Periodontal microbiota in a cohort of Egyptian patients with rheumatoid arthritis and their relation to serum and gingival anticitrullinated peptide protein antibodies and different disease parameters
Eiman Solimana, Abeer Abdelati, Rania Fahmy, Mona Helmy
Departments of *Internal Medicine, Faculty of Medicine, Alexandria University, Egypt; †Oral Medicine and Periodontology, Faculty of Dentistry, Alexandria University, Alexandria, Egypt
Correspondence to Abeer Abdelati, MD, Sidi Gaber Street, Alexandria, 21523, Egypt. Tel: 002034265910; e-mail: abiraliabir@yahoo.com
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**Objective**
The possibility of infectious trigger at the gingival site in rheumatoid arthritis (RA) was reported in previous studies. The aim of our study is to determine the organisms causing periodontitis (PD) in a cohort of Egyptian patients with RA and their relation to serum and gingival anticitrullinated peptide protein antibodies (ACPA) level and other disease parameters.

**Patients and methods**
A prospective cohort study was conducted on 100 consecutive Egyptian patients with RA. Disease activity was assessed by applying disease activity score-28, and functional status was measured using health assessment questionnaire. Dental examination, serum rheumatoid factor, ACPA in serum and gingival crevicular fluid (GCF), and radiograph of the hands were done for all patients. GCF culture was performed for all cases with PD for *Porphyromonas gingivalis* (**Pg**), *Aggregatibacter actinomycetemcomitans* (**Aa**), and *Prevotella intermedia* (**Pi**).

**Results**
Of the 100 patients, 66 patients had PD; of them, GCF culture was performed, and **Pg**, **Aa**, and **Pi** were found in 60.6, 15.2, and 30.3% of patients with RA with PD, respectively. Gingival ACPA showed significant higher level with **Pg** than **Pi** cases (**P** = 0.047). No statistically significant difference was detected on comparing **Pg** with **Aa** or **Aa** with **Pi**. **Aa**-positive cases were associated with significantly higher level of C-reactive protein than **Pi**-positive cases (**P** = 0.029), whereas no statistical significant difference was detected between **Pg**- and **Aa**-positive or **Pi**-positive cases.

**Conclusion**
Our findings support the relationship between PD and infectious trigger at the gingival site and RA. **Pg** is the most prevalent periodontal microbiota in our cohort of patients with RA with PD that is associated with significant higher level of gingival ACPA. None of the detected organisms correlated with the degree of RA activity or other disease parameters, apart from significantly higher C-reactive protein level with **Aa**.

**Keywords:**
aggregatibacter, microbiota, periodontitis, porphyromonas, rheumatoid arthritis

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**Introduction**
Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease that is characterized by synovial inflammation and erosive arthritis [1]. An infectious cause of RA has been implicated; however, solid evidences are lacking. Microbiome is a term that describes certain bacterial colonies that populate skin and mucosal surfaces [2]. Several data demonstrated the role of microbiome in the pathogenesis of RA. Mucosal sites exposed to bacterial antigens, such as the gingiva and gut, may represent the initial site of immune autoreactivation [3]. Periodontitis (PD) is a chronic inflammatory gingival disease that serves as a reservoir for many organisms and inflammatory mediators [4]. A large surface area of the ulcerated pocket epithelium allows exchange between bacterial and host products. It has been noted that periodontal pathogens are able to invade gingival tissues, apart from the local destruction of the periodontal tissues, and gain access to the systemic circulation [5,6]. Several mechanisms may participate in these interactions including those induced by oral organisms, and those associated with host response factors such as proinflammatory cytokines [7,8].

Both PD and RA have cytokine profiles thought to be involved in the inflammatory processes, including high tumor necrosis factor-α production. Several studies...

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reported significantly higher prevalence of gingival disease in patients with RA compared with the general population. They also reported that systemic inflammation induced by periodontal infection might worsen the local inflammatory reactions in the synovium of patients with RA [9]. This effect is supposed to be mediated through systemic inflammatory response such as acute-phase reactants and immune mediators [10].

Many bacteria have been identified in PD, and the most commonly implicated ones are Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi), Tannerella forsythia, and Aggregatibacter actinomycetemcomitans [11]. Although many pathogens have been linked to pathogenesis of RA, Pg has been the major focus of investigations in RA [12,13]. Moreover, some studies identified Aa as the sole pathogen with the ability to produce the citrullinated antigens found in the RA [14]. These associations, if validated, could lead to the detection of potential risk, early preventive, and therapeutic approaches in the preclinical and early clinical phases of RA.

