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Detection of periodontal bacterial DNA in serum and synovial fluid in refractory rheumatoid arthritis patients

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Abstract

Aim: To identify periodontal bacterial DNA (PBDNA) by PCR in subgingival dental plaque (SDP), serum and synovial fluid (SF) of rheumatoid arthritis (RA) with periodontal disease (PD) patients and to explore the possible PBDNA transport pathways from mouth to joints.

Methods: This cross-sectional prolective study involved 19 subjects with RA and PD. Informed consent, health and dental questionnaires were obtained. SDP, SF and serum samples were obtained, and leucocytes were isolated from blood. DNA was extracted and PCR assays to detect main PD species were carried out. Cultures on agar plates and broth, from each sample, were performed.

Results: Hundred percentage of patients showed PBDNA in SDP and SF and 83.5% in serum. *Prevotella intermedia* (89.4% and 73.6%) and *Porphyromonas gingivalis* (57.8% and 42.1%) were the species most frequently detected in SDP and SF, respectively. In SDP, 4.05 different bacterial species were found followed by 1.19 in serum and 2.26 in SF. Culture onto agar plates and broth did not show any bacterial growth, leucocytes were not positive to PBDNA by PCR.

Conclusion: This study suggests that PBDNA could have a role on the RA aetiology. The possible pathway of transport of PBDNA from mouth to joints could be via the free form of DNA.

Key words: culture media; dental plaque; knee; PCR; periodontal bacteria; periodontal disease; periodontitis; rheumatoid arthritis; serum; synovial fluid

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Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the accumulation of inflammatory infiltrate in the synovial membrane, which leads to destruction of the joint architecture resulting in impaired function. It begins between the fourth or fifth decade of life, with a prevalence of at least of 0.5–1% (Symmons et al. 2002). RA affects females more frequently than males (2–3:1) (Gabriel et al. 1999, Symmons et al. 2002). Functional status of people with RA is compromised; affected people suffer a loss in their capacities involving work and daily activities. In the United States, RA represents about 3.3–14.6% of the work–age disabilities (Mercado et al. 2001). The aetiology of RA is still unknown. It has environmental, genetic and immunological components. Some studies suggest that an infectious agent in a susceptible host could be the trigger factor or is related with relapses of RA; the suggested microorganisms involved are mycoplasma, Epstein–Barr virus, cytomegalovirus, rubeola virus and periodontal bacteria (Bartold et al. 2005).

Periodontal disease (PD) is a chronic asymptomatic infectious disease that affects the supporting tissues around the teeth and is associated with a great number of different Gram-negative bacterial species. It is a multifactorial disease; the aetiological factors involved in this disease being environmental, genetics, immunological and bacteriological (Bartold et al. 2005). PD is the second most frequent oral disease and involves

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a progressive loss of alveolar bone and can lead to tooth loss; its prevalence is about 34.5% (Mercado et al. 2001). Periodontitis has other clinical signs such as gingivitis, periodontal pockets, attachment loss, dental mobility and it ends with loss of teeth. The chronic form begins around the third decade of life, and the aggressive form appears in younger patients (Ximenez-Fyvie et al. 2006).

Inside the periodontal pocket, there are several bacterial species that include different groups, the red complex (*Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticola*) being specific for active periodontitis. Recently, clinical studies have associated periodontopathic bacteria with some systemic conditions, like myocardial infarction (Doan et al. 2005), preterm births (Pitiphat et al. 2008), atherosclerosis and stroke (Haraszthy et al. 2000, Padilla et al. 2007) and rheumatoid arthritis (Moen et al. 2006). These associations are based on the fact that the prevalence of periodontitis is high in subjects affected with these systemic conditions, and some studies have reported the identification of periodontal bacteria in atheromas, amniotic fluid and synovial fluid (Haraszthy et al. 2000, Moen et al. 2006, Padilla et al. 2007).

