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Identification of key determinants in *Porphyromonas gingivalis* host-cell invasion assays

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The periodontal pathogen *Porphyromonas gingivalis* can invade host cells, a virulence trait which may contribute to the persistence of infection at subgingival sites. Whilst the antibiotic protection assay has been commonly employed to investigate and quantify *P. gingivalis* invasion, data obtained have varied widely and a thorough investigation of the factors influencing this is lacking. We investigated the role of a number of bacterial and host-cell factors and report that the growth phase of *P. gingivalis*, source (laboratory strain vs. clinical strain), host-cell identity (cell line vs. primary), host-cell lysis method, and host-cell passage number had no significant effect on bacterial invasion. However, incubation time, host-cell seeding density, method of quantification (viable count vs. DNA), and whether host cells were plated or in suspension, were shown to influence invasion. Also, cells isolated by rapid adhesion to fibronectin exhibited higher levels of *P. gingivalis* invasion, possibly as a result of increased levels of active $\alpha5\beta1$ integrin. Interestingly, this may represent a population of cells with stem cell-like properties. This study provides important new information by identifying the most important factors that influence *P. gingivalis* invasion assays and may help to explain variations in the levels previously reported.

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Porphyromonas gingivalis is a gram-negative anaerobe, the presence of which is associated with the more severe forms of periodontal disease. Its presence shifts the microbiome in the periodontal pocket to a dysbiotic one and hence it is referred to as a 'keystone' pathogen (1). *Porphyromonas gingivalis* has the ability to invade host cells, where it can escape the action of the immune system or therapeutic agents, such as antibiotics (2). This characteristic is thought to be a key virulence factor contributing to the persistence of infection and resultant periodontal disease, along with the presence of a bacterial capsule, lipopolysaccharide, and secretion of proteolytic enzymes (gingipains) (2).

Invasion of host cells is thought to result from interaction between host-cell $\alpha5\beta1$ integrins and *P. gingivalis* fimbriae, resulting in internalization of *P. gingivalis* into the cytoplasm where they can be visualized in the perinuclear region (3). Some reports suggest that *P. gingivalis* is aerotolerant enough to divide with the host cell (3). Studies from our laboratory have shown that host-cell invasion is not homogenous and that, at any one time, a subpopulation of both bacteria and host cells are capable of invading/being invaded (4, 5).

A number of image or culture-based methods can be used to assess bacterial invasion. A commonly used approach is the antibiotic protection assay, whereby incubation of host cells with a suspension of bacteria is followed by treatment with antibiotic to kill extracellular bacteria. Host cells are then lysed and viable counting is performed to quantify the number of intracellular bacteria, often expressed as a percentage of the original inoculum. This method has been used in a number of studies to quantify *P. gingivalis* invasion of host cells; values obtained range from 1% (6) to 8% (5), and even to 25% (3). This considerable variation complicates comparisons between published work and may relate to subtle variations in the experimental protocol. In this study we investigated the importance of a number of bacterial, host-cell, and experimental factors on the invasion of *P. gingivalis* so that workers in the field can be aware of what these are and can work towards a consensus on the way such studies should be performed. In the future this will allow direct comparisons to be made between studies of *P. gingivalis* invasion.

Materials and methods

All materials were purchased from Sigma (Gillingham, UK) unless specified otherwise.

Cell culture

The squamous cell carcinoma (SCC)-derived cell line H357 (kindly provided by Professor S. Prime, University of Bristol) was used along with normal human oral keratinocytes (NOK) isolated with ethical permission and informed consent during minor oral surgery (09/H1308/66). Both cell types were cultured in keratinocyte growth medium (KGM) at 37°C, in 5% CO_2. The KGM comprised a 3:1 ratio of Dulbecco's modified Eagle's medium (DMEM) and Nutrient Mixture F-12 (Ham's F12) containing 10% (v/v) fetal calf serum, L-glutamine (2 mM), adenine (0.18 mM), cholera toxin (0.1 nM), hydrocortisone (5 μg ml^{-1}), insulin (5 μg ml^{-1}), epidermal growth factor (10 ng ml^{-1}), penicillin (100 IU ml^{-1}), streptomycin (100 IU ml^{-1}), and amphotericin B (2.5 μg ml^{-1}).

