Studies on the Role of *Chlamydia trachomatis* Infection in the Pathogenesis of Reactive Arthritis / Undifferentiated Spondyloarthropathy

THESIS
Submitted in partial fulfilment
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DOCTOR OF PHILOSOPHY

by
PRAVEEN KUMAR

Under the Supervision of
Dr. Sangita Rastogi, M.Phil, Ph.D.

And Co-supervision of
Prof. Vishal Saxena, Ph.D.

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on the Role of *Chlamydia trachomatis* Infection in the Pathogenesis of Reactive Arthritis / Undifferentiated Spondyloarthropathy"** and submitted by **Mr. PRAVEEN KUMAR** ID No **2013PHXF108P** for award of Ph.D. of the Institute embodies original work done by him under my supervision.

Signature of the Supervisor
(Dr. SANGITA RASTOGI, M.Phil, Ph.D.)
Scientist 'F' & Deputy Director (Senior Grade)
National Institute of Pathology
(Indian Council of Medical Research)
Safdarjung hospital campus,
New Delhi, India

Date:

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

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Signature of the Co-Supervisor
(Prof. VISHAL SAXENA, Ph.D.)
Associate Professor
Department of Biological Sciences,
Birla Institute of Science and Technology,
Pilani, Rajasthan, India

Date:

“I want to dedicate this work to my belated beloved father, I miss you”.

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Abstract

Reactive Arthritis/ undifferentiated spondyloarthropathy due to *Chlamydia trachomatis* is largely asymptomatic hence get unnoticed further, it causes chronic infection in major joints of male and female patients. Role of *Chlamydia trachomatis* in the pathogenesis in ReA/ uSpA patients was studied by estimating the frequency of infection. Intra-articular infection for either *C. trachomatis* MOMP/ plasmid was found in 24.4% (11/45) ReA/ uSpA patients by either snPCR/ nPCR; plasmid and MOMP were found in 11/45 (24.4%) and 7/45 (15.5%) patients, respectively. 28.5% (6/21) ReA and 20.8% (5/24) uSpA patients were found positive for either MOMP/ plasmid gene of *C. trachomatis*. The plasmid gene was also detected by PCR (conventional) in the urine of 17.3% (6/35) uSpA patients without effusions. Cytological detection by DFA showed the presence of *C. trachomatis* EBs in the SF of 31.1% (14/45) ReA/ uSpA patients; while in the urine of uSpA patients without effusions, 13.3% (6/45) were *C. trachomatis*-positive. Although anti-chlamydial antibodies (IgM, IgG, IgA) were detected in SF/ serum of ReA/ uSpA patients, however, only anti-*C. trachomatis* IgA antibodies showed diagnostic importance (SF- 31.1%; 14/45 and serum 15.5%; 7/45). It was concluded that nPCR should be the method of choice for diagnosing intra-articular *C. trachomatis* DNA in SF of chronic ReA/ uSpA patients with effusion. DFA for an initial rapid detection of an acute episode of infection followed by the more confirmatory molecular diagnostic method. Overall, the SF was found to be a more conclusive clinical specimen in both acute and chronically *C. trachomatis*-infected ReA/ uSpA patients with effusion in terms of molecular diagnosis by nPCR, *C. trachomatis* MOMP antigen detection and anti-chlamydial antibody response.

The study further revealed that a non-invasive sample such as urine can also be used for molecular diagnosis of chlamydial infection in acute/ chronic ReA/ uSpA patients with effusion. Also, both clinical samples (serum, urine) can be used for diagnosis of chlamydial IgA antibodies and DNA, respectively in uSpA patients, in whom full blown symptoms are unapparent.

IgG antibodies to chsp60 were found in the serum of 28.5% (10/35) uSpA patients without effusion. Furthermore, levels of hsCRP were positively correlated with the COI of chsp60 IgG antibodies-positive uSpA patients without effusion, hence this finding delineated that both these inflammatory markers get enhanced simultaneously in *C. trachomatis*-positive uSpA patients; this association may prove to be useful as a diagnostic/ prognostic marker.

A pro-inflammatory response was evident in the SF of ReA/ uSpA patients with effusion. This was concluded on the basis of significantly increased IFN-gamma, IL-4 and IL-17 levels in the SF of these patients in comparison to OA patients (controls). IFN-gamma increases synergistically in SF and serum. Serum IL-6 was significantly enhanced in *C. trachomatis*-infected ReA/ uSpA patients with effusion in comparison to the uninfected ReA/ uSpA and controls (RA/ OA). Hence, it was concluded that serum IL-6 plays a role in the pro-inflammatory cascade while SF IL-6 was positively correlated with IFN-gamma. Moreover, in chsp60-positive uSpA patients without effusion, IFN-gamma and IL-17 were increased significantly in comparison to IL-6.

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Abbreviations

ACR	American College of Rheumatology
chsp60	*Chlamydia trachomatis* Heat Shock Protein-60
COI	Cut-off Indices
DFA	Direct fluorescence assay
EB	Elementary bodies
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESR	Erythrocyte Sedimentation Rate
ESSG	European Spondyloarthropathy Study Group Criteria
EtBr	Ethidium bromide
HLA	Human leucocyte antigen
hsCRP	High Sensitive C-reactive protein
MOMP	Major Outer Membrane Protein
NAAT	Nucleic Acid Amplification Test
nPCR	Nested PCR
OA	Osteoarthritis
OD	Optical Density
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
RA	Rheumatoid Arthritis
RB	Reticulate bodies
ReA	Reactive Arthritis
SF	Synovial Fluid
snPCR	Semi-nested PCR
TAE	Tris-acetate-EDTA
uSpA	Undifferentiated spondyloarthropathy

Chapter 1

INTRODUCTION

1.1 Introduction

Reactive Arthritis (ReA) is an inflammatory arthritis which belongs to the group of arthritidies known as the '*spondyloarthropathies*' **(Carter and Hudson, 2009).** The spondyloarthropathies include ankylosing spondylitis, ReA (including Reiter's syndrome), psoriatic arthritis, inflammatory bowel disease–associated spondyloarthropathy, and Undifferentiated Spondyloarthropathy (uSpA). The classic Reiter's syndrome is a triad of symptoms, including the urethra, conjunctiva, and synovium; however, the majority of patients do not present with this classic triad **(Parker and Thomas, 2000).** ReA usually begins one to four weeks after genitourinary or gastrointestinal tract infection. ReA patients may present with a mono-, oligo- or poly-arthritis, but oligoarthritis is the most typical pattern observed overall. Enthesitis is a common feature of reactive arthritis resulting from any etiologic agent. Other organs including the skin and mucous membranes are often involved **(Sieper, 2001; Inman *et al*., 2008).** The symptoms may persist for long periods and may cause long-term disability in some cases.

The causative microorganisms of ReA include either genitourinary *(Chlamydia trachomatis)* or enteric (*Shigella, Salmonella, Yersinia* and *Campylobacter)* pathogens. Many other organisms *Ureaplasma urealyticum, Mycoplasma hominis, Neisseria gonorrhoeae, Helicobacter pylori* have been implicated as potential causes of ReA. The majority of these are of single cases or small series of studies **(Carter and Hudson, 2009).** However, post-enteric and post-chlamydial ReA are distinct entities, in that viable and metabolically active microorganisms can be detected in the joints of patients with *Chlamydia*-induced ReA but not in those with post-enteric ReA **(Gracey and Inman,**

2012; Carter and Hudson, 2010; Gerard *et al.*, 2013). Infection with *C. trachomatis,* the most common agent of sexually transmitted disease, represents one of the most important causes of genitourinary ReA **(Carter *et al*., 2009; Carter and Inman, 2011);** *C. pneumoniae* has also been implicated in ReA, but far less frequently than *C. trachomatis* **(Gerard *et al*., 2010)**. *C. trachomatis*-induced ReA, represents a significant health burden, yet is poorly understood **(Gracey and Inman, 2012).** The term "uSpA" is used to designate patients with clinical and radiographic features consistent with spondyloarthropathy who do not fulfil the classification criteria for any of the established disease categories; these patients considered to be *forme fruste* of *C. trachomatis*-induced ReA **(Carter *et al*., 2009).** The most compelling explanation for the underdiagnosis of *C. trachomatis*-induced ReA relates to the fact that asymptomatic *C. trachomatis* infection might account for 78 – 88% of cases of *C. trachomatis*-induced ReA **(Carter *et al*., 2009).** A study showed that patients with chronic uSpA had 42% prevalence of *C. trachomatis* in the synovial tissue by PCR **(Carter *et al*., 2009).** Approximately 4 - 15% of those with genital *C. trachomatis* infection subsequently develop genitourinary infection induced ReA **(Sieper, 2001).** The worldwide incidence of ReA is approximately 30 - 200 cases per 100,000 population, but this varies greatly among different geographic locations. Some of studies have demonstrated *C. trachomatis* DNA in ReA patients in rather high (80 - 100%) or low frequencies (25%) **(Taylor-Robinson *et al*., 1992; Siala *et al*., 2009, Bas *et al*., 1995)**, while others have shown that it is not present at all **(Piot *et al*., 1994).** Global prevalence of ReA is difficult to quantify because of its asymptomatic nature and there is no specific diagnostic criteria to detect *C. trachomatis* **(Senior, 2012).**

Infection-related rheumatic diseases (*viz:* Chikungunya, Brucellosis) as well as infections in patients with rheumatic diseases (*viz:* Osteoarticular tuberculosis, Lupus, Lyme disease) is major challenge in India **(Handa, 2015).** While there are few studies reported from India on enteric ReA **(Prasad *et al*., 1991)**, yet, to the best of our knowledge, there is lack of data from our country till date on the exact prevalence rate of *C. trachomatis*-induced ReA. In a study conducted in north India, the prevalence of serum IgA antibodies to *C. trachomatis* in ReA patients with symptoms suggestive of genitourinary infection has been reported as 25% **(Chandrashekara *et al*., 2004).** In another study conducted by **Aggarwal *et al*., (1997)** on uSpA patients, the prevalence of serum IgA antibodies to *C. trachomatis* has been reported as 14.2% using ELISA. Since genital infection with *C. trachomatis* is a major health problem in India because of its high prevalence **(Rastogi *et al*., 2002, 2003; Mittal *et al*., 2004)**, hence there exists a possibility that genitourinary *C. trachomatis*-induced ReA may be probably underdiagnosed, underestimated and is not well defined. Thus, there exists a definitive need for finding a more reliable estimate of *C. trachomatis* infection in patients with ReA/ uSpA.

Chlamydiae presumably reach the joint *via* extravasated infected monocytic cells originating at the site of initial infection. Various molecular as well as non-molecular techniques have been used for diagnosis of *C. trachomatis* in the synovial joints of patients with ReA **(Bas and Vischer, 1998; Nelson and Helfan, 2001),** as the microorganism can rarely be cultured from the joint. Chlamydial antigen, rRNA and DNA have been detected in the inflamed joint **(Bas *et al*., 1995; Nanagara *et al*., 1995),** while intact chlamydial forms have been identified by electron microscopy and DNA in

chlamydial cells has been found by *in situ* hybridization **(Bas *et al*., 1995; Taylor-Robinson *et al*., 1992).** 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. Viable, metabolically active although non-culturable *C. trachomatis* organisms persist intra-articularly for several years **(Beutler *et al*., 1997).**

Chlamydial DNA is more likely to be found in the synovial tissue than in the Synovial Fluid (SF) **(Nanagara *et al*., 1995; Branigan *et al*., 1996).** The difficulty in growing *Chlamydia* from joint fluid or tissue may be explained by the fact that the microorganism persists as intracellular Reticulate Bodies (RBs) rather than as infectious Elementary Bodies (EBs). Live but non-cultivable intracellular organisms may be driving the inflammation in chlamydial ReA. The discovery of mRNA and rRNA of *C. trachomatis* using reverse transcriptase PCR in synovial biopsy specimens provided evidence of the occurrence of transcription and hence active multiplication of the bacteria **(Gerard *et al*., 1998).** *C. trachomatis* was also found in the cells of the peripheral blood but not in the serum by PCR in samples from patients with early *Chlamydia*-induced ReA **(Kuipers *et al*., 1998)**, implicating that monocytes may be involved as the vehicle transporting chlamydiae from the genital epithelium to the synovium **(Nanagara *et al*., 1995).** Chlamydial DNA in the joints of ReA patients does not correlate with the immune response, whether measured by antibodies or by lymphocyte proliferation **(Sieper *et al*., 1999).**

Reportedly, antibodies to *Chlamydia* may be found both in serum and in joint fluid samples. The specificity and sensitivity for determination of IgG antibodies in joints is 80%, however, the specificity reaches 90% with the determination of SF IgA

antibodies **(Bas and Vischer, 1998).** The IgA class antibodies were slightly more relevant than those of the IgG class for differentiation of patients with ReA from those with uncomplicated genitourinary infection **(Bas *et al*., 2001).** A study conducted in Finland further revealed that serum antibodies against *C. trachomatis* were most common in patients with undifferentiated arthritis **(Savolainen *et al*., 2009).** Serum samples are relatively easily obtained whereas tissue samples from the site of persistent infection are not readily available **(Puolakkainen, 2013).** As molecular biology techniques like PCR and ligase chain reaction are considered to be the most sensitive and specific methods for confirming *Chlamydia* infection since the sensitivity and specificity of these methods is approximately 100% **(Nelson and Helfan, 2001),** hence these techniques ought to be utilized for detection of *Chlamydia* DNA in urine/ SF/ synovial membrane biopsy/ peripheral blood monocytes in patients with persistent infection **(Schnitger *et al*., 2007).**

The immunopathogenesis of *C. trachomatis* in the progression of joint damage in ReA is poorly studied. It has been reported that an imbalance between type 1 and type 2 immune responses could be critical in determining susceptibility to disease in ReA/ uSpA patients **(Gracey and Inman, 2012).** It is likely that cytokines play critical roles in the persistent infection of *C. trachomatis* as high levels of IFN-gamma and low levels of interleukin IL-4 have been reported **(Kotake *et al*., 1999).** IFN-gamma, the main cytokine produced by Th-1 and natural killer cells, may have a dual role in chlamydial infection, potentially mediating both host resistance and immunopathology within the synovial tissue. However, Yin *et al*., (1997) reported that a Th-2 cytokine pattern predominates in the joints of patients with ReA, and since Th-1 cytokines are necessary for elimination of bacteria, Th-2 cytokines might contribute to bacterial persistence in the

joint. In addition, Yin *et al*., (1997) suggested that the IL-10/ IL-12 balance is crucial for the regulation of the cytokine pattern in the joints of the patients with ReA. Thus, Th-1 cytokine response (production of IFN-gamma, IL-2 and IL-12) is impaired in favour of a Th-2 response (IL-4 and IL-10) **(Kotake *et al*., 1999; Yang *et al*., 1999);** and, in the absence of a "good" anti-bacterial reaction, the microbes can survive. However, little is known about the pathogenesis of this Th-1/ Th-2 imbalance in ReA/ uSpA patients. In a German study, it has been demonstrated that the level of TNF-alpha secretion by T-cells at the onset of ReA is inversely proportional to the disease duration and severity. Although all reports agree on the presence of IFN-gamma, it has been claimed that T-cells making IL-4 are also present in the joint, in contrast with Rheumatoid Arthritis (RA) where IL-4 is clearly absent **(Simon *et al*., 1994).** Since immunity to chlamydiae requires a Th-1 response, it has been argued that the presence of IL-4 could indicate an inadequate Th-1 type response to chlamydiae which could lead to persistence of the microorganism in the joint **(Simon *et al*., 1993; Kotake *et al*., 1999).** Jendro *et al*., (2005) evaluated interleukin (IL)-1β, tumor necrosis factor (TNF)-alpha, IL-6, IL-1 receptor antagonist, and soluble TNF receptor p75 to characterize the differences which might be of diagnostic relevance and to investigate the possible association between synovial identification of *C. trachomatis* by PCR and a typical cytokine profile. This group observed that there were no statistically significant differences in cytokine levels between patients with *C. trachomatis*-associated arthritis or the other arthritis forms. Also, comparison between *C. trachomatis*-induced ReA patients with and without *Chlamydia* DNA in SF revealed no significant differences for these cytokines**.**

Prolonged host-pathogen interaction during persistent *C. trachomatis* infection during ReA indicates the involvement of HLA-B27 as a genetic factor (**Kuipers *et al*., 2003).** Furthermore, several studies have revealed that HLA-B27 modulates antigen presentation to T-cells during cell mediated immunity and it has been speculated that HLA-B27 is directly involved in persistence and is associated with the chronic form of *C. trachomatis*-induced ReA **(Kuipers *et al*., 2003; Ramos and De castro, 2002).** Bas et al., (2003) reported that the lower IFN-gamma concentrations in HLA-B27-positive patients with *C. trachomatis*-induced ReA could be related to the tendency of these patients to have more severe or chronic arthritis that can contribute to the persistence of infected cells, which can serve as depots of antigen capable of stimulating a sustained inflammatory response. Data on IL-17 levels and IL-17+ T-cells is limited in ReA/ other forms of spondyloarthritis, and is still contradictory **(Bas *et al*., 2009; Singh *et al*., 2011).** Bas et al., (2009) reported the presence of both IFN-γ and IL-17 in the SF of patients with *C. trachomatis*-induced ReA and found a positive correlation between their levels indicating possible similar regulation by the local cytokine milieu in *C. trachomatis* ReA joints.

In addition, the role of both chlamydial heat shock protein 60 (chsp60) and C-reactive protein (CRP) during chlamydial persistence in ReA/ uSpA patients needs to be further elucidated. It is apparent that the basic immune mechanisms underlying ReA and specifically the joint injury that follows intra-articular *C. trachomatis* infection have not been fully elucidated. It is hypothesized that cytokine imbalance probably has a major role in the failure to eliminate the triggering bacteria and the microbial antigen, leading to the disease manifestations and chronicity. Hence, more detailed knowledge of the host-

bacteria interaction is needed for the development of more efficacious and new therapeutic concepts **(Elicabe & Genaro, 2014).**

1.2 Review of literature

1.2.1 Overview

C. trachomatis is the most common obligate intracellular bacterium that infects over 90 million people each year worldwide by sexual transmission **(Moss and Darougar, 2001; Lanjouw *et al*., 2010).** The wide spread of these infections and their chronic oligosymptomatic or asymptomatic course pose a great clinical and epidemiological problem. These "*silent infections*" **(Stamm, 2007)** lead to dangerous, frequently irreversible complications both in men and women whose treatment is extremely expensive. It is a gram negative intracellular obligate bacteria with the availability of multiple serovars and biovars and has a biphasic life cycle: intracellular metabolic form recognized as Reticulate Bodies (RBs) while extracellular infectious form are called as Elementary Bodies (EBs).

C. trachomatis causes a gamut of diseases, including blinding trachoma, urogenital tract infections leading to urethritis, cervicitis, proctitis and systemic lymphogranuloma venereum disease **(Schachter, 1999).** In genital tract infection, *C. trachomatis* primarily replicates within the epithelium of the urethra of men and endocervix of women causing inflammation, edema, and mucosal discharge. It has been associated with cervical cancer. More recently, it has been associated with ocular adnexal lymphoma, consequently, its screening is necessary to identify and treat the infection **(Papp *et al*, 2014).**

1.2.2 Taxonomy

The order *Chlamydiales* encompasses a large group of bacteria characterized by their obligate growth in eukaryotic cells. Division of the family *Chlamydiaceae* includes genera, *Chlamydia* and *Chlamydophila* and the inclusion of non-Chlamydiaceae such as Simkaniaceae, Waddliaceae and Parachlamydiaceae **(Everett *et al*., 1999).** *Chlamydiaceae* are the etiological agents of many important human and animal diseases **(Fig. 1.1)**.

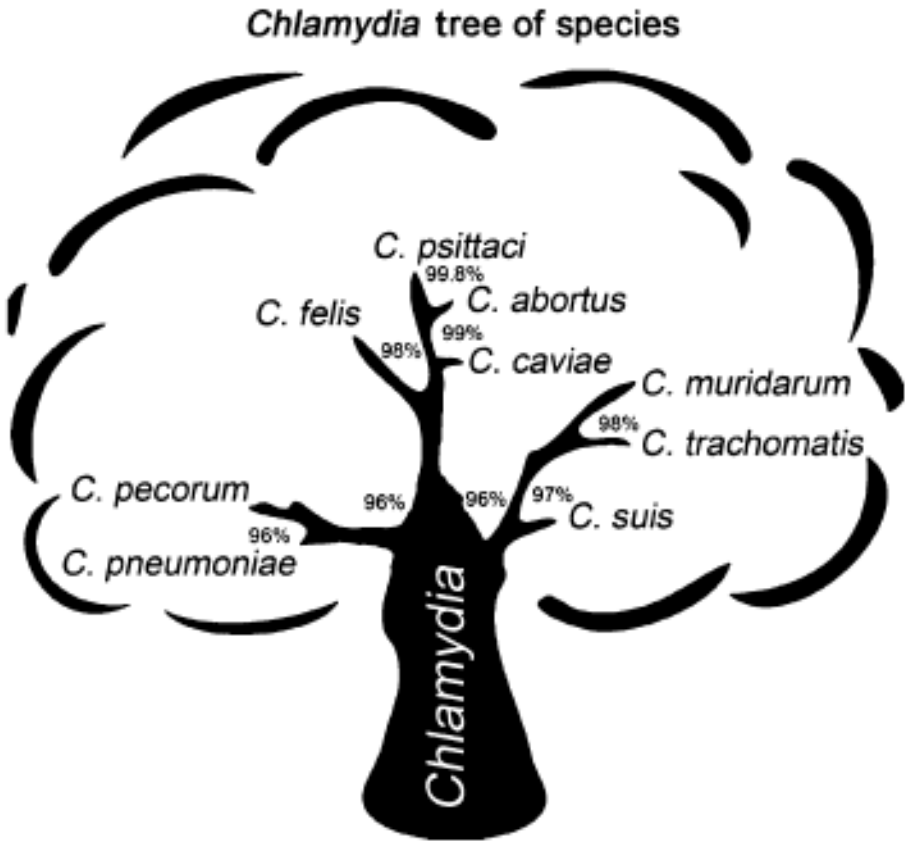


Fig. 1.1.: *Chlamydia* species showing % 16S rRNA gene similarity between species.

(Everett *et al.,* (1999); Int J Syst Bacteriol 49: 415–40)

1.2.3 Developmental cycle of *Chlamydia trachomatis*

C. trachomatis developmental cycle consists of transitions between two main cell types, the infectious non-dividing extracellular form, known as the elementary body (EB), and an intracellular replicative form, called the RB **(Moulder, 1991; Abdelrahman and Belland, 2005)**. The concept of chlamydial development has major implications on chlamydial physiology. Following endocytosis, EBs remain within a membrane-bound compartment termed as an inclusion where they begin to differentiate to RBs. RBs divide by binary fission for the duration of the intracellular developmental cycle but asynchronously begin to differentiate back into EBs, which accumulate within the inclusion until release from the host cell by lysis or extrusion (**Todd and Caldwell, 1985; Lutter *et al*., 2013).** Newly formed EBs are eventually released by cell lysis and/ or extrusion to initiate new rounds of infection **(Dautry-Varsat *et al*., 2005)**

1.2.4 History of *Chlamydia trachomatis*-induced reactive arthritis and other forms of ReA, *viz.:* uSpA

In 460 BC, Hippocrates wrote, *'A youth does not suffer from gout until sexual intercourse'* **(Llydce, 1978)**. In the ancient medical literature, the term '*gout*' was used rather indiscriminately for any type of acute arthritis. Could Hippocrates actually have been describing *C. trachomatis*-induced ReA? Moving forward in the medical literature, several noted physicians suggested a potential link between *Chlamydia trachomatis* and arthritis. In 1507, Pierre van Forest noted patients with '*secondary arthritis and urethritis*' **(Sharp, 1979)**. Baron Yvan described a French captain who developed '*ophthalmia*' and inflammatory arthritis of the lower extremities after a

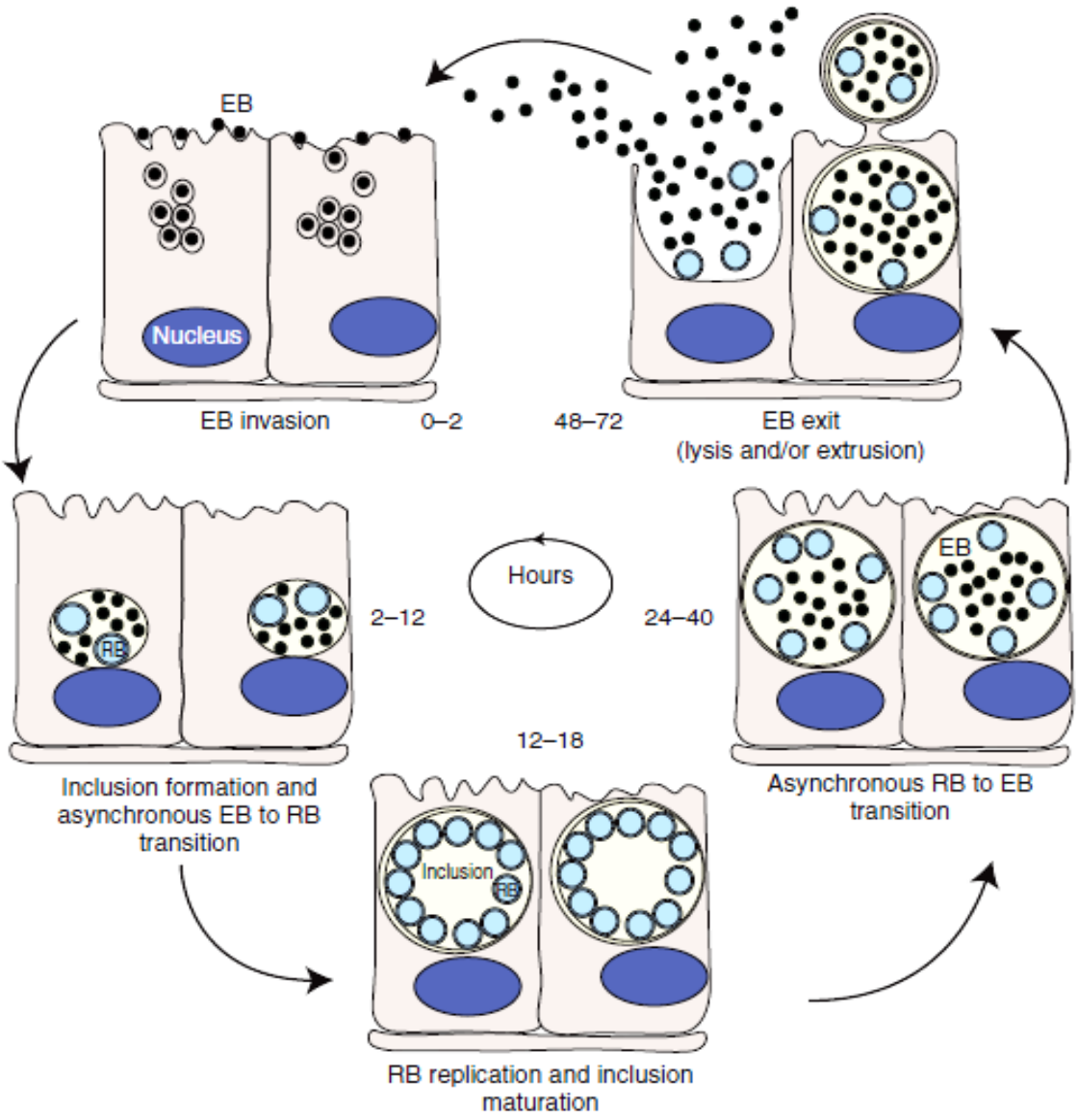


Fig.1.2: Life cycle of *Chlamydia trachomatis*. Within the first 2 h following internalization into cells, elementary bodies (EBs) fuse to form a nascent inclusion. Between 2 and 6 h postinternalization, EBs begin to differentiate into reticulate bodies (RBs). By 12 h postinfection (hpi) RBs can be observed dividing by binary fission and by 18– 24 hpi they peak in numbers. Increasing numbers of RBs differentiate back to EBs around 24 hpi and continue differentiating until lysis or release occurs 48–72 hpi. **(Bastidas *et al*, 2013,Cold Spring Harb Perspect Med doi: 10.1101/cshperspect.a010256)**

venereal infection. Sir Benjamin Brodie described five patients with probable *C. trachomatis*-induced ReA in his book- *Pathological and Surgical Observations on the Disease of the Joints*, which was published in 1818; these five patients developed conjunctivitis and arthritis after episodes of urethritis. He described the '*train of symptoms*' that ensued in all five of these subjects, and he duly noted that four of the five patients developed chronic disease with a '*relapsing course*' **(Brodie, 1818)**.

In 1978, a group of rheumatoid factor-negative inflammatory arthritidies were recognized as a unified entity and termed seronegative polyarthrtitis **(Wright, 1978)**. Spondyloarthropathies (SpA) are a group of arthritidies that share clinical and radiographic features. The SpA's include ankylosing spondylitis, psoriatic arthritis, inflammatory bowel disease-related arthritis, ReA and uSpA. The term uSpA is used to designate patients with clinical and radiographic features consistent with the SpA's, but who do not fulfill the classification criteria for any of the established disease categories. uSpA is also designated as a "*forme fruste*" of ReA **(Aggarwal *et al*., 1997).** Because ReA is a type of SpA and the majority of patients with ReA do not present with the classic triad of symptoms **(Carter, 2006),** the contention that *C. trachomatis* could function etiologically to engender uSpA is reasonable.

1.2.5 Epidemiology of *Chlamydia trachomatis* infection

1.2.5.1 International status

Genital infections with *C. trachomatis* are among the most common sexually transmitted infections in Europe and America, and they primarily affect young people. > 1.3 million *C. trachomatis* infections in USA were reported to CDC in 2010 **(Papp *et al*., 2014).** Studies in USA that followed patients after venereal chlamydial infection demonstrated that 4.1 – 8.1% of patients developed ReA **(Carter and Inman, 2011; Rich *et al*., 1996).** In Norway and Finland, the incidence of *Chlamydia*-induced ReA was determined to be 3 – 5.4/ 100000 population/ year**. (Kvien *et al*., 1994; Savolainen *et al*., 2003).** *C. trachomatis*-induced ReA was seen in approximately 20% of patients, with nearly equal sex ratio, in individuals with undiagnosed arthritis attending an early synovitis clinic **(Wollenhaupt *et al*., 1995).** If accurate, the estimated annual incidence of *Chlamydia*-induced arthritis in the USA is approximately equal to, and perhaps somewhat higher than, that of RA **(Doran *et al*., 2002).** Consistent with this contention, a 2002 study in Sweden found the annual incidence of chlamydial arthritis to be higher than that of RA **(Soderlin *et al*., 2002; Senior, 2012).** The epidemiologic trends of reactive arthritis can only be approached by approximation due to the lack of a precise disease definition and specific diagnostic criteria **(Braun *et al*., 2000).** Furthermore, the estimated incidence and prevalence of ReA differ significantly from study to study. Both incidence and prevalence of the disorder are influenced by genetic differences between populations, the environmental conditions of each country and the lifestyles of populations (sanitary conditions etc.) **(Carter and Hudson, 2009).**

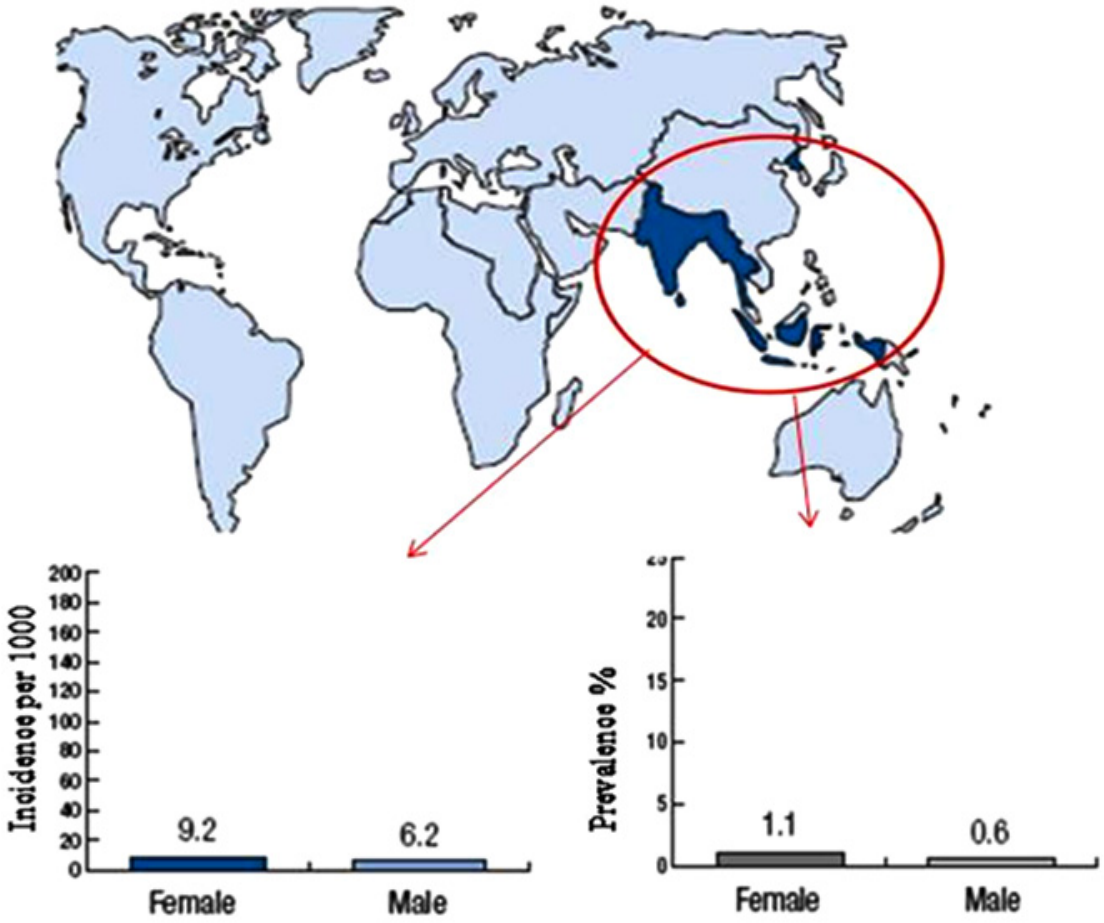


Fig. 1.3: Prevalence and Incidence of *Chlamydia trachomatis*. The WHO South-East Asia Region comprises 11 countries with an estimated population in 2008 of 945.2 million adults between the ages of 15 and 49. 7.2 million incidence of *C. trachomatis* were estimated in 2008 while 8.0 million adults were infected with *C. trachomatis.* Bar chart showing Female: Male ratio of *C. trachomatis* infection. **(***Global incidence and prevalence of selected curable sexually transmitted infection – 2008. WHO 2012)*

Comparable prevalence has been reported from several rheumatological clinics, with identification of *C. trachomatis* as the causative pathogen in 16% of those with undifferentiated oligoarthritis and a much higher prevalence of 63% in urogenic ReA **(Fendler *et al.*, 2001).** Importantly, the preceding genital chlamydial infection is often

asymptomatic; In one study it was obtained in 30% of patients with CReA, and it was more frequent in women (61% of the asymptomatic patients) than in men **(Wollenhaupt *et al*., 1995)**. Two other studies of patients with oligoarthritis of undetermined origin reported evidence of undetected, asymptomatic *C. trachomatis* infection in 36% and 17% of individuals studied, respectively **(Weyand and Goronzy, 1992; Schnarr *et al*., 2001)**.Silent infection, frequent spontaneous remission and reluctance to reveal *C. trachomatis* genital infection may be major reasons why *Chlamydia*-induced ReA is often underdiagnosed. 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 uSpA **(Poole *et al*., 1992; Bas *et al*., 1995; Kuipers *et al*., 2009; Siala *et al*., 2009; Carter *et al*., 2009)**.