PD and RA have been reported having similar physiopathogenic mechanisms because periodontal bacteria and their virulence factors produce an immune response, mediated by neutrophils, monocytes and lymphocytes T and B, and these reactions lead to proteinases, cytokines and prostaglandins release, stimulating osteoclast activity and originating bone erosion, similar to the physiopathology of RA. There are reports that emphasize the clinical association between RA and PD (Mercado et al. 2001), some studies identify antibodies against periodontal bacteria in serum and synovial fluid (Ogrendik et al. 2005, Mikuls et al. 2009) but only a few studies detect periodontal bacterial DNA in RA in affected joints (Moen et al. 2006). Periodontitis and RA are prevalent diseases. It has been suggested that the bacteria involved with PD are also active in the pathogenesis of RA. It is important to study the potential role of periodontal bacterial DNA in the natural evolution of RA.

The purpose of this study was to detect periodontal bacterial DNA from subgingival dental plaque (SDP), serum (SE) and synovial fluid (SF) from patients affected by RA and periodontitis. Additionally, the possible pathways of periodontal bacterial DNA (PBDNA) transport from periodontal pockets to affected joints were explored.

Methods

This cross-sectional prolective study involved 19 subjects with periodontitis and refractory RA despite intensive treatment with disease-modifying antirheumatic drugs (DMARDs) (methotrexate, sulfasalazine, leflunomide and chloroquine); all of them were living in San Luis Potosi (northern-central region), Mexico. Subject recruitment was undertaken at the Regional Unit of Rheumatology and Osteoporosis and Oral Medicine Clinic of San Luis Potosi University. Subjects completed a health questionnaire that included information about systemic health and oral diseases. Informed and voluntary written consent from patients was obtained before clinical examination according to the ethical principles of declaration of Helsinki (Mikuls et al. 2009). A non-probabilistic consecutive sampling was performed from January 2007 to December 2008; >500 patients were evaluated but only 19 fulfilled the inclusion criteria. Subjects involved in this study were males and females >18 years old with an evidence of persistent activity (refractory) of RA, under DMARDs treatment and with effusion of knee synovial fluid, without other systemic diseases and affected by periodontitis. Subjects with antibiotic therapy in the three previous months, those with previous treatment of PD, pregnant and breastfeeding women were excluded from the study.

Rheumatoid arthritis diagnosis

Diagnosis of RA was performed by a rheumatologist in accordance with the criteria of the American College of Rheumatology, 1987 (Arnett et al. 1988). All patients were under two or more DMARDs, most of them with nonsteroidal anti-inflammatory drugs and low doses of steroids.

Periodontitis diagnosis

Diagnosis of periodontitis was determined by a calibrated examiner measuring pocket depth and clinical attachment loss indexes (Rodríguez-Martínez et al. 2006). These indexes were obtained by using a Michigan periodontal probe graduated in millimetres (0–10 mm). The probe was inserted parallel to the teeth long axis and crossed each tooth's surface circumferentially. Clinical attach ment loss index was measured from the epithelial attachment to the cement–enamel junction. The diagnosis of periodontitis was determined when the pocket depth was ≥ 3 mm and the attachment loss was ≥ 2 mm in at least 10 sites (Davila-Perez et al. 2007).

Synovial fluid sample

SF was obtained from the affected knee when the physician was able to determine the evidence of SF effusion. A rheumatologist, in accordance with international standards, obtained the samples. The extraction of SF was performed by a lateral approach, inserting a disposable needle no. 20, one lateral fingerbreadth and above the patella following the asepsis of the punction area (Zuber 2002). Approximately 5 ml of synovial fluid was obtained to perform the molecular and microbiological assays. The synovial fluid was placed in a citrated vacuum tube.

Serum sample

Ten millilitres of peripheral blood from the cubital vein was obtained within the citrated vacuum tubes. It was centrifuged at 275 *g* for 8 min. to obtain the serum.

