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ORIGINAL ARTICLE

In Vitro Invasion and Survival of *Porphyromonas gingivalis* in Gingival Fibroblasts; Role of the Capsule

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Abstract *Porphyromonas gingivalis* is a Gram-negative, anaerobic bacterium involved in periodontitis and peri-implantitis that can invade and survive inside host cells in vitro. *P. gingivalis* can invade human gingival fibroblasts (GF), but no data are available about the role of *P. gingivalis*' capsule in GF invasion. In the current study, we aimed to determine the ability of three strains of *P. gingivalis* (encapsulated wild type W83, non-encapsulated HG91 and the non-encapsulated insertional isogenic knockout mutant of W83, ΔEpsC) to invade GF and the ability of internalized *P. gingivalis* to survive in vitro antibiotic treatment. The ability of *P. gingivalis* strains to invade GF was tested using an antibiotic protection assay at multiplicity of infection (MOI) 100 and 1000. The survival of internalized *P. gingivalis* cells was further analyzed by subsequent in vitro treatment with either metronidazole or amoxicillin alone or a combination of metronidazole and amoxicillin and anaerobic culture viability counts. All strains of *P. gingivalis* used in this study were able to invade GFs. The non-encapsulated mutant of W83 (ΔEpsC mutant) was significantly more invasive than the wild type W83 at MOI 100 (p value 0.025) and MOI 1000 (p value 0.038). Furthermore, internalized *P. gingivalis* was able to resist in vitro antibiotic treatment. As demonstrated by the differences in invasion efficiencies of *P. gingivalis* strain W83 and its isogenic mutant ΔEpsC, the capsule of *P. gingivalis* makes it less efficient in invading gingival fibroblasts. Moreover, internalized *P. gingivalis* can survive antibiotic treatment in vitro.

Keywords Periodontitis · Mechanism of antibiotic resistance · Internalization · Capsule

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Introduction

Porphyromonas gingivalis is a Gram-negative, anaerobic, non-motile rod shaped bacterium found in sub-gingival plaque that has been associated with chronic adult periodontitis (Lamont and Jenkinson 1998) and peri-implantitis (Botero et al. 2005). *P. gingivalis* is equipped with a broad array of virulence factors (Holt et al. 1999) potentially involved in tissue colonization and destruction. *P. gingivalis* capsule is an important virulence factor and it has been shown that encapsulated *P. gingivalis* strains are more virulent than non-encapsulated strains in a mouse model (Laine et al. 1997), non-encapsulated strains adhere more to epithelial cells compared to encapsulated strains (Njoroge et al. 1997) and the capsule may be immunosuppressive for human gingival fibroblasts (GF) in vitro (Brunner et al. 2010). On the basis of capsular polysaccharides, seven capsular (K1-K7) and a non-capsular serotype of *P. gingivalis* have been described previously (Laine et al. 1996; Sims et al. 2001).

Invasion into host cells is one of the multiple ways in which pathogens interact with the host immunity (Theriot 1995). *P. gingivalis* has been shown to invade into a variety

of host cells in several in vitro studies (Andrian et al. 2006; Eick et al. 2002; Deshpande et al. 1998; Amornchat et al. 2003), and to persist and multiply within the epithelial cells (Madianos et al. 1996). The capsule may impede the process of invading host cells by *P. gingivalis*, as partial dissolution of *P. gingivalis* capsule with amyloglucosidase has been shown to increase the invasion efficiency of *P. gingivalis* into endothelial cells (Deshpande et al. 1998).

Systemic metronidazole is most commonly used either alone or in combination with amoxicillin as an adjunct to periodontitis and peri-implantititis treatment (Seymour and Heasman 1995). Recurrence of periodontitis may still be a problem in some patients after the use of systemic antibiotics, probably due to a hampered eradication of periodontal pathogens such as *P. gingivalis* (Magnusson and Walker 1996). Intracellular presence of *P. gingivalis* in epithelial cells collected from patients after systemic antibiotic treatment has been reported (Colombo et al. 2007), and may reflect inadequacy of conventional antibiotics to eliminate internalized *P. gingivalis*.

Fibroblasts are the most abundant stromal cell type in the connective tissue of gingiva (Schroeder et al. 1973) and important cells in the pathogenesis of periodontitis (Lekic et al. 1997). *P. gingivalis* has been shown to penetrate multilayered epithelial cells and reach the underlying connective tissue in an in vitro model (Andrian et al. 2004), which is also consistent with in vivo observations (Saglie et al. 1988). To the best of our knowledge, the role of *P. gingivalis* capsule in invading GF is not known and we hypothesize that the non-encapsulated *P. gingivalis* may be internalized more efficiently into GFs and survive antibiotic treatment inside GF. Therefore, the aim of our current study was to evaluate the effects of *P. gingivalis* capsule on its ability to invade GF and to investigate the in vitro effectiveness of metronidazole, amoxicillin and combination of metronidazole and amoxicillin, on intracellular *P. gingivalis*.

