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Research Article

Patients with prior Infection by *Chlamydia pneumoniae* are vulnerable to Infection by *Chlamydia trachomatis*
Serum IgA Antibodies to Diagnose early-onset Chlamydia-induced reactive Arthritis

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ABSTRACT

Background: Reactive Arthritis (ReA), a subtype of spondyloarthritis, is defined as sterile, transient, typically lower-limb oligoarthritis following distant mucosal, genitourinary or gastrointestinal infection. The accurate diagnosis of early-onset Chlamydia-induced Reactive Arthritis (Chl-i ReA) is important because antibiotics are effective against Chl-i ReA in the early stage. However, diagnosing early-onset Chl-i ReA is challenging because patients frequently exhibit asymptomatic genital infections. Thus, in the current study, we hypothesized that it is important to detect serum IgA against *Chlamydia trachomatis* and *Chlamydia pneumoniae* to diagnose early-onset Chl-i ReA without urogenital or respiratory symptoms (Hypothesis #1). In addition, it has been reported that prior exposure to infection by *C. pneumoniae* can influence the T-cell mediated response to *C. trachomatis*. Thus, we also hypothesized that patients with prior exposure to infection by *C. pneumoniae* are vulnerable to infection by *C. trachomatis* (Hypothesis #2).

Patients and Methods: We performed a retrospective study using Enzyme-linked Immunosorbent Assay (ELISA) to detect serum IgA or IgG against *C. trachomatis* or *C. pneumoniae* in patients with arthralgia or arthritis who visited our outpatient clinic for 2 years. ELISA demonstrated high sensitivity and specificity, which was also confirmed to have no cross-reactivity with antibodies.

Results: Eleven (7.1%) Chl-i ReA patients, including 8 Chl-i ReA patients who were *C. pneumonia* IgA-positive and *C. trachomatis* IgA-negative, were diagnosed among the 155 total patients. The rate of positivity of *C. pneumoniae* IgA was significantly higher in patients with *C. trachomatis* IgA than in those without *C. trachomatis* IgA.

Conclusions: This study supported both Hypotheses #1 and #2. The prevention or treatment of *C. pneumoniae* infection can prevent infection by *C. trachomatis* inducing ReA, infertile, and visual impairment by trachoma.

Keywords: *Chlamydia-induced Reactive Arthritis*, *Chlamydia trachomatis*, *Chlamydia pneumonia*

INTRODUCTION

Reactive Arthritis (ReA), a subtype of Spondyloarthritis (SpA), is generally defined as sterile, transient, typically lower-limb oligoarthritis following distant mucosal, genitourinary, or gastrointestinal infection, although it was recently suggested that this concept be reconsidered [1, 2]. The primary etiologic agents for ReA are Chlamydia trachomatis and *Chlamydia pneumoniae* for which no effective vaccine exists [3]. Previous studies suggested that arthritis is elicited by chlamydia infecting synovial tissue in an abnormal biological state of persistence [1, 4, 5, 6]. In addition, *C. trachomatis* is present and metabolically active in synovium during the remitting phase of chronic Chlamydia-induced ReA (Chl-i ReA) [7]. The intracellular developmental cycle of *C. trachomatis* can be reversibly arrested by environmental factors and stresses [3]. Under these conditions, *C. trachomatis* transitions to aberrantly enlarged, non-dividing `persistent' forms. Persistence may represent a stealthy approach to evade the immune system of the host [3]. This persistence plays a role in the chronic inflammation and hallmarks of the chlamydial disease. Thus, ReA induced by chlamydia infection was recently termed Chl-i ReA, although it was previously described as chlamydia-associated arthritis [5, 8, 9, 10].

The accurate diagnosis and treatment of chlamydial infection and

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early-onset Chl-i ReA are important for the proper management in outpatient clinics because antibiotics may be effective against chlamydial infection or Chl-i ReA in the early stage. The prevalence of Chl-i ReA among patients with C. *trachomatis* genital infections is lower than that reported in a previous prospective study; one of the reasons for this discrepancy may be the early effective treatment using antibiotics [11]. The declining involvement of C. *trachomatis* in ReA was observed in previous studies, which may be explained by wider screening for sexually transmitted infections, and earlier and more systemic treatment despite increasing chlamydia diagnoses [12]. If an infectious agent has been identified as a trigger for ReA, antimicrobial therapy is strongly recommended, often for a long term of 3 to 6 months [13]. It can significantly shorten the time to remission [14]. In addition, antibiotic treatment for chronic Chl-i ReA has not been established.

However, diagnosing early-onset Chl-i ReA is challenging because 70% of female and 25% of male patients exhibit asymptomatic chlamydial genital infection. Furthermore, no universal diagnostic or classification criteria for early-onset Chl-i ReA have been established [13-16]. Thus, we hypothesized that it is important to perform a screening test to detect serum IgA and IgG against C. *trachomatis* and C. *pneumoniae* to diagnose early-onset Chl-i ReA without urogenital or respiratory symptoms (Hypothesis #1).

We used the diagnostic criteria for ReA reported at the Third International Workshop on Reactive Arthritis on September 23-26, 1995, in Berlin, Germany, published in 1996 [17] in the screening test to detect serum IgG and IgA against C. *trachomatis* and C. *pneumoniae* in patients with arthralgia or arthritis to diagnosis early-onset Chl-i ReA. In the current retrospective study, we used Enzyme-linked Immunosorbent Assay (ELISA) [18] with high sensitivity and specificity. It was also confirmed that the ELISA for C. *trachomatis* IgA or IgG does not have cross-reactivity with C. pneumoniae IgA or IgG.

In addition, in 2006, Telyatnicova et al. reported that prior infection by C. *pneumoniae* can influence the T-cell mediated response to C. *trachomatis* using T cell clones derived from synovial fluid lymphocytes of patients with Chl-i ReA (Telyatnikova N and Gaston JS, FEMS 2006 [43]). Thus, we hypothesized that patients with prior infection by C. *pneumoniae* are vulnerable to infection or reactive arthritis induced by C. *trachomatis* (Hypothesis #2).

To address these 2 hypotheses, we analyzed the data of 155 patients with arthralgia or arthritis who visited our outpatient clinic in this 2-year study. We diagnosed 11 Chl-i ReA patients, including 3 who were C. *trachomatis* IgA-positive (one C. *pneumoniae*-positive and 2 C. *pneumoniae*-negative) and 8 patients who were C. *trachomatis* IgA-negative and C. *pneumoniae* IgA-positive. The number of Chl-i ReA patients by C. *pneumoniae* was greater than that by C. trachomatis. The rate of anti-C. *pneumoniae* IgA positivity was significantly higher in patients who were C. *trachomatis* IgA-positive than in those who were C. *trachomatis* IgA-negative. This study supported both Hypotheses #1 and #2, demonstrating the importance and usefulness of serological tests.