Subgingival plaque sample

Subgingival dental plaque was always collected after obtaining SF and blood samples, to avoid transitory bacteraemia that may influence the presence of the different bacterial species in serum and SF. After cleaning of the tooth crown with a sterile sponge, SDP was collected with a Gracey curette from the vestibular, mesial, palatine and distal sulcus and placed into an eppendorf tube with 1 ml of phosphate-buffered saline (PBS). SDP was obtained from the upper right first molar, lower right central incisor and lower left premolar because they were some of the most affected sites by periodontitis, and the possibility of obtaining 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 tooth brushing (Davila-Perez et al. 2007).

SDP, peripheral blood and SF samples were transported on ice and were stored at −40°C until PCR and microbiological evaluations were performed.

DNA extraction and PCR

All samples were processed with aseptic requirements to prevent contamination from both environment and during the DNA extraction method for PCR assays. Five hundred microlitres of SE, 500 μl of SF and 900 μl of PBS with SDP (300 μl from tube of each sample) were centrifuged (16,000 *g*) in an eppendorf tube to obtain the cell pellet and then washed in 1 ml of PBS (pH 7.4). The pellet was suspended in 200 μl of cell lysis buffer (1.0% Triton X-100, 20 mM Tris-HCl, 2 mM EDTA (pH 8.0) and incubated at 85°C for 10 min. Then, 100 μl of 200 U/ml of mutanolysin (Sigma, St. Louis, MO, USA) was added and incubated at 50°C for 1 h, followed by treatment with 80 μl of the cell lysis solution (Puregene DNA isolation kit, Gentra Systems, Minneapolis, MN, USA) at 80°C for 10 min. Then, 60 μl of protein precipitation solution (Puregene DNA isolation kit, Gentra Systems) was added and the proteins were then, removed by centrifugation (16,000 *g* for 10 min.). The DNA was purified by phenol–chloroform–isoamyl alcohol (25:24:1, v/v; Invitrogen, Carlsbad, CA, USA) extraction and isopropanol precipitation. The extracted DNA was dissolved in 50 μl of DNA hydration solution (Puregene DNA isolation kit, Gentra Systems) and the DNA concentration was measured by fluorimetry (Genius, TECAN systems Inc., San Jose, CA, USA), where 198 μl of picogreen 1:400 and 2 μl of the DNA sample or standard (1, 5 and 50 ng/μl) were used.

PCR assay was carried out in 25 μl of a reaction mixture containing 1.0 U Taq DNA polymerase (Roche, Indianapolis, IN, USA), 0.5 μM of oligonucleotides, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$ and 10 ng of DNA template. Specific primers for each species used in the study are indicated in the Table 1. PCR reaction was performed in a thermal cycler (iCycler; Bio-Rad laboratories, Hercules, CA, USA) with the cycling parameters reported (Ashimoto et al. 1996, Tran & Rudney 1996, Stubbs et al. 1999, Suzuki et al. 2001). Positive controls were included in each PCR set by using DNA of the following bacterial strains: *P. gingivalis* (ATCC 33277 and HG1691), *T. forsythensis* (ATCC 43037), *Prevotella intermedia* (ATCC 25611) *Agregatibacter actinomycetemcomitans* (ATCC 29523 y HK1651), *P. nigrescens* (ATCC 25261) and *T. denticola* (ATCC 35405). A negative control was also included in each PCR set, it was a blank sample with only deionized water (instead of patient sample) to know if unspecific products were amplified. The PCR products were analysed by electrophoresis in a 2% agarose gel using Tris–boric acid–EDTA buffer, using a 100 bp DNA ladder marker (New England Biolab, Beverly, MA, USA) to estimate the molecular size. Each gel was stained with ethidium bromide (0.5 μg/ml) and photographed under UV light (Chemi Doc, Bio-Rad laboratories).

Microbiological assays

All SF samples were inoculated in different culture media, (agar plates and broth cultures), such as Brain Heart infusion (Difco-BD, Franklin Lakes, NJ, USA), Soy tripticase (Difco-BD) and Dentaid-1 medium (Alsina et al. 2001). Hundred microlitres of SF were inoculated onto agar plates and 50 μl of sample were inoculated into broth culture media. They were incubated under aerobic and anaerobic conditions at 37°C for 24, 48 and 72 h. Reference strains were used as positive controls in each experiment set. Measuring by spectrophotometry and comparing the optical density with sterile broth determined negative results in broth cultures. Agar plates were observed under stereomicroscopy (Olympus Optical Co., Tokyo, Japan) and colony-forming units (CFU) were determined.