Materials and Methods

Bacterial Strains

Porphyromonas gingivalis strains W83 (K1 serotype), HG91 (K- serotype) and an isogenic non-encapsulated knockout mutant of W83 (ΔEpsC mutant, K-serotype), were grown anaerobically (80 % N_2, 10 % H_2, and at 10 %CO_2 at 37 °C) until log growth phase in brain–heart infusion broth (BHI, Bacto™ Brain Heart Infusion, Becton, Dickinson and Company, USA) enriched with hemin (5 μg/ml) and menadione (1 μg/ml). Purity of the cultures was checked by Gram-staining. The ΔEpsC mutant is an insertional isogenic W83 knockout in the epimerase-coding gene EpsC that is located at the end of the capsular biosynthesis locus (Brunner et al. 2010).

Porphyromonas gingivalis was harvested by centrifugation at 5,000 rpm, for 15 min. Bacterial pellets were washed twice in sterile phosphate-buffered saline (PBS) and resuspended in DMEM containing 10 % fetal bovine serum (FBS, HyClone, Logan, UT, USA) without antibiotics. The optical density (OD_{690}) of the fluid bacterial culture in BHI was measured at 690 nm wavelength. An $OD_{690} = 0.8$ corresponding to 2×10^9 CFUs/ml on bacterial growth curves for different *P. gingivalis* strains (data not shown), was used to establish the number of colony-forming units (CFUs) and infect GFs.

Human Gingival Fibroblasts (GF)

Gingival fibroblasts isolated from an extracted third molar of a 62 years old, periodontally healthy, non-smoking female during a previous study (Scheres et al. 2010) were used. The donor had given a written informed consent and the study was approved by the VUmc (Vrije Universiteit medical centre) medical ethical committee. Briefly, the tissue sample was taken by collecting the free gingiva around the tooth. The tissue sample was washed and maintained as explants in Dulbecco's modified Eagle medium (DMEM; 4.5 g/L glucose + L-glutamine + pyruvate; Invitrogen/Gibco Paisley, UK) supplemented with 10 % fetal bovine serum (FBS; Invitrogen/Gibco, Paisley, Scotland, UK) and 2 % antibiotics/antimycotics (PSF; 100 U/ml of penicillin, 100 μg/ml streptomycin and 250 ng/ml of amphotericin B; Sigma, St Louis, MO, USA) in a humidified environment with 5 % CO_2 at 37 °C until growth of fibroblasts was observed under the microscope. These fibroblasts were further expanded in DMEM containing 10 % FBS and 1 % PSF and stored frozen in liquid nitrogen at passage 4. All experiments were performed using fibroblasts between passages 4 and 10.

Minimum Inhibitory and Minimum Bactericidal Concentrations

Minimum inhibitory and minimum bactericidal concentrations values of the *P. gingivalis* strains W83, HG91 and ΔEpsC mutant were determined by a standard broth dilution method (Hecht et al. 2007). The MIC and MBC values were determined both in BHI and in DMEM enriched with 10 % FBS. Briefly, *P. gingivalis* strains were grown anaerobically in BHI containing hemin and menadione for 18 h at 37 °C, the OD_{690} was determined and based on the standard growth curves, the number of bacteria was calculated. Metronidazole (Sigma-Aldrich, the Netherlands) and amoxicillin (Beecham research laboratories, Beecham

Pharma, Amstelveen, the Netherlands) solutions were prepared in sterile distilled water and filter sterilized with sterile polypropylene syringe filters (Whatman® FP 30/0.2, 0.2 μm pore size, GE Healthcare Europe GmbH, Belgium). Metronidazole and amoxicillin were serially diluted in a 24-well plate in BHI enriched with hemin and menadione and an inoculum containing 1×10^8 *P. gingivalis* was added to each well. *P. gingivalis* strains without antibiotics and antibiotics without *P. gingivalis* served as controls. Four different concentrations of metronidazole: 2, 1, 0.5 and 0.25 μg/ml and amoxicillin: 0.5, 0.25, 0.125 and 0.062 μg/ml were used for MIC and MBC determination. Each concentration of metronidazole (2, 1, 0.5 and 0.25 μg/ml) was combined with respective concentration of amoxicillin (0.5, 0.25, 0.125 and 0.062 μg/ml) for the in vitro combination of antibiotic treatment.