**Objective**

The aim of our study was to determine the organisms causing PD in a cohort of Egyptian patients with RA and their relation to serum and gingival anticitrullinated peptide protein antibodies (ACPA) level and other disease parameters.

**Patients and methods**

This study was carried out on a cohort of 100 consecutive Egyptian patients with RA fulfilling the 2010 American college of rheumatology/European league against rheumatism classification criteria for RA (ACR/EULAR) [15]. All patients were recruited from Rheumatology Outpatient Clinic and Rheumatology Inpatient Unit of Alexandria Main University Hospital (AMUH). Smokers, pregnant and lactating females, patients with diabetes mellitus, individuals who had received antibiotic therapy over the past 4 months as well as faulty prosthesis and fillings all were excluded from the study. Informed consent was obtained, and all patients were subjected to history taking, musculoskeletal examination with assessment of disease activity by applying the disease activity score (DAS-28/ESR version) [16], and functional state by applying health assessment questionnaire (HAQ) [17].

Dental examination was performed for all participants for the presence of PD according to American Academy of Periodontology 1999 [18]. Laboratory investigations included complete blood count, blood urea, serum creatinine, aspartate aminotransferase, alanine aminotransferase, erythrocyte sedimentation rate, and C-reactive protein (CRP). Radiograph of hands and wrists was performed, assessed, and globally scored for erosions and joint space narrowing by using the modified-Larsen scoring system [19]. Immunological profile included rheumatoid factor (RF) titer (Rose Waaler test) [20], serum, and gingival crevicular fluid (GCF) ACPA (using ELISA/Demeditec Diagnostics GmbH, Germany) [21]. For those with proven PD, GCF culture was performed for Pg, Aa, and Pi.

**Sampling of periodontal fluid**

GCF collection was performed as described by Goutoudi et al. [22]. For complete isolation and to prevent salivary contamination, cotton isolation and a suction were used mandatory. Each tooth was then isolated using cotton placed in its buccal vestibule. The tooth was then dried gently with air on the marginal gingival areas from all aspects. GCF samples were collected by filter paper strips which were cut into standardized size of 2×10 mm. A twiser was used to insert each paper strip into the gingival crevice until slight resistance was felt. The paper strip was left in place for about 60 s, then it was removed and examined.

**Molecular detection of Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, and Prevotella intermedia** [23]

A 16 s rRNA-based PCR detection method was used to determine the presence of Aa, Pg, and Pi in periodontal fluid taken from patients with PD. The samples in PBS were warmed to 37°C for 10 min and mixed well on a Vortex mixer; 0.2 ml of the microbial suspension was washed three times with distilled water. The bacterial pellets were resuspended in 0.1 ml of sterile eppendorf tube, 5 μl of bacterial DNA was mixed with 0.5 μl of each primer (forward and reverse) and 12.5 μl DreamTaq™ Green PCR master mix (Fermentas – Thermo, USA), and completed to a final reaction volume of 25 μl with nuclease-free sterile water. PCR amplification was done on Sensoquest thermal cycler according to the following protocol:

1. Pg and ubiquitous primers included an initial denaturation step at 95°C for 2 min, followed by 36 cycles of a denaturation step at 95°C for 30 s, a primer annealing step at 60°C for 1 min, an
extension step at 72°C for 1 min, and a final step of 72°C for 2 min.

(2) Aa and Pi included an initial step of 95°C/2 min, followed by 36 cycles of 94°C/30 s, 55°C/1 min and 72°C/2 min, and a final step of 72°C/10 min.

Overall, 12 μl of PCR product was applied on a 2% agarose gel along with a 100–3000 base pair DNA ladder (Axygen, USA). Electrophoresis was done at 100 Volt for a 45 min period. Bands were detected using ethidium bromide in ultraviolet light (Fig. 1).

Statistical analysis
Data were analyzed using IBM SPSS version 20 (Armonk, NY: IBM Corp.) [24]. Qualitative data were described using number and percentage. Quantitative data were described using range (minimum and maximum), mean, SD, and median. The tests used were Spearman’s correlation coefficient, χ²-test, Mann–Whitney test, Kruskal–Wallis test, Fisher’s exact or Monte-Carlo test, Student’s t-test, and F-test (analysis of variance). P less than 0.05 was accepted as statistically significant. The diagnostic ability of GCF ACPA was identified using the receiver operating characteristic curve (ROC curve). ROC curves with the area under the curve were used to assess the diagnostic performance of GCF ACPA, ‘an area=1.00 (100%) denoting (a gold standard–like) performance, whereas an area=0.5 (50%) denoting (a chance-like) performance’. Significant areas (P<0.05) indicate that the diagnostic performance is significantly better than chance. Cut-off values were chosen as the points that maximizes the sensitivity and specificity.

