Study on the Prevalence of Periodontopathogenic Bacteria in Serum and Subgingival Bacterial Plaque in Patients with Rheumatoid Arthritis

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The purpose of this study was to detect bacterial periodontal DNA from subgingival dental plaque and serum in patients affected by rheumatoid arthritis and periodontitis. The study group included 19 patients with periodontitis and refractory rheumatoid arthritis. The patients were clinically examined and diagnosed and the bacterial DNA was detected in the subgingival bacterial plate and serum by PCR. Severe chronic periodontitis was the most commonly diagnosed (42.2%). The DNA of periodontopathogenic bacteria was detected 100% in subgingival plate samples, and in serum samples it was identified in 84.2% of cases. The most commonly found species in subgingival plate samples were P. intermedia (100%), T. denticola (84.2%) and P. gingivalis (78.9%). In serum samples, the most frequently detected species were P. intermedia (89.4% and 73.6% respectively) and P. gingivalis (57.8% and 42.1%, respectively). A. actinomycetemcomitans and P. gingivalis did not show statistically significant differences between samples. This finding suggests that it could be an association because the same bacteria species detected in the serum were present in bacterial plaque samples. Patients with rheumatoid arthritis contain levels of oral pathogens in the serum and subgingival plaque that are common to red complex organisms, namely Porphyromonas gingivalis, Tannerella forsythia and Prevotella intermedia.

Keywords: subgingival bacterial plaque, periodontitis, rheumatoid arthritis

Inflammation is a complex biological process that occurs in response to infection, autoimmunity, stress and/or other tissue damage. Alveolar bone loss that complicates periodontal inflammation (e.g. periodontitis) is the most common form of clinically significant osteopenia that occurs in humans [1-3]. This is largely due to the fact that periodontal tissues show a significant number of bacterial strains, including anaerobes with pathogenic potential, thus making these tissues prone to infection.

In the subgingival plaque, over 500 species of bacteria have been identified as a complex ecological niche. Under the influence of local or systemic factors, some bacteria in the subgingival dental biofilm become the primary etiologic agents of periodontal disease. These polymicrobial infections in most cases involve gram-negative anaerobic periodontal pathogens that act synergistically [4]. The most commonly involved bacteria are: *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola, Peptostreptococcus micros, Fusobacterium nucleatum, Eikenella corrodens, Campylobacter rectus, Eubacterium nodatum, Capnocytophaga* spp.

Periodontopathogens can produce many virulence factors, leading to the destruction of periodontal tissues [5, 6]. Clinical studies have associated periodontopathic bacteria with some systemic disorders such as myocardial infarction [7], premature birth, atherosclerosis [8, 9], chronic kidney disease [10] and cerebral vascular accident and rheumatoid arthritis [11].

These associations are based on the fact that the prevalence of periodontitis is high in subjects affected by these systemic conditions, and some studies have reported the identification of periodontal bacteria in atheromas, amniotic fluid and synovial fluid [11].

Periodontal disease and rheumatoid arthritis have been reported to have similar physiopathological mechanisms because periodontal bacteria and their virulence factors produce neutrophil, monocyte and T and B mediated immune responses and lead to the release of proteinases, cytokines and prostaglandins, causing bone osteoclast activity and bone erosion, similar to the pathophysiology of rheumatoid arthritis [12].

There are reports that emphasize the clinical association between rheumatoid arthritis and periodontal disease [13], and some studies identify antibodies against periodontal bacteria in serum and synovial fluid [14], but only few studies detect periodontal bacterial DNA in joints affected by rheumatoid arthritis [11].

Periodontitis and rheumatoid arthritis are predominant diseases. It has been suggested that the bacteria involved in periodontal disease are also active in the pathogenesis of rheumatoid arthritis. It is important to study the potential role of periodontal bacterial DNA in the natural evolution of rheumatoid arthritis.

The purpose of this study was to detect bacterial periodontal DNA in the subgingival dental plaque and serum in patients affected by rheumatoid arthritis and periodontitis.

Experimental part

The study group included 19 patients with periodontitis and refractory rheumatoid arthritis despite intensive treatment with anti-rheumatic diseases (DMARD) (methotrexate, sulfasalazine, leflunomide and chloroquine).

The patients completed a health questionnaire that included information on systemic health and oral diseases. Written and informed consent from patients was obtained prior to clinical examination in accordance with the ethical principles of the Helsinki Declaration.