Bacterial culture

Porphyromonas gingivalis NCTC 11834 (ATCC 33277; LGC Standards, Teddington, UK) was used along with a clinical isolate of *P. gingivalis* from the subgingival plaque of an individual with periodontal disease (ethical permission was obtained: study number 13/YH/0114). Both strains were grown and maintained on fastidious anaerobe agar (FAA; LabM, Bury, UK), supplemented with 5% (v/v) oxalated horse blood (Oxoid, Basingstoke, UK). Bacterial subculture and incubation were performed under anaerobic conditions (10% H_2, 10% CO_2, and 80% N_2), at 37°C, 2 d before starting the experiments. For the purpose of testing *P. gingivalis* growth phases, the bacteria were grown in brain–heart infusion (BHI) broth supplemented with yeast extract (5 mg ml^{-1}), haemin (5 μg ml^{-1}), vitamin K (1 μg ml^{-1}), and cysteine (0.5 mg ml^{-1}). Gram staining was performed to confirm the purity of the bacterial culture before starting the experiments.

Antibiotic protection assay

Bacteria were harvested from agar plates using a sterile swab, washed with PBS, and centrifuged at 13,000 ***g*** for 2 min. The bacteria were counted using a Helber chamber (Hawksley, Lancing, UK), resuspended in DMEM, and adjusted to achieve a multiplicity of infection (MOI) of 1:100. H357 cells were seeded into 24-well plates at 5×10^4 cells per well, incubated for 48 h, and the adherent cells were washed three times with PBS before addition of 2% (w/v) BSA in DMEM for 1 h at 37°C to block the non-specific binding sites for bacteria. To count the exact number of cells in each well before commencing the invasion assay, at least three wells were trypsinized (0.05% trypsin, 0.02% EDTA) and the number of cells in each well were counted using a haemocytometer.

Effect of host-cell density and passage number on bacterial invasion

To determine whether *P. gingivalis* invasion is affected by the confluency of the cells, H357 cells were seeded at 2.5×10^4, 5×10^4, and 7.5×10^4 cells per well in a 24-well plate at 37°C, in 5% CO_2, overnight. To determine whether the number of culture passages of H357 cells has an effect on their sensitivity to *P. gingivalis* invasion, comparison was made between cultures of cells with a low passage number (p56-58) and cultures of cells that had been passaged many times (p218-220).

H357 cells were incubated with bacteria for 90 min at 37°C and then washed three times with PBS to remove the non-adherent bacteria. To kill the extracellular adherent bacteria, 200 μg ml^{-1} of metronidazole in DMEM was added for 1 h at 37°C (5). Cells were then washed three times with PBS, and 1 ml per well of distilled water was added to lyse the cells for 20–30 min followed by 1 min of agitation using a sterile pipette tip to ensure release of the internalized bacteria.

Influence of method of host-cell lysis on bacterial recovery

In some experiments, cell lysis was performed using 0.1% (w/v) saponin dissolved in PBS, and the results were compared with those using distilled water. The resultant bacterial suspension was then serially diluted and plated in duplicate on FAA supplemented with 5% horse blood and incubated for 5 d under anaerobic conditions at 37°C. Viable counting was conducted and the percentage of invasion was expressed as the proportion of the number of viable bacteria recovered after 5 d of incubation, divided by the Helber chamber count of the main bacterial inoculum, multiplied by 100. All invasion assays were performed at least three times in triplicate, and the final results were presented as mean ± SD.

