The Relationship between *Porphyromonas gingivalis* and Rheumatoid Arthritis: a Cross-Sectional Clinical, Microbiological, and Molecular Approach

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Abstract

Background: The relationship between rheumatoid arthritis and poor oral hygiene has long been proven. Colonization of *Porphyromonas gingivalis* in the oral cavity can play an important role in rheumatoid arthritis. The aim of this study was to determine whether there is a relationship between rheumatoid arthritis disease and the presence of P. *gingivalis*.

Methods: sixty patients with rheumatoid arthritis and 75 controls with matched age, sex and smoking and oral health status were selected. Provided samples from dental plaque were assessed for the presence of P. *gingivalis* by using bacterial culture and polymerase chain reaction (PCR).

Results: Results of bacterial culture and PCR showed no significant difference between rheumatoid arthritis patients and control group in terms of the presence of P. *gingivalis* (P = 0.9 and P = 0.1, respectively). There was also no relationship between the number of bacterial copies and the activities of rheumatoid arthritis.

Conclusion: Findings showed that there was no significant relationship between the presence of P. *gingivalis* in dental plaque and rheumatoid arthritis.

Introduction

Rheumatoid arthritis (RA) is a common, autoimmune, and systemic disease that affects about 0.5-1% of the world's population and can lead to systemic disabilities and early death (1, 2).

The cause of this disease is unknown and since the disease can cause physical problems and imposes financial cost on societies, it is very helpful to understand the mechanisms of the disease. Both genetic factors and environmental factors are involved in the pathogenesis of this disease. One of the

environmental factors that contributes to the development of RA is oral and dental disease. An increased prevalence of periodontitis (PD) and a higher rate of tooth loss have been observed in individuals with RA compared to the general population (3-5). Besides, several studies have demonstrated that RA has been more prevalent among patients with oral disease/*PD* (6-8). Periodontitis is a series of oral and dental diseases caused by a series of infections and, if it is developed, it leads to inflammation and loss of the tooth. There has been an increase in the incidence of periodontal disease in patients with active and chronic RA compared with healthy people, and on the other hand,

RA incidence is higher in periodontal patients than those without oral and dental disease (9). Approximately, twenty bacteria have been determined to be the pathogens of oral and dental diseases, wherein the most common ones are genital organs P. denticola, P. corpori, and *P. gingivalis. P. gingivalis* is a gram-negative bacilli bacterium, asaccharolytic, anaerobic, motionless, spor-less, which grows in brown or black colonies in the blood agar medium. P.gingivalis has the highest proteolytic activity among bacteria leading to oral and dental diseases and has a high virulence potential (10). This bacterium is capable of producing virulent factors that play a role in the continuation of the presence of this bacterium in the mouth, resulting in the appearance of a physio-pathologic manifestation of this chronic infection (10). This bacterium produces peptidyl arginine deaminase (PPAD) and is able to citrullinate the arginine of the end of the protein (11). Therefore, the chronic presence of these autoantibodies in inflamed periodontal tissues may produce localized citrullinated peptides and the oral environment in these conditions may also be full of pro-inflammatory cytokines such as α-TNF and 1β-IL, which facilitates the presentation of citrullinated antigens into T cells. The enolase enzyme is one of the candidate antigens because the citrullinated enolase is one of the autoantigens identified in rheumatoid arthritis (12). P.gingivalis titer in patients with RA is directly related to the concentration of anti-citrulline protein (13). P.gingivalis may citrullinate the host proteins, and antigenicity changes and triggering of autoimmune reactions may result in RA in predisposed individuals (11). Animal studies have confirmed this finding, and enolase caused arthritis in transgenic-IE-DR4 mice (14).

Higher antibody titers against *P. gingivalis* in RA patients and a positive correlation with Anti-citrulinated protein antibody (ACPA) suggest that infection with this periodontal pathogen may play a role in the risk and progression of RA. Since, RA causes a lot of problems for patients and healthcare system, clarifying the etiology of the disease development could help better treatment and prevention of this disease. Therefore, this study aimed to investigate whether the association between PD and RA is dependent on *P. gingivalis.*

Materials and Methods
Subjects

One hundred and thirty-five consecutive patients with rheumatoid arthritis and healthy controls were invited to participate in this case-control study. RA disease was classified using the 2010 classification criteria of American College of Rheumatology/ European League Against Rheumatism (ACR/EULAR) (25). Inclusion criterion was seropositive Anti ccp-Ab RA. Case group included 60 patients with RA referred to rheumatology clinic of Firoozgar Hospital

