Diagnosis of *Chlamydia trachomatis* in patients with reactive arthritis and undifferentiated spondyloarthropathy

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*Abstract*

Introduction: There is a paucity of information on the frequency of *Chlamydia trachomatis*-induced reactive arthritis (ReA) and undifferentiated spondyloarthropathy (uSpA) in India. In this study, arthritic patients suffering from ReA, uSpA, and rheumatoid arthritis (RA) were screened to investigate the presence of *C. trachomatis* infection in the synovial fluid (SF) or serum by molecular and non-molecular methods.

Methodology: A total of 76 arthritic patients with ReA (n = 16) and uSpA (n = 22) composed the study group while those with RA (n = 38) served as controls. The detection of *C. trachomatis* DNA was done by semi-nested PCR (snPCR) and nested PCR (nPCR) targeting two different genes of *C. trachomatis*, namely major outer membrane protein and plasmid, respectively. The presence of serum or SF immunoglobulin IgG and IgA antibodies against *C. trachomatis* was studied by commercial enzyme-linked immunosorbent assay kits.

Results: The SF from 9 of 38 (23.6%) patients (5 with ReA and 4 with uSpA) was positive for at least one *C. trachomatis* DNA by snPCR or nPCR in comparison to RA (1/38 [2.6%]; p value < 0.05). There was no correlation between the snPCR or nPCR and the serological results of patients with ReA or uSpA.

Conclusions: As molecular diagnostic techniques established intra-articular *C. trachomatis* infection among this group of seronegative spondyloarthropathies in India, these findings should be viewed with concern, and snPCR or nPCR should be considered for a more reliable diagnosis.

*Key words:* Chlamydia trachomatis; reactive arthritis; undifferentiated spondyloarthropathy; synovial fluid


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*Introduction*

*Chlamydia trachomatis* is the most common sexually transmitted disease globally and has emerged as a major etiologic cause of Reiter’s syndrome and reactive arthritis (ReA) [1-5]. Intra-articular persistence of viable, although non-culturatable, *C. trachomatis* is considered to be the cause of arthritis [6]. Urogenital infection is often asymptomatic (in up to 80% of women and 40% of men), making treatment difficult [7]. Also, the intracellular localization creates an additional challenge for diagnosis of *C. trachomatis* in asymptomatic and in chronic or persistent infections where the pathogen load is low. In cases of chronic infection, *C. trachomatis* shows an unusual profile of gene expression that includes the attenuation and up regulation of certain set of genes. [5,8]. A recent study confirmed the presence of metabolically active chlamydiae during the remitting phase in synovial tissues from patients with chronic *C. trachomatis*-induced ReA [9].

Different techniques, such as immunofluorescence, electron microscopy, serology, and nucleic acid amplification tests, have been used to detect *C. trachomatis* infection in the synovial compartment of ReA patients, as the microorganism can rarely be cultured from the joint. As molecular tests serve as sensitive and specific tools for the diagnosis of *C. trachomatis* in joint samples, rRNA hybridization and, PCR have been used to detect several *C. trachomatis* components such as major outer membrane protein (MOMP), rRNA, and plasmid DNA in the affected joints of ReA patients. Published data on the use of PCR for the detection of intra-articular *C. trachomatis* varies between 0% and 100% positivity in patients with ReA and undifferentiated spondyloarthropathy (uSpA) [10-13]. Furthermore, serological tests can be helpful in establishing past or present chlamydial infections, although serology can never replace molecular methods of chlamydial infection detection. Also, in chronically infected patients in whom the
bacteria are no longer detectable locally in the synovium, a positive serological test may be the only indication of chlamydial involvement. In a study conducted in northern India, the prevalence of serum IgA antibodies to \textit{C. trachomatis} in patients with ReA with symptoms suggestive of genitourinary infection has been reported to be 25\% [14], while Aggarwal \textit{et al.} reported the prevalence of serum IgA antibodies to \textit{C. trachomatis} to be 14.2\% using enzyme-linked immunosorbent assay (ELISA) in uSpA patients [15]. As studies conducted on \textit{Chlamydia}-induced ReA are meagre in India and are based on serological detection of antibodies to \textit{C. trachomatis} [16], there is a need for a more reliable diagnosis of the pathogen by molecular methods. Also, as uSpA is a \textit{forme fruste} of ReA, and taken together, both ReA as well as uSpA constitute the seronegative spondyloarthropathy group, patients categorized as uSpA by a rheumatologist were also included in the study. Furthermore, despite a high prevalence of genital \textit{C. trachomatis} in our country [17-25], this pathogen appears to be overlooked in seronegative spondyloarthropathies such as ReA and uSpA in India. In order to find the magnitude of the problem, we investigated patients with ReA or uSpA in a case-control study by molecular and non-molecular methods for the intra-articular presence of \textit{C. trachomatis} MOMP, plasmid genes, and local or systemic anti-chlamydial IgA or IgG antibodies.

