Received: 18th October 2015 Accepted: 19th January 2016 Conflict of Interest: None

Source of Support: Nil

Original Research

Doi: 10.2047/jioh-08-03-10

Assessment of Bacterial Pathogens in Human Volunteers with Rheumatoid Arthritis and Chronic Periodontitis: A Molecular-based Analysis

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How to cite the article:

Thilagar S, Mathew D, Sacket P, Ram S, Sekar H. Assessment of bacterial pathogens in human volunteers with rheumatoid arthritis and chronic periodontitis: A molecular-based analysis. J Int Oral Health 2016;8(3):351-356.

Abstract:

Background: Rheumatoid arthritis (RA) and chronic periodontitis (CP) are chronic destructive inflammatory disorders which result from deregulation of the host inflammatory response. Both conditions are potentiated by an exaggerated inflammatory response featuring an increase in local and perhaps circulating pro-inflammatory mediators, resulting in the destruction of the soft and hard tissue surrounding the synovial joints and periodontium. To detect the periodontal bacterial DNA in the subgingival dental plaque of RA and CP patients using polymerase chain reaction (PCR).

Materials and Methods: In this study, 80 subjects were selected based on the inclusion and exclusion criteria and divided into four groups. Group I (healthy - 20), Group II (CP - 20), Group III (RA - 20), and Group IV (RA and CP - 20). Subgingival plaque sample was collected with sterile paper points, and micro-organisms were analyzed using PCR.

Result: All four groups showed a statistical significant of ($P \leq 0.001$) with the difference in the detection of the number of organisms, with the increase in the level of Tannerella forsythia in the RA + CP group.

Conclusion: The results of our study suggested that periodontal bacterial DNA may have a major pathological role in the development of RA.

Key Words: Chronic periodontitis, periodontal pathogens, polymerized chain reactions, rheumatoid arthritis

Introduction

There are several chronic inflammatory disorders in the human body wherein an active host response plays a major role. Both rheumatoid arthritis (RA) and chronic periodontitis (CP) are two such commonly observed multifactorial anomalies which justify their chronic nature of onset. Exaggerated inflammatory response in RA, targets the synovial joints whereas, the gingival, periodontal ligament, cementum and alveolar bone undergo derogatory changes in CP.[1-4]

Patients suffering from both RA and CP have been shown to undergo, excessive tooth loss. This has been substantiated by several studies.[5,6] Patients suffering from CP also shows an exaggerated condition, pertaining to RA.[7,8] It has been postulated that both RA and periodontal disease (PD) share a similar mechanisms because the virulence factor produced by the periodontal bacteria produce an immune response which is mediated by neutrophils, monocytes and the B and T lymphocytes which lead to an increase in the level of prostaglandin release which stimulates osteoclastic activity leading to bone erosion, similar to the mechanism involved in RA.[9]

A common microbial pathogen involved in both RA and CP is *Porphyromonas gingivalis*.[10,11] Deimination of body proteins is facilitated by a peptidyl arginine deiminase (PAD) enzyme,[12] released by *P. gingivalis*. This, in turn, causes a pro-inflammatory response to citrullinated body proteins. This biochemical reaction is a vital factor in disease progression in RA.[13]

RA patients contain elevated levels of oral pathogenic antibodies which are common to the organisms of the red complex namely *P. gingivalis*, *Tannerella forsythia*, and *Prevotella intermedia*.[14-16] DNA-based techniques, hybridization probes, polymerase chain reaction (PCR)-based techniques provide quick results.[17]

The above-mentioned organisms of the red complex are extremely difficult to isolate. Therefore, the feasibility of the above process is also scarce. Treatment failures often result due to difficulties in thorough elimination of the above organisms. To the best of my knowledge, this is one of the first studies to be done in the south Indian population to assess the periodontal pathogens.

The aim of the study is to:

- Detect the predominant periodontal bacteria DNA from the subgingival plaque samples taken from patients with CP and RA and to compare with that of the controls using PCR.