Invasion Efficiency

An antibiotic protection assay for *P. gingivalis* (Deshpande et al. 1998) was used to quantify the invasion efficiency of the three *P. gingivalis* strains. GFs were seeded into 24-well plates at a cell density of 1×10^4 cells/well, in antibiotic-free DMEM containing 10 % FBS and grown until sub-confluence. *P. gingivalis* was grown overnight in BHI broth with hemin and menadione, and harvested at the log phase (OD_{690} of 0.8) by centrifugation. These *P. gingivalis* were washed first with PBS (pH 7.4) and then with DMEM without antibiotics before suspending them in DMEM containing 10 % FBS without antibiotics. The OD_{690} was again measured in DMEM to establish the CFUs. The numbers of *P. gingivalis* were adjusted to be used subsequently for infecting the fibroblasts with a multiplicity of infection (MOI) 100 (1×10^6/well) and 1000 (1×10^7/well) *P. gingivalis* per GF. Only DMEM containing 10 % FBS was added to the control GFs. *P. gingivalis* without GFs in concentration of 1×10^8/ml was also included for each experiment as control to evaluate the efficiency of the antibiotic used for killing *P. gingivalis*. The bacterial suspensions in DMEM were added to the sub-confluent monolayers of GFs and co-incubated in humidified aerobic atmosphere for 90 min, in 5 % CO_2 at 37 °C. After co-incubation, the unattached bacteria were removed by washing each well twice with PBS. Metronidazole (100 μg/ml) was added to the monolayers and incubated in DMEM for another 60 min to kill the external adherent bacteria. This concentration of metronidazole was shown in pilot experiments to kill 10^8 *P. gingivalis*/ml during 60 min exposure in BHI as well as DMEM and also reported elsewhere (Deshpande et al. 1998; Njoroge et al. 1997). Monolayers were washed twice with PBS and 1 ml of sterile distilled water was added to each well and further incubated for 30 min to lyse the fibroblasts. The cells were disrupted by vigorous and repeated pipetting. The lysate was plated in serial dilutions for CFUs count on horse blood agar plates (Oxoid no.2, Basingstoke, UK) containing hemin and menadione and incubated anaerobically at 37 °C. The remaining samples were stored at −80 °C. CFU counts were determined after 14 days of anaerobic incubation. Invasion efficiency was expressed as a percentage of the initial inoculum recovered after GF lysis (Lamont et al. 1995).

Survival of Internalized *P. gingivalis*

To study the survival of internalized *P. gingivalis*, the most invasive among the three *P. gingivalis* strains, i.e., ΔEpsC mutant was chosen for further experiments. The highest invasion efficiency was seen with MOI 100, therefore only MOI 100 was used in further experiments.

This assay was performed in two steps. The first step was performed similar to the antibiotic protection assay except that the GFs were not lysed at the end of the experiment. In the second step, GFs were washed twice with PBS and either metronidazole (100 μg/ml) alone, amoxicillin alone (12.5 μg/ml) or combination of metronidazole (100 μg/ml) and amoxicillin (12.5 μg/ml) were added to the wells containing GFs and incubated for 60 min under humidified aerobic conditions at 37 °C. Concentrations of antibiotics used in this step were equal to 100 times the MIC. Antibiotic-free DMEM was added to the control GFs. After incubation the GFs were washed twice with sterile PBS and lysed as described previously.

Porphyromonas gingivalis Quantitation by Real Time Polymerase Chain Reaction (RT-PCR)

Porphyromonas gingivalis DNA was isolated from the lysate by MagNA Pure DNA Isolation kit III (Roche, Molecular Diagnostics, Almere, the Netherlands) as mentioned elsewhere (Boutaga et al. 2006). The primer/probe sets and PCR conditions have been described earlier (Boutaga et al. 2005). Briefly, RT-PCR amplification was performed in a total reaction mixture volume of 20 μL. The reaction mixtures contained 10 μL of LightCycler® 480 SYBR Green I Master mix, 1.8 pmol of *P. gingivalis* specific forward and reverse primers each, 0.4 pmol of LightCycler® 480 CYAN 500 Labeling Reagent and 4 μL of purified *P. gingivalis* DNA from the samples. The samples were subjected to an initial amplification cycle of 50 °C for 2 min and 95 °C for 10 min followed by 45 cycles at 95 °C for 15 s and 60°C for 1 min using the LightCycler® 480 Instrument. Data analysis was done with LightCycler® 480 software release 1.5.0 SP4.

Statistical Analysis

Mean and standard error of the mean (SEM) were calculated from three separate experiments for antibiotic protection assays. Data from the culture and RT-PCR were compared and tested for significance either using a two-tailed, non-parametric Mann–Whitney's test or a non-parametric Kruskal–Wallis and Dunn's multiple comparison post hoc tests. Data analysis was performed with GraphPad Prism software (version 5.00 for Windows, San Diego California, USA). Differences were considered statistically significant at a p value <0.05.

Results

MIC and MBC

Table 1 presents the MIC and MBC values for different *P. gingivalis* strains as determined by broth dilution method. The MIC and MBC values of amoxicillin alone for the three *P. gingivalis* strains ranged between 0.125 and 0.25 μg/ml. MIC and MBC values of metronidazole alone for the three *P. gingivalis* strains ranged between 1 and 2 μg/ml. MIC and MBC values for the combination ranged between 1 μg/ml metronidazole plus 0.125 μg/ml amoxicillin and 2 μg/ml metronidazole plus 0.25 μg/ml amoxicillin. There were no significant differences in susceptibility to metronidazole and amoxicillin between the encapsulated and non-encapsulated *P. gingivalis* strains W83, HG91 and ΔEpsC mutant.

Invasion of GF by *P. gingivalis*

Porphyromonas gingivalis strains used in this study were able to invade and survive inside GF. Figure 1 shows the invasion efficiencies of the three *P. gingivalis* strains at MOI 100 and 1000, after 90 min of infection, as determined by anaerobic culture. Invasion efficiencies of W83 wild type, HG91 and ΔEpsC mutant at MOI 100 were 0.007, 0.07 and 0.15 %, respectively. At MOI 1000, respective invasion efficiencies of W83 wild type, HG91 and ΔEpsC mutant were 0.016, 0.04 and 0.09 %, respectively. Invasion efficiencies of the non-encapsulated strains between MOI 100 and MOI 1000 were not statistically different.

