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ORIGINAL ARTICLE

Virulence of major periodontal pathogens and lack of humoral immune protection in a rat model of periodontal disease

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OBJECTIVE: This study was designed to test the hypothesis that periodontal pathogens *Tannerella forsythia* and *Porphyromonas gingivalis* are synergistic in terms of virulence potential using a model of mixed-microbial infection in rats.

MATERIALS AND METHODS: Three groups of rats were infected orally with either *T. forsythia* or *P. gingivalis* in mono-bacterial infections or as mixed-microbial infections for 12 weeks and a sham-infected group were used as a control. This study examined bacterial infection, inflammation, immunity, and alveolar bone loss changes with disease progression.

RESULTS: *Tannerella forsythia* and *P. gingivalis* genomic DNA was detected in microbial samples from infected rats by PCR indicating their colonization in the rat oral cavity. Primary infection induced significantly high IgG, IgG2b, IgG1, and IgG2a antibody levels indicating activation of mixed Th1 and Th2 immune responses. Rats infected with the mixed-microbial consortium exhibited significantly increased palatal horizontal and interproximal alveolar bone loss. Histological examinations indicated significant hyperplasia of the gingival epithelium with moderate inflammatory infiltration and apical migration of junctional epithelium. The results observed differ compared to uninfected controls.

CONCLUSION: Our results indicated that *T. forsythia* and *P. gingivalis* exhibit virulence, but not virulence synergy, resulting in the immuno-inflammatory responses and lack of humoral immune protection during periodontitis in rats.

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Keywords: *T. forsythia; P. gingivalis;* periodontal disease; Th1 & Th2 immune response; alveolar bone loss; inflammation

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Introduction

Periodontal diseases are polymicrobial immuno-inflammatory infectious diseases leading to the destruction of periodontal ligament and adjacent supportive alveolar bone. The subgingival sulcus harbors more than 500 species and reflects a sequential colonization of a broad array of bacteria in transitioning from a healthy to disease subgingival biofilm (Socransky *et al*, 1998; Kuramitsu 2003; Socransky and Haffajee 2005) the molecular mechanisms and bacterial synergism among these genera and species in disease are not well understood. Among the periodontal pathogens, *Porphyromonas gingivalis* and *Tannerella forsythia* are commonly co-isolated in subgingival biofilm samples from chronic periodontitis lesions (Socransky *et al*, 1998; Tanner *et al*, 1998; Socransky and Haffajee, 2005). Both *P. gingivalis* and *T. forsythia* are gram-negative anaerobes present in subgingival plaques in patients with severe periodontitis, have been recognized as defined periodontal pathogens, and are strongly associated with chronic human periodontitis (Tanner *et al*, 1998; Narayanan *et al*, 2005). Furthermore, immune responses to *T. forsythia* antigens in patients with periodontal disease have been detected (Yoo *et al*, 2007). However, its mere presence may be insufficient for periodontal disease initiation and/or progression. *Tannerella forsythia* produces a cysteine protease, encoded by the *prtH* gene and *P. gingivalis* express the potent virulence factors gingipains, three cysteine proteases that bind and cleave a wide range of host proteins (Potempa *et al*, 2000), which may play a role in the transition from a commensal organism to opportunistic pathogens. Higher levels of the prtH genotype were associated significantly with future attachment loss (Hamlet *et al*, 2008). Sialidase activity is another putative virulence factor of the anaerobic

periodontal pathogen *T. forsythia* (Ishikura *et al*, 2003). This bacterium also expresses a cell surface-associated and secreted protein BspA (Sharma *et al*, 1998), which has been recognized as an important virulence factor in inducing alveolar bone loss in mice (Sharma *et al*, 2005a). Furthermore, BspA is an important modulator of host innate immune responses through activation of TLR2 in human gingival epithelial cells in cooperation with TLR1 (Onishi *et al*, 2008).

The consistent co-existence of *P. gingivalis* with *T. forsythia* suggests that a strong ecological relationship involving complex interactions between periodontal pathogens may exist among these two microbial species. Several studies reported periodontal pathogen synergism (enhanced pathogenicity) in abscess (Yoneda *et al*, 2001) and chamber models (Metzger *et al*, 2009) but not in a periodontal disease model. Specifically, *T. forsythia* produced relatively small localized abscesses in mice at the sites of monoinfection and co-infection with *P. gingivalis* or *Fusobacterium nucleatum* produced synergistically larger skin lesions, suggesting the existence of pathogenic microbial synergism (Takemoto *et al*, 1997; Yoneda *et al*, 2001). A few *in vitro* studies also reported that sonicated cell extracts from *T. forsythia* stimulated the growth of *P. gingivalis* under nutrition-limited conditions (Yoneda *et al*, 2001) and *P. gingivalis* outer membrane vesicles enhanced the attachment and invasion of *T. forsythia* into epithelial cells (Inagaki *et al*, 2006). *In vitro* biofilm experiments indicated that *T. forsythia* and *F. nucleatum* exhibited synergy in growth and biofilm formation compared to the individual organisms (Sharma *et al*, 2005b). These bacteria generally represent commensal opportunistic pathogens found in low numbers in healthy sites; however, under the appropriate microenvironmental conditions, currently not well understood, they induce in host cells several proinflammatory molecules which are thought to cause, directly or indirectly, irreversible loss of periodontal supportive tissues (Lamont and Jenkinson, 1998; Holt *et al*, 1999).

The mechanisms of interaction between these two predominant species in the subgingival environment and whether such interactions lead to a synergistic induction of periodontitis is not known (Kolenbrander, 2000). Recently, we have demonstrated that *P. gingivalis*, *T. denticola*, and *T. forsythia* with and without *F. nucleatum* not only exist as a consortium that is associated with chronic periodontitis in humans but also exhibit synergistic virulence resulting in the colonization of the rat oral cavity, induction of enhanced IgG immune responses, and significant alveolar bone resorption characteristic of polymicrobial (three pathogens and more) periodontitis (Kesavalu *et al*, 2007).

