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Invasive differences among *Porphyromonas gingivalis* strains from healthy and diseased periodontal sites

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Abstract

Background and Objective—The purpose of this study was to determine any difference between *Porphyromonas gingivalis* isolates from periodontally healthy sites as compared to those from diseased sites with respect to the ability to invade host cells.

Material and Methods—Subgingival plaque samples were obtained from periodontally healthy and diseased sites using paper points. *P. gingivalis* colonies were isolated and tested, using an antibiotic protection assay, for their ability to invade KB cells. *P. gingivalis* 381 and *Escherichia coli* MC1061 were used as controls.

Results—Mean values of $16.79 \pm 0.86 \times 10^3$ colony-forming units/mL and $26.14 \pm 2.11 \times 10^3$ colony-forming units/mL were observed in invasion assays for isolates from periodontally healthy and diseased sites, respectively. *P. gingivalis* present in diseased sites had significantly greater invasive abilities than strains isolated from healthy sites. No statistical difference was noted between male or female subjects concerning the degree of invasion; isolates from diseased sites from both genders had significantly greater invasion abilities than those from healthy sites. A significant correlation was found between the increased invasive capabilities of *P. gingivalis* isolates vs. an increased probing depth.

Conclusion—The increased invasion noted with *P. gingivalis* isolates from diseased sites vs. healthy sites, and the increased invasive capabilities with increasing probing depth, indicate that *P. gingivalis* isolates have a varying ability to invade host cells in the periodontal pocket.

Keywords

Porphyromonas gingivalis; invasion; periodontal disease; virulence

Periodontitis is a disease of the supporting structures of the teeth, causing loss of attachment to the alveolar bone with eventual exfoliation of teeth (1). Severe, generalized periodontitis affects up to 20% of the population, although mild-to-moderate periodontitis is observed in a majority of adults (2). Gram-negative bacteria play an important role in the pathogenesis of human periodontal diseases (3–5) but *Porphyromonas gingivalis* is one of the species most strongly implicated in periodontal disease (6,7).

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Several putative virulence factors have been identified for *P. gingivalis*. These include extracellular proteolytic enzymes that can degrade host tissues and immune response mediators, toxic metabolites and adherence factors that promote colonization (8). For example, *Porphyromonas* strains elaborate proteases with the potential to degrade opsonizing immunoglobulin and complement proteins (9,10). *P. gingivalis* has the capacity to inhibit neutrophil migration and possesses a capsule with antiphagocytic properties (11,12). Epithelial cells act as the first barrier of defence against bacteria, and the invasion of these cells is an early step in the establishment of infection by a microorganism. The invasion of oral epithelial cells by pathogenic bacteria thus probably represents an important virulence factor in the progression of periodontal disease, as the epithelial cells provide protection from the host immune system. *In vitro*, *P. gingivalis* has been shown to attach to and invade several cell types, including gingival epithelial cells and KB cells (13–17). KB cells were once thought to be derived from an oral cancer, but in fact they were found to be derived from a glandular cancer of the cervix (18). Nevertheless, KB cells have been used extensively as a model to study the interaction between *P. gingivalis* and oral host cells (13–15,17,19–29). In KB cells, *P. gingivalis* isolates have been classified as having high, moderate or low invasive properties, or to be non-invasive (30). *P. gingivalis* invasion of epithelial cells is correlated with the activation of calcium-dependent host cell signaling systems and has been reported to require the release of calcium from intracellular storage and a subsequent increase in the concentration of cytosolic calcium (31).

The tooth surface and the periodontal pocket harbor approximately 10^{10} bacteria per gram net weight (32). The composition of the subgingival microflora varies considerably in periodontal health and in various forms of periodontal disease. The pathogenic nature of *P. gingivalis* is suggested by the recovery of these organisms in higher proportions from progressive periodontitis lesions than from quiescent periodontal sites, as well as by the elevated antibody levels found against *P. gingivalis* (serum and gingival crevicular fluid) in patients with periodontitis compared to normal controls. Studies have shown that *P. gingivalis* occurs with greater frequency and at higher levels in sites that appear to be disease active (6,33–38) and that certain periodontal health indicators in individuals are inversely correlated with the presence or with the levels of *P. gingivalis* (39–43). However, other studies have suggested that *P. gingivalis* is found more frequently in periodontally healthy sites than in sites of periodontal disease (44,45).