The non-encapsulated *P. gingivalis* ΔEpsC mutant was significantly more invasive than the encapsulated W83 parent strain at MOI 100 (p value 0.025) as well as MOI 1000 (p value 0.038) (Fig. 1). Invasion efficiency of the non-encapsulated HG91 strain was also higher than the encapsulated W83 strain at MOI 100 and 1000, although the differences did not reach statistical significance.

Overall, the RT-PCR data show comparable results as seen for anaerobic culture (Fig. 2). There were no statistically significant differences in the amounts of *P. gingivalis* detected by RT-PCR either between W83 and HG91 or HG91 and ΔEpsC mutant at MOI of 100 (Fig. 2). However, at MOI 1000, the detected amounts of ΔEpsC were significantly higher than the wild type W83 (p value 0.027).

In Vitro Survival of Internalized *P. gingivalis*

The internalized ΔEpsC mutant was further treated with different regimens of antibiotics. After the additional in vitro antibiotic treatment of internalized ΔEpsC mutant, viable *P. gingivalis* could still be recovered from the GFs regardless of the antibiotics used. When compared to the total amounts of internalized bacteria (i.e., bacteria left after the first antibiotic treatment), the mean percentages ranged from 13.5 to 17.8 % depending on the antibiotic(s) used (Fig. 3), differences were not significant. Survival of the internalized ΔEpsC mutant was significantly (p value 0.034) reduced compared to control, if a combination of metronidazole and amoxicillin was used (Fig. 4). Either metronidazole alone or amoxicillin alone was not able to significantly reduce the internalized load of *P. gingivalis* (Fig. 4).

Discussion

Clinical studies on periodontitis and peri-implantitis patients treated with antibiotics have reported inability of antibiotics to completely eradicate *P. gingivalis* (von Troil-Linden et al. 1996; Mombelli et al. 2001). This observation is in line with the finding that periodontal pathogens internalized into host cells might act as a potential reservoir

Table 1 MIC and MBC of antibiotics as determined by broth dilution method

P. gingivalis strain (serotype)	MIC/MBC (μg/ml)		
	Metronidazole	Amoxicillin	Metronidazole + Amoxicillin
W83 (K1)	1.0/2.0	0.25/0.25	1 + 0.125/2 + 0.25
G91(K^-)	2.0/2.0	0.25/0.25	1 + 0.125/1 + 0.125
ΔEpsC (K^-)	1.0/2.0	0.125/0.25	1 + 0.125/1 + 0.25

MIC minimum inhibitory concentration, *MBC* minimum bactericidal concentration

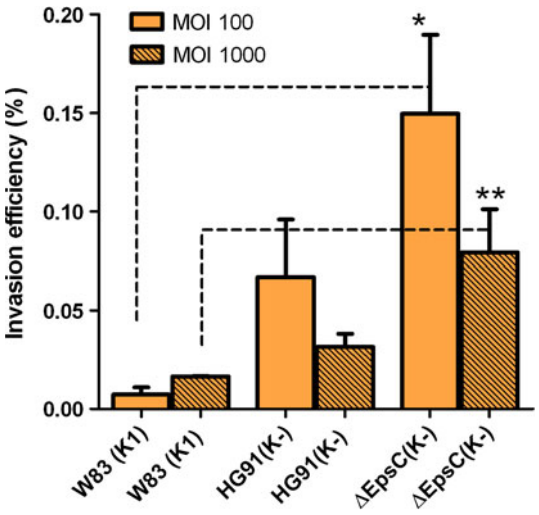

Fig. 1 Invasion efficiencies of *P. gingivalis* strains into GF at MOI 100 and 1000, as determined by anaerobic culture. The non-encapsulated mutant of W83 (ΔEpsC) was significantly more efficient in invading GF compared to the encapsulated wild type (W83) both at MOI 100 (*p value 0.025) and MOI 1000 (**p value 0.038). Results are presented as the mean and SEM of triplicate cultures and represent three independent experiments

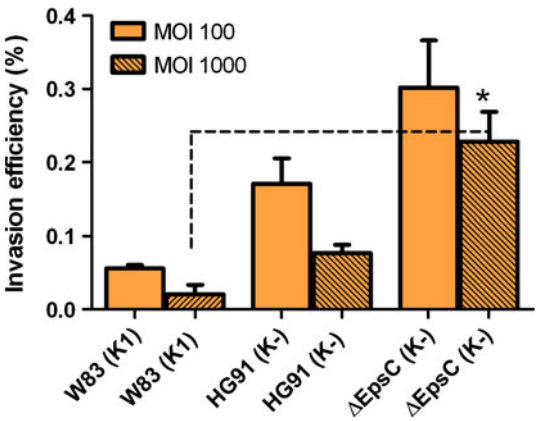

Fig. 2 Invasion efficiencies of *P. gingivalis* at MOI 100 and 1000 as determined by RT-PCR. The invasion efficiency of ΔEpsC mutant into GFs was significantly higher than the wild type W83 at MOI 1000 (*p value 0.027). Results are presented as the mean and SEM of triplicate cultures and represent three independent experiments

for re-infection in periodontitis (Rudney et al. 2005). The re-emergence of *P. gingivalis* has particularly been attributed to failed eradication (Mombelli et al. 2002). Invasion of host cells is one of the possible mechanisms that can offer protection to bacteria against antibiotic pressure (Johnson et al. 2008). GF appear to be important host cells in this regard, because periodontal pathogens have been previously shown to invade into and survive inside GFs (Amornchat et al. 2003; Dabija-Wolter et al. 2009). Intracellular *P. gingivalis* undergoes morphologic changes and has been shown by scanning electron microscopy in the