Monobacterial (single pathogen) periodontal infections primarily using *P. gingivalis*, *T. denticola*, *T. forsythia*, or *F. nucleatum* have been studied in rats and mice (Klausen 1991; Baker *et al*, 1999; Lalla *et al*, 2003; Sharma *et al*, 2005a; Kesavalu *et al*, 2007). While these studies have generally used monoinfections in animal models, increasing evidence supports the concept that bacterial interactions among selected members of the subgingival microbiota at any one time during periodontal disease progression are important. However, to our knowledge there are no published studies establishing a mixed microbial periodontal disease in rodents for examining the potential synergistic effects between *T. forsythia* and *P. gingivalis*. This investigation examined mixed microbial (two species) periodontal disease using *T. forsythia* and *P. gingivalis* and examined their colonization/infection characteristics, induced inflammation, immune response profiles, cytokine/chemokine responses, and induction of alveolar bone resorption. Accordingly, we hypothesize that *T. forsythia* and *P. gingivalis* demonstrate synergistic virulence (colonization/infection) for inducing chronic periodontal inflammation leading to enhanced alveolar bone resorption.

Materials and methods

Bacterial strains and microbial inocula

Porphyromonas gingivalis strain 381 and *T. forsythia* ATCC 43037 were used in this study and were cultured as well as maintained for rat infection as described previously (Kesavalu *et al*, 2006, 2007). The *P. gingivalis* strain 381 was chosen due to its known role in alveolar bone resorption in adult periodontitis and its proven ability to colonize the oral cavity of rodents (Klausen 1991; Evans *et al*, 1992; Lalla *et al*, 2003; Kesavalu *et al*, 2007). The amount of each bacterium was determined quantitatively and the organism resuspended at 2×10^{10} bacteria per ml. For mono infection, each bacterium (2×10^{10} cells per ml) was mixed with equal volumes of sterile 2% (w/v) low viscosity carboxymethylcellulose (CMC; Sigma, St Louis, MO, USA) (Baker *et al*, 1999; Lalla *et al*, 2003; Lee *et al*, 2006; Kesavalu *et al*, 2007) with PBS and 0.5 ml was used for infection (5×10^9 cells) by oral gavage as described previously (Baker *et al*, 1999; Rajapakse *et al*, 2002; Lalla *et al*, 2003; Sharma *et al*, 2005a; Lee *et al*, 2006; Kesavalu *et al*, 2007). For oral mixed microbial infection, *T. forsythia* (2×10^{10} cells per ml) was gently mixed with an equal volume of *P. gingivalis* (2×10^{10} cells per ml), and allowed to interact for 5 min. An equal volume of sterile 2% CMC was added, mixed thoroughly, and 0.5 ml (2.5×10^9 cells of *T. forsythia*, 2.5×10^9 cells of *P. gingivalis*) was administered by oral gavage.

P. gingivalis and T. forsythia oral infections and sampling

Female Sprague-Dawley rats (8–9 weeks old, Charles River laboratories, Boston, MA, USA) were maintained in groups and housed in microisolator cages. Rats were fed standard powdered chow and water *ad libitum*. All animal procedures were performed in accordance with the approved protocol (#E900) guidelines set forth by the Institutional Animal Care and Use Committee of the University of Florida at Gainesville. In addition, adequate measures were taken to minimize pain or discomfort to rats during oral infection and sampling. Rats were administered kanamycin (20 mg) and ampicillin (20 mg) daily for 4 days in the drinking water (Kesavalu *et al*, 2006, 2007; Sathishkumar *et al*, 2008) and the oral

cavity was swabbed with 0.12% chlorhexidine gluconate (Peridex: 3M ESPE Dental Products, St Paul, MN, USA) mouth rinse (Kesavalu *et al*, 2007; Sathishkumar *et al*, 2008) to inhibit the endogenous organisms and to promote subsequent colonization of *P. gingivalis* and *T. forsythia* (Kesavalu *et al*, 2006). After a 3-day antibiotic washout period, rats were randomized into four groups ($n = 9$) followed by oral infection. The mono- and mixed microbial inocula were administered by oral gavage for 4 consecutive days per week on 6 alternate weeks for a total of 24 inoculations during 12 weeks of the experimental infection period. Control uninfected rats received vehicle (sterile 2% CMC) only. Oral microbial samples from isoflurane anesthetized rats were collected at pre- and post-infections as described previously (Kesavalu *et al*, 2007). In order to monitor the colonization/infection with minimal disruption of the biofilms that would develop to enhance the periodontal disease outcomes, a total of three post-infection microbial samples (four, five, sixth infections) were collected following week (8, 10, 12th) from all infected rats. Blood was collected and sera were stored at -20°C for immunoglobulin G (IgG), IgA, IgM, and IgG isotype antibody analysis. Rat skulls were then removed, autoclaved, and mechanically defleshed with a periodontal scaler for evaluation of alveolar bone resorption by morphometric and radiographic analysis. The rat jaws were also suspended in 10% buffered formalin and decalicified for histology and histomorphometry.

PCR determination of T. forsythia and P. gingivalis colonization

DNA was isolated from rat oral microbial samples using a Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) as described previously (Kesavalu *et al*, 2007). The standard genomic DNA for *T. forsythia* and *P. gingivalis* were also extracted following the same procedure from their respective 24–48 h pure cultures (Kesavalu *et al*, 2006, 2007; Sathishkumar *et al*, 2008). Subsequently, PCR was performed using 16S rRNA gene species-specific PCR oligonucleotide primers with a Bio-Rad thermal cycler as described previously (Kesavalu *et al*, 2006, 2007; Sathishkumar *et al*, 2008). PCR products were separated by agarose (1.5%) gel electrophoresis and ethidium bromide staining. The genomic DNA extracted from *P. gingivalis* and *T. forsythia* served as positive controls and PCR performed with no template DNA made up the negative control. Each PCR assay with the standard DNA was sensitive enough to detect 0.05 pg of DNA (*P. gingivalis* 30 cells; *T. forsythia* 19 cells). Different PCR cycles from 35 to 40 were standardized to produce detectable amplicons with the least amount (0.05 pg) of template DNA (Kesavalu *et al*, 2006, 2007).