Results
In this study, a cohort of 100 consecutive Egyptian patients with RA was enrolled. The mean age of cases was 44.80±9.88 years, with 81 females and 19 males. Twenty-four (24%) patients presented with early RA, and 76 (76%) with established RA with mean disease duration of 21.36±20.65 months. The mean ACR/EULAR criteria at the time of enrollment were 8.0±1.22 (Table 1). The mean DAS was 5.76±1.47, with 3% of patients were in remission, 3% had low disease activity, 31% had moderate disease activity, and 63% had high disease activity. However, the mean HAQ score was 1.59±0.91, with 34% of patients showed no disability, 25% had mild disability, 8% had moderate disability, and 33% had severe disability. Laboratory findings in patients with RA (n=100) are shown in Table 2.

PD was found in 66 (66%) patients; of them, GCF culture was performed, and Pg, Aa, and Pi were found in 40 (60.6%) patients, 10 (15.2%) patients, and 20 (30.3%) patients, respectively. Twenty-two (55%) of Pg-positive cases (n=40) had positive RF, whereas 20 (76.9%) of Pg-negative cases (n=26) had positive RF. Comparing the two groups revealed no statistical significant difference (P=0.070; Table 3). Eight (80%) of Aa-positive cases (n=10) had positive RF. On the contrary, 34 (60.7%) of Aa-negative cases (n=56) had positive RF. Comparing the two groups revealed no statistical significant difference (P=0.306; Table 1).

Table 1 Descriptive analysis of patients with rheumatoid arthritis (n=100)

<table>
<thead>
<tr>
<th>Variables</th>
<th>n (%)</th>
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<tbody>
<tr>
<td>Sex</td>
<td></td>
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<tr>
<td>Male</td>
<td>19 (19.0)</td>
</tr>
<tr>
<td>Female</td>
<td>81 (81.0)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
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<tr>
<td>Minimum–maximum</td>
<td>21.0–65.0</td>
</tr>
<tr>
<td>Means±SD</td>
<td>44.80±9.88</td>
</tr>
<tr>
<td>Median</td>
<td>46.0</td>
</tr>
<tr>
<td>Duration of disease (months)</td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>24 (24.0)</td>
</tr>
<tr>
<td>Established</td>
<td>76 (76.0)</td>
</tr>
<tr>
<td>Minimum–maximum</td>
<td>0.03–60.0</td>
</tr>
<tr>
<td>Means±SD</td>
<td>21.36±20.65</td>
</tr>
<tr>
<td>Median</td>
<td>12.0</td>
</tr>
<tr>
<td>ACR/EULAR criteria at enrollment (6–10)</td>
<td></td>
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<tr>
<td>Minimum–maximum</td>
<td>6.0–10.0</td>
</tr>
<tr>
<td>Means±SD</td>
<td>8.0±1.22</td>
</tr>
<tr>
<td>Median</td>
<td>7.50</td>
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</table>

ACR/EULAR, American college of rheumatology/European league against rheumatism classification criteria for RA.
Fourteen (70%) of Pi-positive cases \((n=20)\) had positive RF, whereas 28 (60.9%) of Pi-negative cases \((n=46)\) had positive RF. Comparing the two groups revealed no statistical significant difference \((P=0.479; \text{Table 5})\).

Eighty-two (82%) patients had positive serum ACPA \((\geq 20 \text{ U/ml})\) with mean level of 296.01±500.02 U/ml. However, the mean gingival fluid ACPA level was 5.95 ±13.40 U/ml. PD was higher in ACPA-positive cases, as 54 (81.8%) of PD cases had positive serum ACPA with mean level of 254.66±458.15 U/ml in PD cases. ROC curve of GCF ACPA (Fig. 2) shows that GCF ACPA can significantly diagnose PD \((\text{area under the curve}=0.730*, \text{P}=0.001)\). Periodontitis is diagnosed at the cut-off level greater than 2.11 U/ml.

