ORIGINAL ARTICLE

# Simultaneous detection of *Chlamydia pneumoniae*, *Mycoplasma pneumoniae,* and *Legionella pneumophila* in patients hospitalised for community-acquired pneumonia in South Jordan

*Wael A Al-Zereini*

## ABSTRACT

**Objectives**: To detect *Chlamydia pneumoniae, Mycoplasma pneumoniae,* and *Legionella pneumophila* simultaneously in hospitalized community-acquired pneumonia (CAP) patients in Southern Jordan; the diagnostic utility of PCR and ELISA methods in determining their prevalence, detection of acute infection and identification of the causal agent from a single serum.

**Methods**: Blood sera and nasopharyngeal samples were collected from 200 participants (100 individuals from each of CAP patients and controls). Seroprevalences of IgG and IgM antibodies raised against the three pathogens was analysed in collected sera by ELISA, while presence of their DNA in nasopharyngeal samples was detected by standard PCR. Concurrent infection was detected by multiplex PCR.

**Results**: Based on ELISA-IgG, the general prevalence rates of *C. pneumoniae* and *M. pneumoniae* were significantly higher in CAP cases than controls ($p$= 0.02 and $p\leq$ 0.001, respectively); anti-*L. pneumophila* IgG was not detected in all participants. Based on ELISA-IgM and PCR in detecting acute infections, significant higher detection frequencies of anti-*C. pneumoniae* IgM and DNA were noticed in CAP patients compared to control cohort ($p$= 0.01 and $p\leq$ 0.001, respectively); an insignificant difference in prevalence rates of *M. pneumoniae* and *L. pneumophila* between patients and controls were reported in both assays. Concurrent detection of the three pathogens was noticed in 30% of entire CAP cases.

**Conclusions**: Simultaneous use of ELISA and PCR assays may allow rapidity and improvement in detection of CAP etiology in acute diseases; *C. pneumoniae* is the most possible etiological agent for CAP in Southern Jordan population during the study period.

**Keywords:** *Chlamydia pneumoniae*, *Mycoplasma pneumoniae, Legionella pneumophila,* enzyme-linked immunosorbent assay (ELSA), polymerase chain reaction (PCR), Jordan.

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## INTRODUCTION

Community acquired pneumonia (CAP) is a respiratory infection that causes morbidity and mortality worldwide. Atypical pathogens such as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae,* and *Legionella pneumophila* have a role in CAP establishment. They account for 22% of CAP worldwide (1,2) and 40%-60% of hospitalised patients (3,4). *C. pneumoniae* infections are evident in about 1%-22% of all pneumonia cases; *M. pneumonia* in 8%-30% which might reach to 50% in outbreaks, and *Legionella* species caused pneumonia in 2%-9% of patients (2,5,6). Respiratory infections due to these pathogens have similar clinical features and their isolation and culturing require complex procedures, rendering it difficult managing pneumonia patients and the rapid detection of etiological agents. Furthermore, false positive cross reactions may occur in patients infected with other bacteria due to delayed or abated immune response (7). As more than 50% of CAP cases are treated with antibiotics based on the most likely causing pathogen without identifying the main etiological agent, it is necessary to have approaches that can detect and differentiate these respiratory pathogens using the same sample and the same assay.

Several previous studies in Jordan have highlighted the prevalence of these bacteria by serological detection of immunoglobulins IgG and/or IgM using micro-immunofluorescence (MIF) as a gold standard diagnostic method, or ELISA assays alone or with PCR. Serological studies revealed prevalence of *C. pneumoniae* in 54.4%-61% of apparently healthy individuals (8,9) and in 23%-70% of adult patients with respiratory tract infections (10,11). Antibodies raised against *L. pneumophila* and *M. pneumoniae* were noticed in 6% (11) and 7%-8% of CAP patients (12), respectively. However, the presence of *C. pneumoniae* DNA was evident from 8.8% of adult CAP cases (11) and 4.5% of children (13) using PCR assay; PCR failed to detect the three pathogens in children younger than 2 years old (14) and *M. pneumoniae* in nasopharyngeal samples from adult patients (12). All the aforementioned studies were carried out in central and north regions of Jordan; a large part of these did not include controls to establish a baseline of seropositivity. In a recent study performed in Southern Jordan, prevalence of *C. pneumoniae* was reported in 44.3%, 27%, and 40% of hospitalised CAP individuals based on IgG, IgM, and PCR respectively (6); the authors did not consider the concurrent infection with other atypical pathogens.

The current study is the first that address simultaneous detection of *C. pneumoniae, M. pneumoniae,* and *L. pneumophila* with evaluating their prevalence in hospitalised CAP patients from Southern Jordan, especially from Al-Karak Governorate. This was assessed using ELISA and PCR assays as rapid techniques; ELISA-IgG was employed to detect the presumptive role of each pathogen in CAP occurrence or in past-infections while both ELISA-IgM and PCR were used in revealing recent (current) infection by these bacteria.

## MATERIAL AND METHODS

### Study subjects and sample collection

The study was performed from January 2016 to December 2017 and included 100 CAP patients and 100 asymptomatic controls. Patients were those hospitalized in Al-Karak Governmental Hospital with a clinical and radiological diagnosis of CAP (i.e. fever, sputum production, cough, dyspnea, pulmonary infiltrates on chest x-ray, etc.).

