

## Original Article

**Role of *Chlamydia pneumoniae* in community-acquired pneumonia in hospitalized Jordanian adults**Suzan N Al-Aydie<sup>1</sup>, Nathir M Obeidat<sup>2</sup>, Hesham M Al-Younes<sup>1,3</sup><sup>1</sup> Department of Biological Sciences, Faculty of Science, the University of Jordan, Amman, Jordan<sup>2</sup> Department of Internal Medicine, Faculty of Medicine, the University of Jordan, Amman, Jordan<sup>3</sup> Department of Clinical Laboratory Sciences, Al-Ghad International Colleges for Applied Medical Sciences, Al-Madinah, Kingdom of Saudi Arabia**Abstract**

**Introduction:** This study investigated the role of *Chlamydia pneumoniae* in the etiology of community-acquired pneumonia (CAP) in Jordanian adults.

**Methodology:** Eighty hospitalized CAP patients and 110 healthy adults were enrolled. Overall prevalences of *C. pneumoniae* IgG antibodies in sera and the rate of acute infection were estimated, using the microimmunofluorescence method (MIF), at titers of 1:16 and 1:512, respectively. Moreover, a nested polymerase chain reaction (PCR) was used to detect *C. pneumoniae* DNA in nasopharyngeal and blood Buffy coat samples.

**Results:** Overall chlamydial IgG prevalence was higher in CAP cases than controls (70% versus 61.8%). Similarly, higher rate of acute infection was found in patients than in controls (16.3% versus 5.5%). By focusing on subjects testing positive at 1:16, acute infection was detectable in 23.2% of CAP cases, compared with 8.8% of seropositive controls. Chlamydial DNA was confirmed in 8.2% and 8.8% of nasopharyngeal specimens from controls and patients, respectively. Moreover, 10.9% and 7.5% of Buffy coats from controls and cases, respectively, were PCR-positive. When performances of both assays for detection of the pathogen were assessed, the sensitivities of MIF and PCR were low and comparable. However, MIF demonstrated higher specificity, positive predictive value, and negative predictive value than PCR.

**Conclusions:** MIF-based data indicate that *C. pneumoniae* could be a potential causal agent of CAP in Jordan. This study may serve as a basis to elucidate the exact role *C. pneumoniae* and other co-infecting pathogens in the etiology of respiratory tract disease.

**Key words:** *Chlamydia pneumoniae*; Jordan; microimmunofluorescence; PCR; pneumonia; respiratory disease.

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**Introduction**

Chlamydiae are Gram-negative, obligate intracellular pathogens that infect a variety of vertebrate organisms [1]. This bacterial group includes *Chlamydia pneumoniae* (synonym *Chlamydophila pneumoniae*) that is primarily a human pathogen and has been widely implicated in both lower and upper respiratory tract infections, such as community-acquired pneumonia (CAP), bronchitis, asthma, and pharyngitis [2,3]. It has been also associated with cardiovascular and neurodegenerative illnesses [4,5].

Seroepidemiological studies performed worldwide have demonstrated that 40%–86% of asymptomatic adults have antibodies to *C. pneumoniae* [6-9]. In Jordan, a single study demonstrated 61.1% seroprevalence of *C. pneumoniae*-specific IgG antibodies among apparently healthy male adults [10]. More recently, the IgG seropositivity was investigated

in a population-based survey, which included asymptomatic Jordanian nationals with ages ranging from 2 to 86 years of both sexes [11]. The overall detection rate of IgG against *C. pneumoniae* was 54.9% [11].

CAP is an important lower respiratory tract disease and has been shown to have a yearly incidence between 5 and 11 cases per 1,000 adults as estimated by some population-based studies in the United States and Europe [12]. In the last two decades, *C. pneumoniae* has been recognized to play a role in the etiology of CAP worldwide. According to epidemiological studies, *C. pneumoniae* accounts for 1%–22% of patients suffering from CAP [13-16]. It has been suggested that this pathogen could play an etiological role for CAP in 6% of pediatric outpatients between 6 months and 12 years of age [17]. Moreover, elderly patients are also among

those at high risk of CAP due to *C. pneumoniae* infection [18].