Much of the data on the prevalence and annual incidence of ReA in populations come from the Scandinavian countries where the annual incidence varies between 5 and 28 per 100000 **(Aho and Ahvonen, 1975)**. Among patients with any of the spondylo-arthritis variants seen by rheumatologists, those with ReA are a very small minority. This was illustrated in a study of 1,379 Spanish spondyloarthritis patients, of whom only 16 (1.2%) were diagnosed with ReA **(Collantes *et al*., 2007)**. Most cases of ReA appear sporadically, but outbreaks may follow single-source infections. Approximately 1–3% patients with chlamydial urethritis will show ReA and near to 50% of clinically diagnosed ReA could be diagnosed as *Chlamydia*-induced arthritis. There appears to be some geographical variation in the major organisms associated with ReA. In Minnesota and Oregon in the USA, for example, the incidence of ReA increased following *Campylobacter* and *Salmonella* infections (**Townes *et al*., 2008)**, whereas in Finland and

Sweden the most frequent causative organisms were *C. trachomatis* and *C. jejuni*, respectively (**Soderlin *et al*., 2002)**. Although it is recognized that young people should remain the focus of sexual health programs, recent findings indicate that sexual risk-taking behaviour is not confined to the young but can also occur among older people (**Bodley-Tickell *et al*., 2008).**

1.2.5.2 National status

The burden of genital chlamydial infection is high in India ranging from 30 - 81% **(Malhotra *et al*., 2013; Rastogi *et al*., 2002, 2003; Mittal *et al*., 1995, 2004; Ray *et al*., 1993).** It is argued recently that *Rheumatology* has been a neglected sub-speciality in India and infectious diseases have dominated the medical scene in India for a long time, with tuberculosis, malaria, typhoid, polio, kala-azar, leprosy and other diseases as traditional foes **(Handa, 2015).** Studies on *C. trachomatis*-induced ReA is rare in India mainly focusing on enteric infection induced ReA while studies regarding other organisms are lacking **(Chandrasekaran, 1993).** Study conducted at a tertiary care hospital in north India showed 14 - 25% antibodies against *C. trachomatis* in serum **(Aggarwal *et al*., 1997).**

1.2.6 *C. trachomatis* infections in genital regions

Reportedly, upto 80% of chlamydial infections are asymptomatic **(Zimmerman *et al*., 1990).** In women, clinical manifestations of *C. trachomatis* include cervicitis, urethritis, endometritis, PID or abscess of the Bartholin glands (**Bleker *et al*., 1990; Centers for Disease Control and Prevention, 1993).** Although the initial site of

infection is usually the cervix, the urethra and rectum may also be infected (**Dunlop *et al*., 1985).** The prevalence of *C. trachomatis* infection in pregnant women ranges from 2 to 35% **(Martin *et al*., 1982; Ryan *et al*., 1990; Sweet *et al*., 1987).** Pregnant women with chlamydial infections are at increased risk for adverse outcomes of pregnancy, and postpartum PID. Pregnant women with *C. trachomatis* infection were 10-fold more likely to have outcomes of stillbirth and neonatal death and gestation periods were also significantly shorter in infected women **(Martin *et al*., 1982).** Also, a sub-population of women were found to be at higher risk for preterm delivery and premature rupture of the membranes (**Harrison *et al*., 1983; Sweet *et al*., 1987).** Ascending uterine infections can lead to pelvic inflammatory disease, tubal scarring, ectopic pregnancies and infertility **(Bebear and de Barbeyrac, 2009).**

In men, chlamydial infections are usually urethral and upto 50% are asymptomatic **(Stamm and Cole, 1986; Zelin *et al*., 1995).** Untreated infections may lead to arthritis or Reiter's syndrome. Epididymitis, or infection of the sperm ducts of the testicles, is most often due to *C. trachomatis* or *N. gonorrhoeae* in young, sexually active men less than 35 years of age **(Berger *et al*., 1978; Paavonen and Eggert-Kruse, 1999).** Mild to moderate clear to white urethral discharge is seen in the morning before the patient voids. In epididymitis, history of unilateral testicular pain with scrotal erythema, tenderness or swelling over the epididymis may be elicited **(Malhotra *et al*., 2013).**

Chlamydia is known to spread throughout the body via monocytic cells **(Moazed *et al*., 1998)** to a diverse range of tissues, including the spleen, liver, peritoneum and lungs **(Cotter *et al*., 1997)**. *Chlamydia* is often detected long after the initial infection in

selected tissues, such as the genital tract and synovium **(Gracey and Inman, 2012).** Recently, infection with *Chlamydia* has been associated with an increased risk for subsequent development of invasive squamous cell carcinoma of the uterine cervix **(Koskela *et al*., 2000)**; ocular adnexal lymphoma and Alzheimer's brain **(Balin *et al*., 1998)**.

1.2.7 *Chlamydia trachomatis*-induced reactive arthritis

Historically, *C. trachomatis*-induced ReA has been defined as a non-purulent arthritis that develops during or soon after extra-articular bacterial infections elsewhere in the body, but in which the micro-organism cannot be recovered from the joint. However, it is well accepted today that *C. trachomatis*-induced ReA is caused by intra-articular persistent infection and therefore is of post-infectious origin.

1.2.8 Clinical manifestations in *Chlamydia trachomatis*-induced reactive arthritis

Description of the specific demographics and clinical spectrum of *C. trachomatis*-induced ReA in patients providing a definite diagnosis of chlamydial infection has been rare **(Hannu *et al*., 2006; Wollenhaupt *et al*., 1995; Carter *et al*., 2009)**. *C. trachomatis*-induced ReA, like other ReA and SpA, combines four syndromes: peripheral arthritis, enthesopathy, axial involvement and extra musculoskeletal manifestations. Combination of these four varies from patient to patient and in any given patient during the disease course. The arthritis usually manifests itself in *C. trachomatis*-induced ReA as monoarthritis or oligoarthritis; polyarticular involvement is rare. Large joints, especially of the lower extremities, are most commonly affected, and the pattern of disease is

usually asymmetrical **(Wollenhaupt *et al*., 1995; Carter *et al*., 2009)**. Other manifestations are enthesopathy and dactylitis. Sacroiliitis and axial manifestations of spondyloarthropathy also are frequent. Extra-articular organ manifestations most commonly affect skin and eye, more rarely the heart, kidneys or muscle, and central nervous systems. This includes the well-known Reiter's syndrome with the triad of urethritis, conjunctivitis and arthritis; this term should no longer be used because of the Nazi past of Hans Reiter and the obvious lack of evidence for a separate disease entity **(Zeidler, 2002; Panush *et al*., 2007)**. Other less easily assessed sites of primary infection with *C. trachomatis* include the conjunctivae, a result of transmission from genitalia to eye by smear infection; this can lead to follicular conjunctivitis as well as epithelial and sub-epithelial infiltration of the cornea with mucopurulent or serous reaction **(Haller-Schober *et al*., 2002)**. In some patients *C. trachomatis* has been isolated from pharyngeal swabs, nasal and sinusoidal discharges, and bronchoalveolar lavage probably due to transmission of the infection by orogenital sexual contact. Clinical manifestations include tonsillitis, sore throat, sinusitis, bronchitis and pneumonia, the latter especially in immunocompromised subjects (**Lauhio *et al*., 1991; Munoz Rodríguez *et al*., 1996; Samra *et al*., 1988; Paran *et al*., 1986)**. It is, however, clear that post-enteric and post-chlamydial ReA are distinct entities, in that viable organisms can be detected in the joints of patients with *Chlamydia*-induced ReA but not in those with post-enteric ReA **(Carter and Hudson, 2010).**

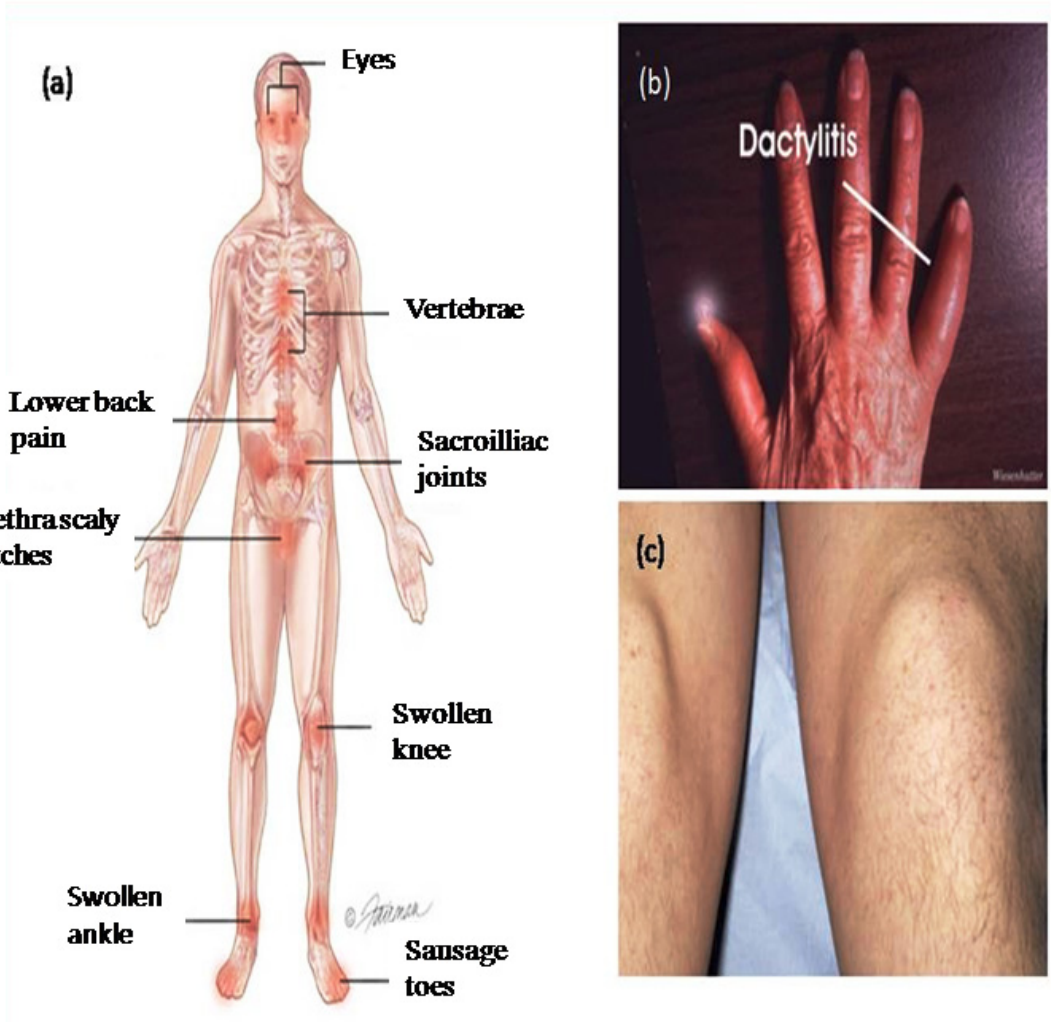


Fig. 1.4: Clinical manifestation and pattern of joint involvement in *Chlamydia trachomatis*-induced reactive arthritis. (pictures taken from http://diseasespictures.com/dactylitis/)

A- Articular & extra-articular symptoms during Reactive Arthritis & Undifferentiated spondyloarthropathy

B- Dactylitis: Inflammation in small fingers

C- Synovitis: Joint swelling

1.2.9 *Chlamydia trachomatis* serovars in genitourinary-induced reactive arthritis

Chlamydiae of serovars A, B, Ba, and C are ocular (trachoma) agents, while those of serovars D-K are recognized as standard genital agents. A separate biovar includes the chlamydiae responsible for lymphogranulosum venereum designated biovars $L_1 - L_3$ **(Yuan *et al*., 1989; Ward, 1999).** Importantly, organisms of ocular serovars can cause genital infections, and *viceversa*, under some conditions**.** However, genital infections by ocular serovars are rare **(Dean *et al*., 2008; Suchland *et al*., 2003; Gao *et al*., 2007).** Because *Chlamydia*-associated arthritis is by definition a sequela of genital infection, it has been assumed that the organisms that disseminate from the urogenital system to the joint belong to the genital serovar group. Contrary to that expectation, only ocular serovar group chlamydiae were identified in synovial biopsies from arthritis patients PCR-positive for *C. trachomatis* DNA in that tissue **(Gerard *et al*., 2010).** Whether and if so how, chlamydiae of the ocular serovars are uniquely arthritogenic as opposed to those of genital serovars remains to be established. Theory of mixed inoculum of oculogenital serovars has been coined as this is not clear why a small fraction of genital chlamydial infection transform in to the intra-articular infection **(Rihl *et al*., 2006).**

1.2.10 *C. trachomatis* route of transmission from primary site

Although the primary infection of the urogenital system is cleared by the immune system or antibiotic treatment or both, the initial inflammatory response in the genital system elicited by the infection attracts mononuclear cells; these become infected, are subsequently extravasated, and via the general circulation make their way to the joint to establish chlamydial infection of synovial tissue (**Gerard *et al*., 2009; Ward, 1999).** A

relatively small proportion of individuals who acquire a genital infection with *C. trachomatis* develop acute inflammatory (reactive) arthritis, and only a portion of those patients proceed to chronic disease **(Schumacher, 2000)**. It has never been clear why some *Chlamydia*-infected individuals develop arthritis whereas others do not, and we do not understand why only a subset of patients who do develop acute disease proceed to chronicity. Clearly, both aspects are at least partly a function of host genetic background, but there may also be as yet unknown genetic or other characteristics of the infecting chlamydiae that contribute. Studies have shown that *C. trachomatis* reaches the joint from the urogenital system *via* circulating monocytes **(Whittum-Hudson *et al*., 2007)**, and that monocytes/ macrophages are the common host cells for persistent organisms during long-term infection of synovial tissue. It is not known how long a period is required for transit to the joint, whether *C. trachomatis* cells are already persistent on arrival in the joint, or whether that state develops only after the organisms are stably in place. However, we do know from *in vitro* studies that *C. trachomatis* cells enter the persistent state within about 48 hours post-infection of normal human peripheral monocytes and alter expression of several important genes, including increasing transcription of their hsp60 gene **(Kohler *et al*., 1997; Gerard *et al*., 2001),** and that acute arthritis only develops after 1 week or more of post-genital infection **(Schumacher, 2000).**

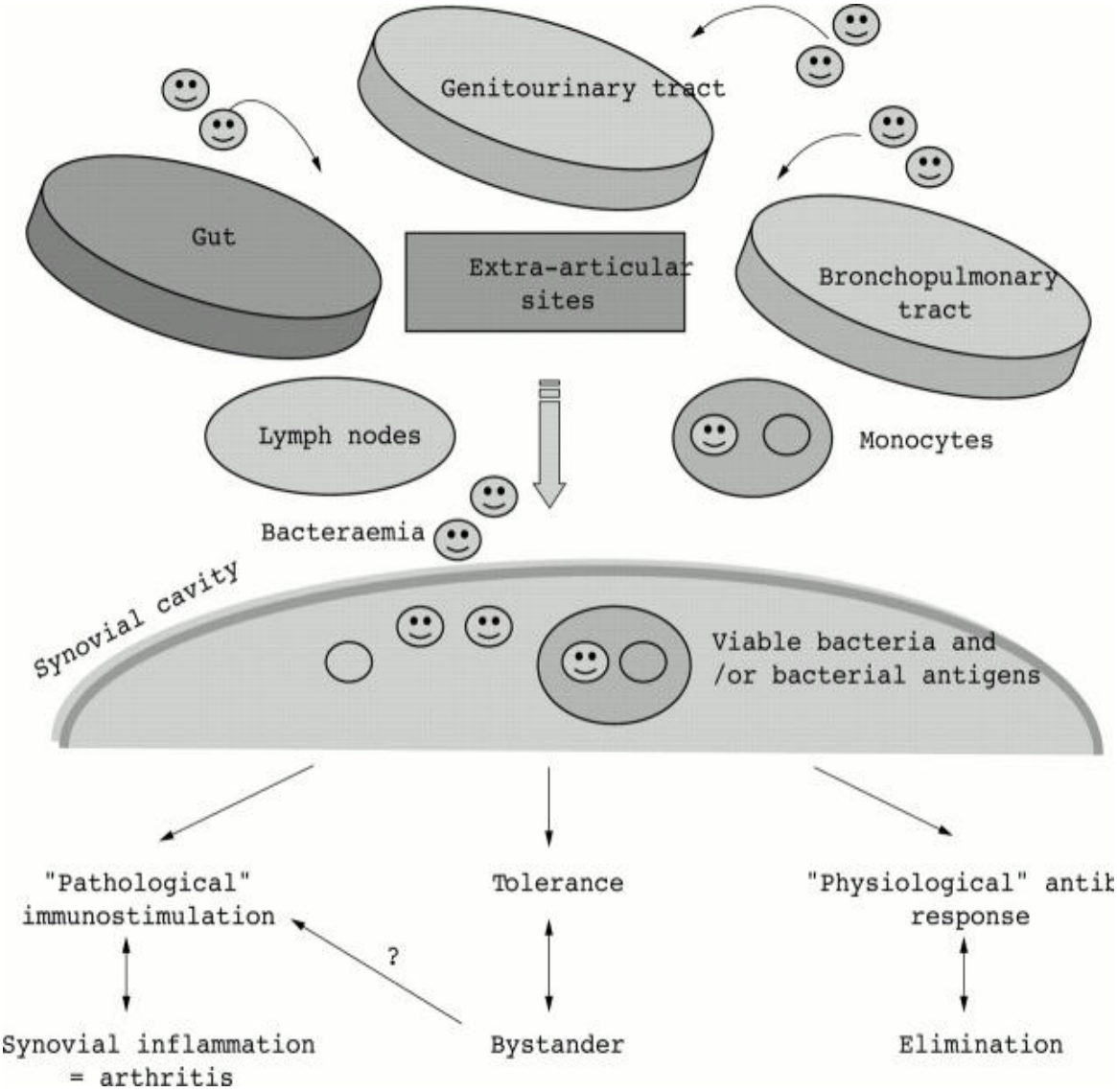


Fig. 1.5: Natural history of arthritogenic infections in reactive arthritis.

Route of transmission of *C. trachomatis* and other bacterial infection from primary site in reactive arthritis. (Sibilia and Limbach, Ann Rheum Dis 61: 580-7 (2002)

1.2.11 Persistent infection of *Chlamydia trachomatis* in genitourinary-induced reactive arthritis

C. trachomatis-induced ReA is a function not of the normal processes of growth and cellular development for *C. trachomatis*, but rather of an unusual infection state of the organism designated '*persistence*' **(Zeidler *et al*., 2004; Rihl *et al.*, 2006; Gerard *et al*., 2009; Whittum-Hudson *et al*., 2007).** In the late 1980s and early 1990s, studies **(Schumacher *et al*., 1988; Nanagara *et al*., 1995)** utilizing more advanced electron microscope techniques analyzing the synovial tissue of patients with *C. trachomatis*-induced ReA demonstrated aberrant intracellular chlamydial forms. These aberrant forms appeared very similar to aberrant chlamydiae produced by an *in-vitro* model upon exposure to penicillin or IFN-gamma **(Beatty *et al*., 1993)**. The chlamydial cycle in this *in-vitro* model is in a state of arrested developmental, known as chlamydial persistence, displaying attenuated production of new EBs. Important for the concept of *C. trachomatis*-induced ReA is the fact that chlamydial persistence is now well documented by PCR and real-time (RT)-PCR analyses involving synovial samples of patients with *C. trachomatis*-induced ReA **(Gerard *et al*., 1998, 2000).** The pattern of gene expression is attenuated and significantly different from that seen during normal active infection. For example, during persistence of *C. trachomatis*, expression of the major outer membrane protein (omp1) gene and several genes required for the cell division process are severely downregulated. This is coupled with differential upregulation of the three paralog genes (Ct110, Ct604, and Ct755) specifying chsp60 **(Gerard *et al*., 2004).** Molecular studies on other bacteria **(Hoffmann *et al*., 2004; Bohn *et al*., 2003)** have shown that persistence is a multifactorial, orchestrated process reflected by corresponding changes of the gene or

protein expression pattern of the infected host cells. The screening of *Chlamydia*-infected cells by microarrays and the detailed analyses by real time reverse transcriptase-polymerase chain reaction or enzyme linked immunosorbent assay showed that innate immunity and other pathological mechanisms have a role in this interaction **(Rihl *et al*., 2006).** Experimental approaches have involved gene expression studies comparing productive and persistent infection. One major finding was the downregulation of an outer membrane protein (omp1) in persistent infection, probably accounting for the aberrant morphology of persistent *Chlamydia* found in the arthritic joint. Another finding was the upregulation of the hsp60 gene encoding for a highly immunogenic protein and thus contributing to the inflammatory response mounted against persistent *Chlamydia* **(Kuipers *et al*., 2003).**

hsps, in general, are paramount to the persistent state of both *C. trachomatis* and *C. pneumoniae*; they provide many functions involved with cell survival. The HSP-60 molecule, specifically, has many functions that appear to be important to the pathophysiology of ReA. HSP-60 has been shown to be pivotal in the inability of *Chlamydia*-infected cells to undergo apoptosis **(Dean and Powers, 2001).** Other study had demonstrated that hsp60 plays a role in eliciting the host immune response **(Curry *et al*., 2000).** The *C. trachomatis* and *C. pneumoniae* genomes each specify three hsp60-encoding genes **(Gerard *et al*., 2004**; **Read *et al*., 2000).** However, in terms of hsp60 gene expression, there are differences even within the *Chlamydia* genus. With *C. trachomatis*, the authentic hsp60 gene, *groEL*, is designated Ct110 in the genome sequence and resides in an operon with *groES*, as in *Escherichia coli* and other bacteria. The two additional paralogs are designated Ct604 and Ct755. The groEL hsp60 protein is

believed to be important in synovial pathogenesis. Each of the three *C. trachomatis* hsp60 genes is transcribed independently during active infection **(Gerard *et al*., 2004).** In acutely infected cells, Ct604 and Ct755 are expressed at higher levels than Ct110. However, expression of Ct110 and Ct755 is attenuated during persistence in the monocyte model, whereas transcript levels from Ct604 are increased significantly in this state. This suggests that the product of Ct604 functions to maintain persistence. Analyses of synovial biopsy samples from patients with *C. trachomatis*-induced ReA also demonstrate high mRNA levels from Ct604 and extremely low levels from Ct755. These observations might explain, in part, the continued elicitation of inflammation in patients with chronic *C. trachomatis*-induced ReA. They also suggest that the Ct604 gene product is involved in the transition from normal active to persistent infection. Conversely, the Ct755 gene product appears to function only during the acute infection state.

Another incompletely explained aspect of the host–pathogen interaction during CiReA is the observation that *Chlamydia* adopts an atypical, persistent state within the joint. *Chlamydia* spp. are obligate intracellular pathogens that exist in two distinct states during their lifecycle: the extracellular, infectious elementary body, and the intracellular, replicative reticulate body. When appropriate stress is applied during the intracellular stage, such as IFN-γ exposure, antibiotic treatment or infection of monocytic cells, *Chlamydia* enters a nonreplicative and unculturable, yet viable, persistent state **(Wyrick, 2010).** The persistent state differs from the normal intracellular state in that the RBs fail to divide or differentiate into infectious particles, have a reduced metabolism, and are immune-evasive **(Belland *et al*., 2003).** *Is persistent Chlamydia the cause of ReA, or the effect of host–pathogen adaptation* **(Gracey and Inman, 2012)***?* A mechanistic

hypothesis to address *Chlamydia* as the instigator of ReA postulates that chlamydial persistence provides a continuous source of bacterial components that stimulate the immune system, resulting in chronic inflammation and tissue damage **(Gerard *et al*., 2010).** These components could be pathogen-associated molecular patterns that stimulate host innate receptors, or microbial antigens that elicit adaptive immune responses. On the other hand, it is equally plausible that persistence represents the host's best attempt at controlling *Chlamydia*, which

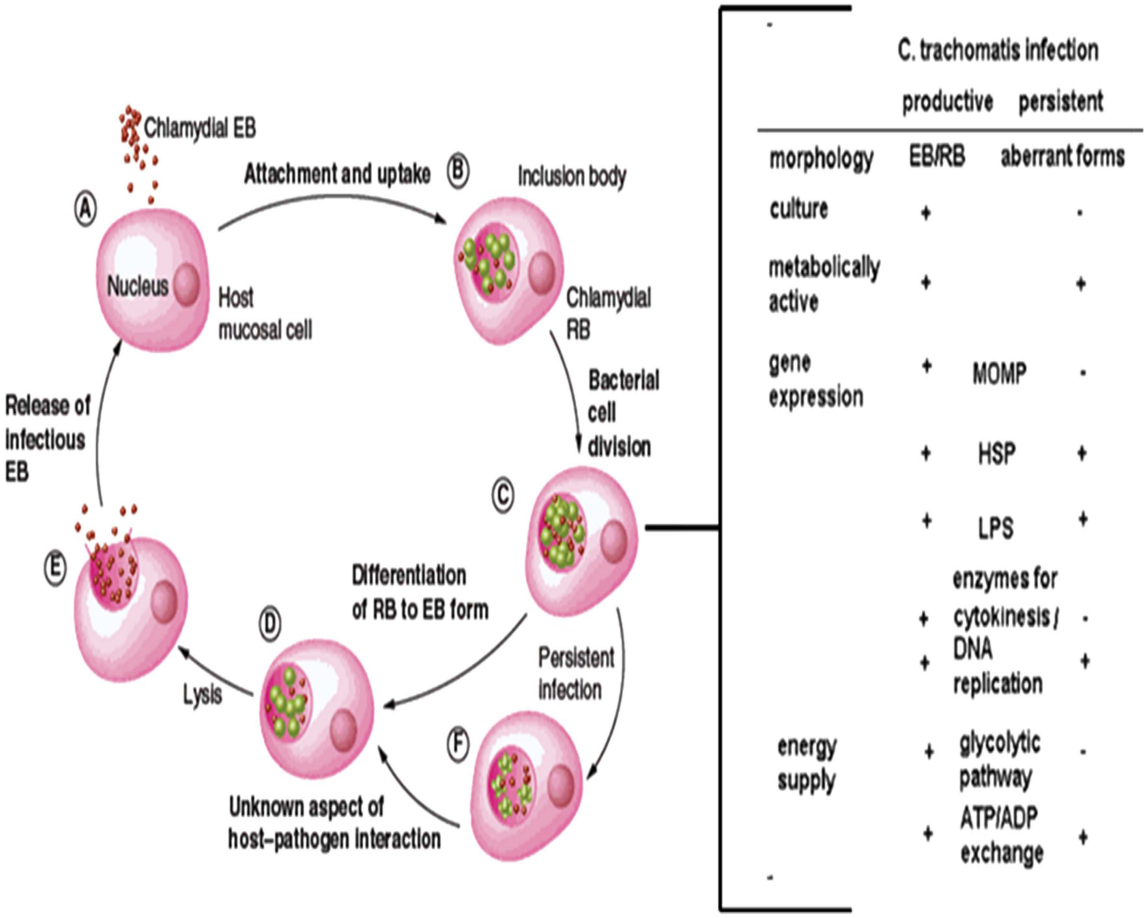


Fig. 1.6: Infectious life cycle of *Chlamydia* with main morphological and metabolic features of normal productive *vs* persistent *Chlamydia trachomatis* infection (Zeidler and Hudson, Ann Rheum Dis Apr; 73(4):637-44 (2013)

Left: *schematic description of persistent life Chlamydia arrested at partially known state of the normal life cycle.*

Right: *the morphologies, the results of culture-based detection of Chlamydia, their metabolic state, their gene expression profiles, and energy supply of Chlamydia during productive infection and in a persistent state.*

Abbreviations: *EB, elementary body; RB: reticulate body; CT, Chlamydia trachomatis; MOMP, major outer membrane protein; HSP, heat shock protein; LPS, lipopolysaccharide; + indicates detection; − indicates lack of the corresponding messenger RNA.*

itself has developed specialized mechanisms to avoid the immune response, particularly the establishment of the immuno-evasive persistent state. In support of this theory, chronic chlamydial infection elicits little immune stimulation relative to that seen during acute infection (**Droemann *et al*., 2007).**

1.2.12 Association between ReA and HLA-B27

The association between ReA and HLA-B27 remains one of the strongest links between an HLA determinant and disease **(Shikawa *et al*., 1986; Keat *et al*., 1978).** This finding helped to confirm the concept of an incomplete Reiter's Syndrome, in which arthritis can occur in the absence of urethritis and conjunctivitis. The prevalence of HLA-B27 in patients with ReA is generally < 50% and HLA-B27 testing does not appear to be of importance in predicting the severity or duration of disease. Thus, HLA-B27 testing, in isolation, has little diagnostic value for ReA. However, it is suggested to reserve HLA-B27 testing for those patients who have an intermediate likelihood of having ReA. Furthermore, HLA-B27 seems to influence severity and persistence rather than just susceptibility **(Owlia and Eley, 2010; Gaston, 2000).** The frequency of HLA-B27 in a healthy general population is around 5 – 7% (**Davatchi *et al*., 1977).** HLA-B27 typing is not a diagnostic tool even in full-blown cases of seronegative spondylo-arthropathies

(i.e., ankylosing spondylitis) in which the association of more than 90% is predicted. Patients who are HLA-B27 positive have about a 50-fold increased chance of developing ReA, but this syndrome can occur in patients who are HLA-B27 negative **(Kobayashi *et al*., 1999).** However, the mechanism of the interaction of the inciting organism with the host (often HLA-B27 positive) leading to the development of ReA is not known. Patients with HLA-B27, as well as those with a strong family clustering of the disease, tend to develop more severe and long-term disease **(Owlia and Eley, 2010).**

1.3 Laboratory diagnosis of *Chlamydia trachomatis* in ReA/ uSpA

1.3.1 Molecular diagnosis of *Chlamydia trachomatis* infection

In order to identify *Chlamydia* as the triggering organism and to establish the diagnosis of *Chlamydia*-induced arthritis, the utilization of PCR for detection of bacterial DNA in the inflamed synovial compartment has increasingly gained importance over the last few years. This was attributed to the problem that direct detection at the primary infectious site is often not possible due to the late presentation of the patient and/or due to the fact that other tests such as serology have a low sensitivity **(Kuipers *et al*., 1995; Freise *et al*., 2001).** Several PCR assays amplifying chlamydial DNA have been developed to examine the presence of *C. trachomatis* DNA in the synovial tissue or fluid of ReA, undifferentiated oligoarthritis and other forms of arthropathies **(Taylor-Robinson *et al*., 1992; Bas *et al*., 1995; Wilkinson *et al*., 1998).** Some of these analyses have demonstrated *C. trachomatis* DNA in ReA patients in rather high (80%) **(Taylor-Robinson *et al*., 1992)** or low frequencies (25%) **(Bas *et al*., 1995). Siala *et al*., (2009)** demonstrated that *C. trachomatis* DNA was present in 100% ReA patients and in 64%

undifferentiated oligoarthritis patients. **Carter *et al*., (2009)** showed 62% uSpA patients were infected with *C. trachomatis* while others have shown that it is not present at all **(Piot *et al*., 1994).**

1.3.2 Non-molecular diagnosis of *Chlamydia trachomatis* infection

1.3.2.1 Serological diagnosis

Serology may be of some value in cases where laboratory testing to identify *Chlamydia* in the joint are not available. For interpretation of chlamydial serology, the temporal occurrence and combination of IgG, IgA and IgM antibodies are important. In particular, the simultaneous detection of IgG and IgA antibodies is typical for fresh or persistent infections and indicates the diagnosis of probable *C. trachomatis* ReA in patients with corresponding clinical history and symptoms **(Bas *et al*., 1996, 2002; Bas and Vischer, 1998; Rihl *et al*., 2006).** The detection of IgM antibodies is of diagnostic relevance in primary infection with *C pneumoniae*, indicating a fresh, acute infection. Interestingly, recent *C. pneumoniae* infections occur frequently in patients with ankylosing spondylitis and *C. pneumoniae* IgM antibody is correlated with active disease, as reported in a Chinese cohort **(Feng *et al*., 2011).** Commercially available serological diagnostics, however, are limited by gender-specific cross-reactivity test systems, non-specific stimulation of anti-chlamydial antibodies, poor sensitivity, and in cases of simultaneous or consecutive exposure to both chlamydial species. Further, diagnostic accuracy is limited by an increasing prevalence of antibodies against *C. trachomatis* and/ or *C. pneumoniae* with age in the healthy population **(Ni *et al*., 1996; Bellido-Casado *et al*., 2006).** The best association of sensitivity (76%) and specificity (85%) was obtained

when IgG and/or IgA reactivity to two *C. trachomatis* antigens was determined **(Bas *et al*., 2002);** these were derived from species-specific epitopes in variable domain IV of the major outer membrane protein and a recombinant polypeptide encoded by open reading frame 3 on the chlamydial plasmid. However, the diagnosis of *Chlamydia*-induced ReA by serology alone is always debatable.