(Tehran, Iran) and 75 healthy individuals were selected of patients referred to orthopedic and geriatric clinics for accidental traumatic fractures or periodic geriatric

Examinations. Case and control groups were matched for age, gender, as well as periodontal and smoking status. The criteria for exclusion were diabetes, malignancy, pregnancy and antibiotic use within 3 months prior to the study. The information including age, sex, the degree of disease activity,

duration of the disease, and laboratory data of patients were extracted from medical documents. Periodontal assessments and matching was performed by a dentist. A rheumatologist determined the activity of RA disease based on the Disease Activity Score 28 joint

Count (DAS28) (15). The DAS28 is a system developed and validated by the

EULAR (European League against Rheumatism) to measure the progress and improvement of Rheumatoid Arthritis and according to that, the disease activity is categorized to <2.6: remission, 2.6-3.2: low activity, 3.2-5.1: moderate activity and >5.1: high activity. Other parameters including duration of RA disease, smoking status, BMI, and RA medication were also considered. Information about WBC, Plt, Hb, ESR was also gathered (Table 1).

Ethics approval

Informed consent forms were taken from all participants prior to the enrollment according to the Declaration of Helsinki. This study was confirmed by Medical Ethics Committee of Iran

University of Medical Sciences (ethical code: IR.iums.rec.1394.25395)

Laboratory procedures
Sampling, microbiological sampling and culture

Microbiological samples were processed by using standard anaerobic culture techniques. Samples were taken from dental plaque of patients with RA and control subjects through putting point papers along with the dental plaques for 16 seconds and 10-fold serial dilutions (100 μL) in PBS were plated on blood agar plates supplemented with horse blood (5% vol/vol), hemin (5 mg/L), and menadione (1 mg/L) incubated in 80% N2, 10% H2, and 10% CO2 at 37°C for up to 14 days. Microscopic morphology, Gram reaction, and the production of a set of metabolic enzymes were considered as the identification

criteria. Finally, the total number of colony-forming units per sample was determined.

DNA extraction and Quantitative polymerase chain reaction (PCR) assay

A single colony of each isolate or strain was inoculated into 7 - 10 mL of Wilkins-Chalgren anaerobe broth (Oxoid, UK) and grown overnight at 28°C. DNA was extracted from the bacterial suspensions using a High pure PCR templete preparation Kit (Roche Germany) according to the manufacturer's instructions. A conserved sequence within the 16S rRNA gene was identified for P. gingivalis strains and detection of P. gingivalis was performed according to a previously reported approach (16) by using a specific primer pair including Forward: 5'-GAGGAACCTTACCCGGGAT–3' and Reverse: 5'- ATGCAGCACCTACATAGAAGC – 3' to amplify a 404-bp fragment of the 16S rRNA gene. 6FAM-TAGATGACTGATGGTGAAAACCGTCTTCC-BHQ1 was used as the probe. Amplification reactions were carried out in a total volume of 20 μl consisting of 10 μl of 2× TaqMan universal PCR master mix (Applied Biosystems, Foster City, USA), 1 μl of each primer and probe (in a final concentration of 0·25 μmol l−1), 2 μl of PCR water, and 5 μl of extracted DNA from plaque samples. PCR amplification was performed in a Rotor-Gene thermal cycler (Corbett 6000; Australia) based thermocycler based on the following cycling program: an initial amplification cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s (denaturation) and 60°C for 1 min (annealing and elongation). The CT (cycle threshold) was defined as number of cycles required for the fluorescent signal to cross the threshold. A 10-fold dilution series of DNA extracted from a P. gingivalis starting suspension was prepared to construct of the standard curve. P. *gingivalis* ATCC 33277 cells and 5 μl of water were used as a positive and negative control.

Statistical analysis

Different data including age, sex, duration of disease activity, the degree of the disease activity, the presence of dental plaque and laboratory data were entered into SPSS for data analysis. Frequency, mean, and standard deviation were used for descriptive analysis. For comparisons between two groups, the unpaired two-tailed, for variables with normal distribution, t-test and for skewed variables, the two-tailed Mann-Whitney test were used.

Results

In this study, 60 patients with RA in the case group and 75 subjects in the control group were evaluated. Mean age of patients in the case group was 49.3 years. The minimum age was 17 and the maximum age was 73 years. Among patients with RA, 10 subjects (16.7%) were male and 50 ones (83.3%) were female. Mean duration of the disease in patients with RA was 7.3 (SD = 6.6) years. Mean disease activity score (DAS-28) was 2.6. There was no significant correlation between positive culture of P. *gingivalis* and RA (P = 0.9). Thirty subjects in the case group (51.66%) and 31 ones in the control group (41.33%) showed positive culture for P. *gingivalis*. There was no positive correlation between positive culture for P.*gingivalis* and the duration of RA (P = 0.1). Mean disease duration was 12.0 years in positive culture and 8.55 years in negative culture patients.