Leucocytes isolation from whole blood

Leucocytes were isolated from whole blood using the gradient technique: 1 ml of Ficoll–Histopaque (Sigma) was added to an eppendorf tube, then 0.5 ml of whole blood was added and tube was kept in ice for 20 min. Red cell pellets precipitate and leucocytes remain in the uppermost layer. Then DNA was extracted from leucocytes with a commercial kit (Wizard DNA isolation kit, Promega, Madison, WI, USA) by the whole blood protocol and a PCR assay was performed to detect the different bacterial species.

Statistical analysis

The examiner was calibrated in all variables with an expert in periodontology through intraclass correlation coefficient. All data are expressed as mean, standard deviation and range. Qualitative data are expressed as frequency and

Table 1. Specific oligonucleotides used in this study

Bacteria	Sequence (5′–3′)	MS (bp)	References
Porphyromonas gingivalis	A TGT AGA TGA CTG ATG GTG AAA ACC ACG TCA TCC CCA CCT TCC TC	197	Tran & Rudney (1996)
Aggregatibacter actinomycetem comitans	CTA GGT ATT GCG AAA CAA TTT G CCT GAA ATT AAG CTG GTA ATC	262	Suzuki et al. (2001)
Prevotella intermedia	CAA AGA TTC ATC GGT GGA GCC GGT CCT TAT TCG AAG	307	Stubbs et al. (1999)
Tanerella forsythensis	GCG TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACC T	641	Ashimoto et al. (1996)
Prevotella nigrescens	ATG AAA CAA AGG TTT TCC GGT AAG CCC ACG TCT CTG TGG GCT GCG A	804	Ashimoto et al. (1996)
Treponema denticola	TAA TAC CGA ATG TGC TCA TTT ACA T TCA AAG AAG CAT TCC CTC TTC TTC TTA	316	Ashimoto et al. (1996)

MS, molecular size.

proportion. To detect statistical differences in the detection of periodontal bacterial DNA among dental plaque, serum and synovial fluid samples, Fisher's exact test was used, and statistical significance was set at $p < 0.05$.

Results

Before starting the study, the examiner was calibrated in the diagnosis of periodontitis through intraclass correlation coefficient obtaining 0.90. Five hundred and two AR patients were examined but only 19 fulfilled the inclusion criteria.

Age, gender and RA evolution time

The mean age of patients was 55.7 (± 15.8) years old, with a range from 21 to 88 years old and 16 (84.2%) of the subjects were female patients. The evolution time of RA was 8.71 (± 5.99) years, with a range of 0.5–20 years from the RA clinical diagnosis.

Periodontitis type, pocket depth, attachment loss and dental presence

The most frequent kind of periodontitis detected was the chronic form found in 18 subjects (94.7%); the aggressive form was present only in one (5.3%) of the 19 subjects. Severe stage of chronic periodontitis was more frequently diagnosed (42.2%) than moderate and mild stages (36.8% and 21.1%, respectively). The general mean pocket depth was 3.9 (± 0.81) mm, but considering the deepest pocket of each tooth, the mean was 4.2 (± 0.79) mm. Regarding the attachment loss, the mean was 3.63 (± 0.90) mm, with the upper molars, being the most affected teeth with a mean of 3.85 (± 0.83) mm. Subjects showed 63.8% of teeth in mouth; the lower anterior and lower premolars being the most frequently present teeth (75.3 and 73.5, respectively); the lower molars were the most frequently absent teeth (46%) (Table 2).