In Jordan, few reports have analyzed the possible role of *C. pneumoniae* in CAP. Al-Ali *et al.* detected *C. pneumoniae* IgM antibodies in 23% and 14% of hospitalized adults and children, respectively [19]. Another report confirmed the presence of *Chlamydia* DNA in 4.5% of Jordanian children younger than five years of age and hospitalized with acute respiratory infection, based on *Chlamydia* spp.-specific polymerase chain reaction (PCR) for nasopharyngeal samples [20]. However, a more recent study could not find *C. pneumoniae* DNA by multiplex PCR in throat and nasal swabs collected from Jordanian children younger than two years of age and hospitalized with acute respiratory disease (A. Shehabi, personal communication, November 13, 2014). It is noteworthy to mention that these reports were without controls and did not include attempts to analyze the *C. pneumoniae*-specific IgG antibody. Additionally, the serological findings have not been correlated with molecular (DNA) prevalence of *C. pneumoniae*. Furthermore, the presence of the bacterial DNA in the blood of CAP cases has not yet been screened. The main goal of the present study was to unveil the possible causal role of *C. pneumoniae* in CAP based on the detection of both the bacterial DNA and *C. pneumoniae*-specific IgG antibodies in clinical specimens collected from hospitalized adults suffering from CAP.

## Methodology

### *Study subjects*

This case-control study was conducted between February 2013 and January 2014. A total of 80 patients with a diagnosis of CAP and who were hospitalized at the University of Jordan Hospital, Amman, were enrolled in this study. This group of CAP cases comprised patients of 18–92 years of age and included 53 males and 27 females with a mean age of 57.4 years  $\pm$  17.5. Diagnosis of CAP was based on chest radiography, performed within 24–48 hours after admission, with signs compatible with acute lower respiratory tract infection and new pulmonary infiltrates on chest X-ray. Other clinical and respiratory symptoms of CAP included cough, fever, dyspnea, tachypnea, and pleuritic chest pain. Control subjects were healthy blood donors, laboratory personnel, and students at the Department of Biological Sciences, the University of Jordan. The ages of controls ranged from 18 to 87 years (mean age 40.2 years  $\pm$  14.5) and consisted of 67 males and 43 females. Control individuals were excluded from the study if they had symptoms of respiratory

disease or received antibiotics within the last three months before enrollment. Controls and CAP patients were divided into four age groups: 18–32 years, 33–48 years, 49–64 years, and  $\geq$  65 years.

The study was approved by the scientific research committees at the Department of Biological Sciences and Faculty of Postgraduate Studies, in addition to the ethics committee of the University of Jordan Hospital. Signed informed consent was obtained from each participating person.

### *Data collection and clinical information*

All available clinical data and radiological diagnosis for each patient were documented on a standardized questionnaire. The same form was also utilized to record all available information from controls eligible for the study.

### *Sample collection and transport*

A nasopharyngeal specimen and two venous blood samples (3–4 mL each) were obtained from each enrolled patient within 48 hours of admission. Prior to admission and sample collections, about 39% of patients had initiated antibiotic treatment. Nasopharyngeal specimens were obtained based chiefly on previously published protocol [21]. Briefly, samples were taken using sterile plastic-shafted Dacron-tipped swabs and collected in 1 mL commercially available sterile *C. pneumoniae* transport medium (Vircell MICROBIOLOGISTS, Santa Fe, Spain) and then stored at  $-72^{\circ}\text{C}$  until analysis of the presence of *C. pneumoniae* DNA. A blood sample drawn from each individual was used to separate serum that was then stored at  $-20^{\circ}\text{C}$  until tested in the serological test. The second blood specimen was used to harvest Buffy coats according to the protocol provided with G-spin Total DNA Extraction Kit (iNtRON Biotechnology, Gyeonggi-Do, Korea). Fractions corresponding to Buffy coats were carefully aspirated and transferred into 1.5 mL Eppendorf tubes and kept at  $-20^{\circ}\text{C}$  until used.

### *Antigen preparation*

The *C. pneumoniae* strain VR1310, a generous gift from Thomas F. Meyer, Max Planck Institute for Infection Biology, Berlin, Germany, was utilized for the antigen preparation in microimmunofluorescence (MIF). Antigens were whole elementary bodies prepared as described previously [22].