The number of sites required for an accurate microbiological assessment of periodontitis patients can be significantly decreased by the selection of deep pockets (46). Sites within subjects do not appear to be actively progressing at all times, so that the time of sampling may play as critical a role as the site to be sampled for understanding the pathogenesis of disease (47).

Griffen *et al.* identified 11 heteroduplex types of *P. gingivalis*, some of which were more strongly associated with disease than others (48). An association between *P. gingivalis* strains harboring the *prtC*$^+$ *fimA*$^+$ genotype and predominance in deep pockets or serious attachment loss was also recently suggested (49). This indicates that virulence in human periodontitis varies among strains of *P. gingivalis*, and that subgroups of highly virulent strains may be present at diseased sites. The fact that there are major differences in the degree of virulence of different isolates of *P. gingivalis* suggests that in some instances when suspected pathogens are found in periodontally healthy sites, the strains may be avirulent. An inability to distinguish virulent from avirulent clonal types has impeded the understanding of the pathogenesis of disease. The purpose of this study was to determine if fresh isolates of *P. gingivalis* from the human oral cavity have varying invasive abilities, and, if so, if this variation is related to their presence in healthy and diseased periodontal sites.

Material and methods

Sampling methodology

Study population—This study was approved by the Institutional Review Board of the University of Florida. Subjects for this study were recruited from the clinics of the College of Dentistry at the University of Florida. Potential subjects were screened, examined and selected for participation if they met criteria for sites of both periodontal health and disease in their oral cavity. For the purposes of this study, periodontally healthy sites included pocket depths and attachment levels that were < 5.0 mm and did not bleed upon probing. Periodontal disease sites were those sites with loss of attachment and pocket depths of ≥ 5.0 mm and which bled upon probing. Probing depth measurements were recorded by a single examiner to the nearest 0.5 mm using a Michigan 'O' periodontal probe with Williams markings. Exclusion criteria for the study were: (i) antibiotic therapy within the previous 6 mo; (ii) oral prophylaxis or periodontal surgery in the sites to be sampled within the last 6 mo; (iii) current pregnancy; and (iv) patients with a history of diabetes, blood dyscrasias, or rheumatic fever.

Bacterial sampling—Excess saliva was removed using a sterile gauze pad to minimize the collection of transient contaminating bacteria from an exogenous source. Any apparent supragingival plaque was removed from the surface of the tooth to be sampled using a Gracey curet. A sterile, fine endodontic paper point (Caulk-Dentsply, Milford, DE, USA) was placed, using cotton pliers, in the sulcus of each site for each tooth to be tested until resistance was felt and then left in place for 10 s. Paper points were then placed in a Corning plastic tube (Corning Inc., Corning, NY, USA) containing 1.0 mL of pre-reduced, anaerobically sterilized Ringers transport media and then transported to the laboratory for processing. After a brief vortex, a series of 10-fold serial dilutions of each sample was prepared. The suspended bacteria were streaked onto tryptic soy agar (Difco Laboratories, Detroit, MI, USA) supplemented with 5% sheep blood (Lampire Biological Laboratories, Pipersville, PA, USA), 0.5% yeast extract (Difco), hemin (5 μg/mL) and menadione (5 μg/mL). The plates were then placed at 37°C in a Coy anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA) with an atmosphere of 5% CO_2, 10% H_2 and 85% N_2.

Identification of P. gingivalis isolates—Colonies that were black pigmented after 5–7 d of incubation were identified as *P. gingivalis* as follows: (i) they did not show red fluorescence under long-wave ultraviolet light; (ii) they had pigmentation in the center of the colony; and (iii) they were trypsin positive when tested with *N*-cL-benzoyl-L-arginine-7-amido-4-methylcoumarin HCl. Asingle *P. gingivalis* colony was randomly selected from each sample and restreaked onto agar plates as described above. Colony polymerase chain reaction (PCR) was performed to confirm these colonies as *P. gingivalis* strains. Briefly, one or two colonies were resuspended in QuickExtract DNA extraction solution (Epicenter, Madison, WI, USA) and incubated at 65°C and then at 98°C for 4 min each. The lysate solution was centrifuged and the supernatant analyzed by nested PCR using the Hot Star *Taq* DNA polymerase, as described previously, with specific primers for the 16S rRNA gene of *P. gingivalis* (50).