Detection of periodontal bacterial DNA by PCR assay

PBDNA was detected in 100% of both SDP and SF samples, in SE samples bacterial DNA was identified in 84.2%. Regarding the number of bacterial species identified, in SBP samples, 4.05 different bacterial species were detected followed by SF samples where 2.26 species and 1.19 species were detected in SE. The species most frequently detected in SDP samples were *P. intermedia* (100%), *T. denticola* (84.2%) and *P. gingivalis* (78.9%). In SF and SE samples, the most frequently detected species were *P. intermedia* (89.4% and 73.6%, respectively) and *P. gingivalis* (57.8% and 42.1%, respectively). The less frequently detected species in SDP, SE and SF samples was *A. actinomycetemcomitans* (21%, 0% and 15.7%, respectively). Comparing the three different samples, *A. actinomycetemcomitans* and *P. gingivalis* did not show significant statistical differences among SDP, SE and SF samples. On the other hand, *P. intermedia*, *T. forsythensis, P. nigrescens* and *T. denticola* showed a statistically significant difference (Table 3).

The species most frequently detected in the all samples at the same time were *P. intermedia* and *P. gingivalis* (63.1% and 36.8%, respectively). There was not any negative subject for *P. intermedia* in the three samples sites (Table 4). The species most frequently absent in all samples at the same time was *A. actinomycetemcomitans* (78.9%).

Possible pathways of DNA transport from periodontal sites to affected joints

The inoculation of SF on agar plates and in broth culture, under aerobic and anaerobic conditions indicated no evidence of bacterial growth in any subject but only in the positive controls at 24, 48 and 72 h at 37°C. After the leucocytes were isolated from whole blood, a PCR assay was performed, but there was not any positive sample to any periodontal bacterial species studied.

Discussion

Periodontitis is a multifactorial disease, which includes different aetiological factors, Gram-negative bacteria being an important component. PD has been associated with RA whose aetiology is still unknown, although some reports have indicated that an infectious agent in a susceptible host could be one possible trigger factor (Carty et al. 2004). There are reports that show that RA patients have a higher periodontal pocket depth [odds ratio (OR) = 2.47] and higher severity of periodontitis (OR = 2.27), and patients with both diseases have higher functional loss (OR = 2.37) (Mercado et al. 2001) There is a recent case–control study that involved 52 healthy subjects and 57 RA patients and showed a positive association (OR = 8.05) between PD and RA (Pischon et al. 2008).

Table 2. Pocket depth, attachment loss and dental presence

	Mean	SD	Range
Pocket depth (mm)	3.9	0.81	2.6–5.7
Attachment loss (mm)	3.63	0.9	1–4
	Presence (%)	SD	Range
Dental presence (teeth number)	17.89 (63.8)	8.93	3–27
Upper anterior (6)	4.10 (68.3)	2.33	0–6
Lower anterior (6)	4.52 (75.3)	1.92	1–6
Upper premolars (4)	2.36 (59)	1.60	0–4
Lower premolars (4)	2.94 (73.5)	1.31	0–4
Upper molars (4)	2.10 (52.5)	1.37	0–4
Lower molars (4)	1.84 (46)	1.57	0–4

$n = 19$.
SD, standard deviation.

Table 3. Periodontal bacterial DNA detected in DP, SE and SF

	Dental plaque		Serum		Synovial fluid		p^*
	frequency	%	frequency	%	frequency	%	
Prevotella intermedia	19	100	14	73.6	17	89.4	0.0453
Tanerella forsythensis	10	52.6	6	31.5	2	10.5	0.0203
Prevotella nigrescens	13	68.4	0	0	4	21.0	<0.0001
Aggregatibacter actinomycetem comitans	4	21.0	0	0	3	15.7	0.1204
Porphyromonas gingivalis	15	78.9	8	42.1	11	57.8	0.0674
Treponema denticola	16	84.2	4	21	6	31.5	0.0004

$n = 19$. *Fisher's exact test.
DP, dental plaque; SE, serum; SF, synovial fluid.