Effect on *P. gingivalis* internalization of host-cell adherence/suspension

To determine whether *P. gingivalis* internalization into H357 oral keratinocytes is affected by whether the host cells are adherent or in suspension, a modified antibiotic-protection assay was conducted. Subconfluent H357 cells were washed with PBS, detached with trypsin/EDTA, washed with 5 ml of DMEM, and centrifuged at 200 ***g*** for 5 min. The cell pellet was resuspended in 1 ml of DMEM, then the number of cells was counted and adjusted to 1×10^6 cells ml^{-1} in triplicate. Cells were centrifuged at 200 ***g*** for 5 min, resuspended with 1 ml of 2% (w/v) BSA in DMEM, and incubated for 1 h at 37°C, in 5% CO_2, on an end-over-end mixer. Cells were counted again to monitor the final cell number before centrifugation at 200 ***g*** for 5 min and resuspension with bacteria at an MOI of 1:100. Cells and bacteria were incubated on an end-over-end mixer for 90 min at 37°C, in 5% CO_2, centrifuged at 200 ***g*** for 5 min, resuspended with 1 ml of DMEM containing metronidazole to kill the adherent bacteria, and incubated for a further hour at 37°C, in 5% CO_2. Cells were washed twice with PBS and centrifuged, before cell lysis, on an end-over-end mixer for 30 min at room temperature. Finally, cells were vortexed at maximum speed for 20 min as an extra step to disrupt the cells and release the bacteria. The lysates were then serially diluted, inoculated on FAA containing 5% horse blood, and viable counting was performed as described above. In parallel, a standard antibiotic protection assay was performed with adherent cell cultures.

Susceptibility, to *P. gingivalis* invasion, of host cells that rapidly adhere to fibronectin

Tissue-culture flasks were coated with 75 μg ml^{-1} of human plasma fibronectin in PBS for 1 h at 37°C and washed with PBS before adding 1% (w/v) BSA in DMEM

for 1 h at 37°C. Following removal of this solution and a final wash with PBS, approximately 2.5×10^7 H357 cells were then seeded for 10 min at 37°C. Non-adherent cells were removed, then the adherent cells were gently washed with PBS and incubated in KGM for 24 h. Adherent cells were then trypsinized, counted, and seeded into 24-well plates for the bacterial invasion assay, along with H357 cells that had not undergone this process.

Influence of method of *P. gingivalis* detection on the invasion assay

To determine the invasion efficiency of *P. gingivalis* using a method not dependent on viable counting, the invasion percentage was calculated using a DNA quantification method (7,8). H357 cells were seeded and infected with *P. gingivalis* NCTC 11834, as described above. Following the cell-lysis step, the lysate was centrifuged at 13,000 ***g*** for 2 min. Then, *P. gingivalis* DNA was extracted and purified using a QIAamp DNA mini kit (Qiagen, Manchester, UK), according to the manufacturer's instructions, using lysozyme, mutanolysin, and lysostaphin for bacterial lysis and proteinase K and RNase A to remove contaminants.

Before calculating the concentration of *P. gingivalis* DNA, a standard curve of *P. gingivalis* DNA concentrations was created using *P. gingivalis* universal primers. The primer sequences used were: GCG AGA GCC TGA ACC AGC CA (forward) and ACT CGT ATC GCC CGT TAT TCC CGT A (reverse) (7). Amplification was conducted in triplicate using the 7900HT Fast Real-Time PCR Detection System in a 96-well plate format (Applied Biosystems, Life Technologies, Paisley, UK). The real-time PCR (qPCR) cycle consisted of an initial denaturation for 5 min at 95°C, followed by 40 cycles of amplification (comprising denaturation of DNA at 95°C, annealing and extension at 72°C for 1 min each, and a final extension for 7 min at 72°C). The reaction mixture was prepared in a total volume of 20 μl containing 10 μl of SYBR Green Master Mix (Thermo Fisher, Runcorn, UK), 7 μl of nuclease-free water, 1 μl each of forward and reverse primers (5 nM), and 1 μl of DNA template.

The DNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher) at 260/280 nm, and adjusted to 1 ng μl^{-1} with nuclease-free water. A standard curve of 1 to 10^{-5} ng of DNA was created using 10-fold serial dilutions of DNA, by plotting the known DNA concentrations against the corresponding point at which the *P. gingivalis* primers showed high linearity ($R > 0.99$). To determine the efficiency of *P. gingivalis* primers used for quantification of genomic DNA, the standard curve slope value was −3.542, indicating an efficiency of 90–100%. The final DNA concentration of *P. gingivalis* was determined by interpolation from the standard curve (Fig. 1A) and the percentage invasion was calculated by comparison with the DNA concentration of the bacterial suspension applied to the cells.

Effect of the growth phase of *P. gingivalis* on invasion

Two-day-old cultures of *P. gingivalis* NCTC 11834 were inoculated in 5 ml of supplemented BHI broth, which was maintained under anaerobic conditions at 37°C overnight. The next day, 1 ml of the culture was transferred into 20 ml of supplemented BHI broth, and the first optical density (OD) measurement (time zero) at 600 nm was recorded using a spectrophotometer (Bio-Rad, Watford, UK). The OD measurements were repeated every 2 h until 30 h of incubation so that a growth curve could be generated (Fig. 1C). Adherent H357 cells were prepared as previously described and exposed to PBS-washed cultures of *P. gingivalis* which had been grown in broth for 12 h (corresponding to the midlog phase) and 30 h (corresponding to the stationary phase).

Statistical methods

All experiments were carried out three times in triplicate and the data were presented using GRAPHPAD PRISM version 7 (GraphPad Software, La Jolla, USA) as mean ± SD. Comparisons were made using paired *t*-tests when $P < 0.05$ was considered significant.

Results

When incubated with *P. gingivalis* for differing periods of time, the level of H357 cell invasion increased from 0.33% (±0.08) at 30 min, to 3.83% (±1.2) at 90 min, and to 5.82% (±1.55) at 240 min (Fig. 2A). A similar, positive correlation was seen when seeding density was increased from 2.5×10^4 cells per well, to 5.0×10^4 cells per well, and then to 7.5×10^4 cells per well, resulting in invasion of 0.23% (±0.03), 0.79% (±0.06) and 1.8% (±0.11), respectively (Fig. 2B). However, neither detergent (3.6% ± 0.4) nor water-based (4.33% ± 0.4) host-cell lysis, or the use of H357 cells at early (2.85% ± 0.24) and late passage (2.32% ± 0.27) numbers, had a significant influence on invasion (Fig. 2C,D). A significant difference in the level of invasion was observed when viable counting methods were used to quantify invasion (2.0% ± 0.22) compared with the use of DNA extraction and quantification (3.8% ± 0.16; Fig. 1B), implying that some bacteria were uncultivable or dead. Growth phase did not significantly influence invasion, as shown by the results obtained for use of *P. gingivalis* at midlog (1.2% ± 1.2) and stationary (2.0% ± 0.9) phases (Fig. 1D).

When H357 cells were exposed to bacteria in suspension, the level of invasion (12.6% ± 0.97) was significantly higher ($P = 0.006$) than when H357 cells were exposed to adherent bacterial cells (5.68% ± 0.9, Fig. 3A). In an attempt to establish the relevance of cell lines in invasion assays, we compared the invasion of H357 cells (3.14% ± 0.4) with primary NOKs (5.26% ± 1.0) and found no statistically significant difference ($P = 0.17$, Fig. 3B). In a similar way, laboratory strains showed no difference in invasive ability (2.03% ± 0.32) compared with a clinical strain (1.39% ± 0.26, $P = 0.6$, Fig. 3C). Finally, H357 cells that rapidly adhered to fibronectin showed higher levels of invasion (5.3% ± 0.78) compared with unsorted cells (3.1% ± 0.9, $P = 0.03$, Fig. 3D).