Their demographics, comorbidities, and clinical data were documented. Selected control subjects were asymptomatic blood donors, laboratory personnel, and co-workers at Al-Karak Hospital. Control participants did not have respiratory diseases or took antibiotics during the three months preceded their enrolment in this study. CAP patients and controls were divided into four age groups: 17–32 years, 33–48 years, 49–64 years, and ≥65 years.

Clinical samples (blood and nasopharyngeal swabs) from CAP patients were collected within 48 h of hospital admission. Venous blood samples (3-4 ml each) were withdrawn from all participants into gel-containing plain tubes (AMPulab$^{TM}$, Germany). Clotted blood samples were centrifuged at 3,500 rpm for 10 minutes (Combi-514R, South Korea) and the resulted sera were placed in sterile Eppendorf tubes; they were stored at -20 °C and used to detect the antibodies raised specific against each pathogen. However, nasopharyngeal specimens collected by sterile plastic-shafted Dacron-tipped swabs were immediately placed in a sterile screw-capped tubes containing 1 ml of transport medium (Vircell Microbiologists, Spain) and stored at -70°C until analysed for presence of the pathogens' DNA.

The Scientific Research Committees at the Department of Biological Sciences, Faculty of Scientific Research, and the Scientific Ethics Committee at Department of Medicine, Mutah University-Al-Karak, Jordan, approved the study (no. 201514). The research was performed in accordance with the Helsinki declaration.

**Immunoglobulin G (IgG) and immunoglobulin M (IgM) detection by ELISA**

Anti-*C. pneumoniae,* anti-*L. pneumophila,* and anti-*M. pneumoniae* IgG and IgM antibodies were detected using commercial kits (Vircell Microbiologists, Spain) and following manufacturer's instructions. Detection was performed spectrophotometric using microplate reader (BioTek ELx800, South Korea) at $A_{450/630\ nm}$. The IgG sorbent was added to each sample well in IgM antibody detection assays to avoid false positive results due to rheumatoid factor and false negative results due to an excess of IgG antibodies.

**Nucleic acid extraction and PCR assays**

DNA was extracted from collected nasopharyngeal samples using G-spin$^{TM}$ total DNA Extraction kit (iNtRON Biotechnology, Korea). DNA concentration and purity was determined spectrophotometric at $A_{260/280nm}$. The forward and reverse primers (Midland Company Inc., USA) used in the PCR assay were from literature (4) targeting the *C. pneumoniae* specific *Pst1* fragment, the macrophage infectivity potentiator gene (*mip*) of *L. pneumophila,* and the P1 cytadhesion of *M. pneumoniae.* The amplification reaction in single PCR contained 300 ng of extracted DNA, 10 µl of 2x PCR master mix solution (i-Max II, iNtRON Biotechnology, Korea), 1.5 µl of each forward and reverse primer (10 pmol/µl) and completed with nuclease-free water to 20 µl. Whilst, multiplex PCR reaction contained 500 ng of template DNA, 10 µl of 2x master mix (MultiMAX, iNtRON Biotechnology, Korea), 6 µl of primer mixture (10 pmol/µl) and completed with nuclease-free water to 20 µl; primer mixture included 1.5 µl from each forward and reverse *pst1*, *mip* and P1 primers.

The cycling conditions were as follow: denaturation for 5 minutes at 95 °C followed by 45 amplification cycles. Each cycle consisted of a denaturation step for 1 minute at 95 °C, annealing for 1 minute at 50 °C (single PCR) or 55°C (multiplex PCR), extension for 1 minute at 72 °C and a final elongation step for 7 minutes at 72 °C. In every PCR run, a negative (nuclease-free water) and positive (AMPLIRUN® *Chlamydophila pneumonia* DNA, AMPLIRUN® *Legionella pneumophila* DNA, and AMPLIRUN® *Mycoplasma pneumoniae* DNA) (Vircell Microbiologists, Spain) controls were used instead of the DNA harvested from the clinical specimens. Amplification products were analyzed by 1.5% agarose gel electrophoresis at 5 V.cm$^{-1}$ and visualized under UV.

**Criteria for etiological diagnosis**

Patients eligible for inclusion in the current study were those with symptoms and signs of fever (temperature ≥38°C), sputum production, cough, chest pain, and dyspnea. They fulfilled the definition of CAP and the presence of new pulmonary infiltrates on chest x-ray confirmed the diagnosis. Patients with nosocomial pneumonia, active tuberculosis, or discharged from hospital a month prior their current hospitalisation due to pneumonia were excluded.

According to the Vircell kits' instructions, IgG or IgM arbitrary index >11 was considered as a serological marker for seropositivity. IgG was regarded as an indication on presumptive bacterial infection, whilst detection of IgM in sera or bacterial DNA in nasopharyngeal samples were indicative of a current (acute) infection.

**Statistical analysis**

All data were analyzed using the Statistical Package for the Social Sciences software, version 14.0.1 (SPSS Inc., Chicago, USA). Calculation of the specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) was performed for ELISA-IgM and PCR tests in CAP cases with acute infection. Multiple correlation coefficients were calculated for all methods used in infection detection among CAP patients. Data were statistically analysed using Chi-squared test ($\chi 2$) and Fisher's exact test. $P$ <0.05 was considered statistically significant.

## *RESULTS*

One hundred from each of hospitalised CAP patients (53 males and 47 females, mean age 45.3±23.5 years) and asymptomatic controls (51 males and 49 females, mean age 44.5 ± 13.9 years) were included in the current study. All CAP cases have fulfilled the inclusion criteria. Insignificant statistical differences were detected between the mean ages of CAP cases and controls ($p$=0.28) as well as between individual numbers from each gender in both cohorts ($p$=0.94), indicating almost age and sex matching between controls and patients.