Materials and Methods

Four groups of patients in the range of 35-60 years were used in this cross-sectional study. 80 patients were included in the study. Each group consisted of 20 patients. Group I - Consisted of healthy patients, Group II - Consisted of patients with CP, samples were collected in the out-patient pool of Department of Periodontics, Thai Moogambigai Dental College, Chennai. With inclusion criteria for periodontitis was determined with the pocket depth of ≥3 mm and attachment loss of ≥2 mm in at least 10 teeth.[18] Group III (based on RA criteria no 6) patients with RA were collected from the outpatient pool of the Department of Rheumatology (Kilpauk Medical College and Hospital). The inclusion criterion was fulfilling the American College of Rheumatology classification criteria for RA.[19] Group IV (based on RA criteria no 7) patients with both RA and CP (RA + CP) which included both the inclusion criteria of CP and RA. Exclusion criteria in the study consisted of subjects with age <18 years, edentulous, diabetes, thyroid disease, the presence of malignancy, cardiovascular diseases, pregnancy, breastfeeding and the use of antibiotic <3 months prior to the study. After the diagnosis process, subgingival plaque samples were collected with the sterile paper points from the upper first molar, lower central incisors, and lower premolars. The collected samples were stored in eppendorf tube with 1ml of phosphate-buffered saline (PBS). The subgingival plaque samples were transported on ice and sent to Regenix multispecialty laboratory, Chennai and were stored at −40°C until PCR and other microbiological evaluations were performed.

Ethics approval

The Ethical Committee for medical and dental research in Kilpauk Medical College and Thai Moogambigai Dental College, Chennai was obtained from them, before proceeding with the study.

DNA extraction and PCR

The present study was conducted under purely aseptic conditions to avoid environment contamination while separating the DNA from samples for PCR assays. 900 µl of PBS with SDP were centrifuged 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 ethylenediaminetetraacetic acid (EDTA) (pH 8.0) and incubated at 85°C for 10 min. Then, 100 µl of 200 u/ml of mutanolysin 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) 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 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. 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 0.5 µM of oligonucleotides, 0.2 Mm of deoxynucleotides, 1.5 Mm of $MgCl_2$ and 10 ng of DNA template. Specific primers for each species used in the study are indicated in Table 1. PCR reaction was performed in a thermal cycler with the cycling parameters reported.[20-22]

Positive controls were included in each PCR set by using DNA of the following bacterial strains: *Prevotella nigrescens* (ATCC 25261), *Treponema denticola* (ATCC 35405). *P. gingivalis* (ATCC 33277 and HG1691) *P. intermedia* (ATCC 25611), and *Tanerella forsythensis* (ATCC 43037).

A blank sample of de-ionized water (instead of patients' blood sample) was included in every PCR set, as a negative control. The PCR products were analyzed by electrophoresis in a 2% agarose gel using Tris-boric acid - EDTA buffer, using a 100 bp DNA ladder marker to estimate the molecular size. Each gel was stained with ethidium bromide (0.5 µg/ml) and photographed under ultraviolet light (Table 1).

Statistical analysis

A statistical analysis of the results was performed with SPSS software by one-way ANOVA method. $P < 0.05$ is considered significant. Intergroup comparison was done *post-hoc* analysis using Turkey test. Detection of periodontal bacterial DNA was done using Fisher exact test $P < 0.05$ is considered to be statistically significant.

Results

In Table 2, we have compared the mean of age, periodontal pocket depth (PPD), and plaque index (PI) in CP, CP + RA and healthy subjects. In accordance to age, overall all the groups show a highly significant value ($P < 0.001$).

Table 1: Specific oligonucleotides used in the study.

Bacteria	Sequence (5`-3`)	MS (bp)	References
Porphyromonas gingivalis	A TGT AGA TGA CTG ATG GTG AAA ACC ACG TCA CCA CCT TCC TC	197	Tran and Rudney
Prevotella intermedia	CAA AGA TTC ATC GGT GGA GCC GGT CCT TAT TCG AAG	307	Stubbs *et al.*
Tanerella forsythensis	GCG TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACC T	641	Ashimoto *et al.*
Prevotella nigrescens	ATG AAA CAA AGG TTT TCC GGT AAG CCC ACG TCT CTG TGG GCT GCG A	804	Ashimoto *et al.*
Treponema denticola	TAA TAC CGA ATG TGC TCA TTT ACA T TCA AAG AAG CAT TCC CTC TTC TTC TTA	316	Ashimoto *et al.*

Table 2: Comparison of mean age and mean probing depth among participants with CP, CP+RA and healthy subjects.