Bacterial strains and growth conditions

Bacterial subculture—*P. gingivalis* strain 381, originally isolated by A. Tanner (The Forsyth Institute, Boston, MA, USA), was subcultured on tryptic soy agar (Difco) supplemented with 5% sheep blood (Lampire), 0.5% yeast extract (Difco), hemin (5 μg/mL), vitamin K_1 (5 μg/mL) and gentamicin (50 μg/mL) (Sigma Chemical Co., St Louis, MO, USA). All *P. gingivalis* cultures were incubated in the anaerobic chamber. *Escherichia coli* strain MC1061 (kindly provided by A.S. Bleiweis, Gainesville, FL, USA) was subcultured on Luria–Bertani medium consisting of Bacto-Agar (15 g/L; Difco), Bacto-Tryptone (10 g/L; Difco),

yeast extract (5 g/L) and sodium chloride (10 g/L; Fisher Scientific, Springfield, NJ, USA) at 37°C aerobically.

Cell culture—KB cells (ATCC CCL-17) were maintained in Eagle's minimal essential medium (Mediatech, Herndon, VA, USA) supplemented with 10% (v/v) fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT, USA), 200 mM L-glutamine (Sigma) and 100 μg/mL of penicillin–streptomycin (Sigma). KB cells were cultured in 75-cm^2 flasks (Sarstedt, Newton, NC, USA) at 37°C in a humidified atmosphere of 5% CO_2. Confluent mono-layers of KB cells were split by treatment with Hank's balanced salt solution (Mediatech) and trypsin-versene (BioWhittaker, Walkersville, MD, USA).

Antibiotic protection assay

The antibiotic protection assay used in this study was performed as described previously for gingival epithelial cells (26,51). Approximately 10^5 KB cells per well were seeded in 24-well tissue culture plates (Sarstedt), washed three times with phosphate-buffered saline and then infected by the addition of a resuspended, overnight broth culture of 10^7 bacteria per mL of antibiotic-free medium at 37°C. After 90 min of incubation, the medium was removed from the infected cells and the cells were washed three times with phosphate-buffered saline. Medium containing 300 μg/mL of gentamicin (Sigma) and 200 μg/mL of metronidazole (Sigma) was then added to each well and the plates were incubated aerobically at 37°C for an additional 60 min. Control wells containing no cells were also included to establish that the antibiotic treatment was effective at killing the extracellular bacteria. Finally, the medium was removed, the cells were washed three times with phosphate-buffered saline and the cells were lysed by the addition of sterile distilled water and incubated for 20 min at 37°C under aerobic conditions. Dilutions of the cell lysates infected with bacteria were plated in triplicate on agar plates and incubated. *P. gingivalis* 381 and *E. coli* MC1061 were used as positive and negative controls, respectively for each invasion assay. The numbers of colony-forming units of invasive bacteria were then enumerated.

Statistical analysis

A simple linear regression model was used to compare invasion eficiency for periodontally healthy vs. periodontal diseased sites. The relationship between degree of invasion eficiency was compared with gender differences and probing depths using analysis of variance. Pearson product moment correlations were used to determine the relationship between invasion and probing depth. P values of < 0.05 were considered significant. All values were enumerated as mean ± standard error of the mean (52).

Results

Thirty-six subjects (16 male, 20 female) provided bacterial samples for potential *P. gingivalis* isolation, of which 30 subjects (14 male, 16 female) had culturable *P. gingivalis* at both healthy and diseased sites. The results for both periodontally healthy and diseased sites (including gender, tooth site sampled, probing depth and average colony-forming units per milliliter), which were compiled from three independent invasion assays, were analysed. When tested in KB cells, the invasion efficiencies of the strains isolated during this study were primarily in the moderate invasion efficiency range, with a few samples in the low invasion efficiency range, according to the criteria previously described by Dorn *et al.* (30). *P. gingivalis* 381 (the positive control) and *E. coli* MC1061 (the negative control) demonstrated high invasion efficiency or non-invasiveness, respectively.