**Overall seroprevalence of *C. pneumoniae*, *M. pneumoniae*, and *L. pneumophila* in controls and cases**

The general prevalence of the three pathogens in CAP and control cohorts was assessed by detecting the anti-pathogen specific IgG antibodies in collected sera (Table 1). A significant 1.6 times higher prevalence of anti-*C. pneumoniae* IgG was observed in CAP cases than in control group (40% vs 25%, $p$= 0.02). The highest detection frequency of the antibody was noticed in controls age group ≥65 years (37.5%), whilst it was > 80% in CAP age groups over 48 years. Overall seroprevalence of *M. pneumoniae* IgG was almost four times higher in CAP patients compared to controls (22% vs 5%, $p$= < 0.001); it was the highest at age group (49-64 years, 30%) in CAP cases and in controls age bracket (≥65 years, 12.5%). Noticeably, anti-*L. pneumophila* IgG was not detected in both patients and controls.

**Detection rate of immunoglobulin M (IgM) in controls and CAP patients**

Anti-*C. pneumoniae* IgM was detected at a significantly higher rate (2.5 times) in CAP patients compared to the control cohort (19% vs 7%, $p$=0.01); the detection rate also increased with age in the CAP groups and reached a peak of 100% in the age bracket ≥65 years). In the control group, the highest IgM seroprevalence was reported in the age group 33-48 years (13.6%). However, an insignificant difference was noticed in anti-*M. pneumoniae* IgM between the patient and control groups (6% vs 3%, $p$=0.25); it was detected in one age group of controls (17-32 years) and in two patients' age groups (33-48 years and 49-64 years). Intriguingly, anti-*L. pneumophila* IgM was absent in just one CAP cases age group (≥65 years); its seroprevalence was statistically insignificant between the two included cohorts (11% vs 14%, $p$=0.3) (Table 2).

**Table 1.** Seroprevalence of anti-*C. pneumonia,* anti-*M. pneumoniae* and anti-*L. pneumophila* based on detection of IgG antibodies in patients and controls and their age-wise distribution.

| Age group (years) | Number positive (% positivity) | | *P** |
|---|---|---|---|
| | Controls (n=100) | Patients (n=100) | |
| ***C. pneumonia*** | | | |
| 17-32 | 12/55 (21.8) | 21/62 (33.9) | 0.11 |
| 33-48 | 6/22 (27.3) | 6/23 (26.1) | 0.5 |
| 49-64 | 4/15 (26.7) | 8/10 (80) | 0.01 |
| ≥65 | 3/8 (37.5) | 5/5 (100) | 0.04 |
| Total positive | 25 (25) | 40 (40) | 0.02 |
| ***M. pneumoniae*** | | | |
| 17-32 | 1/55 (1.8) | 14/62 (22.6) | < 0.001 |
| 33-48 | 2/22 (9.1) | 5/23 (21.7) | 0.2 |
| 49-64 | 1/15 (6.7) | 3/10 (30) | 0.16 |
| ≥65 | 1/8 (12.5) | 0/5 (0) | 0.6 |
| Total positive | 5 (5) | 22 (22) | < 0.001 |
| ***L. pneumophila*** | ND | ND | - |

* Statistically significant differences compared to the control group ($P<0.05$). ND: not detected.

**Table 2.** Age distribution of anti-*C. pneumoniae,* anti-*M. pneumoniae* and anti-*L. pneumophila* IgM antibodies and nasopharyngeal PCR positivity for determination of acute infections in CAP patients and controls.

| Age group (years) | Number positive (% positivity) | | | | | |
|---|---|---|---|---|---|---|
| | IgM | | *P** | PCR | | *P** |
| | Controls (n=100) | Patients (n=100) | | Controls (n=100) | Patients (n=100) | |
| ***C. pneumonia*** | | | | | | |
| 17-32 | 3/55 (5.4) | 9/62 (14.5) | 0.09 | 12/55 (21.8) | 23/62 (37.1) | 0.05 |
| 33-48 | 3/22 (13.6) | 3/23 (13) | 0.6 | 3/22 (13.6) | 11/23 (47.8) | 0.01 |
| 49-64 | 1/15 (6.7) | 2/10 (20) | 0.3 | 4/15 (26.7) | 6/10 (60) | 0.1 |
| ≥65 | 0/8 (0) | 5/5 (100) | < 0.001 | 1/8 (3) | 3/5 (60) | 0.1 |
| Total positive | 7 (7) | 19 (19) | 0.01 | 20 (20) | 43 (43) | < 0.001 |
| ***M. pneumoniae*** | | | | ND | ND | - |
| 17-32 | 3/55 (5.4) | 0/62 (0) | 0.1 | | | |
| 33-48 | 0/22 (0) | 5/23 (21.7) | 0.03 | | | |
| 49-64 | 0/15 (0) | 1/10 (10) | 0.4 | | | |
| ≥65 | 0/8 (0) | 0/5 (0) | 1 | | | |
| Total positive | 3 (3) | 6 (6) | 0.25 | | | |
| ***L. pneumophila*** | | | | | | |
| 17-32 | 4/55 (7.2) | 8/62 (12.9) | 0.2 | 1/55 (1.8) | 4/62 (6.5) | 0.2 |
| 33-48 | 4/22 (18.2) | 5/23 (21.7) | 0.5 | 2/22 (9.1) | 2/23 (8.7) | 0.7 |
| 49-64 | 1/15 (6.7) | 1/10 (10) | 0.6 | 1/15 (6.7) | 1/10 (10) | 0.6 |
| ≥65 | 2/8 (25) | 0/5 (0) | 0.4 | 0/8 (0) | 0/5 (0) | 1 |
| Total positive | 11 (11) | 14 (14) | 0.3 | 4 (4) | 7 (7) | 0.3 |