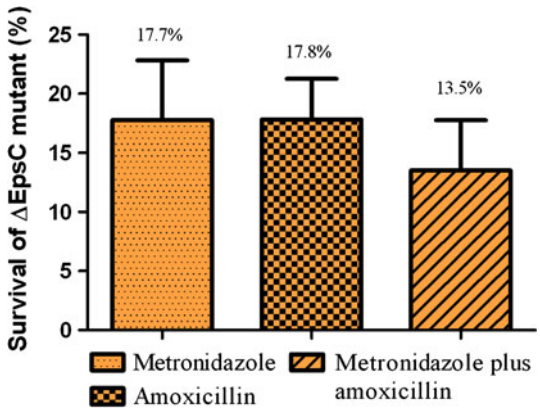

Fig. 3 ΔEpsC *P. gingivalis* recovered by anaerobic culture after in vitro challenge of the internalized bacteria with metronidazole (100 μg/ml), amoxicillin (12.5 μg/ml) or combination of metronidazole (100 μg/ml) and amoxicillin (12.5 μg/ml), is shown here as mean percentages of the initially internalized bacteria at MOI 100. No viable *P. gingivalis* was recovered from the control

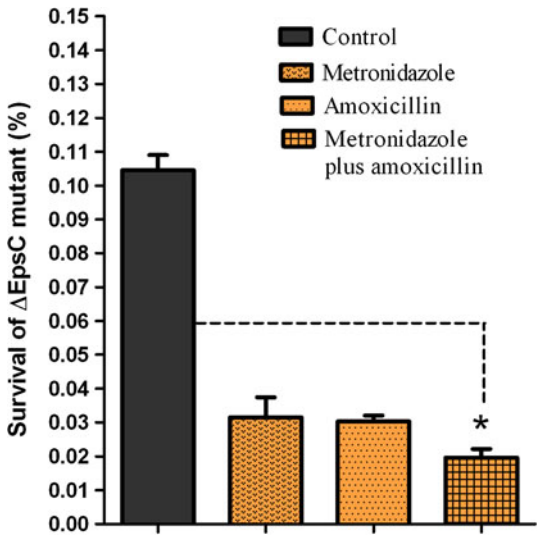

Fig. 4 Survival of *P. gingivalis* (ΔEpsC mutant) recovered by anaerobic culture after in vitro challenge of the internalized bacteria with metronidazole (100 μg/ml), amoxicillin (12.5 μg/ml) or combination of metronidazole (100 μg/ml) and amoxicillin (12.5 μg/ml). A significant reduction in the survival of internalized bacteria was observed after combination of metronidazole and amoxicillin treatment, compared to control (*p value 0.034). Results are presented as the mean and SEM of triplicate cultures and represent three independent experiments

cytoplasm without membrane surrounding (Amornchat et al. 2003).

In the current study, both encapsulated and non-encapsulated *P. gingivalis* strains were able to invade into GFs; however, differences were found in their invasion efficiencies. The non-encapsulated ΔEpsC mutant was significantly more invasive than its encapsulated wild type strain W83. Invasion efficiency of the naturally non-

encapsulated HG91 strain also tended to be higher than the encapsulated W83, but the difference did not reach statistical significance. This indicates that the capsule of W83 is mainly responsible for the differences in the invasion efficiencies between the parent W83 strain and its non-encapsulated isogenic ΔEpsC mutant. Factors other than the capsule may confound a comparison between a non-encapsulated *P. gingivalis* strain, e.g., HG91, with an encapsulated one, e.g., W83. Furthermore, in the current study we found the highest invasion efficiency with MOI 100, which is consistent with previous studies (Amornchat et al. 2003; Lamont et al. 1995). There may be a threshold for a fibroblast to optimally internalize a certain number of *P. gingivalis* and increasing the number of bacteria beyond the threshold may not further enhance invasion (Lamont et al. 1995). The non-encapsulated ΔEpsC mutant, being the most invasive of the three *P. gingivalis* strains used, was chosen for further experiments to study the survival of internalized *P. gingivalis* to in vitro antibiotics treatment. The results show that *P. gingivalis* can resist antibiotic treatment inside fibroblasts. Persistence and/or multiplication of *P. gingivalis* after internalization and survival in GF, was not evaluated in the current study although other studies have shown that *P. gingivalis* can persist and multiply inside host cells after internalization (Madianos et al. 1996). In the current study, combination of metronidazole and amoxicillin was found to be significantly more effective in reducing the amounts of internalized *P. gingivalis* compared to control. Clinical isolates of *P. gingivalis* may be more difficult to eradicate compared to laboratory strains, with the antibiotic therapy for periodontitis and peri-implantitis because clinical isolates of *P. gingivalis* have been found to be considerably more resistant to antibiotic treatment inside host cells (Eick and Pfister 2004). Since growth of fibroblasts can be affected by anaerobic conditions (Clark 1964), antibiotic protection assays in the current study were carried out under aerobic conditions according to the standard protocol used in previous studies (Murakami et al. 2008).

