

## Original Article

# Identification of Anaerobic Bacteria in patients with Rheumatoid Arthritis

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## Abstract

**Background & Objective:** There is a report about the 2-5% prevalence for septic arthritis by anaerobic bacteria. The relevance between synovitis and intestinal microbial flora has been a hypothesis for ten years. The PCR with sensitivity and specificity 99% for microorganism detection in acute, chronic and relapse forms of septic arthritis is helpful. In this research we aimed to diagnose intestinal anaerobic bacteria which are able to occur in bacteremia or septicemia.

**Methods:** The amplification of the 16srRNA and narG genes of anaerobic bacteria was carried out by three PCR reactions. Here we used universal primers for their detection.

**Results:** From 100 patients with septic arthritis, 61% were bacterial arthritis and 18% were infected by anaerobic bacteria.

**Conclusion:** On the base of nitrate reductase coding, the positive samples were identified as *Enterobacter cloacae* and *Methylovorus* sp. O157 *Escherichia coli* and *Borrelia garinii*.

**Keywords:** Septic arthritis, anaerobic bacteria, PCR, NarG

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## Introduction

The 1987 American College of Rheumatology (ACR) criteria for the classification of rheumatoid arthritis (RA) is the current standard for identifying subjects with RA for research studies. The greater incidence of RA among females, which is more apparent before menopause, suggests an influence of reproductive and hormonal factors. Classic epidemiologic evidence of an infectious etiology for RA has not been forthcoming. Incident cases of RA do not cluster in space or time. However these observations cannot rule out infection

as a potential cause. It is conceivable that a ubiquitous infectious agent may be responsible for the disease in a genetically susceptible host<sup>1</sup>. Clinical studies of RA and periodontal disease have provided evidence for a significant association between the two disorders. High levels of oral anaerobic bacterial antibodies have been found in the serum and synovial fluid of RA patients. *Porphyromonas gingivalis*, *Tannerella forsythensis*, and *Prevotella intermedia* have been identified in RA synovial fluid. They are gram negative bacteria and are considered to be directly responsible for human infections<sup>2</sup>. The most common etiologic organisms

observed in these earlier reports were *Enterobacteriaceae*, usually *Proteus mirabilis*, *Escherichia coli*, *Serratia marcescens*, and the *Salmonellae*; the *Pseudomonadaceae* were infrequently noted<sup>3</sup>.

Anaerobes are the predominant components of the normal human skin and mucous membranes bacterial flora<sup>1</sup> and, therefore, are a common cause of bacterial infections of endogenous origin. Because of their fastidious nature, these organisms are difficult to isolate from infectious sites and are often overlooked. Their exact frequency is difficult to ascertain because of the inconsistent use of methods for isolation and identification. The lack of adequate therapy against these organisms may lead to clinical failures. DNA-based techniques, hybridization probes, polymerase chain reaction (PCR)-based techniques and protein detection by mass spectroscopy provide quick results<sup>4</sup>. Treatment of anaerobic infection is complicated by the slow growth of these organisms, by their poly microbial nature and by the growing resistance of anaerobic bacteria to antimicrobials.

*Escherichia coli* can respire anaerobically by using a number of alternative terminal electron acceptors, such as nitrate, nitrite, dimethyl sulfoxide, trimethylamine-N-oxide, and fumarate, in order to generate energy by electron transport-linked phosphorylation reactions. Dissimilatory denitrification refers to the respiratory reduction of nitrate and/or nitrite to di-nitrogen via nitric oxide and nitrous oxide. This alternative anaerobic process of energy conservation is phylogenetically widespread in bacteria<sup>5</sup>. Furthermore, regarding the nitrate reductase encoded by *narG* it was recently shown that the *narGHJI* operon encodes the major respiratory nitrate reductase located in the cytoplasmic membrane<sup>6</sup>. In this research we designed the diagnosis of bacterial infections and anaerobic bacterial infections of rheumatoid arthritis. We have performed it from 16srRNA and *narG* gene amplification by universal primers in synovial fluid samples of Iranian patients with rheumatoid arthritis.