**Detec tion of nucleic acid by PCR for the pathogens**

Nasopharyngeal samples from enrolled subjects were tested for the presence of each pathogen's DNA as a criterion of recent infection by conventional PCR; multiplex PCR was performed on all samples to reveal concurrent detection of more than one pathogen-specific DNA in a single sample (Figure 1). Optimized PCR condition in both conventional and multiplex assays gave the same outcomes. Surprisingly, PCR failed to detect *M. pneumonia* DNA in all tested nasopharyngeal samples; it was less efficient in amplifying the DNA of positive control (well no# 4). *C. pneumoniae* DNA was detected in all age groups of controls and CAP participants; the detection rate was significantly 2.15 folds higher in CAP cases than controls

(43% vs 20%, $p< 0.001$). An insignificant difference was noticed in detection rate of *L. pneumophila* DNA between patient and control individuals (7% and 4% respectively, $p=0.3$) with absence of the bacterium nucleic acid in age group ≥65 years of both cohorts (Table 2). Overall, these data verify the results of IgM that relate between *C. pneumoniae* and CAP in Southern Jordan. Noteworthy, all nasopharyngeal samples of CAP cases and 75% of controls that were positive to *L. pneumophila* DNA were also positive to *C. pneumoniae* in multiplex PCR.

**Comparison between IgM antibody and PCR in detection of acute infection with correlation of ELISA-IgM and molecular results**

In the entire CAP patients, 19 individuals demonstrated seropositivity to anti-*C. pneumoniae* IgM and 43 nasopharyngeal samples revealed presence of *C. pneumoniae* DNA. Meanwhile, anti-*M. pneumoniae* IgM was detected in six patients with absence of its DNA in the nasopharyngeal samples; *L. pneumophila* was identified in 14 and seven CAP cases based on IgM and PCR, respectively. In controls, seven sera were positive to anti-*C. pneumoniae* IgM and 20 nasopharyngeal samples demonstrated the presence of the bacterial DNA. Furthermore, three sera were anti-*M. pneumoniae* IgM positive with PCR being less efficient in detecting bacterial DNA in the nasopharyngeal samples; anti-*L. pneumophila* IgM and bacterium nucleic acid were evident in 11 sera and four nasopharyngeal samples, respectively, indicating a cross reactivity in ELISA assay and false-positive PCR results as asymptomatic carrier of *L. pneumophila* is not recognised. Considering ELISA-IgG seropositive results in CAP patients, 45% (18/40) and 22.5% (9/40) of *C. pneumoniae* positive cases were PCR- and IgM-positive, respectively; all of *M. pneumoniae* positive persons were PCR negative and 18% (4/22) revealed positivity to IgM (Figure 2). However, in asymptomatic controls, 4% (1/25) and 48% (12/25) of individuals with seropositivity to *C. pneumoniae* IgG were positive to IgM and PCR, respectively. Remarkably, none of the controls carried *M. pneumoniae* DNA in the respiratory tract.

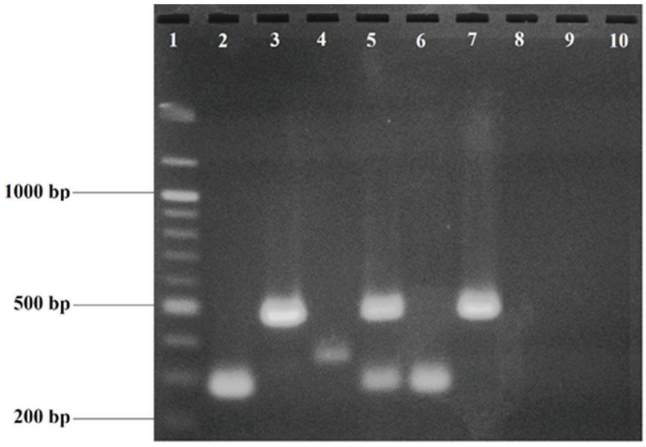


**Figure 1.** Gel-electrophoresis revealing the amplification of atypical pathogenic bacteria, single PCR for positive controls and multiplex PCR for examples from CAP cases. Lane 1: molecular size DNA ladder 100 bp, lanes 2: single PCR reaction for *Pst1* fragment of *C. pneumoniae* (283 bp), lanes 3: single PCR reaction for *mip* fragment of *L. pneumonophila* (487 bp) and lane 4: single PCR reaction for P1 fragment of *M. pneumoniae* (360 bp). Multiplex PCR of genes combination, lane 5: sample # 2; lane 6: sample # 10 and lanes 7: sample # 5. *M. pneumoniae* DNA was not detected in CAP or control samples.

