



A Pilot Study for Detection of Intra-articular Chromosomal and Extra-Chromosomal Genes of *Chlamydia trachomatis* among Genitourinary Reactive Arthritis Patients in India

Kumar Praveen¹, Khanna Geetika², Batra Sumit², Sharma Vinod Kumar² and Rastogi Sangita^{1*}

¹Microbiology laboratory, National Institute of Pathology (ICMR), Safdarjung hospital campus, Post Box No. 4909, New Delhi-110 029, INDIA

²Central Institute of Orthopedics (CIO), Vardhaman Mahavir Medical College (VMMC) & Safdarjung hospital, New Delhi-110 029, INDIA

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Abstract

Chlamydia trachomatis is reportedly the most frequent sexually transmitted pathogen associated with genitourinary Reactive Arthritis (gReA) in developed countries. However, there is a paucity of reliable data on the intra-articular presence of *C. trachomatis* in seronegative spondyloarthropathies such as reactive arthritis from developing countries like India. As genital infection with *C. trachomatis* is fairly common in our country, it is quite possible that *C. trachomatis* is either underdiagnosed or else, underestimated in gReA patients. The aim of the present study was to investigate *C. trachomatis* in joint fluid of gReA patients by targeting *C. trachomatis*-specific chromosomal and extra-chromosomal genes by Polymerase Chain Reaction (PCR) assay. A case control pilot study was designed and carried out in collaboration with a tertiary care large government hospital in New Delhi (India) to calculate the frequency of *C. trachomatis* in gReA while patients with Rheumatoid Arthritis (RA) served as controls. Joint fluid was aspirated during arthrocentesis in 57 arthritic (gReA/ RA) age-matched patients. DNA was isolated using a commercially available kit (QIA Amp DNA Blood mini kit; Qiagen, Germany). PCR was performed using primers for *C. trachomatis* 16s-rRNA (150 bp) and plasmid (201 bp) genes. 7/25 (28%) gReA patients were found positive for plasmid/ 16s-rRNA gene while 3.1% (1/32) RA patients were also found to be *C. trachomatis*-positive. The presence of intra-articular infection with *C. trachomatis* in patients with gReA is cause of concern in Indian set-up. We recommend screening of larger number of gReA patients for determining the exact magnitude of *C. trachomatis* infection and to evaluate the effect of treatment on the persistent state of chlamydial infection in the synovium.

Keywords: *Chlamydia trachomatis*, genitourinary reactive arthritis, synovial fluid, polymerase chain reaction.

Introduction

Chlamydia trachomatis is the most widely prevalent sexually transmitted pathogen in the world including India¹⁻⁴. Infection with this agent is generally asymptomatic, making both diagnosis and treatment difficult. Undetected genital infections may evolve into complications such as ectopic pregnancy, pelvic inflammatory disease, salpingitis with tubal scarring and infertility in female patients while in men, arthritis and epididymitis may result in urethral obstruction and decreased fertility. Furthermore, in epidemiological studies reported largely from western countries, *C. trachomatis* has been implicated as the most common bacteria triggering genitourinary-induced Reactive Arthritis (gReA)⁵. In the case of *Chlamydia*-induced gReA, circulating monocytes/ macrophages are the common host cells for the persistent microorganisms during long term infection and recent articles further reveal that *C. trachomatis* is metabolically active during the remitting phase in synovial tissues from patients with chronic *C. trachomatis*-induced gReA⁶. The means by which persistently infecting chlamydiae engender pathology is poorly understood, although it is clear that they do elicit a strong immunopathogenic response.