Dependent variable	*n*	Mean	SD	95% confidence interval for mean		Minimum	Maximum	*P* value[#]
				Lower bound	Upper bound			
Age								
CP	20	46.1000	4.99368	43.7629	48.4371	38.00	56.00	<0.001*
CP+RA	20	48.8000	4.43194	46.7258	50.8742	41.00	57.00	
Healthy	20	42.4500	3.81789	40.6632	44.2368	35.00	48.00	
RA	20	44.8000	4.56070	42.6655	46.9345	35.00	53.00	
PD								<0.001*
CP	20	6.40	1.142	5.87	6.93	5	9	
CP+RA	20	6.75	1.860	5.88	7.62	4	12	
Healthy	20	1.60	0.503	1.36	1.84	1	2	
RA	20	3.30	1.302	2.69	3.91	2	6	
PI								<0.001*
CP	20	2.1500	0.36635	1.9785	2.3215	2.00	3.00	
CP+RA	20	2.1000	0.30779	1.9559	2.2441	2.00	3.00	
Healthy	20	2.9000	0.30779	2.7559	3.0441	2.00	3.00	
RA	20	0.8000	0.41039	0.6079	0.9921	0.00	1.00	

[#]One-way ANOVA, *$P<0.05$ is considered significant. SD: Standard deviation, RA: Rheumatoid arthritis, CP: Chronic periodontitis, PI: Plaque index, PD: Periodontal disease

Table 3 Intragroup comparison of mean age between the groups.

On comparing the mean age of all the groups, showed statistically significant difference between Group IV and Group I, Group IV and Group III, Group III and Group I only.

Intragroup comparison of PPD and PI between the groups.

On comparing the of PPD and PI of all the groups, showed statistical significant difference between Group II and Group I, Group II and Group III, Group IV and Group I, Group III and Group I, Group III and Group IV only showing a $P = 0.000$.

In Table 4, comparison is done between bacterial DNA and CP, RA, RA + CP and healthy.

When *P. intermedia* is compared with CP (33.3%), RA (29.6%), CP + RA (37.0%), and in healthy it is completely (0%), there was an increase in the CP + RA group. overall it showed a highly significant value ($P < 0.00$).

When *T. forsythia* is compared with CP (34.2%), RA (13.2%), CP + RA (44.7%) and healthy (7.9%), there was an increase observed in the CP + RA group, and overall it showed a highly significant value ($P < 0.00$).

When *P. nigrescens* is compared with CP (29.3%), RA (29.3%), CP + RA (36.6%) and in healthy it is (4.9%), an increase was observed in the CP + RA group and overall it showed a highly significant value ($P < 0.00$).

When *P. gingivalis* is compared with CP (32.1%), RA (32.1%), CP + RA (35.8%) and healthy it is completely (0%), showed an increase in the CP + RA group and the overall it showed a highly significant value ($P < 0.00$).

When *T. denticola* is compared with CP (34.0%), RA (30.0%), CP + RA (36.0%) and in healthy it is completely (0%). Which showed an increase in CP + RA group, and the overall it showed a highly significant value ($P < 0.00$).

Overall there was an increase in the bacterial DNA in the CP + RA group of which *P. intermedia* and *P. gingivalis* was detected in most of the subgingival samples in this group.

Discussion

It has been inferred from many studies that an anaerobic Gram-negative infection plays a common role in both RA and CP. Clinical periodontal parameters like bleeding on probing, periodontal pocket depth and clinical attachment level were found to be elevated in RA patients, compared to healthy subjects.[23]

The main focus of these association studies is on the antibody detection against various bacteria. Periodontitis was a more common in patients with RA positive for anti-cyclic citrullinated peptide (CCP); there was an association between periodontitis and the number of inflamed joints and RF Antibodies specific for anti-CCP were higher in patients with *P. gingivalis* subgingival plaque.[24] Detection of periodontal bacterial DNA was shown to be more important than the detection of antibodies in patients suffering from RA. This clearly indicated a translocation of bacterial DNA from sites of periodontal infections to the synovial joints of RA patients.