Table 4. Periodontal bacterial DNA detected in different combinations

	Frequency (%)				
	positives DP, SE and SF	negatives DP, SE and SF	positives DP and SE	positives DP and SF	positives only DP
Prevotella intermedia	12 (63.1)	0 (0)	2 (10.5)	5 (26.1)	0 (0)
Tannerella forsythensis	2 (10.5)	9 (47.3)	4 (21.0)	0 (0)	4 (21.0)
Prevotella nigrescens	0 (0)	5 (26.3)	0 (0)	4 (21.0)	9 (47.3)
Aggregatibacter actinomycetem comitans	0 (0)	15 (78.9)	0 (0)	3 (15.8)	1 (5.2)
Porphyromonas gingivalis	7 (36.8)	4 (21.0)	1 (5.2)	4 (21.0)	3 (15.78)
Treponema denticola	3 (15.78)	3 (15.78)	1 (5.2)	3 (15.78)	9 (47.3)

$n = 19$.
DP, dental plaque; SE, serum; SF, synovial fluid.

There have been studies exploring the association of periodontal bacteria with RA. They are mainly focused in the detection of antibodies against different bacteria in SF and SE. In a case–control study, serum antibodies against disease-producing periodontal bacteria were identified more frequently in subjects affected by RA and periodontitis than controls (Ogrendik et al. 2005). However, it is important to consider that the detection of periodontal bacterial DNA in SF in RA patients is more important than the detection of antibodies because it suggests the transport of bacterial DNA from periodontal infections to the joints of RA patients.

In this study, only 19 patients were included because of the difficulty of finding patients that fulfilled the inclusion criteria. Patients with refractory RA under DMARDs treatment were selected because this condition was necessary to obtain a synovial fluid sample as controlled RA patients do not show effussion of synovial fluid in joints. The ideal design for this study should include a control group integrated by patients with RA and knee effusion without periodontitis to compare between groups, but unfortunately, after the examination of more than 500 RA patients, most of them with periodontitis, there were not enough number of patients with these features; there were two edentulous patients with knee effusion, but they were not included because of the impossibility to obtain dental plaque samples. This finding could affirm the fact that periodontal bacterial DNA could be one possible factor that activates the disease and this is the reason of the presence of knee effusion in spite of intense treatment of rheumatoid arthritis with DMARDs. Most patients were females (84.2%), and it was in accordance with the knowledge that RA affects more females than males. The most frequent form of periodontitis found in this study was the chronic form; this situation can be due to the age of the patients included. The mean age was 55.7 years and the aggressive form of periodontitis appears mainly in young patients in the second or third decade of life. The severity of periodontitis in the subjects was high, but the attachment loss mean was not that high because healthy sites were included; therefore, they showed a high tooth loss (63.8%); the lower molars being the teeth more frequently absent (46% lower molars present in mouth).

In the present study, periodontal bacterial DNA was detected in 100% of SDP and SF samples and in 84.2% of serum samples. Regarding the number of bacterial species detected, a higher number (4.05) of bacterial species in SDP were identified, followed by SF (2.26) species and serum (1.19). The fact that in SE there is less presence of PBDNA can be explained by the dilution of PBDNA in the blood stream by renal filtration, but in SF, bacterial content is accumulated over time in the affected joints. These data are in accordance with other studies where PBDNA was detected by DNA–DNA checkerboard hybridization in 93% of SF samples and in 100% of the SE samples, and more bacterial species in SF than serum of the 16 patients with RA were detected (Moen et al. 2006). The species more frequently identified in SF and SE were *P. intermedia*, *P. gingivalis* and *T. denticola*; two of them belong to the red complex species, which is associated with destructive disease. 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 in accordance with previous reports (Moen et al. 2006).