RESULTS

Detection of serum IgG and IgA against *C. trachomatis* and *C. pneumoniae* in the first group

Serum IgG and IgA against C. *trachomatis* were detected in 6 (8.0%)

Table 1.1: The first group of patients (2018.8 ~2019.7). Profiles of 7 patients with positive serum IgA to *Chlamydia trachomatis.*

Patient	Age (yr)/sex	Diagnosis	Duration of arthralgia/ arthritis	Serum IgA to *C. Trachomatis* (Titer and positivity)	Serum IgA to *C. Pneumoniae* (Titer and positivity)	Associated clinical features
1	36/M	Chl-i ReA	5y	3.25 +	4 -	Polyarthritis. Ankle, knee, elbow
2	68/F	SLE	4m	2.52 +	20 +	Monoarthritis. Lt, ankle
3	25/M	MCTD	3w	0.92 +/-	8 +	Polyarthritis
4	26/F	Behcet	4w	1.02 +/-	12 +	Oligoarthritis. Lt knee
5	35/F	Sjogren	4y	1.18 +	15 +	Polyarthralgia. PIP, MCP, wrist
6	31/M	Behcet	1.5y	1.02 +/-	9 +/-	Polyarthritis. Knee, wrist, lt elbow, PIP, MCP
7	57/F	Sjogren	7m	1.98 +	8 +/-	Polyarthralgia. MCP

Chl-i ReA, Chlamydia-induced Reactive Arthritis; SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease.
M, male; F, female.

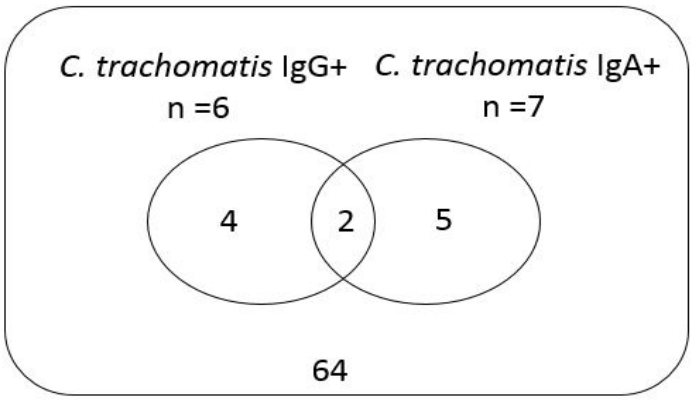


Figure 1A: C. *trachomatis* IgG and IgA.

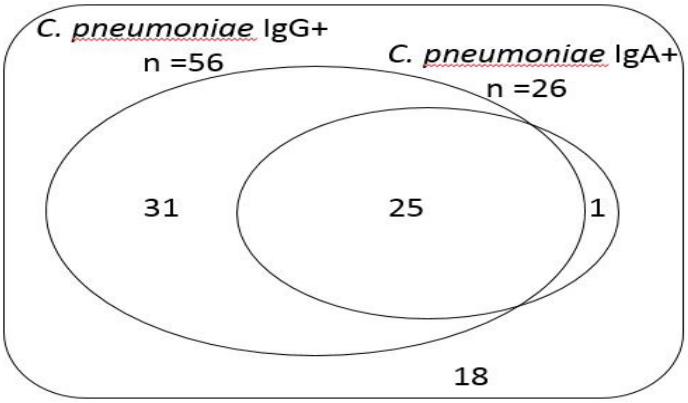


Figure 1B: C. *pneumoniae* IgG and IgA.

and 7 (9.3%) patients, respectively, of the 75 total patients (Figure 1A). Both serum IgG and IgA against C. *trachomatis* were detected in 2 (2.7%) patients, Patients #3 and #7 (Table 1.1, Figure 1A). Serum IgG and IgA against C. *pneumoniae* were detected in 56 (74.7%) and 26 (34.7%) patients, respectively, of the 75 total patients (Figure 1B). Both serum IgG and IgA against C. *pneumoniae* were detected in 25 (33.3%) patients (Table 1.1, Figure 1B). Serum IgA against both C. *trachomatis* and C. *pneumoniae* was detected in 6 patients (Table 1.1, Figure 1C). Of the 26 patients who were C. *pneumoniae* IgA-positive, 20 (78%) were C. *trachomatis* IgA-negative (Figure 1C).

In the first group, one patient was diagnosed with Chl-i ReA among 7 *C. trachomatis* IgA-positive patients

The profiles of 7 patients positive for serum IgA against C. trachomatis are shown in Table 1. Six of the 7 patients were positive for serum IgA against *Chlamydia pneumoniae* (Table 1.1, Figure 1C). One patient (Patient #1 in Table 1) was diagnosed with Chl-i ReA among the 7 C. *trachomatis* IgA-positive patients in the first group.

In the first group, 4 patients were diagnosed with Chl-i ReA, including probable and possible, among the *C. pneumoniae* IgA-positive and *C. trachomatis* IgA-negative patients

The profiles of 4 patients who were C. *pneumoniae* IgA-positive and C. *trachomatis*-negative are shown in Table 1.2 (Patients #8, #9, #10 and #11 in Table 1.2). Patient #9 was diagnosed with 'Chl-i ReA, probable' according to our original criteria modified from the diagnosis criteria for ReA (Kingsley et al, 1996), as shown in the Methods section, because arthritis was observed in the upper limb, but not the lower limb; ReA induced by C. *trachomatis* typically presents in the lower limb, although it remains unclear whether ReA induced by C. *pneumoniae* is typically in the lower limb. Patient #11 was diagnosed with 'Chl-i ReA, possible' according to our original criteria modified from the diagnosis criteria for ReA (Kingsley et al, 1996), as shown in the Methods section, because the patient was diagnosed with and treated for Inflammatory Bowel Disease-associated Arthritis (IBD-AA) when she visited our outpatient clinic. In the diagnotic criteria for ReA (Kingsley et al. 1996), the 'Exclusion criteria' include patients with other known causes of mono/oligoarthritis such as other defined spondyloarthropathies. However, Patient #11 (Table 1.2) was diagnosed with both type-2 IBD-AA (UC) and 'Chl-i ReA, possible' because arthritis in the wrist may have been induced by C. *pneumonia*. Thus, Chl-i ReA, including probable and possible, was diagnosed in 5 of 75 (6.7%) patients in the first group.