Around 1% of patients with non-gonococcal urethritis and up to 3% of patients with bacterial enteric infection subsequently develop Reactive Arthritis (ReA)⁷. *C. trachomatis* infection accounts for 42 - 69% of patients with gReA in USA whereas in developing countries, enterically acquired ReA is more common⁸. A report from south India indicated *Shigella* and *Salmonella* to be responsible for triggering enteric ReA in 14% and 1.2% respectively⁹. According to Sinha et al¹⁰, *Salmonella typhimurium* accounts for one-third of cases in our community in contrast to *Yersinia* and *Chlamydia* in the west. Although the presence of serum anti-chlamydial antibodies has been reported from India in patients with gReA/ unclassifiable spondyloarthropathies^{11,12}, however, to the best of our knowledge, there appears to be limited data on the exact frequency of infection in the synovial fluid or membrane with this pathogen in patients with gReA. As there is reportedly high prevalence of genitourinary infection with *C. trachomatis* in our country, it is quite possible that *C. trachomatis* is underestimated and studies on intra-articular detection of chlamydial infection are needed to assess the magnitude of infection in gReA. In this regard, the use of accurate and reliable molecular diagnostic technique such as Polymerase Chain Reaction (PCR) assay should prove to be useful for

establishing chlamydial infection in the synovial compartment of gReA. Hence, the aim of this pilot study was to screen gReA patients by PCR targeting *C. trachomatis*-specific extra-chromosomal/ chromosomal genes in the joint aspirate.

Material and Methods

With the permission of hospital ethics committees and after taking informed written consent from each patient, 57 age-matched spondyloarthropathy (*viz.*: those with gReA and Rheumatoid Arthritis (RA) patients presenting at Central Institute of Orthopedics (CIO), Safdarjung hospital, New Delhi were enrolled for conducting a case-control pilot study. The detailed history of each patient was recorded in a standardized questionnaire which included details of urogenital infection and treatment, *viz.*: NSAID, antibiotics, steroids, etc. taken during the current/ previous infection. ESSG (European Spondyloarthropathy Study Group) criteria was followed for the selection of 25 gReA patients^{13,14} while age-matched RA patients (n= 32) were selected as controls following the American College of Rheumatology (ACR) criteria¹⁵. gReA was defined as asymmetrical lower limb oligoarthritis preceded by h/o urogenital infection in the previous four weeks. RA was defined as bilateral, symmetrical, inflammatory polyarthritis affecting hand joints and duration of symptoms exceeding six weeks.

Patients with other arthropathies like inflammatory bowel disease/ psoriasis/ systemic lupus erythematous/ ankylosing spondylitis/ preceding enteric infection, were excluded from the study. Patients found positive for other infections like tuberculosis/ viral infection were also excluded.

Collection of samples from arthritic patients: Approximately, 5-7 ml of Synovial Fluid (SF) was aspirated by the rheumatologist under aseptic conditions using local anesthesia. SF was transported on ice to the laboratory and centrifuged at x15,000 rpm. Subsequently, the cell pellet and the supernatant were collected and stored at -80°C for use in various assays.

DNA isolation from SF: DNA was extracted from the SF of patients by the QIA Amp DNA Blood Mini Kit (*Qiagen, Germany*). Briefly, 500 µl of synovial fluid was centrifuged at 15,000 rpm for 10 minutes and cells were pelleted. Following this, 20 µl 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 and quantity of DNA was checked by gel electrophoresis and nanodrop. Subsequently, the DNA samples were stored at -80°C.

Diagnosis of *C. trachomatis* in SF by PCR assays: Subsequent to DNA isolation, PCR assays were performed for the molecular diagnosis in the SF by targeting both 16srRNA and plasmid genes of *C. trachomatis*. The primers used in the PCR assays have been

shortlisted in table 1 and were synthesized by m/s Bioline, New Delhi (India).

Detection of *C. trachomatis* plasmid PCR: PCR was performed for the detection of *C. trachomatis* DNA (200 ng per reaction) in the SF. All DNA samples were screened for the presence of beta globin gene. The detection of *C. trachomatis* plasmid was done by targeting 201 bp genes in the SF DNA¹⁶. PCR was performed in 25 µl reaction mixture containing 2.5 µl of 10X buffer (containing 100 mM Tris-HCl, 50 mM KCl and non-idet P40 (*Fermentas*), 1.5 µl of 25 mM MgCl₂, 0.5 µl of 2 mM of dNTP mix, 0.25 µl of Taq DNA polymerase (*Fermentas*), 0.5 µl each of 10 pmole/ µl forward and reverse primers, 3.0 µl of template DNA 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.

