

Original article

Assessment of gut microbiota in rheumatoid arthritis patients

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ABSTRACT

Background: Pathophysiology of rheumatoid arthritis (RA) is an undetermined complex mechanism which include interaction between genetic, environmental and immunological factors. Recent studies suggest a possible role for gut microbiome in the pathogenesis. **Objectives:** To assess the alteration of fecal *Bacteroids*, *Lactobacilli*, and *Prevotella* genera among RA patients. **Methods:** Stool samples were collected from 25 patients newly diagnosed as RA (RA group) and 25 healthy controls (control group) for quantitation of *Bacteroids*, *Lactobacilli*, and *Prevotella* genera by real time PCR. **Results:** The intestinal microbiome of RA group was significantly altered. We reported a significant enrichment of the bacterial *Lactobacilli* genus in RA patients (16.206% vs 1.5%; $P < 0.001$). *Bacteroids* and *Prevotella* genera showed lower percentages in RA patients compared to the controls (1.113% vs 2.7 %; 3.157% vs 17% respectively, $P = 0.018$, $P < 0.001$ respectively). **Conclusion:** Predominance of *Lactobacilli* genus with the lower levels of *Bacteroids* and *Prevotella*, in recently diagnosed RA patients, could indicate the role of gut dysbiosis in the pathophysiology of RA.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease manifested by an autoimmune response with destruction and inflammation of different joints [1]. Development of RA involves a complex interaction between the immune system, genes, and environmental factors [2]. These factors related to the environment that affect the pathophysiology of RA are still unclear. Anti-citrullinated protein IgA antibody (ACPA) is recently found in sera of RA patients before the onset of arthritis, providing an evidence that RA pathogenesis starts at mucosal surfaces, such as the gut [3,4].

Several citrullinated peptides of a colonic origin were isolated from the synovial fluids of patients with RA and these citrullinated proteins were goals for ACPAs, suggesting that mucosa of the

colon might be a possible cut out area for immune tolerance to these proteins [5].

Dysbiosis in the mucosal surfaces progresses to immune changes and cut outs in the tolerance to citrullinated autoantigens [2] where gut dysbiosis incriminated in the pathogenesis of RA [2, 6-10]. Therefore, we aimed to assess the alteration of fecal *Bacteroids*, *Lactobacilli*, and *Prevotella* genera in RA patients.

Patients and Methods

Study design and setting

Our case-control study was conducted over six months, from March 2020 to September 2020. It was carried out in Medical Microbiology & Immunology Department, and Rheumatology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt.

It was approved by the institutional review board (IRB) no 6373, Faculty of medicine, Zagazig University. The study was done according to the revised Declaration of Helsinki. Informed written consents were acquired from all participants.

Study subjects

Twenty-five new onset, treatment-naïve adult RA patients (≥ 18 years old) were enrolled in RA group and 25 apparently healthy participants of matched sex and age were registered in the control group. Patients were recruited by systemic random sampling from those attending Rheumatology outpatient clinic. Rheumatoid arthritis diagnosed according to the criteria of 2010 American college of Rheumatology (ACR)/ European league Against Rheumatism classification (EULAR) [11].

We excluded participants suffering from recent (less than three months) bacterial or parasitic infections of the gastrointestinal tract. Patients received antibiotics, corticosteroids, or non-steroidal anti-inflammatory drugs (NSAIDs) in the previous three months were excluded from the study. Pregnancy and breast feeding were also excluded.

Rheumatoid arthritis participants were undergone: detailed history, clinical examination, serum inflammatory markers as C-reactive proteins (CRP) and erythrocytes sedimentation rate (ESR), liver function tests, complete blood picture, kidney function tests, rheumatoid factor titre (RF), and anti-cyclic citrullinated peptide antibody (Anti-CCP). Fecal samples were gathered from all RA patients and apparently healthy individuals to estimate *Bacteroides*, *Lactobacilli*, and *Prevotella* families by real time PCR.

Sample collection

Table 1. Primers used in PCR.

Target	Sequence (5' to 3')	Product size (bp)	Reference
All bacteria	F- ACTCCTACGGGAGGCAGCAGT R- GTATTACCGCGGCTGCTGGCAC	200	[12]
<i>Bacteroides</i>	F- GTCAGTTGTGAAAGTTTGC R- CAATCGGGAGTTCTTCGTG	127	[13]
<i>Lactobacillus</i>	F- GCAGCAGTAGGGAATCTTCCA R- GCATTACACCGCTACACATG	340	[14]
<i>Prevotella</i>	F- CACCAAGGCGACGATCA R- GGATAACGCCCGGACCT	283	[15]

Fresh stool samples were collected from the participant. The samples were stored at -80°C until PCR analysis.