Metronidazole is used to treat periodontal and peri-implant infections either alone or in combination with amoxicillin (Seymour and Heasman 1995). Once taken up by the bacterial cell, metronidazole reacts with bacterial DNA, resulting in bacterial cell death (Muller 1983). Although we have not tested entry of metronidazole into gingival fibroblasts in the current study, metronidazole is known to cross cell membrane of gingival fibroblasts (Yu et al. 2009). It has been shown in an in vitro study that *P. gingivalis* internalized into host cells can survive metronidazole concentrations of up to 100 times its MIC (Eick and Pfister 2004). Amoxicillin is a β-lactam antibiotic, which is bactericidal and act by inhibiting bacterial cell wall synthesis (Yocum et al. 1980). Penicillins do not readily cross plasma membrane, which limits their ability to kill intracellular bacteria (Schentag et al. 1985). The inability of metronidazole and amoxicillin to effectively eliminate internalized *P. gingivalis* is in line with clinical studies (Colombo et al. 2007; Magnusson and Walker 1996).

Evasion of the host defence mechanisms and invasion of host tissues to maintain a successful infection are the prerequisites for a successful chronic infection. The capsule of *P. gingivalis* has been particularly implicated in the reduction of host immune response and increase in virulence (Singh et al. 2011). Recent studies have confirmed the immunomodulatory role of *P. gingivalis* capsule in interaction with host cells (Vernal et al. 2009). Invasion of non-phagocytic host cells is yet another important mechanism of *P. gingivalis* to maintain a successful infection. The role of *P. gingivalis* capsule in its ability to invade GF has not been reported previously. Polysaccharide capsule of *P. gingivalis* may interfere with the initial step of bacterial binding to host cell membrane. For example, the polysaccharide capsule of *P. gingivalis* decreases their surface hydrophobicity (van Winkelhoff et al. 1993), which may partly explain the higher affinity and increased invasion efficiency of non-encapsulated strains to host cells (Andrian et al. 2006; Dierickx et al. 2003). The capsule has also been shown to impede host cell invasion in other pathogens such as *Klebsiella pneumoniae* (Sahly et al. 2000) and *Haemophilus influenzae* (St Geme et al. 1992). In case of GF, the mechanism of invasion by *P. gingivalis* is not fully understood, although *P. gingivalis* has been reported to exploit host cell signaling pathways and cytoskeleton for its internalization into gingival epithelial cells (Lamont et al. 1995; Yilmaz et al. 2003). Other invasive bacterial species have also been shown to rely on the host cell actin cytoskeleton for a successful infection (Amieva et al. 2002). Once inside host cell, *P. gingivalis* can promote its survival by activating a variety of apoptotic pathways (Handfield et al. 2005) and regulation of distinctive *P. gingivalis* proteins and genes (Zhang et al. 2005). These observations show the strategies that *P. gingivalis* has evolved to increase its chances of survival inside the host cell although their relevance for *P. gingivalis*-fibroblast interactions has yet to be determined. The in vitro inability of high concentrations of antibiotics to kill *P. gingivalis* internalized into GF, shown in this study, provides further support to the idea that GF may play an important role in *P. gingivalis* resistance to antibiotics. Although the evidence that *P. gingivalis*, to a certain extent, can invade host cells and resist antibiotic treatment is in line with clinical studies, in vitro studies may not be directly extrapolated to in vivo situation.

Within the limitations of the study, we conclude that *P. gingivalis* can internalize into human gingival fibroblasts

in vitro, non-encapsulated *P. gingivalis* is more efficient in invading GFs than encapsulated *P. gingivalis* and once inside the host cell, *P. gingivalis* can survive antibiotic treatment.

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References

Amieva MR, Salama NR, Tompkins LS, Falkow S (2002) *Helicobacter pylori* enter and survive within multivesicular vacuoles of epithelial cells. Cell Microbiol 4(10):677–690

Amornchat C, Rassameemasmaung S, Sripairojthikoon W, Swasdison S (2003) Invasion of *Porphyromonas gingivalis* into human gingival fibroblasts in vitro. J Int Acad Periodontol 5(4):98–105

Andrian E, Grenier D, Rouabhia M (2004) In vitro models of tissue penetration and destruction by *Porphyromonas gingivalis*. Infect Immun 72(8):4689–4698. doi:10.1128/IAI.72.8.4689-4698.2004

Andrian E, Grenier D, Rouabhia M (2006) *Porphyromonas gingivalis*-epithelial cell interactions in periodontitis. J Dent Res 85(5):392–403

Botero JE, Gonzalez AM, Mercado RA, Olave G, Contreras A (2005) Subgingival microbiota in peri-implant mucosa lesions and adjacent teeth in partially edentulous patients. J Periodontol 76(9):1490–1495. doi:10.1902/jop.2005.76.9.1490