### Serological assay

MIF method detects indirectly human IgG antibodies raised against *C. pneumoniae* using labeled anti-human IgG antibodies [22]. The antibodies used were fluorescein-isothiocyanate (FITC)-labeled goat anti-human IgG (Bio-Rad, Hercules, USA). The serological test was performed as previously described [10,11]. *C. pneumoniae*-specific IgG in sera was first screened at 1:16, a dilution widely considered a marker for *C. pneumoniae* positivity [7,8,10,11,21,23-25]. If sera were reactive at 1:16, they were further tested at serial twofold dilutions (from 1:16 to 1:512) for IgG antibody titer determination. Chlamydial IgG titer of 1:512 was considered indicative of acute or recent infection [7,23-27].

### DNA extraction

Nucleic acids from 200  $\mu$ L of each nasopharyngeal sample were harvested according to a protocol specific for harvesting DNA from body fluids provided with the G-spin Total DNA Extraction Kit. DNA from Buffy coats was extracted by the same kit according to a procedure recommended by the manufacturer. DNA was eluted in a final volume of 50  $\mu$ L of a buffer provided with the kit, aliquoted, and stored at -20°C until used. The amount of DNA was measured spectrophotometrically.

### PCR assay

A nested PCR method targeting the *C. pneumoniae* gene CPn\_0809, which encodes the species-specific 53-kDa protein, was used for the detection of DNA in all clinical specimens collected. Primers used were those previously designed and published by Fukano [28]. Sequences of primers in the two amplification rounds as follows. Outer forward primer: 5'-ATG ATC GCG GTT TCT GTT GCC A-3'; outer reverse primer: 5'-GAC CGA CGT TTT GTT GCA TCT C-3'; inner forward primer: 5'-TGT CCA AGC GGT GAA ACA AG-3' and inner reverse primer: 5'-CAA CCG TGA CCC ATT TAC TG-3'. Each reaction mixture had a total volume of approximately 20  $\mu$ L. The first (outer) amplification reaction contained 10  $\mu$ L of 2x PCR master mix solution (i-MAX II; iNtRON Biotechnology) supplemented with DNA polymerase, 1  $\mu$ L (25 pmol) forward primer, 1  $\mu$ L (25 pmol) reverse primer and 500 ng extracted DNA (1-4  $\mu$ L). The total volume was completed to 20  $\mu$ L by addition of nuclease-free water. The same components were used to prepare second (inner) amplification reaction, except that 2  $\mu$ L from the first amplification step after PCR cycling was used instead of extracted DNA. PCR

amplifications were performed according to previously described protocols [28]. For the outer amplification, cycling consisted of 30 seconds at 95°C followed by 40 cycles of 1 minute at 95°C, 1 minute at 56°C, and 1 minute at 72°C. The inner amplification cycling consisted of 30 seconds at 95°C followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 1 minute at 72°C. In every PCR run, negative and positive controls were included; the negative control PCR mixture contained all components of the amplification mixture and distilled water instead of the extracted DNA. Heat-treated lysed *C. pneumoniae* served as a positive control. Amplification products were analyzed by agarose gel electrophoresis. Amplicon sizes produced in the first and second amplification rounds were 499 and 239 bp, respectively.

### Calculation of sensitivity and specificity of diagnostic methods and statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) software, version 14.0.1. The diagnostic sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for MIF and nested-PCR using clinically diagnosed CAP cases with an indication of acute infection as the gold standard. The statistical analysis of the data obtained was determined using Chi-square test. A probability value (*p*) of < 0.05 was considered statistically significant.

## Results

### Demographic and clinical profiles of enrolled subjects

A total of 110 Jordanian individuals were enrolled as controls (67 males and 43 females; mean age 40.2 years  $\pm$  14.5). The total number of hospitalized CAP patients was 80 (53 males and 27 females; mean age 57.4 years  $\pm$  17.5). The difference between ages of CAP cases and controls was statistically significant (*p* = 0.000). Table 1 demonstrates the demographic features of study subjects in addition to the major symptoms and clinical characteristics of patients at hospital admission. The frequency of cough and sputum production in patients was 92.5% and 77.5%, respectively. Fever was observed in about half of the CAP patients. Approximately 67% and 52% of patients had dyspnea and chest pain, respectively. Chest X-ray examination revealed new pulmonary infiltrates in 90% of CAP cases.

**Table 1.** Demographic characteristics of study individuals and clinical features of Jordanian adults suffering from community-acquired pneumonia (CAP) who were eligible for this study.