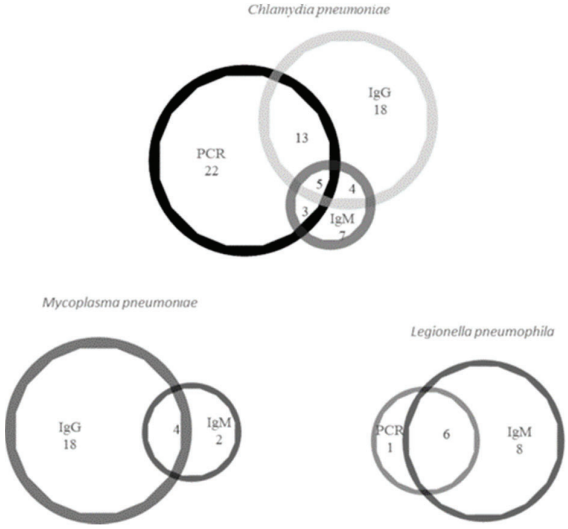


**Figure 2.** Venn diagram revealing the relation between diagnostic tests employed for detection of pneumonia infection in CAP patients.

All these data indicated a strong correlation between PCR and both ELISA-IgG and –IgM (r = 0.74 and 0.88, respectively) but a very week correlation between ELISA-IgG and –IgM (r = 0.33) in detection of infections in CAP patients. Furthermore, PPV, NPV, sensitivities, and specificities of both assays were calculated for diagnosis of acute infections using diagnosed CAP cases as the gold standard (Table 3). ELISA-IgM was less sensitive but more specific than PCR in detecting *C. pneumoniae* infections (19% vs 43% and 93% vs 80%, respectively); it has improved PPV over PCR (73.08% over 68.25%) as indicative on acute infections. PCR and IgM were highly specific to *M. pneumoniae* (97%-100%) with a very low sensitivity (0-6%); meanwhile, IgM was more sensitive than PCR in detecting *L. pneumophila* with a comparable specificity. Worth noting, PCR had high PPV than anti-*L. pneumophila* IgM as indicative on occurrence of acute infections.

**Table 3.** Sensitivity, specificity, PPV and NPV of the ELISA-IgM and PCR assays for the determination of acute infection in patients suffering from CAP due to *C. pneumoniae, M. pneumoniae* and *L. pneumophila.*

| Assay | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|
| | 95% CI* | | | |
| ***C. pneumoniae*** | | | | |
| **IgM** | 19 (11.84-28.07) | 93 (86.11-97.14) | 73.08 (54.42-86.0) | 53.45 (50.73-56.15) |
| **PCR** | 43 (33.14-53.29) | 80 (70.82-87.33) | 68.25 (57.77-77.17) | 58.39 (53.56-63.07) |
| ***M. pneumonia*** | | | | |
| **IgM** | 6 (2.23-12.60) | 97 (91.48-99.38) | 66.67 (33.97-88.61) | 50.79 (49.28-52.29) |
| **PCR** | 0 (0.00-3.62) | 100 (96.38-100.00) | NA | NA |
| ***L. pneumophila*** | | | | |
| **IgM** | 14 (7.87-22.37) | 89 (81.17-94.38) | 56 (37.79-72.72) | 50.86 (48.24-53.47) |
| **PCR** | 7 (2.86-13.89) | 96 (90.07-98.90) | 63.64 (34.59-85.28) | 50.79 (49.12-52.47) |

*Data percentage with 95% confidence interval. NA: not applicable (ignored values).

## DISCUSSION

Overall, seroprevalence of the three atypical pathogens in participating patients was 64%; involved 26% *C. pneumoniae,* 8% *M. pneumoniae,* and 30% concurrent detection of two or more pathogens in CAP patients. The general prevalence of these agents in hospitalised CAP cases worldwide was lower than our findings (40%-60%) (3,4,15-17). Based on ELISA-IgG, the prevalence rate of *C. pneumoniae* (40%) was lower than than reported in adult patients from central Jordan and other countries (50%-70%) (11,18,19); but it was analogous to the recently published infection frequency in patients from Southern Jordan (6). Whilst the herein documented prevalence rate of *M. pneumoniae* (22%) in patients was in accordance with worldwide reported rates (3%-40%) (17,20-23), but higher than that stated for patients of central Jordan (12). Intriguingly,
*L. pneumophila* has been detected in 1%-16% of atypical pneumonia cases in different countries (17,22-24). Relatedly, reports from several countries demonstrated detection of anti-*M. pneumoniae* and anti-*L. pneumophila* IgG in healthy adults at rates (30%-86%) and (6-20%), respectively (6,8,11,16,25,26); frequencies that were relatively higher than our findings (Table 1). A significant 1.6 folds ($p$=0.02) and 4-folds ($p$<0.001) increase in the overall anti-*C. pneumoniae* and anti-*M. pneumoniae* IgG positivity in CAP cohort over asymptomatic controls indicated a possible association between these two pathogens and CAP in individuals of Southern Jordan.

Diagnosis of acute infections due to atypical agents is based on detecting a fourfold increase in IgG titer between acute-phase and convalescent serum, IgG titer ≥1:512 and/or IgM titer ≥1:16 in a single serum were adopted as serological criteria worldwide including population studies in Jordan (1,6,9,11,27-29). PCR is considered a second method to detect acute infections. Production of antibodies may delay in some infections and obtaining a paired serum requires 3-4 weeks (2); the level of IgM may not increase in response to re-infections in adults (29,30) and an acute increase in IgG titers in patients could be due to past exposure and not indicative of acute infection (31). These criteria are important in retrospective studies but clinically inconvenient when rapid detection of the etiological agent is compulsory and initiation of proper treatment protocols in a timely manner is demanding.