IgG and IgG subclass antibody analysis

Serum from monobacterial ($n = 9$) or mixed microbial infected rats ($n = 9$) at 12 weeks was used to determine IgG, IgM, IgA, and IgG subclass (IgG1, IgG2a, IgG2b, IgG2c) antibody concentrations against whole cells of *P. gingivalis* and *T. forsythia* or to recombinant cell surface-associated and secreted protein BspA (kind gift from Dr Ashu Sharma, University at Buffalo, Buffalo, NY, USA) from *T. forsythia* using a standard ELISA protocol (Kesavalu *et al*, 2006, 2007), and provided an additional marker of infection. Briefly, *P. gingivalis* and *T. forsythia* were grown in liquid cultures as described before (Kesavalu *et al*, 2007) and cells were treated overnight with 0.5% formalin in buffered saline (FK cells), washed, and diluted to $OD_{600nm} = 0.3$ as coating antigen. Diluted infected rat serum (1:100 for IgG and 1:20 for IgM, IgA and IgG subclass) was incubated in wells of FK whole-cell antigen or rBspA protein (1 μg/100 μl/well) (Sharma *et al*, 2005a) coated microtiter plates, along with a purified rat IgG (Sigma) standard to create a gravimetric curve for antibody quantization. After washing, affinity purified goat anti-rat IgG conjugated to alkaline phosphates (1:5000), IgM and IgA (Bethyl Laboratories, Montgomery, TX, USA) was added (1:500) to the plates and the assay developed with *p*-nitrophenolphosphate (Sigma). The assay reactions were stopped by the addition of 3M NaOH and analyzed at $OD_{405\ nm}$ using a Bio-Rad Microplate Reader. For IgG subclass antibody analysis, alkaline phosphatase-conjugated goat anti-rat IgG1, IgG2a, IgG2b, and IgG2c (Bethyl Laboratories, Montgomery, TX, USA) were used (1:500). The infected rat serum antibody concentration was quantified using appropriate standard curves.

Analysis of alveolar bone resorption

The pattern of alveolar bone resorption (horizontal or intrabony defects) induced by *P. gingivalis* and *T. forsythia* were measured by both morphometric and radiographic methods, respectively. The mono- and mixed microbial infected rat maxilla and mandible ($n = 6$) were immersed in 3% (v/v) hydrogen peroxide overnight, washed with deionized water, air dried, and stained for one minute in an aqueous solution of 0.1% (w/v) methylene blue to delineate the cemento-enamel junction (CEJ) (Rajapakse *et al*, 2002; Lee *et al*, 2006). The digital images of both buccal and lingual root surfaces of all molar teeth were captured after superimposition of buccal and lingual cusps to ensure reproducibility and consistency. The line tool was used to make horizontal bone resorption measurements from the CEJ to the alveolar bone crest (ABC). The surface perimeters of CEJ and ABC were traced using the calibrated line tool. Two blinded investigators were used and all measurements were done two times by the same examiner at separate times and the mean of measurements was obtained for each of the four quadrants.

The hemisected maxillae and mandibles were trimmed to reduce the bucco-lingual dimensions in order to allow close proximity of the teeth to the digital Kodak 6000 sensor (CareStream Health, Rochester, NY, USA). Digital radiographs of distal and mesial surfaces of the molars were acquired using orthogonal projection geometry and an exposure time of 0.08s at 60 kVp and 15 mA. All radiographic images were exported into the TACT workbench (Tuned Aperture Computed Tomography®) and histograms-equalized. The line tool was used to make vertical bone resorption (intrabony

defects) measurements on the distal and mesial side of each interproximal surface [two sites (mesial and distal) per tooth] for each of the molars in each quadrant from the CEJ to the ABC (i.e. resorption) as the primary outcome parameter of the study (Kesavalu *et al*, 2006, 2007; Sathishkumar *et al*, 2008). To ensure agreement between observers, the measurements were made by investigators blinded as to the group designation. The summation of alveolar bone resorption in mm was tabulated and analyzed for intra and inter-group differences (Kesavalu *et al*, 2006, 2007; Sathishkumar *et al*, 2008).

Histological evaluation of inflammation and bone loss
Maxillae and mandibles of mono- and mixed microbial infected rat jaws ($n = 3$) were randomly removed and fixed in 10% buffered formalin for 24 h. Maxillae and mandibles were decalcified with Immunocal (Decal Chemical Corporation, Tallman, NY, USA) for 28 days at 4°C. The decalcified tissue blocks were embedded in paraffin and sections were prepared at 5 μm followed by staining with hematoxylin and eosin. The interproximal areas between the molars in each specimen at consecutive sections or levels one, 10, and 20 were examined (Liu *et al*, 2006). The images were captured at 10 × magnification. Inflammation was evaluated based on an inflammatory score of PMN/mononuclear cell infiltration in the supra-crestal gingival connective tissue: zero, no inflammatory cells; one, minimal inflammation [scattered inflammatory cells close to the junctional epithelium (JE)]; two, moderate inflammation (numerous inflammatory cells in the gingival connective tissue); and three, severe inflammation (numerous inflammatory cells, elongation of rete ridge, and apical migration of junctional epithelium). Histomorphometric analysis of interdental crestal alveolar bone loss (linear distances between the CEJ and ABC: mm) were determined by using the AxioVision software as described above.

Statistical analysis
The IgG antibody and alveolar bone resorption data were presented as means ± standard deviations (Prism five, GraphPad Software, La Jolla, CA, USA). *P* values were calculated using the Mann–Whitney Student's *t*-test. The Mann–Whitney Student's *t*-test assumes that the data sets do not confirm to a Gaussian distribution. *P* values of 0.05 were considered statistically significant.

Results

Oral mono- and mixed bacterial infections
We examined all rats prior to infection with *T. forsythia* and *P. gingivalis* using appropriate bacterium-specific primers by PCR and showed all rats were negative for *T. forsythia* and *P. gingivalis*. PCR evaluations of the oral microbial samples collected at three time points demonstrated that 100% of rats (monomicrobial Gr I, 9/9; mixed microbial Gr III, 9/9) were infected with *P. gingivalis* during 12 weeks of experimental periodontal disease period. Similarly, the PCR results also demonstrated an appropriately sized amplicon for *T. forsythia* (426 bp) present in DNA isolated from 100% of the rats in oral microbial samples following mono- and mixed microbial infection (monomicrobial Gr II, 9/9; mixed microbial Gr III, 9/9). The *T. forsythia* and *P. gingivalis* co-infection data clearly indicated their colonization in the rat oral cavity. None of the uninfected control rats were positive for *P. gingivalis* and *T. forsythia* infection during the study period.