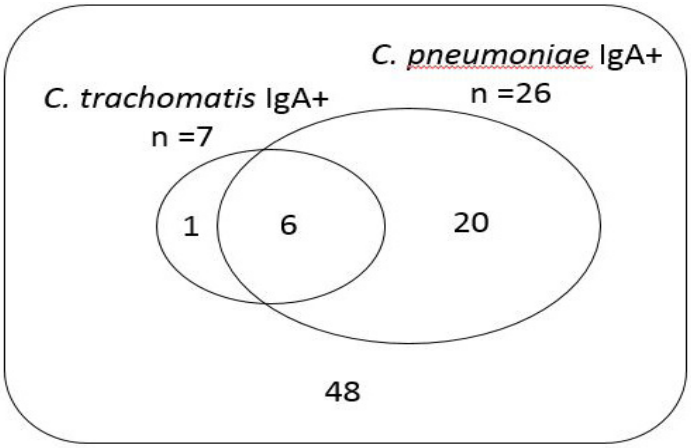


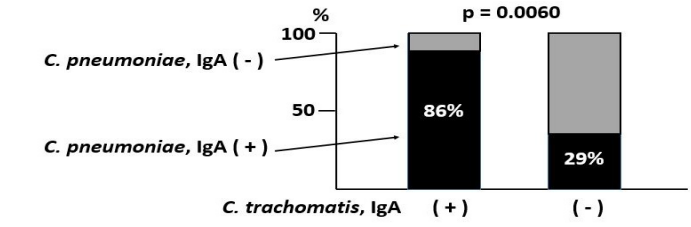
Figure 1C: C. *trachomatis* IgA and C. *pneumoniae* IgA

Increased rate of positivity of anti-*C. pneumoniae* IgA in the patients with anti-*C. trachomatis* IgA in the first group

Six (86%) of 7 patients who were C. *trachomatis* IgA-positive were C. *pneumoniae* IgA-positive, whereas 20 (29%) of 68 patients who were C. *trachomatis* IgA-negative were C. *pneumoniae* IgA-positive (Figure 1D). Thus, the positive rate of C. *pneumoniae* IgA was significantly higher among patients who were C. *trachomatis* IgA-positive than among those who were C. *trachomatis* IgA-negative in the first group (Fisher's exact test, p = 0.0060).

Detection of serum IgG and IgA against *C. trachomatis* and *C. pneumoniae* in the second group of patients

Serum IgG and IgA against C. *trachomatis* were detected in 14 (17.5%) and 15 (18.8%) patients, respectively, of the 80 total patients (Figure 2A). Both serum IgG and IgA against C. *trachomatis* were detected in 7 (8.8%) patients (Table 2.1, Figure 2A). Serum IgG and IgA against C. *pneumoniae* were detected in 53 (66.3%) and 31 (38.8%) patients, respectively, of the 80 total patients (Figure 2B). Both serum IgG and IgA against C. *pneumoniae* were detected



C. trachomatis, IgA	(+)	(-)	Total
C. pneumoniae, IgA (-)	1	48	49
IgA (+)	6	20	26
Total	7	68	75

Fisher's exact test: p = 0.0060

Figure 1D: Positive rate of anti-C. *pneumoniae* IgA among patients with anti-C. *trachomatis* IgA

Table 1.2: The first group of patients (2018.8 ~2019.7). Profiles of 7 patients with positive serum IgA to *Chlamydia trachomatis*.

Patient	Age (yr)/sex	Diagnosis	Duration of arthralgia/ arthritis	Serum IgA to *C. Trachomatis* (Titer and positivity)	Associated clinical features	Treatment after diagnosis
8	76/M	Chl-i ReA	4m	19 +	Monoarthritis. Rt knee	SASP
9	86/M	Chl-i ReA, prob.	3w	19 +	Monoarthritis. Lt, wrist	SASP
10	75/M	Chl-i ReA	3y	13 +	Oligoarthritis. Rt hip, knee	SASP
11	41/F	IBD-AA (UC), type 2. Chl-i ReA, pos.	2y(UC) wrist:4m	11 +	Oligoarthritis. Bil, wrist	MTX (5-ASA, 2y)*

Chl-i ReA, Chlamydia-induced Reactive Arthritis; SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease.
M, male; F, female ; SASP, salazosulfapyridine; MTX, methotrexate; 5-ASA; IBD-AA, inflammatory bowel disease-associated Arthritis; w, weeks; m, months; y, years; pos, possible.
*, The patient had been diagnosed and treated at the first visit to our outpatient clinic.

in 27 (33.8%) patients (Figure 2B). Serum IgA against both C. *trachomatis* and C. *pneumoniae* was detected in 10 (12.5%) patients (Table 2.1, Figure 2C). Of 29 patients who were C. *pneumoniae* IgA-positive in the second group, 19 (66%) were C. *trachomatis* IgA-negative (Figure 2C). Thus, of 55 patients who were C. *pneumoniae* IgA-positive among the 155 in the first and second groups, 39 (71%) were C. *trachomatis* IgA-negative (Figure 1C, Figure 2C).

In the second group, two patients were diagnosed with Chl-i ReA, including probable, among 13 *C. trachomatis* IgA-positive patients

The profiles of 13 patients positive for serum IgA against C. *trachomatis* are shown in Table 2.1. All of the 13 patients had not been diagnosed or treated when they visited our outpatient clinic. Two patients included among the 15 C. *trachomatis* IgA-positive patients shown in Figure 2A were excluded because they had been diagnosed and treated before they visited our outpatient clinic. Eight of these 13 patients were positive for serum IgA against C. *pneumoniae* (Table 2.1).

Patient #1 (Table 2.1) was diagnosed with 'Chl-i ReA, probable' according to our original criteria modified from the diagnosis criteria for ReA [17], as shown in the Methods section, because arthritis was observed in the upper limb, but not in the lower limb; ReA induced by C. *trachomatis* typically causes arthritis in the lower limb, although it remains unclear whether ReA induced by C. *pneumoniae* causes arthritis in the lower limb. Patient #1 (Table 2.1) was positive for serum IgA against C. *trachomatis* and C. *pneumoniae*. Patient #2 (Table 2.1) was treated using azithromycin because she had C. *trachomatis* infection in the vaginal cervical canal.

Thus, 2 patients were diagnosed with Chl-i ReA, including probable, among 13 C. *trachomatis* IgA-positive patients (Table 2.1).

Table 2.1: The second group of patients (2019.8 ~2020.7). Profiles of 13 patients with positive serum IgA to *Chlamydia trachomatis*.