The patients included in this study were males and females over 18 years of age who had persistent

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rheumatoid arthritis with synovial fluid effusion on their knees without other systemic diseases and affected by periodontitis.

Patients who received antibiotic therapy at least three months prior to the study, those who received pre-treatment for periodontal disease, pregnant women and nursing mothers were excluded from the study.

All patients were treated with rheumatic DMARD-modifying medication, most of them also benefited from non-steroidal anti-inflammatory medication and low-dose steroids.

The diagnosis of periodontitis was determined by measuring the depth of the periodontal pocket and the clinical index of loss of attachment. These indices were obtained using a periodontal probe, the Merrit B (Hu-Friedy) probe graduated in millimetres (0-10 mm).

Ten millilitres of peripheral blood from the cubital vein were collected from each patient and placed inside vacuum tubes and citrate medium. They were centrifuged at 275 g for 8 min to obtain the serum.

The subgingival dental plaque has always been collected after obtaining blood samples to avoid transient bacteraemia that may influence the presence of different bacterial species in the serum. After cleaning the crown of the tooth with a sterile sponge, a subgingival plate was collected with a Gracey curette from the vestibular, medial, palatin, and distal sulcus. This was placed in an Eppendorf tube with 1 ml of phosphate buffered saline (PBS). The subgingival plaque was harvested from the first upper molar in quadrant I, from the lower central incisor in quadrant III, and from the lower premolar in quadrant IV as they present the most affected areas of periodontitis, and the possibility of obtaining the subgingival dental plaque was more feasible. The sample from each tooth was placed in a different tube; the sample was collected 2 h after the last meal and dental brushing.

The subgingival plate and peripheral blood were transported with ice and stored at -40°C until polymerase chain reaction (PCR) and microbiological assessments were performed. All samples were processed under aseptic requirements to prevent contamination of both the environment and the DNA extraction method for PCR assays.

Positive controls were included in each PCR set using DNA from the following bacterial strains: *P. gingivalis* (ATCC 33277 and HG1691), *T. forsythia* (ATCC 43037), *Prevotella intermedia* (ATCC 25611), *Aggregatibacter actinomycetemcomitans* (ATCC 29523 and HK1651), *P. nigrescens* (ATCC 25261) and *T. denticola* (ATCC 35405).

A control (negative) test sample was also included in each PCR set containing a sample only with deionized water (instead of a patient sample) to know if non-specific products were amplified.

For the statistical analysis, the ESPE SS 12 package was used, and the α value was set to 0.05. Normally distributed variables were reported as standard deviations. The Chi-Square and Fisher tests with Anova were used to compare the data.

To determine the statistical differences in the periodontal bacterial DNA detection near the dental plaque and serum, Fisher's exact test was also used and the statistical significance was established at $p < 0.05$.

Results and discussions

The mean age of patients was 55.7 (± 15.8) years, with a range of 21 to 88 years of age. Sixteen patients (84.2%) were females. The time course of rheumatoid arthritis was 8.71 (± 5.99) years, with a range of 0.5-20 years from the initial clinical diagnosis of rheumatoid arthritis. The most common type of periodontitis detected was the chronic form found in 18 subjects (94.7%); the aggressive form was present in only one subject (5.3%) of the 19 subjects.

The severe phase of chronic periodontitis was more commonly diagnosed (42.2%) than the moderate and mild stages of chronic periodontitis (36.8% and 21.1%, respectively). The average overall depth of the pocket was 3.9 (± 0.81) mm, but given the deepest depth of the pocket of each tooth, the average was 4.2 (± 0.79) mm. In terms of loss of attachment, the average was 3.63 (± 0.90) mm, the upper molars being the most affected teeth, with an average of 3.85 (± 0.83) mm.

Subjects presented 63.8% of teeth present in the arcade; lower molars were the most frequently absent teeth (46%) (table 1).

The DNA of periodontopathogenic bacteria was detected in 100% of the subgingival plate samples, and in serum samples it was identified in 84.2% of cases.

Regarding the number of identified bacteria species, 4.05 different bacterial species were detected in subgingival samples and 1.19 species were detected in serum samples. The most commonly found species in subgingival samples were *P. Intermedia* (100%), *T. denticola* (84.2%) and *P. gingivalis* (78.9%). In serum samples, the most frequently detected species were *P. Intermedia* (89.4% and 73.6% respectively) and *P. gingivalis* (57.8% and 42.1%, respectively) (table 2).