## Methods

**Samples:** Synovial fluid samples were collected from cases with infectious joint which referred to Taleghani,

Emam Khomeini, Shariati, Akhtar Hospitals of Tehran. This group consisted of 100 men and women without any contamination from an intra-articular injection or a penetrating wound who referred to physician for treatment. After the diagnosis process, synovial fluid samples were collected and sent to Cellular and Molecular Biology Research Center.

### Primer designing:

We used the *16srDNA* and *narG* fragments of bacteria in NCBI genbank. After comparison between these sequences, we could obtain two groups of the most similar ones. The universal primers for *16srDNA* and semi-universal ones for *narG* fragments of bacteria were designed by gene-runner software. The amplified products were 1400bp for *16srRNA* and 300-2000 bp for *narG* gene in anaerobic bacteria.

Table1: Universal and Semi-universal primers used in this research

Primer name	Primer seq.	Base pairs	Annealing T. °C
16srRNA	F 5'gtttgatcmtggctcag 3' R5' aaggagggtwtccarcc 3'	1400 bp	60
Group one	F1 5' atattccattacca 3' R1 5' cctgctgtacacg 3'	300-2000bp	40
Group two	F2 5' taccgatgatgcgca 3' R2 5' tcgectcgacggtgctc 3'	300-2000bp	40

The bacteria contain *narG* gene were grouped by similarity of gene sequence as follow:

Group one: *Klebsiella pneumonia* subsp. *rhinoscleromatis*, *Corynebacterium efficiens* YS-314, *Lactobacillus antri* DSM 16041, *Nakamurella multipartite* DSM 44233, *Alcanivorax borkumensis* SK2, *Staphylococcus epidermidis* M23864, *Lactobacillus reuteri* MM2-3, *Catenulispora acidiphila* DSM 44928, *Borrelia garinii*, *Escherichia coli*

Group two: *Staphylococcus aureus* subsp. *aureus* MRSA252, *Ralstonia solanacearum* GMI1000;H, *Desulfobacterium autotrophicum* HRM2, *Mycobacterium tuberculosis* H37Rv, *Mycobacterium avium* subsp. *paratuberculosis* K-10, *Brucella abortus* bv. 6 str.870, *Enterobacter cloacae*, *Methylovorus* sp.

### DNA Extraction and Isolation of bacterial samples by PCR:

DNA was extracted from Synovial fluid samples using phenol chloroform as previously described<sup>(7)</sup>. PCR reaction was carried out by *16srRNA* primers (Table1).

The suspected DNA samples were amplified by *I6srRNA* universal primers. PCR reaction included 1µg of DNA, 150 µM dNTP, 4 pmol each of forward and reverse primers, 1.5 mM MgCl<sub>2</sub>, 1X PCR buffer and 1.25 units of Taq DNA polymerase in 30µl final volume. PCR amplification was performed by following parameters: denaturing at 94 °C for 30sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec. These processes were repeated for 30 cycles. Reaction was settled at 94°C and 72°C for 5 min before and after PCR cycling, respectively.

#### Detection of samples with anaerobic agents:

The positive DNA samples were studied by semi-universal primers (table1). The amount of PCR reactions were like above and annealing temperature of primers was 40 °C.

#### Detection of PCR Product:

The 1400bp from *I6srRNA* and 300-2000 bp from *narG gene* PCR products were analyzed by electrophoresis on 2% agarose gel and DNA band was observed by UV Trans-illuminator after SyBr green staining in both steps. Statistical analysis of the results was performed with SPSS software by Anova method.

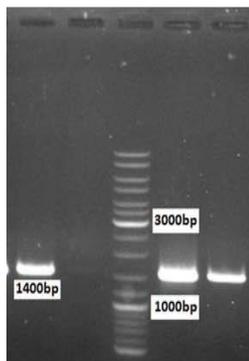
## Results

### Morphology of scaffolds

DNA samples obtained from synovial fluid were analyzed by PCR.

#### 1- Detection of bacterial infection in the samples:

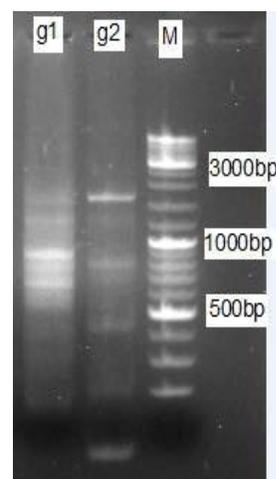
This part of diagnosis was carried out by *I6srRNA* gene amplification in infectious samples (fig.1).