The present study was divided into four groups with each group comprising of 20 patients. Both males and females were included in the study with a higher proportion of females (total no of females=48). The age groups of the subjects in the study were in the range of 35-60 years.

Subgingival dental plaque samples in the study revealed a 100% pathogenicity in the periodontal bacterial DNA. The

Table 3: *Post-hoc* analysis using - Tukey test HSD.

Dependent variable	(I) Group	(J) Group	Mean difference (I-J)	*P* value	95% confidence interval	
					Lower bound	Upper bound
Age	CP	CP+RA	−2.70000	0.233	−6.4138	1.0138
		Healthy	3.65000	0.056	−0.0638	7.3638
		RA	1.30000	0.795	−2.4138	5.0138
	CP+RA	CP	2.70000	0.233	−1.0138	6.4138
		Healthy	6.35000*	0.000	2.6362	10.0638
		RA	4.00000*	0.030	0.2862	7.7138
	Healthy	CP	−3.65000	0.056	−7.3638	0.0638
		CP+RA	−6.35000*	0.000	−10.0638	−2.6362
		RA	−2.35000	0.351	−6.0638	1.3638
	RA	CP	−1.30000	0.795	−5.0138	2.4138
		CP+RA	−4.00000*	0.030	−7.7138	−0.2862
		Healthy	2.35000	0.351	−1.3638	6.0638
PD	CP	CP+RA	−0.350	0.828	−1.43	0.73
		Healthy	4.800*	0.000	3.72	5.88
		RA	3.100*	0.000	2.02	4.18
	CP+RA	CP	0.350	0.828	−0.73	1.43
		Healthy	5.150*	0.000	4.07	6.23
		RA	3.450*	0.000	2.37	4.53
	Healthy	CP	−4.800*	0.000	−5.88	−3.72
		CP+RA	−5.150*	0.000	−6.23	−4.07
		RA	−1.700*	0.000	−2.78	−0.62
	RA	CP	−3.100*	0.000	−4.18	−2.02
		CP+RA	−3.450*	0.000	−4.53	−2.37
		Healthy	1.700*	0.000	0.62	2.78
PI	CP	CP+RA	0.05000	0.969	−0.2414	0.3414
		Healthy	−0.75000*	0.000	−1.0414	−0.4586
		RA	1.35000*	0.000	1.0586	1.6414
	CP+RA	CP	−0.05000	0.969	−0.3414	0.2414
		Healthy	−0.80000*	0.000	−1.0914	−0.5086
		RA	1.30000*	0.000	1.0086	1.5914
	Healthy	CP	0.75000*	0.000	0.4586	1.0414
		CP+RA	0.80000*	0.000	0.5086	1.0914
		RA	2.10000*	0.000	1.8086	2.3914
	RA	CP	−1.35000*	0.000	−1.6414	−1.0586
		CP+RA	−1.30000*	0.000	−1.5914	−1.0086
		Healthy	−2.10000*	0.000	−2.3914	−1.8086

RA: Rheumatoid arthritis, CP: Chronic periodontitis, PI: Plaque index, PD: Periodontal disease

subgingival plaque samples revealed more virulent species compared to the supragingival samples. Members of the red complex namely *P. gingivalis*, *T. denticola* and *P. intermedia* were more frequently identified in the subgingival plaque samples. These results were in accordance to a previous study conducted by Martinez-Martinez RE *et al.*, 2009[25] and Moen *et al.*, 2006.[26]

T. forsythia (44.7%) and *P. intermedia* (37.0%) are the most frequently identified microorganism in our study in the subgingival dental plaque, in the CP + RA group with a $P < 0.001$ which showed a statistically significant result. There was a difference in the detection of periodontal bacterial DNA among *P. intermedia* (37.0%), *T. forsythia* (44.7%), *P. nigrescens* (36.6%), *P. gingivalis* (35.8%) and *T. denticola* (36.0%) in the CP + RA group in the subgingival dental plaque, but all the organism showed a statistical significance with a $P < 0.001$.