Discussion

Invasion of host cells by *P. gingivalis* is a key virulence trait and a number of studies have presented

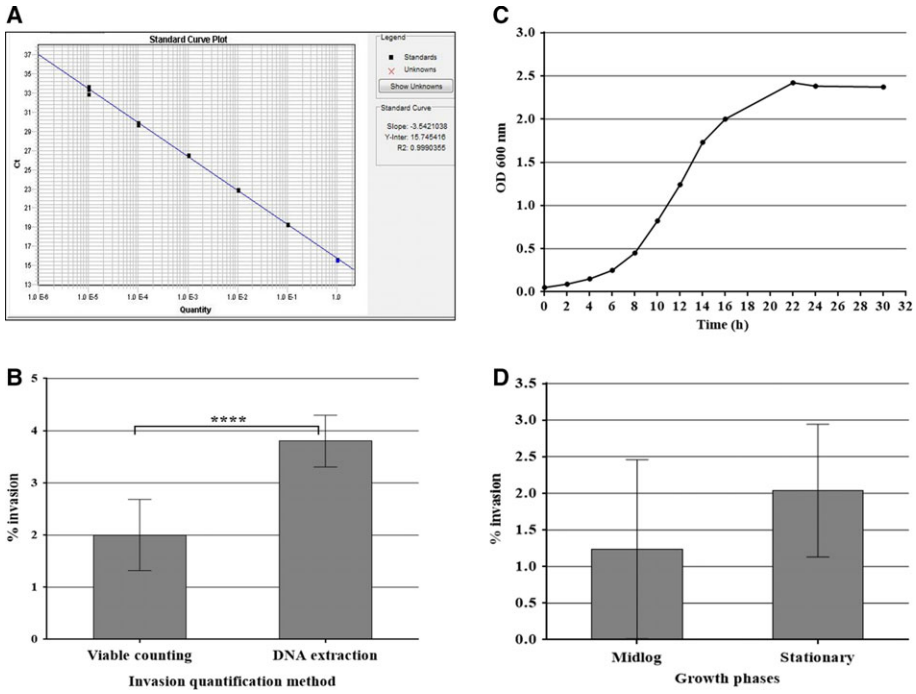

Fig. 1. (A) Standard curve of the real-time PCR (qPCR) cycle threshold (C_t) value against log *Porphyromonas gingivalis* DNA concentration that was used to quantify invasion based on the recovery of DNA from host cells. (B) Comparison of DNA-based and viable counting methods for the detection of invaded *P. gingivalis* (****$P < 0.0001$). (C) Growth curve of optical density at 600 nm (OD 600 nm) with time, used to assess midlog and stationary growth phases. (D) Percentage invasion of host cells by *P. gingivalis* in midlog and stationary phases. Bar charts show mean and SD of three experiments performed in triplicate.