Detection of *C. trachomatis*-specific 16s-rRNA: PCR was performed in 25 µl reaction mixture containing 2.5 µl of 10X buffer (100 mM Tris-HCl, 50 mM KCl and non-idet P40 (*Fermentas*), 1.5 µl of 25 mM of MgCl₂, 0.5 µl of 2 mM of dNTP mix, 0.25 µl of Taq DNA polymerase (*Fermentas*), 0.5 µl each of 10 pmole/ µl forward and reverse primers (150 bp), 3.0 µl of template DNA and 16.25 µl of distilled water. Initially, DNA was denatured at 95°C for 5 minutes and thereafter, for 30 seconds per cycle while annealing was done at 48°C for 1 minute. Extension was done at 45 seconds and the final extension was done for 10 minutes. The PCR was subjected to 35 cycles. The reaction was processed in a Gene Amp PCR system 9700 (*Applied Biosystems*) for 35 cycles¹⁷.

Detection of PCR products: The amplicons were detected by performing electrophoresis of the reaction mixture in an ethidium bromide-stained 1.2% agarose gel. 1-kilobase DNA ladder (*Bangalore Genei*) was used as DNA size standard. The DNA was visualized on an UV-Transilluminator and photographed.

Statistical analysis: Statistical analysis was performed with GraphPad Prism software (version 5.0; GraphPad Software, Inc., San Diego, Calif., USA). Fisher exact test were performed for different variables.

Results and Discussion

57 arthritic patients (*viz.*: gReA/ RA) were investigated for various *C. trachomatis* genes in the synovial aspirate. Among the study group (*i.e.* gReA), the range of age was 21 - 44 years, while the disease duration varied between 3 - 36 months with male: female ratio being 19: 6. The majority of gReA patients had oligoarthritis pattern of joint involvement while 11/25 (44%) had low backache and 2/25 (8%) had enthesitis in the small joints of hand. All *C. trachomatis*-positive ReA patients had raised levels of serum CRP. Further clinical details have been summarized in table 2 and table 3.

Table-1
List of Primer Sequences in PCR Assays

| <i>C. trachomatis</i> Genes | Reference Number | Primer Sequence (5'-3') | Amplicon Size |
|-----------------------------|------------------|--|---------------|
| Plasmid | 16 | 5'-TAGTAACTGCCACTTCATCA-3' 5'-TTCCCCTTGTAATTCGTTGC-3' | 201 bp |
| 16s-rRNA | 17 | 5'-TGAGGCATGCAAGTCGAAC-3' 5'-TTACTCGGATGCCCAAATATC-3' | 150 bp |

Table-2
Baseline and Clinical Details of Arthritic Patients (N=57)

| Characteristics of Arthritic Patients | Genitourinary Reactive Arthritis (n=25) | Rheumatoid Arthritis (n=32) |
|---|---|-----------------------------|
| Mean Age \pm SD (years) | 34.2 \pm 6.83 | 36.6 \pm 7.73 |
| Male: Female | | |
| • Number | 19: 6 | 6: 26 |
| • Ratio | 76: 24 | 19: 81 |
| Urogenital Symptoms | 25 (100) | 00 |
| Mean Disease Duration \pm SD (months) | 15 \pm 9.98 | 36 \pm 16.41 |
| Monoarthritis | 5 (20) | 00 |
| Oligoarthritis | 18 (72) | 2 (6) |
| Polyarthritis | 2 (8) | 30 (94) |
| Synovitis | 25 (100) | 32 (100) |

Note: Figures in parenthesis are percentages

Table-3
Clinical Profile of *Chlamydia trachomatis*-Positive Reactive Arthritis Patients (n=7)

| S. No. | Age/ Sex | Pattern of Arthritis | Disease Duration (months) | Urogenital Symptoms | Category of Effusion | Serum C-Reactive Protein (μ g/ml) | <i>C. trachomatis</i> Plasmid (201 bp) | <i>C. trachomatis</i> 16s-rRNA (150 bp) |
|--------|----------|----------------------|---------------------------|---------------------|----------------------|--|--|---|
| 1. | 32/ M | oligoarthritis | 12 | Present | severe | 83 | Absent | Positive |
| 2. | 22/ F | oligoarthritis | 03 | Present | severe | 75 | Absent | Positive |
| 3. | 28/ M | oligoarthritis | 36 | Present | severe | 62 | Positive | Positive |
| 4. | 38/ M | monoarthritis | 06 | Present | moderate | 37 | Positive | Positive |
| 5. | 21/ M | oligoarthritis | 03 | Present | moderate | 53 | Positive | Absent |
| 6. | 34/ F | oligoarthritis | 08 | Present | moderate | 31 | Positive | Absent |
| 7. | 29/ F | oligoarthritis | 03 | Present | mild | 62.5 | Positive | Absent |

