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Coexistence of *Porphyromonas gingivalis* , *Tannerella forsythia* and *Treponema denticola* in the Red Bacterial Complex in Chronic Periodontitis Subjects

Coexistencia de *Porphyromonas gingivalis* , *Tannerella forsythia* y *Treponema denticola* en el Complejo Rojo Bacteriano en Sujetos con Periodontitis Crónica

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ABSTRACT: Previous reports showed that periodontitis is associated with different microorganisms rather than individual periodontopathogens in the dental biofilm. The purpose of the current study was to evaluate the coexistence and relationship among *Porphyromonas gingivalis, Tanerella forsythia*, and *Treponema denticola* in the red complex, noting its association with the severity of periodontitis. In this cross sectional study, 96 subjects, aged 33 to 82 years (with ≥18 residual teeth) with chronic periodontitis who attended the dental clinics of the Universidad de Antioquia in Medellín, Colombia were invited to participate. The presence or absence of bleeding on probing and plaque were registered. Probing depth and clinical attachment level were measured at all approximal, buccal and lingual surfaces. Microbial sampling on periodontitis patients was performed on pockets >5 mm. The presence of *P. gingivalis, T. forsythia*, and *T. denticola* was detected by PCR using primers designed to target the respective 16S rRNA gene sequences. The coexistence of the three periodontopathogens was the most frequent (25 subjects). A statistically significant association between the three bacteria was observed (*P. gingivalis* and *T. forsythia*, P<0.0001; *P. gingivalis* and *T. denticola*, P=0.001; *T. forsythia* and *T. denticola*, P<0.0001). Similarly, the logistic regression analysis showed a significant association among periodontopathogens. The most relevant was observed between *P. gingivalis* and *T. forsythia* (OR=6.1). In conclusion, the present study found a significant association in the coexistence of *P. gingivalis, T. forsythia* and *T. denticola*, and they related strongly to clinical parameters of inflammation and periodontal destruction.

KEY WORDS: periodontitis, *tannerella forsythia, porphyromonas gingivalis, treponema denticola*, coaggregation.

INTRODUCTION

Periodontitis is an oral inflammatory disease provoked principally by gram-negative microorganisms that will induce a local and systemic inflammatory response, leading to periodontal tissue damage. Previous report showed that periodontitis is associated with different microorganisms rather than individual periodontopathogens in the dental biofilm, defining five microbial complexes. The red complex, considered the most pathogenic, appears later in the biofilm including three pathogens: *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* (Socranski *et al*., 1998). Red complex bacteria have been shown to employ neuraminidases to scavenge host sialic acid for use as an embellishing molecule. This method supports the periodontal pathogens to avoid host immune defenses (Amano *et al*., 2014). *P. gingivalis* can locally invade periodontal tissues and evade the host defense mechanisms and utilizes a panel of virulence factors that cause deregulation of the innate immune and inflammatory responses. The ability of *P. gingivalis* to cause chronic periodontitis is resolute

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by its store of virulence factors. Biofilm development and bacterial dipeptidyl peptidase IV activity provide to its pathogenic potential (Clais *et al*., 2014). *T. forsythia* virulence factors are beginning to be adequately identified and characterized, including the surface antigen BspA, a hemagglutinin, cell envelope lipoproteins, cell surface proteolytic enzymes, glycosidases , and the cell surface layer. Besides that, lipopolysaccharide (LPS), which is present in the outer membrane of most Gram-negative bacteria for both its structural and functional integrity, is a well-known immunostimulatory agent serving as one of the primary targets of the innate arm of the mammalian immune system (Posch *et al*., 2013). *T. denticola* outer membrane revealed the presence of a lipooligosaccharide, similar in overall structure and function to the LPS; it has a distinctly different pattern of sugar molecules and lacks the lipid A component of a typical LPS, becoming a primary activator of inflammatory responses.