	CAP group	Control group	P value*
<b>Age (years)</b>			
Range	18-92	18-87	0.000
Mean ( $\pm$ SD)	57.4 $\pm$ 17.5	40.2 $\pm$ 14.5	0.049
<b>Age groups (n; %)</b>			
18–32 years	10 (12.5)	37 (33.6)	0.035
33–48 years	14 (17.5)	47 (42.7)	0.817
49–64 years	20 (25.0)	18 (16.4)	0.035
$\geq$ 65 years	36 (45.0)	8 (7.3)	
<b>Gender (n; %)</b>			
Male	53 (66.25)	67 (60.9)	0.201
Female	27 (33.75)	43 (39.1)	0.000
<b>Symptoms (n; %)</b>			
Fever ( $\geq$ 38°C)	43 (53.75)		
Cough	74 (92.5)		
Rigors	32 (40.0)		
Dyspnea	54 (67.5)		
Chest pain	42 (52.5)		
Chills	36 (45.0)		
Sputum production	62 (77.5)		
<b>Radiographic findings (n; %)</b>			
New infiltrate	72 (90.0)		
Normal	8 (10.0)		
<b>Comorbidities (n; %)</b> (COPD, asthma, heart failure)	27 (33.75)		
<b>Antibiotic prior to admission (n; %)</b>	31 (38.75)		

\*Differences in positivity percentages between patients and controls were statistically significant when  $p < 0.05$ ; COPD: chronic obstructive pulmonary disease

**Table 2.** Age-specific prevalences of anti-*C. pneumoniae* IgG, determined at a titer of 1:16, among Jordanian controls and community-acquired pneumonia (CAP) cases.

Age group (years)	Controls		CAP patients		P value*
	No. positive	%	No. positive	%	
18–32	15/37	40.5	3/10	30.0	0.543
33–48	32/47	68.1	9/14	64.3	0.790
49–64	14/18	77.8	14/20	70.0	0.587
$\geq$ 65	7/8	87.5	30/36	83.3	0.771
Total	68/110	61.8	56/80	70.0	0.242

\*Differences between positivity rates of patients and controls within the same age group were statistically significant when  $p < 0.05$

**Table 3.** Positive cases of *C. pneumoniae* confirmed by nested polymerase chain reaction (PCR) for nasopharyngeal and Buffy coat specimens for Jordanian adults suffering from community-acquired pneumonia (CAP) and control subjects.

Specimen used for PCR	No. and percentage of PCR-positive samples				P value*
	CAP cases		Controls		
	No. positive	% positive	No. positive	% positive	
Nasopharyngeal swab	7	8.8	9	8.2	0.889
Buffy coat	6	7.5	12	10.9	0.425
Total	13	16.25	21	19.1	0.563

\*Differences in positivity percentages between patients and controls were statistically significant when  $p < 0.05$

**Overall seroprevalence of *C. pneumoniae* in controls and patient cases**

To initially determine the general seroprevalence of *C. pneumoniae* in patient and control groups enrolled in this study, existing or preexisting infections were screened by testing the IgG seropositivity at a cut-off value of 1:16. The overall seroprevalence of IgG antibody in healthy control individuals tested was 61.8% (68/110). Noticeably, the antibody was detected in 56 out of 80 CAP patients, resulting in a higher overall prevalence (70%;  $p = 0.242$ ).

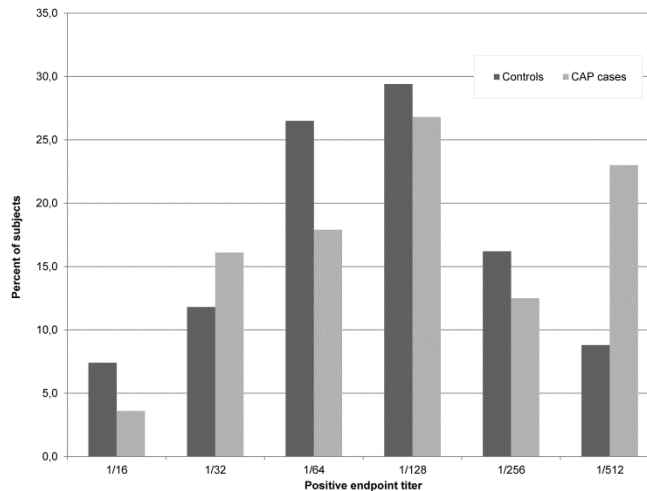
**Age distribution of *C. pneumoniae*-seropositive cases**

The overall age-related seroprevalences of *C. pneumoniae* IgG, determined at a titer of 1:16, are summarized in Table 2. In the control cohort, the IgG seropositivity was the lowest (40.5%) in the 18-32 year age group and increased steadily in the subsequent age groups to reach a peak of 87.5% in the oldest controls. A comparable pattern of age-related seropositivities was noticed in CAP patients (Table 2).