DNA extraction and real time PCR

DNA was extracted from fecal samples using extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Quantitative Real Time PCR technique was used for quantitation of *Bacteroides*, *Lactobacilli*, *Prevotella genera* in the fecal samples.

The fecal microbiota was assessed in feces using applied biosystem real-time PCR (Step one TM, real-time PCR system, Applied Biosystems Inc, USA). All DNA extracts of the RA patients and controls were diluted 1:10. The standard curve was constructed from pooled stool samples of healthy individuals, with two-fold serial dilution of the extracted bacterial DNA. The standard curve was constructed for total bacterial, *Lactobaclii*, *Prevotella*, and *Bacteroides*. Each bacterial family was relatively expressed as a ratio in comparison to the total fecal bacteria of each sample. The used primers (Willowfort, Birmingham) were listed in **table (1)**. The amplifications were done in a final reaction volume of 20 ul containing 10 ul of 2x SYBR mix (Thermo Fisher scientific inc, Maxima SYBR Green qPCR Master Mix (2X), USA), 1 ul of each primer, 8 ul of bacterial DNA. The amplification protocol started with an initial DNA denaturation step at 95°C for 10 minutes, followed by 10 cycles of annealing at 60°C for 55 seconds, then 30 cycles of annealing at 50°C for 30 seconds and elongation at 72°C for 45 seconds. The process was ended with an elongation step at 72°C for 10 minutes.

Statistical analysis

Data analysis was performed using the software SPSS (Statistical Package for the Social Sciences) version 20. Quantitative variables were described using their means and standard deviations. Categorical variables were described using their absolute frequencies and were compared using chi square test. Kolmogorov-Smirnov (distribution-type) and Levene (homogeneity of variances) tests were used to verify assumptions for use in parametric tests. To compare quantitative non-parametric data and medians of categorical discrete data between two groups, Mann Whitney test was used. Independent sample t test was used to compare two groups concerning quantitative normally distributed continuous data. The level statistical significance was set at $P < 0.05$.

Results

Demographic and clinical data

There were statistically non-significant difference between RA and control groups regarding age or sex ($P = 0.221$ and 0.306 respectively). There was

statistically non-significant difference between both groups regarding smoking, ALT, AST or serum creatinine levels. CRP, and ESR were significantly higher among RA group while hemoglobin was significantly lower among them on comparing these parameters with healthy control group (Table 2).

Disease specific data

Anti-CCP ranged from 26 to 113 with median 110. Morning stiffness of joints ranged from 10 to 60 minutes with median 30 minutes. On examination, tender joints ranged from 3 to 28 joints and swollen joints ranged from 0 to 4 joints (Table 2).

Microbiome analysis

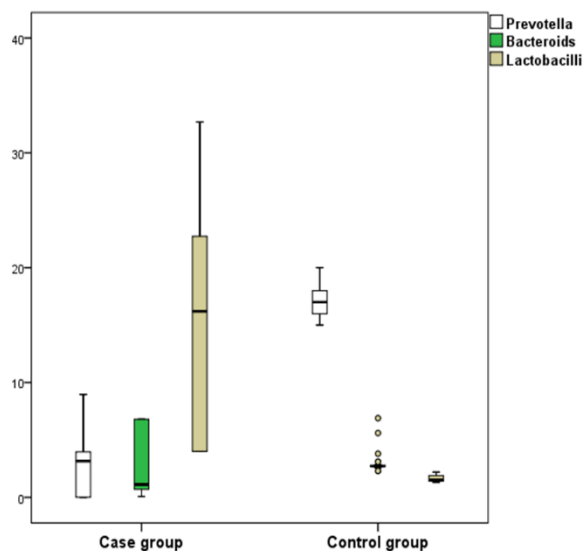
We found statistically significant percent variation among the studied genera of bacterial microbiota in RA group compared to the control group. *Lactobacilli* were significantly enriched in RA patients (16.206% vs 1.5%; $P < 0.001$). Percent of *Bacteroids* and *Prevotella* were significantly lower in RA group compared to the control group (1.113% vs 2.7%, 3.157% vs 17% respectively, $P = 0.018$, $P < 0.001$ respectively) (Figure 1).

Table 2. Demographic and clinical data of the study groups

	RA group (n= 25)	Control group (n=25)	P-value
Age (years)	39.4 ± 12.692	35.2 ± 11.210	0.221 [‡]
Female sex, no (%)	21 (84%)	18 (72%)	0.306 [¥]
Smoking habit, no (%)	20 (80%)	19 (76%)	0.733 [¥]
Swollen joint on examination	1 (0 – 4)	-	
Tender joint on examination	15 (3 – 28)		
Hemoglobin (g/dL)	10.828 ± 0.881	12.908 ± 1.069	<0.001 ^{‡*}
ESR (mm/h)	64.44 ± 19.369	10.08 ± 1.38	<0.001 ^{‡*}
CRP (mg/l)	16 (2.76 – 75)	4 (2 – 6)	<0.001 ^{∞*}
ALT	19.94 ± 8.25	19.4 ± 3.39	0.762 [‡]
AST	18 (8.8 – 32)	18 (16 – 21)	0.784 [∞]
Creatinine (mg/dL)	0.83 ± 0.137	0.81 ± 0.111	0.590 [‡]
Morning stiffness (minutes)	30 (10 – 60)	-	
Anti-CCP	110 (26 – 113)		
RF titer	43 (29.8 – 164)		