When a comparison was made between isolates from periodontally healthy and diseased sites, the mean degree of invasion for strains from periodontally healthy sites was 16.79 ± 0.86 ×

10^3 colony-forming units/mL compared with 26.14 ±2.11 × 10^3 colony-forming units/mL for strains from periodontally diseased sites (Fig. 1). Thus, the level of invasion of strains from periodontally diseased sites was significantly higher ($p < 0.0001$) than the level of invasion of strains from the periodontally healthy sites. All strains with invasion frequencies in the low invasion range were isolated from healthy sites, while strains with the highest invasion level (at the high end of the moderate invasion efficiency category) were all isolated from periodontally diseased sites. While making a site-to-site comparison of degree of invasion within each patient, 82.7% of the diseased sites contained strains that exhibited a higher degree of invasion efficiency than the strains at healthy sites for each patient (Fig. 2). Thus, only 17.3% of healthy sites contained strains that exhibited slightly higher levels (average = 4000 (at the high end of the moderate invasion efficiency category) colony-forming units/mL higher) of invasion than their corresponding diseased site.

No statistical difference ($p = 0.43$) was noted between female (21.18 ± 1.60 colony-forming units/mL) and male (21.60 ± 2.01 colony-forming units/mL) subjects regarding degree of invasion. Both male and female subjects exhibited a strong difference in degree of invasion between healthy and diseased sites ($p < 0.0001$). The mean invasion efficiency of *P. gingivalis* for each probing depth was 16.76 ± 1.12 × 10^3 colony-forming units/mL for female healthy sites, 16.82 ± 1.33 × 10^3 colony-forming units/mL for male healthy sites, 25.53 ± 2.70 × 10^3 colony-forming units/mL for female diseased sites and 26.81 ± 3.48 × 10^3 colony-forming units/mL for male diseased sites (Fig. 3). These results indicate that the degree of *P. gingivalis* invasion efficiency is not gender dependent, but is dependent on the individual host/bacterial interactions.

A significant correlation between probing depth and degree of invasion of *P. gingivalis* was observed. The coefficient of correlation (r) was 0.457 (95% confidence interval 0.290–0.597, $p < 0.0001$). (Fig. 4). The mean degree of invasion efficiency of *P. gingivalis* isolated from each probing depth was 16.69 ± 1.85 × 10^3 colony-forming units/mL for 2 mm, 16.55 ± 1.01 × 10^3 colony-forming units/mL for 3 mm, 22.08 ± 0.71 × 10^3 colony-forming units/mL for 4 mm, 30.16 ± 6.56 × 10^3 colony-forming units/mL for 5 mm, 23.36 ± 3.16 × 10^3 colony-forming units/mL for 6 mm, 28.75 ± 5.05 × 10^3 colony-forming units/mL for 7 mm, 20.94 ± 2.37 × 10^3 colony-forming units/mL for 8 mm, 26.66 ± 1.33 × 10^3 colony-forming units/mL for 9 mm and 30.70 × 10^3 colony-forming units/mL for 10 mm probing depth sites.

Discussion

Animal test systems have shown that there are strain differences (clonality) in the virulence of *P. gingivalis* isolates (53–55). This is also observed with invasion (i.e. different laboratory strains exhibit varying invasion abilities). Dorn *et al.* (30) categorized *P. gingivalis* invasion efficiencies into four groups: high (×10^5), moderate (×10^4), low (×10^3) and noninvasive (≤ 10^2). Of 26 *P. gingivalis* strains tested by Dorn *et al.* (30), only *P. gingivalis* AJW4 was noninvasive, and *P. gingivalis* strain 381 possessed a 1000-fold greater ability than *P. gingivalis* AJW4 to invade KB cells. The invasion efficiencies obtained in this present study using fresh, clinical isolates were predominantly in the moderate range, with a few in the low range, and one strain from a disease site showed invasiveness close to the high efficiency level. Most periodontally diseased sites contained isolates that had greater invasion capabilities than did strains from the healthy sites, but some of the healthy sites contained strains with slightly greater invasion efficiencies than the corresponding diseased site. This increased invasion efficiency of strains from some healthy sites could be explained by the sites being in an ‘early disease stage’ at which the sites may be on the verge of a periodontal disease burst, but where sufficient attachment loss to classify the site as diseased, according to the criteria of the present study, has not yet occurred. Conversely, according to the burst hypothesis, the *P. gingivalis* strains present in the diseased sites may have, at the time of sampling, entered a quiescent phase

of disease. In order to determine if this was the case, each diseased site in each patient would have to be evaluated longitudinally for disease progression related to the degree of *P. gingivalis* invasion efficiency. An additional and likely possibility for the difference in invasive efficiencies of strains between the healthy and diseased sites could be the presence of differing strains (clonal types) of *P. gingivalis* present at each site. Future studies will use genomic (such as arbitrary PCR) and phenotypic (such as serotyping, and measuring collagenolytic and proteolotic activities) analyses to determine if the isolates obtained from the healthy and diseased sites are in fact different strains. Griffen *et al.* (48) reported that the level of virulence among strains of *P. gingivalis* from human periodontitis varies, and that certain *P. gingivalis* subgroups are highly virulent. These virulent strains may be responsible for the attachment loss present at diseased sites in this study, whereas less virulent strains may be present in the same individual and may have colonized the healthy sites, explaining why these sites exhibit less attachment loss.