The less common species detected was *A. actinomycetemcomitans* (15.7%). Comparing the two types of biological samples we can see that *A. actinomycetemcomitans* and *P. gingivalis* did not show significant statistical differences in the samples. On the other hand, *P. Intermedia, T. forsythia, P. nigrescens* and

	Mean value	Standard Deviation (SD)	Interval
Pocket depth (mm)	3.9	0.81	2.6-5.7
Present teeth (n)	17.89 (63.8)	8.93	3-27
Superior anterior teeth (6)	4.10 (68.3)	2.33	0-6
Inferior anterior teeth (6)	4.52 (75.3)	1.92	1-6
Superior premolars (4)	2.36 (59)	1.60	0-4
Inferior premolars (4)	2.94 (73.5)	1.31	0-4
Superior molars (4)	2.10 (52.5)	1.37	0-4
Inferior molars (4)	1.84 (46)	1.57	0-4
n= 19.			

Table1
DENTAL PARAMETERS ON NUMBER OF PATIENTS

Table 2
SUBGINGIVAL AND SERUM PERIODONTAL BACTERIAL DNA

	Dental plaque		Serum		p-value
	Frequency	%	Frequency	%	
Prevotella intermedia	19	100	14	73.6	0.0453
Tannerella forsythia	10	52.6	6	31.5	0.0203
Prevotella nigrescens	13	68.4	0	0	<0.0001
Aggregatibacter	4	21.0	0	0	0.1204
Porphyromonas gingivalis	15	78.9	8	42.1	0.0674
Treponema denticola	16	84.2	4	21	0.0004

T. denticola statistically showed a significant difference (table 2).

The most commonly found species in all types of serum samples were *P. intermedia* and *P. gingivalis* (63.1% and 36.8%, respectively). There was no negative topic for *P. intermedia* (table 3). The most common species absent in all samples was *A. actinomycetemcomitans* (78.9%).

Periodontitis is a multifactorial disease, which includes various etiological factors, gram-negative bacteria being a decisive component in triggering the disease. Bacterial plaque microorganisms colonize tooth surfaces as biofilms, a phenomenon that makes the treatment of these types of infections a challenge. Another factor that complicates the control of periodontitis is invasion of the gingival tissues of periodontal microorganisms.

Periodontal disease was associated with rheumatoid arthritis, whose etiology is still not fully elucidated, although some reports indicated that an infectious agent in a susceptible host could be a possible trigger factor [15].

Both rheumatoid arthritis and periodontal disease share similar immunopathological mechanisms, because the virulence factors produced by periodontal bacteria produce an immune response that is mediated by neutrophils, monocytes, B and T lymphocytes which lead to an increase in the release level of prostaglandins that stimulate osteoclastic activity and lead to bone erosion, similar to the mechanism involved in rheumatoid arthritis [2].

A common microbial pathogen involved in both pathologies, rheumatoid arthritis and periodontal disease is *P. gingivalis*. Protein deamination is facilitated by a PAD of the peptidyl enzyme arginine deaminase, an enzyme released by *P. gingivalis*. This, in turn, causes a pro-inflammatory response to citrulline proteins. This biochemical reaction is a vital factor in the progression of rheumatoid arthritis.

There have been studies exploring associations between periodontal bacteria and rheumatoid arthritis. They are mainly concentrated on detecting antibodies against various bacteria, especially in serum. In a case-control study, serum antibodies against periodontal bacteria and subsequently causing disease were identified more frequently in subjects affected by rheumatoid arthritis and periodontitis than in control subjects [14]. In any case, it is important to bear in mind that the detection of periodontal bacterial DNA in patients with rheumatoid arthritis is more important than the detection of antibodies because it suggests the transport of bacterial DNA from periodontal infections to the joints of patients with rheumatoid arthritis.

In this study, only 19 patients were included due to the difficulty of finding patients who met the inclusion criteria. Patients with refractory rheumatoid arthritis treated with DMARD-modifying disease of the periodontal disease were selected because this condition was necessary to obtain a serum sample with significant characteristics.