IgG immune response to oral infections and BspA protein
To provide additional documentation of oral infection and to demonstrate specific immunological responses to *T. forsythia* and *P. gingivalis* infection, we measured the levels of pathogen specific IgG, IgM, IgA, and IgG subclass (IgG1, IgG2a, IgG2b, IgGc) antibodies in rat serum (Figure 1a–e). All rats in the *P. gingivalis* infected group (9/9) induced significantly robust levels of anti-whole cell specific IgG antibody ($P < 0.0005$) following oral infection, compared with sham-infected control rats (Figure 1a). In addition, all rats (9/9) infected with *T. forsythia* also induced significant levels of anti-whole cell specific IgG antibody ($P < 0.0005$) than levels of sham-infected control rats during 12 weeks of periodontal disease (Figure 1c). The detection and levels of antibody in the serum paralleled the detection of *P. gingivalis* and *T. forsythia* in the oral microbial samples (individual rat antibody data not shown). Similarly, all rats (9/9) in the mixed microbial groups showed significant robust serum IgG antibody responses to *P. gingivalis* and *T. forsythia* whole-cell antigens compared to the levels in sham-infected control rats (Figure 1b,d). Furthermore, only *T. forsythia* induced significant ($P < 0.0001$) anti-BspA antibodies during mono- and mixed microbial infection in rats compared to the levels in sham-infected control rats (Figure 1e). None of the *T. forsythia* and *P. gingivalis* infected rats induced IgA and IgM antibodies during 12 weeks of infection.

IgG subclass responses to primary infection
Thus, we had observed that *T. forsythia* and *P. gingivalis* are antigenic in the rats, which resulted in robust levels of serum IgG antibodies. In order to more fully evaluate the characteristics of the IgG antibody, we determined the IgG subclass distribution of the humoral immune response following primary oral infection (Table 1). During 12 weeks of *P. gingivalis* mono microbial infection, the IgG2b subclass levels ($P < 0.005$) were higher than the IgG1 ($P < 0.05$) and IgG2a antibody levels (Figure 1a). Similarly, in *T. forsythia* mono microbial infection, the IgG2b subclass levels ($P < 0.0005$) were higher than the IgG1 and IgG2a antibody levels and significantly $P < 0.05$) greater than in control rats (Figure 1c). Additionally, *T. forsythia* as mono microbial infection induced high-concentration of IgG subclass antibodies than that induced by *P. gingivalis*. Furthermore, *T. forsythia* induced significant IgG subclass profiles (IgG2b > IgG1 > IgG2a > IgG2c) during mixed infection (Figure 1d) whereas *P. gingivalis* induced moderate IgG2b and IgG1 subclass antibodies and undetectable levels of IgG2a and IgG2c antibodies during mixed microbial infection (Figure 1b).

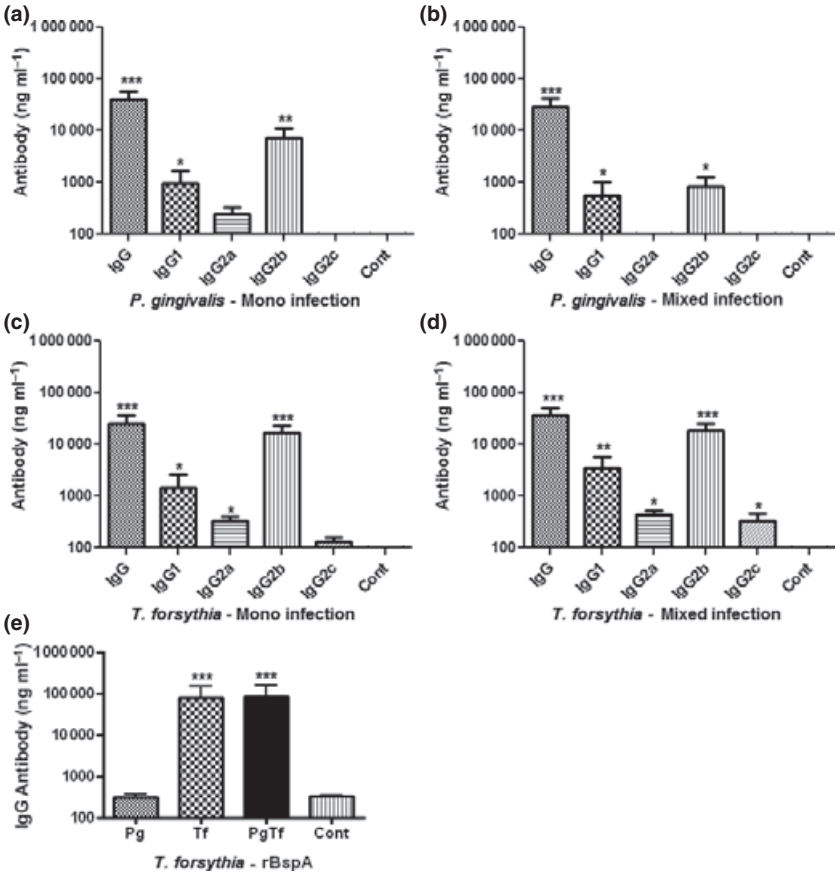

Figure 1 Serum IgG, IgG subclass (IgG1, IgG2a, IgG2b, IgG2c) antibody levels. Serum IgG antibody levels in serum from rats following monomicrobial infection ($n = 9$) or mixed microbial infection ($n = 9$) with *T. forsythia* and/or *P. gingivalis*. The graphs show the results for IgG and IgG subclass antibody (anti-whole-cell) reactive with each of the two species of bacteria as well as with the rBspA (*T. forsythia*) protein. The bars indicate the mean antibody concentrations in serum from rats orally infected with the individual bacteria, with the mixed microbial bacteria, purified rBspA protein, or from control uninfected rats. The error bars indicate one standard deviation from the mean. An asterisk indicates that a value is significantly different (*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$) from that for control uninfected rats. *Pg*, *P. gingivalis*; *Tf*, *T. forsythia*; Cont, control uninfected

Table 1 IgG isotype immune response profiles in rats after primary infection with *T. forsythia* and *P. gingivalis*

IgG Isotype (Th response)	*T. forsythia*	*P. gingivalis*	*T. forsythia + P. gingivalis*	
			T. forsythia	*P. gingivalis*
IgG1 (Th2)	+	+	+ +	+
IgG2a (Th2)	+	+	+	–
IgG2b (Th1)	+ + +	+ +	+ + +	+
IgG2c (Th1)	+	–	+	–

+ + +, indicate significantly high antibody concentration ($P < 0.0005$); + +, indicate moderate concentration ($P < 0.005$); +, indicate low antibody concentration ($P < 0.05$); –, indicate undetectable antibody concentration.