1.3.2.1 Detection by Direct Fluorescence Assay (DFA)

Chlamydial EBs were found in the joints of five of eight patients with Sexually Acquired Reactive Arthritis (SARA) by using a specific anti-chlamydial fluorescein conjugated monoclonal antibody **(Keat *et al*., 1987; Taylor-Robinson *et al*., 1988).** The finding of chlamydial EBs in the joints of one third of the women with arthritis was strong evidence for an aetiological association. Detection by DFA offers a chance of confirming the diagnosis rapidly and simply by examining a synovial fluid cell deposit, even when the disease is not of recent onset **(Taylor-Robinson *et al*., 1988).** In a study published by **Taylor-Robinson *et al*., (1992),** 80% concordance was reported between DFA and PCR results. These authors further suggested that the discordant results in the remaining 20% of patients could be due to loss of chlamydial EBs during storage or in the process of transfer from slide to PCR reaction mixture **(Taylor-Robinson *et al*., 1992).**

1.4 Pathogenesis of *Chlamydia trachomatis* in genitourinary-induced ReA/ uSpA

a) *Chlamydia trachomatis* T-cell mediated immune responses

Chlamydiae are obligate intracellular pathogens and as such require T-cell mediated immunity to control the infection. Antibody may have a role in preventing

reinfection, especially when it is produced locally in the genital tract, but in most circumstances has little if any role in clearing the organism. It is not surprising therefore that T-cell mediated immune responses have been readily demonstrated in the joints of patients with *Chlamydia*-induced ReA. Indeed, characterizing this immune response has contributed substantially to what is known about cell mediated responses to *C. trachomatis* in humans, with the identification of several of the antigenic components of chlamydiae which elicit T-cell mediated responses. CD4+ T-cells play the major part in controlling chlamydial infection, probably through their production on interferon-gamma, but protective CD8+ T-cells have also been described in mice, and more recently in humans. The relative importance of responses by these subsets in humans has not yet been established, and chlamydia specific CD8+ T-cells have not yet been isolated from human joints. Nevertheless, CD8+ T-cells are activated in the joint and also produce a similar set of cytokines as CD4+ T-cells **(Beacock-Sharp & Gaston, *unpublished data*),** and the failure to isolate *chlamydia* specific T-cells may simply reflect technical difficulties in working with these cells.

b) *Chlamydia trachomatis* and cytokine production in the reactive arthritis joint

If T-lymphocytes encounter bacterial antigen in the joint they get activated to produce the cytokines which ultimately control joint inflammation and destruction. Synovial T- lymphocytes from ReA joints analysed *ex-vivo* show spontaneous production of interferon gamma, generally regarded as a proinflammatory cytokine, and also the critical factor in overcoming chlamydial infection. Although all reports agree on the presence of interferon-gamma, it has been claimed that T-cells making interleukin 4 (IL-4) are also present in the joint in contrast with RA where IL-4 is clearly absent **(Simon *et***

***al*., 1994).** This is relevant to pathogenesis, using the model which classifies T-cells into two categories according to their pattern of cytokine production—Th1 cells dominated by interferon gamma and Th2 cells dominated by IL-4, the two subsets of T-cells being mutually antagonistic **(Mosmann and Sad, 1996).** Since immunity to chlamydiae requires a Th1 response, it has been argued that the presence of IL-4 could indicate an inadequate Th1 type response to chlamydiae which could lead to persistence of the organism in the joint. Studies in mice lacking the interferon gamma gene indicate that apart from an inability to clear chlamydial infection, such mice may sustain a vigorous and potentially tissue damaging delayed type hypersensitivity response driven by Th2 cytokines, particularly IL-4. IL-10 is another cytokine which antagonises Th1 responses, and this is also present in the joint, but whether the amounts are appropriate is not known. In animal models of chlamydial infection, clearance of the organism is affected by the relative balance between interferon-gamma and IL-10 production **(Kotake *et al*., 1999)** and IL-10 gene knockout mice clear chlamydial infection more rapidly than normal **(Yang *et al*., 1999).** One of the most interesting T-cell derived cytokines likely to be relevant to joint destruction is the recently described IL-17, which is present in ReA joints. IL-17 can mimic many of the properties of IL-1 and tumour necrosis factor alpha (TNF-alpha) which lead to cartilage breakdown and bone erosion **(Kotake *et al*., 1999).** In addition to cytokines derived from T-cells, and those whose production is induced by T- cells, proinflammatory cytokines may also be produced by cells in the synovium as a result of infection itself. The cells include both macrophage-like synoviocytes which can make IL-1 and TNF-alpha and fibroblast-like synoviocytes which also have the ability to make cytokines **(Rodel *et al*., 1998).** These effects of infection in the joint may parallel

those at the initial site of infection, where chlamydiae induce cytokine production by epithelial cells**.**

However, even in situations where antigen persists, the immune system has mechanisms for downregulating immune responses appropriately so that inflammation is not sustained unnecessarily. These include the death of effector cells by apoptosis and the production of anti-inflammatory cytokines such as IL-4, IL-10, IL-13, and TGF-β. Determination of cytokines in arthritis patients has yielded important information about molecular mechanisms leading to and perpetuating synovitis, as highlighted by the breakthrough of anti-tumor necrosis factor alpha (anti-TNF alpha) therapies **(Braun *et al*., 1999; Feldmann *et al*., 1999; Kanik *et al*., 1998)**. However, conflicting data exist about the specificity of cytokine profiles for different arthritis forms. Whereas, in some studies, lower levels of cytokines were described for patients with ReA than with RA, others found higher levels and still others were unable to detect differences between arthritides at all **(Lettesjo *et al*., 1998; Schlaak *et al*., 1996; Steiner *et al*., 1995; Steiner *et al*., 1999)**. In a study conducted by **Bas *et al*., (2003)** higher interleukin (IL)-10 levels and interferon gamma (IFN-gamma) to IL-10 ratios were found in SF than RA patients. In most studies, there was no differentiation between patients with post-urethritic or post-enteritic ReA, nor were patients with spondylarthropathy and ReA separately analyzed **(Lettesjo *et al*., 1998; Schlaak *et al*., 1996; Steiner *et al*., 1995; Steiner *et al*., 1999).** In addition, the intra-articular presence of *C. trachomatis* was not determined by PCR. *C. trachomatis*-induced arthritis is characterized by persistent intra-articular *C. trachomatis* infection, and *C. trachomatis* infection of macrophages *in vitro* leads to marked secretion of cytokines such as IL-1 beta or TNF-alpha **(Rodel *et al*., 1998).**

In a study conducted by **Jendro *et al*., (2005),** the cytokine profile of *C. trachomatis induced*-ReA patients was compared with that of undifferentiated oligoarthritis patients in whom *C. trachomatis* infection was not identified. However, the two profiles were not different and authors concluded that determining these cytokines does not aid in differentiating between undifferentiated oligoarthritis patients with and without *C. trachomatis* infection as there was no significant differences were found in cytokines in both groups. Synovitis in *C. trachomatis*-induced arthritis, undifferentiated oligoarthritis and RA is cytokine-driven, without gross differences in the cytokine pattern; therefore, authors concluded that determination of these cytokines is not helpful for etiologic differentiation of these arthritis forms **(Jendro *et al*., 2005).**

c) Immune responses to chsp60

In addition to a physiological role in protein folding, extracellular hsp60 may be recognised by myeloid cells, resulting in the production of pro-inflammatory cytokines, in a manner similar to lipopolysaccharide **(Chen *et al*., 1999).** This effect has been shown for chsp60 **(Kol *et al*., 1999, 2000).** However, immune recognition of hsp60 is also very common in infection, particularly where intracellular organisms are concerned **(Gaston, 1997).** Studies of T-cell recognition of hsp60 in ReA have produced two major findings. Firstly, although it has been postulated that T-cells reactive to chsp60 might cross react with the human counterpart, precise mapping of the peptides (epitopes) within chsp60 which are recognised by CD4+ T-cells has so far not shown any evidence of cross reactivity with human hsp60. Secondly, in two instances the epitope identified in *C. trachomatis* hsp60 was identical or nearly identical in *C. pneumoniae* hsp60 **(Deane *et al*., 1997).**

1.5 Gaps in existing research

Epidemiological data suggest that *Chlamydia*-induced ReA is a more common condition than previously thought, and that clinicians often fail to recognize it **(Owlia and Eley, 2010; Carter *et al*., 2011)** also, asymptomatic uSpA patients without effusion are an additional challenge. However, due to its intracellular obligate nature, biphasic life cycle, multiple serovars, asymptomatic infection and lack of standardized diagnostic methods, there exists a diagnostic dilemma till date to diagnose this pathogen during acute or chronic infection in ReA/ uSpA patients and hence, it is underdiagnosed globally **(Senior, 2012).** There is need for standardization of diagnostic methodologies in terms of sensitivity, specificity and establish agreement between the molecular and non-molecular methods of diagnosis. Detection of *C. trachomatis* in non-invasive clinical specimens such as urine, serum and their concordance with molecular methods is also not explored. Furthermore, there is no reliable estimate on the exact frequency of *C. trachomatis*-induced ReA/ uSpA in India. Also, persistence and pathogenesis of *C. trachomatis* in ReA/ uSpA patients still needs to be explored for understanding the immunological mechanism whereby the bacteria escapes the immune system. In this regard, the Th-1/ Th-2/ Th-17 cytokine nexus during *C. trachomatis* infection in joint needs further studies. There is a lack of data on *C. trachomatis*- induced ReA and existing data is contraindicated **(Singh *et al*., 2011; Gracey and Inman, 2012; Eliçabe and Genaro, 2014).**

HLA-B27 plays an important role in host-pathogen interaction and is closely associated with disease chronicity, however, the role of genetic factor HLA-B27 is not completely elucidated. Also, chsp60 is the second most abundant protein secreted by host

cells during the persistent phase and its role needs further to be studied in ReA/ uSpA. Hence, this study reveals the association of *C. trachomatis* and ReA/ uSpA and adds towards a better understanding of *C. trachomatis* pathogenesis.

1.6 Aims and Objectives

The aims and objectives of the study were as follows:

1. **To determine the frequency of *Chlamydia trachomatis* infection in patients with reactive arthritis/ undifferentiated spondyloarthropathy:**
    - To undertake *C. trachomatis* screening in the synovial fluid/ urine/ serum of ReA/ uSpA patients for estimating the exact frequency of chlamydial infection in our patient population by targeting C. trachomatis chromosomal/ extra-chromosomal genes, localization of MOMP antigen and determination of circulatory/ localized anti-*C. trachomatis* antibodies
    - To determine the frequency of *C. trachomatis* infection in uSpA patients without effusion in non-invasive samples, viz.: urine and serum
2. **To compare molecular *versus* non-molecular methods of *Chlamydia trachomatis* diagnosis during the course of reactive arthritis/ undifferentiated spondyloarthropathy:**
    - To compare molecular/ non-molecular diagnostic techniques for detection of chlamydial infection in terms of sensitivity, specificity, positive predictive value and negative predictive value in ReA/ uSpA

- To find whether there is correlation between various methods of diagnosis for establishing reliable detection of *C. trachomatis* infection in ReA/ uSpA patients
- To find suitable diagnosis method for differentiating between acute and chronic *C. trachomatis* infection in ReA/ uSpA patients

3. **To investigate the association between circulatory inflammatory markers, *viz.: Chlamydia trachomatis* Heat Shock Protein 60 (chsp60) and high sensitive C-reactive protein (hsCRP) in ReA/ uSpA patients:**
    - To investigate whether the presence of chsp60 can serve as a disease prognostic/ predictive marker in ReA/ uSpA patients
    - To study the role of hsCRP in *C. trachomatis*-induced ReA/ uSpA

4. **To ascertain the expression of few Th1/ Th2/ Th17 cytokines, *viz.*: IFN-γ, IL-4, IL-17 and IL-6 in the immunopathogenesis of *C. trachomatis*-induced reactive arthritis/ undifferentiated spondyloarthropathy:**
    - To determine the expression of signature Th-1/ Th-2/ Th-17 cytokines, viz.: IFN-gamma, IL-4 and IL-17 for elucidating the pattern of immune response in *C. trachomatis*-induced ReA/ uSpA
    - To elucidate the role of the pleiotropic cytokine, viz.: IL-6 in the pathogenesis of *C. trachomatis*-associated ReA/ uSpA
    - To find the effect of the presence of HLA B27 on the cytokine expression in *C. trachomatis*-induced ReA/ uSpA

Chapter-2

MATERIALS AND METHODS

Materials and methods

2.1 Enrollment of patients

A total of 150 arthritic patients with ReA/ uSpA/ RA and OA were enrolled from Out-Patients' Department (OPD) of Central Institute of Orthopedics (CIO), Safdarjung hospital, New Delhi, India and Department of Rheumatology and Clinical Immunology, Army hospital *(Research and Referral)*, New Delhi, India **(Fig. 2.1).**

2.2 Ethics statement

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 arthritic patient was in no way influenced by the outcome of the test performed.

2.3 Detailed history and data collection

The detailed history of each patient, *viz*.: age, disease duration, extra-articular manifestations, family history of arthritis was recorded in a standardized questionnaire that included details of urogenital infection and treatment, namely NSAIDs/ antibiotics/ steroids taken during the current or previous infection(s). Female patients with pregnancy or any gynecological disorder were not included in the study.

2.4 Inclusion criteria

(a) European Spondyloarthropathy Study Group (ESSG) criteria

The ESSG criteria included both clinical and radiographic features and has been demonstrated to be 87% sensitive and specific for the diagnosis of spondyloarthropathy (**Dougados *et al*., 1991**). It is somewhat less well suited for the diagnosis of early disease

but is extremely useful for the established disease. In 1995, the Amor criteria was further developed for

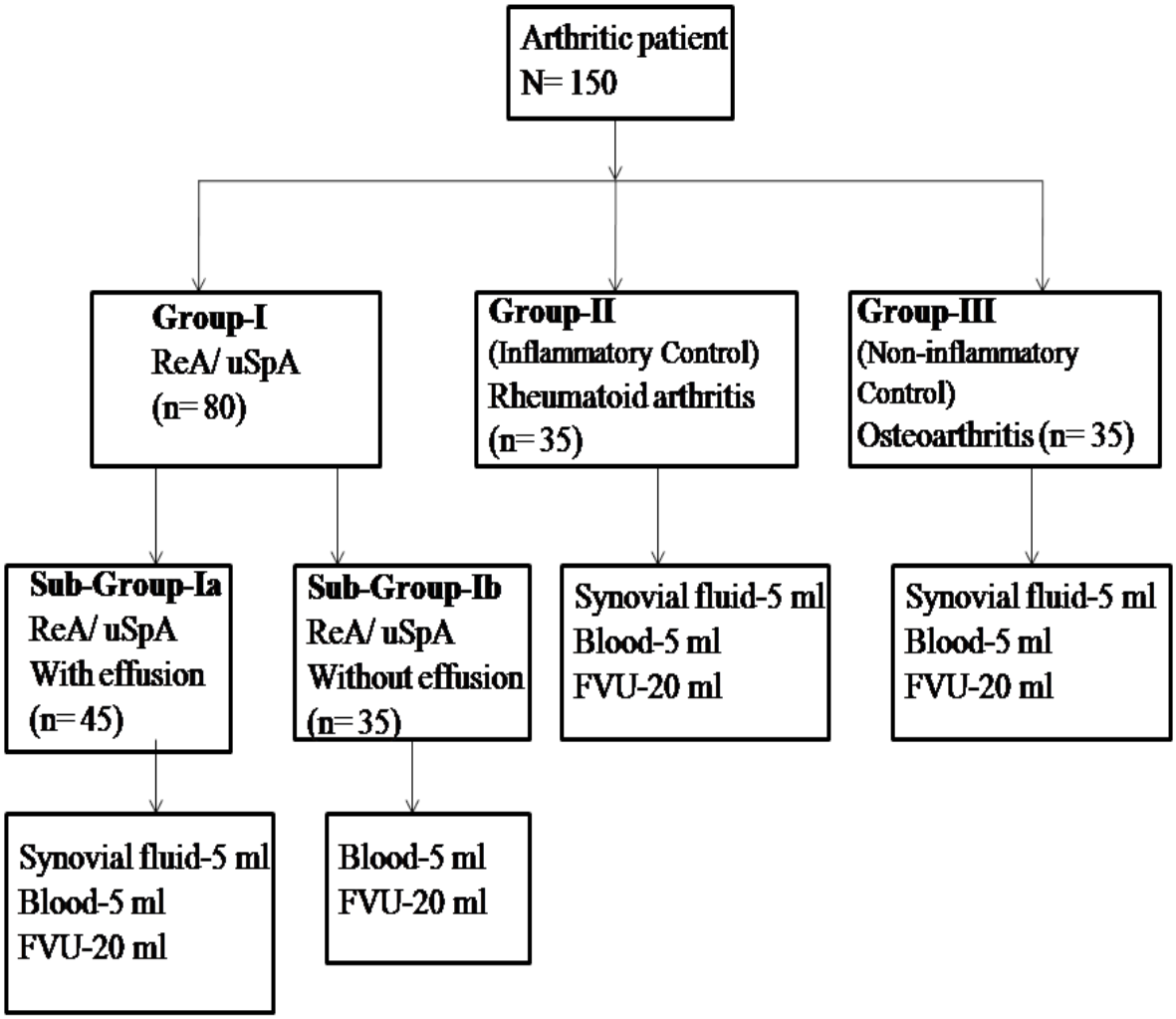


Fig. 2.1: Flow chart of sample collection in arthritic patients.

Abbreviations: ReA- Reactive arthritis patients; uSpA- undifferentiated spondyloarthropathy patients; FVU- First void urine

spondyloarthropathy, which employed many of the same clinical and radiographic features as the ESSG criteria, but also included HLA-B27 as part of the diagnostic panel **(Amor et al, 1995)**.The subjects could be male or female, at least 18 years of age and symptoms had to be present for at least 6 months.

Subjects had to meet the following inclusion criteria:

A. Inflammatory spinal pain (spinal pain in back, dorsal or cervical region, with at least four of the following:

(a) onset before the age of 45,

(b) insidious onset,

(c) improved by exercise,

(d) associated with morning stiffness,

(e) at least 3 month duration

Or

B. Synovitis (inflmmation of joint, effusion) and one or more of the following (criteria C-G)

C. Positive family history (presence in first-degree or second-degree relatives of any of the following:

(a) ankylosing spondylitis (Incidous inflammatory back pain radiate to gluteal region)

(b) psoriasis (skin manifestations precede joint Involvement)

(c) acute uveitis (Inflammation in eye)

(d) reactive arthritis (arthritis due to enteric or genitourinary infection)

(e) inflammatory bowel disease (inflammatory conditions of the colon and small intestine)

D. Urethritis (Burning in micturation or Inflammation of urethra) or cervicitis within 1 month before arthritis

E. Alternating buttock pain (pain alternating between the two buttocks)

F. Enthesopathy (past or present spontaneous pain or tenderness on examination of the site of the insertion of the Achilles tendon or plantar fascia)

G. Sacroiliitis (inflammation of sacroiliac joint, at least grade 2 unilateral; radiographic grading system: 0 = normal, 1 = possible, 2 = minimal, 3 = moderate, 4 = ankylosis)

(b) American College of Rheumatology (ACR) criteria (**Arnett *et al*., 1988**)

ACR criteria was followed for the enrollment of RA patients.

(c) Inclusion criteria for Osteoarthritis

Enrollment of OA patients was done on the basis of clinical and radiological evidence.

2.5 Exclusion criteria

Patients with history of tuberculosis, ankylosing spondylitis, inflammatory bowel disease, psoriasis and with history of preceding enteric infection were excluded. Also, patients who received antibiotic treatment in the previous 03 months were not included in the study. Exclusion criteria also included use of anti-coagulates, current pregnancy (in females) and previous prolonged exposure to antibiotics (> 2 weeks) as a specific therapy for possible ReA.

2.6 Categorization of arthritic patients

Arthritic patients **(N- 150)** enrolled in the study were divided as follows:

- **Group I (Study Group):** ReA/ uSpA patients **(n=80)**
  - **Sub-group I a:** ReA and uSpA patients with effusion **(n= 45)**

- **Sub-group II b:** uSpA patients without effusion **(n=35)**

- **Group II (inflammatory control group):** Rheumatoid Arthritis (RA) patients **(n= 35)**
- **Group III (non-inflammatory control group):** Osteoarthritis patients (OA) **(n= 35)**

2.7 Collection of clinical specimens from arthritic patients

SF, non-heparinized venous blood and First Void Volume (FVU) of urine were collected from ReA/ uSpA/ RA/ OA patients **(Fig. 2.1).** Details are as follows:

2.7.1 Synovial fluid

Approximately, 5-7 ml of SF was aspirated from knee joints of patients in sub-group I a, and groups II - III by the clinician under aseptic conditions using local anesthesia. Storage of the SF (neat) was done in aliquots, for performing DNA extraction, DFA, cytokine estimation, etc. to avoid frequent freezing and thawing. The SF showing RBC contamination was not processed further. SF was centrifuged at 20916 Relative Centrifugal Force (RCF) for DNA isolation. Subsequently, the cell pellet and the supernatant were collected and stored at -80^{0}C for use in various assays.

2.7.2 Blood

5 ml of non-heparinized venous blood was collected in sterilized vials. Serum was separated and stored at -20^{0}C in aliquots for use in different assays.

2.7.3 Urine

FVU of morning urine sample (20 ml) was collected and centrifuged at 13386 (RCF). The pellet was washed twice by phosphate buffered saline, centrifuged and stored at -20^{0}C.

2.8 Molecular detection of *Chlamydia trachomatis* infection

2.8.1 DNA isolation from synovial fluid

DNA was extracted from the SF of arthritic patients in sub-group I a and in groups II - III by using a commercial kit- QIA Amp DNA Blood Mini kit *(Qiagen, Hilden, Germany)* as per the manufacturer's guidelines. Briefly, 500 µl of SF 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 in washing buffer and eluted.

2.8.2 Viral RNA isolation from urine

Nucleic acid isolation was done in the urine of groups I - III patients by using commercial RNA kit, *(QIAamp Viral RNA mini kit, Hilden, Germany)* as per the manufacturer's guidelines. For the assay, 560 µl of AVL buffer and 5.6 µl carrier RNA were added followed by 140 µl of urine. Subsequent to incubation and centrifugation, 560 µl of ethanol (96 - 100%) was added, 630 µl solution was transferred to a mini-column and centrifuged at 5949 RCF. After discarding the tube containing the filtrate, the above procedure was repeated. Thereafter, 500 µl washing buffer was added and centrifugations were carried out at 5949 RCF and 18220 RCF. Finally, 60 µl of AVE buffer was added

and centrifuged at 5949 RCF. The final elution was obtained in two fractions of 40 µl each.

2.8.3 Qualitative and quantitative analysis

Quantification of DNA was done by running 0.2% gel to ensure the quality of DNA on a nano-drop spectrophotometer *(Fermentas-Thermo Fisher Scientific, Waltham, USA)* at 260 nm **(Fig. 2.2a)** while for qualitative check **(Fig. 2.2b)**, DNA samples were screened for the presence of human beta-globin gene (**Nikkari *et al*., 1994)**, while for negative control, the DNA template was not added to the reaction mixture. Negative control was included in each set of reaction.

2.8.4 Selection of primers for PCR

Endogenous plasmid sequence of *C. trachomatis* (200 bp) was targeted to detect the infection in urine specimens by conventional PCR **(Joyee *et al*., 2003).** The *C. trachomatis*-specific chromosomal MOMP gene and the extra-chromosomal plasmid gene were targeted for semi-nested (snPCR) and nested PCR (nPCR) assays, respectively. The sequences for primer pairs of *C. trachomatis* MOMP **(Palmer et al., 1991)** and endogenous plasmid **(Bas *et al*., 1995; Griffais and Thibon 1989)** were taken from published literature for the commercial synthesis of primers by *m/s Biolink, New Delhi, India.* List of primers for molecular diagnosis of *C. trachomatis* in SF and urine of arthritic patients is given in **Table 2.1.**

Table 2.1: Primers for molecular diagnosis of *Chlamydia trachomatis* in synovial fluid and urine of arthritic patients

Genes of *C. trachomatis*	Primer sequences: 5'-3'	Amplicon Size	References
Plasmid (nPCR in SF)	TAGTAACTGCCACTTCATCA (A) *(Outer PCR)* TTCCCCTTGTAATTCGTTGC (B)	201 bp	*Griffais and Thibon, (1989)*
	CCACCTTGAAAATCAGAAGT (A') *(Inner PCR)* CTTTGGATAGCTGCTAATGC (B')	141 bp	*Bas et al., (1995)*
MOMP (snPCR in SF)	TAT ACA AAA ATG GCT CTC TGC TTT AT *(Outer PCR)* CCC ATT TGG AAT TCT TTA TTC ACA TC	537 bp	*Palmer et al., (1991)*
	CCC ATT TGG AAT TCT TTA TTC ACA TC *(Inner PCR)* TTG CCA GAC AAT CCT CAG GC	380 bp	*Palmer et al., (1991)*
Endogenous plasmid gene (conventional PCR in urine)	CTA GGC GTT TGT ACT CCG TCA TCC TCA GGA GTT TAT GCA CT	200 bp	*Joyee et al., (2003)*
Beta globin (conventional PCR in each DNA sample)	CAA CTT CAT CCA CGT TCA CC GAA GAG CCA AGG ACA GGT AC	268 bp	*Nikkari et al., (1994)*

Abbreviation: MOMP- major outer membrane protein of *Chlamydia trachomatis*

To exclude possible false negative results caused by inhibitory factors, the presence of human DNA in the SF was verified by collateral amplification of human

beta-globin gene using primers **(Nikkari *et al*., 1994)** which amplified a 268 bp segment of human beta-globin encoding genome **(Fig. 2.2).**

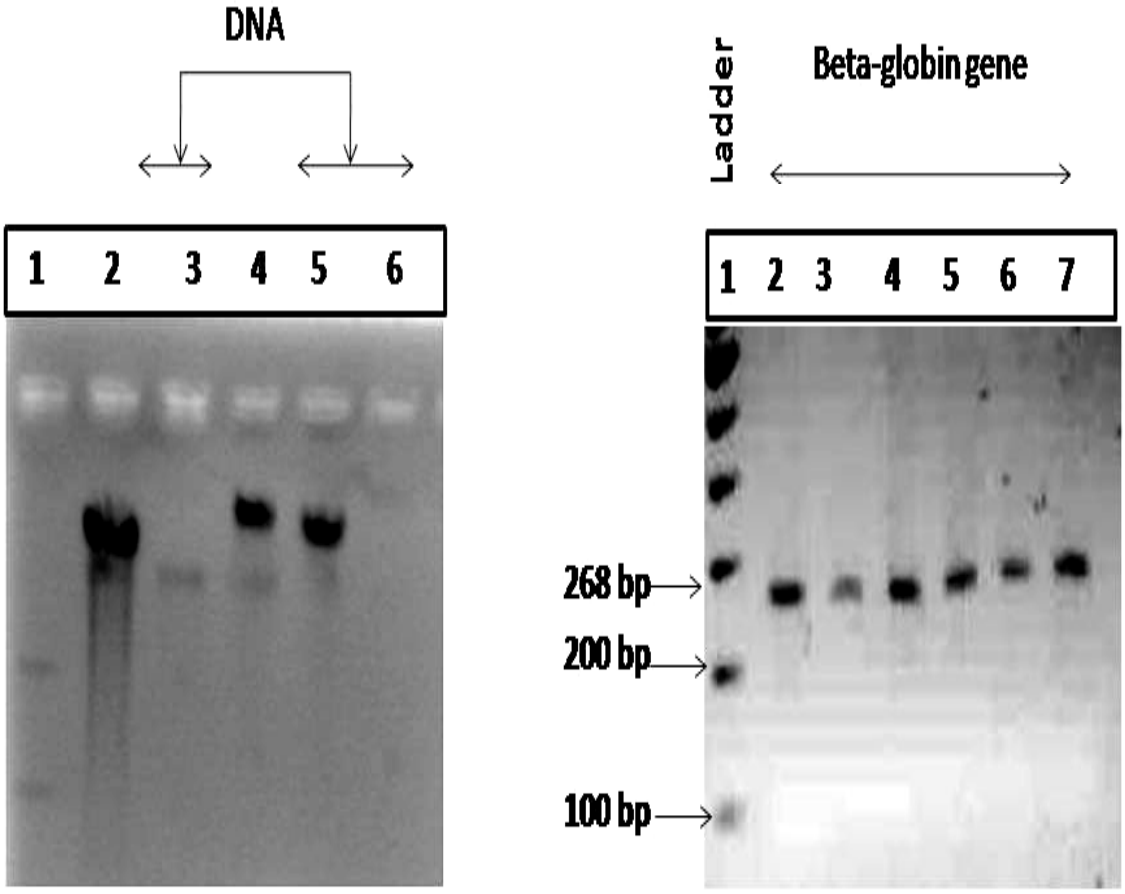


Fig. 2.2: Agarose gel electrophoresis products visualized under UV-Transilluminator.

(a) Qualitative analysis of purified DNA in synovial fluid (lane 2, 4, 5 had DNA while in lane 1, 3, 6 DNA was absent

(b) Lanes 2 - 7 shows different clinical specimen DNA with beta-globin gene (268bp) by PCR

2.9 Nucleic acid amplification tests

PCR assays were performed using thermal cycler *(Applied Bio System, USA)*. The amplicons were detected by performing electrophoresis of the amplified product in an ethidium bromide-stained 1.2% agarose gel. A one kb DNA ladder *(Bangalore Genei, Bangalore, India)* was used as the DNA size standard. The DNA was visualized on an UV-Transilluminator.

2.9.1 Detection of *Chlamydia trachomatis* endogenous plasmid in urine by polymerase chain reaction (conventional) assay

Endogenous plasmid gene sequence of *C. trachomatis* (200 bp) **(Joyee *et al*., 2003)** was targeted to diagnose infection in urine specimens of groups I - III. The target DNA was amplified using 0.5 µl each of 10 pmole/ µl of the forward and reverse primers in 25 µl volume containing 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH-8.4), 0.2 mM dNTPs *(Fermentas-Thermo Fisher Scientific, Waltham, USA)* 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.

2.9.2 Detection of *Chlamydia trachomatis* chromosomal MOMP gene in synovial fluid by semi-nested polymerase chain reaction assay (snPCR)

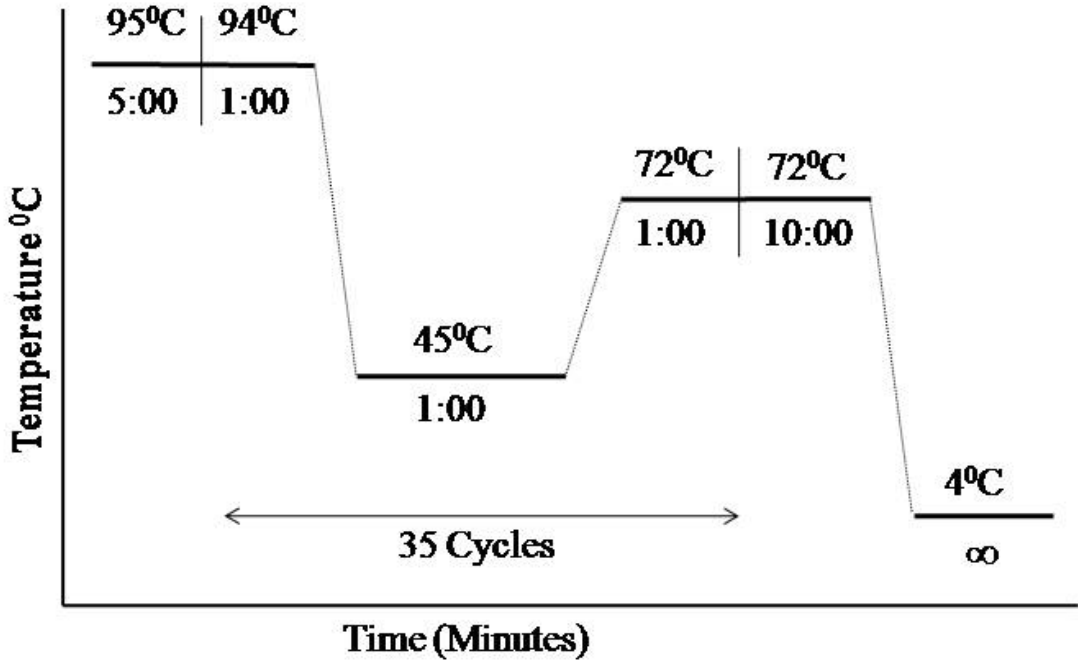
snPCR was performed to detect *C. trachomatis* MOMP, in which a 380 bp DNA fragment was amplified in sub-group I A and groups II - III. The oligonucleotide primers were used to amplify a fragment within the conserved region of the MOMP gene of *C.*

trachomatis. The target DNA was amplified using 0.5 µl each of 10 pmole/ µl of the primers HP-1 and HP-2 **(Palmer *et al.*, 1991)** in 25 µl volume containing 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH- 8.4), 0.2 mM dNTPs, 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 using 4 µl of PCR product from the first reaction as a template **(Table 2.2, Fig.2.3).**

Table 2.2: PCR reaction mixture (25 µl) for semi-nested PCR

Distilled water (molecular grade)	14.5 µl
$MgCl_2$ (2 mM)	1.5 µl
KCl (50 mM) & Tris-HCl (10 mM) (pH - 8.4)	2.5 µl
dNTPs (0.2 mM)	0.5 µl
Forward primer (10 picomole/ µl)	0.5 µl
Reverse primer (10 picomole/ µl)	0.5 µl
DNA Taq polymerase (0.4 unit)	0.25 µl
Template DNA	5 µl

Fig 2.3: PCR conditions for semi-nested PCR



2.9.3 Detection of *Chlamydia trachomatis* extra-chromosomal plasmid gene in synovial fluid by nested polymerase chain reaction assay (nPCR)

Primers that amplify a plasmid sequence of 201 bp **(Griffais and Thibon, 1989)** were used for the first amplification in sub-group I a and in groups II - III. 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 $MgCl_2$, 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, 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 thereafter for 30 seconds for each cycle, annealing was done at 57°C for 1

minute while extension was done for 45 seconds, 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 were added **(Bas *et al.*, 1995)**. Samples were further subjected to 35 cycles under the same conditions except for the initial denaturation, which was done for 1 minute **(Table 2.3, Fig. 2.4).**

2.9.4 Sequencing of PCR products

After confirmation of intra-articular *C. trachomatis* infection by snPCR/ nPCR, the PCR products were subjected to sequence analysis. A commercial QIAquick Gel Extraction kit *(Qiagen, Hilden, Germany)* was used to extract DNA from the agarose gel. DNA fragment was excised from the gel with a clean, sharp scalpel and the gel slice was collected in a colourless tube after weighing. Purified product was used for DNA sequencing in an Automated Sequencer *(ABI PRISM 3130 XL, Foster City, CA)*. Resulted sequences were blasted in NCBI through non-redundant blast against *C. trachomatis* genome database..

2.10 Non-molecular detection of *Chlamydia trachomatis* infection

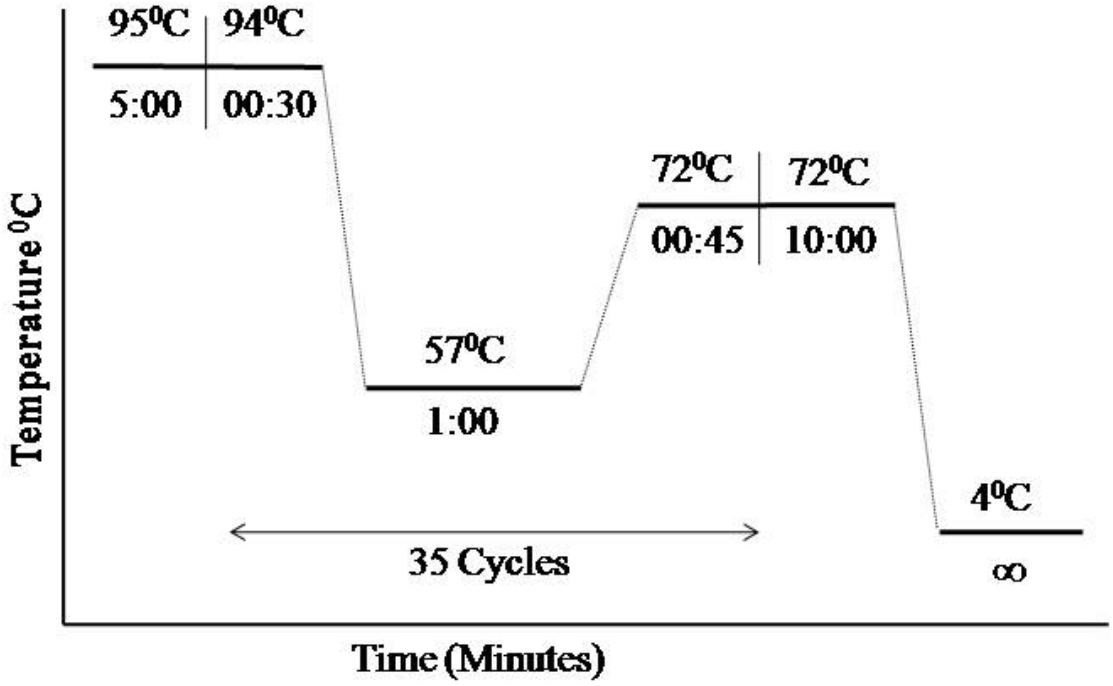
2.10.1 Detection of *Chlamydia trachomatis* antigen in synovial fluid (sub-group I a and groups II - III) and urine (groups I - III) by immunofluorescence microscopy

After centrifugation at x 600 g for 10 minutes, the cell deposit from SF/ urine was spun onto clean glass microscope slides using Cytospin *(Shandon, Astmoor Run, UK)*.