The distribution and occurrence of periodontal pathogens change depending on geographic situations highlighting the importance of studying different locations (Haffajee *et al*., 2004). However correlation between coinfection of these three microbes with severity of periodontitis is not adequately documented in Latin America. Various studies have shown the prevalence of periodontal pathogens (Haffajee *et al*.; Ardila *et al*., 2012; Lafaurie *et al*., 2007) but they do not explicit their relationship properly and some of them have used only cultivation techniques that are not appropriate to identify them, this is the case of *T. forsythia*, periodontal pathogen that has remained an under investigation because of its fastidious growth and recalcitrant nature to genetic manipulation (Amano *et al*.); on the other hand, *T. denticola* is the difficulty in cultivating them, moreover, progress in molecular analysis of specific *T. denticola* behaviors has been considerably slowed by the limitations of currently available genetic systems for this organism (Fenno, 2012) Moreover, a better understanding of the composition of the subgingival plaque and the association of periodontal pathogens with periodontal status in a particular population are crucial to carry out the most effective periodontal treatment.

Thus, the objective of this investigation was to evaluate the coexistence and relationship among *P. gingivalis*, *T. forsythia*, and *T. denticola* in the red complex, noting its association with the severity of periodontitis.

MATERIAL AND METHOD

In this cross sectional study, 96 subjects, aged 33 to 82 years (with ≥18 residual teeth) with chronic periodontitis who attended the dental clinics of the Universidad de Antioquia in Medellín, Colombia were invited to participate between January 2009 and December 2011. Informed and written consent was obtained from each participant. The study design was approved by the Ethics Committee on Human Research of the School of Dentistry of the University of Antioquia according to the Declaration of Helsinki on experimentation involving human subjects.

Exclusion criteria included diagnosed diabetes and autoimmune diseases. Pregnant women, previous (six months) consumption of systemic antimicrobials, non-steroidal analgesics or anti-inflammatory drugs, and previous periodontal therapy also served as exclusion criteria.

Medical history and clinical and radiographic examination were conducted for each patient. The diagnosis of chronic periodontitis was made based on criteria defined by Eke *et al*. (2012); subjects were classified as moderate periodontitis by ≥2 interproximal sides with clinical attachment level (CAL) ≥4 mm, or by ≥2 interproximal sides with probing depth (PD) ≥5 mm (not at the same tooth). Severe periodontitis was characterized by ≥2 interproximal sides with CAL ≥6 mm and ≥1 interproximal side with PD ≥5 mm (not at the same tooth). Subjects with no evidence of mild, moderate, or severe periodontitis were used as control group.

A trained and calibrated clinician performed all clinical examinations. The intra-examiner reproducibility was assessed before and during the study. The intra-class correlation coefficients for mean PD and CAL were 0.92 and 0.91, respectively; the intra-evaluator kappa index were in the range 0.85–0.96 The presence or absence of bleeding on probing (BOP) and plaque were registered. PD and CAL were measured at all proximal, buccal and lingual surfaces to the nearest millimeter by a calibrated standard probe (UNC-15, Hu-Friedy, Chicago, IL).

Microbial Sampling. Microbial sampling on periodontitis patients was performed on pockets > 5 mm. The deepest six pockets were selected for sampling. After removing supragingival plaque with curette and isolating the area with cotton pellets, the

paper points (Maillefer, Ballaigues, Switzerland) were inserted into each periodontal pocket for 20 seconds. One paper point from each site was introduced into an empty 1.5 ml micro-fuge tube for polymerase chain reaction (PCR) analysis.

The presence of *P. gingivalis*, *T. forsythia*, and *T. denticola* was detected by PCR using primers designed to target the respective 16S rRNA gene sequences, according to the method of Ashimoto *et al*. (1996). Briefly, PCR mixtures (50 mL) were prepared with 5 mL of bacterial DNA (GoTaq Flexi DNA Polymerase, Promega), 0.5 mM species-specific primers, 10 mL of 5 PCR buffer (Deoxynucleotide Triphosphates, Promega),1.25 U of Taq DNA polymerase, # 0.2 mM dNTP mix, and 1.5 mM MgCl2. Gene-specific amplification was performed in a thermal cycler (MyCycler® Termal Cycler), Bio-Rad with the following thermal profiles: *T. forsythia* and *T. denticola*, initial denaturation step at 95°C for 2 minutes, followed by 36 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute and extension at 72°C for 1 minute, and a final extension at 72°C for 2 minutes. PCR products were electrophoresed on 1% agarose gels and stained with 0.5 mg/mL ethidium bromide, and the presence of target bands for each bacterium was confirmed.

