Detection of Bacterial DNA in Peripheral Blood Mononuclear Cells of Patients with Reactive Arthritis

Bahaa E. Ibrahiem, Naglaa F. Abd El Haliem, Nashwa El-Khouly, Walaa F. El-Bazz, Mona Abd Al-Raoof, Zinab N. Said

Departments of Internal Medicine and Microbiology & Immunology, Faculty of Medicine (for girls), Al-Azhar University, Cairo, Egypt.

Reactive arthritis (ReA) is an infection-induced systemic illness characterized by an aseptic inflammatory joint involvement and occurring in genetically predisposed patients with a bacterial infection localized in a distant organ or system. We evaluated the possible role of bacterial infection in the etiology of reactive arthritis by testing peripheral blood monocytes (PBMCs) for the presence of prokaryotic 16S ribosomal RNA genes which are known as 16S rDNA. PBMCs were isolated from 40 patients with ReA and 10 healthy controls. Clinical, laboratory and radiological evaluations were carried out for all patients and controls. Bacterial DNA was extracted from the PBMCs and subjected to PCR amplification of the 16S rDNA gene followed by DNA sequencing and database comparative analysis. Bacterial DNA was detected in 16/40 (40%) patients and 3/10 (30%) healthy controls. No significant difference was detected between the PCR +ve and -ve groups of patients as regards arthritis, arthralgia, sacroiliitis, low back pain and enthesopathies (P >0.05), while a significant difference was detected in the PCR +ve females with gynaecological infection (P<0.05). A significant difference of the pattern of arthritis was also observed between the two groups of patients. Comparative analysis of 7 16S rDNA sequences from patients and controls using Basic Local Alignment Search Tool (BLAST) and the National Center for Biotechnology Information (NCBI) database revealed high % similarity with potential pathogens and nonpathogenic bacteria. Further studies are needed to establish the exact role of these organisms in the pathogenesis of ReA.

Reactive arthritis (ReA) refers to an infection-induced systemic illness, characterized by an aseptic inflammatory joint involvement and occurring in a genetically predisposed patients with a bacterial infection localized in a distant organ or system (Flores et al., 2003). Although reactive arthritis is considered a sterile arthritis, accumulated evidence has shown that bacterial degradation products and even bacterial DNA are present in the synovium of patients with this disease (Colmegna & Espinoza, 2005).

Chlamydia trachomatis (C. trachomatis) is found in 50% of patients who develop ReA with preceding urogenital tract infection and occurred in 0-15% of patients after infection with Salmonella, Shigella, Campylobacter, or Yersinia (Hannu et al., 2002). It is often stated that susceptibility to ReA is strongly associated with human leukocyte antigen-B27. HLA-B27 is present in 72-84% of patients with ReA (Townes et al., 2008).

The classic syndrome of ReA is a triad of symptoms, including urethritis, conjunctivaitis, and synovitis; Reiter's syndrome, however, the majority of patients do not present with this classic triad (Carter & Hudson, 2009).

Macrophages and Monocytes seem to be likely candidates for transportation of bacterial DNA to the joint where it may act as immunostimulant and contribute to disease pathogenesis (Sibilia & Limbach, 2002). The pathogenesis of ReA follows a primary extraarticular infection and is characterized by an immune-mediated synovitis with intraarticular persistence of viable but nonculturable bacteria and/or immunogenic bacterial antigens (Townes, 2010). This pathogenesis involves the modification of host cells by pathogen-associated molecular
patterns (PAMPs), e.g. lipopolysaccharide, bacterial effector proteins, the adaptive immune system, and the genetic background, and up to 30% of the patients develop chronic symptoms (Rihl et al., 2006).

16S ribosomal RNA (or 16S rRNA) is a component of the 30S small subunit of prokaryotic ribosomes, and the genes coding for it are referred to as 16S rDNA. The prokaryotic 16S rRNA genes consist of widely conserved regions and highly variable regions. The nucleotide sequences in the divergent region provide distinctive characteristics that has been extensively used by the researchers for bacterial identification and found to have discriminatory power. And this unique character can be detected by molecular biology (Maity et al., 2008).

