

# A Study on Blood Microbiome Using 16S rRNA Gene and Sequencing in Rheumatoid Arthritis Patients

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## Abstract

**Background:** Recent studies suggest that bacteria may play a role in triggering rheumatoid arthritis. Considering the recent reports on viable but non-culturable cells (VBNC) in the synovial fluid and blood of patients, the role of bacteria has become more prominent.

**Objective:** The present study aimed to use general and specific primers to detect microbiome in the blood of patients with rheumatoid arthritis.

**Methods:** A general primer, called 16S rRNA was used in the present study to detect a wide range of bacteria in the blood of patients with rheumatoid arthritis. Specific primers were designed and used such as *nuc* and *rfbE* to trace *Staphylococcus aureus* and *Escherichia coli* in patients' blood. Examining 102 blood samples and performing the genomic extraction separately for each sample, PCR was then performed using general and specific primers, and the results were sequenced. The findings were analyzed using descriptive statistics.

**Results:** According to the results, in 102 blood samples of rheumatoid arthritis patients, who were negative for bacteriological culture, there were 74 cases (72.54%) of 16S rRNA gene, and 54 cases (52.9%) of *Staphylococcus aureus nuc* gene, respectively. Moreover, a specific gene, *rfbE*, was traced in 12 samples.

**Conclusion:** The findings of the present study indicated the presence of microbiome in the blood of patients with rheumatoid arthritis. Because the blast sequencing results of the PCR product showed a wide range of bacterial genomes. These findings may potentially improve the management of rheumatoid arthritis diagnosis and treatment.

**Keywords:** Microbiome, Rheumatoid arthritis, Blood, PCR

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## 1. Introduction

Rheumatoid arthritis (RA) is an inflammatory disease with an unknown origin, affecting about 1% of the world's population<sup>1</sup>. Autoimmunity and infection due to local fever in patients with rheumatoid arthritis are among the causes stated for this disease. However, the cause of RA is still unknown. It may also be due to the complex interaction between different factors<sup>2</sup>. Recent studies indicate that intestinal microbiota and their collective metabolic excretion may have impacts on the human immune system, and RA disease. However, the role of the intestinal microbial metabolites in RA pathogenesis is still unknown<sup>3</sup>. Hence, some researchers believe that RA is a

complex disease, and various genetic and environmental factors play roles in its development and progression<sup>4</sup>. Despite significant advances in understanding the genetic, cellular, and molecular aspects of RA, may relatively few factors have been known that are important in terms of sensitivity to RA and their interaction with host genetics in the development of RA<sup>3,5</sup>. Some reports indicate the role of *Staphylococcus aureus* superantigen infection in the pathogenesis of rheumatoid arthritis<sup>6</sup>. According to the findings, microbes, especially those derived from the urinary tract, the microbial imbalance of the gastrointestinal tract, microbial changes in the mouth, as

well as tooth and gum infections contribute to the development of RA <sup>7-9</sup>. Furthermore, the roles of viral infections have been considered in the development of rheumatoid arthritis. recently, EBV virus was detected and reported in synovial fluid of rheumatoid arthritis patients <sup>10</sup>. Using the Uniplex PCR and Multiplex PCR methods, the researchers have shown the presence of specific genes of *Mycoplasma arthritis*, *Mycoplasma pneumoniae*, and *Mycoplasma hominis* in the synovial fluid of rheumatoid arthritis patients. Moreover, some mycoplasmas were isolated by preparing a specific culture medium inoculated with the above samples <sup>11, 12</sup>. However, there is evidence of abnormal innate and acquired immune responses affecting the pathogenesis of rheumatoid arthritis disease <sup>13, 14</sup>. Since rheumatoid arthritis is an inflammatory phenomenon, and bacterial superantigens are the most important inflammatory factors <sup>15</sup>, several studies have been conducted to indicate the presence of common superantigens in synovial fluid and blood of rheumatoid arthritis patients <sup>16</sup>. Moreover, the existence of encoding genes of A, C, E, and D superantigens <sup>17-18</sup> has been showed in the blood and synovial fluid of patients. Although the bacteriological cultures of blood and synovial fluid have been reported to be negative, the origin of these superantigens in synovial fluid and blood is unclear, and there are arising numerous unanswered questions in this regard. For instance, it is not clear why the routine bacteriological culture of synovial fluid and blood have been reported to be negative, but exist superantigens.