**Analysis of *C. pneumoniae* endpoint titers in seropositive controls and patients**

To determine the endpoint titers of IgG (the highest positive dilutions) in sera, which were reactive at 1:16, serial twofold dilutions (1:16 to 1:512) were further tested by the serological assay. Distribution of IgG titers for *C. pneumoniae* in seropositive controls and patients is demonstrated in Figure 1. An IgG titer of

**Figure 1.** Distribution of endpoint titers of *C. pneumoniae* IgG antibody in sera of 56 community-acquired pneumonia (CAP) patients and 68 apparently healthy Jordanian adults that showed seropositivity by microimmunofluorescence (MIF) at 1:16. Acute *C. pneumoniae* infection, estimated at a cut-off value of 1:512, was detected in 23.2% and 8.8% of the total seropositive CAP patients and controls, respectively. Light grey bars represent CAP cases, while dark grey ones indicate controls. Difference in prevalences of acute infection between patients and controls are statistically significant ( $p = 0.014$ ).



1:512 was considered here as a sign of acute infection. Data shown in the figure demonstrate that acute infection was remarkably found in about a quarter (23.2%; 13/56) of the total number of seropositive CAP patients. Interestingly, only 8.8% (6/68) of acute infection prevalence was noticed among seropositive

**Table 4.** Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the microimmunofluorescence (MIF) assay and nested polymerase chain reaction (PCR) for determination of acute *C. pneumoniae* infections in patients suffering from community-acquired pneumonia (CAP) and requiring hospital admission.

Assay	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
IgG MIF	16.25	94.55	68.42	60.82
	(8.95–26.19)	(88.5–97.96)	(43.46–87.35)	(53.07–68.18)
PCR	15.00	81.82	37.50	56.96
	(8.01–24.74)	(73.32–88.52)	(21.12–56.30)	(48.86–64.80)

Data are percentages (95% confidence intervals)

**Table 5.** Distribution of nasopharyngeal and Buffy coat polymerase chain reaction (PCR)-positive patients and controls in correlation to microimmunofluorescence (MIF) findings. The PCR findings of nasopharyngeal and Buffy coat samples are combined.

MIF titer	No. and percentage of PCR-positive cases						P value*
	CAP patients			Controls			
	No. examined	No. positive	% positive	No. examined	No. positive	% positive	
Negative	24	1	4.2	42	10	23.8	0.039
1:16–1:256	43	9	20.9	62	10	16.1	0.530
≥ 512	13	2	15.4	6	0	0.0	0.310
<b>Total</b>	<b>80</b>	<b>12</b>	<b>15.0</b>	<b>110</b>	<b>20</b>	<b>18.2</b>	

\*Differences in positivity percentages between patients and controls were statistically significant when  $p < 0.05$

controls. The difference in the prevalence of acute infection between seropositive patients and controls was statistically significant ( $p = 0.014$ ).

#### *Overall seroprevalence of acute C. pneumoniae infection in the entire populations of controls and patients*

As shown above, analysis of endpoint titers in controls and patients, tested positive at a titer of 1:16, revealed significant elevation in the rate of acute infection in CAP cases. Here, the involvement of *C. pneumoniae* in CAP was further analyzed by estimating the overall prevalence of *C. pneumoniae* acute infection among all individuals included in both study cohorts. About 5.5% (6/110) of the control population had an IgG titer of 1:512. Intriguingly, 16.3% (13/80) of CAP cases had high IgG titers, indicative of acute chlamydial infection. The difference in detection rates of acute infection in CAP patients and the control group was statistically significant ( $p = 0.036$ ). Collectively, the cut-off value of 1:512 suggests a role of *C. pneumoniae* in the etiology of CAP in Jordan.