Patient	Age (yr)/ sex	Diagnosis	Duration of arthralgia/ arthritis	Serum IgA to *C. Trachomatis* (Titer and positivity)	Serum IgA to *C. Pneumoniae* (Titer and positivity)	Associated clinical features	Treatment after diagnosis
1	77/M	Chl-i ReA, prob.	6w	1.20 +	18 +	Polyarthritis. MCP, PIP, wrist	SASP
2	57/F	Chl-i ReA	2m	2.10 +	5 -	Oligoarthritis, CM, ankle, MTP. vaginal *C. tra.* & gono. PCR (-)	SASP Gynecology:Azithromycin
3	69/M	MPA	1m	1.44 +	21 +	Oligoarthralgia	
4	46/F	SLE, Sjogren	6y	9.86 +	5 -	Oligoarthralgia. Knee.	
5	64/F	MCTD	1m	0.90 +/-	10 +/-	Oligoarthralgia, Shoulder	
6	57M	APS	6m	1.73 +	35 +	Headache, Af	
7	65/M	Sjogren, Psoriasis	2y	3.41 +	24 +	Dry eye	
8	64/M	Behcet	1y	1.07 +/-	4 -	Oligoarthralgia	
9	38/F	EAC	5w	1.85 +	4 -	Oligoarthralgia. Rt PIP	
10	19/F	SLE	3m	1.92 +	9 +/-	Polyarthritis. PIP, MCP, wrist, rt elbow	
11	73/M	Seronega. EORA	3y	1.55 +	3 -	Monoarthritis. Lt I MTP.	
12	65/F	RA	7m	1.10 +	18 +	Oligoarthritis. Rt wrist, lt MTP. ACPA 432, RF 272	
13	51/F	AS	5y	1.25 +	14 +	LBP, enthesitis, uveitis, SIJ. HLA-B35+	

Chl-i ReA, Chlamydia-induced Reactive Arthritis; SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease.
M, male; F, female ; ECM, erythema annulare centrifugum; EORA, elderly-onset RA; AS, ankylosing spondylitis.; prob., probable;

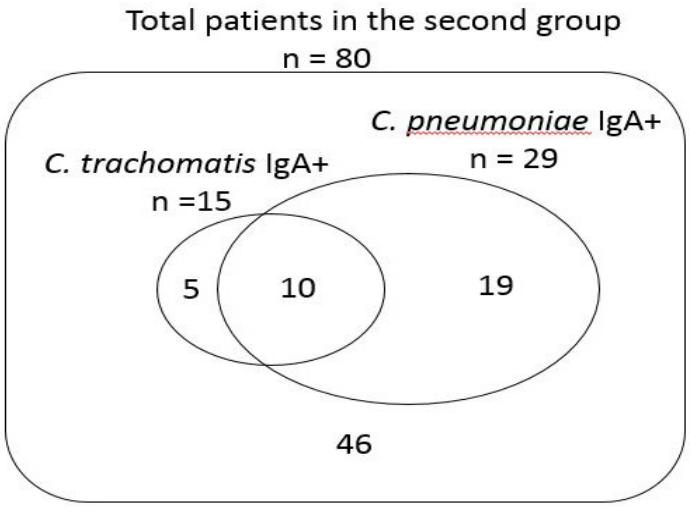


Figure 2A: C. *trachomatis* IgG and IgA

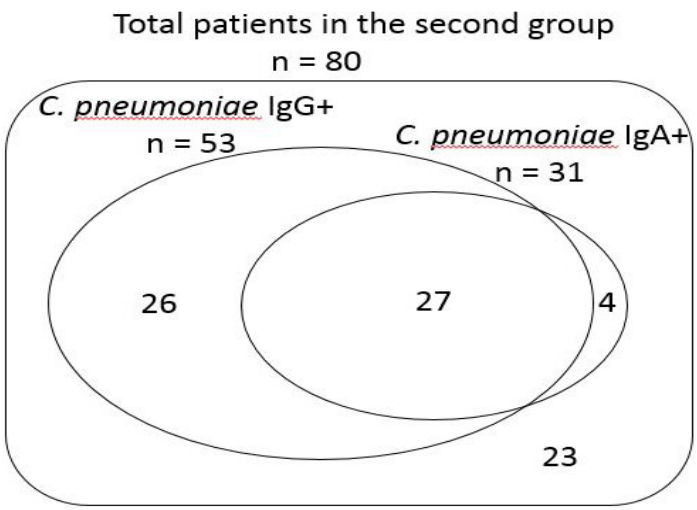


Figure 2B: C. *pneumoniae* IgG and IgA

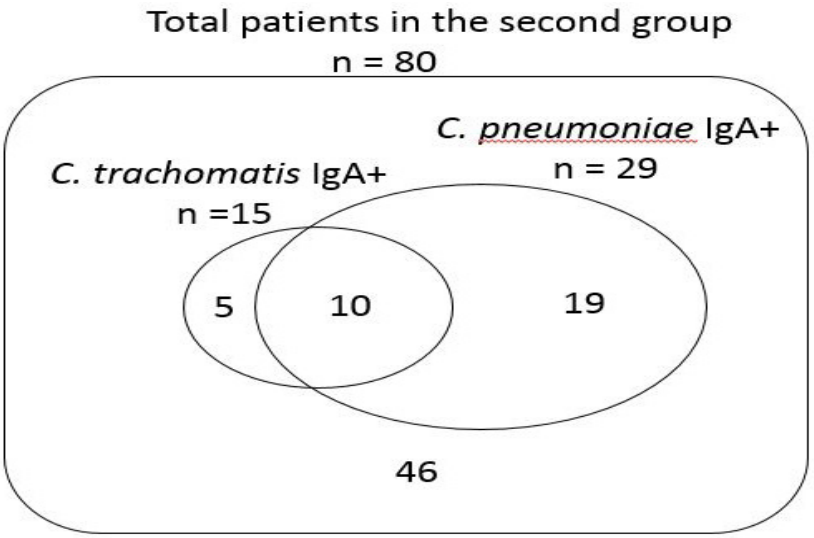


Figure 2C: C. *trachomatis* IgA and C. *pneumoniae* IgA

Table 2.2: The second group of patients (2019.8 ~2020.7). Profiles of Chl-i ReA patients who were C. *pneumoniae* IgA positive and C. *trachomatis* IgA negative.

Patient	Age (yr)/sex	Diagnosis	Duration of arthralgia/ arthritis	Serum IgA to *C. Pneumoniae* (Titer and positivity)	Associated clinical features	Treatment after diagnosis
14	44/M	IBD-AA (Cro.), type 2. Chl-i ReA, pos.	29y	21 +	Oligoarthritis. Knee, ankle	(AZA, 5-ASA, ADA)*
15	61/F	Chl-i ReA, SpA	2m	26 +	Polyarthritis. Rt elb, Lt I MP Polyenthesitis. Achilles tendinitis, plantar fasciitis, knee, elbow.	SASP
16	33/F	Chl-i ReA	3m	42 +	Polyarthralgia. PIP, ankle	NSAID
17	55/M	Chl-i ReA, SpA	8y	14 +	Oligoarthritis. ankle Polyenthesitis. Achilles tendinitis, plantar fasciitis.Newton test+, IBP+	NSAID

Chl-i ReA, Chlamydia-induced Reactive Arthritis; SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease. M, male; F, female ; SASP, salazosulfapyridine; MTX, methotrexate; 5-ASA ; IBD-AA, inflammatory bowel disease-associated arthritis; w, weeks; m, months; y, years; Cro., Crohn's disease;
*, The patient had been diagnosed and treated at the first visit to our outpatient clinic.