A significant correlation between probing depth and *P. gingivalis* degree of invasion was observed. As the pocket depth increases there are ecological changes in the pocket, which then result in changes within the biofilm. Investigations have previously shown that bacterial characteristics may be influenced by the surrounding biofilm. For example, *Streptococcus cristatus*, a gram-positive bacterium, is capable of modulating virulence expression of *P. gingivalis* through repression of the *P. gingivalis* fimbrial gene (*fimA*). Thus, *P. gingivalis* was unable to form biofilm microcolonies with *S. cristatus* (56). The biofilm interactions present at shallow probing depths may have an inhibitory effect on the invasive capabilities of *P. gingivalis*, whereas at deeper probing depths the bacteria present may assist the survival of more invasive *P. gingivalis* strains. In addition, host mechanisms may be present that select for strains which are more invasive at deeper probing depths and that may not be present in shallow pockets. Takemoto *et al.* found, using a rabbit disease model, that mono-inoculation with *Tannerella forsythia* (opinion on name change from *Tannerella forsythensis* pending; formerly *Bacteroides forsythus*), *P. gingivalis* or *Fusobacterium nucleatum* did not cause abscess formation. However, when *T. forsythia* was co-inoculated with either *P. gingivalis* or *F. nucleatum*, abscess formation occurred at 100% and 75% of the sites, respectively (57). Thus, biofilm interactions may trigger changes that modulate the survival/colonization of a particular *P. gingivalis* strain at deeper pocket depths, which may shift the predominant strains to those that are more invasive. Invariably, the host defense mechanisms may promote the emergence of *P. gingivalis* strains with increased invasion abilities and inevitably greater tissue destruction.

Adherence and invasion are active processes in which microorganisms may use host proteins and enzymes to gain entry into the cell, thus stimulating their own uptake. *P. gingivalis* has developed invasion strategies and mechanisms similar to those of other pathogens for both epithelial and endothelial cells. A common strategy among invasive bacteria is to trigger the host cell to undergo cytoskeletal rearrangements mediated by actin polymerization (58). *P. gingivalis* invasion has been shown to be inhibited by thapsigargin and 1,2-bis(2-aminophenoxy) ethane-*N*,*N*, *N*1,*N*1-tetraacetic acid (31), as well as by cytochalasin D, nocodazole, staurosporine, protease inhibitors and sodium azide, thus indicating that cytoskeletal rearrangements, protein phosphorylation, energy metabolism and *P. gingivalis* proteases are essential for invasion (51,59).

It seems logical to expect greater invasion efficiency of *P. gingivalis* in sites with evidence of periodontal disease, as this would support evidence of the ability of *P. gingivalis* to interact with the host tissues and thus cause disease. Countering the increased invasive abilities of *P. gingivalis* present in deeper periodontal pockets may require multiple subgingival environment disruptions via scaling and root planing, or may require the adjunctive use of subgingival antimicrobial or antibiotic modalities. In addition, monitoring of the subgingival flora for

bacterial strains with greater invasive abilities may be required in order to predict the possibility of future disease progression and which methods of treatment would be best utilized to control the disease process.

In summary, periodontal sampling of *P. gingivalis* at healthy and diseased sites showed that *P. gingivalis* strains isolated from diseased sites possess greater invasion efficiencies than strains from healthy sites. This increased ability to invade cells may promote disease progression. Invasion efficiency differences were not found to be related to gender, but both male and female patients exhibited significantly greater *P. gingivalis* invasion at diseased sites. Also, greater invasion efficiency was noted for increasing probing depth.