Table 2.3: PCR reaction mixture for nested PCR

Distilled water	14.5 µl
$MgCl_2$ (25 mM)	1.5 µl
10X Buffer (KCl (50 mM) & Tris-HCl (100 mM) (pH -8.4) nonidet P40)	2.5 µl
dNTPs (2 mM)	0.5 µl
Forward primer (10 picomole/ µl)	0.5 µl
Reverse primer (10 picomole/ µl)	0.5 µl
DNA Taq polymerase (0.4 Units)	0.25 µl
Template DNA	5 µl

Fig 2.4: PCR conditions for nested PCR



Four slide preparations were made from each sample for detection of *C. trachomatis* antigen by Direct Fluorescence Assay (DFA) using a commercial MicroTrak *C. trachomatis* Direct Specimen Test Kit *(Trinity Biotech, Jamestown, NY, USA)* as per the manufacturer's recommendations. Briefly, slides were fixed in methanol and subsequently incubated at room temperature with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to *C. trachomatis* Major Outer Membrane Protein (MOMP). Evans blue dye was used as control for autofluorescence while FITC-conjugated monoclonal antibody to herpes simplex was used as another control antibody. For confirmation of morphology, a total of 04 slides were prepared for each patient. Each slide was read by 02 independent observers after blinding. Each observer counted Elementary Bodies (EBs) in 05 fields per slide using 100 x oil objective and calculated the average number of EBs. Diagnosis of *C. trachomatis* was based on the presence of a minimum of 07 or more chlamydial EBs per slide which appeared as pinpoints of bright green fluorescence. Likewise, enumeration and an average count of EBs were made by each observer in each of the 04 slides.

2.10.2 Detection of IgM, IgG and IgA antibodies to *C. trachomatis* in synovial fluid and serum by ELISA

ELISAs were performed to detect both circulatory and localized antibodies to *C. trachomatis* in patients belonging to sub-group I a and in groups II – III.

2.10.2.1 Anti-*C. trachomatis* IgM and IgG antibodies

The presence of IgM & IgG antibodies to *C. trachomatis* was estimated in SF and serum using commercial ELISA kit purchased from IBL International *(IBL, Hamburg, Germany)* as per the manufacturer's guidelines. Before the assay, all samples were diluted 1 + 100 with IgM/ IgG sample diluent and then dispensed as follws: 10 µl sample and 1 ml IgM/ IgG sample diluent into tubes for obtaining 1+100 dilution; and vortexed. 100 µl controls and diluted samples (in duplicate) were dispensed into the respective wells. Well A1 was left for the substrate blank. After incubation for 1 hour ± 5 minutes at 37 ± 1°C, each well was washed thrice with 300 µl of washing solution. 100 µl *C. trachomatis* anti-IgM/ IgG conjugate was dispensed into all wells except for the blank well and incubated for 30 minutes at room temperature. After incubation, each well was washed thrice with 300 µl of washing solution. 100 µl TMB substrate solution was dispensed into all wells and incubated for 15 minutes at room temperature in the dark. Thereafter, 100 µl stop solution was dispensed into each well. The blue colour developed during the incubation was found to turn into yellow. The absorbance of all wells was measured at 450 nm with reference to 620 nm within 30 minutes after addition of the stop solution (Labsystem, Multiskan ELISA reader, India). The absorbance values for each control and patient sample were thus recorded. The cut-off was the mean absorbance value of the cut-off control determinations. Samples were considered positive when the absorbance value was higher than 10% over the cut-off **(Table 2.4).**

Units = patient (mean) absorbance value x 10/ cut-off

2.10.2.2 Anti-*C. trachomatis* IgA antibodies

The estimation of anti-chlamydial IgA antibodies was done by a commercial anti-*C. trachomatis* IgA antibodies detection kit *(Savyon Diagnostics, Ashdod, Israel)* as per the manufacturer's guidelines. Each patient serum was diluted in 1/21 ratio with the supplied serum diluent. 50 µl of negative control and positive control and diluted specimens were added to wells. The plate was covered and incubated for 1 hour at 37°C at 100% humidity. Thereafter, wells were washed thrice with wash buffer. 50 µl of 1/300 diluted HRP-conjugate was added to each well and incubated for 1 hour at 37°C at 100% humidity. The washing step was again repeated with wash buffer.100 µl of TMB-substrate was added to each well and the plate was incubated for 15 minutes at room temperature. 100 µl of stop solution was added to each well and the absorbance was recorded at 450 nm. The cut-off value (COV) was calculated for *C. trachomatis*-specific IgA antibodies according to the formula: COV= NC x 2, where NC= average absorbance at 450 nm of the negative control run in duplicate, while the cut-off index (COI) was calculated according to the formula: COI= absorbance of serum sample at 450 nm divided by COV. Patients with COI > 1.1 were considered as positive, COI < 1.0 as negative, while those with COI between 1 - 1.1 were borderline cases **(Table 2.4).**

2.11 Detection of circulatory IgG antibodies to chlamydial heat shock protein 60 in serum of undifferentiated spondyloarthropathy patients without effusion and control patients

Recombinant enzyme immunoassay was performed for the detection of IgG antibodies to chsp60 in the serum by using commercially available ELISA kit (*Medac,*

Hamburg, Germany) as per the manufacturer's instructions. For the assay, 50 µl of each diluted serum sample (1: 50 with sample diluent)/ control was pipetted in wells and incubated at 37^0C.

Table 2.4: Standard cut-off value and performance of ELISA kits for determining antibodies to *Chlamydia trachomatis* (As per manufacture instructions)

Anti-*C. trachomatis* antibodies	**Cut-off indices**	**Result**	**Sensitivity**	**Specificity**	**Interference (mg/ml)**
IgM	<9 U 9-11 U >11 U	Negative Borderline Positive	83.3%	>95%	10*/5**/0.2#
IgG	<9 U 9-11 U >11 U	Negative Borderline Positive	>95%	91.3%	10*/5**/0.2#
IgA	<1.1 COI 1 - 1.1 COI >1.1 COI	Negative Borderline Positive	95%	90%	**ns**

Abbreviations:
U- units; COI- cut-off indices; ns- not specified.
Interferences with hemolytic, lipemic or icteric sera
*10 mg/ ml hemoglobin,
**5 mg/ ml triglycerides
0.2 mg/ ml bilirubin

Each sample was used in duplicate. Thereafter, wells were washed and 50 µl of conjugate was added followed by second incubation. Following this, wells were washed, 50 µl of TMB-substrate was added and incubated. Finally, 100 µl stop solution was added to each well and the Optical Density (OD) was read at 450 nm. Qualitative, semi-quantitative and quantitative analysis of OD values was done. Cut-off index values were calculated by

dividing OD values by OD cut-off. Cut-off was calculated by adding 0.350 to mean OD value of the negative control. Interpretation of results was done as shown in **Tables 2.5 – 2.7.**

Table 2.5: Qualitative analysis of IgG antibodies to chsp60*

Results	**Valuation**
OD < grey zone	negative
OD cut-off ± 10%	equivocal
OD > grey zone	positive

- The OD value of the blank has to be < 0.100.
- The mean OD value of the negative control has to be < 0.100.
- The OD value of the positive control has to be > 0.800.
- Cut-off = mean OD value of the negative control + 0.350
- Grey zone = Cut-off ± 10 %

***** Recombinant enzyme immunoassay for the detection of IgG antibodies to chsp60 (*Medac, Hamburg, Germany)*

Table 2.6: Semi-quantitative analysis of IgG antibodies to chsp60*

Cut-off Index: OD sample/ OD cut-off	**Valuation**
< 0.9	negative
0.9 – 1.1	equivocal
> 1.1	positive

***** Recombinant enzyme immunoassay for the detection of IgG antibodies to chsp60 (*Medac, Hamburg, Germany)*

Table 2.7: Quantitative analysis of IgG antibodies to chsp60*

Cut-off Index	IgG	
	Result	**Titer**
<0.90	Negative	<1: 50
0.90 - 1.10	Equivocal	
1.11 - 1.80	Positive	1: 50
1.81 - 3.60	Positive	1: 100
3.61 - 7.20	Positive	1: 200

***** Recombinant enzyme immunoassay for the detection of IgG antibodies to chsp60 (*Medac, Hamburg, Germany)*

Note: Calculation of the endtiter was done using following formula:

1: endtiter = 55.5 x (OD/ cut-off)

2.12 Quantitative detection of high sensitive C-reactive protein (hsCRP) in serum of undifferentiated spondyloarthropathy patients without effusion and control patients

ELISA was performed for the quantitative estimation of hsCRP in the serum of uSpA/ RA/ OA patients by using commercial kit *'CRP high sensitive ELISA' (IBL International, Hamburg, Germany)*. Samples and standards were diluted according to manufacturer's instructions. Briefly, 100 µl of sera was added to each well and incubated. Wells were washed and 100 µl of conjugate was thereafter added followed by incubation. After washing, chromogen was added to each well and finally stop solution was added. OD was read at 450 nanometer. The average absorbance value of each calibrator was plotted against the corresponding hsCRP value and the best calibration log curve was constructed. The average absorbance value of each calibrator was plotted against the

corresponding CRP-value and the best calibration curve (e.g. log/ linear) was constructed. Average absorbance value was used for each patient sample obtained in the hsCRP ELISA to determine the corresponding value by simple interpolation from the curve. The minimal detectable concentration of ELISA was approximately 0.02 µg/ ml **(Fig. 2.5).**

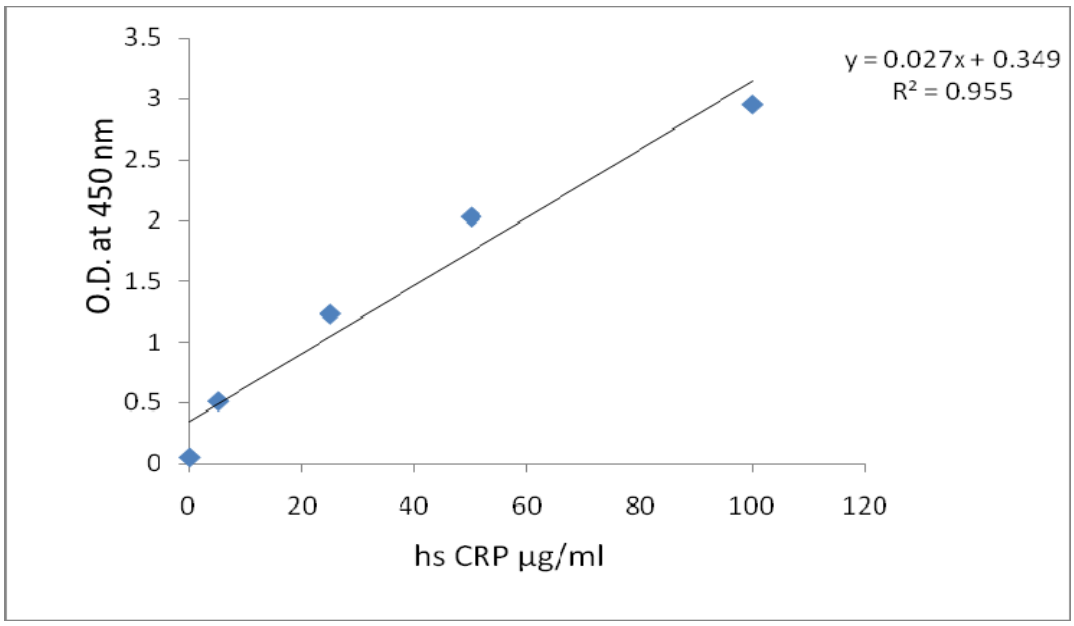


Fig. 2.5: Standard curve obtained by plotting the commercial hsCRP absorbance values of standards

'CRP high sensitive ELISA' (IBL International, Hamburg, Germany) kit

2.13 Total cell count

The total cell count was performed in the SF using the neubauer chamber (haemocytometer). Cell suspension (100 µl) was prepared and stained by tryptophan blue (100 µl). Grid lines of the hemocytometer were focused using 10X objective of the microscope. Healthy unstained cells were counted. The count was done as follows: total count from 4 sets of 16 corners = (cells/ ml x10^4) x 4 squares from one hemocytometer grid. Divided the count by 4 and then multiplied by 2 to adjust for the 1:2 dilutions in trypanblue.

2.14 Detection of HLA B27 gene in synovial fluid DNA

Commercial kit, *viz.:* Morgan TM HLA SSP B27 Typing Kit (*Texas BioGene, USA*) was used for determining HLA alleles using PCR technique with Sequence Specific Primers (SSP) as per the manufacturer's instructions. Allele sequence specific primer pairs were designed to selectively amplify target sequences specific to a single allele or group of alleles. In addition to sequence specific primers, an internal control primer pair, which amplified a conserved region of the house keeping gene, was included in every PCR reaction mix, and the PCR product of the internal control primer pair served as an indication of the integrity of PCR reaction. 10 µl of the Master Buffer/ Taq polymerase (0.35 U/ 10µl) mixture was taken into each reaction tube of the Typing Tray and was diluted (10 - 40 ng/ µl). DNA sample (2 µl) was added into the reaction tube of the Typing Tray. This was followed by centrifugation, PCR and gel electrophoresis. Extreme precautions were taken to avoid cross-contamination between wells by applying the master mix to the walls of the tube **(Table 2.8).**

Table 2.8: PCR conditions for detection of HLA B27 gene*

Steps	**Cycle Number**	**Temperature**	**Time**
1	1	96^0 C	2.5 minutes
2	10	96^0 C	15 seconds
		65^0 C	60 seconds
3	22	96^0 C	15 seconds
		62^0 C	50 seconds
		72^0 C	30 seconds
4	1	4^0 C	until removed

***** Morgan TM HLA SSP B27 Typing Kit (*Texas BioGene, USA*)

2.15 Estimation of Th1/ Th2/ Th17 and IL-6 cytokines

Commercial ELISA kits were used to estimate IFN-gamma (*eBiosciences, USA*), IL-4 (*eBiosciences, USA*), IL-6 (*BD Biosciences, USA*) and IL-17 (*Krishgen BioSystems, CA*) in arthritic patients as per the manufacturer's instructions. Detection limit of these kits were as follows: IFN-gamma: 4 - 500 pg/ ml, IL-4: 2 - 200 pg/ ml, IL-6: 2.2 - 300 pg/ ml, IL-17: 15.6 - 1000 pg/ ml.

Briefly, the ELISA plate was coated with 100 µl/ well of capture antibody and incubated overnight at 4°C. Subsequently, wells were washed, blocked with 200 µl/ well of blocking buffer, incubated and washed. Thereafter, 100 µl standards were added to each well. Two -fold serial dilutions of the top standards was performed to make the standard curve as per the instructions in the kit manual. After adding 100 µl/ well of samples, the plate was incubated, washed and Detection antibody was added to each well and incubated. Thereafter, wells were washed and 100 µl/ well of Avidin-HRP was added to each well and incubated. 100 µl/ well of Substrate Solution was then added to each well and incubated. Finally, 50 µl of Stop solution was added to each well and the plate was read at 450nm.

2.16 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/ RA/ OA were compared by Fisher's exact test. A 'p' value less than or equal to 0.05 was considered to be significant. Odds ratios, sensitivity, specificity, Positive

Predictive Value (PPV) and Negative Predictive Value (NPV) were also calculated to evaluate the diagnostic efficacy. Mean and standard deviations were derived from the numerical data using Column statistics. The degree of agreement was assessed by performing Kappa statistics **(Viera & Garrett, 2005)**. The Kappa values ranging from 0.01 - 0.20 were considered as slight agreement, from 0.21 - 0.40 as fair agreement, from 0.41 - 0.60 as moderate agreement, from 0.61 - 0.80 as substantial agreement and from 0.81 - 0.99 as almost perfect agreement.

For the prediction of post-test probability, Nomograph was created which can determine diagnostic test characteristics (sensitivity, specificity, likelihood ratios) and/or determine the post-test probability of of developing ReA/ uSpA due to *C. trachomatis* infection. A Nomograph was created using the following online analytical tool:

(http://araw.mede.uic.edu/cgibin/testcalc.pl?DT=&Dt=&dT=&dt=&2x2=Compute)

Clinical data, median values and interquartile range were calculated by Colum statics. Non-parametric Spearman's rank correlation test was performed to calculate correlations between different groups. Fisher's Exact test was used to test contingency tables. One-way ANOVA was performed using Kruskal-Wallis test to compare various groups. Differences in cut-off index medians were calculated between the study and control group by non-parametric Mann-Whitney test. Clinical data was analysed by Colum statics. '*p*' value less than <0.05 was considered as significant.

Chapter 3

DETECTION OF CHLAMYDIA TRACHOMATIS INFECTION IN REACTIVE ARTHRITIS/ UNDIFFERENTIATED SPONDYLOARTHROPATHY PATIENTS

3.1 Introduction

The obligate intracellular pathogen *C. trachomatis* has emerged as a major causative agent in genitourinary ReA in various countries **(Zeidler *et al*., 2004; Senior, 2012)** and it has been reported that *C. trachomatis* is present in a metabolically active form during the remitting phase in synovial tissues from patients with chronic *Chlamydia*-induced ReA **(Gerard *et al*., 2013)**. Patients with uSpA may or may not present with typical clinical symptoms like synovitis in joints. Broadly, the only difference with ReA is the absence of urogenital symptoms. *C. trachomatis* causes chronic infection and it becomes persistent and untreatable in joint and this asymptomatic form makes this pathogen an unrecognized cause of ReA **(Senior, 2012).** Intra-articular persistence of viable, although non-culturable, *C. trachomatis* is considered to be the cause of arthritis **(Carter and Hudson, 2010)**. Urogenital infection is often asymptomatic (in up to 80% of women and 40% of men), making treatment difficult **(Gaydos *et al*., 2004)**. Also, the intracellular localization creates an additional challenge for diagnosis of *C. trachomatis* in asymptomatic and in chronic or persistent infections where the pathogenic load is low.

In developing countries, enterically acquired ReA is more common and studies from India have also largely focused on patients with ReA due to enteric pathogens **(Misra, 2008)** and there is a paucity of data on genitourinary-induced ReA. In an earlier study from India, the clinical profile and prevalence of serum immunoglobulin A antibodies against a panel of bacterial antigens, including *C. trachomatis*, in the sera of patients with ReA was reported **(Aggarwal *et al*., 1997)**. Another study from north India reported the prevalence of serum IgA antibodies to *C. trachomatis* in gReA patients as

25% **(Chandrashekhra, 2004)**. As a high prevalence of genitourinary infection with *C. trachomatis* has been reported in our country **(Mittal *et al*., 1995, 2004; Rastogi *et al*., 1999, 2000, 2002, 2003)**, there is a possibility that the association of *C. trachomatis* in genitourinary-induced ReA may well be currently overlooked and there is a need to uncover the magnitude of chlamydial infection in such patients.

No specific recommendations currently exist at the national or international level defining specific clinical and/or laboratory investigations for tests to diagnose *C. trachomatis* induced ReA **(Braun *et al*., 2000; Zeidler and Hudson, 2013).** 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 *C. trachomatis* components such as major outer membrane protein (MOMP), rRNA and plasmid DNA in the affected joints of ReA patients **(Keat *et al*., 1987; Bas *et al*., 1995; Freise *et al*., 2001; Poole and Lamont, 1992; Kuipers *et al*., 2009; Siala *et al*., 2009; Carter *et al*., 2009; Gerard *et al*., 2010).**

Various molecular as well as non-molecular techniques have been used for diagnosis of *C. trachomatis* in the synovial joints of patients with ReA **(Bas and Vischer, 1998**, **Nelson and Helfan, 2001).** Although the isolation of *C. trachomatis* by culture has been largely unsuccessful in ReA **(Beutler *et al*., 1997)** *C. trachomatis* DNA/ RNA/ antigen/ proteins have been detected in the joints **(Taylor-Robinson *et al*., 1992; Bas *et al*., 1995; Wilkinson *et al*., 1998).** PCR is considered to be the most promising tool for

routine diagnosis of *C. trachomatis* infection, yet the results vary between different laboratories. Further, the method used for detection of *C. trachomatis* in the synovium is still a matter of choice as various workers have used different methodologies for detection in various settings and there is no uniformity with regard to sampling and methodology used for detection. In this regard, the use of direct immunofluorescence assay for detection of *C. trachomatis* probably stands out as a more convenient and cost-effective method of initial diagnostic testing and may assist in the detection of *C. trachomatis* in instances where specimens have lost viable chlamydiae through prolonged transport or sub-optimal storage**.**

Direct detection of *Chlamydia* usually relies on the examination of specimens collected from the site of primary infection **(Wollenhaupt *et al*., 1995; Sieper *et al*., 2001; Wollenhaupt and Zeidler, 1998).** Several PCR assays have been developed to examine the presence of *C. trachomatis* DNA in the synovial tissue or fluid of ReA, undifferentiated oligoarthritis and other forms of arthropathies **(Taylor-Robinson *et al*., 1992; Bas *et al*., 1995; Wilkinson *et al*., 1998).** Some of these studies have demonstrated *C. trachomatis* DNA in ReA patients in rather high (80%) (**Taylor-Robinson *et al*., 1992)** or low frequencies (25%) **(Bas *et al*., 1995)**, while others have shown that it is not present **(Piot *et al*., 1994, Poole *et al*., 1992). Bas *et al*., (1995)** reported that *C. trachomatis* DNA was found in SF samples from 25% of the patients with *C. trachomatis*–sexually acquired ReA, 50% of the patients with sexually acquired ReA and 41% of patients with undifferentiated seronegative oligoarthritis. **Siala *et al*., (2009)** used SF and synovial biopsy to demonstrate that *C. trachomatis* DNA was present in 100% ReA patients and in 64% undifferentiated oligoarthritis patients.

Briefly, In the **first section** of this chapter the frequency of *C. trachomatis* infection has been determined in patients with ReA and in uSpA with effusion (sub-group I a and groups II - III) further in **second section** comparison has been done in molecular *versus* non-molecular methods of *C. trachomatis* diagnosis during the course of ReA/ uSpA. In **third section** additional work has been done by determining the frequency of *C. trachomatis* infection in uSpA patients without effusion.

3.2 Results

3.2.1 Section-I

Frequency of *Chlamydia trachomatis* infection in reactive arthritis/ undifferentiated spondyloarthropathy patients with effusion (sub-group I a and groups II - III)

3.2.1.1 Clinical data:

ReA/ uSpA patients with effusion had mean age of 30.5 years. The male-to-female ratio was as follows: 17: 4 for ReA and 18: 6 for uSpA patients. The mean disease duration was 15 months in ReA and 14.1 months in uSpA patients, however, the mean value of ReA/ uSpA was 9.6 months. The majority (77.7%) of ReA/ uSpA patients (35/45) had oligoarthritis, while 6.6% (3/45) were categorized as patients with monoarthritis. 57.7% (26/45) ReA/ uSpA patients had low backache, while 11.1% (5/45) had enthesitis in their small joints. Further clinical details of patients have been summarized in **Table 3.1.**

3.2.1.2 **Detection of *Chlamydia trachomatis* DNA by PCR assays in synovial fluid and urine**

(a) Synovial fluid

Overall, SF from 12 of 115 (10.4%) arthritic patients (6 ReA, 5 uSpA and 1 RA) was positive for at least one *C. trachomatis* DNA (MOMP/ plasmid) by snPCR or nPCR. In Group Ia, 24.4% (11/45) ReA/ uSpA patients were positive for either *C. trachomatis* MOMP/ plasmid by either snPCR or nPCR. Plasmid was found in 11/45 (24.4%), MOMP was found in 7/45 (15.5%) while both MOMP and plasmid genes were found in 7/45 ReA/ uSpA patients (3 ReA- 14.2%; and 4 uSpA- 16.6%)**.** These patients were significantly more likely to be PCR-positive compared to RA (1/35, 2.8%; $p < 0.02$) and OA (0/35; $p < 0.005$). Six of the 21 (28.5%) ReA patients were positive for the plasmid gene, 3/21 (14.2%) had MOMP and 3/21 (14.2%) showed the presence of both genes. *C. trachomatis* DNA was detected in 20.8% (5/24) of synovial samples from the uSpA group of patients. 5/24 (20.8%) uSpA patients were amplified by the plasmid PCR: 4/24 (16.6%) were positive for MOMP gene while 4/24 (16.6%) were positive for both genes **(Figs. 3.1, 3.2; Tables 3.2, 3.3).**

(b) Urine

In group I a, the endogenous plasmid gene of *C. trachomatis* (200 bp) was detected in the urine of 20% (9/45) ReA/ uSpA patients; among these, 28.5% (6/21) and 12.5 (3/24) were ReA and uSpA patients, respectively. In the urine, none of control patients was found to be positive for *C. trachomatis* plasmid gene (**Fig. 3.3; Table 3.2).**

Table 3.1: Clinical profile of arthritic patients (n=115)

Clinical Characteristics	ReA/ uSpA patients with effusion (n=45)	RA patients (n=35)	OA patients (n= 35)
Age (mean ± SD) (years)	30.5 ± 9.8	37.4 ± 5.2	44.5 ± 9.7
Male: Female	35: 10 (77.7: 23.3)	09: 26 (25.7: 74.2)	19:16 (54.2: 45.7)
Disease duration (mean ± SD) (months)	9.6 ± 9.9	10.8 ± 9.6	17.6 ± 15.6
Arthritic pattern			
○ monoarthritis	03 (6.6)	0 (0)	13 (37.1)
○ oligoarthritis	35 (77.7)	05 (14.2)	22 (62.8)
○ polyarthritis	07 (15.5)	26 (74.2)	0
Enthesitis	05 (11.1)	0	0
Low-back ache	26 (57.7)	0	0
Urogenital symptoms	21 (46.6.)	0	0
Family history	4 (5.5)	2 (5.7)	0
Rheumatoid factor	0	31 (100)	0

Abbreviations: ReA/ uSpA- reactive arthritis/ undifferentiated spondyloarthropathy; RA- rheumatoid arthritis; OA- osteoarthritis

Note: Data in parenthesis represents percentages

Table 3.2: Detection of *Chlamydia trachomatis* by snPCR and nPCR in synovial fluid and urine of arthritic patients

PCR	**ReA/ uSpA (n=45)**	**RA (n=35)**	**OA (n=35)**	**Odd Ratio (95% CI)**	**'p' value ***
snPCR in SF (MOMP)	7 (15.5%)	1 (2.8%)	0	5.4 (0.63-46.3)	0.13# (with RA) 0.03 (with OA)
nPCR in SF (Plasmid)	11 (24.4%)	0	0	17.9 (0.83-246)	0.005(with RA) 0.005(with OA)
Conventional PCR in urine (Plasmid)	09 (20%)	0	0	14.82 (0.83-263)	0.01 (with RA) 0.01 (with OA)

Agreement of PCR findings in synovial fluid and urine				
PCR	**'k'**	**SE of kappa**	**95% CI**	**Strength**
n SF vs urine	0.5	0.165	0.222-0.886	***moderate***

Abbreviations: PCR- polymerase chain reaction assay; snPCR- semi nested PCR; nPCR- nested PCR

* Comparison done with control patients (Rheumatoid Arthritis (RA) n- 35, Osteoarthritis (OA) n- 35)

Not significant

Table 3.3: Clinical features of *Chlamydia trachomatis* PCR-positive ReA/ uSpA patients

Patient	Age (years)/ Sex	DD	Joint pattern	Joints involved	Morning stiffness	LBA	Urogenital symptoms	Enthesitis
ReA	26/M	10	O	knees, ankles	-	+	+	+
ReA	19/M	24	O	knees, ankles	+	-	+	-
ReA	38/M	06	O	knees, shoulder-hips		-	+	-
ReA	23/M	03	O	knee	-	-	+	-
ReA	31/M	09	M	knee	-	-	+	-
ReA	24/F	10	O	knees	-	-	+	+
uSpA	23/M	18	O	knees	-	+	-	-
uSpA	19/M	06	O	knees, ankle	+	+	-	-
uSpA	18/M	06	O	knees, ankle	+	-	-	-
uSpA	49/M	12	O	knees, ankle small joints	+	-	-	-
uSpA	44/M	36	O	knees, small joints	-	+	-	-

Abbreviations: ReA- reactive arthritis; uSpA- undifferentiated spondyloarthropathy; M- male; F- female; O- oligoarticular; LBA- low backache; DD- disease duration.

***** ReA/ uSpA patients with effusion and *C. trachomatis*-positive

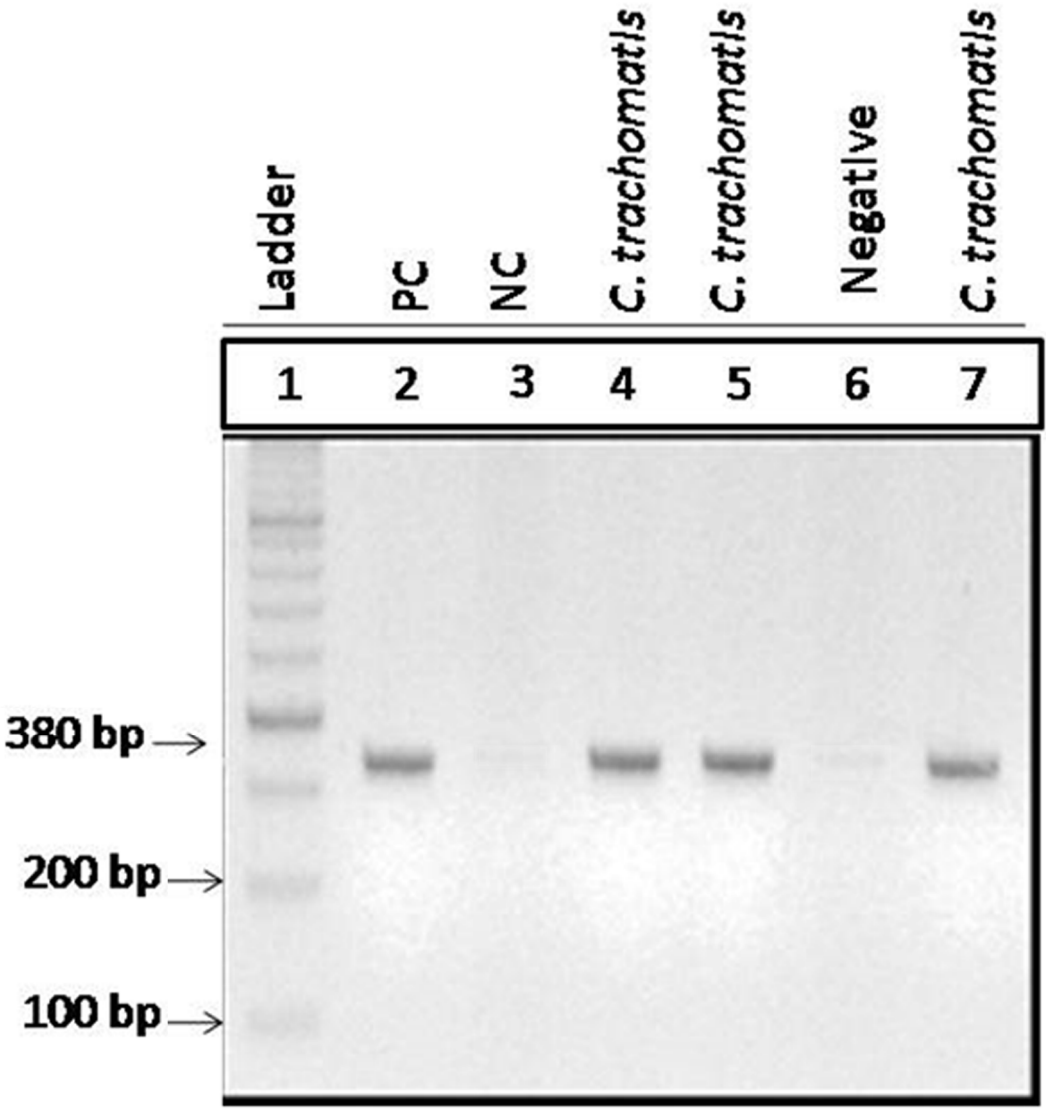


Fig.3.1: Semi-nested PCR for major outer membrane protein gene (380 bp) of *Chlamydia trachomatis* in ReA/ uSpA patients.

Lane 1 shows 100 bp ladder, lanes 2 and 3 show positive (PC) and negative (NC) controls for *Chlamydia trachomatis* while lanes 4 - 5 and 7 show *Chlamydia trachomatis*-positive DNA in synovial fluid.

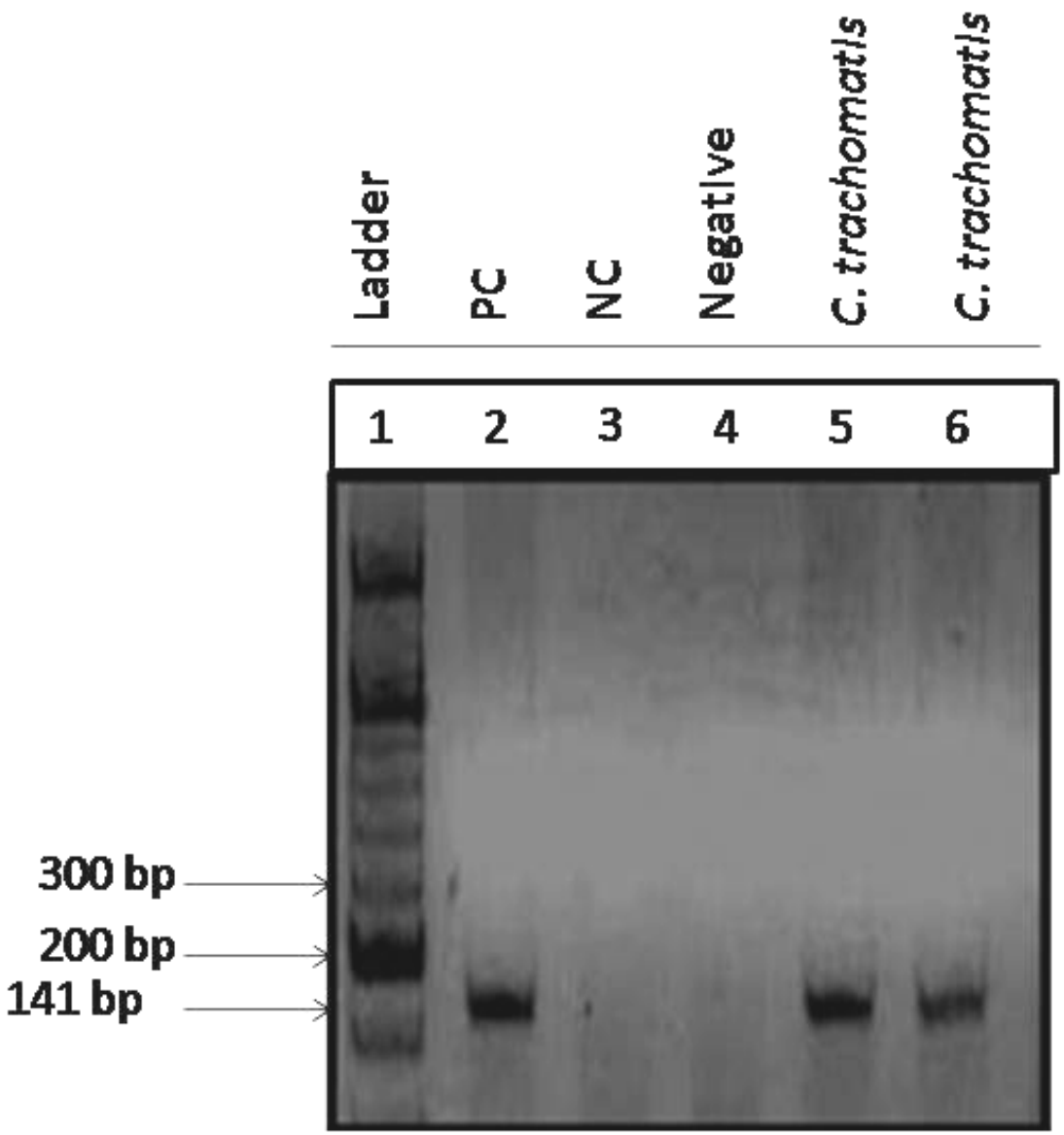


Fig.3.2: Nested PCR product for plasmid gene (141bp) of *Chlamydia trachomatis* in ReA/ uSpA patients.