A. actinomycetemcomitans and *P. gingivalis* did not show statistical significant differences among SDP, SE and SF samples. This finding suggests that there could be an association because the same bacterial species detected in SDP were present in SF and SE samples. On the other hand, *P. intermedia*, *T. forsythensis, P. nigrescens* and *T. denticola* showed statistical differences between samples because in SDP, there was more frequency of detection of PBDNA than SE and SF. *P. intermedia* and *P. gingivalis* were the species most frequently detected in the three samples sites. It is important to notice that subjects who were positive to any bacteria in serum and/or SF, the same species was always detected in SDP as well. *P. gingivalis* produces a microbial enzyme, peptidyl arginine deaminase (PAD), which is the human equivalent of this enzyme has been related as a susceptibility factor for RA, because the antigens generated by PAD lead the production of rheumatoid factor and local inflammation of both gingival and synovium (Rosenstein et al. 2004). PAD leads to the citrullination of RA autoantigen such as fibrin in the synovium joint, which in association with major histocompatibility complex molecules by antigen-presenting cells, leads to the production of the anti-CCP antibody (Liao et al. 2009).

On the other hand, antibodies against heat shock proteins (hsp 70) of *P. intermedia* have been found in the periodontal tissue as well as the synovial tissue of patients with RA (Moen et al. 2003). It has also been shown that when the hsp 70 expressions induced with certain stress-stimulating factors, proinflammatory cytokines are induced in the synovium of RA patients (Schett et al. 1998). These findings are important, as in this study, *P. intermedia* was the most frequently isolated bacteria in SF of RA patients.

It has been suggested that there are three different ways to transport PBDNA from periodontal sites to affected joints such as free DNA, whole viable bacterial cell and intracellular capture of immune cells (lymphocytes and macrophages) (Moen et al. 2006). In order to explore these pathways of transport, several experiments were carried out: SF was inoculated onto agar plates and into broth cultures, with different culture media, such as BHI, soy trypticase and Dentaid-1 (specific culture medium for *A. actinomycetemcomitans*) (Alsina et al. 2001). Agar plates were incubated under aerobic and anaerobic conditions and CFU were estimated at 24, 48 and 72 h. There was no bacterial growth in any culture media. This result suggests that there were no viable bacterial cells in the samples studied; however, in the positive control, there was evidence of bacterial growth in both agar plates and broth cultures. The other pathway of transport is the intracellular form and it was suggested that leucocytes may contain different materials that includes bacterial DNA (Stahl et al. 2000). To explore this pathway of bacterial DNA transport from the periodontal pocket to affected joints, leucocytes were isolated from whole blood and a PCR assay was carried out and there were no positive samples to any bacterial species. These results could suggest that DNA does not travel from the periodontal sites to joints neither inside immunological cells nor as viable cells. Therefore, these results may suggest that the transport of bacterial DNA is in the free DNA form. There is evidence that the bacterial DNA contains CpG motifs that have received special attention in the aetiology of inflammatory diseases due to their strong immunostimulatory effects. After stimulation, the innate immune system is activated involving toll-like receptors, and these mechanisms have been shown to initiate a synovial inflammation in mice, with subsequent cytokines release, leading to bone destruction (Krieg 1998, Heikenwalder et al. 2004). This mechanism could be a key point in the immunopathogenesis of RA and periodontitis.

In conclusion because PBDNA was detected in SF of patients with RA and periodontitis, we suggest that PBDNA may play a role in the pathogenesis of rheumatic disease. *P. intermedia*, *P. gingivalis* and *T. denticola* were the species more frequently detected in SF, two of them part of the red complex. The transportation of DNA from periodontal pockets to joints could be as free DNA, because there was no evidence of either viability of bacterial cells or presence of PBDNA inside leucocytes. This information can be valuable for future studies to elucidate if PBDNA could be one possible trigger for RA development.

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Clinical Relevance

Scientific rationale for the study: Periodontal bacteria have been associated with systemic diseases such as rheumatoid arthritis whose aetiology is still unknown. Therefore, it is important to provide information about the role of periodontopathic bacteria in association with rheumatoid arthritis-affected joints.

Principal findings: Periodontal bacterial DNA was identified in the synovial fluid and serum samples of 100% of refractory rheumatoid arthritis and periodontitis patients. Besides, there is evidence that the transport pathway could be as a free DNA form.

Practical implications: It is important to establish periodontitis preventive measures, because periodontal bacteria could play a role in the pathogenesis of rheumatoid arthritis.