Interestingly, the cut-off value 1:512 used here to indicate acute infection seems to correlate with clinical and respiratory symptoms reported for all CAP cases (Table 1). Percentages of CAP patients, who were seropositive at 1:512, with either fever, rigors, dyspnea, chest pain, chills, or sputum production were 84.6%, 61.5%, 84.6%, 61.5%, 53.8%, and 100%, respectively. These percentages were higher than those reported for the entire population of CAP cases (Table 1). The percentage of CAP patients who had positivity at 1:512 and cough was high (92.3%) and comparable to that reported in Table 1 for the general CAP population. These findings may support the credibility of MIF and, specifically, the use of 1:512 cut-off value in determination of *C. pneumoniae* acute infection.

#### *Prevalence of C. pneumoniae determined by PCR*

Specimens collected from the nasopharynxes and blood of the study subjects were investigated for the presence of *C. pneumoniae* DNA using nested PCR. Nine (8.2%) and 12 (10.9%) of the 110 control individuals showed positive PCR results in nasopharyngeal and Buffy coat specimens, respectively (Table 3), whereas chlamydial DNA was detected in seven (about 8.8%) nasopharyngeal samples and six (7.5%) Buffy coats from patients suffering from CAP (Table 3). The slightly elevated level of PCR positivity shown in nasopharyngeal swabs from CAP patients is not significant when compared to that of control subjects ( $p = 0.889$ ). The detection rate of chlamydial

DNA in Buffy coats from CAP cases was less than that in Buffy coats from control subjects. Of note, only one individual from each of the study groups was *C. pneumoniae* DNA positive in both nasopharyngeal and Buffy coat specimens.

#### *Comparison between PCR and MIF for assessment of C. pneumoniae acute infection and correlation of MIF and PCR results*

Using clinically defined CAP cases as the gold standard, sensitivities, specificities, PPV, and NPV of used assays were calculated for diagnosis of acute *C. pneumoniae* infections. A total of 12 CAP cases out of 80 had the pathogen DNA in one or both types of clinical samples collected, whereas 13 cases had serological results indicative of acute infection. As summarized in Table 4, sensitivities for both assays were low and comparable (16.25% and 15.00% for MIF and PCR, respectively). However, higher specificity was observed for MIF compared to that for PCR (94.55% and 81.82%, respectively). In addition, the MIF test had much higher PPV (68.42%) than did PCR (37.50%). The NPV for MIF was 60.82%, a slightly higher value than that for PCR (56.96%) (Table 4). The distribution of PCR-positive subjects based on their serological distribution is depicted in Table 5. Only one (4.2%) PCR-positive CAP patient was seronegative in MIF. Unexpectedly, 10 (23.8%) out of 42 control individuals with negative sera were found to be positive by PCR. Percentages of 20.9% and 16.1% of CAP cases and control individuals, respectively, who had moderate *Chlamydia* IgG titers (1:16–1:256), were found to be positive by PCR. None of the control subject who had an IgG titer of 1:512 were positive by PCR. In comparison, 2 (15.4%) out of 13 CAP cases with sera positive at a titer of 1:512 were reactive in PCR (Table 5). Taken together, the present data show no correlation of PCR with MIF findings.

## **Discussion**

The present study utilized the MIF in conjunction with nested PCR assay to address the overall prevalence and the etiologic role of *C. pneumoniae* in CAP in adult Jordanian nationals. Using a cut-off titer of 1:16, a higher overall seropositivity of *C. pneumoniae* IgG was found in CAP patients compared with healthy controls. The IgG seropositivity in Jordanian CAP patients is considered one of the highest compared to rates reported previously for adult CAP populations in many regions of the world, which ranged from 50% to 70% [16,29-32]. On the other hand, the rate of IgG detection in apparently healthy controls estimated here is

analogous to overall *C. pneumoniae* seroprevalence previously recorded in healthy Jordanian adults [10,11].

Epidemiological reports proposed controversial diagnostic criteria for acute infection with *C. pneumoniae*. Using single serum sample, a chlamydial IgM titer of  $\geq 1:16$  [21], IgG  $\geq 1:256$  [8] or more frequently  $\geq 1:512$  [7,23-27,33] were defined as evidence for acute infection. However, the Centers for Disease Control and Prevention (CDC) in the United States and Canada have recommended the use of paired serum samples to detect a fourfold increase in the IgG titer as a diagnostic criterion for acute infection [21]. The second (convalescent) serum sample should be obtained within four to six weeks to detect rises in IgG level. Based on a single serum sample, CDC considered an IgG titer of  $\geq 1:512$  suggestive of possible acute infection [21]. Obtaining a second serum sample was technically not possible and remained a major challenge during this study. Nonetheless, serodiagnosis of acute *C. pneumoniae* infection based on a single serum sample seems to be more realistic, as decisions of treatment of chlamydial infections are based often on a single serum sample and the convalescent serum is useful in only a retrospective diagnosis [14,27].