Statistical Analysis. Differences in continuous and categorical variables were examined with independent t test (data were distributed normally) and X2 test, respectively. Associations between periodontal pathogens were assessed by logistic regression analysis. The OR and corresponding 95% confidence intervals were calculated for each microorganism. P values of <0.05 were considered statistically significant. All analyses were performed using statistical software (SPSS version 15.0; SPSS, Chicago, IL).

RESULTS

A total of 72 women and 24 men with chronic periodontitis were studied. Table I depicts the demographic characteristics and periodontal parameters of the subjects. A higher number of women and non-smokers were observed. When comparing smokers and non-smokers higher PD and CAL lost were observed.

Table II shows coexistence of microorganisms in the red complex and their frequency in patients with chronic periodontitis. The coexistence of the three periodontopathogens was the most frequent (25 subjects). A statistically significant association between the three bacteria was observed (*P. gingivalis* and *T. forsythia*, P<0.0001; *P. gingivalis* and *T. denticola*, P=0.001; *T. forsythia* and *T. denticola*, P<0.0001). Interestingly, in 22 subjects the presence of any bacteria was identified. In summary, in coexistence of the three bacteria, higher PD and CAL lost were noticed (Table III).

On the other hand, in bivariate analysis a strong association between smoking and *T. forsythia* was observed (P<0.0001). Consistent with this result, the logistic regression analysis (crude model) confirmed this association (OR=2.72, 95% confidence interval [CI] = 1.09-6.8; P=0.031). This statistically significant association remained after adjustment for age and sex (OR= 2.75, 95% CI= 1.09-6.9; P=0.032).

Similarly, the logistic regression analysis showed a significant association among periodontopathogens (Table IV). The most relevant was observed between *P. gingivalis* and *T. forsythia* (OR=6.1).

Table I. Demographic characteristics and periodontal parameters of the patients with periodontitis.

Parameter		**Periodontitis subjects (n=96)**
Age (years±SD)		46±9.3
Sex	Female	75%
	Male	25%
Smoking	Non-smokers	72%
	Smokers	28%
PD (mm±SD)		3.6±1.1
CAL (mm±SD)		4.3±1.4
Plaque		54%
BOP		47%

SD=Standard Deviation.

Table II. Coexistence of microorganisms in the red complex and their frequency in patients with chronic periodontitis.

Periodontopathogens	Frequency	%
P. gingivalis, T. forsythia and *T. denticola*	25	26
P. gingivalis and *T. forsythia*	19	19.8
P. gingivalis and *T. denticola*	4	4.2
T. forsythia and *T. denticola*	4	4.2
P. gingivalis	12	12.5
T. forsythia	8	8.3
T. denticola	2	2.1
None	22	22.9

Table III. Coexistence of red complex microorganisms and periodontal parameters.

Microorganisms	PD (mm±SD)	CAL (mm±SD)	Plaque (%±SD)	BOP (%±SD)
P. gingivalis, T. forsythia and *T. denticola*	3.8±0.8	4.7±1.1	46±28	50±27
P. gingivalis and *T. forsythia*	3.7±1.5	4.5±1.7	51±32	49±31
P. gingivalis and *T. denticola*	3.3±1.5	3.9±1.2	35±18	30±15
T. forsythia and *T. denticola*	3.5±1.1	4.6±1.4	66±34	40±20
P. gingivalis	3.7±1.1	4.5±1.5	55±38	47±37
T. forsythia	3.2±1.2	3.5±1.1	49±32	39±30
T. denticola	2.3±1.1	2.1±1.2	63±51	70±27

SD=Standard Deviation.

Table IV. Associations among periodontal pathogens evaluated.

	T. forsythia			*T. denticola*		
	OR	95% IC	*P* value	OR	95% IC	*P* value
P. gingivalis	6.1	2.6-14.2	<0.0001	4.7	1.8-12	0.001
T. forsythia	---	---	---	5	1.9-12.3	0.001

DISCUSSION

The outcomes of this investigation specify that the associations among coexistence of *P. gingivalis*, *T. forsythia* and *T. denticola*, namely the red complex and CAL, PD and BOP were significant in chronic periodontitis, suggesting an important role in the progression of chronic periodontitis. Confirming our results, previous investigations showed similar relationships (Haffajee *et al*.; Ardila *et al*.; Lafaurie *et al*.); however they did not report the coexistence and its relation to periodontal parameters in detail as was done in this study. Thus, in sites with higher PD and CAL lost a higher frequency of the coexistence *P. gingivalis*, *T. forsythia* and *T. denticola* was shown, ratifying the solid association between chronic periodontitis and red complex species, previously presented (Haffajee *et al*.; Socransky *et al*.).