Boutaga K, van Winkelhoff AJ, Vandenbroucke-Grauls CM, Savelkoul PH (2005) Periodontal pathogens: a quantitative comparison of anaerobic culture and real-time PCR. FEMS Immunol Med Microbiol 45(2):191–199

Boutaga K, van Winkelhoff AJ, Vandenbroucke-Grauls CM, Savelkoul PH (2006) The additional value of real-time PCR in the quantitative detection of periodontal pathogens. J Clin Periodontol 33(6):427–433

Brunner J, Scheres N, El Idrissi NB, Deng DM, Laine ML, van Winkelhoff AJ, Crielaard W (2010) The capsule of *Porphyromonas gingivalis* reduces the immune response of human gingival fibroblasts. BMC Microbiol 10:5. doi:10.1186/1471-2180-10-5

Clark ME (1964) Growth and morphology of adult mouse fibroblasts under anaerobic conditions and at limiting oxygen tensions. Exp Cell Res 36:548–560

Colombo AV, da Silva CM, Haffajee A, Colombo AP (2007) Identification of intracellular oral species within human crevicular epithelial cells from subjects with chronic periodontitis by fluorescence in situ hybridization. J Periodontal Res 42(3):236–243. doi:10.1111/j.1600-0765.2006.00938.x

Dabija-Wolter G, Cimpan MR, Costea DE, Johannessen AC, Sornes S, Neppelberg E, Al-Haroni M, Skaug N, Bakken V (2009) *Fusobacterium nucleatum* enters normal human oral fibroblasts in vitro. J Periodontol 80(7):1174–1183. doi:10.1902/jop.2009.090051

Deshpande RG, Khan MB, Genco CA (1998) Invasion of aortic and heart endothelial cells by *Porphyromonas gingivalis*. Infect Immun 66(11):5337–5343

Dierickx K, Pauwels M, Laine ML, Van Eldere J, Cassiman JJ, van Winkelhoff AJ, van Steenberghe D, Quirynen M (2003) Adhesion of *Porphyromonas gingivalis* serotypes to pocket epithelium. J Periodontol 74(6):844–848. doi:10.1902/jop.2003.74.6.844

Eick S, Pfister W (2004) Efficacy of antibiotics against periodontopathogenic bacteria within epithelial cells: an in vitro study. J Periodontol 75(10):1327–1334. doi:10.1902/jop.2004.75.10.1327

Eick S, Rodel J, Einax JW, Pfister W (2002) Interaction of *Porphyromonas gingivalis* with KB cells: comparison of different clinical isolates. Oral Microbiol Immunol 17(4):201–208 (pii:omi170401)

Handfield M, Mans JJ, Zheng G, Lopez MC, Mao S, Progulske-Fox A, Narasimhan G, Baker HV, Lamont RJ (2005) Distinct transcriptional profiles characterize oral epithelium–microbiota interactions. Cell Microbiol 7(6):811–823

Hecht DW, Citron DM, Cox M, Jacobus N, Jenkins SG, Onderdonk A, Roe-Carpenter D, Rosenblatt JE, Wexler HM (2007) Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard-seventh edition. Clinical and Laboratory Standards Institute (CLSI). http://www.clsi.org/source/orders/free/m11a7.pdf. Accessed 10 Jan 2012

Holt SC, Kesavalu L, Walker S, Genco CA (1999) Virulence factors of *Porphyromonas gingivalis*. Periodontology 2000(20):168–238

Johnson JD, Chen R, Lenton PA, Zhang G, Hinrichs JE, Rudney JD (2008) Persistence of extracrevicular bacterial reservoirs after treatment of aggressive periodontitis. J Periodontol 79(12):2305–2312. doi:10.1902/jop.2008.080254

Laine ML, Appelmelk BJ, van Winkelhoff AJ (1996) Novel polysaccharide capsular serotypes in *Porphyromonas gingivalis*. J Periodontal Res 31(4):278–284

Laine ML, Appelmelk BJ, van Winkelhoff AJ (1997) Prevalence and distribution of six capsular serotypes of *Porphyromonas gingivalis* in periodontitis patients. J Dent Res 76(12):1840–1844

Lamont RJ, Jenkinson HF (1998) Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev 62(4):1244–1263

Lamont RJ, Chan A, Belton CM, Izutsu KT, Vasel D, Weinberg A (1995) *Porphyromonas gingivalis* invasion of gingival epithelial cells. Infect Immun 63(10):3878–3885

Lekic PC, Pender N, McCulloch CA (1997) Is fibroblast heterogeneity relevant to the health, diseases, and treatments of periodontal tissues? Crit Rev Oral Biol Med 8(3):253–268

Yu L, Liu H, E L, Wu X, Wang D (2009) Uptake of metronidazole by human gingival fibroblasts. J Periodontol 80(6):993–998

Madianos PN, Papapanou PN, Nannmark U, Dahlen G, Sandros J (1996) *Porphyromonas gingivalis* FDC381 multiplies and persists within human oral epithelial cells in vitro. Infect Immun 64(2):660–664

Magnusson I, Walker CB (1996) Refractory periodontitis or recurrence of disease. J Clin Periodontol 23(3 Pt 2):289–292