*C. pneumoniae* has consistently been recognized as a common cause of CAP. Between 1% and 22% of CAP cases have been attributed to this pathogen [13-16,18,34]. Clearly, frequency of acute infection (obtained here based on MIF) among CAP patients correlates well with available epidemiological data. Remarkably, the overall prevalence of acute infections assessed in CAP cases was significantly threefold higher than that in the control group. This detection rate in controls may be due to the asymptomatic course of either primary infections or reinfections with *C. pneumoniae*. Moreover, acute infection with *C. pneumoniae* was found in approximately a quarter of the total number of seropositive CAP cases, compared with 8.8% among seropositive controls. It is noteworthy to mention that the vast majority of studies on the epidemiology of CAP due to *C. pneumoniae* had no control subjects. This is extremely confusing, as the significance of the seroprevalences of patient groups reported in those studies cannot be known. Overall, MIF results may provide evidence of the role of *C. pneumoniae* of CAP in Jordan.

In Jordan, few investigations with no control groups attempted to link *C. pneumoniae* with CAP. The percentages of CAP cases with *C. pneumoniae* infection in these reports ranged from 0% to 23%, as assessed by PCR or IgM level determination [19,20]. Here, the prevalence of *C. pneumoniae* acute infection in

Jordanian CAP cases was 16.3%. This high degree of heterogeneity in the detection rates of *Chlamydia* in Jordan can be explained by variations in clinical samples examined, size of the population studied, ages of population subjects, and inconsistencies of the diagnostic methods.

PCR and bacterial culturing are the second most widely used techniques for *C. pneumoniae* screening after MIF. The PCR technique is basically used to rapidly recognize the pathogen in various clinical specimens [2,14,21]. The nested PCR used here was previously shown to be the most sensitive assay that led to chlamydial DNA detection in 23.4% of peripheral blood mononuclear cell (PBMC) specimens collected from healthy blood donors, compared with four other PCR assays [28]. In this report, however, this assay was able to detect *C. pneumoniae* DNA in only about 11% and 8% of harvested Buffy coats obtained from controls and CAP cases, respectively. These contradicting results between the present study and the previous one [28] may be related to variations in study subjects, geographic locations, sample processing, and DNA extraction methods. Further, Fukano [28] utilized purified PBMCs, while this study included Buffy coat samples that are rich with blood nucleated cells. These nucleated cells are usually utilized by *C. pneumoniae* as vehicles for its dissemination from the lungs to non-pulmonary tissues [35]. Other reports used various PCR methods to analyze the presence of *C. pneumoniae* DNA in PBMCs of healthy subjects and showed variable detection rates that exceeded 45% [5,36-38]. However, two reports showed that PBMCs were *C. pneumoniae* DNA positive only in the minority of patients with acute respiratory tract infections [39,40]. Here, PCR findings failed to correlate *C. pneumoniae* with CAP. The present PCR data, along with those reported before [39,40], strongly suggest that blood-based PCR is not a suitable diagnostic tool for respiratory tract infections caused by *C. pneumoniae*.

Similarly, PCR-based analysis of chlamydial nucleic acids in respiratory clinical samples led to conflicting results with regard to the role of *C. pneumoniae* and respiratory diseases. In investigations without controls, *C. pneumoniae* DNA was found in 5% to 26% of patients diagnosed with acute respiratory infections such as CAP or bronchitis [20,39,41,42]. Miyashita and colleagues found that 8% of patients with acute respiratory disease had DNA in their respiratory samples, compared with 3% of asymptomatic controls [40]. In contrast, other studies that did not include controls demonstrated DNA prevalences ranging from 0 to 0.9% in CAP patients, suggesting no role or a minor

role of *C. pneumoniae* in the etiology of this disease [16,43,44]. Consistently, Liu *et al.* [45] showed an apparent low detection rate of the pathogen DNA in CAP patients (3.5%), which was almost similar to that obtained in asymptomatic controls (2.1%). Our findings are in agreement with previous data that were unable to lend support for the role of *C. pneumoniae* in acute respiratory infections based on PCR. In general, reported variations in chlamydial DNA prevalence in respiratory samples may be related to the use of different PCR assays, specimens, protocols for specimen collection and processing and DNA extraction methods. Unexpectedly, the percentage of controls with positive PCR results was higher than that of CAP patients. This finding could be an overestimation due to the problems with contamination that may be impossible to avoid in nested PCR assays [2,14,21].