The aim of this work is to evaluate possible role of bacterial infection in the etiology of reactive arthritis by detecting bacterial DNA in peripheral blood monocytes (PBMCs) using PCR targeting the prokaryotic 16S rDNA genes.

Patients and Methods

Patients

Patients were selected from the out-patients clinic of the Internal Medicine Department, Al-zahraa University Hospital, Cairo. The study population were all seronegative for rheumatoid factor (RF) and antinuclear antibodies (ANA), have mono, oligo or polyarthritis or arthralgia undiagnosed definitely, their age ranged between 18-60 years, and have positive history of preceding infection; urinary tract infection (UTI), diarrhea and gynecological infection, 1-2 months preceding the symptoms. All patients were subjected to full clinical examination of musculoskeletal system, laboratory and radiological investigations.

Methods

All patients and controls were subjected to venipuncture under complete aseptic conditions; 5cc blood was collected from each, divided into two aliquots; one for routine laboratory investigations including complete blood picture (CBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), blood sugar, blood urea, serum creatinine, liver enzymes, bilirubin and alkaline phosphatase. Rheumatoid factor (RF) by rose wailer test and antinuclear antibody (ANA) were also, evaluated.

The other aliquot was received in EDTA screw capped sterile tube for isolation of peripheral blood monocytes (PBMCs) by Ficoll-Hypaque method (Boyum, 1968).

- PCR amplification

Bacterial DNA was extracted from 200 µl of PBMCs using commercially available kit (QIAamp Extraction kits, QIAGEN - USA) (Catalogue No. 51104). Then 5 µl of the extracted DNA was subjected to the detection of bacterial 16S rDNA according to Maity et al., (2008). The PCR total reaction volume was 50 µl containing 50 pmole of each primer and 25 µl of Taq PCR Master Mix (QIAGEN - USA). Primers were selected from highly conserved region of the 16S ribosomal gene according to Maity et al., (2008); forward primer was: 515F 5′-GTGCCAGCAGCGGGTAAT-3′ (20) and reverse primer was: 1390R 5′-AGGCCCGGAACGTATTCACC-3′ (20) based on complete 16S rRNA gene sequence of E. coli.

Amplification conditions were 95°C for 10 minutes for one cycle, 95°C for 30 seconds, 64°C for 1 minute, 72°C for 30 seconds for 30 cycles and a final extension step of 72°C for 7 minutes after the last cycle in an automated thermal cycler (T1 - Biometra – Germany).

The PCR DNA products were separated by 1.5% agarose gel electrophoresis stained with 5µg/ml ethidium bromide and visualized using UV trans-illuminator. The size of amplified DNA was determined using molecular DNA marker, 100-1500 bp, (0.25 µg/µL) (GmbH-Germany). The expected size for the amplicon was 860 bp (Figure-1). Full precautions were taken to prevent contamination and standard bacterial strains (Eschericia Coli - ATCC.
DNA sequencing was carried out for seven PCR positive samples using the same prokaryotic 16s rDNA specific primers. PCR products were purified using QIAquick Purification kits, QIAGEN - USA. Cycle sequencing PCR reaction was performed according to Cuchacovich et al, (2002) by (Big Dye Terminator v3.1 Cycle Sequencing Kit – USA). Using Centri-Sep™ spin columns (Applied Biosystems) (Catalogue No. 401763) for cycle sequencing products purification, and automated sequencer (ABI PRISM 310 Genetic Analyzer – USA) for sample electrophoresis and analysis. Comparative analysis was performed by Basic Local Alignment Search Tool (BLAST application) to determine the possible bacterial species of the seven sequences by calculating % similarity with bacterial sequences of reference strains in the National Center for Biotechnology Information (NCBI) database.

Statistical Analysis
Statistical Package for Social Sciences (SPSS / version 15) software was done. Results were expressed by arithmetic mean and standard deviation (±SD). Comparisons were performed using “t” test, Chi-square (X²) and Z test. P value < 0.05 was considered significant.