In addition, 3 (15%) of the 20 total C. *trachomatis*-positive patients were diagnosed with Chl-i ReA (Table 1.1, 2.1).

In the second group, 4 patients were diagnosed with Chl-i ReA, including possible, among the *C. pneumoniae* IgA-positive and *C. trachomatis* IgA-negative patients.

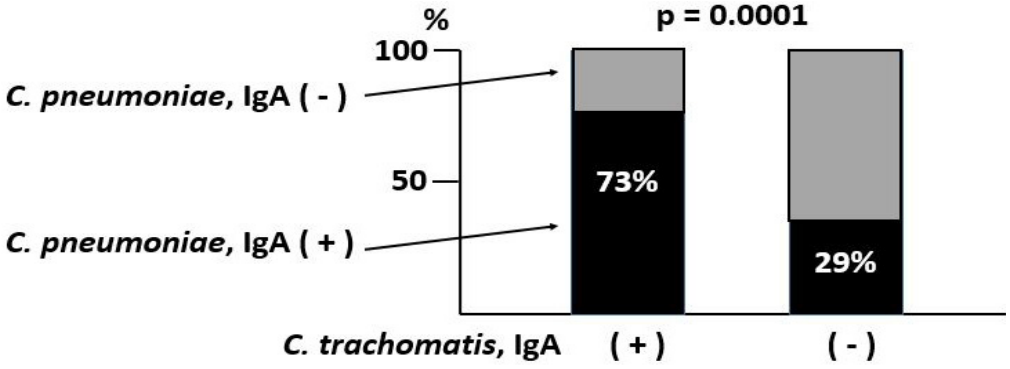
The profiles of 4 patients who were C. *pneumoniae* IgA-positive and C. trachomatis-negative are shown in Table 2.2 (Patients #14, #15, #16, and #17 in Table 2.2). Patient #14 (Table 2.2) was diagnosed with 'Chl-i ReA, possible' according to our original criteria modified from the diagnosis criteria for ReA [17], as shown in the Methods section. Patient #14 had been diagnosed with and treated for IBD-AA, type 2, when he visited our outpatient clinic. In the diagnosis criteria for ReA [17], it is mentioned in the 'Exclusion criteria' that patients with other known causes of mono/oligoarthritis, such as other defined spondyloarthropathies, be excluded. However, Patient #14 (Table 2.2) was diagnosed with both IBD-AA (Crohn's disease), type 2, and Chl-i ReA, possible.

Patients #15 and #16 were diagnosed with both 'Chl-i ReA' and spondyloarthritis (SpA) because they met the criteria for ReA [17] and SpA [19].

Thus, Chl-i ReA, including probable and possible, was diagnosed in 6 of 80, i.e., 7.5%, patients of the second group we analyzed. Of the 155 total patients in the first and second groups, 11, i.e., 7.1%, were diagnosed with Chl-i ReA, including probable and possible. The 11 Chl-i ReA patients included 3 who were C. *trachomatis* IgA-positive (one C. *pneumoniae*-positive and 2 C. *pneumoniae*-negative) and 8 who were C. *trachomatis* IgA-negative and C. *pneumoniae* IgA-positive.

Increased rate of positivity of anti-*C. pneumoniae* IgA in the patients with anti-*C. trachomatis* IgA in the second group

Ten (67%) of 15 patients who were C. *trachomatis* IgA-positive were C. *pneumoniae* IgA-positive, whereas 19 (29%) of 65 patients who were C. *trachomatis* IgA-negative were C. *pneumoniae* IgA-positive



C. trachomatis, IgA	(+)	(-)	Total
C. pneumoniae, IgA (-)	6	94	100
IgA (+)	16	39	55
Total	22	133	155

Fisher's exact test: p = 0.0001

Figure 3: Positive rate of anti-C. *pneumoniae* IgA among patients with anti-C. *trachomatis* IgA among 155 total patients during 2 years.

(Figure 1C). Thus, the positive rate of C. *pneumoniae* IgA was significantly higher among patients who were C. *trachomatis* IgA-positive than among those who were C. *trachomatis* IgA-negative in the second group (Fisher's exact test, p = 0.0086).

Increased rate of positivity of anti-*C. pneumoniae* IgA in the patients with anti-*C. trachomatis* IgA among 155 patients during 2 years

Sixteen (73%) of 22 patients who were C. *trachomatis* IgA-positive were C. *pneumoniae* IgA-positive, whereas 39 (29%) of 133 patients who were C. *trachomatis* IgA-negative were C. *pneumoniae* IgA-positive (Figure 3). Thus, the positive rate of C. *pneumoniae* IgA was significantly higher among patients who were C. *trachomatis* IgA-positive than among those who were C. *trachomatis* IgA-negative among the 155 total patients during 2 years (Figure 3, Fisher's exact test, p = 0.0001).

DISCUSSION

In the current retrospective 2-year study, 11 (7.1%) Chl-i ReA, including possible and probable, patients were diagnosed among the 155 total patients with arthralgia or arthritis who visited our outpatient clinic. In addition, 3 (15%) of 20 C. *trachomatis*-positive patients with arthralgia or arthritis were diagnosed with Chl-i ReA. Significant differences were not detected between the groups. To our knowledge, this is the first study to report the rate of ReA patients who visited the first outpatient rheumatology clinic during 2 years. The incidence of sexually acquired ReA is 3.0%-8.1% according to a systemic literature review [20]. In Japan, only one (0.8%) patient was diagnosed with ReA among 123 with C. *trachomatis* infection [11]. However, it is difficult to calculate the accurate incidence of ReA because chlamydia infection is asymptomatic in 70% of females and 25% of males [15]. In addition, no universal diagnostic or classification criteria have been established for ReA [20]. A study from Sweden in 2002 stated that rates of ReA were higher than rates of rheumatoid arthritis, suggesting that ReA is significant, but potentially under-recognized [21]. In chronic and recurrent C. *trachomatis* infections, in particular, the isolation or detection of C. *trachomatis* at the site of primary infection may be difficult because the organism has often disappeared from the primary site of infection or limited assay sensitivity [18]. Therefore, advanced unstandardized tests may be needed to detect chlamydia infection to determine the accurate incidence of Chl-i ReA. For example, Kumar et al. recommended the usage of semi-nested Polymerase Chain Reaction (PCR) and nested PCR targeting genes of C. *trachomatis* because its sensitivity and specificity are markedly high, which we also previously demonstrated [4, 5, 6, 22].