Patients with positive culture and negative culture showed no significant difference in WBC (7535 and 7564 respectively, P= 0.9), Hb level (13 and 12.63 respectively, P = 0.1) and mean ESR (26 and 28 respectively, P = 0.09). The median of DAS-28, which is the indicator of disease activity, was 2.39 in the positive and 2.8 in the negative culture groups that shows no significant difference (P = 0.07).

According to the cycle of threshold (CT), subjects were divided into the three groups of CT>37, 37>CT>32, and CT<32. The average copy number of the bacteria in the mentioned three groups was 10446, 33, and 1.7, respectively that showed significant difference (P < 0.05) and was indicative of the correct performance of PCR. The number of patient samples in the three groups of 37 <CT, 37>CT>32, and 37> CT, were 30, 14, and 16, respectively and the number of control subjects were 17, 37 and 21 ones respectively. The average copy number in the control subjects was also significantly different in the three groups (CT <32: 23096, 32> CT> 37: 15 and CT> 37: 0.4, P = 0.02). Association between P. gingivalis and RA has been summarized in Table 2.

The disease activity was evaluated in the three groups based on CT, where the mean DAS-28 in group 32 <CT and copy number = 10446 was 2.70. DAS-28 was 2.66 and 2.28 in group 32> Ct > 37 and copy number = 33 and in group CT> 37 and copy number = 1.7, respectively. These findings showed that there is no significant difference among the three groups in terms of disease activity (P > 0.05). Association of the disease activity and *P. gingivalis* has been shown in Table 3.

ESR was also assessed on the basis of CT groups. The average of ESR in CT> 37, 32> CT> 37, and Ct <37 was 23.3, 24, and 17, respectively, where in there was no significant differences in ESR between three groups (P = 0.4).

Duration of the disease was respectively 5.18 and 8.9 years in the 37> Ct and 37 <Ct, groups. That shows no significant difference between the two groups (P = 0.1). Mean WBC was 7666 in group 37> Ct and 7637 in group 37 <CT, which was not significantly different between the two groups (P = 0.9). The average platelet index in the CT> 37, and CT <37groups was also not significantly different (P >0.05).

Table 1. Demographic data of studied subjects

	RA patients	controls
Mean age (year)	49.3	42
Sex (female/male)	(50/10)	40/20
Disease duration(month)	7.3	_
Diseae activity score (DAS-28)	2.6	_
CRP (mg/dl)	1.10	_
ESR (mm/hour)	23.33	_
Mean WBC (count/µl)		_
Hb (g/dl)		_
IgM-RF (IU/ml)	+	_
Anti-CCP Ab (units/ml)	+	_

Table 2. The association between P. gingivalis and RA based on culture and qPCR data

Groups	Culture		OR (95% CI)	P-Value	qPCR			P-Value
	Positive	Negative			CT<37	32<CT<37	CT>37	
Control	31	44	0.70 (0.35-1.39)	0.31	21	37	17	P > 0.05
RA	30	30	1.41 (0.71-2.81)		16	14	30	

Significant ORs are shown in bold. CI: confidence interval; OR: odds ratio

Table 3. The association between P. gingivalis and RA disease activity (DAS-28 index) based on culture and qPCR data

Groups	Culture		PCR	
	Positive	Negative	CT>37	CT<37
Average DAS-28	2.39	2.8	2.28	2.68
Standard deviation (SD)	0.94	1.28	1	1.1
Number	31	29	29	31
P-Value	0.07		0.2	

According to Mann-Whitney-U test, the average DAS -28 was not significantly different between groups.

Discussion

The obtained results showed no significant difference in P. gingivalis presence on dental plaques between the rheumatoid arthritis patients and the normal population. This finding was achieved through comparing anaerobic bacteria culture and colonization between these two groups. In addition, PCR has been done in order to confirm the presence of this bacterium on dental plaques. Similarly, there was no meaningful difference between the two groups. So, the result was compatible with the outcome of bacterial culture which also showed no difference. Likewise, there was no significant difference between patient's group and normal population in regard to the activity of

rheumatic arthritis patients with proved presence of *P. gingivalis* bacteria on dental plaques based on bacterial culture and PCR result. Also, the activity level of rheumatoid arthritis according to DAS-28 score in patients with *P. gingivalis* on dental plaques (based on bacterial culture and PCR) was not significantly different from that of the negative bacterial group and the severity of the disease was not significantly different too.