Results
Fourty patients with arthritis or arthralgia were included in this study. Their age ranged between 15 to 55 years, with mean (±SD), 35.48 (±9.95) years. The disease duration ranged between 1.5 to 48.0 months with mean (±SD), 9.22 (10.3) months. Thirty four patients were females. Ten healthy volunteer control subjects of comparable age and sex were included.

Sixteen out of fourty patients (40%) were positive for bacterial 16S rDNA and three out of ten healthy controls (30%) were also positive (Figure 1).

Figure 1. Ethidium bromide stained 1.5% agarose gel electrophoresis showing: MW DNA marker of 100 bp ladder in lane 1, positive and negative controls in lane 2&3, respectively, PCR- positive samples for bacterial 16S rDNA in PBMCS in lanes: 5, 7, 8 and PCR-negative samples in lanes: 4, 6 & 9. The expected size for the amplicon is 860 bp.
Based on PCR results, patients were divided into two groups, group A who were positive for bacterial 16S rDNA in PBMCs and group B who were bacterial 16S rDNA negative. Both groups A and B patients were compared as regard: age, sex and disease duration, where no significant difference was detected between both groups \((P > 0.05)\).

As regard the history of preceding infection, urinary tract infection (UTI) was the commonest in both groups; 7/16 (43.8%) in group A and 11/24 (45.8%) in group B, with no significant difference between both groups \((P > 0.05)\), while history of gynaecological infections was found in 15/34 (44%) female patients; 7/15 (46.6%) in group A and 8/19 (42%) in group B with no significant difference between both groups \((P > 0.05)\) (Table 1).

<table>
<thead>
<tr>
<th>History of preceding infections</th>
<th>Patients with ReA N=40</th>
<th>Group A &quot;n=16&quot;</th>
<th>Group B &quot;n=24&quot;</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary tract infections</td>
<td>18 (45%)</td>
<td>7 (43.8%)</td>
<td>11 (45.8%)</td>
<td>NS</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>7 (15%)</td>
<td>2 (12.5%)</td>
<td>5 (20.8%)</td>
<td>NS</td>
</tr>
<tr>
<td>Gynecological* infections</td>
<td>15 (44%)</td>
<td>7 (46.6%)</td>
<td>8 (42%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Total number of female patients = 34/40; 15 in group A and 19 in group B.

History of gynaecological infections = 15/34; 7/15 in group A and 8/19 in group B

\(P < 0.05\) is significant. NS = not significant

On the other hand, clinical examination of patients of group A showed that 12/16 (75%) had arthritis and 4/16 (25%) had arthralgia, 3 (18.8%) with enthesopathies and one (6.3%) with sacroiliitis. Also, in group B, 18/24 (75%) had arthritis and 6/24 (25%) had arthralgia, 3 (12.5%) patients with sacroiliitis and 3 (12.5%) with enthesopathies. Enthesitis was presented in 5/16 (31.3%) in group A and 11/24 (46%) in group B. Low back pain was presented in 7/16 (43.8%) in group A and 13/24 (54%) in group B, with no significant difference between both groups, \((P > 0.05)\).

Pattern of arthritis showed that asymmetrical oligoarthritis or arthralgia was the commonest pattern in both groups, it was presented in 11/16 (68.5%) patients of group A and of 18/24 (75%) patients of group B. The most common affected joint in both groups was the knee joint in 12/16 (75%) patients of the group A and 18/24 (75%) patients of group B, while ankle was affected in 10/16 (65%) patients of group A and 16/24 (66.7%) patients of group B, with no significant difference between both groups, \((P > 0.05)\).

Evaluation of frequency of associated clinical symptoms in both groups showed that gynaecological infections (vaginitis or cervicitis diagnosed by gynecologists) were the commonest. It was found in 15/34 (44%) females patients; 8/15 (53.3 %) in group A and 7/19 (36.8%) in group B with significant difference between both groups \((P < 0.05)\). Followed by dysurea 7/16 (43.8) in group A and 10/24 (42%) in group B (Table 2).
Table 2. Major clinical findings in patients with reactive arthritis.