### 1.1. Objective

Consequently, the present study aimed to examine the presence of blood microbiome in patients with RA by using the 16S rRNA gene and also, *nuc*, and *rfbE* genes.

## 2. Materials and methods

### 2.1. Patient selection and sampling

To conduct the present study, the blood samples were separately taken from 102 rheumatoid arthritis patients referring to the rheumatology clinic of Baqiyatallah

Hospital, whose disease was confirmed by a rheumatologist. The inclusion or exclusion criteria of patients were based on previous criteria <sup>19</sup>. Blood sampling was performed from certain patients within 6 months, and the samples were transferred to the laboratory under aseptic conditions and assayed.

### 2.2. DNA Extraction

The DNA extraction protocol was according to the MAXCell kit. Briefly, 200 µl of the patient blood sample was put into a 1.5 ml microtube. Then, 300 µl of lysis buffer, 20 µl of Proteinase K, and 10 µl of carrier DNA were added. After a few spins, the incubation was performed at a temperature of 57 °C for 20 min. Next, adding 100 µl isopropanol, it was gently vortexed. The content was then transferred to a column-containing microtube and centrifuged at 12000 rpm for 2 min. Adding 400 µl of wash buffer 1 to the column, it was re-centrifuged at 12000 rpm for 2 min. Then, the content was discharged again under the column and 600 µl of wash buffer 2 was added to the column, and it was centrifuged at 12000 rpm for another 2 min. The stage was repeated with 400 µl of wash buffer 2, and finally, the contents were discharged below the column. For drying, centrifugation was performed at 12000 rpm for 2 min. After this stage, the content was discarded under the column and the column was transferred to a 1.5 ml sterile microtube. At this stage, 35 µl of Elution buffer was added to the center of the column and incubated at room temperature for 2 min. Centrifuging at 12000 rpm for 1 min, the column was removed from the microtube, and the available solution containing DNA was examined in terms of quality and quantity.

### 2.3. Primers

In the present study, As we have shown in [table 1](#), two pairs of primers from previous sources were used. A pair primer was also designed in this research, which was synthesized according to looking for the sequence of the considered reference gene in the NCBI website.

**Table 1.** The specifications of primer in the research

Name	Seq. (5' _3')	Tm	GC %	Ref.
Universal-F	AGAGTTTGATCMTGGCTCAG	56	45 to 50	(20)
Universal-R	AAGGAGGTGWTCCARCC	52	53 to 59	
<i>nuc</i> - F	ATGGACGTGGCTTAGCGTAT	57	50	(19)
<i>nuc</i> - R	GCGTTGTCTTCGCTCCAAAT	57	50	
<i>rfbE</i> - F	GCCCAGTTAGAACAAGCTGA	57	50	In this study
<i>rfbE</i> - R	CTTTCCTCTGCGGTCCTAGT	59	55	

## 2.4. PCR Establishment by Target Primers

After designing the primer, and extraction of DNA from the blood, PCR was performed according to the set uped

protocol which have shown in [table 2](#) for all samples and 45 cycles.