Horizontal alveolar bone resorption

To evaluate the potential synergism between the *T. forsythia* and *P. gingivalis* consortia in periodontal disease progression in the rats, we assessed the effect of oral infection on the maxilla and mandible horizontal alveolar bone resorption. *P. gingivalis* and *T. forsythia* as monoinfections and *P. gingivalis* + *T. forsythia* as mixed microbial infections induced both buccal and palatal areas of alveolar bone resorption which was measured morphometrically and compared with that of sham-infected rats (Table 2; Figure 2). Here, the maxillary and mandibular bone loss in the rats infected with *P. gingivalis*, *T. forsythia*, and *P. gingivalis* + *T. forsythia* were significantly greater ($P < 0.05$) than that of the control uninfected group (Table 2). In addition, mandibular bone loss was generally higher than maxillary bone loss in both buccal and palatal surfaces (Table 2). Both monoinfections induced significantly pronounced palatal (mandibular and maxillary) horizontal alveolar bone loss areas compared to buccal area alveolar bone loss. Similarly, mixed infection with *P. gingivalis* and *T. forsythia* induced significantly more bone loss in both maxilla and mandibles, palatal and buccal surfaces than control uninfected rats (Table 2).

Interproximal alveolar bone resorption

Furthermore, to confirm our observations of horizontal alveolar bone loss, digital radiographic analysis of vertical alveolar bone loss was also performed. All interproximal bone loss measurements were taken from the CEJ to the most coronal level of the crestal alveolar bone to the mesial and distal sides of the three molars. Confirming our previous observations, monobacterial infections with *T. forsythia* or *P. gingivalis* and mixed microbial infection with *T. forsythia* + *P. gingivalis* resulted in significantly increased maxillary, mandibular, and total interproximal alveolar bone loss at 12 weeks of periodontal disease compared with sham-infected rats ($P < 0.05$) (Figure 3). In addition, mixed infection demonstrated a significant increase in maxillary, mandibular, and total vertical bone loss compared to any of

Table 2 Morphometric measurements of horizontal alveolar bone loss area in rats

Infection groups		*Control* n = 6	*P. gingivalis* n = 6 (P-*value*)	*T. forsythia* n = 6 (P-*value*)	*P. gingivalis* + *T. forsythia* n = 6 (P-*value*)
Maxilla	Buccal	3.3 ± 0.37[a]	3.8 ± 0.41 (0.041)[b]	3.7 ± 0.54 (0.393)[b] (0.6)[c]	4.6 ± 0.64 (0.008)[b] (0.041)[d], (0.015)[e]
	Palatal	5.5 ± 0.29	6.7 ± 0.89 (0.004)	6.3 ± 0.62 (0.041) (0.94)	7.1 ± 0.57 (0.002) (0.31), (0.065)
Mandible	Buccal	3.5 ± 0.31	3.9 ± 0.34 (0.026)	3.8 ± 0.54 (0.484) (0.5)	4.8 ± 0.85 (0.008) (0.093), (0.041)
	Palatal	6.6 ± 0.60	8.9 ± 0.87 (0.002)	8.2 ± 0.93 (0.004) (0.132)	9.2 ± 0.69 (0.002) (0.59), (0.002)

[a] Mean (mm^2) and standard deviation from six rats per group measured using AxioVision line tool software as described in methods section.
[b] Numbers in the parenthesis indicate significant differences of horizontal alveolar bone loss area between uninfected control and infected rats ($P < 0.05$).
[c] Numbers in the parenthesis indicate significant differences of alveolar bone loss between *P. gingivalis* and *T. forsythia* infected rats ($P < 0.05$).
[d] Numbers in the parenthesis indicate significant differences of alveolar bone loss between *P. gingivalis* and mixed microbial infected rats ($P < 0.05$).
[e] Numbers in the parenthesis indicate significant differences of alveolar bone loss between *T. forsythia* and mixed microbial infected rats ($P < 0.05$).

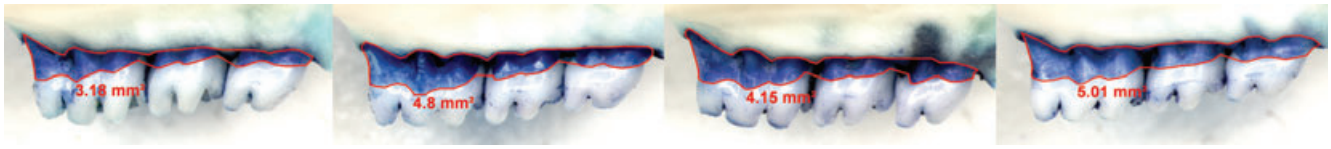

Figure 2 Morphometric evaluation of alveolar bone loss. Representative rat left mandible of a control rat (a) showing the palatal horizontal bone loss area by morphometry. The area outlined (AxioVision image analysis) between CEJ-ABC represents the area of horizontal alveolar bone resorption in mm^2. (b). Rat left mandible infected with *P. gingivalis*, (c) *T. forsythia*, and (d) *P. gingivalis* + *T. forsythia* during 12 weeks of infection showing extensive horizontal alveolar bone loss. M1, M2, M3 are molars. The digital images were captured under a 10 × stereo dissecting microscope (SteReo Discovery V8; Carl Zeiss Microimaging, Inc.) with a color video camera (AxioCam MRc, Carl Zeiss) and connected to a computer with an image analysis morphometry software program (AxioVision LE 29A software version 4.6.3.)