<table>
<thead>
<tr>
<th>Clinical findings</th>
<th>Patients with ReA No=40</th>
<th>Group A &quot;n=16&quot;</th>
<th>Group B &quot;n=24&quot;</th>
<th>*P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Dysurea</td>
<td>17</td>
<td>42.5</td>
<td>7</td>
<td>43.8</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Gynecological infections</td>
<td>15</td>
<td>44</td>
<td>8</td>
<td>53.3</td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td>4</td>
<td>10</td>
<td>1</td>
<td>6.3</td>
</tr>
<tr>
<td>Mouth ulcer</td>
<td>1</td>
<td>2.5</td>
<td>1</td>
<td>6.3</td>
</tr>
</tbody>
</table>

*Total number of female patients = 34/40; 15 in group A and 19 in group B
Gynaecological infections = 15/34; 8/15 in group A and 7/19 in group B
* *P < 0.05 is significant. NS= not significant.

Routine laboratory investigations including ESR, CRP, Hemoglobin (Hg), blood sugar, blood urea, serum creatinine, liver enzymes (AST & ALT), uric acid showed no significant difference between both groups A & B (*P > 0.05). Patients of both groups were negative for rheumatoid factor (RF), and Anti-Nuclear antibodies (ANA).

Radiological and imaging studies of the affected joints showed abnormalities in majority of patients 25/40 (65.5%) with no significant difference between both groups (*P > 0.05), but significant difference was present in Plain X-ray of the sacroiliac, lumbosacral and cervical joints (*P < 0.05) (Table 3).

Table 3. Radiological and imaging data in patients with reactive arthritis

<table>
<thead>
<tr>
<th>Types of radiological studies</th>
<th>Patients with ReA No=40</th>
<th>Group A &quot;n=16&quot;</th>
<th>Group B &quot;n=24&quot;</th>
<th>*P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>X- Ray of the affected joints</td>
<td>9</td>
<td>22.5</td>
<td>4</td>
<td>25.0</td>
</tr>
<tr>
<td>Pelvis X-Ray AP (for sacroilitis)</td>
<td>5</td>
<td>12.5</td>
<td>1</td>
<td>6.3</td>
</tr>
<tr>
<td>Dorsolumbosacral spine AP &amp; Lateral</td>
<td>6</td>
<td>15</td>
<td>2</td>
<td>12.5</td>
</tr>
<tr>
<td>MRI, CAT scan of lumbosacral and cervical X-ray</td>
<td>5</td>
<td>12.5</td>
<td>4</td>
<td>25.0</td>
</tr>
</tbody>
</table>

* *P < 0.05 is significant. NS=not significant
Sequencing analysis of positive bacterial 16S rDNA of five patients and two controls revealed that two patients and one control subject had potentially pathogenic organisms; *Stenotrophomonas* and *Pseudomonas* respectively. Also, DNA products from environmental bacteria previously detected in arthritis as *Proteobacterium* and *Pantoea* species were detected in two patients and one control subject respectively (Table 4).

Table 4. Bacterial species identified by sequencing of the 16S rDNA genes.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Gene bank Accession number</th>
<th>Length of sequence</th>
<th>% similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Uncultured gamma proteobacterium</td>
<td>FJ659124.1</td>
<td>406bp</td>
<td>94%</td>
</tr>
<tr>
<td>2 Uncultured alpha proteobacterium</td>
<td>AY822200.1</td>
<td>756bp</td>
<td>83%</td>
</tr>
<tr>
<td>3 Uncultured bacterium Toolik clone</td>
<td>DQ509208.1</td>
<td>761bp</td>
<td>94%</td>
</tr>
<tr>
<td>4 Stenotrophomonas maltophilia</td>
<td>HQ166115.1</td>
<td>1445bp</td>
<td>82%</td>
</tr>
<tr>
<td>5 Stenotrophomonas maltophilia</td>
<td>AJ131914.1</td>
<td>1305bp</td>
<td>86%</td>
</tr>
<tr>
<td>6 Pseudomonas fluorescens</td>
<td>AY900171.1</td>
<td>1435bp</td>
<td>79%</td>
</tr>
<tr>
<td>7 Pantoea agglomerans</td>
<td>AY920524.1</td>
<td>761bp</td>
<td>76%</td>
</tr>
</tbody>
</table>

N.B: Samples 1-5 were from patients, while 6 & 7 were from control subjects.