**Table 2:** Polymerase chain reaction protocol used in this study.

Target gene	Stage 1 Primary denaturation	Stage 2 Denaturation	Stage 3 Annealing	Stage 4 Extension	Stage 5 Final Extension
NUC Gene	C'95	C'93	C'57	C'72	C'72
	3 min	45 seconds	60 seconds	60 seconds	5min
Universal Gene	C'95	C'93	C'58	C'72	C'72
	3 min	45 seconds	60 seconds	60 seconds	5min
rfbE Gene	C'95	C'93	C'58	C'72	C'72
	3 min	45 seconds	60 seconds	60 seconds	5min

### 2.4.1. PCR product electrophoresis

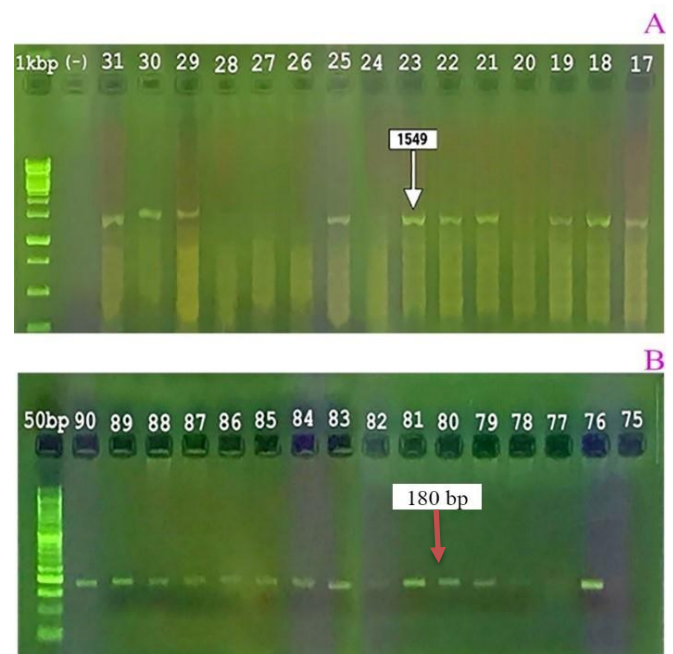
To evaluate the quality of PCR products, we used the agarose gel method. The gel concentration was 1.5% for the general primer, and 2% with 80 volts, and 50 min for the specific primer. The bands were observed and evaluated with UV light radiation. Then, a sample of each band was sent to Pishgam or Sinaclon Company for sequencing. The bioinformatical examination was performed for the sequencing results.