Lane 1- 100 bp ladder, lane 2- positive DNA control (PC), lane 3- negative DNA control (NC), lanes 5 and 6- *Chlamydia trachomatis*- positive DNA in synovial fluid.

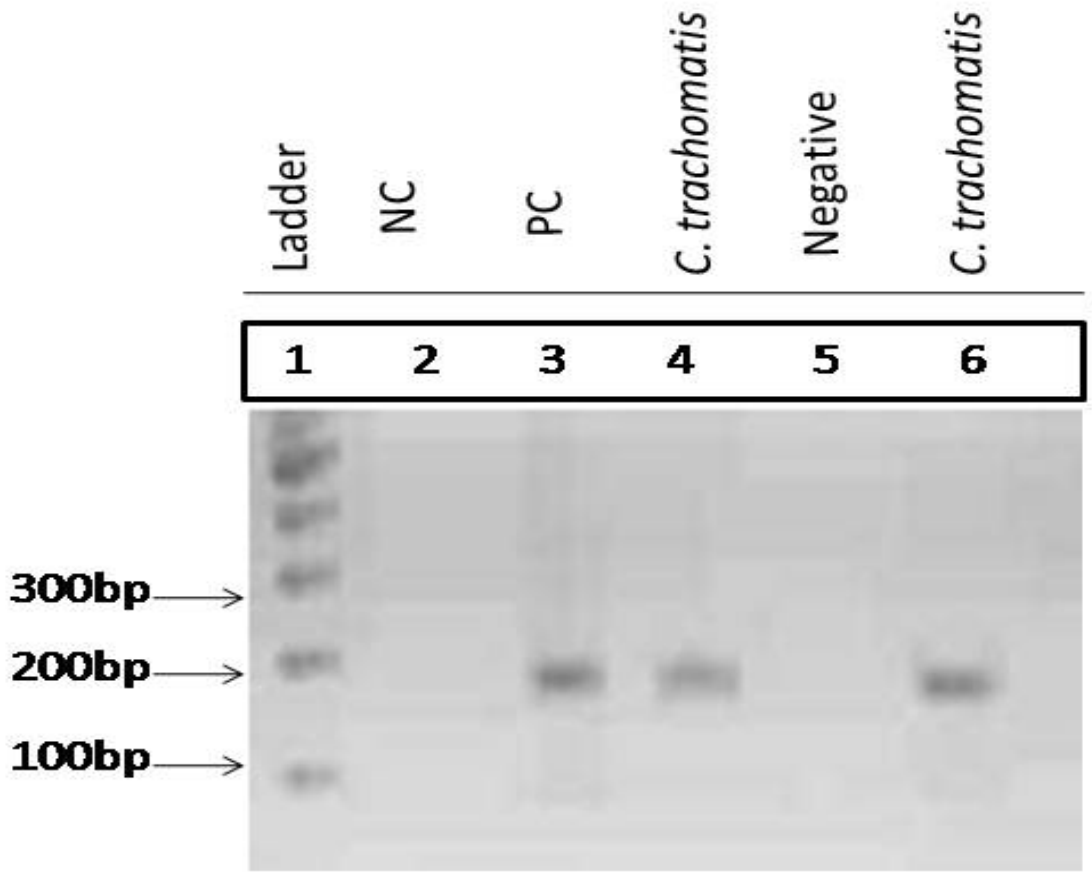


Fig. 3.3: PCR (*conventional*) for detection of plasmid gene (200 bp) of *Chlamydia trachomatis* in urine of ReA/ uSpA patients.

Lane 1 shows 100 bp ladder; lanes 2 and 3 show negative and positive controls (NC and PC, respectively) for *Chlamydia trachomatis* and lanes 4 and 6 show *Chlamydia trachomatis*-positive DNA.

3.2.1.3 Sequencing of PCR products

PCR products of *C. trachomatis*-positive patients were purified and sequenced. The resulting DNA sequences were compared with sequences in the NCBI database using BLAST. DNA findings to *C. trachomatis* pathogen had percent similarity > 98%, the best score and the maximum length of alignment between the sequenced positive PCR product and the corresponding sequence in the database.

3.2.1.4 Concordance of PCR results in SF and urine

Six ReA/ uSpA patients were *C. trachomatis*-positive in both SF and urine by PCR while 03 patients were positive for chlamydial DNA in urine only and were otherwise negative in the SF. Kappa coefficient was calculated for this discrepancy and a **"moderate agreement"** (k- 0.5; SE of kappa- 0.165; 95% CI- 0.222 - 0.886) was observed between *C. trachomatis* detection in SF/ urine in ReA/ uSpA patients **(Table 3.2).**

3.2.1.5 Localization of *Chlamydia trachomatis* antigen (EBs) by direct fluorescence assay in synovial fluid and urine

(a) Synovial fluid

The range of EBs seen in the SF cell deposits by each observer varied from at least a minimum of 07 and a maximum of 10 - 15 EBs per slide. Among ReA/ uSpA patients (n- 45), 14 (31.1%) were found to be *C. trachomatis*-positive, 28 (62.2%) were negative, while 03 (6.6%) could not be categorized as either positive/ negative. Hence,

Kappa statistics was applied for inter-reader agreement and an overall **"good agreement"** (k- 0.852; 95% CI: 0.692 – 1.0) was found.

Among 14 infected patients with ReA/ uSpA in Group I, 38% (8/21) had ReA while 25% (6/24) had uSpA. Of these, 14 (08 ReA and 06 uSpA) patients, 11 were male (78.57%, 11/14) and 03 were female patients (21.42%, 3/14) who found to be *C. trachomatis*-positive. All positive patients (n-14) exhibited an oligoarticular clinical picture with knee joint involvement. 2.8% (1/35) RA patients were also found to be *C. trachomatis*-positive by DFA **(Fig. 3.4; Table 3.4).**

(b) Urine

C. trachomatis EBs were observed in the urine of 13.3% (6/45) patients in Group I a. Among the latter, *C. trachomatis* positivity was found in 19% ReA (4/21) and 8.3% uSpA (2/24) patients. None of the control patients showed the presence of *C. trachomatis* EBs in the urine **(Table 3.4)**.

(c) Concordance of DFA findings in synovial fluid and urine

As out of 6 DFA-positive patients in urine sample, only 3 patients showed concordance with SF results, hence kappa statistics was applied for agreement between both methods. There was **"poor concordance"** between the DFA findings in SF and urine. **(Table 3.4)**.

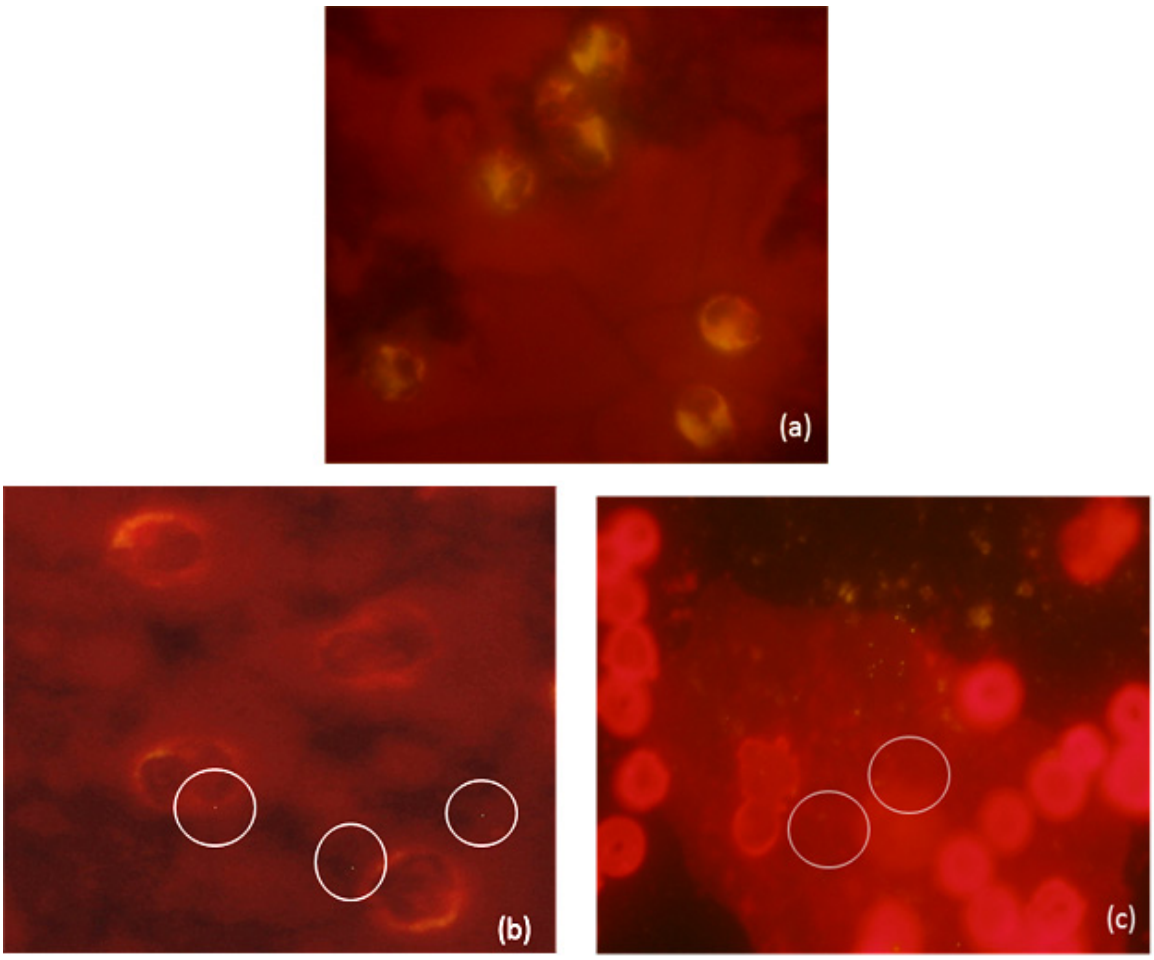


Fig.3.4: Photograph shows fluorescent *Chlamydia trachomatis* Elementary Bodies (EBs) in synovial fluid of ReA/ uSpA patients (100 X oil immersion objective magnification)

(a) Negative control shows synovial cells

(b) EBs in synovial fluid of a reactive arthritis patient (encircled)

(c) Extracellular EBs in synovial fluid of an undifferentiated spondyloarthropathy patient (encircled)

Table 3.4: Performance of Direct Fluorescence Assay (DFA) in synovial fluid and agreement with urine DFA in ReA/ uSpA patients

	ReA/ uSpA patients (n=45)	**RA patients (n=35)**	**OA patients (n=35)**	**Odds Ratio (95% CI)**	**'p' value**
DFA in SF	14 (31.1%)	1 (2.8%)	0	10.89 (1.3-86.3)	0.007 (with RA) 0.003(with OA)
DFA in urine	06 (13.3%)	0	0	10.14 (0.55-186)	0.07# (with RA) 0.07# (with OA)
Agreement between synovial fluid and urine DFA					
	'k'	**SE of kappa**	**95% CI**	**Strength**	
DFA in SF vs Urine	0.139	0.143	0.140-0.419	***poor***	

Abbreviations: ReA- reactive arthritis; uSpA- undifferentiated spondyloarthropathy; RA- rheumatoid arthritis; OA- osteoarthritis

***** Comparison done with control patients (rheumatoid arthritis n- 35, osteoarthritis n- 35)

Not significant

3.2.1.6 Detection of anti-*Chlamydia trachomatis* antibodies by ELISA in synovial fluid and serum

(a) Synovial fluid

In Group I a (n- 45), intra-articular *C. trachomatis* IgM antibodies were detected in the SF of 1/45 (2.2%) uSpA patient. 6.6% (3/45) ReA/ uSpA patients had anti-chlamydial IgG antibodies while 28.8% (13/45) patients (ReA- 9/21, 42.8% and uSpA- 4/24, 16.6%) showed the presence of IgA antibodies. None of the control patients with RA (Group II)/ OA (Group III) had IgG/ IgM/ IgA antibodies to *C. trachomatis* in the SF **(Table 3.5).**

(b) Serum

Serology results showed the presence of *C. trachomatis* IgG antibodies in 13.3% (6/45) ReA/ uSpA patients. Serum IgM antibodies against *C. trachomatis* were detected in 4.4% (2/45) ReA/ uSpA patients while serum IgA antibodies against *C. trachomatis* was detected in 15.5% (7/45) ReA/ uSpA patients. 2.8% (1/35) RA patients were positive for serum IgA antibodies **(Table 3.5)**.

(c) Concordance between presence of anti-*C. trachomatis* antibodies in synovial fluid and serum

There was **"poor concordance"** between IgM, IgG and IgA antibodies in SF and serum **(Table 3.5).**

Table 3.5: Anti-*Chlamydia trachomatis* antibodies in the synovial fluid and serum of arthritic patients

	ReA/ uSpA patients with effusion (n=45)	**RA patients (n=35)**	**OA patients (n=35)**	**Odds Ratio (95% CI)**	**'p' value**
Anti-*Chlamydia trachomatis* antibodies in the synovial fluid					
IgM	01 (2.2%)	0	0	2.3 (1.21 - 355)	1.0 (ns) (with RA) 1.0 (ns) (with OA)
IgG	03 (6.6%)	0	0	12.7 (0.72 - 223)	0.2 (ns) (with RA) 0.2 (ns) (with OA)
IgA	13 (28.8%)	1 (2.8%)	0	21.07 (1.2 - 366)	**0.001** (with RA) **0.001** (with OA)
Anti-*Chlamydia trachomatis* antibodies in the serum*					
IgM	02 (4.4%)	0	0	3.9 (0.18 - 83.9)	0.5 (ns) (with RA) 0.5 (ns) (with OA)
IgG	06 (13.3%)	0	0	10.1 (0.55-186.3)	0.07 (ns) (with RA) 0.07 (ns) (with OA)
IgA	07 (15.5%)	1 (2.8%)	0	5.4 (0.63 - 46.3)	0.13 (ns) (with RA) **0.03** (with OA)
Agreement between SF and serum antibodies detection					

Method	**'k'**	**SE of kappa**	**95% CI**	**Strength**
Antibodies to *Chlamydia trachomatis* in SF vs serum	0.123	0.150	-0.171-0.416	***poor***

*In serum, comparison was done with control patients (rheumatoid arthritis n-35 and osteoarthritis n-35); in serum IgA- One RA patient was positive, NS- not significant.

3.2.2 Section II

3.2.2.1 Comparison between molecular (nPCR) *versus* non-molecular (DFA and anti-*C. trachomatis* antibodies) diagnostic methods

Comparison of molecular *versus* non-molecular methods to detect *C. trachomatis* was done by calculating the degree of *'kappa agreement'*. To evaluate the efficacy of molecular and non-molecular methods, sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were calculated.

(a) Sensitivity, specificity, PPV and NPV

Considering nPCR as gold standard, the sensitivity, specificity, PPV and NPV were calculated. 11 patients were found positive for *C. trachomatis* by nPCR while snPCR was able to detect only 07 patients. Among the latter, 04 were false negative patients.

In comparison to the gold standard nPCR assay (11 patients were found positive for *C. trachomatis* by nPCR), 08 DFA-positive patients were found to be nPCR-positive, 06 patients were false positives, and 03 patients were observed as false negatives (in comparison to nPCR). Anti-*C. trachomatis* IgA antibodies-positive ReA/ uSpA patients in SF and serum showed concordance with nPCR (7 and 2 patients respectively).

In comparison to nPCR, snPCR was found to be 100% specific, however, it had a poor sensitivity (63.6%). DFA was found to be a promising method to detect *C. trachomatis* as it showed good sensitivity (72.7%) and specificity (82.3%) in comparison to nPCR (100%).

Anti-*C. trachomatis* IgA antibodies in SF showed 63.6% sensitivity and 82.3% specificity while anti-*C. trachomatis* IgA antibodies in serum had poor sensitivity (18.1%) with 85.2% specificity in comparison to nPCR.

PPV value was highest (100%) in nPCR *versus* snPCR followed by DFA (57.1%) and anti-*C. trachomatis* IgA in SF (53.8%) and serum (28.5%). NPV was comparable in all groups with highest value in nPCR *versus* DFA (90.3%) followed by snPCR (89.4%), anti-*C. trachomatis* IgA in SF (87.5%) and serum (76.3%).

Conventional PCR assay in urine had 54.5% sensitivity, 91.1% specificity, 66.6% PPV and 86.1% NPV in comparison to nPCR **(Table 3.6)**.

(b) Kappa agreement

Considering nPCR as a gold standard, diagnostic agreement between molecular *versus* non-molecular methods was estimated. DFA finding was compared to the gold standard nPCR method; 8 ReA/ uSpA patients were positive for *C. trachomatis* infection by both nPCR and DFA, hence DFA in SF showed **"moderate agreement"** with nPCR. Anti-*C. trachomatis* IgA antibodies were found in the SF of 7/11 nPCR-positive ReA/ uSpA patients and a **"moderate agreement"** was observed with nPCR. However, anti-*C. trachomatis* IgA antibodies in serum showed **"poor agreement"** with nPCR **(Table 3.7)**.

Table 3.6: Diagnostic efficacy of different diagnostic methods for *Chlamydia trachomatis* detection in reactive arthritis/ undifferentiated spondyloarthropathy spondyloarthropathy

Methods	**Sensitivity**	**Specificity**	**Positive Predictive Value (PPV)**	**Negative Predictive Value (NPV)**
n-PCR vs sn-PCR in synovial fluid (95% CI)	63.6% (30.8 - 88.8)	100% (89.6 - 100)	100% (58.9 - 100)	89.4% (75.1 - 96.9)
nPCR vs DFA in synovial fluid (95% CI)	72.7% (39 - 93.9)	82.3% (65.4 - 93.2)	57.1% (28.2 - 82.3)	90.3% (74.2 - 97.9)
nPCR vs IgA to *Chlamydia trachomatis* in synovial fluid (95% CI)	63.6% (30.7 - 89)	82.3% (65.4 - 93.2)	53.8% (25.1 - 80.7)	87.5% (71 - 96.4)
nPCR vs IgA to *Chlamydia trachomatis* in serum (95% CI)	18.1% (2.2 - 51.7)	85.2% (68.9 - 95)	28.5% (3.6 - 70.9)	76.3% (59.7 - 88.5)

Abbreviations:

CI- confidence interval; PPV- positive predictive value; NPV- negative predictive value; snPCR- semi nested PCR; nPCR- nested PCR

Table 3.7: Agreement on diagnosis of nPCR with snPCR, serology, DFA considering n-PCR as a gold standard

Methods	Kappa coefficient ('k')	SE of kappa	95% CI	Strength of agreement
nPCR - snPCR in synovial fluid	0.726	0.126	0.479 - 0.972	***good***
nPCR - DFA in synovial fluid	0.504	0.142	0.226 - 0.782	***moderate***
nPCR - IgA to *Chlamydia trachomatis* in synovial fluid	0.433	0.150	0.139 - 0.727	***moderate***
nPCR in synovial fluid - IgA to *Chlamydia trachomatis* in serum	0.040	0.149	0.253 - 0.332	***poor***

Degree of agreement was assessed by doing Kappa statistics.

Kappa values ranging from 0.01-0.20 were considered as slight agreement, from 0.21-0.40 as fair agreement, from 0.41-0.60 as moderate agreement, from 0.61-0.80 as good agreement and from 0.81-0.99 as almost perfect agreement.

Abbreviations: snPCR- semi nested PCR; nPCR- nested PCR

(c) Prediction of post-test probability of developing *C. trachomatis* infection by 'Nomograph'

The findings of molecular/ non-molecular methods were further interpreted using a mathematical model, *viz.:* 'Nomograph'. The latter predicts the post-test probability of developing infection in the disease group. Results obtained by NAATs, DFA and Elisa for antibody detection were used to evaluate the post-test probability which revealed that patients positive for *C. trachomatis* by NAATs have the maximum probability of developing ReA/ uSpA followed by DFA and Elisa serum antibody detection **(Fig. 3.5)**

As per the observation the prior problity was detected between 20-30% in each category by ether molecular or non-molecular method. However, the post test probability was restricted in non-molecular method in comparison to molecular methods. For DFA it was observed between 10-60% followed by serum anti-*Chlamydia trachomatis* antibody <10-60% however by molecular method it was obseverd between 10-99%.

3.2.2.2 Diagnosis of *C. trachomatis* infection in acute and chronic ReA/ uSpA patients

Data obtained from various molecular/ non-molecular methods in SF/ serum/ urine was further analyzed to differentiate between acute and chronic ReA/ uSpA patients (n=80). It was observed that out of 11 *C. trachomatis* nPCR-positive patients, the maximum (90%) **nPCR**-positive patients with *C. trachomatis* in SF were males who were **chronically infected (with longer disease duration (> 06 months).** These patients belonged to 18 - 30 years age group. **DFA** in urine was more conclusive to diagnose *C. trachomatis* in **acute disease (< 06 months)** as it was able to detect 83.3% ReA/ uSpA patients.

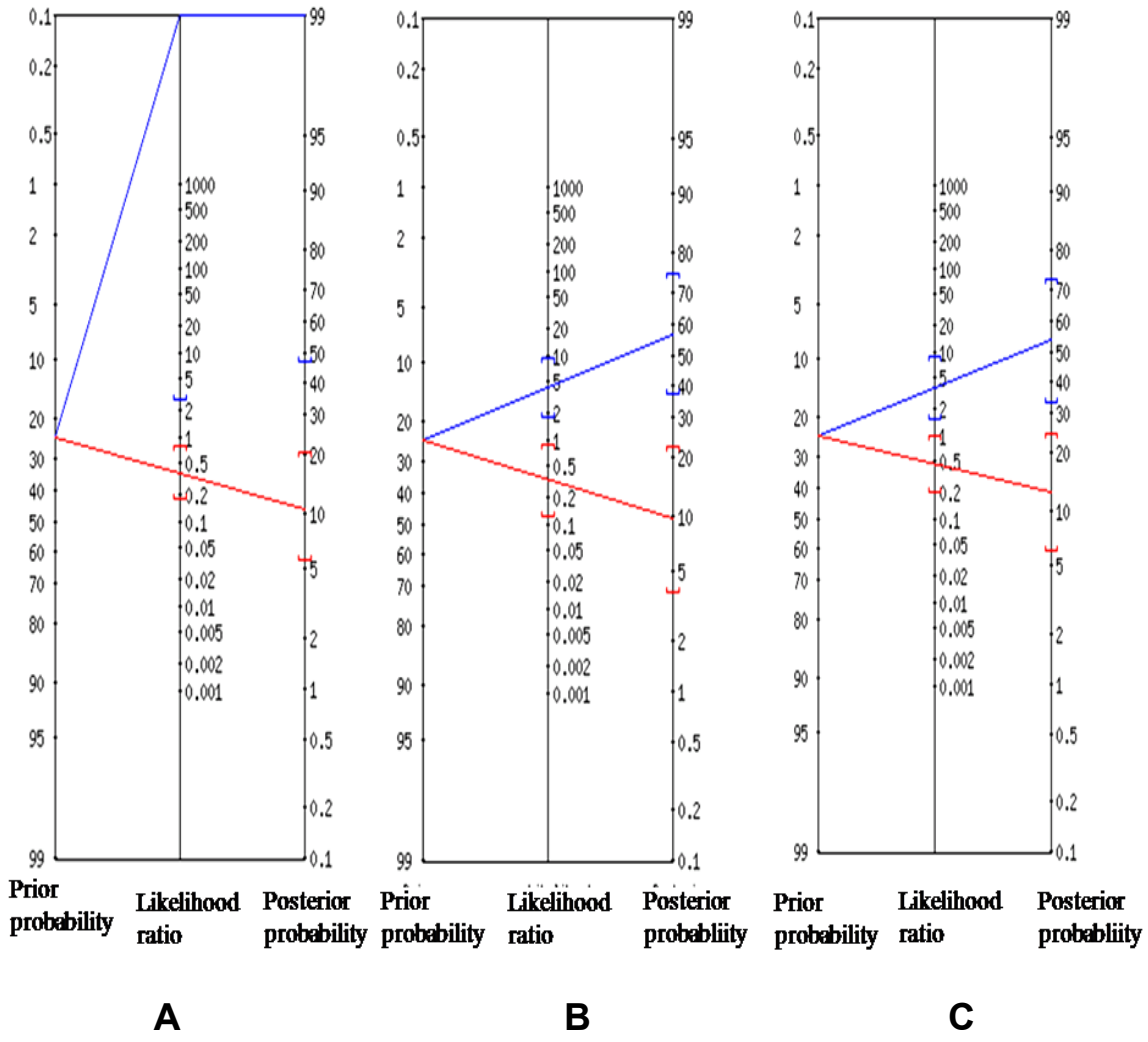


Fig. 3.5: Nomograph showing sensitivity, specificity and precision between:

(A) nested PCR -- semi-nested PCR;

(B) PCR -- DFA

(C) PCR -- serology

DFA in SF for *C. trachomatis* detection did not show any specific pattern, however, majority of the patients (64.2%) had chronic infection. Anti-*C. trachomatis* antibodies in SF were also frequent in symptomatic patients in comparison to asymptomatic patients. Anti-*C. trachomatis* antibodies in serum did not show any specific pattern in these ReA/

uSpA patients. Majority of these patients (77%) were chronically infected. **(Tables 3.8 and 3.9).**

Table 3.8: Diagnostic importance of molecular *versus* non-molecular methods to differentiate disease status in *Chlamydia trachomatis*-induced ReA/ uSpA with effusion

Characteristics of *C. trachomatis* infected ReA/ uSpA	**nPCR in SF (n=11)**	**PCR in urine (n= 9)**	**DFA in SF (n= 14)**	**DFA in urine (n=6)**	**Anti-*C. trachomatis* antibodies in SF (n=13)**	**Anti-*C. trachomatis* antibodies in serum (n=7)**
Age (years) • 18-30 • 30-40 • 40-50	**7 (63.6)** 2 (18.1) 2 (18.1)	5 (55.5) 3 (33.3) 1 (11.1)	5 (35.7) 6 (42.8) 2 (14.2)	**5 (83.3)** 1 (16.6) 0 (0)	5 (38.4) 7 (53.8) 1 (7.6)	3 (42.8) 3 (42.8) 1 (14.2)
Male: Female	10: 1 (91: 9)	7: 2 (78: 22)	11: 3 (79: 21)	3: 3 (50: 50)	10 : 3 (77: 23)	4 : 3 (57 : 43)
ReA patients symptomatic for urogenital infection	6 (54.5)	6 (66.6)	8 (57.1)	4 (66.6)	9 (69.2)	3 (42.8)
uSpA patients asymptomatic for urogenital infection	5 (45.4)	3 (33.3)	6 (42.8)	2 (33.3)	4 (30.7)	4 (57)
Acute patients (disease duration < 6 months)	1 (9)	4 (44.4)	5 (35.7)	**5 (83.3)**	3 (23)	4 (57)
Chronic patients (disease duration > 6 months)	**10 (91)**	5 (55.5)	9 (64.2)	1 (16.6)	**10 (77)**	3 (42.8)

Abbreviations: ReA- reactive arthritis; uSpA- undifferentiated spondyloarthropathy; SF-synovial fluid

Table 3.9: Evaluation of diagnostic methods for *Chlamydia trachomatis* detection versus clinical specimens

	Synovial fluid	**Urine**	**Serum**	**Strength of agreement**
Polymerase chain reaction	24.4% (11/45)	20% (9/45)	Not applicable	***moderate*** (between SF and urine)
Direct fluorescence assay	31.1% (14/45)	13.3% (6/45)	Not applicable	***poor*** (between SF and urine)
Anti-*Chlamydia trachomatis* IgA antibodies	28.8% (13/45)	Not applicable	15.5% (7/45)	***poor*** (between SF and serum)

3.2.3 Section III

3.2.3.1 Frequency of detection of *Chlamydia trachomatis* infection in undifferentiated spondyloarthropathy patients without effusion (Sub-group I b and Groups II - III)

35 uSpA patients without effusion in their joints were further included in the study. Serum and urine specimens were investigated for *anti-C. trachomatis* antibodies by Elisa and antigen detection by DFA.

(a) Clinical characteristics

uSpA patients without effusion had mean age of 30.5 years. The mean disease duration in these patients was 13.6 months. The male: female ratio was found to be 24:

11. 88.5 % uSpA patients without effusion complained of low backache while enthesitis was observed in 14.2 % patients. The majority of patients had oligoarthritis followed by polyarthritis and monoarthritis.

(b) Detection of *Chlamydia trachomatis* DNA by PCR assay in urine

uSpA patients without effusion of SF were investigated for the presence of *C. trachomatis* plasmid gene in urine samples of these patients. *C. trachomatis* plasmid DNA was detected in 17.35% (6/35) patients **(Table 3.10)**.

(c) Localization of *Chlamydia trachomatis* antigen in urine by DFA

DFA was performed in urine cells sediments and it was found that 22.8% (8/35) uSpA patients were positive for *C. trachomatis* EBs **(Table 3.10).**

(d) Detection of anti-*C. trachomatis* antibodies by Elisa in serum

Overall, the presence of serum antibodies against *C. trachomatis* in uSpA patients without effusion was higher in comparison to uSpA patients with effusion. Anti-*C.trachomatis* IgM was detected in 11.42% (4/35) while IgG was found in 22.8% (8/35) patients. Anti- *C.trachomatis* IgA found to be in 25.7% (9/35) patients **(Table 3.10)**.

(e) Agreement of PCR with DFA and anti-*C. trachomatis* IgA findings in urine

There was 'fair' concordance observed between the results of PCR and DFA in urine. As out of 8 DFA-positive patients in urine, only 3 showed concordance with PCR in urine, hence, kappa statics was applied for the agreement between both methods. IgG antibodies against *C. trachomatis* in serum were common in 3 PCR-positive patients

while IgA antibodies were common in 4 PCR patients in urine; 'fair' to 'moderate' agreement was observed with these antibodies (**Table 3.11)**.

Table 3.10: Non-invasive detection of *Chlamydia trachomatis* in urine and serum of uSpA patients without effusion

Method	**uSpA (n=35)**	**RA (n=35)**	**OA (n=35)**	**Odds Ratio (95% CI)**	**'p' value**
Urine PCR	06 (17.35%)	0	0	13 (0.70-239)	**0.02**(with RA) **0.02** (with OA)
Urine DFA	08 (22.8%)	0	0	17 (0.94-306.1)	**0.007** (with RA) **0.007** (with OA)
Serum IgM	04 (11.42%)	0	0	09 (0.46-173.6)	0.1* (with RA) 0.3* (with OA)
Serum IgG	08 (22.8%)	0	0	17 (0.94-306.1)	**0.007** (with RA) **0.007** (with OA)
Serum IgA	09 (25.7%)	1 (2.8%)	0	09 (1-74.9)	**0.01** (with RA) **0.003** (with OA)

Abbreviations: PCR- polymerase chain reaction; DFA- direct fluorescence assay

* statistically non-significant

Table 3.11: Degree of agreement between PCR, DFA and anti-*Chlamydia trachomatis* antibodies in uSpA patients without effusion

	Kappa ('k') agreement	**SE of kappa**	**95% CI**	**Strength**
PCR and DFA in urine	0.32	0.184	-0.035 – 0.687	***Fair***
PCR and IgG antibodies to *Chlamydia trachomatis* in serum	0.32	0.184	-0.035 – 0.687	***Fair***
PCR and IgA antibodies to *Chlamydia trachomatis* in serum	0.44	0.174	0.104-0.785	***Moderate***

3.3 Discussion

Reactive arthritis or undifferentiated spondyloarthropathy due to *C. trachomatis* is widely underestimated in various parts of the world and the global burden of ReA due to *C. trachomatis* is impossible to quantify accurately because there is no specific diagnostic test to diagnose the infection **(Owlia and Eley, 2010; Senior, 2012)**. Practical problems in isolating *Chlamydia* from joint aspirates include the insensitivity of chlamydial culture compared with other detection methods **(Ostergaard *et al*., 1990; Ossewaarde *et al*., 1992)**, the toxicity of SF for tissue culture cells, and the possibility that chlamydial

replication may be reversibly inhibited by IFN-gamma, leading to only an intermittent release of infectious chlamydial EBs **(Beatty *et al*., 1995; Ward, 1999)**. With the advent of non-viability dependent techniques for the detection of chlamydial infection, it is became clear that chlamydial antigen or nucleic acid reaches the affected joint, at least in ReA patients selected for the likelihood of having *C. trachomatis* infection. Thus, *C. trachomatis* antigen has been detected in SF or biopsy tissue by immunofluorescence and/or electron microscopy **(Keat *et al*., 1987; Schumacher *et al*., 1988).**

In India *C .trachomatis* screening is not a routine diagnostic procedure in clinical practice either in Rheumatology Clinic or elsewhere **(Handa, 2015)**. There may be so many reasons for this such as lack of awareness for *C. trachomatis* screening, initial asymptomatic infection, difficulties in diagnosis **(Senior, 2012)**. Diagnosis of *C. trachomatis* is slightly difficult in CiReA in comparison to urogenital infection because, there is no international consensus on this further there are several changes takes place in *C. trachomatis* biology as it becomes uncultivable, abherent morphology, major outer membrane protein gene get attenuated **(Schumacher *et al*., 1988; Rihl *et al*., 2006)**. Despite all these hurdles this pathogen is still surviving in synovium and it is metabolically active and shows many gene transcript of liveliness **(Gerard *et al*., 2013)**. Genetic manipulation in *C. trachomatis* genome hinders the approaches for developing new tools for *C. trachomatis* detection **(Bastidas *et al*., 2013)** hence it was reasonable to put a syndromic approach to detect this pathogen in ReA/uSpA by using conventional method so that it can be adopted by resource limited centers in developing countries as these methods are easy to perform, cost effective with good sensitivity and specificity.