**Figure1:** 1400bp PCR product of *I6srRNA* against of 100bp DNA ladder

#### 2- Detection of anaerobic bacterial infection in the positive samples:

The positive samples were analyzed by *narG gene* amplification (fig.2).



**Figure2:** PCR products of Synovial samples DNA by g1 and g2 primers (g1, g2), 100bp DNA ladder (M)

#### 3- Sequencing results of PCR products:

The PCR products of positive samples in group two was similar to *Enterobacter cloacae* and *Methylovorus* sp. but *O157 Escherichia coli* and *Borrelia garinii* in group one were detected with 92% identity.

## Discussion

Anaerobes have rarely been reported as a cause of septic arthritis in children. In this study, 100 synovial fluid samples from patients with rheumatoid arthritis were analyzed. We reported 61% with bacterial infection and of whom 18% were anaerobic isolates by *narG* amplification. Another research by Feigin et al.<sup>8</sup> have reported two children with septic arthritis caused by clostridia, here we isolated 4 different bacteria from the synovial samples. They had similarities to *Enterobacter cloacae* and *Methylovorus* sp. and also *O157 Escherichia coli* and *Borrelia garinii* sequences in genbank with 92% identity.

The isolated bacteria can respire anaerobically by using a number of alternative terminal electron acceptors, such as nitrate, nitrite, dimethyl sulfoxide, trimethylamine-N-oxide, and fumarate, in order to generate energy by electron transport-linked phosphorylation reactions. This alternative anaerobic

process of energy conservation is phylogenetically widespread in bacteria<sup>5</sup>. Furthermore, regarding the nitrate reductase encoded by *narG* it was recently shown that the *narGHJI* operon encodes the major respiratory nitrate reductase located in the cytoplasmic membrane<sup>6</sup>.

*Borrelia garinii* and *Escherichia coli* can be anaerobic or microaerophilic<sup>9</sup>. *Methylovorus* sp. are strictly aerobic, gram-negative, asporogenous, non-motile rods that multiply by binary fission, are mesophilic and neutrophilic and synthesize indole-3-acetic acid and exopolysaccharide<sup>10</sup>. *Enterobacter cloacae* is a member of the normal gut flora of many humans and is not usually a primary pathogen. It is sometimes associated with urinary tract and respiratory tract infections<sup>11</sup>.

There are many reports from other countries about isolation of anaerobic bacteria from septic arthritis. Nelson and Koontz<sup>12, 13</sup> reported three patients of 219 with septic arthritis: one with *Clostridium novyi*, one with *Clostridium bifermentans*, and one with *Bacteroides funduliformis*. Sanders and Stevenson reviewed the literature published before 1968 of *Bacteroides* infections in children, and reported five patients, of whom two were their patients, and three were reported by others<sup>14</sup>. Fine gold<sup>14</sup> revealed 1236 joint infections involving anaerobic bacteria. The majority of these cases were reported from the pre-antimicrobial era, and the most common anaerobe was *Fusobacterium necrophorum*, which accounted for a third of the anaerobes recovered from these patients. Sternoclavicular joint infection due to *Prevotella oralis* was reported<sup>15</sup>. Hip arthritis due to *Fusobacterium necrophorum* was described after tonsillectomy in a 9 year-old boy<sup>16</sup>. *Propionibacterium acnes* is associated with arthritis in prosthetic joints<sup>17</sup>. Brook<sup>14</sup> studied 65 infected joints (patients aged 12 weeks to 79 years) for aerobic and anaerobic bacteria. Seventy-four organisms (1/1 isolates/ specimen), consisting of 67 anaerobic bacteria and seven facultative or aerobic bacteria, were isolated from 65 joint specimens. The predominant anaerobes were *P. acnes* (24 isolates), anaerobic cocci, *Bacteroides* sp, and *Clostridium spp.* Thus bacterial infections or normal flora can be arthritis agents.

Therefore universal and semi-universal PCR design can help on-time detection and diagnosis of infection agents like anaerobic bacteria.

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