PAD, a specific microbial enzyme detected in *P. gingivalis*, was found to be associated strongly with RA. This shows the correlation between CP and RA since subjects with CP had higher levels of *P. gingivalis* in their plaque samples. Similarly RA, also had higher levels of PAD, which increases the signs and symptoms of periodontal tissue destruction in subjects with CP. This PAD engendered antigens leads to the production of rheumatoid factor-containing immune complexes and provoke local inflammation, both in gingiva and synovium via FC and C5a receptors.[10]

These PAD engendered antigens, which is presented in association with major histocompatibility complex molecules by antigen-presenting cells, will lead to the production of the anti-CCP antibody. Anti CCP antibodies, in turn, form immune complexes with de-iminated proteins. These are bound by pro-inflammatory cells via, FC receptor molecules. This, in turn, results in the release of certain pro-inflammatory receptors which causes clinical signs of RA like swelling and arthritic degeneration of the joints.[27]

Table 4: Periodontal bacterial DNA in CP, CP+RA, HP, RA patients using fisher exact test.

Organisms	Groups *n* (%)				*P* value
	CP	RA	RA+CP	Health	
Prevotella intermedia					
No	2 (7.7)	4 (15.4)	0 (0.0)	20 (76.9)	<0.001*
Yes	18 (33.3)	16 (29.6)	20 (37.0)	0 (0.0)	
Tanerella forsythus					
No	3 (7.1)	15 (35.7)	7 (16.7)	17 (40.5)	<0.001*
Yes	13 (34.2)	5 (13.2)	17 (44.7)	3 (7.9)	
Prevotella nigrescens					
No	8 (20.5)	8 (20.5)	5 (12.8)	18 (46.2)	<0.001*
Yes	12 (29.3)	12 (29.3)	15 (36.6)	2 (4.9)	
Porphyromonas gingivalis					
No	1 (3.7)	3 (11.1)	3 (11.1)	20 (74.1)	<0.001*
Yes	17 (32.1)	17 (32.1)	19 (35.8)	0 (0.0)	
Treponema denticola					
No	3 (10.0)	5 (16.7)	2 (6.7)	20 (66.7)	<0.001*
Yes	17 (34.0)	15 (30.0)	18 (36.0)	0 (0.0)	

*P<0.05 is considered statistically significant. RA: Rheumatoid arthritis, CP: Chronic periodontitis

T. forsythia, a non-mobile spindle-shaped anaerobe produces proteolytic enzymes which have a hazardous effect on the immune system, resulting in severe bone and tissue destruction in both the synovial joints and the periodontal tissues. The unique quality of apoptosis induction by *T. forsythia* also aggravates the condition in RA.

It has been found that bacterial DNA contains CpG motifs which are involved in the etiology of inflammatory diseases due to their strong immunostimulatory effects. On stimulation cells of the innate immune system, activates a pathway involving Toll-like receptor-9 these mechanisms have proven to initiate synovial inflammation, which promotes the activation of antigen-specific B-cells and the secretion of T-helper-1-like cytokines, leading to bone destruction.[28,29] This mechanism could suggest a possible immunopathogenesis of CP and RA.

Conclusion

In conclusion, the periodontal bacterial gene material was detected in the subgingival plaque of RA patients, It has been suggested that periodontal bacterial DNA plays a major pathological role in the severity of RA. *P. intermedia*, *T. forsythia*, and *P. gingivalis* were the species more frequently detected in the subgingival dental plaque with the two predominant red complex organisms which is involved in the bone destruction leading to Periodontitis. The data's obtained in this study collectively provide evidence for a possible link of RA with PD. Since there is a close association between RA and PD we could observe in this study an inter dependable change between RA and CP in this study. More longitudinal studies with the larger sample size are required to have a better understanding of the interrelation ship between RA and CP. The information obtained in this study could be useful to the dentist to assess the health status and prevent the further progression of the disease.

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