Various PCR assays amplifying chlamydial DNA have been developed to examine the presence of *C. trachomatis* DNA in the synovial tissue or fluid of ReA, Undifferentiated Oligoarthritis and other forms of arthropathies **(Taylor-Robinson *et al*., 1992; Bas *et al*., 1995; Wilkinson *et al*., 1998)**. Studies have demonstrated *C. trachomatis* DNA in ReA patients in rather high (80%) **(Taylor-Robinson *et al*., 1992)** or low frequencies (25%) **(Bas *et al*., 1995)**, while others have shown that it is not present at all **(Piot *et al*., 1994)**. Using a nested PCR targeting three different *C. trachomatis* genes in synovial fluid and synovial tissue, a study in Tunisia demonstrated that *C. trachomatis* DNA was present in 100% ReA patients and in 64% UOA patients **(Siala *et al*., 2009)**. This discrepancy might have arisen in part from considerable differences in the sensitivity of the various methods used and could also possibly be due to the particular template sequence selected for amplification.

3.3.1 *Chlamydia trachomatis* in ReA/ uSpA patients with effusion

In the present study, 24.4% (11/45) ReA/ uSpA patients were found positive in the SF for *C. trachomatis* infection by two-step PCR, either nested or semi-nested PCR assay. PCR in urine was able to detect 20% *C. trachomatis*-positive (9/45) ReA/ uSpA patients. Concordance between both PCRs showed "moderate agreement" which revealed that urine sampling can serve as an alternate non-invasive specimen, which may be investigated for diagnosis of *C. trachomatis* in ReA/ uSpA patients. In previous studies, PCR showed good sensitivity to urine in comparison to SF **(Kuipers *et al*., 1995)** while the specificity reached 97% in both sexes **(Cook *et al*., 2005)**. In SF of ReA/ uSpA patients', endogenous plasmid gene of *C. trachomatis* was more detectable in comparison to MOMP gene as copy number of plasmid gene is more in number than MOMP **(Siala *et***

***al*., 2009)**. These findings were further confirmed by the sequence analysis of PCR products to confirm the findings. The low percentage prevalence of *C. trachomatis* may be attributed to the low copy number available in SF in comparison to the synovial tissue **(Bas *et al*., 1995; Branigan *et al*., 1996).** Two (most sensitive) PCR systems **(Bas *et al*., 1995; Palmer, 1991)** capable of detecting very low copy number (5 - 10 copies, 10 fg of DNA) of *C. trachomatis* were used as maximum sensitivity was desirable for the amplification of potentially low bacterial copy number for reliable diagnosis and rapid initiation of an appropriate drug treatment **(Bas *et al*., 1995; Wilkinson *et al*., 1998; Rihl and Zeidler, 2007)**. Majority of *C. trachomatis*-positive patients by PCR were males (91%; 9/11) in comparison to female patients (9%; 1/11). This may be due to the fact that the long-term effect of *C. trachomatis* infection in men is widely regarded as negligible, but it might be the main and unrecognized cause of ReA **(Senior, 2012; Bas *et al*., 2001).**

DFA is a promising method to detect *C. trachomatis* in urogenital specimens with good sensitivity and specificity; however, few studies showed its efficacy in SF **(Keat *et al*., 1987; Taylor and Robinson, 1992)**. In our study, fair detection rate of *C. trachomatis* was achieved by PCR/ DFA targeting the MOMP gene. Our findings were different from previous studies which reported that the plasmid gene was more detectable than the chromosomal MOMP gene **(Mahony *et al*., 1993; Roosendaal *et al*., 1993)**. In the present study, we diagnosed *C. trachomatis* EBs in the SF of 31.1% (14/45) patients using DFA. Among these 14 patients, 8 were PCR-positive, however, 6 were false positives in comparison to PCR findings. Hence, it was assumed that this might have occurred due to the poor sensitivity of PCR as argued earlier by others **(Taylor Robinson *et al*., 1992)**. As urine has very few number of cells in either symptomatic or in

asymptomatic patients, hence DFA has limited role in the diagnosis of *C. trachomatis* and was unable to give any conclusive result in urine. The MOMP gene is reported to be attenuated during the chronic phase; however, in our study, MOMP was found in fair number of ReA/ uSpA patients. 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 **(Gupta *et al*., 2008)**.

Although diagnosis of anti-*C. trachomatis* antibodies is considered to be less sensitive and specific in comparison to the other methods like culture, DFA and NAATs, yet the presence of antibodies in the serum or SF is the only method for indication of disease chronicity and the stage of infection due to *C. trachomatis* **(Bas *et al*., 1996, 2001; Puolakkainen, 2013).**

In our study, circulatory IgM antibodies against *C. trachomatis* were found in 4.4% ReA/ uSpA patients, 13.3% were found positive for IgG antibodies while secretary IgA antibodies to *C. trachomatis* were present in 15.5% patients. **Wollenhaupt *et al*., (1989)** reported that the IgA-class antibodies against chlamydial antigens were positive in 96% among IgG-positive patients. In SF of ReA/ uSpA patients, IgM and IgG were present in 2.2% and 6.6% patients while secretory IgA antibodies were increased in fair number of patients (28.8%). These results corroborate with others **(Inman *et al*., 1989; Hughes *et al*., 1989)**. Furthermore, there was "moderate agreement" between the findings of PCR and anti-*C. trachomatis* antibodies in our study. Higher prevalence of IgG (33%) and IgA (44%) antibodies had been also observed by others, however, no concordance was found between PCR and serology; hence, the authors stated that the shorter disease

duration (1.8 months) was the reason for this discrepancy **(Siala *et al*., 2009)**. There is currently no recommendation for *Chlamydia*-induced ReA treatment merely on the basis of anti-chlamydial antibodies detection in ReA/ uSpA patients; however, as the subclinical stage of *C. trachomatis* is a matter of concern, antibodies might probably be used as surveillance marker suggestive of infection **(Wilkinson *et al*., 1998)**. IgA to *C. trachomatis* was found in higher percentage in SF while serum had increased level of IgM and IgG in comparison to SF. Serum antibodies against *C. trachomatis* in asymptomatic uSpA patients without effusion were higher in percentage in comparison to uSpA patients with effusion; anti- *C. trachomatis* IgM was detected in 11.4% (4/35), while IgG was found in 22.8% (8/35) patients. Anti- *C. trachomatis* IgA was found in 25.7% (9/35) uSpA patients.

It was suggested that very high level of IgG, IgA and IgM isotypes of chlamydial antibodies might be of use in the diagnosis of ReA **(Bas *et al*., 1996)**. However, others believed that the presence of *Chlamydia* in ReA/ uSpA patients is strongly dependent on the prevalence of actual chlamydial joint infection in the population tested; therefore, the presence of chlamydial antibodies should be considered to be suggestive but not diagnostic **(Wilkinson *et al*., 1998)** of infection.

In comparison to nPCR, considered as gold standard for the diagnosis of *C. trachomatis* in ReA/ uSpA patients, snPCR was found to be 100% specific. However, snPCR has poor sensitivity in comparison DFA. The latter is a promising method that shows good sensitivity (72.7%) in comparison to snPCR (63.6%), however, DFA was less specific (82.3%) than snPCR (100%). In our study, the sensitivity of anti-*C. trachomatis* IgA antibodies in SF was lower in comparison to nPCR (63.6%) while the

specificity was comparable (82.3%) to DFA. The percentage of positive predictive value was highest (100%) in nPCR versus snPCR followed by DFA (57.1%) and anti-*C. trachomatis* IgA in SF (53.8%). The negative predictive value was comparable in all groups with the highest value in nPCR - anti-*C. trachomatis* IgA antibodies (90.3%). The sensitivity (18.1%) and specificity (85.2%) of anti-*C. trachomatis* IgA antibodies in serum was lowest in comparison to nPCR.

3.3.2 *Chlamydia trachomatis* in uSpA patients without effusion in major joints

uSpA is one of the most common presentation of all the spondyloarthropathies **(Boyer *et al*., 1999)** and is considered to be a *forme fruste* of ReA **(Aggarwal *et al*., 1997)**. To the best of our knowledge, till date, the detection of *C. trachomatis* infection in uSpA patients without effusion in major joints has never been reported. Probably in such patients, the diagnosis of *Chlamydia* becomes difficult because of the lack of the SF.

In this particular category of uSpA patients, the presence of serum antibodies to *C. trachomatis* might prove to be helpful to diagnose *C. trachomatis*. Serum anti-chlamydial antibodies were higher in uSpA patients without effusion in comparison to those with effusion: Anti-*C. trachomatis* IgM was detected in 11.4% (4/35) while IgG was found in 22.8% (8/35) patients. Anti-*C. trachomatis* IgA found positive in 25.7% (9/35) patients.

Chapter-4

CHLAMYDIAL HEAT SHOCK PROTEIN 60 IGG ANTIBODIES AND HIGH SENSITIVE C-REACTIVE PROTEIN IN UNDIFFERENTIATED SPONDYLOARTHROPATHY PATIENTS WITHOUT EFFUSIONS

4.1 Introduction

C. trachomatis is the most prevalent sexually transmitted bacterium and it causes chronic, persistent infection in the synovium resulting in chronic inflammation and synovitis. 78 - 88% *C. trachomatis*-induced ReA patients are asymptomatic and thus remained unrecognized **(Sieper, 2001).** *C. trachomatis* infection can persist for very long periods because, usually, the human immune system cannot eliminate pathogens that remain hidden but virulent at focal sites; thus, these silent foci represent a high risk for complications **(Swanborg *et al*., 2006)**.

In India, *C. trachomatis* infection is underestimated in ReA/ uSpA patients despite high prevalence in genital infection **(Mittal *et al*., 1995, 2004; Rastogi *et al*., 2002, 2003)**. Studies till date have largely focussed on enteric infection-induced arthritis **(Aggarwal *et al*., 1997; Chandrashekhra, 2004; Singh *et al*., 2013; Malviya *et al*., 2014**). A recent review article states that India faces the twin challenge of infection-related rheumatic diseases and infections in patients with rheumatic diseases **(Handa, 2015).** Currently, there are no specific recommendations at the national or international level defining specific clinical and/ or laboratory investigation for tests to diagnose *Chlamydia*-induced ReA **(Braun *et al*., 2000).** Hence, reliable tests for joint specimens are needed urgently. Moreover, the development of new practical and sensitive methods for detection of *Chlamydia* in blood samples will allow simpler, more rapid and specific diagnosis in patients where joint samples cannot be obtained **(Zeidler and Hudson, 2013).**

Human hsps are highly conserved throughout evolution with considerable sequence homology between various species, and crossreactivity between bacterial and human hsp60 has been correlated with a number of inflammatory disorders **(Raska and Weigl, 2005; Pockley *et al*., 1999)**. chsp60 is the second most abundant protein in *C. trachomatis* lysates, present in both EBs and in Reticulate Bodies (RBs); also, chsp60 is the only protein produced following induction of a non-culturable persistent state. It is released into the extracellular milieu during persistence **(Linhares and Witkin, 2010).** The microorganism penetrates inside epithelial cells as an EB and then converts into RB, the replicating form of the pathogen. During persistent infection, *C. trachomatis* produces a large quantity of hsp60 (chsp60) **(Bavoil *et al*., 1990)**, which has been implicated in the pathogenesis of autoimmune disorders such as ReA **(Van Eden *et al*., 1988; Anderton *et al*., 1995)**. Rheumatologic studies have primarily focused on the role of hsp60 in RA **(Huang *et al*., 2010).** Studies indicate that the concurrence of persistent *C. trachomatis* infection and appearance of cell surface hsp60 can promote an autoimmune aggression towards stressed cells and the development of diseases such as autoimmune arthritis, multiple sclerosis, atherosclerosis, vasculitis, diabetes, thyroiditis, etc. **(Cappello *et al*., 2009),** however, the role of chsp60 is not clear in ReA/ uSpA patients**.** In humans, elevated antibody responses to chsp60 have been strongly correlated with the presence of pelvic inflammatory disease, ectopic pregnancy, scarring trachoma, and tubal infertility **(Neuer *et al*., 1997; Peeling *et al*., 1998; Jakus *et al*., 2008; Mascellino *et al*., 2008; Ondondo *et al*., 2009).**

chsp60 is a *Chlamydia* genus-specific protein, serving as a strong antigenic target for the immune system **(Morrison *et al*., 1989; Kaufmann, 1990)**. Antibodies to chsp60

have been suggested as markers of chronic inflammation **(Kaufmann, 1990)** and considered to be good predictors for the risk of tubal pathology. Under persistence-inducing conditions, the chsp60 is reportedly highly upregulated. chsp60 has pro-inflammatory effects by directly activating mononuclear leukocytes, which mediate the inflammatory response. Reinfection or persistent state causes pathological changes. Likewise, increased expression of human hsp60 in the synovium of patients with RA has been demonstrated **(Sharif *et al*., 1992; Schett *et al*., 2001).**

Another important inflammatory marker C- reactive protein (CRP) is an acute-phase reactant synthesized mainly by hepatocytes in response to cytokines such as Interleukin-6 (IL-6), IL-1 and Tumor Necrosis Factor (TNF-alpha). Elevation of CRP level is an essential component of the acute-phase response to a variety of cellular abnormalities such as infection, inflammation, tissue trauma, and malignancies **(Gabay and Kushner, 1999).** CRP binds to polysaccharides of microorganisms and plays a role in the activation of the classical complement pathway, as well as in the clearance of apoptotic cells **(Rhodes *et al*., 2011).** In chronic rheumatic diseases such as RA and systemic vasculitis, the CRP level correlates with disease activity. CRP is in fact one of the components of many disease activity indices used for assessment of inflammatory arthritis **(Wells *et al*., 2009).** A conventional CRP assay typically measures CRP at levels above 3 mg/l. liter. However, a novel high-sensitivity (hs) CRP assay can now detect CRP at a level as low as 0.2 µg/ ml.

As hsCRP is a good marker of inflammation in various diseases and in certain bacterial infections, hence, it was hypothesized that both inflammatory markers, *viz.:*

hsCRP and chsp60 possibly serve as biomarkers of *C. trachomatis* infection in ReA/ uSpA patients also.

In this chapter**,** the frequency of serum IgG antibodies to chsp60 in uSpA patients (without effusion) was investigated and serum hsCRP was quantified further these parameter **(**hsCRP and chsp60) were clinically correlated.

4.2 Results

4.2.1 Clinical data

35 uSpA patients in the age group 18 - 48 years were screened for circulatory IgG antibodies to chsp60 and hsCRP in the serum. Patients showed monoarthritis/ oligoarthritis/ polyarthritis profile with or without effusion in their joints. Majority of uSpA patients (80%) in study group showed an oligoarthritic profile **(Table 4.1).**

4.2.2 Estimation of chlamydial heat shock protein 60 (chsp60) in serum of undifferentiated spondyloarthropathy patients without effusion and control patients

chsp60 results were analysed qualitatively, semi-quantitatively and quantitatively. Overall 28.5% (10/ 35) uSpA patients had circulatory IgG antibodies in serum against chsp60 ('p' < 0.004; Odds Ratio: 21; CI: 1.191 - 372.2 vs RA/ OA). Qualitative analysis showed 3 patients in 'grey zone; titer calculation was done for quantitative analysis and it was found that among 10 chsp60 IgG-positive uSpA patients, 8 had titer of 1: 50 while 2 patients had titer of 1: 200 and 1: 100, respectively. There was a significant difference

between the Cut-Off indices (COI) values between chsp60-positive and -negative uSpA patients as well as RA patients **(Table 4.2; Fig. 4.1).**

Table 4.1: Clinical and demographic profile of undifferentiated spondyloarthropathy (without effusions), rheumatoid arthritis and osteoarthritis patients

Clinical characteristics	**uSpA patients (n=35)**	**RA patients (n=35)**	**OA patients (n=35)**
Age (mean ± SD) (years)	30.5 ± 9.8	37.4 ± 5.2	44.5 ± 9.7
Male: Female	24: 11 (68: 32)	08: 27 (22.8: 77.1)	24: 11 (68.5: 31.4)
Disease duration (mean ± SD) (months)	9.6 ± 9.9	10.8 ± 9.6	17.6 ± 15.6
Arthritic pattern			
Monoarthritis	03 (8.5)	0 (0)	06 (17.1)
Oligoarthritis	28 (80)	03 (8.5)	29 (82.8)
Polyarthritis	04 (11.4)	32 (91.4)	0
Enthesitis	05 (14.2)	03 (8.5)	0
Low backache	30 (85.7)	0	0

Abbreviations: uSpA- undifferentiated spondyloarthropathy; RA- rheumatoid arthritis; OA- osteoarthritis.

Note: Data in parenthesis represents percentage value

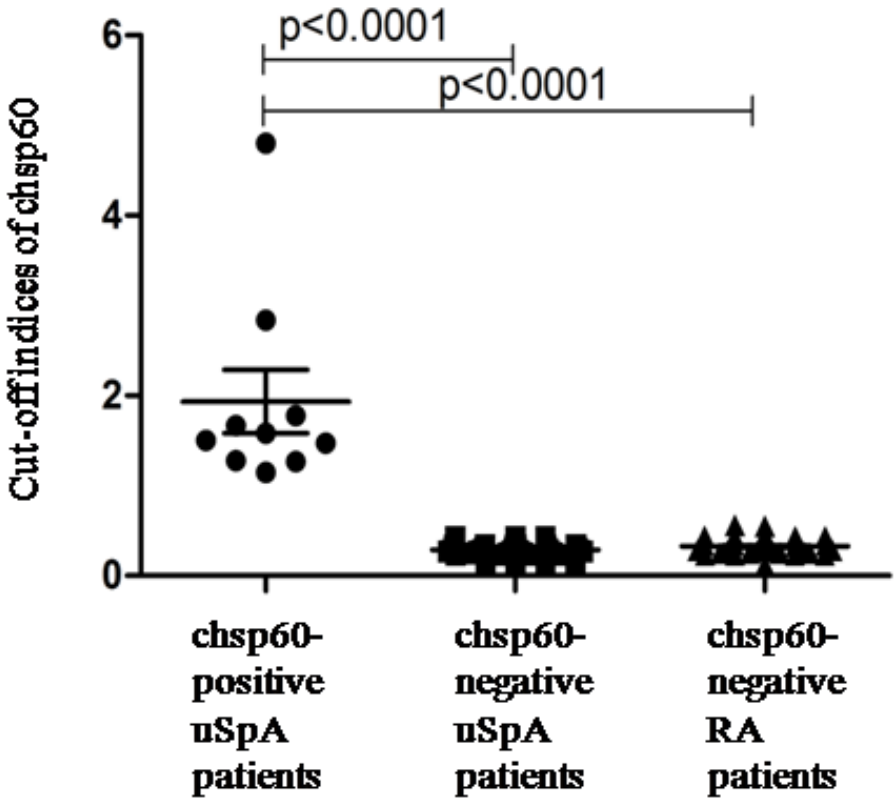


Fig. 4.1: Cut-off indices (COI) were compared between the chsp60-positive/ negative undifferentiated spondyloarthropathy (without effusions) and rheumatoid arthritis patients. Significant differences were observed between these groups. (Line between the bar shows mean with standard error of mean)

Abbreviations: chsp60+, chlamydial heat shock protein 60 IgG antibody positive undifferentiated spondyloarthropathy; chsp60-, chlamydial heat shock protein 60 IgG antibody negative undifferentiated spondyloarthropathy; RA, rheumatoid arthritis patients

Table 4.2: Qualitative, semi-quantitative and quantitative analysis of chlamydial heat shock protein 60 in serum of undifferentiated spondyloarthropathy patients (without effusions).

Patient	Sex	Arthritic pattern	Qualitative analysis	Semi-quantitative analysis (COI)	Quantitative analysis (titer)
Patient# 1	male	oligoarthritis	positive	1.58	1: 50
Patient # 2	female	oligoarthritis	positive	1.67	1: 50
Patient # 3	female	polyarthritis	positive	1.27	1: 50
Patient # 4	female	oligoarthritis	positive	1.47	1: 50
Patient # 5	male	oligoarthritis	positive	1.50	1: 50
Patient # 6	male	monoarthritis	positive	4.80	1: 200
Patient # 7	male	monoarthritis	positive	1.15	1: 50
Patient # 8	male	polyarthritis	positive	1.28	1: 50
Patient # 9	male	monoarthritis	positive	2.84	1: 100
Patient # 10	male	oligoarthritis	positive	1.78	1: 50
Patient # 11	male	oligoarthritis	grey zone	< 1.1	Equivocal
Patient # 12	male	oligoarthritis	grey zone	< 1.1	Equivocal
Patient # 13	female	polyarthritis	grey zone	< 1.1	Equivocal

Abbreviations: uSpA, undifferentiated spondyloarthropathy; COI, cut-off indices.

4.2.3 Determination of high sensitive C-reactive protein in serum of undifferentiated spondyloarthropathy patients without effusion and control patients

Level of hsCRP in uSpA patients varied between 0.0 - 84.5 µg/ ml (mean ± S.D. = 35.2 ± 28.6 µg/ ml) while in chsp60-positive uSpA patients, it was between 19 - 81.5 µg/ ml (mean ± S.D. = 48.1 ± 22.8 µg/ ml). hsCRP was higher in chsp60-positive uSpA patients as compared to chsp60-negative patients, however, it was not significant ('p' = 0.17). It was observed that hsCRP value in chsp60-positive patients was significantly higher than both inflammatory and non-inflammatory controls ('p' value < 0.05 vs RA, 'p' value < 0.0001 vs OA). In uSpA patients found to be chsp60-negative, hsCRP was significantly higher than in non-inflammatory control OA patients (mean ± S.D = 2.0 ± 1.22 µg/ ml; 'p' = 0.0001), however, there was only marginal difference with inflammatory controls (mean ± SD = 25.87 ± 21.4 µg/ ml; 'p' = 0.23). None of the control patients was positive for the chsp60 IgG antibodies **(Fig. 4.2).**

4.2.4 Clinical correlations in chsp60-positive uSpA patients

Age, disease duration, cut-off index of chsp60 IgG antibodies and hsCRP protein in chsp60-positive uSpA patients were compared using one-way ANOVA (Kruskal-Wallis test). All four parameters, comprising of age, disease duration, cut-off index of chsp60 IgG antibodies and hsCRP, showed significant difference in their median values ('p' < 0.0001). A significant positive correlation (r - 0.64; 'p' value < 0.05) was found between hsCRP and cut-off index of chsp60 by Spearman's rank correlation test **(Figs. 4.3 – 4.4).**

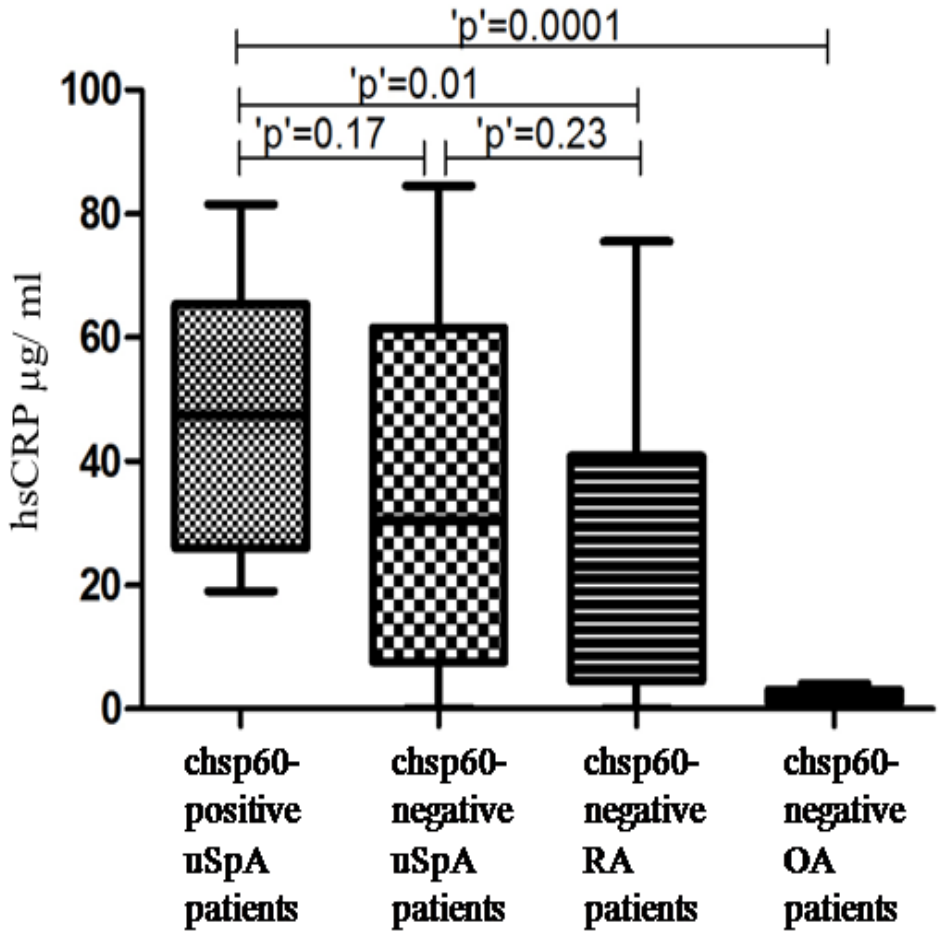
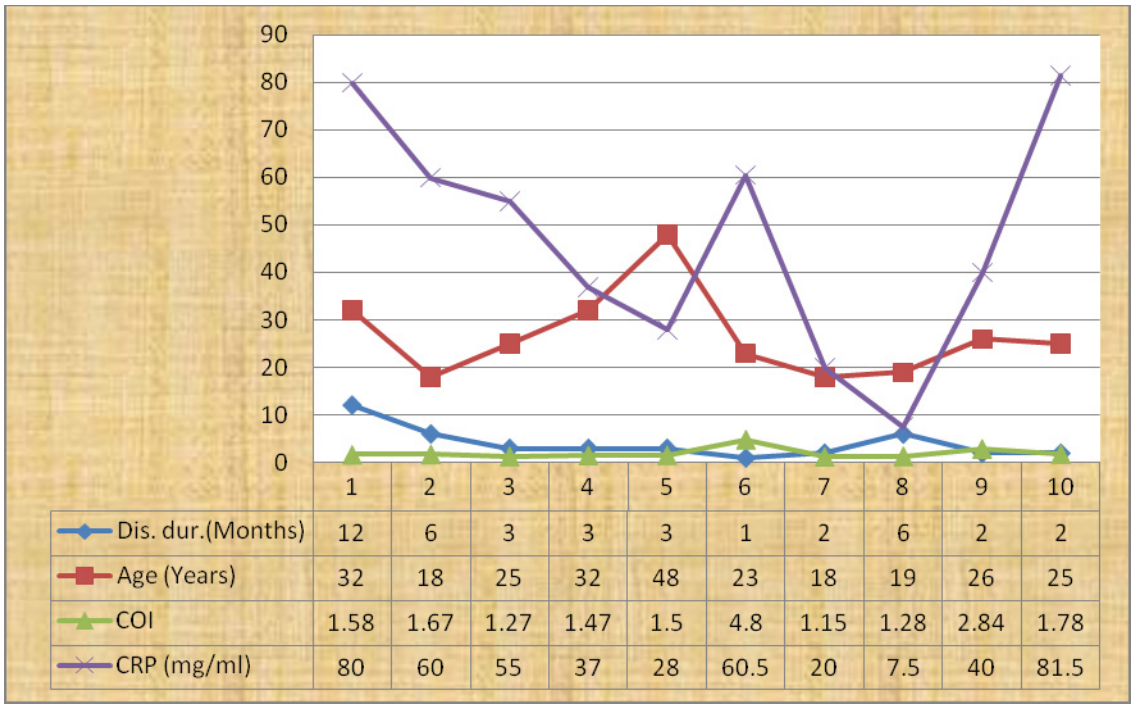


Fig. 4.2: Level of high sensitive C-reactive protein in chsp60 IgG-positive uSpA patients, chsp60 IgG-negative uSpA patients, chsp60 IgG-negative rheumatod arthritis patients and chsp60 IgG-negative osteoarthritis patients. Data in box plot is showing minimum to maximum hsCRP value and mean (in center) of hsCRP. 'p' value < 0.05 was considered to be significant.

Abbreviations: uSpA- undifferentiated spondyloarthropathy , RA- Rheumatoid Arthritis, OA - Osteoarthritis

The erythrocyte sedimentation rate was found to be high in uSpA patients without effusions. The disease duration in these patients varied from 1 - 84 (mean ± SD = 9.6 ± 9.9) months while chsp60-positive uSpA patients had disease duration of 1 - 12 months (mean ± SD = 4 ± 3.2). It was observed that among 10 chsp60-positive uSpA patients, 9 (90%) had disease duration of equal to or less than 6 months while 1 patient (10%) reported disease duration of 12 months ('p' < 0.01 vs chsp60-negative uSpA patients; Odds Ratio- 9; CI- 1.091 - 74.26). 70% of chsp60-positive uSpA patients (7/ 10) belonged to 18-28 years age group, while 20% (2/10) patients were in 28-33 years age group while one patient was 48 year old **(Tables 4.3 – 4.4; Figs. 4.3 - 4.4).**



	1	2	3	4	5	6	7	8	9	10
Dis. dur.(Months)	12	6	3	3	3	1	2	6	2	2
Age (Years)	32	18	25	32	48	23	18	19	26	25
COI	1.58	1.67	1.27	1.47	1.5	4.8	1.15	1.28	2.84	1.78
CRP (mg/ml)	80	60	55	37	28	60.5	20	7.5	40	81.5

Fig. 4.3: Graph shows high sensitive C-reactive protein (CRP), cut-off index (COI), age, disease duration (Dis dur) in chsp60-positive uSpA patients (n- 10). (Y-axis shows newmerical values of different parameter while on X-axis first row shows chsp60 positive patients)

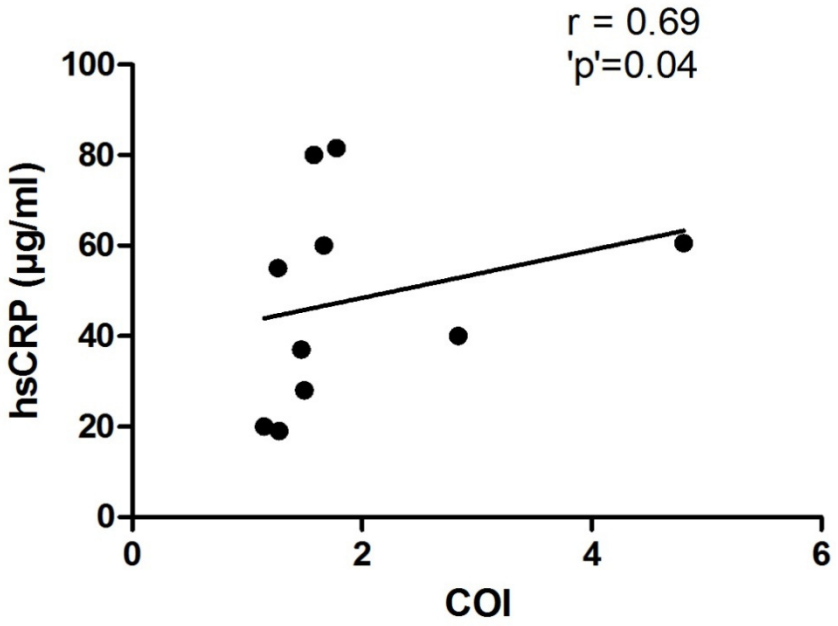


Fig. 4.4: Correlation between high sensitive C- reactive protein and cut-off index of chsp60-positive uSpA patients (r = 0.64; 'p' = 0.04) (Spearman's rank correlation test)

Table 4.3: Distribution and comparison of chlamydial heat shock protein 60-positive undifferentiated spondyloarthropathy patients on the basis of age

Groups	Age (years)	uSpA patients (n=35)	chsp60-positive uSpA patients (n=10)
Group I	18-23	9 (25.7)	4 (44.4)
Group II	23-28	8 (22.8)	3 (37.5)
Group III	28-33	5 (14.2)	2 (40)
Group IV	33-38	5 (14.2)	0
Group V	38-43	4 (11.4)	0
Group VI	43-48	4 (11.4)	1 (25)

Data in parenthesis depicts percentage

Table 4.4: Distribution and comparison of chlamydial heat shock protein 60-positive undifferentiated spondyloarthropathy patients on the basis of disease duration

Groups	Disease Duration (months)	chsp60-positive uSpA patients
Group I	1 - 6	9*
Group II	6 - 12	1
Group III	12 - 18	0
Group IV	18 - 23	0
Group V	23 - 28	0
Group VI	28 - 36	0

* 'p' value < 0.05 (between Groups I and II)

4.3 Discussion

C. trachomatis represents the most common single cause of genitourinary ReA while it has also been found in uSpA patients **(Carter *et al*., 2009)**. Due to asymptomatic *C. trachomatis* infection in uSpA and ReA patients and lack of reliable diagnostic methods, it is hugely underdiagnosed globally **(Senior, 2012).** Furthermore, past chlamydial infection is difficult to diagnose because of the absence of genetic material while conventional antibodies determination against *C. trachomatis* also does not give the conclusive picture **(Bas *et al*., 1998).** Hence, it was aimed to diagnose chsp60 IgG antibodies in serum along with hsCRP which is considered to be a very sensitive disease activity marker in various inflammatory diseases such as cardiovascular disease, RA, systemic lupus erythematic etc. **(Wells *et al*., 2009; Mok *et al*., 2013).** In the present

study, overall 28.5% (10/ 35) uSpA patients had circulatory IgG antibodies in the serum against chsp60.

Since *C. trachomatis* infection may go undetected or be misdiagnosed, and may be long lasting, anti-hsp60 antibodies are very likely to be causing disease silently for a long time without the physician being aware of potentially very damaging situation **(Cappello *et al*., 2009).** Elevated levels of IgA antibodies and CRP, in the absence of an acute infection, have been suggested to be markers of chronic inflammation and infection. In chlamydial infection, IgM response appears 2–3 weeks after the first symptoms of the illness while IgG response occurs after 6–8 weeks. IgA antibodies are only occasionally found during the primary infection but appear frequently during reinfection. The main antigens triggering an antibody-mediated response are LPS and hsp60. Although protective value of these antibodies is still to be established but hsp60 may be used as diagnostic or prognostic marker in chronic, persistent infection**.** uSpA patients are at greater risk of developing ReA due to asymptomatic infection with *C. trachomatis* **(Aggarwal *et al*., 1997).** Our data shows that uSpA patients were positive for anti- *C. trachomatis* IgA and IgG antibodies**.** In the present study 28.5% (10/35) patients with uSpA were found positive for chsp60 IgG antibodies which indicate that this protein is more abundantly found in these asymptomatic patients. chsp60, which is one of the most immunogenic *C. trachomatis* proteins, is found in the EB's and RB's, where it is encoded by 3 genes that are expressed independently and differentially in active versus persistent *C. trachomatis* infection **(Gerard *et al*., 2004).**

Further, chsp60-positive patients were differentiated on the basis of age and disease duration to find whether these factors were associated with the *C. trachomatis*-

positive uSpA patients. It was observed that young patients (18 - 28 years) were more prone to be positive for chsp60 IgG antibodies in comparison to older individuals (80% patients with chsp60 IgG were below the 28 years). Among 10 chsp60-positive patients, the male: female ratio was 7: 3, however, it was quite close (29.1%: 27.2%) when compared with the total male: female ratio (24: 11). High positivity of chsp60 has been observed in subclinical female infertility patients where anti-chsp60 antibodies are significantly more prevalent (45 – 75%) as compared with those without tubal disease or fertile controls (8 – 20%) **(Freidank *et al*., 1995; Claman *et al*., 1997; Persson *et al*., 1999; den Hartog *et al*., 2005)**. Another feature of the study was that the disease duration of the chsp60 patients was less than six months. Collectively, the study suggested that chsp60 might serve as a better serological marker in persistent infection and other parameters *viz.*: age, disease duration may also be important factors in diagnosing *C. trachomatis*. Another important aspect of this study is that the majority of patients develop synovitis at a later stage, hence this approach can be used for while making an initial diagnosis as well as can be used as post-therapy prognostic marker. Thus, positive correlation between hsCRP and chsp60 may have implications as a prognostic/ diagnostic marker in spondyloarthropathy patients. To the best of our knowledge till date, this is probably the first study evaluating the importance of chsp60 IgG antibodies and hsCRP along with age and disease duration in uSpA patients.