## 3. Results

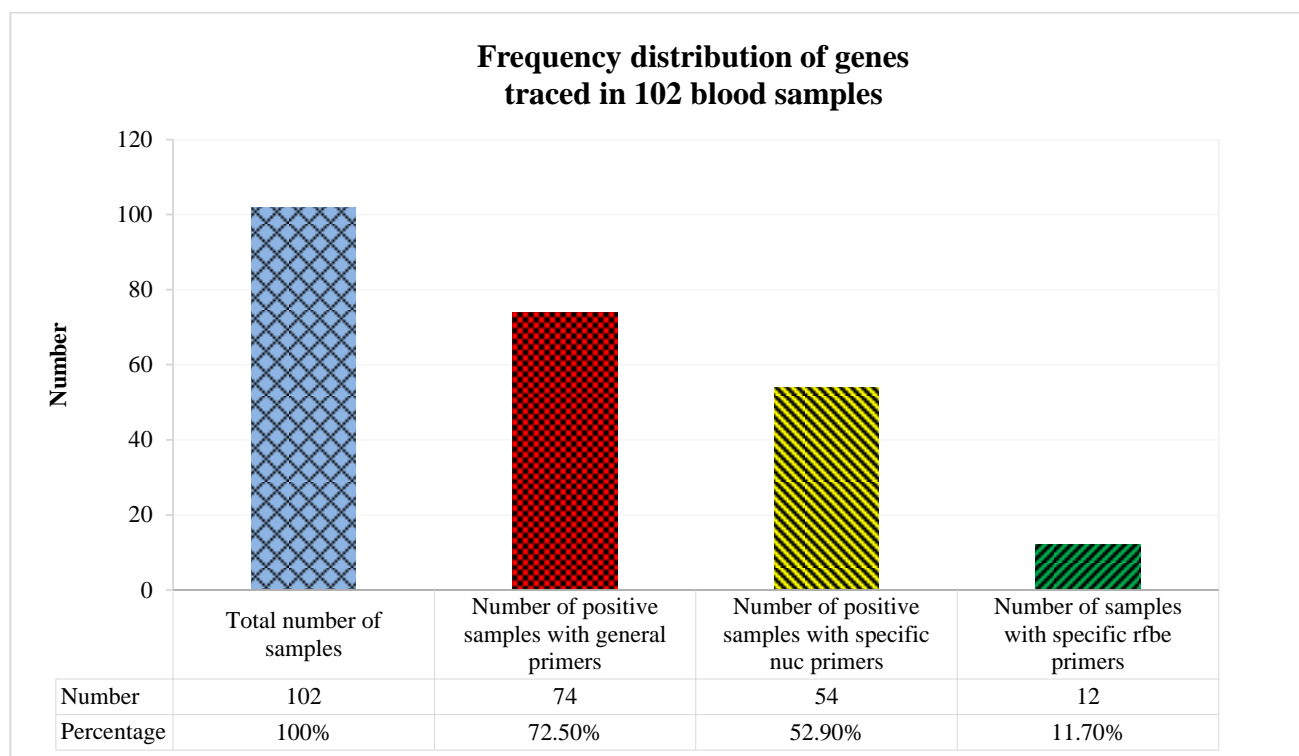
The results of DNA extraction from patients' blood samples indicated the extraction of sufficient amounts of DNA. Based on the concentration of DNA and NanoDrop data, the concentration homogenization of DNA was prepared for each sample and 1  $\mu$ L of the concentration was used as a template for each PCR.

In the study, the molecular method, PCR was utilized to trace a wide range of bacteria. The results of PCR ([Fig.1](#)) of DNA indicated that 74 (about 75%) out of 102 blood samples of patients with rheumatoid arthritis had 16S rRNA genes, and there was *Staphylococcus aureus* specific gene (*nuc*) in 54 samples. Furthermore, the specific *Escherichia coli* gene (*rfbE*) was duplicated in 12 blood samples of rheumatoid arthritis patients. [Fig.2](#) shows the percentage of genes traced in the blood samples of rheumatoid arthritis patients.

The results of determining the sequence of PCR product obtained from the duplication of 16S rRNA, nuc, and rfbE genes, and the examination of their homology with the reference genes in the gene bank revealed a wide range of gram-positive and gram-negative bacteria, and *Staphylococcus* and *Escherichia coli* strains.



**Figure 1.** The samples of PCR product electrophoresis of 16S rRNA, and nuc genes: a) the electrophoresis of PCR product and several blood samples with the fragment duplication of about 1549 bp; b) The duplication of nuc gene and the creation of a 180 bp fragment



**Figure 2.** The frequency distribution of genes traced in blood samples of patients with rheumatoid arthritis

#### 4. Discussion

The determination of the etiology of rheumatoid arthritis has been considered by researchers for many years. However, the cause of this disease is still unknown. Studies indicate that superantigenic enterotoxins are effective in the pathogenesis of chronic inflammatory diseases <sup>21</sup>. Furthermore, injection of some bacterial superantigens into rats is associated with higher production of laboratory diagnostic factors for rheumatoid arthritis <sup>22</sup>. In addition, the role of the microbiome in the development of rheumatoid arthritis has been considered in recent years <sup>23</sup>. Most studies indicate an association between gastrointestinal or oral microbiome with rheumatoid arthritis <sup>24</sup> although no report exists on blood microbiome in patients. Thus, the present study sought to indicate the presence of genes of microbes in the blood of rheumatoid arthritis patients.