The detection of serum C. *trachomatis* IgA is important for screening because it means that C. *trachomatis* infection exists under the mucous membrane. Klingebiel et al. reported that ReA following infection by C. *trachomatis* is an important differential diagnosis in atraumatic joint swelling based on 2 athlete patients with monoarthritis in the knee as typical Chl-i ReA patients [15]. They also reported that to make a diagnosis, specific anamnesis and direct detection of the pathogen in the specimen of synovial fluid by PCR are essential. However, the standard method to diagnose chlamydia infection in Chl-i ReA is the detection of chlamydia DNA in the urogenital specimens by PCR. In addition, the specificity of PCR is generally high, but the sensitivity is not. On the other hand, active chlamydia infections are linked to serum C. *trachomatis* IgG and serum C. *trachomatis* IgA levels [23]. Thus, the detection of serum I IgA is important for screening because it means that C. *trachomatis* infection exists under the mucous membrane.

C. *trachomatis* IgA also plays an important role in the immunity of patients who had Chl-i ReA. IgA antibodies against C. *trachomatis* have a protective role in humoral immunity in ReA patients [24]. Anti-C. *trachomatis* IgA antibodies exhibit neutralizing activity, which limits the spread of infection but does not eliminate the microorganisms from the body [25]. Higher levels of secretory IgA are associated with a low disease activity index in patients with ReA [26]. Furthermore, in the sera of 4 patients who were culture positive for C. trachomatis ReA, an increase and decrease in C. *trachomatis* antibody values of both IgA and IgG were associated with prolonged arthritis and remission of arthritis, respectively [18]. Thus, it is important to measure the level of serum IgA against C. *trachomatis* in ReA patients.

In the current study, of 55 patients who were C. *pneumoniae* IgA-positive among 155 total patients of the first and second groups, 39 (71%) were C. *trachomatis* IgA-negative (Figure 1C, Figure 2C). Eight (21%) of the 39 total patients who were C. *trachomatis* IgA-negative and C. *pneumoniae* IgA-positive were diagnosed with Chl-i ReA, being a higher ratio than expected. This supports Hypothesis

#1 that it is important to perform screening tests to detect serum IgA and IgG against C. trachomatis and C. *pneumoniae* to diagnose early-onset Chl-i ReA without urogenital or respiratory symptoms.

C. *pneumoniae* is the most common intracellular bacterium, which is mainly involved in respiratory infections [27]. It has a 50% antibody prevalence by 20 years of age, which increases to 80% by the age of 60 to 70 years, suggesting that the infection is usually asymptomatic, and people are infected and reinfected throughout life [28]. In 1993, Gran et al. reported the first clinical description of ReA caused by C. *pneumoniae*; A 37-year-old HLA B27-negative man developed erythema nodosum, pneumonia, myocarditis and oligoarthritis due to C. *pneumoniae* [29]. As reported in this patient, ReA induced by C. *pneumoniae* is usually diagnosed when the patient exhibits respiratory symptoms. Thus, it is difficult to diagnose early-onset Chl-i ReA with asymptomatic infection due to C. *pneumoniae*. In addition, no epidemiological information is available for Chl-i ReA by C. *pneumoniae*; a causative entity of chlamydial infection in Chl-i ReA has been described only in case studies [30, 31]. In 2016, Zeidler et al. reported that studies on C. *pneumoniae* are sparse but suggestive, and further studies of this pathogen in joint disease are likely to provide clinically significant information [9]. Our study on Chl-i ReA, which may be induced by C. *pneumoniae*, is therefore important.

Serological tests for C. *trachomatis* and C. *pneumoniae* in patients with early-onset Chl-i ReA can help diagnose Chl-i ReA, as demonstrated in the current study. Indeed, Patient #2 in Table 2.1 who was diagnosed with Chl-i ReA was treated using azithromycin in the gynecology department. Serological tests may not replace molecular methods of chlamydial infection detection, but those using serum are the most easily available in the outpatient clinic for screening. It has been reported that ReA patients who were C. *trachomatis*-negative by PCR were positive for anti-C. *trachomatis* IgA in synovial fluid or serum [22]. One reason for this discrepancy is that such patients may have had an earlier short episode of urogenital infection leading to arthritis [22]. Concurrent direct studies of the urogenital tract also reported that the Direct Immunofluorescence (DIF) method or PCR and a serological blood test increase the chance of detecting C. *trachomatis* infection because no correlations between detecting the presence of C. *trachomatis* in the urogenital tract and the presence of specific antibodies in the serum of ReA patients were observed [32]. Thus, serological tests are practically useful in diagnosing early-onset Chl-i ReA, although there may be discrepancies between serological tests and PCR.

We used the diagnostic criteria for ReA reported at the Third International Workshop on Reactive Arthritis in1995 [17], but not the European Spondyloarthropathy Study Group (ESSG) classification criteria [33] or Assessment of Spondyloarthritis International Society (ASAS) criteria [19, 34]. ESSG [33] and ASAS criteria [19, 34] require the classification of Spondyloarthritis (SpA) before the classification of ReA. Thus, it is difficult to diagnose or classify early-onset ReA using these criteria. On the other hand, in the diagnostic criteria for ReA [17], it is not necessary to diagnose SpA or confirm HLA-B27 positivity to diagnose ReA. HLA-B27 can be measured as it correlates with the severity of the ReA [35] but is not diagnostic for ReA [13]. It is recommended that HLA-B27 not be used as a diagnostic tool for the diagnosis of acute ReA [13]. Thus, the diagnostic criteria for ReA reported in1995 [17] are useful to diagnose early-onset ReA.

We slightly modified the diagnostic criteria for ReA from 1995 for two reasons. First, it is necessary to diagnose Chl-i ReA as early as possible considering treatment. Thus, we diagnosed the patients with 'Chl-i ReA', 'Chl-i ReA, probable', or 'Chl-i ReA, possible', as mentioned in Methods. The patient who was C. *pneumoniae* IgA-positive and had arthritis in the upper limb was diagnosed with 'Chl-i ReA, probable' because ReA induced by C. *trachomatis* typically causes arthritis in the lower limb. However, it remains unclear whether ReA induced by C. *pneumoniae* typically causes arthritis in the lower limb, although it has been reported that clinical aspects of Chl-i ReA by C. *pneumoniae* mirror those of Chl-i ReA by C. *trachomatis* to a certain extent [30]. Indeed, ReA typically occurs in the lower extremities; however, upper extremity involvement, including the small joints of the hands, is not uncommon [16]. In addition, there were areas of disagreement in the 4th International Workshop on Reactive Arthritis such as 'Is arthritis essential for the diagnosis of ReA ?' or 'Is it oligoarthritis or any arthritis?' [36]. Undifferentiated SpA may also be a forme fruste of ReA because salmonella-derived protein specific to T cells increases in ReA and undifferentiated SpA [37]. Thus, we slightly modified the criteria in the current study.

Misra et al. suggested the need for a more pragmatic case definition of ReA [2]. The diagnostic criteria for ReA are too rigid to be practical. Indeed, most ReA cases [38] are best labeled as 'probable' or 'very likely' based on fulfilling most of the diagnostic criteria [2].