The acute phase protein, CRP is a serological marker of inflammation. CRP levels are >10 mg/l in acute infections and <1 mg/l in the absence of an infection. CRP levels between 1 and 10 mg/l are assumed to reflect a low-grade inflammation **(Pearson *et al*., 2003)** and can be detected using a high-sensitivity (hs) CRP test. The role of elevated

hsCRP levels as markers of an ongoing low-grade inflammation has been evaluated in studies on the relationship between persistent *C. pneumoniae* infections and cardiovascular diseases. These studies have shown that the known association between *C. pneumoniae* and cardiovascular diseases is even stronger in the presence of slightly elevated hsCRP levels **(Roivainen *et al*., 2000; Gattone *et al*., 2001; Johnston *et al*., 2001),** however the role of hsCRP is not evaluated in *C. trachomatis*-induced ReA/uSpA. hsCRP has proved as a most important disease activity marker in various diseases. In this study, hsCRP was increased (not significantly) in chsp60 IgG antibodies-positive uSpA patients in comparison to chsp60 IgG antibodies-negative uSpA patients, however, it was significantly higher than the inflammatory control RA and the non-inflammatory control OA. Levels of hsCRP were positively correlated with the COI of chsp60 IgG antibodies-positive uSpA patients, hence this finding delineates that both these inflammatory markers are enhanced simultaneously in *C. trachomatis*-positive uSpA patients. This association may prove to be useful as a diagnostic/ prognostic marker.

Chapter 5

ROLE OF CYTOKINES IN THE PATHOGENESIS OF CHLAMYDIA TRACHOMATIS-ASSOCIATED REACTIVE ARTHRITIS/ UNDIFFERENTIATED SPONDYLOARTHROPATHY

5.1 Introduction

ReA, previously known as Reiter's syndrome, is an inflammatory arthritis preceded by infection with enteric pathogens, such as *Salmonella* spp., *Yersinia* spp., *Shigella* spp. and *Campylobacter* spp., or the oculogenital pathogen, *viz.: Chlamydia* spp. **(Gracey and Inman, 2012)**. *C. trachomatis* represents the most common single cause of ReA **(Gerard *et al*., 2010).** Further among all the causative agents of ReA, viable organisms can be detected in the joints of patients with *Chlamydia*-induced ReA but not in those with post-enteric ReA **(Carter and Hudson, 2010).** It has been shown that *C. trachomatis* reaches the joints from the urogenital system through circulating monocytes and those monocytes/ macrophages are the common host cells for persistent organisms during long term infection, with a major role in the induction of inflammation **(Rihl *et al*., 2006).** The existence of persistent pathogens in the arthritic joint is known, but their relevance remains elusive. Research is focused currently on understanding the underlying mechanism of ReA, wherein an imbalance between type 1 and type 2 immune responses seems to be critical in determining susceptibility to disease **(Gracey and Inman, 2012)**.

It is likely that cytokines play a critical role in the pathogenesis of arthritis. A predominant Th1 cytokine profile, i.e., high concentrations of IFN-gamma and low concentrations of IL-4, has been reported **(Lahesmaa *et al*., 1992; Simon *et al*., 1993; Schlaak *et al*., 1996; Kotake *et al*., 1999; Ribbens *et al*., 2000)**. In contrast to the above reports, other studies have shown relative predominance of immunosuppressive cytokines, *viz.:* IL-4 and IL-10 and relative lack of IFN-gamma and tumor necrosis factor-α (TNF-alpha) in the synovial membrane **(Simon *et al*., 1994; Yin *et al*., 1999)**

and SF **(Yin *et al*., 1997, 1999)** of ReA patients compared with RA. Temporal relationships of these different Th1 and Th2 cytokines or blunting of initial cytokine response might also be important in the disease manifestation and its maintenance.

It has been argued that clearance of ReA-associated bacteria requires the production of appropriate levels of IFN-gamma and TNF-alpha, while IL-10 acts by suppressing these responses **(Yin *et al*., 1997; Autenrieth *et al*., 1994; Sieper and Braun, 1995; Holland *et al*., 1996; Yang *et al*., 1996; Sieper *et al*., 2000).** Indeed, IL-10 is a regulatory cytokine that inhibits the synthesis of IL-12 and TNF-alpha by activated macrophages **(de Waal Malefyt *et al*., 1991; Hart *et al*., 1995; Chomarat *et al*., 1995)** and of IFN-gamma by T-cells **(Yin *et al*., 1997; Macatonia *et al*., 1993).** However, IFN-gamma-inducing cytokines, such as IL-12 and IL-18 **(Scott, 1993; Dinarello, 1999; Mastroeni *et al*., 1999; Lammas *et al*., 2000; Yamamura *et al*., 2001)**, may also play a role in ReA. Thus, **Yin *et al*., (1997**) assigned a crucial role to the IL-10/IL-12 balance in the regulation of the cytokine pattern in the joints of patients with ReA. High levels of the inflammatory cytokine IL-6 were also reported in plasma and SF of patients with ReA **(Schlaak *et al*., 1996; Claudepierre *et al*., 1997).**

Th1 cells secrete IFN-gamma and regulate cellular immunity, while Th2 cells produce IL-4, IL-5, and IL-13 and mediate humoral responses. Recently, a third category of IL-17-producing T-helper cells (Th17) has been identified **(Park *et al*., 2005; Harrington *et al*., 2005).** Differentiation of Th0 cells to Th17 cells is mediated by simultaneous presence of TGF-ßeta and IL-6 **(Veldhoen *et al*., 2006, Bettelli *et al*., 2006)**. Th17 cells have been shown to be key mediator of chronic inflammation in animal models of RA, multiple sclerosis and inflammatory bowel disease **(Sallusto and**

Lanzavecchia, 2009). In immune response against microbes, IL-17 has antibacterial and antifungal properties both in humans and mice (**Sallusto and Lanzavecchia, 2009**, **Chabaud *et al*., 1999)**. Increased IL-17, IL-6 and TGF-ß, with good correlation between their levels has been reported in ReA/ uSpA patients and it has been suggested that this leads to the generation of proinflammatory Th17 cells **(Singh *et al*., 2007)**. In a recent report, IL-17 in *C. trachomatis* positive ReA patients was found to be positively correlated to IFN-gamma **(Bas *et al*., 2009)**. However, the status and role of IL-17 cytokine is still not clear and contraindicated in terms of its protective or destructive role in *C. trachomatis*-induced ReA/ uSpA. IL-6 is produced by monocytes and macrophages immediately after the stimulation with distinct pathogen-associated molecular patterns. In non-infectious inflammation, damage-associated molecular patterns from damaged or dying cells stimulate monocytes and macrophages to produce IL-6. The pathogenic role of IL-6 in rheumatic diseases like RA has been well established. The critical role for IL-6 in the pathogenesis of RA is provided by clinical trials, in which tocilizumab, specific for IL-6R, has been shown to suppress disease activity and erosive progression in patients with RA **(Smolen *et al*., 2008).** In ReA, the status of tocilizumab in this regard is unclear, however, elevated IL-6 concentrations in the plasma and sera of patients has been reported **(Metsärinne *et al*., 1992; Straub *et al*., 2002).**

CD4+ T-cells mediate immunity as a balance between different lineages of T helper Th1, Th2, Th3 and Th17 which secrete IFN-gamma, IL-4, TGF-beta and IL-17, respectively, as the main cytokine for each profile. Some studies revealed low levels of Th1 cytokines in ReA, especially TNF-alpha and also IFN-gamma in peripheral blood and synovium **(Yin *et al*., 1997; Simon *et al*., 1994; Smeets *et al*., 1998; Kotake *et al*.,**

1990; Appel *et al*., 2004; van Holten *et al*., 2005; Braun *et al*., 1999). IL-17 is readily detected in the SF and synovium of RA patients **(Chabaud *et al*., 1998).** Several studies using mouse models of RA have demonstrated a key role for IL-17 in the progression of disease **(Murphy *et al*., 2003; Nakae *et al*., 2003 (a/b); Lubberts *et al*., 2004; Ruddy *et al*., 2004)**. A blockade of IL-17 after disease onset effectively prevents bone and cartilage erosion and reduces the severity of clinical symptoms (**Lubberts *et al*., 2004)**. The broad involvement of IL-17 in many autoimmune diseases makes this cytokine an ideal drug target. Thus IL-17 may have a pathogenic role in ReA/ uSpA, however, studies on its role in animal models of ReA are still not available. Characterization of the role of Th17 cells in the synovium of ReA may provide further information about the role of IL-17 in ReA.

It has been demonstrated by various researchers that *C. trachomatis*-infected ReA/ uSpA patients with HLA B27 gene face more chronic infection in comparison to HLA B27-negative *C. trachomatis*-infected ReA/ uSpA patients **(Kuipers *et al*., 2002; Gaston, 2000)**. Low levels of IFN-gamma have been observed in *C. trachomatis*-infected ReA/ uSpA patients with HLA B27 gene in comparison to HLA B27-negative individuals **(Bas *et al*., 2003)**. A cytokine imbalance and the interaction between bacteria and HLA B27 are thought to have a major role in the failure to eliminate the triggering *C. trachomatis* and the microbial antigen, leading to the disease manifestations and chronicity **(Rihl *et al*., 2006)**.

chsp60 is an inflammatory protein which plays protective role during *C. trachomatis* infection; however, it is still unknown how this protective role takes place in synergism of cytokine regulation **(Gaston, 1997)**. Also, the role of inflammatory high

sensitive C-reactive protein in the microenvironment of synovium of ReA/uSpA patients is not yet explored.

This chapter deals with the estimation and evaluation the role of localized and circulatory Th1/ Th2/ Th17 (IFN-gamma, IL-4, IL-17 and IL-6) cytokines as well as the role of inflammatory protein (*viz.:* chsp60, hsCRP) and genetic factor (HLA B27) in the pathogenesis of *C. trachomatis*-induced ReA/ uSpA.

5.2 Results

5.2.1 Clinical details

In ***C. trachomatis*-infected ReA/ uSpA patients,** the median age was 26 years (IQR 21 -33); median number of SF leucocytes was 14200/ mm^3 (range: 8200 - 24600) while in ***Chlamydia*-negative patients,** the median age was 26.5 years (Inter-Quartile Range (IQR): 21.5 - 41.5); median number of SF leucocytes was 11200/ mm^3 (8400 - 24800). The median age of **RA patients** was 38 years (IQR 31 - 42); median number of SF leucocytes was 8300/ mm^3 (IQR 6300 - 12900), while in **OA patients,** the median age was 48 years (IQR 45 - 60); while median number of SF leucocytes was 400/ mm^3 (IQR 165 - 465).

5.2.2 Estimation of cytokines in synovial fluid

Level of **IFN-gamma** in SF of *C. trachomatis*-positive ReA/ uSpA patients (median-45.15 pg/ ml; IQR: 39.7 - 62.8) was significantly higher ('p' = 0.0003) than the negative patients (median - 23.5 pg/ ml; IQR: 12.8 - 33.3) as well as inflammatory control RA (median-22 pg/ ml; IQR: 21 - 27; 'p' = 0.0001) and non-inflammatory control

OA (median 1.32 pg/ ml; IQR: 0.3 - 2.1; 'p' = 0.0001). **IL-4** was significantly higher ('p' = 0.01) in *C. trachomatis*-infected ReA/ uSpA patients (median- 19.3 pg/ ml; IQR: 14.1 - 23.4) in comparison to non-infected ReA/ uSpA patients (median- 13.7 pg/ ml; IQR: 10.5 - 19.2). The level of IL-4 in *C. trachomatis* infected ReA/ uSpA patients was also significantly upregulated in comparison to RA (median- 10 pg/ ml; IQR: 8.4 - 12.2; 'p' = 0.0001) and OA (median- 1 pg/ ml; IQR: 0.24 - 2.1; 'p' = 0.0001).

There was no significant difference observed in the level of the pleiotropic cytokine **IL-6** in *C. trachomatis*-infected ReA/ uSpA patients (median- 265.8 pg/ ml; IQR: 225.6 - 275.2; 'p' = 0.6) in comparison to non-infected patients (median- 261.1 pg/ ml; IQR: 216 - 295.8), however, it was significantly higher than both control groups (RA; median- 156.3 pg/ ml; IQR: 139.8 - 201.3; 'p' = 0.0001 and OA; median- 1.9 pg/ ml; IQR: 0.0 - 4.8; 'p' = 0.0001).

IL-17 was comparable ('p' = 0.9) between the *C. trachomatis*-infected ReA/ uSpA patients (median- 77.6 pg/ ml; IQR: 48.8 - 91.3; 'p' = 0.0001) and non-infected patients (median- 77.3 pg/ ml; IQR: 48.8 - 91.3) and RA patients (median- 77.3 pg/ ml; IQR: 39.9 - 102.4; 'p' = 0.8). However, it was significantly high in comparison to OA patients (median- 1.8 pg/ ml; IQR: 1.1 - 2.5; 'p' = 0.0001) **(Table 5.1; Figs. 5.1 and 5.2).**

5.2.3 Estimation of cytokine levels in serum

IFN-gamma level in serum of *C. trachomatis*-infected ReA/ uSpA patients was higher ('p' = 0.06 (non-significant), median- 29 pg/ ml; IQR: 21.8 - 40.9) than the

Table 5.1: Estimation of cytokines in the synovial fluid of arthritic patients

Cytokine	CT-infected ReA/ uSpA patients (n= 12)	CT-non infected ReA/ uSpA patients (n= 33)	RA patients (n= 35)	OA patients (n= 35)	'p' value
IFN-gamma (pg/ ml)	13.6 - 77 45.1 (39.4 - 62.7)	2.0 - 95.8 23.5 (12.8 - 33.3)	9.7 - 69.2 22.0 (21 - 27.5)	1.2 - 3.2 1.32 (0.3 - 2.1)	0.0003* 0.0001# 0.0001$
IL-4 (pg/ ml)	13.3 – 33 19.3 (14.1 - 23.4)	6.4-31.6 13.7 (10.5 -19.2)	2.3 - 23.2 10 (8.4 - 12.2)	0.23 - 3.1 1.0 (0.24 - 2.1)	0.01* 0.0001# 0.0001$
IL-6 (pg/ ml)	42.1 - 295.8 265.8 (225.6 -275.2)	42.1 - 295.8 261.1 (216 - 295.8)	92.6 - 247.1 156.3 (139.8 -201.3)	0.0 - 4.8 1.9 (1.8 - 2.3)	0.6 vs* 0.0001# 0.0001$
IL-17 (pg/ ml)	20.6 - 146 (77.6) (48.8 - 91.3)	20.6 - 146 (77.3) (48.8 - 91.3)	20.6 - 130.2 (77.3) (39.9 - 102.4)	0.0 - 4.2 (1.8) (1.1 - 2.5)	0.9* 0.5# 0.0001$

Data represented shows range followed by median value and interquartile range (IQR) in parenthesis.

'p' value < 0.05 is considered to be significant.

Abbreviations: CT- *Chlamydia trachomatis;* ReA- Reactive Arthritis, uSpA- Undifferentiated Spondyloarthropathy, RA- Rheumatoid arthritis; OA- Osteoarthritis.

* *C. trachomatis*-positive ReA/ uSpA versus *C. trachomatis* non-infected ReA/ uSpA patients;
\# *C. trachomatis*- positive ReA/ uSpA versus RA patients;
$ *C. trachomatis*- positive ReA/ uSpA versus OA patients.

C. trachomatis-non-infected patients (median- 21.1 pg/ ml; IQR: 12.3 - 27.7). Level of serum IFN-gamma in *C. trachomatis*-infected ReA/ uSpA patients was significantly upregulated in comparison to RA ('p' = 0.009; median- 19.6 pg/ ml; IQR: 11.8 - 23.1) and OA ('p' = 0.0001; median- 1.32 pg/ ml; IQR: 0.3 - 2.1). Serum levels of **IL-4** in *C.*

trachomatis-infected ReA/ uSpA patients were significantly elevated ('p' = 0.006; median- 14.1 pg/ ml; IQR: 10.4 - 22.2) than the non-infected ReA/ uSpA patients (median- 10.5 pg/ ml, IQR: 8.3 - 11.7) Furthermore, the level of IL-4 was significantly high in the *C. trachomatis*-positive ReA/ uSpA patients in comparison to RA ('p' = 0.001; median- 9.2 pg/ ml; IQR: 7.4 -11.6) and OA ('p' = 0.0001; median- 1 pg/ ml; IQR: 0.24 - 2.1). In contrast to the synovial findings, the pleiotropic cytokine **IL-6** was significantly enhanced in *C. trachomatis*-infected ReA/ uSpA patients ('p' = 0.04; median- 15.2 pg/ ml; IQR: 8.6 - 28.1) in comparison to patients without *C. trachomatis* infection (median- 6.96 pg/ ml; IQR: 3.5 - 21.5). Also, *C. trachomatis*-infected ReA/ uSpA patients had higher levels of IL-6 in comparison to RA ('p' = 0.04; median- 6.98 pg/ ml; IQR: 4.4 - 21.6) and OA ('p' = 0.0001; median- 2.3 pg/ ml; IQR: 1.8 - 4.8). **IL-17** levels were differential in contrast to SF findings but there was no significant difference between *C. trachomatis*-infected ('p' = 0.9; median- 100 pg/ ml; IQR: 41.9 - 149.6) and non-infected ReA/ uSpA (median- 58 pg/ ml; IQR: 39.2 - 152.3) patients. It was found that the level of IL-17 in infected ReA/ uSpA was non-significant in comparison to RA ('p' = 0.16; median- 77.3 pg/ ml; IQR: 39.9 - 102.4) while it was significantly elevated as compared to OA ('p' = 0.0001; median- 1.8 pg/ ml; IQR: 1.1 - 2.5) **(Table 5.2; Figs. 5.1-5.2).**

Table 5.2: Estimation of serum cytokines in arthritic patients

Cytokine	CT-infected ReA/ uSpA patients (n=12)	CT non-infected ReA/ uSpA patients (n=33)	RA (n=35)	OA (n=35)	'p' value
IFN-gamma (pg/ ml)	5.3 - 65 29.0 (21.8 - 40.9)	5.3 - 45 21.1 (12.3 - 27.7)	7.2 - 69.2 19.6 (11.8 - 23.1)	1.2 - 3.2 1.32 (0.3 - 2.1)	0.06* 0.009# 0.000$
IL-4 (pg/ ml)	7.3-33 14.17 (10.4 - 22.2)	3.4 - 14.1 10.5 (8.3 - 11.7)	3.4 - 15 9.2 (7.4 - 11.6)	0.23 - 3.1 1.0 (0.24 - 2.1)	0.006* 0.001# 0.0001$
IL-6 (pg/ ml)	6.1 - 39.2 15.2 (8.6 - 28.1)	1.5 - 78.9 6.96 (3.5 - 21.5)	1.5 - 134 6.98 (4.4 - 21.0)	0.2 - 4.9 2.3 (1.8 - 4.8)	0.04* 0.02# 0.0001$
IL-17 (pg/ ml)	16.1 - 317 100 (41.9-149.6)	3.07 - 424.9 58 (39.2 - 152.3)	20.7 - 130.2 77.3 (39.9 -102.4)	0.0 - 4.2 (1.8) (1.1 - 2.5)	0.6* 0.07# 0.000$

Data represented shows range followed by median value and interquartile range (IQR) in parenthesis.

'p' value < 0.05 is considered to be significant.

Abbreviations: CT- *Chlamydia trachomatis;* ReA- Reactive Arthritis, uSpA- Undifferentiated Spondyloarthropathy, RA- Rheumatoid arthritis; OA- Osteoarthritis.

* *C. trachomatis*-positive ReA/ uSpA versus *C. trachomatis* non-infected ReA/ uSpA patients;
# *C. trachomatis*- positive ReA/ uSpA versus RA patients;
$ *C. trachomatis*- positive ReA/ uSpA versus OA patients.

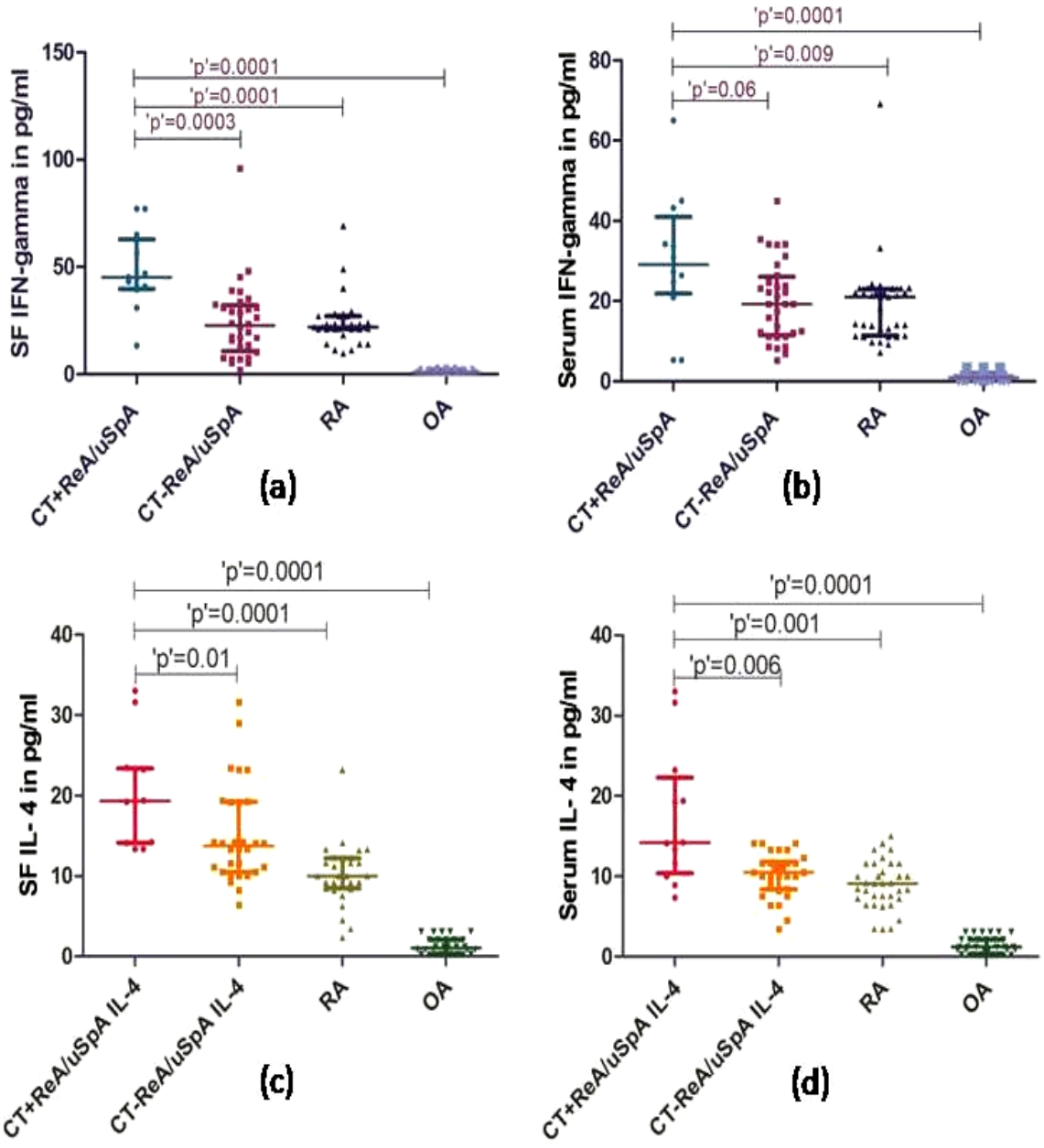


Fig.5.1: IFN-gamma level in (a) synovial fluid and (b) serum of arthritic patients & IL-4 levels in (c) synovial fluid and (d) serum of arthritic patients

Abbreviations: CT- *Chlamydia trachomatis;* ReA- Reactive Arthritis; uSpA- Undifferentiated Spondyloarthropathy; RA- Rheumatoid arthritis; OA- Osteoarthritis; CT+ *C. trachomatis*-positive; CT- *C. trachomatis* non-infected

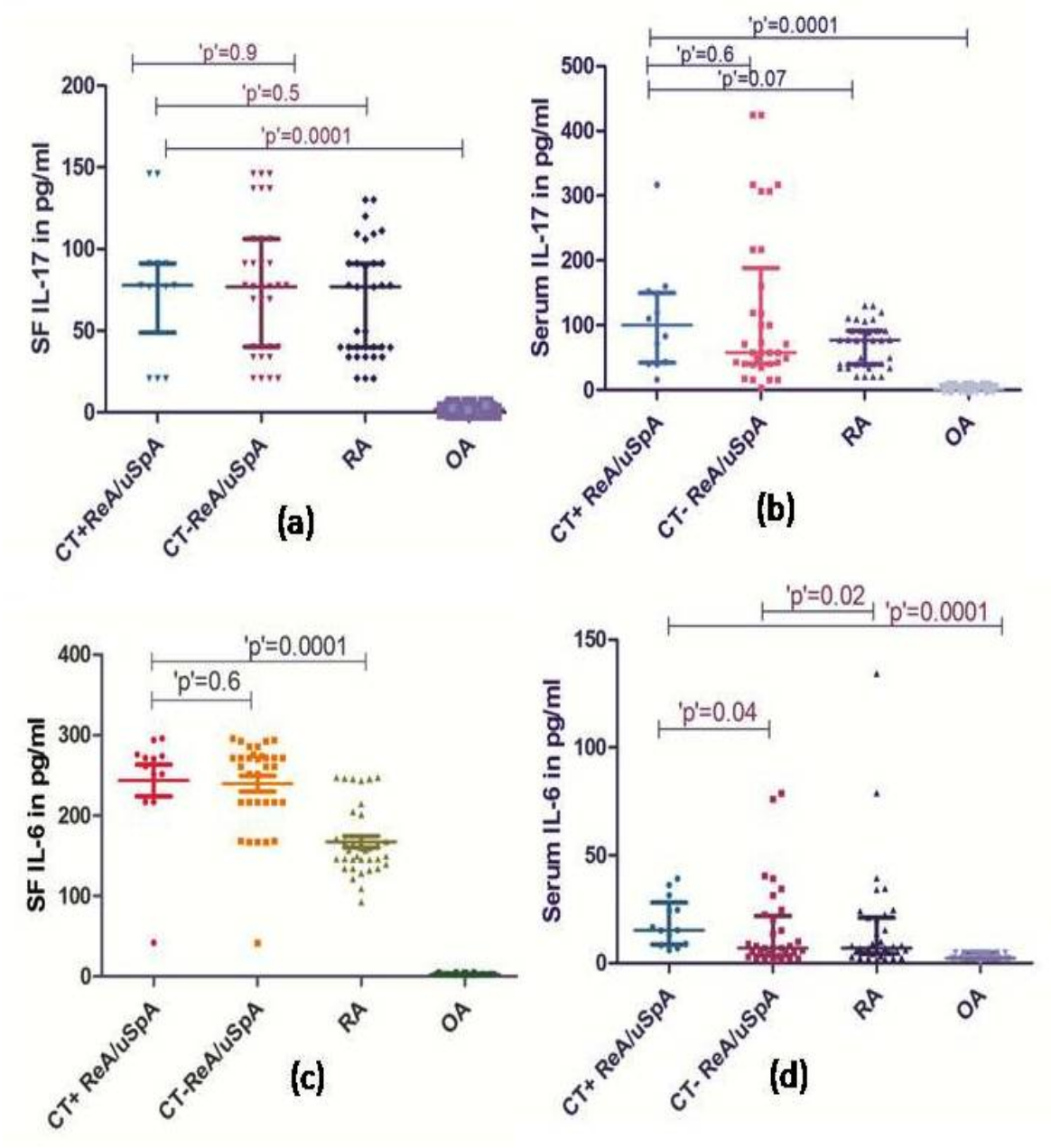


Fig. 5.2: IL-17 levels in (a) synovial fluid and (b) serum of arthritic patients & IL-6 level in (c) synovial fluid and (d) serum of arthritic patients. Abbreviations: CT- *Chlamydia trachomatis;* ReA- Reactive Arthritis; uSpA- Undifferentiated Spondyloarthropathy; RA- Rheumatoid arthritis; OA- Osteoarthritis; CT+ *C. trachomatis*-positive; CT- *C. trachomatis* non-infected

5.2.4 **Correlation between synovial fluid and serum cytokines**

Cytokine findings in *C. trachomatis*-infected ReA/ uSpA patients (n- 12) were compared in both SF and serum by Kruskal Wallis test ('p' = 0.0001) and correlated to each other using Spearman's rank correlation test. In SF, IL-6 was found to be positively correlated (r = 0.72; 95% CI: 0.23 - 0.91; 'p' = 0.007) with IFN-gamma. Also, SF IFN-gamma was positively correlated with serum IFN-gamma (r = 0.28; $p < 0.05$).

Cytokine levels in SF and serum of *C. trachomatis*-positive ReA/ uSpA patients were further analyzed using non-parametric Mann-Whitney test. Upon analysis, both IFN-gamma and IL-6 were increased ('p' = 0.4 and 'p' = 0.0001, respectively) in SF in comparison to serum; while IL-17 was higher ('p' = 0.4) in serum than in SF. However, IL-4 was comparable ('p' = 0.7) in both SF and serum (**Figs.5.3– 5.4; Table 5.3).**

Table 5.3: Correlation among Th1/ Th2/ Th17 cytokines in synovial fluid and serum of *C. trachomatis*-infected reactive arthritis/ undifferentiated spondyloarthropathy patients

S. No.	Correlation between cytokines	Correlation coefficient (r)	'p' value
Synovial fluid			
1.	IL-6 vs IL-17	0.14	0.6
2.	IL-17 vs IL-4	0.06	0.83
3.	IL-17 vs IFN gamma	-0.42	0.12
4.	IL-6 vs IFN gamma	0.72	0.007**
Serum			
1.	IL-6 vs IL-17	0.10	0.7
2.	IL-17 vs IL-4	-0.05	0.8
3.	IL-17 vs IFN gamma	-0.31	0.25
4.	IL-6 vs IFN gamma	-0.47	0.12

Spearman's rank correlation test was applied for comparison.

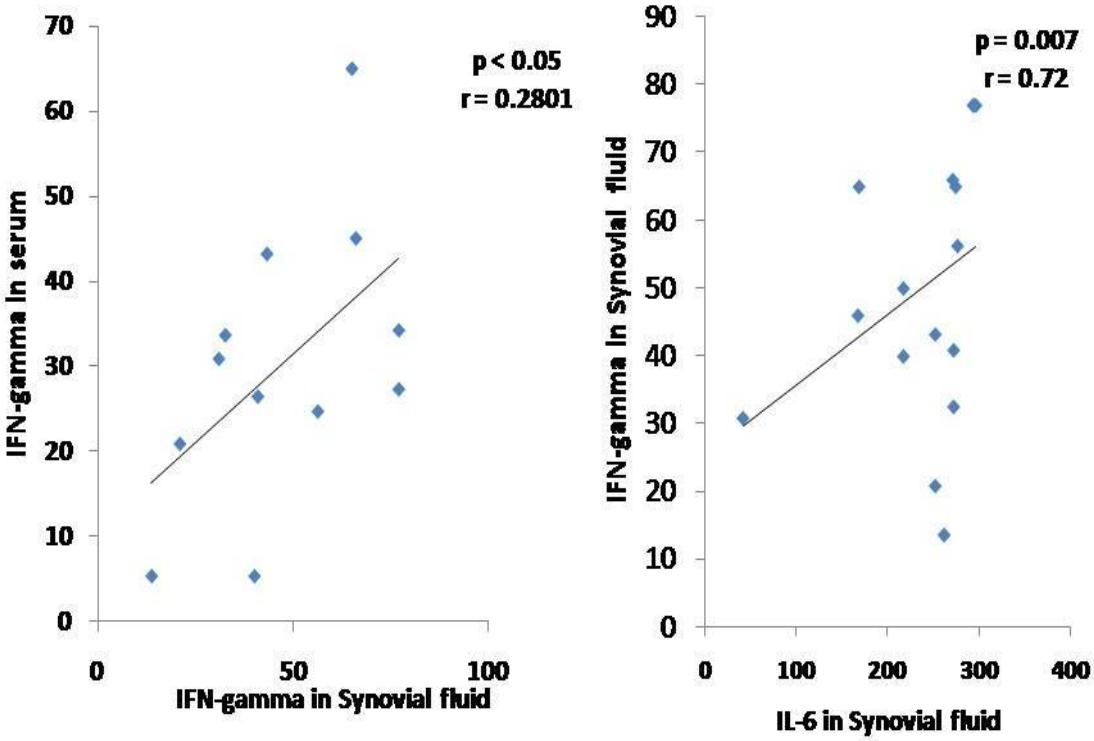


Fig.5.3: Correlation between (a) IFN-gamma and (b) IL-6 cytokines in synovial fluid and serum in *Chlamydia trachomatis*-positive reactive arthritis/ undifferentiated spondyloarthropathy patients by Spearman's Rank Correlation test.