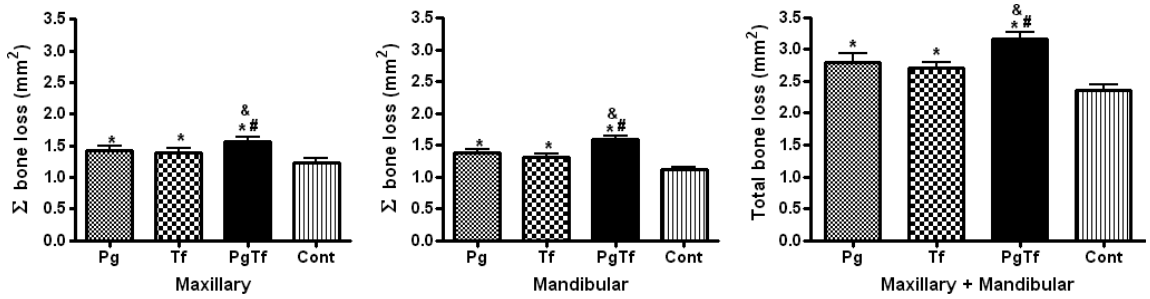

Figure 3 Interproximal evaluation of alveolar bone loss. Radiographic vertical alveolar bone resorption (maxillary, mandibular and total bone loss: 12 weeks of infection) in rats ($n = 6$) following mono bacterial infection with *P. gingivalis* or *T. forsythia* and with the mixed microbial infection containing *P. gingivalis* + *T. forsythia*. Each bar indicates the mean interproximal alveolar bone resorption for distance measured between the CEJ and ABC at mesial and distal sites of three molar teeth (six sites). The error bars indicate standard deviation from the mean. An asterisk denotes significantly different from uninfected control rats ($P < 0.05$). The '#' sign indicates significant difference comparing between monobacterial *P. gingivalis* and the mixed bacterial infected rats ($P < 0.05$). An '&' sign indicates significant difference between *T. forsythia* and the mixed microbial infected rats ($P < 0.05$). *Pg*, *P. gingivalis*; *Tf*, *T. forsythia*; Cont; control uninfected

the monobacterial and control infections ($P < 0.05$) (Figure 3). These data confirm the *in vivo* virulence potential with a *T. forsythia* and *P. gingivalis* consortium.

Histological evaluation of inflammation and bone loss

In order to determine if the infection protocols induced differing levels of inflammation which could be responsible for the increased alveolar bone resorption observed, the maxilla and mandible sections at consecutive levels one, 10, and 20 of rats infected with *P. gingivalis* and/or *T. forsythia* during 12 weeks of periodontal disease were examined for inflammation. The control uninfected rat maxilla and mandibles (Figure 4a), where the histological sections were observed exhibited minimal hyperplasia of the crevicular epithelium with scattered chronic inflammatory cells (score 0). The rats infected with *P. gingivalis* showed thin non-hyperplastic gingival crevicular epithelium (Figure 4b) with elongation of rete ridges, mild apical migration of junctional epithelium (JE) along with minimal to moderate inflammation (score one to two). Rats infected with *T. forsythia* exhibited significant hyperplasia of the gingival crevicular epithelium with elongation of rete ridges, mild apical migration of JE with moderate inflammatory infiltration (score two) and proliferation of small capillaries. The infiltrate consisted

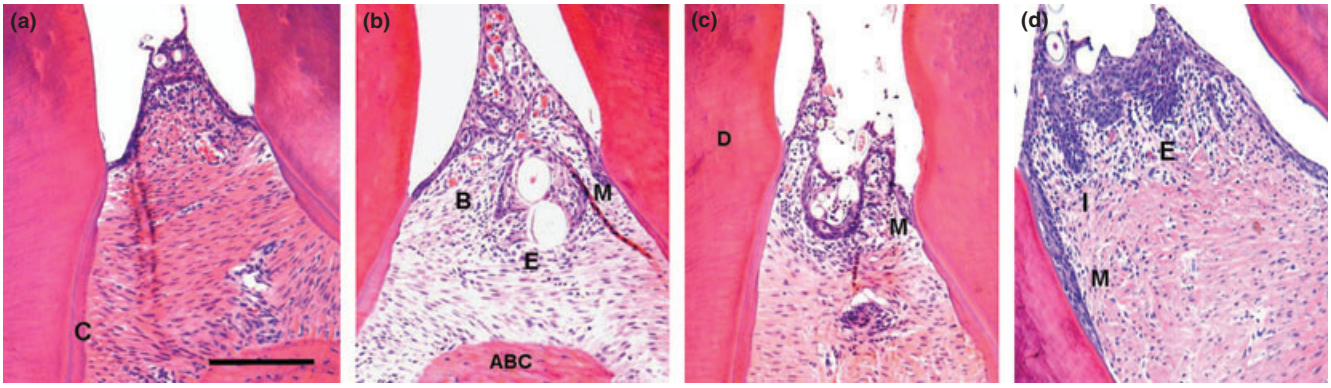

Figure 4 Histopathologic evaluation of inflammation. Comparative maxillary histology (hematoxylin and eosin staining) of alveolar bone sections from the maxilla of rat infected with *P. gingivalis* and/or *T. forsythia* at 12 weeks. **(a)** Section from a control uninfected rat displaying dentin (D) and cementum (C) with minimal inflammation and hyperplasia of the crevicular epithelium. **(b)** Section from the *P. gingivalis* infected rat displaying significant inflammation (I) and hyperplasia of the epithelium with elongation of the rete ridges (E) and increase in small capillary infiltration (B). Apical migration of JE (M) is noted in the section. Alveolar bone crest (ABC) is seen in some of the sections. **(c)** Section from the *T. forsythia* infected rat also displaying features very similar to image B. Apical migration of JE is also noted. **(d)** Section from the mixed infection with *P. gingivalis* + *T. forsythia* exhibiting most severe histological changes namely, significant inflammation (I), hyperplasia of the epithelium with elongation of rete ridges (E), significant apical migration of the JE (M) and numerous dilated capillaries. All images in the panel are at a magnification of 10 ×. Scale bar represents 100 μm