\section*{Methodology}
\subsection*{Patient selection}
After excluding patients who had septic arthritis, traumatic history, tubercular infection, and those with positive or abnormal findings on pyogenic bacterial culture, mantoux test, or differential cell count, 83 arthritic patients (ages ranging from 18 to 45 years) were enrolled in the study in consultation with the rheumatologist at the Department of Rheumatology and Clinical Immunology, Army Hospital (research and referral), New Delhi, India. European Spondyloarthropathy Study Group criteria [26] and American College of Rheumatology criteria [27] were followed for the enrolment of ReA/uSpA and rheumatoid arthritis (RA) patients, respectively. Of these, 7 patients did not provide consent for synovial fluid (SF) sampling; as a result, 76 arthritic patients (male-to-female ratio 32:44) were eventually included in the study.

The study had the permission of the hospital’s ethics committee. Informed written consent was obtained from each enrolled patient. The consent signed by the patient was specific with regard to the fact that treatment of the patient suffering from arthritis from whom synovial fluid was collected was in no way influenced by the outcome of the test performed.

The detailed history of each patient was recorded in a standardized questionnaire that included details of urogenital infection and treatment, namely NSAID, antibiotics and, steroids. Taken during the current or previous infection(s). Patients were age-matched and divided into two groups. Group I included patients with ReA (n = 16; mean age 34.2 years; male-to-female ratio 13:3) and uSpA (n = 22; mean age 30.7; male-to-female ratio 15:7), while Group II included RA patients (n = 38) as the inflammatory control group.

ReA was defined as asymmetrical lower-limb oligoarthritis preceded by a history of urogenital infection in the previous four weeks, while uSpA patients reported inflammatory backache, oligoarticular asymmetrical arthritis, enthesitis with or without radiological evidence of sacroiliitis, but did not fulfil the diagnostic criteria for any currently established diseases such as ankylosing spondylitis, psoriatic arthritis, reactive arthritis, or arthritis associated with chronic inflammatory bowel disease. RA was defined as bilateral, symmetrical, inflammatory polyarthritis affecting hand joints with symptom duration exceeding six weeks.

\section*{Collection of synovial fluid and blood}
Approximately 5-7 mL SF was collected under aseptic conditions by a rheumatologist in a sterile container, without the use of any anticoagulants. Also, 5 mL of non-heparinized blood was collected in sterilized vials. Sera was separated and stored at -20\°C for further use.

\section*{DNA isolation from synovial fluid}
DNA was extracted from the SF of patients in the study group (ReA/uSpA) and in the control group (RA) by a QIA Amp DNA Blood Mini kit (Qiagen, Hilden, Germany) per the manufacturer’s guidelines. Briefly, 500 \(\mu\)L of SF was centrifuged at 15,000 rpm for 10 minutes and cells were pelleted. Following this, 20 \(\mu\)L of protease was added, vortexed, and thereafter, lysis buffer was added and the mixture was kept at 56\°C in a water bath for 10 minutes. After precipitation with ethyl alcohol, DNA was washed twice and eluted. The quality, quantity, and purity of DNA in samples were determined by a nano-drop spectrophotometer (Fermentas-Thermo Fisher Scientific, Waltham, USA). DNA was stored at -80\°C.
Selection of C. trachomatis primers

The C. trachomatis-specific extra-chromosomal plasmid gene and the chromosomal MOMP gene were targeted for semi-nested or nested PCR (snPCR/nPCR) assays; the MOMP gene was amplified by snPCR, while the plasmid gene was amplified by nPCR. The sequences for primer pairs of MOMP [28] and plasmid [11,29] were taken from published literature for the commercial synthesis of primers.