We further evaluated the MIF assay and PCR for the detection of acute *C. pneumoniae* respiratory infection by determining their sensitivity, specificity, PPV, and NPV. These values calculated for MIF were discouraging, although its specificity was high. Even more disappointing findings were observed for the PCR test, whose sensitivity, specificity, PPV, and NPV were shown to be lower than those for MIF. These calculations make diagnostic value of the used PCR assay questionable; therefore, it is not recommended as a tool to analyze the etiologic role of *C. pneumoniae* in CAP. However, several in-house PCR assays have recently shown a high sensitivity and specificity in addition to rapidity and have been recommended for use to diagnose acute *C. pneumoniae* infections in respiratory systems [46-48]. Some researchers considered PCR, especially real-time assays, to hold more promise for diagnosis and may offer advantages over conventional culture and serology [13,46,47]. In this report, the diagnostic utility of nested PCR may be hampered by several limitations like the lack of adequate validation. It may be also influenced by the type of clinical sample, protocols for specimen collection and processing and DNA extraction methods. Another general drawback of nested PCR tests is that they involve significant problems with contamination that may be impossible to avoid, resulting in an overestimation of *C. pneumoniae*-infected cases [2,14,21] and, subsequently, in misleading analytical calculations of sensitivity and other values. This contamination may have occurred, at least in part, in the 10 PCR-positive controls with no serological evidence of infection (Table 5). Another factor that may have affected analytical calculations of nested PCR is the

clearance of the bacterium by the use of antibiotics, which might have decreased chances of detection of the chlamydial DNA in CAP patients.

Findings of our study indicate that MIF is not an entirely satisfactory serological assay for defining acute *C. pneumoniae* respiratory infection. Despite relative higher specificity, PPV, and NPV, MIF showed low sensitivity (about 16%) that was comparable to that of nested PCR. This study is consistent with previous ones, which demonstrated unconvincing diagnostic utility of IgG-detecting MIF in defining CAP cases caused by *C. pneumoniae*. This can be attributed to several limitations of this assay. MIF results may be affected by the antigen preparation used and variations between in-house MIF and commercially available validated ones [21,49]. Other drawbacks of the test include technical complexity, subjective endpoints, the lack of standardized reagents, the timing of serum samples, and the high rate of IgG seropositivity in some populations [14,21,46,49], including a Jordanian one [11]. More importantly, MIF was found to lack specificity during acute infection due to cross-reactivity [49].

It is clear that PCR findings of this study have no agreement with those of MIF, analogous to previously published reports [14,16,49]. We believe that the drawbacks of both tests may significantly contribute to the lack of correlation. Besides, the negative PCR results in some MIF-positive individuals may be due to low numbers of chlamydial genome copies in the clinical samples. Further, some MIF-negative, DNA-positive controls may serve as carriers of the pathogen or have subclinical infections [50]. It could be also possible that some IgG-negative patients, who were PCR-positive, might have contracted a primary *C. pneumoniae* infection when enrolled in this study and might require longer period of time until they could generate MIF-detectable IgG antibodies.

## Conclusions

This case-control study showed that *C. pneumoniae* is common among Jordanian adults and examined the role of *C. pneumoniae* in CAP in Jordanian adults. Although the present IgG-based MIF findings are in favor of the presence of an association between *C. pneumoniae* and CAP in Jordan, they remain, however, not fully conclusive because of significant drawbacks of utilized MIF. Therefore, further investigations are necessary to elucidate the etiology of *C. pneumoniae* in CAP in Jordanian nationals. On the basis of previous findings [41,46,47], reliable, and sensitive diagnostic tests such as validated real-time PCR alone or combined



with specific IgM-detecting method should be used to define cases of acute respiratory *C. pneumoniae* infection. Importantly, *C. pneumoniae* can be found in association with other CAP etiologic agents such as *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* [13]. Thus, a comparative analysis of co-infections with *C. pneumoniae* and other microorganisms is required to evaluate the role of *C. pneumoniae* more precisely.

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