Most patients were female (84.2%), and was consistent with the information that rheumatoid arthritis affects women more than men. The most common forms of

Table 3
PERIODONTAL BACTERIAL DNA DETECTED IN DIFFERENT COMBINATIONS

Bacteria	Negative subgingival and serum samples (absence of bacterial DNA) (no. of samples/percentage values	Positive subgingival and serum samples (presence of bacterial DNA) (no. of samples/percentage values)	Positive subgingival samples (presence of bacterial DNA) (no. of samples/percentage values)
Prevotella intermedia	0 (0)	2 (10.5)	0 (0)
Tannerella forsythia	9 (47.3)	4 (21.0)	4 (21.0)
Prevotella nigrescens	5 (26.3)	0 (0)	9 (47.3)
Aggregatibacter actinomycetemcomitans	15 (78.9)	0 (0)	1 (5.2)
Porphyromonas gingivalis	4 (21.0)	1 (5.2)	3 (15.78)
Treponema denticola	3(15.78)	1 (5.2)	9 (47.3)

periodontitis found in this study were those of chronic form; this may be due to the age of the included patients.

In the present study, periodontal bacterial DNA was detected in 100% of subgingival plaque samples and 84.2% in serum samples. Regarding the number of bacterial species detected, a large number (4.05) of bacterial species was identified in the subgingival plate, followed by serum (1.19). The fact that there is a lower presence of serum bacterial DNA can be explained by its dilution from the blood stream by renal filtration. This data is consistent with other studies where bacterial DNA has been detected by DNA-DNA chess hybridization resulting in 100% positive serum samples [11]. Several species commonly identified in serum were *P. intermedia, P. gingivalis* and *T. denticola*; two of them belong to the red complex, which is associated with destructive diseases. On the other hand, *A. actinomycetemcomitans*, mainly responsible for aggressive periodontitis, was less frequently detected. The reason could be that only one patient was affected by aggressive periodontitis. These data are consistent with previous reports [11].

A. actinomycetemcomitans and *P. gingivalis* did not show statistically significant differences between samples. This finding suggests that it could be an association because the same bacteria species detected in the serum were present in bacterial plaque samples.

On the other hand, there were statistical differences between samples for *P. intermedia, T. forsythia, P. nigrescens* and *T. denticola*. In addition, *P. intermedia* and *P. gingivalis* were the most frequently detected species in the three sample sites.

The tooth associated microflora has been extensively studied. The presence of *Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actinomycetemcomitans* poses an increased risk for periodontitis. The microbiota of healthy periodontal sites and those affected by the disease have been shown to differ from one another. A small number of microorganisms and fewer morphological types can be found in the healthy gingival sulcus [16]. Affected sites have a complex microflora with a high proportion of gram-negative anaerobic microorganisms.

The microbiota of healthy periodontal sites and that of the affected sites have been shown to differ from one another. Reduced numbers of microorganisms and fewer morphological types can be found in healthy gingival sulcus. Affected sites have a complex microflora, with a high proportion of anaerobic gram-negative bacteria [17].

It is important to note that patients who were positive for any bacteria in the serum were also positive when detected in the subgingival plaque. *P. gingivalis* produces a microbial enzyme, peptidyl arginine deaminase (PAD), which is the human equivalent of this enzyme and which has been associated as a susceptible factor for rheumatoid arthritis, since the antigens generated by arginine peptidyl deaminase lead to the production of rheumatoid factor and local inflammation [18].

Antibodies against *P. intermedia* heat shock proteins (hsp 70) were found in periodontal tissue as well as in the synovial tissue of patients with rheumatoid arthritis [19]. It has also been shown that when there are hsp 70 expressions induced by certain stress stimulating factors, proinflammatory cytokines are induced in the synovium of patients with rheumatoid arthritis [20]. These findings are important because in this study, *P. intermedia* was the most commonly isolated bacterium in patients with rheumatoid arthritis.

We therefore suggest that bacterial DNA can play a role in the pathogenesis of rheumatic diseases. The transport of DNA from periodontal pockets to the joints could be the free DNA. This information can be valuable for future studies to elucidate whether periodontal pathogenic DNA might be a possible trigger for rheumatoid arthritis development.

Conclusions

Periodontal bacterial DNA was detected in the subgingival plaque of patients with rheumatoid arthritis. It is therefore suggested that periodontal bacterial DNA plays a major pathological role in the severity of rheumatoid arthritis. *P. intermedia, T. forsythia*, and *P. gingivalis* were the most commonly found species in the subgingival dental plaque, the corresponding and predominant bacteria in the red complex, which is involved in the destruction of the periodontal bone.

Patients with rheumatoid arthritis contain serum levels of antibodies to oral pathogens, which are common to red complex organisms, namely *Porphyromonas gingivalis, Tannerella forsythia* and *Prevotella intermedia.*

The data obtained in this study provides evidence to demonstrate the existence of a link between rheumatoid arthritis and periodontal disease.

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