Assessed by ELISA-IgM as a tool for acute infection detection, the prevalence rate of *C. pneumoniae* was significantly 2.8 folds higher in CAP patients than controls (19% vs 7%, $p$= 0.01); whilst insignificant differences were noticed between detection rates of *M. pneumoniae* and *L. pneumophila* in CAP and control cohorts (6% and 14% versus 3% and 11%, respectively; Table 2). The frequency of *C. pneumoniae* detection reported herein was consonant with the epidemiological data from other countries which ranged 6%-22% (16,30,32); it was lower than previously reported results in uncontrolled and case-control studies in Jordan, which were 23% and 27.1%, respectively (6,9). Meanwhile, *M. pneumoniae* was detected in a frequency analogous with those documented earlier in several studies counting results from central and north Jordan (4%-21%) (10,12,16,22,24). Furthermore, the detection rate of anti-*L. pneumophila* IgM in CAP cases coincided with reports from India (11%-15%) (24,33) but was higher than formerly reported results in different epidemiological studies, including north Jordan (2%-9%) (2,10,23).

The detection rate of *C. pneumoniae* DNA in nasopharyngeal samples was significantly higher in CAP than in control participants (43% vs 20%, $p$< 0.001). Meanwhile, an insignificant difference in detection rate of *L. pneumophila* DNA was observed between patient and control cohorts (7% vs 4%, $p$= 0.3); *M. pneumoniae* was not detected in either CAP or control subjects by PCR (Table 2). Detection frequency of *C. pneumoniae* DNA by PCR in CAP adults is in agreement with recently published data from Southern Jordan (6) but higher than reported frequencies in respiratory tract infected patients worldwide (2%-23%) (15,17,34). Moreover, *L. pneumophila* DNA was detected in an analogous frequency to those reported in other studies (<1%-18%) (15,17,35); noteworthy, its nucleic acid was found in 53.8% of patients in uncontrolled study from Sudan using multiplex PCR (34).

The insignificant difference in detection frequency of anti-*M. pneumoniae* IgM between controls and CAP cases might be attributed either to an early sampling before production of the IgM in CAP patients as well as that the titer of IgM doesn't increase in adults in response to re-infections, or its low prevalence rate in populations from Southern Jordan. The absence of *M. pneumoniae* DNA in nasopharyngeal samples of seropositive CAP individuals might be due to weak efficiency of PCR in detecting *M. pneumoniae* DNA, an undetectable bacterial load, and pathogen shedding from respiratory tracts due to previous antibiotic treatments. Thus, the role of this bacterial agent in CAP occurrence was excluded during the time of this study, particularly as *M. pneumoniae* infection occurs in cyclic outbreaks every 3-7 years (5), its occurrence is rare in Jordanian CAP individuals and it is more common in children than adults (12,14); though, *M. pneumoniae* DNA has been identified in 1%-25% CAP cases worldwide (15,17,21,23,24,34). Moreover, absence of IgG response to *L. pneumophila* with insignificant detection rates of anti-*L. pneumophila* IgM and PCR in both CAP and control cohorts could be due to the insensitivity of used tests in detecting *L. pneumophila* infections, especially as asymptomatic carriage is not fully documented; cross reactivity in IgM detection test; or low prevalence of this pathogen in the studied population. Nevertheless, it was documented that continuous exposure of individuals to environmental sources contaminated with *L. pneumophila* might triggers immune response and causes elevation in Ig titers in 1%-30% of healthy individuals without clinical symptoms (2,36).

PCR assay was more sensitive in the diagnosis of *C. pneumoniae* acute infection than ELISA-IgM (43% vs 19%) but was less specific (80% vs 93%) with comparable diagnostic utility (PPV and NPV values). Conversely, ELISA-IgM was slightly more sensitive than PCR in detecting *M. pneumoniae* (6% vs 0%) and *L. pneumophila* (14% vs 7%) acute infections with comparable specificities but with lower accuracy in diagnostic utility than PCR in case of *L. pneumophila* (lower PPV value). Low sensitivity of IgM with a single serum may attributed to its delayed production in some infections and weak response at reinfections in adults. Therefore, if paired sera were obtained, IgM sensitivity in detecting acute infections might have been improved. Detection of bacterial DNA in serological negative patients was predicted either due to early sampling time before immune response was initiated or because of pathogen persistence. Whilst PCR negative results in serological positive cases could be due to past infections, an undetectable bacterial load, previous antibiotic treatments, pathogen shedding from respiratory tracts, presence of PCR inhibitors, or assay technical problems associated with unsuccessful sample processing.

Overall, concurrent infections were detected in 30% of all CAP patients by serology; *C. pneumoniae* and *L. pneumophila* were detected in 14 patients (14%), *C. pneumoniae* and *M. pneumoniae* or *M. pneumoniae* and *L. pneumophila* were identified in six patients (6%) and the three pathogens were found in four CAP individuals (4%). It was possible to identify the DNA of both *C. pneumoniae* and *L. pneumophila* in seven patients from the entire CAP cohort (7%); all those that were PCR positive to *L. pneumophila* demonstrated positivity to *P. pneumoniae*. The presence of one pathogen might prime the coinfection by the other pathogen; that explains the reported 25%-48% concurrent infections in CAP cases by different studies (37,38).