Semi-nested polymerase chain reaction assay

SnPCR was performed to detect C. trachomatis MOMP, in which a 380 bp DNA fragment was amplified. The oligonucleotide primers were used to amplify a fragment within the conserved region of the MOMP gene of C. trachomatis. The target DNA was amplified using 0.5 μL each of 10 pmole/μL of the primers HP1 and HP2 [28] in 25 μL volume containing 2 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 0.2 mM dNTPs (Fermentas), and 2-5 units of DNA polymerase (Fermentas-Thermo Fisher Scientific, Waltham, USA). The optimum conditions for the reaction were as follows: 1 minute annealing at 45°C, 5 minutes extension at 72°C, and 1-5 minutes denaturation at 94°C for 35 cycles, with extension time increased to 10 minutes during the final cycle. The first round of PCR was performed with HP-1 and HP-2 primers, while the second round of PCR was performed with HP-2 and HP-3 primers [28] using 4 μL of PCR product from the first reaction as a template.

Nested polymerase chain reaction assay

Primers that amplify a plasmid sequence of 201 bp [29] were used for the first amplification. PCR was performed in a 25 μL reaction mixture containing 2.5 μL of 10X buffer containing 100 mM Tris-HCl, 50 mM KCl and nonidet P40 (Fermentas-Thermo Fisher Scientific, Waltham, USA), 1.5 μL of 25 mM MgCl₂, 0.5 μL of 2 mM of dNTP mix, 0.25 μL of Taq DNA polymerase (Fermentas-Thermo Fisher Scientific, Waltham, USA), 0.5 μL each of 10 pmole/μL forward (A) and reverse (B) primers [30], 3.0 μL of template DNA (200 ng per reaction), and 16.25 μL of distilled water. Initially, DNA was denatured at 95°C for 5 minutes and then for 30 seconds for each cycle, annealing was done at 57°C for 1 minute, extension was done at 45 seconds, and final extension was done for 10 minutes. The PCR was subjected to 35 cycles. Thereafter, 5 μL of each sample was transferred to another tube containing fresh reaction mixture and nested primers A’ and B’, which amplified 141 bp [11]. Samples were subjected to 35 cycles again under the same conditions except for the initial denaturation, which was done for 1 minute.

Detection of PCR products

The amplicons were detected by performing electrophoresis of amplified product in an ethidium bromide-stained 1.2% agarose gel. A 1-kilobase DNA ladder (Bangalore Genei, Bangalore, India) was used as the DNA size standard. The DNA was visualized on an UV Transilluminator and photographed.

Anti-C. trachomatis antibody detection by ELISA

ELISA was performed to detect circulatory IgG and localized IgA antibodies to C. trachomatis in arthritic patients. The presence of serum IgG and SF IgA antibodies to C. trachomatis was estimated using commercial ELISA kits purchased from IBL International (IBL, Hamburg, Germany) and Savoy Diagnostics (Savoy Diagnostics, Ashdod, Israel), respectively, following the manufacturers’ guidelines. As per the kit manual, IgG antibodies were calculated in units, and samples containing more than 11 units were considered to be positive, while for IgA, an absorbance value A450 of > 1.1 was considered to be positive.

Statistical analysis

Statistical analysis was performed for different variables with GraphPad Prism software version 5.0 (GraphPad Software, Inc., San Diego, USA). Patients with ReA/uSpA who had intra-articular C. trachomatis infection were compared with RA patients by Fisher’s exact test. A p value less than or equal to 0.05 was considered to be significant.

Results

General characteristics

ReA and uSpA patients had a mean age of 34.2 and 30.7 years, respectively. The male-to-female ratios were 13:3 (ReA) and 15:7 (uSpA). Mean disease duration was 15 months in ReA and 14.1 months in uSpA patients. The majority (71.05%) of ReA and uSpA patients (27/38) had oligoarthritis, while 23.6% (9/38) were categorized as patients with monoarthritis.
A total of 78.9% (30/38) ReA and uSpA patients had low backache in the study group, while 15.7% (6/38) had enthesitis in their small joints. Further clinical details of all patients (Groups I and II) have been summarized in Table 1.

**PCR results**

Overall, SF from 10 of 76 (13.1%) arthritic patients (5 with ReA, 4 with uSpA, and 1 with RA) were positive for at least one *C. trachomatis* DNA (MOMP/plasmid) by snPCR or nPCR. In Group I, 23.6% (9/38) of ReA and uSpA patients were positive for either *C. trachomatis* MOMP/plasmid by snPCR or nPCR. The *C. trachomatis* plasmid was found in 8 of the 38 patients (21.05%), while the *C. trachomatis* MOMP was found in 6 of the 38 patients (15.7%). Both plasmid and MOMP genes were found in 5 of the 38 patients (13.1%; 2 ReA and 3 uSpA) (Table 2). These patients were significantly more likely to be PCR positive compared to RA (1/38 [2.6%]; p < 0.05).