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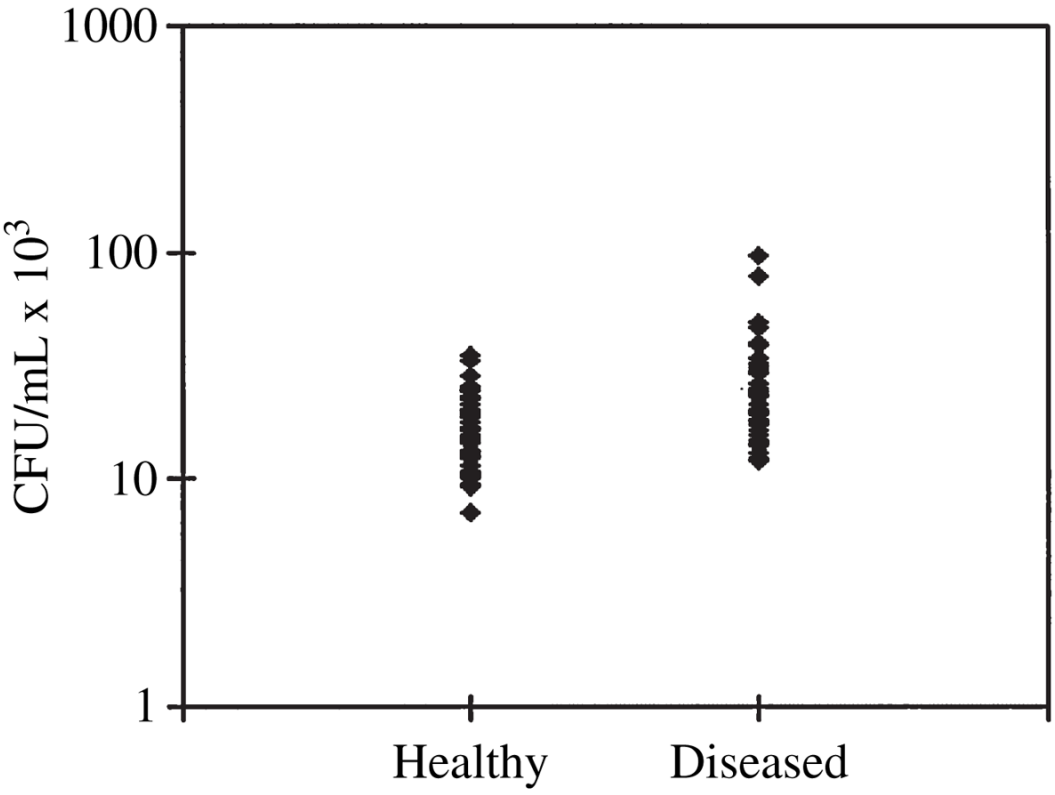

Fig. 1.

Invasion of KB cells by *Porphyromonas gingivalis* isolates from healthy and diseased periodontal sites. *P. gingivalis* isolates from subgingival plaque samples were tested, using an antibiotic protection assay, for their ability to invade KB cells. The mean level of invasion of strains isolated from periodontally healthy and diseased sites was $16.79 \pm 0.86 \times 10^3$ colony-forming units/mL and $26.14 \pm 2.11 \times 10^3$ colony-forming units/mL, respectively ($p < 0.0001$). CFU, colony-forming units.