Zeidler et al. proposed an algorithm using serology and direct detection of chlamydia at the portal of entry for the diagnosis of ReA in 2014 [39]. In the algorithm, the simultaneous detection of IgG and IgA antibodies is typical for fresh or persistent infections and indicates a diagnosis of 'ReA probable' in patients with corresponding clinical history and symptoms. 'ReA definite' is diagnosed if the patient is initially positive for chlamydia. We also referred to the algorithm when we modified the diagnostic criteria for ReA [17].

The second reason we slightly modified the diagnostic criteria from 1995 [17] is as follows: Recently, potential molecular signatures through host-microbe interactions for ReA and Inflammatory Bowel Disease (IBD) have been reported using a combinational approach; ReA and IBD were suggested to 'co-evolve' [40]. In addition, approximately 25% of ReA patients develop chronic disease, and some of these patients develop signs and symptoms of IBD [16, 41]. Thus, we did not use the exclusion criteria when diagnosing Chl-i ReA in patients with IBD-AA.

The positive rate of C. *pneumoniae* IgA was significantly higher among patients who were C. *trachomatis* IgA-positive than among those who were C. *trachomatis* IgA-negative among 155 total patients during 2 years (Figure 3, Fisher's exact test, $p = 0.0001$). This was not due to cross-reactivity of antibodies because the ELISA methods we used in the current study have been confirmed to not have cross-reactivity of antibodies, as described in the Methods section.

Telyatnikova N, et al. reported that prior exposure to C. *pneumoniae* can influence the T-cell-mediated response to C. *trachomatis* [43]. Their study demonstrated that prior C. *pneumoniae* infection primes a Th1 cell response to C. *trachomatis* antigens, suggesting that CD4+ T cells are cross-reactive in their responses to C. *trachomatis* and C. *pneumoniae*. In addition, the pathogenesis of the sequelae of C. *trachomatis* infection, i.e., trachoma, infertility, and arthritis, may be influenced by prior exposure to C. *pneumoniae* [43]. Our current study supports Hypothesis #2 that patients with prior exposure to infection by C. *pneumoniae* are vulnerable to infection or reactive

arthritis by C. *trachomatis*.

In 2016, we demonstrated that Th17 cells differentiated to Th1 cells in the peripheral blood of an early-onset Chl-i ReA patient [48]. In the pathogenesis of ReA, it is possible that the IL-17 and IL-23 axis may play an essential role in the development of Chl-i ReA. After 1999 when we first revealed that IL-17 plays an important role in the pathology of rheumatoid arthritis and osteoclastogenesis [44], we demonstrated that IL-17 and IL-23 play important roles in the pathogenesis of both rheumatoid arthritis and spondyloarthritis [45-52]. In 2016, we demonstrated that Th17 cells differentiated to Th1 cells, i.e., non-classic Th1 cells, in the peripheral blood of an early-onset Chl-i ReA (C. *trachomatis*) patient (43-year-old female. Disease duration, 9 months) [48]. There is a high level of IL-17 in synovial fluid of Chl-i ReA patients (median duration of arthritis, 14 days) [53]. It has been also reported that *Salmonella typhimurium* outer membrane protein A is recognized by synovial fluid CD8+ T cells, which stimulate synovial fluid mononuclear cells to produce IL-17 and IL-23 in patients with ReA [54]. In addition, using a ReA model of SKG mice following infection with *Chlamydia muridarum*, IL-23 produced by neutrophils functioned in the development of ReA [55]. Thus, Th17 or Tc17 cells may play an important role in the initiation phase of ReA. Our study is limited by its retrospective design. However, to our knowledge, this is the first study to report the rate of ReA in patients who visited the outpatient clinic for 2 years.

In conclusion, in the current retrospective study during 2 years, 11 (7.1%) Chl-i ReA, including possible and probable, patients were diagnosed among the 155 total patients with arthralgia or arthritis who visited our outpatient clinic. In addition, 3 (15%) of 20 C. trachomatis patients with arthralgia or arthritis were diagnosed with Chl-i ReA. Eight (21%) of the 39 total patients who were C. *trachomatis* IgA-negative and C. *pneumoniae* IgA-positive were diagnosed with Chl-i ReA; the number of Chl-i ReA cases by C. *pneumociae* was greater than expected. Rheumatologists were able to administer Salazosulfapyridine (SASP) or MTX earlier for these patients before the chronic phase of arthritis. Supporting Hypothesis #1, the current study demonstrated the usefulness of serological tests. In addition, our study highlighted some of the diagnostic difficulties using the classification criteria for ReA.

The positive rate of C. *pneumoniae* IgA was significantly higher among patients who were C. *trachomatis* IgA-positive than among those who were C. *trachomatis* IgA-negative among 155 total patients during 2 years, supporting Hypothesis #2, suggesting the existence of cross-reactive T cells. To our knowledge, this is the first study to analyze a large number of patients and demonstrate that patients with prior infection by C. *pneumoniae* are vulnerable to infection by C. *trachomatis*. The prevention or treatment of C. *pneumoniae* infection can prevent the infection by C. *trachomatis*, which induces ReA, inferitiity, and visual impairment by trachoma.

PATIENTS AND METHODS

rofiles of patients

We retrospectively analyzed the patients at a new outpatient clinic in our hospital who had arthralgia or arthritis. The patients in the first and second groups visited our outpatient clinic during the 12 months between August 2018 and July 2019, and between August 2019 and July 2020, respectively. The number of patients in the first and second groups was 75 and 80, respectively. Serum IgG and IgA against both C. *trachomatis* and C. *pneumoniae* were measured to diagnose Chl-i ReA in the patients with arthralgia or arthritis, which we usually perform as the standard examination in our outpatient clinic.

Chl-i ReA was diagnosed according to the diagnostic criteria for reactive arthritis reported at the Third International Workshop on Reactive Arthritis on September 23-26, 1995, in Berlin, Germany [17]. The diagnostic criteria are as follows:

"Typical peripheral arthritis: Predominantly lower limb, asymmetric oligoarthritis plus evidence of preceding infection:

- Clear clinical diarrhea or urethritis within the preceding four weeks with laboratory confirmation is desirable but not essential.
- No clear clinical infection, but laboratory confirmation of infection is essential.

Exclusion criteria: Patients with other known causes of mono/oligoarthritis, such as other defined spondyloarthropathies, septic arthritis, crystal arthritis, Lyme disease, and streptococcal ReA, should be excluded. The diagnosis of ReA does not require the presence of HLA-B27 or extra-articular features of Reiter's syndrome or typical spondyloarthropathic features (inflammatory back pain, alternating buttock pain, enthesitis, iritis) but these, if present, should be recorded." [17].