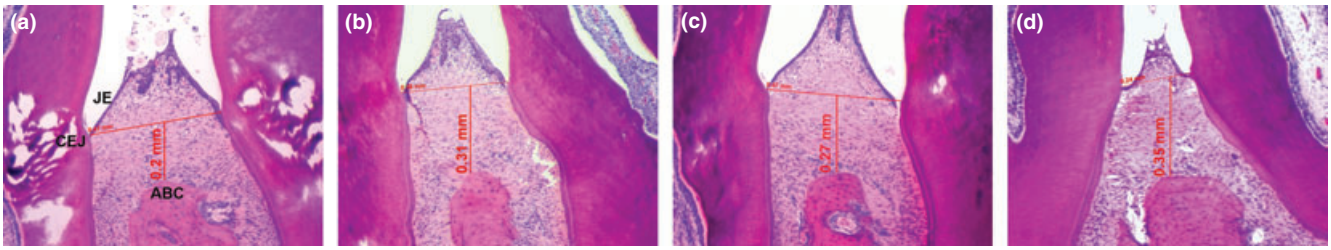

Figure 5 Histometric analysis of bone loss. Photomicrographs illustrating the top of the alveolar bone of the maxilla showing the interdental alveolar bone distance by histometry (hematoxylin and eosin staining). The distances between the ABC and the CEJ were measured in mm using the AxioVision LE 29A software version 4.6.3. Image analysis program. Representative image of a control rat showing the distance between the ABC and CEJ **(a)**. **(b)** Rat right maxilla infected with *P. gingivalis*, **(c)** *T. forsythia*, and **(d)** *P. gingivalis* + *T. forsythia* during 12 weeks of infection showing increased distances between the top of the alveolar bone and the CEJ intersection. JE, junctional epithelium

primarily of lymphocytes and plasma cells (Figure 4c). Similarly, mixed infection specimens exhibited an inflammatory score of 2–3 with significant hyperplasia of the crevicular epithelium, elongation of rete ridge, pronounced apical migration of JE, and significant proliferation of small capillaries in the vicinity (Figure 4d). Histomorphometric analysis of interdental alveolar bone (maxilla) in rats (Figure 5) infected with *P. gingivalis* and *T. forsythia* indicated increased distance (bone loss) between the top of the alveolar bone and the CEJ than monobacterial infection as well as compared to control uninfected rats (data not shown).

Discussion

This study assessed the pathogenesis of *T. forsythia* and *P. gingivalis* as mixed infections in a periodontal disease model and determined their potential *in vivo* virulence synergism. The results demonstrated for the first time, our ability to colonize rats orally with *T. forsythia*/*P. gingivalis* as mixed microbial infections in a periodontal disease model, generation of robust specific systemic IgG immune responses to *T. forsythia* and *P. gingivalis*, induction of characteristic periodontal inflammation and stimulation of enhanced alveolar bone resorption in rats. These two pathogens have been routinely identified as part of the pathogenic biofilm in deep periodontal pockets of chronic periodontitis patients and their coisolation may indicate that the two pathogens interact in the hostile subgingival environment (Socransky *et al*, 1998; Tanner *et al*, 1998; Socransky and Haffajee, 2005; Narayanan *et al*, 2005; Tanner and Izard, 2006). Oral bacterial synergism in progression from health to periodontal disease has been proposed but few *in vivo* studies have documented periodontal pathogen synergism due to the inherent complexity of the subgingival microflora (Simonson *et al*, 1992; Brogden 2002; Kesavalu *et al*, 2007). In spite of several *in vivo* animal model studies (Feuille *et al*, 1996; Takemoto *et al*, 1997; Kesavalu *et al*, 1998; Yoneda *et al*, 2001; Metzger *et al*, 2009) (other than periodontal disease models) and *in vitro* studies demonstrating *P. gingivalis* and *T. forsythia* nutritional interaction (Dzink *et al*, 1987; Yoneda *et al*, 2005), and synergistic IL-6 production by murine macrophage-like J774.1 cells (Kesavalu *et al*, 1998; Tamai *et al*, 2009), no reports

examined the synergistic virulence of two periodontopathic bacteria as mixed infections in a periodontal disease model. Moreover, *in vivo* oral infection studies in periodontal disease models are considered to be crucial in documenting specific periodontal pathogenesis. In addition, periodontal disease models facilitate the quantitative analyses of bacteria-bacteria colonization in the oral cavity, invasion of gingival epithelial cells, bacteria-host interactions, initiation of inflammation, subsequent generation of specific immune responses, and induction of osteoclast-mediated alveolar bone resorption leading to periodontal disease. In a previous study (Kesavalu *et al*, 2007) we had not examined the mixed co-infection of *T. forsythia* and *P. gingivalis* in induction of periodontal inflammation, assessment of both palatal and buccal horizontal bone loss and IgG subclass antibody profiles in induction of periodontal disease. Here we demonstrate this in a mixed microbial periodontal disease model.

Several putative virulence determinants (*P. gingivalis*: gingipain cysteine proteinase; *T. forsythia*: trypsin-like protease, sialidase, BspA protein, alpha-D-glucosidase, *N*-acetyl-beta-glucosaminidase-D-glucosidase) have been identified in *P. gingivalis* and *T. forsythia*, respectively. However, there is little understanding of their interaction with a range of host proteins including fibronectin, laminin, fibrinogen, and their contribution to pathogenesis (colonization/infection) in mixed bacterial infections in an experimental periodontal disease model (Potempa *et al*, 2000).

The mono- and mixed bacterial oral infection of rats indicated that *P. gingivalis* and *T. forsythia* exhibited the ability to colonize/infect the oral cavity with six alternate weekly infections (24 inoculations) during the 12 weeks study establishing oral infection. We have shown previously that infecting rats 15–16 times with *P. gingivalis*, over a similar interval of the experiment, resulted in consistent genomic DNA detection of the microorganism in oral microbial samples (Kesavalu *et al*, 2007). As the microbial sample size was small, the levels of the infecting pathogens have not been quantified in this study. We recognize the limitations in the sample collection procedures in all of the current techniques for microbial sampling from the oral cavity. Moreover, robust induction of significant IgG immune responses and enhanced alveolar bone loss observed in all rats in the present study clearly documents that these rats were infected with *T. forsythia* and *P. gingivalis*. The serum bacterial-specific IgG antibody levels to monobacterial infection during 12 weeks of periodontal disease indicated that *P. gingivalis* and *T. forsythia* are highly effective in colonization and/or are highly immunogenic in the rats. The antibody responses demonstrated substantial specificity for each of the infecting species as well as to the *T. forsythia* rBspA virulence protein.