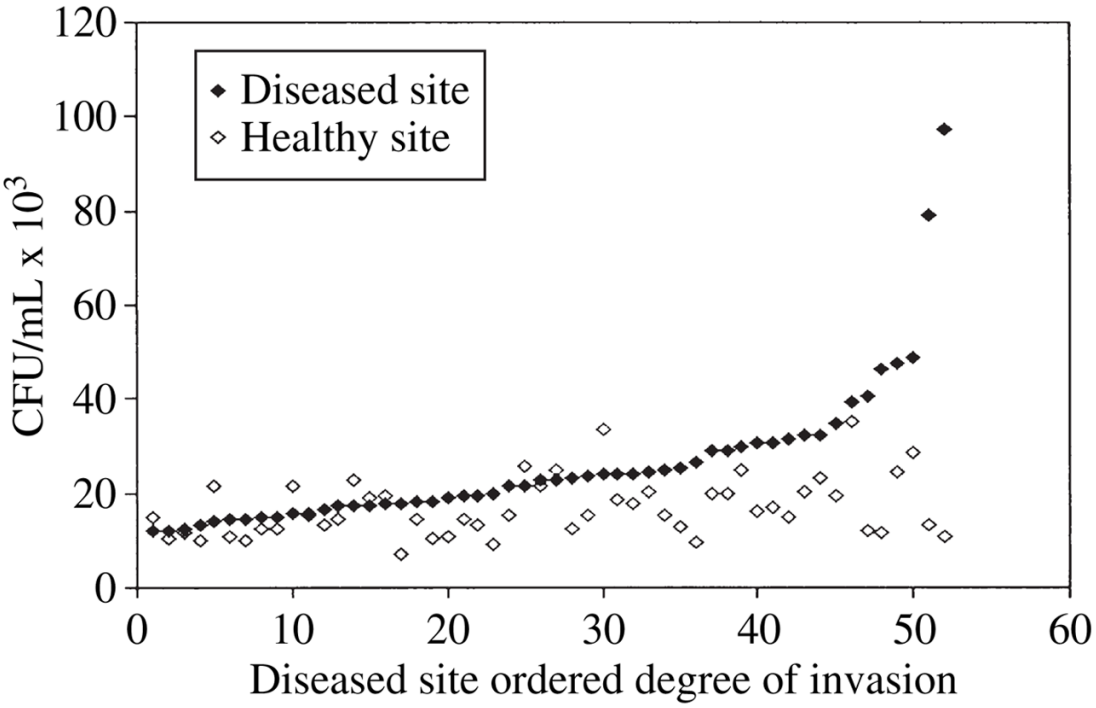

Fig. 2.

Invasion of KB cells by *Porphyromonas gingivalis* from healthy and diseased sites. *P. gingivalis* isolates from healthy and diseased periodontal sites of each patient were compared for their ability to invade KB cells by using an antibiotic protection assay. The degree of invasion of *P. gingivalis* for each diseased site was recorded in increasing order along with the paired data from the corresponding healthy site. CFU, colony-forming units.