Five of the 16 (31.2%) ReA patients were positive for the plasmid gene, 2/16 (12.5%) for MOMP DNA, and 2/16 (12.5%) for both genes. *C. trachomatis* DNA was detected in 18.1% (4/22) of synovial samples from the uSpA group of patients. Three of the four (75%) positive uSpA patients were amplified by the plasmid PCR, 4/4 (100%) were positive for the MOMP gene, and 3/4 (75%) were positive for both genes (Table 2).

**Comparison between PCR and antibody results**

Serology results showed the presence of *C. trachomatis* IgG antibodies in 5 of 38 ReA and uSpA patients (13.1%). A total of 2/16 (12.5%) ReA patients were positive for *C. trachomatis* IgG antibodies, while *C. trachomatis* IgG antibodies were detected in 3 of 22 uSpA (13.6%) patients. Serum IgG antibodies against *C. trachomatis* were detected in 2 of 9 (22.2%) of the PCR-positive ReA and uSpA patients and in 3 of 29 (10.3%) PCR-negative samples.

Intra-articular *C. trachomatis* IgA antibodies were detected in the SF of 3/9 (33.3%) patients (in 6 of 16 ReA [37.5%] and in 3 of 22 [13.6%] uSpA). *C. trachomatis* IgA antibodies were found in the SF of 3 of 9 PCR-positive ReA and uSpA cases (33.3%) and in 6 of 29 (20.6%) PCR-negative samples (Table 3). However, none of the ReA and uSpA patients were positive for both serum IgG and SF IgA antibodies to *C. trachomatis*. Also, none of the RA patients had IgG or IgA antibodies to *C. trachomatis*.

### Table 1. Clinical and demographic details of arthritic patients (N = 76)

<table>
<thead>
<tr>
<th>Features</th>
<th>Reactive arthritis (n = 16)</th>
<th>Undifferentiated spondyloarthropathy (n = 22)</th>
<th>Rheumatoid arthritis (n = 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age ± SD (years)</td>
<td>34.2±8</td>
<td>30.7±9.2</td>
<td>36.6±7.7</td>
</tr>
<tr>
<td>Male : Female (%)</td>
<td>13 : 3 (81 : 19)</td>
<td>15 : 7 (68 : 32)</td>
<td>4 : 34 (10 : 90)</td>
</tr>
<tr>
<td>Urogenital symptoms (%)</td>
<td>16 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Disease duration ± SD (months)</td>
<td>15±9.9</td>
<td>14.1±9.8</td>
<td>36±16.4</td>
</tr>
<tr>
<td>Monoarthritis (%)</td>
<td>3 (18.7)</td>
<td>6 (27)</td>
<td>0</td>
</tr>
<tr>
<td>Oligoarthritis (%)</td>
<td>12 (75)</td>
<td>15 (68)</td>
<td>2 (5.2)</td>
</tr>
<tr>
<td>Polyarthritis (%)</td>
<td>1 (6.2)</td>
<td>1 (4.5)</td>
<td>36 (94.7)</td>
</tr>
<tr>
<td>Synovitis (%)</td>
<td>16 (100)</td>
<td>22 (100)</td>
<td>38 (100)</td>
</tr>
<tr>
<td>C-reactive protein ± SD (µg/mL)</td>
<td>38.1±35.5</td>
<td>29.6±16.1</td>
<td>27.5±24.6</td>
</tr>
<tr>
<td>Rheumatoid factor</td>
<td>0</td>
<td>0</td>
<td>38</td>
</tr>
</tbody>
</table>

### Table 2. Percentage PCR positivity of *C. trachomatis* in reactive arthritis (ReA) and undifferentiated spondyloarthropathy (uSpA) patients