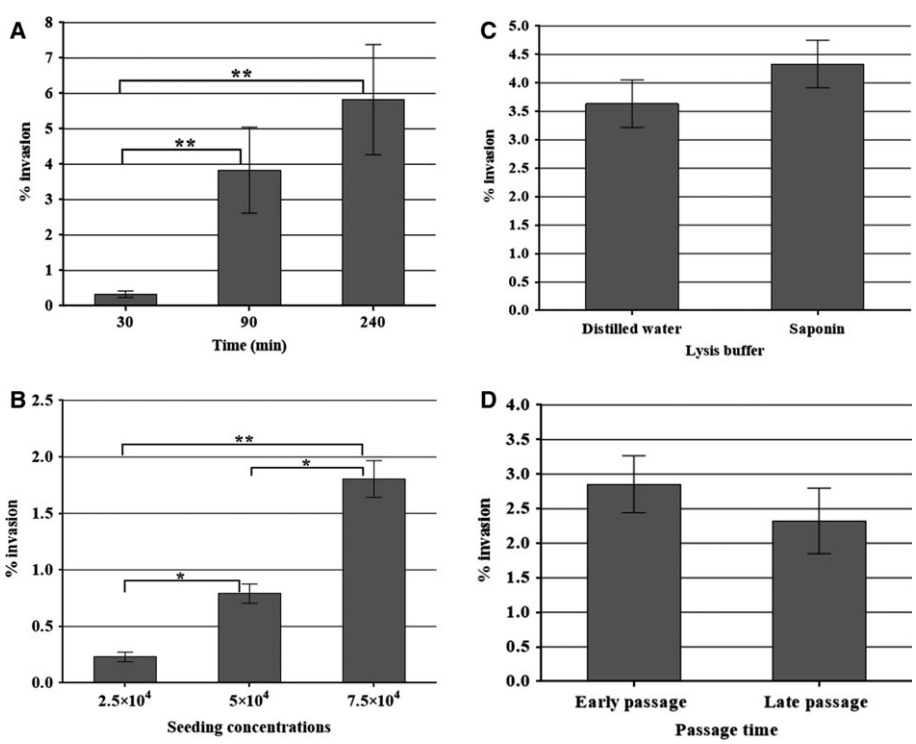

Fig. 2. Effect of incubation time (A), host-cell seeding density (B), host-cell lysis buffer (C), and host-cell passage number (D) on the invasion of *Porphyromonas gingivalis* using an antibiotic protection assay (*$P < 0.05$, **$P < 0.01$). Bar charts show mean and SD of three experiments performed in triplicate.

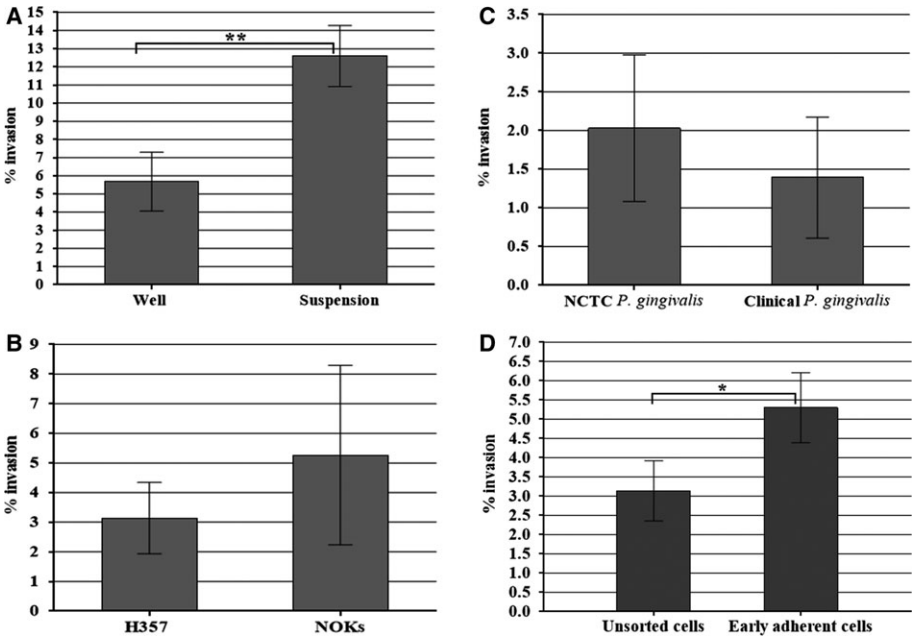

Fig. 3. (A) Comparison of the invasion of host cells (in suspension or adherent) by *Porphyromonas gingivalis* (**$P < 0.001$). (B) Invasion of primary normal oral keratinocytes (NOKs) compared with the H357 cell line. (C) Invasion of H357 cells by a laboratory strain of *P. gingivalis* when compared with a clinical strain isolated from a patient with periodontal disease. (D) Invasion of host cells which were rapidly (10 min) adherent to fibronectin compared with unsorted H357 cells (*$P < 0.05$). Bar charts show mean and SD of three experiments performed in triplicate.