7/25 (28%) ReA patients were found positive for *C. trachomatis* 201 bp endogenous plasmid gene and/ or 150 bp 16s-rRNA gene in the joint fluid sample. Among 7 ReA patients who were found positive for intra-articular *C. trachomatis* infection, 5/25 (20%) patients were positive for plasmid gene while 4/25 (16%) patients were positive for 16s-rRNA gene. Two ReA patients were found positive for both plasmid and 16s-rRNA genes of *C. trachomatis*. In control RA patients, 1/32 (3.1%) showed the presence of *C. trachomatis*. The findings in the study group of *C. trachomatis*-positive ReA patients were found to be statistically significant in comparison to the RA group (28% versus 3.1%; 'p' value < 0.02).

The sequelae of primary genital infection due to *C. trachomatis* are severe including not only chronic ReA¹⁸, but also dysfunction of temporo-mandibular joint¹⁹. The diagnosis of *C. trachomatis* in gReA has been attempted by various methods, viz: serology²⁰, immunohistochemistry and electron

microscopy²¹ and PCR²². However, serology is lacking in specificity and sensitivity in undifferentiated arthritis patients²³. Although, there is no established diagnostic criteria to detect *C. trachomatis* in ReA patients; but still molecular methods are considered to be the gold standard.

Metabolically active and viable chlamydiae persist in joints for long duration in gReA²⁴. These non-replicating microorganisms reside in the monocytes within the synovial compartment of gReA patients and have been reported to exist in typical aberrant chlamydial forms²¹. Recent data suggest that a prolonged combined antimicrobial treatment approach has the potential to eradicate persistent chlamydial infection.

In our country, the incidence of *C. trachomatis* in genital infections is high both in males and females and even asymptomatic patients have been found to be positive^{25,26}. However, there is a paucity of reliable data on the intra-articular

presence of *C. trachomatis* in seronegative spondyloarthropathies such as gReA. To the best of our knowledge, there are no published studies from India on the molecular diagnosis of this pathogen in gReA and only serological studies have been reported in patients with gReA/ unclassifiable spondyloarthropathies, showing the prevalence of anti-chlamydial antibodies varying between 14 - 25%^{11,12}. We targeted two *C. trachomatis*-specific genes, viz.: extra-chromosomal endogenous plasmid as well as chromosomal 16s-rRNA by PCR in the synovial fluid as tests to detect *C. trachomatis* in affected joints utilizing PCR have been gaining importance worldwide over the last few years. The results obtained in this study clearly indicate the intra-articular presence of both extra-chromosomal endogenous plasmid as well as chromosomal 16s-rRNA in 28% patients with gReA. Siala et al²⁷ have also shown that increased levels of detection can be achieved when PCR is performed using several targets.

Gerard et al reported that *C. trachomatis* genes are differentially expressed in active and persistent infections²⁸. Persistently infecting chlamydiae display an unusual profile of gene expression, with transcript levels from some genes attenuated and others upregulated²⁹. In our study, the absence of plasmid gene in two patients who were otherwise *C. trachomatis*-positive for 16s-rRNA gene, might probably indicate plasmid-less variants. The presence of plasmid-less strains of *C. trachomatis* has also been observed by other researchers³⁰. As is apparent from the present pilot study, there is a definite need for determining the exact magnitude of chlamydial infection in gReA.

Conclusion

The present study indicates an underestimation of *C. trachomatis* infection in gReA. This should be viewed with concern and larger number of such patients should be screened and treated at the primary stage of infection thereby preventing them from developing persistent chlamydial infection.

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