Mombelli A, Feloutzis A, Bragger U, Lang NP (2001) Treatment of peri-implantitis by local delivery of tetracycline. Clinical, microbiological and radiological results. Clin Oral Implants Res 12(4):287–294. pii:clr120401

Mombelli A, Schmid B, Rutar A, Lang NP (2002) Local antibiotic therapy guided by microbiological diagnosis. J Clin Periodontol 29(8):743–749

Muller M (1983) Mode of action of metronidazole on anaerobic bacteria and protozoa. Surgery 93(1 Pt 2):165–171

Murakami J, Kato T, Kawai S, Akiyama S, Amano A, Morisaki I (2008) Cellular motility of Down syndrome gingival fibroblasts is susceptible to impairment by *Porphyromonas gingivalis* invasion. J Periodontol 79(4):721–727. doi:10.1902/jop.2008.070400

Njoroge T, Genco RJ, Sojar HT, Hamada N, Genco CA (1997) A role for fimbriae in *Porphyromonas gingivalis* invasion of oral epithelial cells. Infect Immun 65(5):1980–1984

Rudney JD, Chen R, Sedgewick GJ (2005) *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythensis* are components of a polymicrobial intracellular flora within human buccal cells. J Dent Res 84(1):59–63

Saglie FR, Marfany A, Camargo P (1988) Intragingival occurrence of *Actinobacillus actinomycetemcomitans* and *Bacteroides gingivalis* in active destructive periodontal lesions. J Periodontol 59(4):259–265

Sahly H, Podschun R, Oelschlaeger TA, Greiwe M, Parolis H, Hasty D, Kekow J, Ullmann U, Ofek I, Sela S (2000) Capsule impedes adhesion to and invasion of epithelial cells by *Klebsiella pneumoniae*. Infect Immun 68(12):6744–6749

Schentag JJ, Swanson DJ, Smith IL (1985) Dual individualization: antibiotic dosage calculation from the integration of in vitro pharmacodynamics and in vivo pharmacokinetics. J Antimicrob Chemother 15(Suppl A):47–57

Scheres N, Laine ML, de Vries TJ, Everts V, van Winkelhoff AJ (2010) Gingival and periodontal ligament fibroblasts differ in their inflammatory response to viable *Porphyromonas gingivalis*. J Periodontal Res 45(2):262–270

Schroeder HE, Munzel-Pedrazzoli S, Page R (1973) Correlated morphometric and biochemical analysis of gingival tissue in early chronic gingivitis in man. Arch Oral Biol 18(7):899–923

Seymour RA, Heasman PA (1995) Pharmacological control of periodontal disease II. Antimicrobial agents. J Dent 23(1):5–14 pii:0300-5712(95)90654-Z

Sims TJ, Schifferle RE, Ali RW, Skaug N, Page RC (2001) Immunoglobulin G response of periodontitis patients to *Porphyromonas gingivalis* capsular carbohydrate and lipopolysaccharide antigens. Oral Microbiol Immunol 16(4):193–201

Singh A, Wyant T, Anaya-Bergman C, Aduse-Opoku J, Brunner J, Laine ML, Curtis MA, Lewis JP (2011) The capsule of *Porphyromonas gingivalis* leads to a reduction in the host inflammatory response, evasion of phagocytosis, and increase in virulence. Infect Immun 79(11):4533–4542. doi:10.1128/IAI.05016-11

St Geme JW, 3rd, Falkow S (1992) Capsule loss by *Haemophilus influenzae* type b results in enhanced adherence to and entry into human cells. J Infect Dis 165(Suppl 1):S117–118

Theriot JA (1995) The cell biology of infection by intracellular bacterial pathogens. Annu Rev Cell Dev Biol 11:213–239. doi:10.1146/annurev.cb.11.110195.001241

van Winkelhoff AJ, Appelmelk BJ, Kippuw N, de Graaff J (1993) K-antigens in *Porphyromonas gingivalis* are associated with virulence. Oral Microbiol Immunol 8(5):259–265

Vernal R, Leon R, Silva A, van Winkelhoff AJ, Garcia-Sanz JA, Sanz M (2009) Differential cytokine expression by human dendritic cells in response to different *Porphyromonas gingivalis* capsular serotypes. J Clin Periodontol 36(10):823–829. doi:10.1111/j.1600-051X.2009.01462.x

von Troil-Linden B, Saarela M, Matto J, Alaluusua S, Jousimies-Somer H, Asikainen S (1996) Source of suspected periodontal pathogens re-emerging after periodontal treatment. J Clin Periodontol 23(6):601–607

Yilmaz O, Young PA, Lamont RJ, Kenny GE (2003) Gingival epithelial cell signalling and cytoskeletal responses to *Porphyromonas gingivalis* invasion. Microbiology 149(Pt 9):2417–2426

Yocum RR, Rasmussen JR, Strominger JL (1980) The mechanism of action of penicillin. Penicillin acylates the active site of *Bacillus stearothermophilus* D-alanine carboxypeptidase. J Biol Chem 255(9):3977–3986

Zhang Y, Wang T, Chen W, Yilmaz O, Park Y, Jung IY, Hackett M, Lamont RJ (2005) Differential protein expression by *Porphyromonas gingivalis* in response to secreted epithelial cell components. Proteomics 5(1):198–211