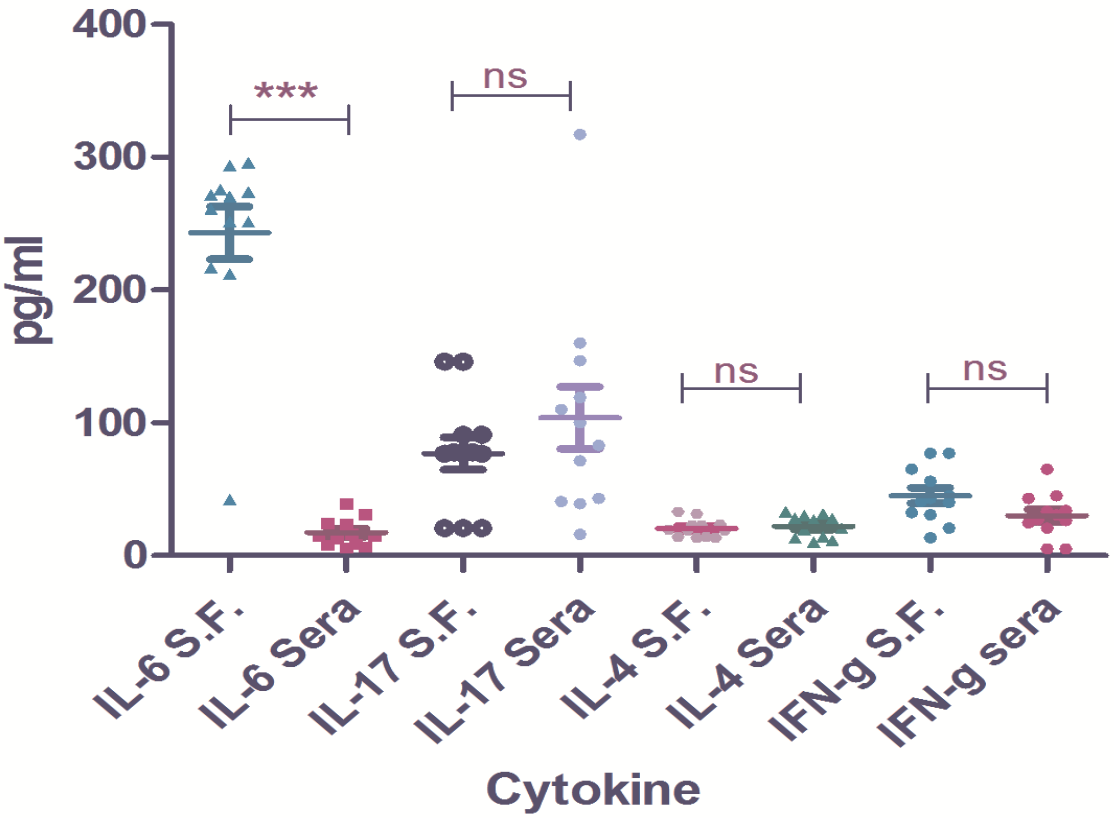


Fig. 5.4: Comparison of synovial fluid and serum cytokines in *Chlamydia trachomatis*-positive reactive arthritis/ undifferentiated spondyloarthropathy patients by Mann-Whitney test.
Abbreviations- ns: non-significant; SF: synovial fluid; IFN-g: interferon gamma

5.2.5 Effect of chsp-60 on Th1/ Th2/ Th17 cytokines and high sensitive C-reactive protein

Level of serum cytokines, *viz.:* IFN-gamma, IL-4, IL-6 and IL-17 were compared between chsp60- positive (n- 10) versus chsp60-negative (n- 32) uSpA patients. IFN-gamma was high ('p' > 0.05, non-significant) in chsp60-positive patients in comparison to the negative patients, however, no difference was found in IL-4, IL-6 and IL-17 levels. IFN-gamma, IL-4, IL-6 and IL-17 were further compared among the positive patients and it was found that levels of IFN-gamma ('p' = 0.04) and IL-17 ('p' = 0.0001) were

significantly higher than IL-6. IL-17 was also significantly higher ('p' = 0.01) than IFN-gamma level. High sensitive CRP was also differential in both groups **(Table 5.4; Fig. 5.5)**.

Table 5.4: Cytokines and high sensitive C-reactive protein levels in chlamydial heat shock protein-positive/ negative undifferentiated spondyloarthropathy patients

Parameter	**chsp60-positive patients (n=10)**	**chsp60-negative patients (n= 32)**	**'p' value**
IFN-g (pg/ ml)	2 - 95 32.5 (10.3 - 56.2)	2 - 48.1 19.5 (11.2 - 29.9)	0.23 ^{ns}
IL-4 (pg/ ml)	7.3 - 33 14.17 (10.4 - 22.2)	3.4 - 14.1 10.5 (8.3 - 11.7)	0.42 ^{ns}
IL-6 (pg/ ml)	1.7-36.2 8.3 (6.1 - 11.2)	1.5 - 78.9 6.96 (3.5 - 21.5)	0.77 ^{ns}
IL-17 (pg/ ml)	16.1 - 160 73.0 (50.8 - 114.6)	3.07 - 424.9 58 (39.2-152.3)	0.95 ^{ns}
hsCRP (µg/ ml)	8.9 - 69 39 (19.6 – 62)	9.2 - 21.2 13.2 (12.2 - 17.2)	< 0.009

Data represented shows range followed by median value and interquartile range (IQR) in parenthesis.

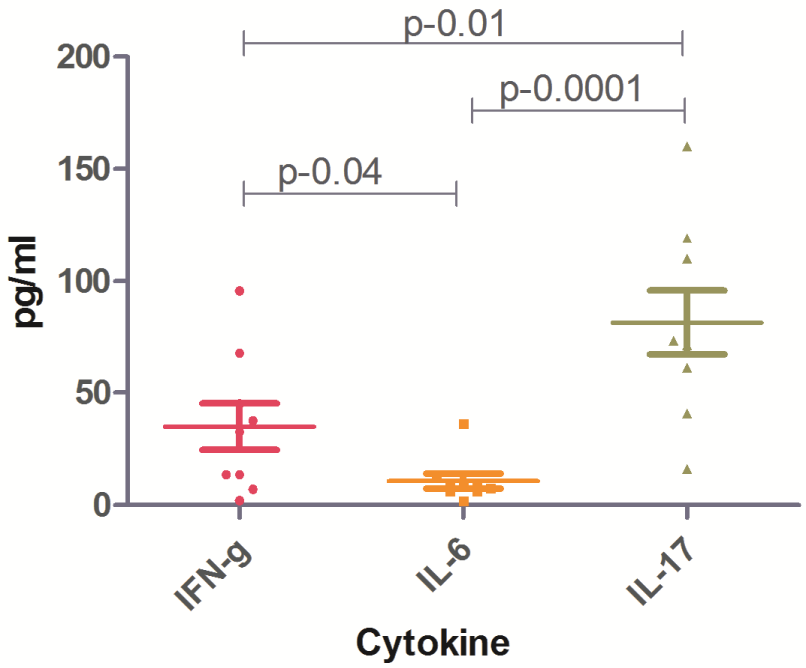


Fig 5.5: Level of cytokines in *Chlamydia trachomatis* heat shock protein 60-positive uSpA patients. Data was compared using Mann-Whitney test. **Abbreviations:** IFN-g: interferon gamma

5.2.6. **Effect of the presence of HLA B27 gene on cytokines in ReA/ uSpA patients**

40% (18/ 45) ReA/ uSpA patients were positive for HLA B27 gene in the SF. Among these, 38.8% (7/18) patients had *C. trachomatis* infection. The pro-inflammatory/ anti-inflammatory cytokines and hsCRP were compared between HLA B27-positive (*C. trachomatis*-positive) and HLA B27-positive (*C. trachomatis* non-infected) patients. Level of hsCRP was significantly higher ('p' = 0.007) in HLAB27-positive (*C. trachomatis*-infected) patients in comparison to the non-infected patients with HLA B27 gene **(Table 5.5)**. No significant difference was found in IFN-gamma, IL-4, IL-6 and IL-17 in both groups, however, IFN-gamma was decreased in HLA B27 patients ('p' > 0.05; non-significant).

Table 5.5: Expression of cytokine profile in the synovial fluid of HLA B27-positive reactive arthritis/ undifferentiated spondyloarthropathy patients with/ without *C. trachomatis* infection.

	HLAB27-positive *C. trachomatis*-positive ReA/ uSpA patients (n= 7)	**HLAB27-negative *C. trachomatis*-non-infected ReA/ uSpA patients (n= 11)**	**'p' value**
IFN-gamma (pg/ ml)	2.3 - 34.3 14.2 (10.3 - 24.2)	2 - 39.1 19.5 (9.2 - 45.9)	0.54 ^{ns}
IL-4 (pg/ ml)	6.3 - 32.6 13.17 (10.4 - 21.2)	2.4 - 13.1 10.1 (8.3 - 12.7)	0.39 ^{ns}
IL-6 (pg/ ml)	1.4 - 39.2 8.9 (5.4 - 11.2)	1.5 - 39.9 7.46 (3.5 - 19.5)	0.9 ^{ns}
IL-17 (pg/ ml)	14.1 - 163 63.0 (49.8 - 109.6)	3.07 - 233.9 58 (39.2 - 152.3)	0.2 ^{ns}
hsCRP (µg/ ml)	10 - 73 49 (20.6 - 62.3)	10.2 - 23.2 14.2 (12.2 - 16.2)	< 0.007

Data represented shows range followed by median value and interquartile range (IQR) in parenthesis.

Abbreviations: ReA- Reactive Arthritis, uSpA- Undifferentiated Spondyloarthropathy

5.3 **Discussion**

ReA is a Th1-dominant, self-limiting disease in the majority of cases. It has been reported that the increased level of IFN-gamma regulates the inflammation caused by Th17 cells **(Park *et al*., 2005; Harrington *et al*., 2005; Cruz *et al*., 2006)**. In present study the levels of Th-1 cytokine IFN-gamma in synovial fluid and serum was significantly upregulated in *C. trachomatis* infected ReA/ uSpA patients in comparison to non-infected ReA/ uSpA patients as well as in inflammatory control patients RA and non-inflammatory control OA patients. These results were in agreement with other investigators **(Lahesmaa *et al*., 1992; Schlaak *et al*., 1992; Simon *et al*., 1993)**. In contrast, the Th-2 cytokine IL-4 was comparatively inhibited in comparison to IFN-gamma levels. However, IL-4 was significantly increased in *C. trachomatis*-infected patients in comparison non-infected RA and OA patients. (**Simon *et al*., 1994; Yin *et al*., 1997, 1999)**

Th17 cells are highly proinflammatory and are involved in various autoimmune diseases like experimental autoimmune encephalitis, RA as well as in host defence **(Sallusto and Lanzavecchia, 2009).** Bas et al., (2009) were the first to report the presence of IL-17 in the SF of patients with *C. trachomatis*-induced ReA. IL-17 has been shown to be pathogenic in *Borrelia*-induced arthritis **(Nardelli *et al*., 2008).** IL-17 is well known for its role in joint destruction **(Lubberts *et al*., 2005)** but recent findings indicate that IL-17 may also contribute to protection against intracellular bacteria such as *Mycobacterium tuberculosis* **(Khadersa *et al*., 2007; Scriba *et al*., 2008)**. In our study, IL-17 was significantly higher than the non-inflammatory control OA patients (Group IV). This profile was evident in both SF and serum, hence it was difficult to differentiate

the exact role of IL-17 in protection or destruction **(Khadersa *et al*., 2007; Scriba *et al*., 2008)**.

Bas *et al*., 2009 detected both IFN-γ and IL-17 in the SF of patients with *C. trachomatis*-induced ReA and a positive correlation was also found between their levels indicating possible similar regulation by the local cytokine milieu in joints. **Beatty *et al*., (1993)** also reported similar finding in reference to IFN-gamma. In our study, negative correlation was found between both these cytokines in SF as well as in serum. This discrepancy in results may be due to the longer disease duration found in ReA/ uSpA patients (more than six months). The alteration in IL-17 and IFN-gamma may be a cause of persistent *C. trachomatis* infection in our patients.

A positive correlation was found between SF IL-6 and IFN-gamma which probably indicates that IL-6 is a key player in the pathogenesis of *C. trachomatis*-induced ReA/ uSpA. In the present study, SF IL-6 was marginally higher (statistically non-significant) in *C. trachomatis*-infected patients in comparison to non-infected ReA/ uSpA patients, however, there was significant difference between infected patients in study group versus the controls (RA and OA). Higher levels of IL-6 have been reported in inflammatory arthritis such as in ReA **(Singh *et al*., 2007)** and in RA **(Schlaak *et al*., 1996).** In the present study, the inflammatory cytokine IFN-gamma was positively correlated in SF and serum. Antibacterial activity of inflammatory cytokine IFN-gamma was supported in both synovial fluid and serum of ReA/ uSpA patients.

Immune recognition of hsp60 is also very common in infection, particularly where intracellular organisms are concerned **(Gaston, 1997)**. Precise mapping of the

peptides (epitopes) within chsp60 which are recognized by CD4+ T-cells has so far not shown any evidence of cross reactivity with human hsp60 **(Gaston, 2000),** however, there exits possibility of cross-reaction with *C. pneumoniae* **(Deane *et al*., 1997)**. There is considerable evidence implicating immune responses to chsp60 in inflammation, as anti-hsp60 antibodies were found to be raised in patients with tubal infertility, delayed type hypersensitivity responses to hsp60 in animal models of trachoma and salpingitis, continued production of hsp60 by chlamydiae in a non-cultivable state, transcription of hsp60 genes by synovial chlamydiae **(Gaston, 2000)**. In our study, asymptomatic uSpA patients with chsp60 IgG antibodies showed slight enhancement in IFN-gamma level in comparison to the chsp60-negative patients, however, when cytokine levels were compared among the chsp60-positive patients, it was found that IL-17 and IFN-gamma were significantly upregulated in comparison to IL-6 while the level of IL-17 was significantly high than IFN-gamma.

Based on these findings, it was concluded that probably chsp60 plays a protective role by upregulation of the pro-inflammatory/protective cytokines IFN-gamma and IL-17 in uSpA patients. However, the low level of IL-6 remains unexplained and it can be assumed that upregulation of IL-17 cytokine takes place by other regulatory pathways.

HLA B27 gene is associated with disease severity/chronicity in *C. trachomatis*-induced ReA/ uSpA **(Sonkar and Usha., 2007)**. In an *in-vitro* model, it was demonstrated that HLA B27 suppressed chlamydial replication **(Kuipers *et al*., 2001);** also soluble HLA B27, which is induced by ReA-triggering bacteria, is able to inhibit HLA B27 restricted T-cells and thus may also promote chlamydial persistence **(Kuipers *et al*., 2002)**. In our study, there was no significant difference in IFN-gamma, IL-4, IL-6

and IL-17 levels in *C. trachomatis*-infected HLA B27-positive patients versus *C. trachomatis*-non-infected HLA B27-positive patients, however, IFN-gamma level was decreased in *C. trachomatis*-infected HLA B27-positive patients. The lower IFN-gamma concentrations in HLA B27-positive patients with *C. trachomatis*-induced ReA could be related to the tendency of these patients to have more severe or chronic arthritis **(Bas *et al*., 2003)**. Braun et al., (1998) reported low level of IFN-gamma secretion by PBMCs from patients having long disease duration and a high frequency of HLA B27 positivity. They also reported a lower level of TNF-alpha secretion by HLA B27-positive than by HLA B27-negative patients and found a significant correlation between low TNF-alpha secretion and a more protracted course of arthritis.

There was marginal difference in SF IL-6 level between *C. trachomatis*-infected and uninfected ReA/ uSpA patients and likewise results were obtained for IL-17 also. It has been reported that physiological factor also affect overall cytokine status of patients **(Stanley and Lacy, 2010)**.

Chapter-6

CONCLUSIONS AND FUTURE SCOPE OF WORK

6.1 Conclusions

In the present study, an attempt was made to **screen ReA/ uSpA** patients for simultaneous detection of the presence of ***C. trachomatis*** infection by both molecular and non-molecular diagnostic methods in various clinical samples, *viz.:* SF, serum and urine for estimating the **frequency** of infection. The **molecular** detection of *C. trachomatis* was done in the SF/ urine by targeting chromosomal MOMP/ extra-chromosomal plasmid genes by conventional PCR/ snPCR/ nPCR. Intra-articular infection for either ***C. trachomatis* MOMP/ plasmid** was found in **24.4%** (11/45) **ReA/ uSpA patients** by either snPCR/ nPCR; plasmid and MOMP were found in 11/45 (24.4%) and 7/45 (15.5%) patients, respectively. 28.5% (6/21) ReA and 20.8% (5/24) uSpA patients were found positive for either MOMP/ plasmid gene of *C. trachomatis*. The **plasmid** gene was also detected by PCR (conventional) in the urine of **17.3%** (6/35) **uSpA patients without effusions.**

Non-molecular diagnosis in SF/ serum/ urine included DFA for chlamydial MOMP antigen and circulatory/ localized antibody (IgM/ IgG/ IgA) detection by ELISA. Cytological detection by **DFA** showed the presence of *C. trachomatis* EBs in the **SF** of **31.1%** (14/45) **ReA/ uSpA** patients; while in the **urine** of **uSpA patients without effusions, 13.3%** (6/45) were *C. trachomatis*-positive. Although anti-chlamydial antibodies (IgM, IgG, IgA) were detected in SF/ serum of ReA/ uSpA patients, however, only **anti-*C. trachomatis* IgA antibodies** showed diagnostic importance (**SF- 31.1%;** 14/45 and **serum 15.5%;** 7/45).

snPCR was found to be 100% specific with respect to the gold standard nPCR, however, it had a poor sensitivity (63.6%); hence it was concluded that **nPCR** should be

the method of choice for diagnosing intra-articular *C. trachomatis* DNA in **SF** of **chronic ReA/ uSpA patients with effusion.** As **DFA** in **SF** showed 87.5% sensitivity, 83.7% specificity and **"*moderate*"** kappa agreement with nPCR findings, hence *C. trachomatis* MOMP diagnosis in the SF by this method can be used in **ReA/ uSpA patients with effusion** for an initial rapid detection of an **acute** episode of infection followed by the more confirmatory molecular diagnostic method. Furthermore, in our study, **"*moderate*"** kappa agreement was observed between PCR and intra-articular **anti-*C. trachomatis* IgA antibodies** in the **SF** of **ReA/ uSpA patients with effusions.** Overall, the **SF** was found to be a more conclusive clinical specimen in **both acute and chronically *C. trachomatis*-infected ReA/ uSpA patients with effusion** in terms of molecular diagnosis by nPCR, *C. trachomatis* MOMP antigen detection and anti-chlamydial antibody response.

The study further revealed "*moderate*" kappa agreement between SF and urine for *C. trachomatis* DNA detection by PCR; hence a **non-invasive sample such as urine** can also be used for molecular diagnosis of chlamydial infection in **acute/ chronic ReA/ uSpA patients with effusion.** Also, "m*oderate"* kappa agreement between IgA anti-chlamydial antibodies in **serum** and presence of *C. trachomatis* DNA in the **urine** of **uSpA patients without effusion** is another important finding of the study which implies that **both clinical samples** can be used for **diagnosis of chlamydial IgA antibodies and DNA,** respectively in uSpA patients, in whom full blown symptoms are unapparent. It is pertinent to mention that our study is the **first attempt** to investigate a non-invasive sample such as urine for the diagnosis of *C. trachomatis* infection in ReA/ uSpA patients with/ without effusion.

IgG antibodies to chsp60 were found in the serum of **28.5%** (10/35) **uSpA patients without effusion.** Majority of chsp60-positive uSpA patients without effusion were from **younger** (18 - 33 year) age group and had **disease duration of < 06 months.** Furthermore, levels of hsCRP were positively correlated with the COI of chsp60 IgG antibodies-positive uSpA patients without effusion, hence this finding delineated that both these inflammatory markers get enhanced simultaneously in *C. trachomatis*-positive uSpA patients; this association may prove to be useful as a **diagnostic/ prognostic marker.** The hsCRP in HLA B27-positive (*C. trachomatis*-infected) was also found to be significantly higher than uninfected HLA B27-positive patients.

To study the role of *C. trachomatis* infection in the **pathogenesis** of ReA/ uSpA patients, signature Th-1/ Th-2/ Th-17 cytokines were estimated in SF and serum. A **pro-inflammatory** response was evident in the **SF** of **ReA/ uSpA patients with effusion.** This was concluded on the basis of **significantly increased IFN-gamma, IL-4 and IL-17 levels** in the **SF** of these patients in comparison to OA patients (controls). Furthermore, SF IFN-gamma was positively correlated with serum IFN-gamma level, providing evidence that **IFN-gamma increases synergistically in SF and serum. Serum IL-6** was significantly enhanced in *C. trachomatis*-infected ReA/ uSpA patients with effusion in comparison to the uninfected ReA/ uSpA and controls (RA/ OA). Hence, it was **concluded that serum IL-6 plays a role in the pro-inflammatory cascade while SF IL-6 was positively correlated with IFN-gamma.** SF IL-17 was comparable between *C. trachomatis*-positive ReA/ uSpA with effusion *versus* the non-infected ReA/uSpA and control RA patients. In **chsp60-positive uSpA patients without effusion,** IFN-gamma and IL-17 were increased significantly in comparison to IL-6.

Specific Conclusions

- ✓ Intra-articular infection for either *C. trachomatis* MOMP/ plasmid was found in 24.4% (11/45) ReA/ uSpA patients by either snPCR/ nPCR
- ✓ The plasmid gene was also detected by PCR (conventional) in the urine of 17.3% (6/35) uSpA patients without effusions.
- ✓ As DFA and anti *C. trachomatis* IgA antibodies in SF showed "*moderate*" kappa agreement with nPCR findings, hence these methods can be used for *C. trachomatis* detection in ReA/ uSpA patients with effusion
- ✓ The study further revealed "*moderate*" kappa agreement between SF and urine for *C. trachomatis* DNA detection by PCR; hence a non-invasive sample such as urine can also be used for molecular diagnosis of chlamydial infection in acute/ chronic ReA/ uSpA patients with effusion.
- ✓ Levels of hsCRP were positively correlated with the COI of chsp60 IgG antibodies-positive uSpA patients without effusion, this association may prove to be useful as a diagnostic/ prognostic marker.
- ✓ A pro-inflammatory response was evident in the SF of ReA/ uSpA patients with effusion. This was concluded on the basis of significantly increased IFN-gamma, IL-4 and IL-17 levels in the SF of these patients in comparison to OA patients (controls).
- ✓ IFN-gamma increases synergistically in SF and serum of *C. trachomatis* induced ReA/ uSpA patients.

- ✓ It was concluded that serum and SF IL-6 plays a role in the pro-inflammatory cascade in *C. trachomatis*-infected ReA/ uSpA patients with effusion.
- ✓ In chsp60-positive uSpA patients without effusion, IFN-gamma and IL-17 were increased significantly in comparison to IL-6.

Future Scope of Work

Due to the lack of standard diagnostic criteria and asymptomatic nature of chlamydial infection, many ReA/ uSpA patients remain unrecognized. Also, it is not evident till date whether a single chlamydial infection or multiple infections are responsible for developing genitourinary-induced ReA. The development of an animal model shall prove to be useful in this regard. Further work is also needed to elucidate the exact cytokine mechanism during the course of ReA/ uSpA and its effect on regulatory mechanism by inflammatory protein and genetic susceptibility factor.

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APPENDIX

List of reagents used in Molecular method

Name of Reagents	Name of the company
$MgCl_2$ (25 mM)	Fermentas-Thermo Fisher Scientific, Waltham, USA
10X Buffer (KCl (50 mM) Tris-HCl (100 mM) (pH-8.4) nonidet P40)	Fermentas-Thermo Fisher Scientific, Waltham, USA
dNTPs (2 mM)	Fermentas-Thermo Fisher Scientific, Waltham, USA
Oligonucleotides	Biolink, New Delhi, India
Oligonucleotides	Biolink, New Delhi, India
DNA Taq polymerase	Fermentas-Thermo Fisher Scientific, Waltham, USA
1-kilobase DNA ladder	Bangalore Genei, Bangalore, India
Agarose	Bangalore Genei, Bangalore, India
Ethidium bromide	Bangalore Genei, Bangalore, India
AMPLIRUN CHLAMYDIA TRACHOMATIS DNA CONTROL	VIRCELL, Granada, Spain

List of Kits/ Reagents used in non-Molecular method

Name of Kit/ Reagent	Name of the company
Anti-*Chlamydia trachomatis* IgM antibodies Elisa kit	IBL International (IBL, Hamburg, Germany)
Anti-*Chlamydia trachomatis* IgG antibodies Elisa kit	IBL International (IBL, Hamburg,

	Germany)
Anti-*Chlamydia trachomatis* IgA antibodies Elisa kit	Savyon Diagnostics (Savyon Diagnostics, Ashdod, Israel)
MicroTrak *C. trachomatis* Direct Specimen Test	Trinity Biotech, Jamestown, NY, USA
Recombinant enzyme immunoassay chlamydial heat shock protein 60 IgG ELISA kit	Medac, Hamburg
Quantitative high sensitive determination of C-reactive protein (CRP)	IBL International, Hamburg, Germany
The Morgan TM HLA SSP B27 Typing Kit	Texas BioGene, USA. Lot-FP12061K

List of cytokine kits

Name of kit	**Name of company**
IL-4	*eBiosciences, USA*
IFN-gamma	*eBiosciences, USA*
IL-6	*BD Biosciences, USA*
IL-17	KRISHGEN BioSystems 15375 Ashley Ct, Whittier, CA 90603

BUFFERS

Preparation of Phosphate Buffer Saline (PBS)

NaCl (8 g/lit), KCl (0.2 g/lit), Na_2HPO_4 (1.42 g/lit), KH_2PO_4 (0.24 g/lit) was dissolved in one liter of distal water and 7.4 pH was maintained.

Preparation of Tris-acetate-EDTA (TAE)

A 50X stock solution was prepared by dissolving 242g Tris base in water, adding 57.1mL glacial acetic acid, and 100mL of 500mM EDTA (pH 8.0) solution, and makeup the final volume up to 1 liter. This stock solution was diluted 50:1 with water to make a 1X working solution. This 1X solution will contain 40mM Tris, 20mM acetic acid, and 1mM EDTA.

Primer dilution

Lyophilized primers were reconstituted in UV treated nuclease free water according to the manufactures instruction.

Precautions

During each assay, care was taken to avoid cross-contamination. For two-step PCR, each sample preparation was done in an isolated laminar flow and gloves were changed at each step to avoid contamination. Sera were prepared from aseptically collected samples using standard techniques. Heat-inactivated sera was not used as growth factor and amino acid get affected. The lipemic, turbid or contaminated sera was not used as the particulate material and precipitates in sera might give erroneous results. Such specimens were clarified by centrifugation or filtration prior to being assayed.

List of Publications

Original research articles

1. **Kumar P**, Khanna G, Batra S, Sharma VK, Rastogi S (2013). A pilot study for detection of intra-articular chromosomal and extra-chromosomal genes of *Chlamydia trachomatis* among genitourinary reactive arthritis patients in India. **International Research Journal of Medical Sciences, 1(4): 16 - 20.**
2. **Kumar P**, Khanna G, Batra S, Sharma VK, Rastogi S (2013). Diagnosis of circulatory antibodies to *Chlamydia trachomatis* among asymptomatic undifferentiated spondyloarthropathy patients in India**. Internet Journal of Rheumatology and Clinical Immunology, 1(S1): SO1**
3. **Kumar P,** Khanna G, Batra S, Sharma VK, Rastogi S (2014). *Chlamydia trachomatis* elementary bodies in synovial fluid of patients with reactive arthritis and undifferentiated spondyloarthropathy in India. **International Journal of Rheumatic Diseases (9 APR 2014 | DOI: 10.1111/1756-185X.12364)**
4. **Kumar P**, Bhakuni DS, Rastogi S (2014). Detection of *Chlamydia trachomatis*: A causative pathogen of reactive arthritis/ undifferentiated spondyloarthropathy. **Journal of Infection in Developing Countries 8 (5): 648 - 654**
5. **Kumar P**, Bhakuni DS, Rastogi S (2015). Do IgA antibodies to *Chlamydia trachomatis* have protective role in humoral immunity: A study in reactive arthritis patients. **Microbes and Infection 17: 806 - 810.**

Abstracts

6. **Kumar P,** Bhakuni DS, Mullick G, Kartik S, Rastogi S (2012). Diagnosis of *Chlamydia trachomatis* in joint fluid of chronic reactive arthritis and undifferentiated spondyloarthropathy patients. **Indian Journal of Rheumatology, S (7): S1 - S6**
7. **Kumar P**, Bhakuni DS, Rastogi S (2014). Clinical significance of circulatory chlamydial heat shock protein-60 IgG antibodies in reactive arthritis (ReA)/ undifferentiated spondyloarthropathy (uSpA) patients. **BMC Infectious Diseases, 14 (Suppl 3): P59**
8. Rastogi S**, Kumar P**, Bhakuni DS (2014). Is the role of *Chlamydia trachomatis* underestimated in reactive arthritis patients in India. **International Journal of Infectious Diseases, 21 (S): 1 – 460**
9. **Kumar P**, Bhakuni DS, Rastogi S (2014). Presence of HLA-B27 gene in reactive arthritis patients and its association with *Chlamydia trachomatis* infection. **Indian Journal of Rheumatology,** 9. DOI:10.1016/j.injr.2014.10.226
10. Rastogi S, **Kumar P**, Bhakuni DS (2015). IL-6: key player in the pathogenesis of *Chlamydia trachomatis*-induced reactive arthritis. **International Journal of Rheumatic Diseases, 18 (Suppl. 1): P 119**

Oral presentations in National/ International Conferences:

- Paper titled, **"*Diagnosis of Chlamydia trachomatis in joint fluid of chronic reactive arthritis and undifferentiated spondyloarthropathy patients*" presented at IRACON-2012 (Indian Rheumatology Association Conference- 2012)** at Ahmedabad, Gujarat (2012).
- Paper titled ***"Is major outer membrane protein of Chlamydia trachomatis a major antigenic protein in genitourinary-induced reactive arthritis?"*** Presented at **(IMMUNOCON-2013)** Delhi University conference centre organized by **Indian Immunology Society & UCMS.**
- Paper titled ***"Circulatory chlamydial heat shock protein-60 and C-reactive protein in reactive arthritis"*** presented at National Institute of Pathology (ICMR), New Delhi on the occasion of foundation day and "Dr Sriramachari Young Scientist Award" Ceremony held on 2nd May 2014.
- Paper titled *"**Presence of HLA-B27 Gene in Reactive Arthritis Patients and Its Association with Chlamydia trachomatis Infection"*** presented at 30th National conference of Indian Rheumatology Association (IRACON-2014), PGIMER, Chandigarh. (28th Nov-30th Nov)
- Paper entitled "**Efficacy of diagnostic methods for detection of *Chlamydia trachomatis* persistent infection in reactive arthritis"** presented at 18th International Union for Sexually Transmitted Infection (IUSTI) conference for Asia Pacific region at Bangkok, Thailand 11th -14th November 2014.

Poster presentations in International Conferences:

- Poster presentation titled, **"IL-6: key player in the pathogenesis of *Chlamydia trachomatis*-induced reactive arthritis"** in APLAR-2015 (17th Asia Pacific League of Associations for Rheumatology Congress), Chennai (6th - 9th Sep 2015)

Biography of the Ph.D. candidate

Mr. Praveen Kumar, M.Sc. (Medical Microbiology)

I received my Bachelor of Science from University Maharaja College (University of Rajasthan) followed by M.Sc. in Medical Microbiology from Bundelkhand University, Jhansi. I did my dissertation at Institute of Genomics and Integrative Biology (CSIR) for one year under the guidance of Dr. Hasi R Das and worked on 'Lectin' protein under the title of ' Lectin as tool for clinical diagnosis of microorganism'. I was associated as a Junior Research Fellow (DBT) in an Indo-UK randomized double blind placebo control clinical trial to study the efficacy of Vitamin D in reducing the morbidity and mortality of low birth weight infants. During this trial I learned various aspect of clinical trial as well as I gained experience in Th-1/Th-2 whole blood cytokine culture assay and work toward the setting up an Immunology Lab.

I was awarded direct senior research fellowship (2009/80/635/ECD-I) from Indian Council of Medical Research to pursue my Ph.D. in *Chlamydia trachomatis*-induced Reactive Arthritis under the supervision of Dr.Sangita Rastogi, Scientist-F at National Institute of Pathology (ICMR), New Delhi. During my ICMR-SRF tenure (4.5 years) I have been able to publish 10 research papers (5-original research article and 5-abstract) from my thesis in peer-reviewed national and international journals with total 12 impact factor. I received hands on traning on several aspects viz: Informatics in medicine, Molecular research technique, cloning and expression of recombinant protein, Flow-cytometry, scientific writing etc. I had opportunity to present my research work as five oral presentations and one poster presentation at national and international conference. I was awarded International Travel Support (ITS) by Department of Science & Technology, Government of India for my oral presentation at IUSTI-2014, Bangkok, ancillary grant for this visit was awarded from 'Immunology Foundation' New Delhi.

Biography of Ph.D. Supervisor

Dr. Sangita Rastogi, M.Sc., M.Phil., Ph.D. (Zoology)

Dr. Sangita Rastogi has been the recipient of '*Young Scientists Gold Medal'* by Society of Biosciences and was awarded direct SRF and direct RA by CSIR for conducting research on endocrinal regulation of hepatocellular toxicity during her Ph.D. (Zoology). Presently, she is working as Scientist 'F' & Senior Deputy Director at National Institute of Pathology (ICMR), New Delhi. She is head of Microbiology Division and her group focuses on immunology of chlamydial infections and comprises of one research scientist and 03 Ph.D. students. She is also Officer-InCharge, Central Animal House Facility at the institute. Since the last 22 years, she is actively working on the immunomolecular mechanism of pathogenesis of genital chlamydiasis in spontaneous aborters and female infertility. Another focus of her research is on genitourinary-induced reactive arthritis. She is member of various international/ national scientific organizations, *viz.:* ISID, ARHP, IIS and STOX. She is teaching faculty for WHO-sponsored training programme for pathologists and technicians, Off-campus Pre-Ph.D. program of BITS Pilani, Symbiosis International University, Pune, GGSIP University and Jamia Hamdard University, New Delhi. She has supervised thesis of post-graduate students, PhD and MS (Obstetrics & Gynecology) students. She has 35 research papers in national and international peer-reviewed journals to her credit and has also contributed one chapter in book. She has handled/ supervised extramural research projects funded by LSRB, DRDO, DOE (MIT), DST and ICMR in varying capacities as PI/ Co-PI/ CoI. She has participated and presented her research work in national/ international conferences in both India and abroad. She is the recipient of two international travel grant awards by ICMR and another by DST for oral/ poster presentations in scientific conferences.

Brief biography of co-supervisor

Prof. Vishal Saxena, Ph.D.

Prof. Vishal Saxena is working as an Associate Professor, Molecular Parasitology & Systems Biology Lab, Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Rajasthan, India. His major thrust area of research is Molecular Biology and Immunology with special emphasis to Genomics and Proteomics of Malaria parasites;*Plasmodium vivax* and *P. falciparum*. His group is focusing on various aspects related to *P. vivax* infections in humans, apicoplast and its genome, metabolic pathways functional in the apicoplast, hypothetical proteins encoded by *P. vivax* nuclear genome. He was a Visiting Scholar at Department of Public Health, College of Global Health, University of South Florida, Tampa, Florida, USA from June- July 2015. Prof. Saxena did B. Pharmacy (University of Rajasthan, Jaipur, August 1999) followed by M. E. Biotechnology (BITS, Pilani, June, 2001) and Ph. D. (Biological Sciences Group, BITS, Pilani, October, 2006). He has a total over 15 years (5 year's Pre-doctoral experience, over 10year's job tenure) of research experience. He is actively involved in teaching and research, has handled projects from various funding agencies, has supervised many graduate and post-graduate student thesis and is currently supervising 5 PhD students. He was a recipient of **Young Scientist Award** for Best Poster Presentation at International Conference on Molecular Epidemiology & Immunology of Malaria and other Vector Borne Diseases at RMRCT, Jabalpur, M. P., INDIA, 2007. He has published more than 15 research paper in international journals and has authored a book on Genetic Engineering. He has participated in many national and international conferences in India and abroad.