**Discussion**

Reactive arthritis (ReA) is a broad term applied to a group of sterile arthritic processes that occur in association with distant corroborated infection and no cultureable bacteria in the affected joints. Thus, there are no universally accepted diagnostic or classification criteria for ReA and case definitions vary widely from one study to another (Townes et al., 2010). ReA might be presented with different clinical pictures. Classical presentation was rare (Townes et al., 2010).

In this study, majority of patients were females with no significant difference in age. Women and men are equally likely to develop ReA in reaction to food-borne infections. However, men are nine times as likely as women in response to sexually transmitted bacteria (NIAMS, 2011).

Kuipers et al., (2003) emphasized that in *Chlamydia trachomatis*-induced arthritis (CIA), infection of the urogenital tract (UGT) is often asymptomatic and it is thought that from the entry site, *Chlamydia trachomatis* is disseminated within monocytes by way of the blood stream into the joints. In accordance, (Colmegna & Espinoza., 2005), also reported that monocytes are the cells that transport the enteric pathogens from the entry site to the joints where *Yersinia* and *Salmonella* bacterial antigens, and only in one report *Enterobacter* DNA have been found.

Maity et al., (2008) illustrated that in PCR based approach, species-specific primer pairs
are used for generation of unique PCR product specific to a particular bacterium. Combining different primers for different species in a multiplex reaction is practically impossible for routine clinical analysis of bacterial diseases because the clinical specimens may contain multiple pathogens. For this, a multiplex PCR has to be standardized containing a large number of primers set or several individual PCR must be run to identify each possible pathogen present in the clinical specimen. Siala et al., (2009) reported that running a multiplex PCR with large number of species-specific primer pairs is a difficult exercise as the optimization of all the primer pairs to anneal at a single annealing temperature is a difficult task, moreover as the number of primers increases in the reaction chances of cross reactivity also increases. Multiple individual PCR reaction could be the alternative but the expenses and complexity of the assay increases. The individual PCR also increases the assay time and thus the diagnosis becomes critical (Townes et al., 2010). To avoid all these possible problems, thus, we make a single PCR with a universal set of primers for 16s rDNA to amplify a particular segment of DNA from any bacterium present followed by sequencing and database gene bank searching to identify the species. This is to evaluate possible role of bacterial infection as a cause of undiagnosed arthritis and PBMCs as a transportation vehicle.

This study showed that the possible triggering agents were detected in PBMCs in 40% of the studied patients. This was in accordance to the reported study of Ozgul et al., (2006) who detected bacterial DNA in PBMCs in 42.9% of patients of their study. Also, Frendler et al., (2001), were reporting detection of bacterial DNA in PBMCs in 50% of ReA patients.

Also, Siala et al., (2009) found that PCR was positive in 20 out of 27 patients (74.10%) indicating the presence of bacterial 16S rDNA within the synovial samples. It is on note that, PCR was positive in five out of five (100%) patients with ReA, nine out of nine (100%) patients with undifferentiated arthritis (UA), three out of seven (43%) patients with rheumatoid arthritis (RA) and three out of six (50%) osteoarthritis (OA) patients.

Zeidler et al., (2004) studied 83 patients with oligoarthritis of undetermined origin to identify asymptomatic infections potentially triggering the inflammatory response in the synovial fluid. 57/83 (69%) of the patients with oligoarthritis and 4/20 (20%) of the control subjects were carriers of clinically silent infections. Also, our results showed that bacterial DNA was detected in PBMCs in 30% of control subjects.

The frequency of joints affection showed that the knee joint was the commonest joints affected (75%) in both groups, followed by ankle joint (62.5%) in group A vs. (66.75%) in group B, with significant differences between both groups. This was in agreement with Ozgul et al., 2006 and Eberl et al., 2003. Also, the least joint affected was hip joint (6.25%) in patients with positive bacterial 16S rDNA, this was in agreement with Eberl et al., (2003).