Table 4 Prevalence of rheumatoid factor in periodontitis cases with *Aggregatibacter actinomycetemcomitans* (Aa) \((n=66)\)

| RF | Negative \((n=56)\) [\(n(\%)\)] | Positive \((n=10)\) [\(n(\%)\)] | \(\chi^2\) | \(P\) | FE | E
<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>22 (39.3)</td>
<td>2 (20.0)</td>
<td>1.364</td>
<td>0.306</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>34 (60.7)</td>
<td>8 (80.0)</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

\(\chi^2, P: \chi^2\) and \(P\) values for \(\chi^2\)-test for comparing between the two groups. FE, Fisher exact for \(\chi^2\)-test; RF, rheumatoid factor.

**Comparison between positive cases for the studied organisms regarding different disease parameters**

In *Pg*-positive cases, 32 (80%) cases were positive for serum ACPA and 34 (85%) had positive CRP with mean GCF ACPA of 8.61±16.58 U/ml. In *Aa*-positive cases, 10 (100%) cases were positive for serum ACPA and had positive CRP, with mean GCF ACPA of 4.77 ±6.10 U/ml. In *Pi*-positive cases, 14 (70%) cases were positive to serum ACPA and 12 (60%) had positive CRP, with mean GCF ACPA of 1.38±1.29 U/ml. There was no statistical significant difference among
the three studied organisms regarding serum ACPA level. Aa-positive cases were associated with positive CRP significantly higher than Pi-positive cases (P=0.029). No statistical significant difference was detected between Pg-positive and Aa-positive or Pi-positive cases. GCF ACPA was significantly higher in Pg-positive cases than Pi-positive cases (P=0.047). No significant difference was detected regarding GCF ACPA between Pg-positive cases and Aa-positive cases or between Aa-positive and Pi-positive cases (Table 6).

On comparing the DAS, in Pg-positive cases, 20 (50%) cases had high disease activity, 18 (45%) had moderate disease activity, and two (5%) had low disease activity. In Aa-positive cases, six (60%) had high disease activity, four (40%) had moderate disease activity, and none of them had low disease activity. In Pi-positive cases, 12 (60%) cases had high disease activity, eight (40%) had moderate disease activity, and none of them had low disease activity.

Regarding HAQ, in Pg-positive cases, 12 (30%) had severe functional disability, four (10%) had moderate disability, 10 (25%) had mild disability, and 14 (35%) had no disability. In Aa-positive cases, six (60%) had severe functional disability and four (40%) had mild disability. In Pi-positive cases, 10 (50%) had severe functional disability, eight (40%) had mild disability, and two (10%) had no disability. No statistical significant difference was detected between the cases of the three studied organisms regarding DAS-28 or HAQ.

According to modified-Larsen radiographic scoring systems used, 12 (12%) patients with RA showed either erosions or joint space narrowing by radiography, whereas 88 (88%) patients had normal radiography of the hands and wrists, with no evidence of erosion or joint space narrowing. In the group of patients with joint damage, eight (66.7%) patients had PD [of them, two (25%) patients had severe generalized PD] and four (33.3%) patients had chronic gingivitis. However, in the group of cases with normal radiography, 58 (65.9%) patients had PD [of them, 26 (44.8%) patients had severe generalized PD] and four (33.3%) patients had chronic gingivitis. No statistical significant difference was detected between the two groups regarding number of patients with PD (P=1.000) or number of severe generalized PD cases (P=0.451). Six (75%) patients with joint damage were positive for Pg.
two (25%) patients were positive to Aa, and two (25%) patients were positive to Pi. On the contrary, in the normal radiography group, 34 (58.6%) patients were positive for Pg, eight (13.8%) patients were positive for Aa, and 18 (31%) patients were positive for Pi. No statistical significant difference was detected between the two groups (normal and abnormal radiography groups) regarding the oral microbiota (Table 7).

### Discussion
Many studies showed an increased prevalence of RA in patients with PD [25,26]. Others demonstrated the presence of RF in the gingiva, saliva, and serum of patients having PD [27,28].