Study limitations include reliance on a single serum without following it with a paired serum (seroconversion), incapability of determining the endpoint titer of antibodies against each atypical pathogen and using nasopharyngeal samples for pathogens' DNA detection instead of sputum might influence the sensitivity of PCR. The study depended on blood samples for detection of *L. pneumophila* without incorporating it with urine antigen test, and there is no agreed evaluation on diagnostic accuracy of ELISA. Obtaining second convalescent sera from patients during hospital stay was impractical and challenging; and delay in seroconversion results may not be useful in acute infection diagnosis or during outbreaks. Nevertheless, serodiagnosis of acute infection based on a single serum sample seems to be more realistic in clinic facilities as the rapid decision for empirical treatment of an infection is based often on a single serum sample.

Based on the current findings, *C. pneumoniae* may play a causal role in CAP infection in population of Sothern Jordan; absence of bacterial DNA in respiratory samples from some of the *C. pneumoniae* seropositive CAP patients indicated past infections. Both *M. pneumoniae* and *L. pneumophila* have low prevalence rates in studied population. The strong correlation between PCR assay and IgM in detecting acute infections points out that PCR could improve the clinical utility of serological methods, with caution in single samples. Simultaneous use of ELISA and PCR in clinical facilities might allow rapidity and improvement in diagnosis of acute respiratory infections and precision in identifying the etiological pathogen that may play role in establishing CAP infections when convalescent samples are unattainable.

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## AUTHOR INFORMATION

Wael A Al-Zereini, PhD, Associate Professor

Department of Biological Sciences, Faculty of Science, Mutah University, Al-Karak 61710, Jordan

**Correspondence.** Dr. W Al-Zereini.
E-mail: wzereini@mutah.edu.jo

## REFERENCES

1. Arnold FW, Summersgill JT, LaJoie AS, et al. A worldwide perspective of atypical pathogens in community-acquired pneumonia. *Am J Respir Crit Care Med* 2007; 175(10): 1086-1093.
2. Mercante JW, Winchell JM. Current and emerging Legionella diagnostics for laboratory and outbreak investigations. *Clin Microbiol Rev* 2015 1;28(1):95-133.
3. Miyashita N, Saito A, Kohno S, et al. Multiplex PCR for the simultaneous detection of Chlamydia pneumoniae, Mycoplasma pneumoniae and Legionella pneumophila in community-acquired pneumonia. *Respir Med* 2004; 98(6): 542-550.
4. McDonough EA, Barrozo CP, Russell KL, Metzgar D. A multiplex PCR for detection of Mycoplasma pneumoniae, Chlamydophila pneumoniae, Legionella pneumophila, and Bordetella pertussis in clinical specimens. *Mol Cell Probes* 2005; 19(5): 314-322.
5. Atkinson TP, Balish MF, Waites KB. Epidemiology, clinical manifestations, pathogenesis and laboratory detection of Mycoplasma pneumoniae infections. *FEMS Microbiol Rev* 2008; 32(6): 956-973.
6. Al-Hajaya TS, Al-Zereini WA, Al-Younes HM. Chlamydia pneumoniae infection in patients hospitalised for community-acquired pneumonia in Southern Jordan. *Indian J Med Microbiol* 2020; 38(3-4): 338-343.
7. Ginevra C, Barranger C, Ros A, et al. Development and evaluation of Chlamylege, a new commercial test allowing simultaneous detection and identification of Legionella, Chlamydophila pneumoniae, and Mycoplasma pneumoniae in clinical respiratory specimens by multiplex PCR. *J Clin Microbiol* 2005; 43(7): 3247-3254.