On the other hand, sacroiliitis showed significant differences between both groups (18.8% in group A vs. 33% in group B). Also, Ozgul et al., (2006) reported sacroiliitis in (34.1%) patients with positive bacterial 16S rDNA, this was in agreement with Eberl et al., (2003).

Regarding the pattern of arthritis in the present study, asymmetrical oligoarthritis was the commonest (68.7% vs 75%) in group A & group B respectively. This is similar to the results obtained by Rita et al., (2002) who found that asymmetrical oligoarthritis was the commonest arthritis pattern in patients with ReA (80%) and patients with other arthropaties (63.3%).

Back pain was 50% in all patients, (43.8% in group A vs. 54% in group B). These results
were in agreement with Ozgul et al., (2006) who found that (51.2 %) of the patients in their study of clinical presentations of chlamydial and non-chlamydial ReA complained of backache. Eberl et al., (2003) reported the percentage of back pain in his study (38%). Also, Rita et al., (2002) found that (45%) of reactive arthritis patients had low back pain and (46.7%) in other arthropathy.

Gynaecological infections (vaginitis or vulvo-vaginitis) were the commonest clinical manifestation. It was reported in (53.3%) and (36.8%) in group A & B respectively, with significance difference between both groups ($P < 0.05$). While, urinary tract infection (dysurea) was (43.8) in group A vs 10/24 (42%) in group B with no significant difference between both groups. Ozgul et al., (2006) reported that (40%) of their studied patients had genitourinary infection symptoms as urethritis, however, Eberl et al., (2003) reported urethritis and cervicits were the most common symptoms in patients with reactive arthritis (38.9%).

The X ray findings, of the sacroiliac joints was abnormal in (12.5%) of all patients, with significant differences between both groups (6.3% in group A vs. 17% in group B). This was in agreement with results obtained by Renisk et al., (2009) who had demonstrated sacroilitis in (10%) of their patients with early disease and eventually up to (70%) in patients with chronic reactive arthritis.

A detailed sequence analysis of the PCR-positive samples of ReA patients and controls revealed DNA of bacteria that have previously been described in human infections but not in arthritis, including: Stenotrophomonas maltophilia and Pseudomonas fluorescens. Also, DNA products from environmental bacteria previously detected in arthritis, such as Proteobacterium and Pantoea species were detected. DNA from bacteria so far not described in human infections such as uncultured bacterium Toolik clone was detected and no common organism was identified for development of ReA in the studied patients. This was in accordance to Cox et al., (2003), who found that numerous bacterial sequences in synovial fluid from reactive arthritis and postinfectious arthritis patients and most of these bacterial sequences were derived from gut and skin commensals. However, organisms known to be causative in the individual patients, such as C. trachomatis, were not identified by this approach.

Also, Siala et al., (2009) examined synovial tissues (ST) samples in patients of reactive arthritis and patients with undifferentiated arthritis—and found a mixture of bacterial DNA in both control and studied patients’ samples. Siala et al., (2009) recognized a wide spectrum of bacteria including those known to be involved in ReA. They reported DNA of bacterial species including Shigella species, Escherichia species, and other coliform bacteria as well as opportunistic pathogens such as Stenotrophomonas maltophilia and Achromobacter were shared in all arthritis patients. Also they found DNA from bacteria not described in human infections such as Bacillus niacini, Paenibacillus humicus, Diaphorobacter species and uncultured bacterium genera incertae sedis.

It could be concluded that careful history taking might explore preceding infection, where in this study history of urinary tract infection was the commonest (45%) followed by history of gynecological infections (44%). Detection of bacterial DNA in peripheral blood mononuclear cells (PBMCs) using bacterial universal primers and further sequencing for full bacterial identification, highlights the occurrence of a spectrum of bacteria which did not known to be involved in ReA and not previously associated with
Arthritis. As a typical ReA patient is previously healthy and seeks help for joint discomfort, the triggering infection may have passed unnoticed. In most cases, triggering the causative organism can be identified. However, further studies are needed to clarify the actual association between the presence of these bacteria and the development of ReA. Proper definition and evaluation of generally accepted criteria for diagnosis of ReA is urgently needed.

References