The aim of this study was to evaluate the prevalence of *P. gingivalis* in the mouth of rheumatoid arthritis patients and the relationship of the activity of this disease based on DAS-28 score with the presence of periodontitis and gingivitis.

In a study by Fulvia Ceccarelli et al in 2018, the presence of *P. gingivalis* and periodontitis in RA patients has been evaluated using PCR. The presence of this bacterium in patients with periodontitis was higher than that of the control group. Moreover, total bacterial genome and RA disease activity based on DAS-28 was higher in patients with periodontitis. The prevalence of *P. gingivalis* in patients in the remission group was lower compared to non- remission groups that demonstrated the correlation between the presence of *P. gingivalis* on tongue biofilm and the activity of rheumatoid arthritis (17).

Another study conducted by Beatriz Rodríguez-Lozano et al in 2019 showed meaningful correlation between periodontitis and rheumatic arthritis and also stronger correlation in higher scores of RA activity (18).

Smit de menke et al performed a study in 2012 in which RA patients with more severe periodontitis had higher titer of IgM and IgG against *p. gingivalis*. Yet, the culture of *P. gingivalis* was not positive in all cases (19).

Arvikar L Sheila et al. showed in a study that IgG level against *P. gingivalis* in both groups of early and late RA were higher than the other group. Also, the severity of the disease was higher in patients with higher titers of antibody (20).

A study by P Ortiz and his colleagues in 2009 showed that periodontitis treatment in RA patients, regardless of the drugs used, improved the symptoms and signs of rheumatoid arthritis, based on the DAS-28 score(21).

P. gingivalis is a bacterium which is capable of Peptidyl arginine deaminase (PAD) production. This capability can cross react with human peptides, and citrulinate fibrinogen and enolase. Consequently, it leads to expression of antibodies against citrulinated proteins that increase anti-ccp titer itself. The chronic presence of these autoantibodies in inflamed periodontal tissue may lead to the production of local and oral citrulinated peptides. The surrounding environment may be full of inflammatory cytokines such as α-TNF 51 and β-IL$_1$.These cytokines facilitate the presentation of citrulinated antigens to T-cells (22).

Previous studies showed a relationship between *P. gingivalis* serology and RA disease. Bender P et al reported in a meta-analysis study in 2017 that there is a significant correlation between positive serology of *P. gingivalis* and RA disease (23).

In 2016 johansson L revealed that level of *P. gingivalis* antibody in patients who had been recently diagnosed with RA was higher in comparison to the control group (24).

There is a point that in all of these studies the relation between RA and *P. gingivalis* bacterium has been shown by serology test assessment which means that high level of *P.gingivalis* anti bodies and periodontitis of patients is

associated with the incidence of RA and its severity based on DAS-28 score.

Findings of the above mentioned studies are not compatible with the results of the present study. In the present study, PCR findings showed no correlation between the number of *P. gingivalis* and the severity of RA disease. PCR showed that patients with an average copy number of 10446 and CT <32, mean copy number = 33 and 32< CT< 37, mean Copy number = 1.7, CT> 33 There was no difference in terms of disease activity based on DAS-28. This indicates that the presence of bacterium on dental plaque could not be considered as a risk factor for RA. Probably there are other associated factors more important than the presence of bacterium on dental plaques and periodontitis which can stimulate the immune system. Many factors could be considered as the cause of this relation. For instance, stimulated immune system due to periodontitis can explain the relationship between the presence of bacterium and RA disease. Eventually, there is no correlation between the presence of bacterium itself on the dental plaques and RA disease. Due to the fact that some studies focused on this issue using PCR to show the correlation is extremely limited, it is highly recommended to perform more studies and trials in this field with larger samples and different modalities. Meantime, one of the limitations in this study was accepting the fact that in group of RA patients most of persons were in low disease-activity status and they were under control. So actually, it can be helpful to perform similar studies based on DAS- 28 over cases with higher RA disease-activity who is struggling with RA recently.

Conclusion

In summary, these results demonstrated an independent relationship between RA and cultivable *P. gingivalis* in established RA patients. qPCR results also confirmed the culture data and no considerable correlation was observed between *P. gingivalis* copy number and RA. Also, there was no significant relationship between *P. gingivalis* and the disease activity and more bacteria in dental plaque alone could not cause more severe forms of RA disease.

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