data – most commonly obtained using antibiotic-protection assays – indicating the level of such invasion. Whilst such behavior can be closely followed in vitro, the literature reports a wide variation and thus we decided to investigate the experimental factors that have the greatest influence on this data. The length of time that the bacteria are left with the host cells results in a significant and linear increase in invasion, presumably by providing more opportunity for interactions between the two to occur. Others have noted increased invasion with time (3), and changes in the host-cell cytoskeleton have been detected in response to *P. gingivalis* after as little as 30 min (9). It is possible that by 240 min the intracellular bacteria are dividing (3), which would have an influence on the recovery when this was assessed using viable counting. Entry of *P. gingivalis* into host cells is thought to be primarily mediated by an interaction between bacterial fimbrae and $\alpha5\beta1$ integrin, the levels of which may vary with degree of confluence (10). However, in the present study this was shown not to be the case as invasion increased in a linear manner with plating density. The characteristics of cells can also change in culture over time but this did not appear to be the case for H357, at least with respect to systems mediating bacterial interactions, as cells from early and late passages exhibited similar levels of invasion.

The method by which invaded bacteria are detected has a dramatic effect on the number quantified. In agreement with others (11), our method, using extraction of DNA, was shown to be an extremely accurate and reproducible way of assessing bacterial load. However, as non-viable and cell-associated bacteria would also be detected it is perhaps not surprising that the results were approximately twice as high as the value obtained using the standard viable counting method. Previous reports using samples collected in vivo have suggested that PCR-based methods result in increases, of up to fivefold, in levels of detection (12). However, such DNA-based approaches may have diagnostic relevance as they have been used as part of a panel of biomarkers that may predict the outcome of periodontal treatment (7). Other approaches used to study the interaction of *P. gingivalis* with host cells include the use of fluorescently labelled bacteria and flow cytometry (13).

The physical state of the host cells had an influence on invasion, and cells in suspension were invaded more than twice as often as were adherent cells. This phenomenon has been reported in our previous studies of *Staphylococcus aureus* invasion (14) and may relate to an upregulation or increased availability of host-cell receptors, such as $\alpha5\beta1$ integrin. The similarity between the levels of invasion using broth- or plate-grown *P. gingivalis* suggest that the physical growth state of the bacteria does not influence their ability to invade. Invasion of *S. aureus* is dependent on the growth phase of the bacteria, as expression levels of fibronectin-binding proteins, which mediate interactions with host cells, vary (15). This does not appear to be the case for *P. gingivalis* as the invasion of midlog and stationary phases of *P. gingivalis* were similar.

Invasion correlates with severity of periodontal disease (16, 17) and antibiotic-protection assays can be used to develop therapeutic strategies to prevent

bacterial internalization or target intracellular bacteria. For such data to be applicable to the in-vivo environment it is important to establish whether the use of cell lines and laboratory bacterial strains is appropriate. Our data suggest that invasion of both an oral SCC cell line and primary NOKs by laboratory and clinical strains of *P. gingivalis* are similar. This supports the use of immortalized cells as a clinically relevant model, and other studies, using *S. aureus*, have shown a wide variation in the levels of invasion of normal human keratinocytes when they are derived from different individuals (18).

A higher degree of invasion of *P. gingivalis* into cells that exhibited rapid adherence to fibronectin was observed, possibly because such cells exhibited an increase in the levels or activity of $\alpha5\beta1$ integrin. This cell-surface receptor mediates interaction not only with fibronectin, but also with *P. gingivalis* and other bacteria (14). Interestingly, such rapidly adherent cells may be a stem cell-like population (19) which may represent a mechanism for persistence of infection at periodontal sites as they are not shed from an epithelium. Unpublished work from our laboratory has shown that H357 cells which are rapidly adherent to fibronectin possess enhanced levels of the stem-cell markers CD44, CD24, and CD49, along with an enhanced ability to form colonies when plated at low density.

This study represents a thorough and systematic investigation of the most important host and bacterial factors that influence *P. gingivalis* invasion assays which is currently lacking in the literature and may help to explain variations in the invasion levels previously reported. The behaviour of cell lines and laboratory strains of *P. gingivalis* appears to be similar to that of normal cells and clinical strains, suggesting such assays model in vivo conditions well. It is, however, important to acknowledge that only one clinical strain was used in this study and that other isolates may vary in the level of fimbriation and display different levels of invasion.

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Conflict of interest – The authors have no conflict of interest.

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