<table>
<thead>
<tr>
<th>Patients (Group I)</th>
<th>snPCR (MOMP)</th>
<th>nPCR (Plasmid)</th>
<th>snPCR + nPCR (MOMP/Plasmid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ReA (n = 16)</td>
<td>2 (12.5%)</td>
<td>5 (31.2%)</td>
<td>2 (12.5%)</td>
</tr>
<tr>
<td>uSpA (n = 22)</td>
<td>4 (18.1%)</td>
<td>3 (13.6%)</td>
<td>3 (13.6%)</td>
</tr>
</tbody>
</table>

snPCR: semi-nested PCR; nPCR: nested PCR; MOMP: major outer membrane protein
Discussion

Genital chlamydial infection causes serious sequelae, one of which is chronic ReA. Reports indicate that approximately 5% of those with a genital chlamydial infection will develop acute ReA, and about half of these will proceed to chronicity [30]. Further, published data indicate that 20%–40% of individuals with inflammatory arthritis but no documented prior genital infection with C. trachomatis who are given a diagnosis of uSpA, oligo- or mono-arthritis are PCR positive in synovial tissue and/or fluid for C. trachomatis DNA [31,32]. A study published in 2009 reported that C. trachomatis DNA in ReA and uSpA patients is rather high (100% and 64%, respectively) [13], while other studies reported relatively low frequencies (25%) [11]. Thus, published data on the use of PCR for the detection of intra-articular C. trachomatis varies greatly in patients with ReA and uSpA [10,33]. Since no results are available on inter-laboratory variability, these differences cannot be simply attributed to varying prevalence, as they may reflect different sensitivities and specificities of the employed PCR.

There are reports suggesting the probability that the prevalence of C. trachomatis-induced ReA is underestimated in various parts of the world. As there are meagre published reports on this aspect from India, an attempt was made to determine the presence of intra-articular C. trachomatis DNA in ReA and uSpA patients by sensitive and specific molecular techniques such as snPCR and nPCR targeting chlamydial MOMP and plasmid genes by primers with good sensitivity [11,28,29] to establish the infection in arthritic patients (both symptomatic for urogenital symptoms as well as asymptomatic) having monoarticular or oligoarticular joint pain in their lower extremities. Patients with inflammatory backache and involvement of joints of the hand were also included in the study, and one RA patient was also diagnosed with Chlamydia infection. Extreme precautions were taken during sample collection and during processing of SF for molecular assays to avoid cross-contamination and false positive results. A total of 23.6% of ReA and uSpA patients were found to be positive for either the C. trachomatis gene in the joint fluid by snPCR or nPCR. It was observed that 31.2% of ReA patients were positive for the MOMP gene, while the plasmid gene of C. trachomatis was detected in 12.5% of ReA patients. The MOMP gene is generally considered to be attenuated during the chronic phase; however, in our study, the opposite was found. The presence of the MOMP gene in SF probably indicates that the transfer of this pathogen through the bloodstream is continuous, while the absence of the plasmid gene in some cases might be showing a plasmid-less variant. The latter has been reported previously from India [34] and might also be present in the SF of patients with ReA or uSpA. While uSpA patients are reportedly asymptomatic for any infection, we found C. trachomatis infection in 18.1% of such patients. However, although the mere intra-articular presence of C. trachomatis DNA does not prove a causal relationship, it provides a valuable clue with regard to the etiology of an undifferentiated oligoarthritis [35]. Once established in the joint, this pathogen does not progress through the normal life or developmental process occurring in the genital tract. Rather, it resides for long periods within monocytic cells in the synovial tissue and displays unusual metabolic characteristics, some of which contribute significantly to pathogenesis.

In our study, C. trachomatis-negative (by PCR) ReA and uSpA patients were also found to be positive for anti-C. trachomatis IgG/IgA antibodies in SF or serum. One reason for this discrepancy could be that such patients might have undergone an earlier short episode of urogenital infection leading to arthritis. The lack of correlation between the detection of intra-articular C. trachomatis DNA in patients with ReA or uSpA by snPCR or nPCR and the serological results has been observed by others [13,36].

The present study shows the presence of C. trachomatis infection in joint fluid by snPCR and nPCR methods in both ReA and uSpA patients in India. There is a definite need to further investigate C. trachomatis in this group of seronegative spondyloarthopathies. Also, the finding of the MOMP gene in our study is remarkable and needs further research, as it is reportedly either absent or else present in an attenuated form during chronic infection. As molecular diagnostic techniques established intra-articular C. trachomatis infection among both ReA and uSpA patients in India, this should be a cause for concern, and snPCR or nPCR should be considered for a more reliable diagnosis.

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References


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### Conflict of interests:  
No conflict of interests is declared.