In the current study, we modified the diagnostic criteria. The patients who met the criteria described above were diagnosed with 'Chl-i ReA, definite'. The patient who was C. *pneumoniae* IgA-positive and had arthritis in the upper limb was diagnosed with 'Chl-i ReA, probable' because ReA induced by C. *trachomatis* typically causes arthritis in the lower limb, although it remains unclear whether ReA induced by C. *pneumoniae* causes arthritis in the lower limb. The patients who were diagnosed and treated with IBD-AA, type 2 at our outpatient clinic were C. *pneumoniae* IgA-positive. In the diagnosis criteria for ReA [17], it is mentioned in the 'Exclusion criteria' that patients with other known causes of mono/oligoarthritis, such as other defined spondyloarthropathies, be excluded. However, these patients were diagnosed with both IBD-AA, type 2 and 'Chl-i ReA, possible'. The trial was performed by following the principles of the Declaration of Helsinki and Good Clinical Practice guidelines. The Institutional Ethics committee approved the research protocol.

Measurement of serum IgA or IgG against *C. trachomatis* or *C. pneumonia*: Plasma was obtained from peripheral blood using heparinized tubes. The measurement was performed at the laboratory of H.U. Group Holdings, Inc. [the Special Reference Laboratory (SRL), Inc. (Tokyo)]. The anti-chlamydia antibody IgA/IgG, LSI Medience Corporation (LSIM) kit (C. *trachomatis* IgA and IgG, 21200AMZ00626000; C. *pneumoniae* IgA, 22500AMX01787000; C. *pneumoniae* IgG, 22500AMX01788000; LSI Medience Corporation, Tokyo) was used for the ELISA method with high sensitivity and specificity. The procedure for C. *trachomatis* IgA or IgG was as follows: The ELISA plate was coated with both the purified antigen derived from C. *trachomatis* L2 strain and the synthetic peptides specific for C. *trachomatis* F strain, i.e., the ELISA method using a cocktail of 2 antigens.

The antigen was purified from C. *trachomatis* L2 strain, which cannot be described in detail according to H.U. Group Holdings, Inc. because of the patent. The method for the purification is a standard biochemical method using a surfactant. The synthetic peptides include the sequence with TTLNPTIAG from Variable Domain (VD) IV of the Major Outer Membrane Protein (MOMP)

of C. trachomatis. However, the sequence of the synthetic peptide for C. *trahomatis* F strain cannot be described according to H.U. Group Holdings, Inc. because of the patent.

In addition, ELISA does not have cross-reactivity with C. *pneumoniae* IgA or IgG. Kajihara S et al. demonstrated that the ELISA kit we used in the current study has no cross-reactivity with C. *pneumoniae* using sera samples from 10 patients who were C. *trachomatis*-positive and C. *pneumoniae*-negative by the micro-immunofluorescence (micro-IF) method; C. *pneumoniae* IgA-negative and C. *pneumoniae* IgG-negative was confirmed in all 10 patients (58).

The sensitivity and specificity of ELISA to discriminate between IgA and IgG against C. *trachomatis* were confirmed as follows: Kajihara S et al. compared 3 ELISA kits, 1) the ELISA kit in the current study, 2) the serodiagnostic kit "HITAZYME Chlamydia ® (Hitachi Kasei Kogyo, Inc.)" for C. *trachomatis* Infection and 3) "P - E L I S A® (Meiji Nyugyo, Inc.)" against the specific peptide of C. *trachomatis* MOMP VDIV region [58]. The measurements by the ELISA kit in the current study were strongly positively correlated with the measurements by the other ELISA kits. In addition, when each measurement by each was not similar, the results of the ELISA kit in the current study were the closest to those of the Western blotting method among the 3 ELISA methods [58].

The genital tract biovar (serovars D–K) is the most prevalent sexually transmitted bacterium. Thus, F strain is one of the most prevalent sexually transmitted strains. In addition, according to H.U. Group Holdings, Inc. they comprehensively investigated the reaction of antibodies and the sequence of peptides from the VD of the major outer membrane protein of each strain of C. *trachomatis*, finding the strongest and the most specific reaction with the peptide from F strain. The Lymphogranuloma Venereum (LGV) biovar (serovars L1–L3) causes invasive urogenital or anorectal infection [3].

Plasma from patients and a standard were added to the plate. Peroxidase-conjugated anti-human IgA antibodies or peroxidase-conjugated anti-human IgG antibodies were added to the plate. Chromogenic reactions proceeded for 30 minutes and were stopped by adding H2SO4. The absorbance was measured using a Bering ELISA processor III at 450 nm (Siemens Healthcare Diagnostics, K.K., Tokyo). Duplicate ELISA tests were performed. The antigen of C. *pneumoniae* was derived from lipopolysaccharide. The sensitivity and specificity of the ELISA for C. *pneumoniae* IgA were 96% and 99%, respectively, compared with MIF and CF methods. ELISA for C. pneumoniae IgG also demonstrated the same sensitivity and specificity as quoted by the manufacturer (Persson K **&**Boman J. Comparison of five serology tests for diagnosis of acute infections by *Chlamydia pneumoniae*. Clinical and Diagnostic Laboratory Immunology. In addition, in a study of cross-reactivity, none of the 16 serum samples positive for C. trachomatis and negative for C. *pneumoniae* was positive according to the ELISA for C. *pneumoniae* IgA (as quoted by the manufacturer). ELISA for C. *pneumoniae* IgG also had no cross-reactivity, as quoted by the manufacturer.

Coefficient Variation (CV) of ELISA for C. *pneumoniae* IgA or IgG: The standard sera for C. *pneumoniae* IgA or IgG were measured 10 times. The levels of CV were less than 10% (as quoted by the manufacturer). Correlation of ELISA for C. *pneumoniae* IgA or IgG: The correlation of IgA or IgG between ELISA used in the current study and ELISA of 'H Inc.' was 83.6% or 88.2%, respectively (as quoted by the manufacturer). The procedure for C. *pneumoniae* IgA or IgG was as follows: The ELISA plate was coated with the purified antigen derived from C. *pneumoniae*. Plasma from patients and a standard were added to the plate. Peroxidase-conjugated sheep anti-human IgA polyclonal antibodies or peroxidase-conjugated sheep anti-human IgG polyclonal anti-human IgG antibodies were added to the plate. Chromogenic reactions proceeded with a 3, 3', 5, 5'-tetramethylbenzidine set and were stopped by adding H2SO4. The absorbance was measured using a Bering ELISA processor III (Siemens Healthcare Diagnostics, K.K., Tokyo).

Positivity was determined as follows:

C. trachomatis IgA/IgG.

$$\text{Index: } <0.9,(-);0.9\,1.09,(+/-);1.10<,(+).$$

C. pneumoniae IgG.

$$\text{Index: } <30,(-);30\ \ 45,(+/-);45<,(+)$$

C. pneumoniae IgA.

$$\text{Index: } <8,(-);8\ \ 12,(+/-);12<,(+)$$

Statistical analysis- Data were analyzed using Fisher's exact test (js-STAR XR release 1.1.3j). A significant difference was defined as $p < 0.05$.

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