‡ Independent sample t test (data is represented as mean±SD), ¥ Chi square test (data is represented as number and percentage), ∞ Mann Whitney test (data is represented as median and range), * $P < 0.05$ is statistically significant, ESR, Erythrocyte sedimentation rate; CRP, C-reactive protein; ALT, Alanine aminotransferase; AST, aspartate aminotransferase; RA, rheumatoid arthritis; Anti-CCP, anti-citrullinated protein autoantibody; RF, rheumatoid factor.

Figure 1. Boxplot showing comparison between the studied groups regarding percent variation of bacteria in fecal microbiota.



Discussion

Previous studies suggested a part of the intestinal microbiome in RA pathophysiology. This started when degradation products of bacterial nucleic acids and cell walls were detected in inflamed joints. Those observations started a hypothesis that antigenic material of gut bacteria leaks to the blood circulation and ends up in joints causing arthritis in genetically susceptible individuals [16].

In animal models, parenteral injection of intestinal bacterial cell-wall fragments has induced arthritis and this effect varied according to the investigated bacterial species [17]. Moreover, alteration of patients' microbiota through, changing the diet habits, reduced the symptoms of arthritis [18]. These accumulating findings suggested that alteration of gut microbiota had a significant role in inducing RA. This theory was further supported by studies carried out in the United States, China and England that reported intestinal dysbiosis in patients with RA [8,19,20].

The composition and diversity of gut microbiota vary according to age, gender, geographic region, and dietary and cultural practices [21,22]. Therefore, Egyptian RA patients are likely to have different composition of their gut microbiota compared to other populations. To the best of our knowledge, this is the first study to assess intestinal microbiota among Egyptian RA patients.

This study reported that the fecal microbiota of RA patients showed significantly less *Bacteroids* and *Prevotella* species compared to the controls. For

Lactobacilli species, they were increased with statistical insignificance.

In agreement with the results of this work, almost all of the previous studies reported decreased *Bacteroids* in rheumatoid subjects whether in human patients [19, 20, 23] or animal models [24]. This is an important indicator of dysbiosis since *Bacteroids* make up the main portion of the mammalian gut microbiota (about 25%) [25].

In accordance with findings of the current study, some previous works reported decreased *Prevotella* species in RA patients [6,19]. In these studies, a common primer for all *Prevotella* species was used. However, some other studies detected certain *Prevotella* species using specific primers. A Japanese study had reported increased numbers of intestinal *Prevotella copri* with a significant effect of these species in inducing arthritis in mice [26]. On the other hand, other study reported a positive effect of *Prevotella. histicola* in preventing the development of arthritis [27] These findings suggested that different *Prevotella* species have variable effects on arthritis.

As regard *Lactobacilli* species, results of previous studies widely varied. Some studies reported declined gut *Lactobacilli* in RA [23], while other studies reported increased *Lactobacilli* [7]. The current study reported statistically significant increase among *Lactobacilli*. This variation may be caused by differences between Egyptian and other populations in dietary habits and practices. Very few studies had correlated intestinal dysbiosis to activity of RA. **Zhang and his coworkers** reported increased *Lactobacillus salivarius* in individuals with RA, specially, in cases with very active disease [8].

Conclusion

We reported alteration in intestinal microbiota of RA patients. *Bacteroids* and *Prevotella* species were significantly decreased while, *Lactobacilli* species were significantly increased. Our findings, together with previous studies on established and recent RA patients, indicated the mucosal origin hypothesis and the role of microbiota dysbiosis in RA pathophysiology. Awareness with the mechanisms that initiate autoimmune process could highlight new intervention strategies that could treat the disease in early stages with better long outcome or even longer periods of drug free remission. Intestinal dysbiosis could be a target for future preventive interventions. This also points to

the possibility of using probiotics in treatment of such cases.

Limitation

This study included only 25 patients and investigated only three genera of microbiota. Further studies are recommended that include more subjects, more investigated microbiota with follow up and monitoring of the therapeutic effect of gut microbiota modulation. Our study did not assess the immune response (innate and adaptive) against *Bacteroids*, *Prevotella*, and *Lactobacilli* families in new onset RA that could give a clear idea about the immune pathogenesis of RA in relation to the altered microbiome.

Conflict of interest

The authors declare no conflict of interest.

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