Molecular PCR methods were utilized in the present work, along with the 16S rRNA encoding gene, as well as specific genes of *Staphylococcus aureus* and *Escherichia coli* to design and implement PCR. The PCR product was sequenced then and the results were compared and analyzed with other studies. Fortunately, recent published research on blood microbiome circulation of rheumatoid arthritis patients have support our research <sup>25</sup>. However, in the present study, duplication of a part of the 16S rRNA gene was revealed in 74% of cases and there was the presence of more than ten bacterial species in blast analysis revealed. But, why despite the large number of

bacteria in the blood, negative bacterial growth is reported?

This may be due to the presence of Viable But Not Culturable (VBNC) of bacteria. Therefore, in order to assay this type of bacteria, as representative VBNC, the *Staphylococcus aureus nuc* and *Escherichia coli rfbE* genes were investigated. The study results have shown that the trace of *Staphylococcus nuc* gene was found in 54% of samples along with the *rfbE* gene of *Escherichia coli* in 12 cases. It should be noted that these studies are the first published paper in this field. The use of a general primer for detecting parts of the 16S rRNA gene is a very sensitive tool in molecular studies. According to the results of a study, there were different species of *Mycoplasma* other than MAM (*Mycoplasma Arthritis Mitogen*) superantigen in mononuclear leukocytes (ML) of blood and SF of patients with RA <sup>26</sup>. In a series of studies, researchers have found increased MAM antibodies in the serum of RA patients. and suggested that MAM, or a molecule such as MAM, may be related to RA <sup>12</sup>. Another study indicated that MAM binding to HLA-DR results in structural changes in the MAM structure, thus enabling its interaction with TLR2 and TLR4, and causing the detection of T cells <sup>12</sup>. Other studies have reported the presence of *Staphylococcus aureus* superantigens in the blood and synovial fluid of patients with rheumatoid arthritis <sup>27</sup>. Tracing these genes in the blood or synovial fluid of rheumatoid arthritis patients has raised many questions, for instance, what is the origin of the

superantigens-encoding genes in the blood or synovial fluid of patients with rheumatoid arthritis? Is it endogenous or exogenous?

Even though the results of other studies indicate the roles of superantigens in the pathogenesis of some inflammatory diseases, including RA <sup>27</sup>, there are many ambiguities about their origin. Nevertheless, the results of a recent study indicate that intraperitoneal injection of C superantigen of *Staphylococci* in rats increases the expression of the inflammatory marker CD18 up to three times <sup>28</sup>. More studies are required in this field. Hence, the present study probably can explain a part of the etiology of rheumatoid arthritis since these findings are a promising tool in the etiological diagnosis of RA patients to improve patient management and select an appropriate and definitive treatment.

## 5. Conclusion

The 16S rRNA gene tracking and bioinformatics analysis of the product in the present study indicated the presence of at least ten bacteria in the blood. The presence of specific genes representing *Staphylococcus aureus* and *Escherichia coli* VBNC was also traced in the blood of rheumatoid arthritis patients. These findings are extremely important since they make it possible to design new protocols for managing the diagnosis and treatment of the disease and create a new perspective in the field of microbiome and its role in rheumatoid arthritis. Further studies are required to answer the question of how there are genes representing bacteria in patients' blood without culturing and isolating the bacteria?

### Highlights

#### What Is Already Known?

Recent studies suggest that bacteria may play a role in triggering rheumatoid arthritis.

#### What Does This Study Add?

the present study indicated the presence of microbiome in the blood of patients with rheumatoid arthritis. These findings may potentially improve the management of rheumatoid arthritis diagnosis and treatment.

## Authors' Contribution

Ramezani R, Ataee R A, developed the original idea and Alishiri GH had Diagnosed of patients whit rheumatoid arthritis diseases and selected, introduce of patients for sampling in this research. Ramezani R, Ataee R A, design the experimental protocol and the laboratory experiments have carried out and data Collection, and prepared the manuscript. Authors have read and approved the manuscript.

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