The predominant response following *T. forsythia* and *P. gingivalis* infection was the IgG2b (T helper type one) and IgG1 subclass (T helper type two), followed by IgG2a (Th2) antibody indicating a stimulation of both Th1 and Th2-activities in development of the humoral immune response to primary bacterial infection. Despite the high bacterial specific IgG antibody levels during 12 weeks of infection, there was no significant antibody protection from alveolar bone loss in rats. Several studies also showed that *P. gingivalis*-induced specific serum antibody response does not prevent alveolar bone loss (Baker *et al*, 1999; DeCarlo *et al*, 2003) suggesting complex mechanisms of antibody protection. In addition, immunization with *P. gingivalis* whole cells induced high-titer serum IgG2a (Th2), moderate-titer IgG2b (Th1) and low-titer IgG1 (Th2) responses and immunization with RgpA-Kgp cysteine proteases of *P. gingivalis* induced high-titer serum IgG2a (Th2) responses which restricted colonization and decreased periodontal bone loss indicating a protective immune response in the rat (Rajapakse *et al*, 2002). Similarly, *P. gingivalis* recombinant hemagglutinin B immunization or immunized and infected rats induced IgG subclass response (IgG1 = IgG2a > IgG2b > IgG2c) suggesting a mixed Th1 and Th2 response and immunized rats had less alveolar bone loss indicating a protective immune response (Katz and Michalek, 1998; Katz *et al*, 1999). Furthermore, that there is involvement of immune cells (B and T lymphocytes) in the course of alveolar bone resorption has been demonstrated by the expression of the receptor activator of nuclear factor-*k*B (RANKL) in the bone resorptive lesion (gingival tissues) of human periodontal disease (Kawai *et al,* 2006). Recently it was also demonstrated that activated antigen-specific B cells can induce periodontal bone resorption in a RANKL-dependent manner using a rat periodontitis model (Han *et al,* 2006). Furthermore, the RANKL mRNA level is highest in the inflammatory cells of advanced periodontitis patients. In addition, abundant RANKL was expressed on $CD3^+$ T cells and also on $CD20^+$ B cells, but only a little, or none, on $CD14^+$ monocytes, implicating a possible involvement of RANKL in the osteoclast-mediated bone destruction in periodontitis (Taubman *et al*, 2005; Kawai *et al,* 2006; Han *et al*, 2007).

While differences in horizontal and interproximal alveolar bone resorption levels were observed following mono- and mixed infection with *P. gingivalis* and *T. forsythia* dependent upon both the differences in the sites of the samples as well as the techniques for measurements, we could not easily compare the magnitude of alveolar bone resorption between these individual bacteria. Both *P. gingivalis* and *T. forsythia* induced similar levels of horizontal and vertical alveolar bone resorption in the rats. Importantly, in testing our hypothesis, oral infection with the mixed microbial consortium of *P. gingivalis/T. forsythia* significantly increased virulence of interproximal alveolar bone resorption. This increased bone loss may be related to enhancement of expression of the virulence of *P. gingivalis* and *T. forsythia* by cooperative abilities of their extracellular potent proteinases (*P. gingivalis* RgpA, RgpB, Kgp gingipains cysteine proteinase) (*T. forsythia* cysteine protease, BspA, sialidiase) to affect host systems through specific cleavage of cell surface receptors and the inactivation of host-defense proteins (Potempa *et al*, 2000; Ishikura *et al*, 2003;

Sharma *et al*, 2005a). Furthermore, the maxillary inflammatory response observed in the mixed-microbial infection rats was consistent with established characteristics of periodontal disease lesions (alveolar bone resorption, gingival inflammation, apical migration of JE). These data clearly indicate a strong correlation between bacterial infection, subsequent immune response and eventual alveolar bone loss in rats. However, alveolar bone resorption data demonstrated that no *in vivo* virulence synergism could be demonstrated between *T. forsythia* and *P. gingivalis* in rats at least with the levels of bacteria used for infection. Whether or not such synergism can be demonstrated using lower levels of infection still remains to be determined. In contrast, co-infection with *P. gingivalis* and *T. forsythia* exhibits virulence synergism in rabbits (Takemoto *et al*, 1997) and in the murine abscess model and showed that the gingipain-encoding genes are responsible for this synergism (Yoneda *et al*, 2001). This mixed infection model will provide an opportunity for further studies to clarify the characteristics and alterations of the host response profiles such as proinflammatory cytokines and matrix metalloproteinase's in periodontal tissues that relate to osteoclastic alveolar bone loss in response to mixed infections.

These data have demonstrated the following: (i) mixed microbial colonization/infection of *T. forsythia* and *P. gingivalis* in the rat oral cavity (gained access to the oral epithelium), (ii) induction of moderate periodontal inflammation and pronounced apical migration of JE in rats, (iii) generation of a specific IgG antibody responses to whole-cell and rBspA protein (engagement of host response mechanisms), stimulation of both Th1- and Th2-like immune responses as reflected by the serum IgG subclass profiles, and antibodies was not protective, (iv) induction of enhanced interproximal crestal alveolar bone resorption in rats as expected (direct result of local infection), and (v) no apparent virulence synergism could be demonstrated between *T. forsythia* and *P. gingivalis* in a rat model.

Competing interests

The authors declare that they have no competing interests.

Author contributions

L Kesavalu conceived the study, designed the research and coordination as well as drafted the manuscript and corresponding author. R Verma carried out the majority of the experiments, and statistical analysis. I Bhattacharyya, I Aukhil and S Pola conducted the histology and histometry analyses. R Verma, A Sevilla, I Lieberman, and S Pola were involved in the morphometry assays. R Verma, M Nair and A Sevilla were responsible for the radiography. R Verma and S Wallet carried out antibody analysis. All authors were involved in analyzing the data as well as reading and approving the final manuscript.

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