). No correlation was found between invasion ability and *fimA* genotype and the distribution of strains according to these variables is shown in Fig. 2B. There was also no correlation between adhesion and invasion abilities, as shown in Fig. 2C. There were no significant differences in mean values of *P. gingivalis* cells of each genotype that were able to adhere or to invade KB cells (anova).

The diversity within *P. gingivalis* isolates may result in increased virulence of certain clones. The *fimA* heterogeneity may reflect the ability of genotypes to adhere and survive within the host, because type II strains may reach higher counts in periodontal sites (18). However, there is no correlation between the detection of genotype II strains and disease severity (4). Since adhesion to epithelial cells is a key step in the colonization process, and invasion may enable the bacteria to escape the host defenses, the present study evaluated

the adhesive and invasive abilities of *P. gingivalis* genotypes on KB cells.

The reference strain HW24D-1 (*fimA* II) showed the highest adhesion efficiency among all tested reference strains, as also shown for Hep-2 cells by using type II recombinant *fimA* II (13). However the high adhesion efficiency was not a trait shared by other *fimA* type II isolates. Among clinical isolates, a genotype I clinical isolate exhibited the highest adhesion efficiency. Although adhesion to epithelial cells can occur in the absence of fimbriae (6) and the expression of *fimA* is not sufficient for invasion (7), the lack of fimbriae drastically reduces *P. gingivalis* attachment to and invasion of epithelial cells (23). On the other hand, the present data suggested that *fimA* variation is not responsible for differences in adhesion efficiency. Beyond the fimbriae, gingipain proteases produced by *P. gingivalis* are also involved in adherence to and invasion of epithelial cells (6, 9). Gingipain catalytic activity, especially that of Arg-gingipains, modulated *P. gingivalis* adhesion, and is required to process prefimbrilin to fimbrilin. Moreover, gingipain adhesins also mediate *P. gingivalis* adherence to epithelial cells (5). Furthermore, an *in vivo* study suggested that *P. gingivalis* fimbriae mediate adherence to the root surface at the bases of human periodontal pockets, rather than to the pocket epithelium (16). Possibly, factors affecting *fimA* expression by different clones and/or other adhesins also play a role in *P. gingivalis* adhesion to epithelial cells. However, it was previously shown that *fimA* was transcribed even in a non-invasive isolate and microscopic examination revealed that the cells exhibited fimbriae (7).

The invasion efficiency of *P. gingivalis* is variable among different strains (13), and except for one clinical strain (genotype IV), all the strains tested in the present study were able to invade KB cells. The reference strain ATCC 33277 (*fimA* I) showed the highest invasion efficiency in KB cells among the reference strains, whereas a genotype II isolate was the most invasive among all the studied strains. Although the genotype II reference strain HW 24D-1 exhibited the highest adhesion efficiency, this strain presented an invasion efficiency lower than 1%, differing from data showing that the recombinant fimbria type II from this strain was the only invasive fimbria in Hep-2 cells (13). These data suggest that the lack of invasion is not related to the absence of *fimA*, nor to the amino acid variation in FimA.

It should be noted, however, that the ability to invade may not correlate with virulence. The presence of the *rag* operon in certain virulent strains such as W50 and W83 was also correlated to virulence (6). However, strain W83 exhibited very low invasive ability in the present study, and strain W50 was also shown to be a low invasive organism (7). Although strain ATCC 33277 is more invasive than strain W83, the latter is considered more virulent, possibly because of genes encoding capsular polysaccharide synthesis, and other open reading frames probably obtained by lateral gene transfer (5). Thus, other characteristics besides the invasion ability of genotype II strains may account for their increased prevalence. In a mouse experimental model, most *P. gingivalis fimA* genotype II strains, but not genotype I strains, caused severe inflammation (15). In addition *fimA* type II strains degrade both paxillin and FAK more quickly than other genotypes, which regulate several functions in eukaryotic cells (2). Type II *fimA P. gingivalis* also led to the highest levels of sialic acid in serum, a quantitative inflammatory parameter (15).

In the present study, the adhesive ability did not correlate with the invasion ability, as also shown by Dorn et al. (7), suggesting that other factors beyond adherence promoted by major fimbriae influenced the ability to invade epithelial cells. This hypothesis is supported by findings in other invasive organisms that showed that the presence, or even the absence, of several gene products is correlated with the invasion process (3, 11, 17).

Therefore, the present data suggest that *fimA* diversity in *P. gingivalis* is not correlated with the ability to adhere or to invade KB cells, indicating that other factors regarding a higher colonization ability or evasion of host response may determine a higher prevalence and proportion of genotype II in periodontal sites.

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