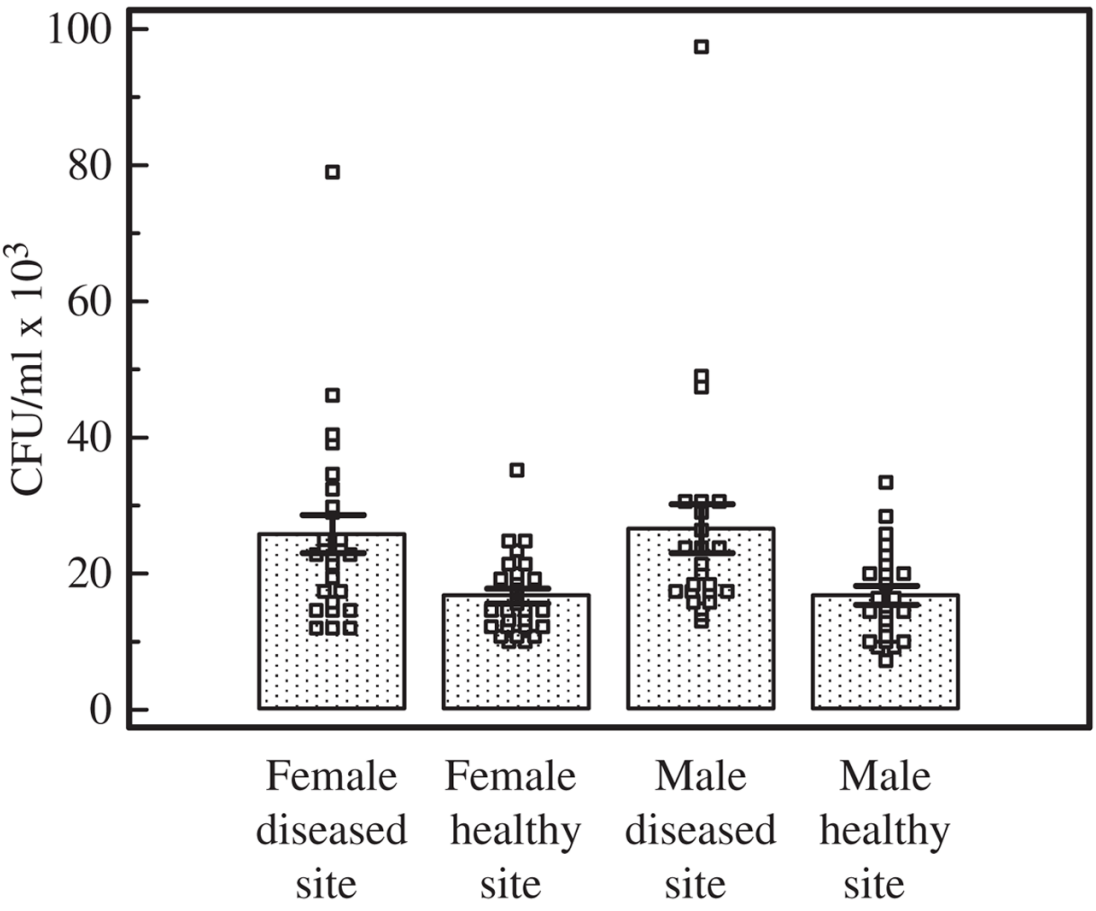

Fig. 3.

Mean degree of invasion (± standard error of the mean) of KB cells by *Porphyromonas gingivalis* from healthy and diseased sites as grouped by gender. *P. gingivalis* isolates were tested, using an antibiotic assay, for their ability to invade KB cells and were then grouped by origin of the site (healthy vs. diseased and gender of the patient). Statistically significant differences were observed between both the male and female subjects vs. their healthy and diseased sites ($p < 0.0001$). CFU, colony-forming units.

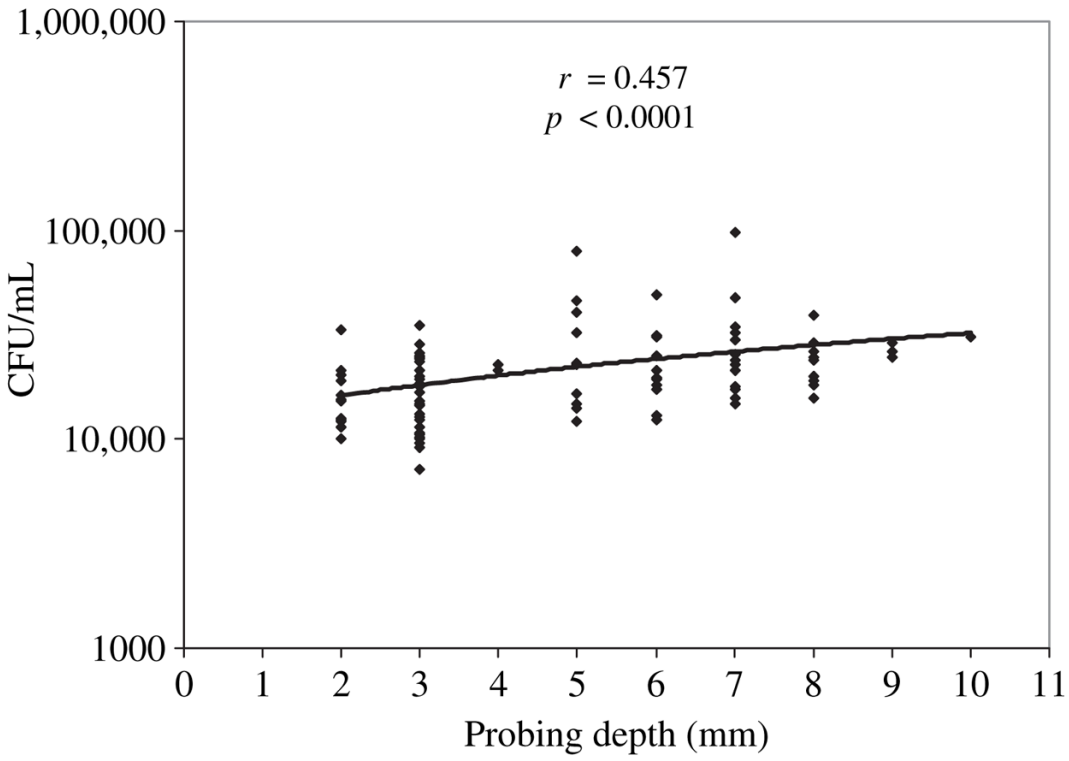

Fig. 4.

Degree of invasion of KB cells by *Porphyromonas gingivalis* vs. probing depth. *P. gingivalis* isolates were tested, using an antibiotic assay, for their ability to invade KB cells and were then grouped according to the probing depth recorded on the day of isolation. A significant correlation was found between the probing depth and the degree of invasion of *P. gingivalis* ($p < 0.0001$). CFU, colony-forming units.