Coaggregation between microorganisms plays a key role in the colonization of the gingival crevice and the organization of periodontopathic biofilms. In this study, the logistic regression analysis showed a significant association among *P. gingivalis*, *T. forsythia* and *T. denticola*. It has been shown that members of this red complex coaggregate strongly in vitro (Yao *et al*., 1996) and one species of the complex may produce growth factors required by another in that complex (Nilius *et al*., 1993). Analogously, Hashimoto *et al*. (2003) suggested that *P. gingivalis fimbriae* and *T. denticola* dentilisin are implicated in the coaggregation of these bacteria; Meuric *et al*. (2013) showed that interactions of *P. gingivalis* with other bacterial species, such as *T. denticola*, induce increased adhesive capacities on various substrata by hemagglutinin adhesion domain-containing proteins. Besides, the surface layer of *T. forsythia* is composed of cell surface glycoproteins, such as TfsA and TfsB, and is known to

play a role in adhesion/invasion and suppression of proinflammatory cytokine expression (Shimotahira *et al*., 2013).

The current study has a particular interest because a strong association between smoking and *T. forsythia* was observed (P<0.0001) which remained after adjustment for age and sex (OR=2.75, 95% CI= 1.09–6.9; P=0.032). Similar results presented Guglielmetti *et al*. (2014), showing that counts of *T. forsythia* were higher in smokers. The correlation observed could be the result of the lower pocket oxygen tension found in smokers with periodontitis, compared to non-smokers (Hanioka *et al*., 2000). Another mechanism of action involved could be the smoking-promoted selective adherence of periodontal pathogens to the epithelial surfaces of periodontal pockets (Guglielmetti *et al*.).

In conclusion, the present study found a significant association in the coexistence of *P. gingivalis*, *T. forsythia* and *T. denticola*, and they related strongly to clinical parameters of inflammation and periodontal destruction. Further studies are needed to find out the molecular mechanisms underlying the coaggregation between periodontal pathogens.

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RESUMEN: Reportes previos mostraron que la periodontitis se asocia con diferentes microorganismos en lugar de periodontopatógenos particulares en la biopelícula dental. El objetivo del presente estudio fue evaluar la coexistencia y relación entre *Porphyromonas gingivalis*, *Tanerella forsythia* y *Treponema denticola* en el complejo rojo, señalando su vinculación con la severidad de la periodontitis. En este estudio transversal, 96 sujetos de 33 a 82 años (con ≥18 dientes residuales) con periodontitis crónica que asistieron a las clínicas dentales de la Universidad de Antioquia en Medellín, Colombia fueron invitados a participar. Se registraron la presencia o ausencia de sangrado al sondaje y placa. La profundidad de sondaje y nivel de inserción clínica se midieron en todas las superficies proximales, bucal y lingual. El muestreo microbiano en pacientes con periodontitis se realizó en los bolsillos mayores a 5 mm. La presencia de *P. gingivalis*, *T. forsythia*, y *T. denticola* se detectó por PCR usando las bolsas periodontales diseñadas para dirigirse a las respectivas secuencias de genes 16S RNAr. La coexistencia de los tres periodontopatógenos fue la más frecuente (25 sujetos). Se observó una asociación estadísticamente significativa entre las tres bacterias (*P. gingivalis* y *T. forsythia*, P<0,0001; *P. gingivalis* y *T. denticola*, P=0,001; *T. forsythia* y *T. denticola*, P<0,0001). Del mismo modo, el análisis de regresión logística mostró una asociación significativa entre periodontopatógenos; la más relevantes se observó entre *P. gingivalis* y *T. forsythia* (OR=6,1). El presente estudio encontró una asociación significativa en la coexistencia de *P. gingivalis*, *T. forsythia* y *T. denticola*, y estuvieron fuertemente relacionadas a los parámetros clínicos de la inflamación y destrucción periodontal.

PALABRAS CLAVE: periodontitis, *Tannerella forsythia, Porphyromonas gingivalis, Treponema denticola*, coagregación.

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