8. Al-Younes HM. Seroprevalence of Chlamydia pneumoniae in male adults in Jordan. *Dirasat* 2009; 36: 1-6.
9. Al-Younes HM. High prevalence of Chlamydia pneumoniae infection in an asymptomatic Jordanian population. *J Microbiol Immunol Infect* 2014; 47(5): 412-417.
10. Al-Ali MK, Batchoun RG, Al-Nour TM. Etiology of community-acquired pneumonia in hospitalized patients in Jordan. *Saudi Med J* 2006; 27(6): 813-816.
11. Al-Aydie SN, Obeidat NM, Al-Younes HM. Role of Chlamydia pneumoniae in community-acquired pneumonia in hospitalized Jordanian adults. *J Infect Dev Ctries* 2016; 10(3): 227-236.
12. Obeidat NM, Qatouseh LF, Shehabi AA. Rare occurrence of Mycoplasma pneumoniae infection among Jordanian adults with respiratory tract infections. *Microb Ecol Health Dis* 2005; 17(4): 216-218.
13. Kaplan NM, Dove W, Abd-Eldayem SA, et al. Molecular epidemiology and disease severity of respiratory syncytial virus in relation to other potential pathogens in children hospitalized with acute respiratory infection in Jordan. *J Med Virol* 2008; 80(1): 168-174.
14. Jayyosi MG, Khuri-Bulos NA, Halasa NB, et al Rare occurrence of Bordetella pertussis among Jordanian children younger than two years old with respiratory tract infections. *J Pediatr Infect Dis* 2015; 10(2): 53-56.
15. Bokhary H, El-Gamal E, El-Fiky S. Detection of Legionella pneumophila, Mycoplasma pneumoniae and Chlamydophila pneumoniae as aetiological agents of community–acquired pneumonia in Holy Makkah, KSA. *Egypt J Med Microbiol* 2006; 15(2): 437-447.
16. Qu J, Cao B. Research progress in atypical pathogens of community acquired pneumonia. *Community Acquir Infect* 2014; 1(1): 11-14.
17. Goodarzi NN, Pourmand MR, Rajabpour M, et al. Frequency of Mycoplasma pneumoniae, Legionella pneumophila and Chlamydia spp. among patients with atypical pneumonia in Tehran. *New Microbes New Infect* 2020; 37: 100744.
18. Chedid MB, Chedid MF, Ilha DO, et al. Community-acquired pneumonia by *Chlamydophila pneumoniae*: a clinical and incidence study in Brazil. *Braz J Infect Dis* 2007; 11(1): 75-82.
19. Charles PG, Whitby M, Fuller AJ, et al. The etiology of community-acquired pneumonia in Australia: why penicillin plus doxycycline or a macrolide is the most appropriate therapy. *Clin Infect Dis* 2008; 46(10): 1513-1521.
20. Phares CR, Wangroongsarb P, Chantra S, et al. Epidemiology of severe pneumonia caused by Legionella longbeachae, Mycoplasma pneumoniae, and Chlamydia pneumoniae: 1-year, population-based surveillance for severe pneumonia in Thailand. *Clin Infect Dis* 2007; 45 (12): e147-155.
21. Martínez MA, Ruiz M, Zunino E, et al. Detection of Mycoplasma pneumoniae in adult community-acquired pneumonia by PCR and serology. *J Med Microbiol* 2008; 57(12): 1491-1495.
22. Ngeow YF, Suwanjutha S, Chantarojanasriri T, et al. An Asian study on the prevalence of atypical respiratory pathogens in community-acquired pneumonia. *Int J Infect Dis* 2005; 9(3): 144-153.
23. Berebichez-Fridman R, Blachman-Braun R, Azrad-Daniel S, et al. Atypical pneumonias caused by Legionella pneumophila, Chlamydophila pneumoniae and Mycoplasma pneumonia. *Rev Med Hosp Gen (Mex)* 2015; 78(4): 188-195.
24. Chaudhry R, Valavane A, Sreenath K, et al. Detection of Mycoplasma pneumoniae and Legionella pneumophila in patients having community-acquired pneumonia: A multicentric study from New Delhi, India. *Am J Trop Med Hyg* 2017; 97(6): 1710-1716.
25. Hyman CL, Roblin PM, Gaydos CA, et al. Prevalence of asymptomatic nasopharyngeal carriage of Chlamydia pneumoniae in subjectively healthy adults: assessment by polymerase chain reaction-enzyme immunoassay and culture. *Clin Infect Dis* 1995; 20(5): 1174-1178.
26. Waites KB, Talkington DF. Mycoplasma pneumoniae and its role as a human pathogen. *Clin Microbiol Rev* 2004; 17 (4): 697-728.
27. Dowell SF, Peeling RW, Boman J, et al. Standardizing Chlamydia pneumoniae assays: recommendations from the centers for disease control and prevention (USA) and the laboratory centre for disease control (Canada). *Clin Infect Dis* 2001; 33(4): 492-503.
28. Puljiz I, Kuzman I, Dakovic-Rode O, et al. Chlamydia pneumoniae and Mycoplasma pneumoniae pneumonia: comparison of clinical, epidemiological characteristics and laboratory profiles. *Epidemiol Infect* 2006; 134(3): 548-555.
29. Kumar S, Hammerschlag MR. Acute respiratory infection due to Chlamydia pneumoniae: current status of diagnostic methods. *Clin Infect Dis* 2007; 44(4): 568-576.
30. Lin LJ, Chang FC, Chi H, et al. The diagnostic value of serological studies in pediatric patients with acute Mycoplasma pneumoniae infection. *J Microbiol Immunol Infect* 2020; 53(2): 351-356.
31. Cunha, BA. The atypical pneumonias: clinical diagnosis and importance. *Clin Microbiol Infect* 2006; 12: 12-24.
32. Miyashita N, Obase Y, Fukuda M, et al. Evaluation of serological tests detecting Chlamydophila pneumoniae-specific immunoglobulin M antibody. *Intern Med* 2006; 45 (20): 1127-1131.
33. Sowjanya G, Amar CS, Swetha G, et al. Prevalence of Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila in LRTI patients in a tertiary care center, Karimnagar. *Int J Curr Microbiol Appl Sci* 2019; 8 (5): 1551- 1556.
34. Mustafa MO, Enan KA, El Hussein AR, Elkhidir IM. Molecular detection of Legionella pneumophila, Mycoplasma pneumoniae and Chlamydia pneumoniae among Sudanese patients with acute respiratory infections in Khartoum State, Sudan. *Ann Microbiol Infect Dis* 2019; 2(3): 7-11.
35. Miyashita N, Higa F, Aoki Y, et al. Clinical presentation of Legionella pneumonia: evaluation of clinical scoring systems and therapeutic efficacy. *J Infect Chemother* 2017; 23(11): 727-732.
36. Graham FF, Hales S, White PS, Baker MG. Review global seroprevalence of legionellosis-A systematic review and meta-analysis. *Sci Rep* 2020; 10(1): 1-11.
37. Plouffe JF. Importance of atypical pathogens of community-acquired pneumonia. *Clin Infect Dis* 2000; 31 (Supplement 2): S35-S39.
38. Lochindarat S, Suwanjutha S, Prapphal N, et al. Mycoplasma pneumoniae and Chlamydophila pneumoniae in children with community-acquired pneumonia in Thailand. *Int J Tuberc Lung Dis* 2007; 11(7): 814-819.