*Porphyromonas gingivalis* was not detected. Scher et al. [30] did not show any relationship between anti-Pg or Pg abundance and the presence of RA autoantibodies, including RF and ACPA, and concluded that overall exposure to Pg was not associated with high serum ACPA level. Furthermore, they found two other organisms, *Prevotella* spp. and *Leptotrichia* spp., were prevalent in new-onset RA. In the study by Ziebolz et al. [31], 11 periodontal pathogens in subgingival plaque profile from patients with RA were examined. There was no association between the presence or abundance of Pg with RF. On the contrary, Mikuls et al. [32] studied the anti-Pg antibodies and they demonstrated that anti-Pg titers were higher in RA than controls. They also showed that Pg level was associated with ACPA. However, they did not show any association between prior infection with Pg and established RA. However, in another study by Mikuls et al. [33], who examined serum ACPA and antibodies against three different periodontopathic bacteria, they showed that anti-Pg was associated with ACPA and RF. This relationship was seen with anti-Pg but not with the other oral bacteria tested, suggesting that Pg exposure was the main susceptibility factor in the development of RA antibodies, even in the preclinical arthritis. In our study, ACPA was detected in all examined GCF samples from PD cases, being of significant higher levels with Pg than Pi. No statistically significant difference was detected on comparing Pg with Aa or Aa with Pi. Interestingly, GCF ACPA can significantly diagnose PD (ROC curve) at the cut-off level greater than 2.11 U/ml. This may point to the origin of ACPA from the gingiva. This might indicate that citrullination of proteins occurs in gingival tissues during periodontal inflammation and raises the possibility that the periodontium may be an extra-articular site of citrullinated proteins. Furthermore, autoantibodies to these citrullinated proteins may be formed in the periodontium, eventually contributing to the RA disease process.

### Table 7 Radiographic findings with regard to periodontitis and periodontal microbiota

<table>
<thead>
<tr>
<th>Radiography findings</th>
<th>Normal (n=58) [n (%)]</th>
<th>Combined erosion and JSN (n=8) [n (%)]</th>
<th>Test of significance</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodontitis (n=66)</td>
<td>58 (65.9)</td>
<td>8 (66.7)</td>
<td>$\chi^2=0.003$</td>
<td>FE=1.000</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
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<tr>
<td>Negative</td>
<td>24 (41.4)</td>
<td>2 (25.0)</td>
<td>$\chi^2=0.790$</td>
<td>FE=0.464</td>
</tr>
<tr>
<td>Positive</td>
<td>34 (58.6)</td>
<td>6 (75.0)</td>
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<tr>
<td><em>Aggregatibacter actinomycetemcomitans</em></td>
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<tr>
<td>Negative</td>
<td>50 (86.2)</td>
<td>6 (75.0)</td>
<td>$\chi^2=0.687$</td>
<td>FE=0.596</td>
</tr>
<tr>
<td>Positive</td>
<td>8 (13.8)</td>
<td>2 (25.0)</td>
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<tr>
<td><em>Prevotella intermedia</em></td>
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<tr>
<td>Negative</td>
<td>40 (69.0)</td>
<td>6 (75.0)</td>
<td>$\chi^2=0.121$</td>
<td>FE=1.000</td>
</tr>
<tr>
<td>Positive</td>
<td>18 (31.0)</td>
<td>2 (25.0)</td>
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</table>

FE, Fisher exact for $\chi^2$-test; JSN, joint space narrowing.
Okada et al. [13] studied relationships between anti-Pg and RA antibodies in Japanese patients with RA and they found that anti-Pg was associated with RF, but not with ACPA. In another study conducted by De Smit et al. [34], antibodies to Pg were associated with higher serum level of ACPAs targeting citrullinated fibrinogen.

Harvey et al. [35] in their study reported that citrullinated proteins and ACPA have been noted in inflamed periodontal tissues. They had excluded RA to rule out the possibility that the presence of citrullinated proteins was owing to ongoing RA. They stated that as none of the participants in their study had RA, so the presence of anti-CCP antibodies in the GCF of patients may indicate preclinical phase of RA.

Regarding Aa, Konig et al. [14] studied the periodontal microenvironment in patients with PD to define mechanisms underlying mucosal inflammation and autoimmunity in RA. Among the microbial species associated with periodontal disease, they identified Aa as the only pathogen with the ability to reproduce the repertoire of citrullinated antigens found in the RA joint. They concluded that Aa may be a primary oral microbe that can trigger autoimmunity in RA.

Our findings support the relationship between PD and infectious trigger at the gingival site and RA. Further studies in this field are recommended aiming, specifically to define the exact origin of ACPA, primarily to initiate preventive measures for this destructive disease.

Acknowledgements
A.A. and E.S contributed in concept; A.A., E.S and M. H. contributed in design; data collection and/or processing was done by A.A., M.H., R.F.; analysis and/or interpretation of the data done by R.F.; literature search was done by A.A., E.S, and R.F; writing manuscript was done by A.A. and E.S; critical review of the article was done by M.H. and E.S.

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Conflicts of interest
There are no conflicts of interest.

References


