

Frequency of *Mycoplasma pneumoniae*, *Legionella pneumophila* and *Chlamydia* spp. among patients with atypical pneumonia in Tehran

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Abstract

Mycoplasma pneumoniae, *Legionella pneumophila* and *Chlamydia pneumoniae* are the most common bacterial agents, which account for 15–40%, 2–15% and 5–10% of atypical community-acquired pneumonia (CAP) respectively. These agents are mostly associated with infection in the outpatient setting. The aim of this study was to evaluate the frequency of these pathogens among patients with CAP attending outpatient clinics in Tehran. A cross-sectional study was carried out of 150 patients attending to educational hospitals in Tehran with CAP. *M. pneumoniae*, *L. pneumophila* and *Chlamydia* spp. were detected by PCR assay, targeting the *PI* adhesion gene, macrophage infectivity potentiator (*mip*) gene and 16S rRNA gene respectively from throat swabs obtained from each patient. A total of 86 (57.3%) of 150 patients were women; median age was 50 years (interquartile range, 35–65 years). *M. pneumoniae*, *L. pneumophila* and *Chlamydia* spp. were detected in 37 (24.7%), 25 (16.7%) and 11 (7.3%) patients respectively; of these, 66 patients (44%) were infected at least by one of these three pathogens. The frequency of *L. pneumophila* was significantly higher among patients over 60 years old (p 0.03). Coinfection was detected in seven patients (4.7%); six were infected by *M. pneumoniae* and *L. pneumophila*, and only one was infected by *L. pneumophila* and *Chlamydia* spp. *M. pneumoniae* was the most prevalent agent of atypical CAP, and *L. pneumophila* was more likely to infect elderly rather than younger people. Further studies on the prevalence of CAP and its aetiological agents are needed to improve the diagnosis and treatment of CAP patients.

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Keywords: Atypical pneumonia, *Chlamydia* spp., *Legionella pneumophila*, *Mycoplasma pneumoniae*, PCR

Original Submission: 23 December 2019; **Revised Submission:** 2 August 2020; **Accepted:** 11 August 2020

Article published online: 14 August 2020

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Introduction

Pneumonia is still a common cause of hospitalization with high mortality. Current clinical guidelines have categorized pneumonia into four types: community acquired (CAP), healthcare associated, hospital acquired [1] and ventilator associated [2]. Among these, CAP is identified as infection in a patient with no recent contact with the healthcare system [3]. *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila* are the most common bacterial agents of atypical CAP [4].

M. pneumoniae and *L. pneumophila* account for 15–40% and 2–15% of atypical CAP in Iran respectively [5,6]. *M. pneumoniae* and *C. pneumoniae* are mostly associated with infection in the outpatient setting [7].

M. pneumoniae causes infections in both the upper and lower respiratory tracts. It occurs in all age groups, especially school-age children and young adults [6]. Symptoms of this infection are usually mild, but in some cases it may lead to hospitalization and even death [8]. Moreover, in some cases severe extrapulmonary complications such as neurologic diseases and haemolytic anaemia may occur [9].

C. pneumoniae is more prevalent in children, but cases of serious infection in adults have also been reported [10]. A previous study demonstrated a significant association between the lung cancer and past *C. pneumoniae* infection [11] as well as an association between this infection and asthma [12,13].

L. pneumophila mainly affects elderly people and more often men than women. This bacterium is rarely differentiated from other atypical pneumonia because it presents similar clinical signs and symptoms [3,10].

The prevalence of these pathogens is usually underestimated as a result of its complex and difficult identification methods [14]. The conventional methods to diagnose these bacteria include culture, rapid antigen testing, serologic methods and molecular techniques [15–17].

M. pneumoniae and *L. pneumophila* are also two slow-growing bacteria which require complex nutrients for cultivation. Because these bacteria are rarely isolated from specimens, they are neglected in clinical laboratories [7]. In addition to the long incubation time needed to successfully deploy the culture method, *M. pneumoniae* colonies on agar cannot be differentiated from other mycoplasmas solely by morphology, and further phenotypic tests are required [16].

The reference standard of *C. pneumoniae* diagnosis by micro immunofluorescence is reported to be time consuming, and it lacks specificity and sensitivity. Serologic kits are commercially available, but these methods also lack specificity and paired sera specimens are required, so they are not optimal for treatment decisions [15,18].

Molecular methods such as PCR are commercially available with high sensitivity and specificity. This method facilitates rapid detection with higher throughput, and results can be obtained in time to guide treatment decisions. Several PCR assays have been developed and have indicated even better sensitivity and specificity than those that use conventional microbiologic methods [19,20].

The aim of this study was to evaluate the frequency of the three most common causes of atypical pneumonia among patients with CAP attending outpatient clinics in Tehran.

Materials and methods

Patient involvement

This study was conducted of patients suspected to have atypical pneumonia who were referred to educational hospitals in Tehran from January to June 2019. The study protocol was

approved by the medical ethics committee of Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1397.126), and written informed consent was obtained from all the patients.

Patients were enrolled onto this study according to the physician's decision taking into account the following: relevant results of clinical examination, abnormal breathing sounds, chest radiography, clinical signs and symptoms (nonproductive cough, headache, chest pain, dyspnoea, sore throat, fever or hypothermia, cervical adenopathy, fatigue and myalgia). All patients with a history of hospital-acquired pneumonia and chronic diseases such as lung transplantation, cancer, cystic fibrosis, bronchitis and tuberculosis were excluded.

Specimen collection

Patients were sampled via throat swabbing with a sterile Dacron swab (Deltalab, Barcelona, Spain), which was placed in a tube containing 2 mL phosphate-buffered saline and transferred to the laboratory in cold packs at 4°C within 2 hours of collection. DNA extraction was carried out immediately when the samples arrived.

DNA extraction

The swab specimens were vortexed for 1 minute and the swabs discarded. Genomic DNA was extracted from 1 mL of each sample using the FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech, Taiwan) following the manufacturer's instructions. DNA was eluted in a final volume of 50 µL, and aliquots were stored at –20°C before performing the PCR assay.

Primer selection

Three sets of primers targeting *PI*, macrophage infectivity potentiator (*mip*) and 16S rRNA were selected to identify *M. pneumoniae*, *L. pneumophila* and *Chlamydia* spp. respectively. The specificity and sensitivity of all three sets of primers have been evaluated and approved in previous studies (Table 1) [8,21,22].

PCR assay

The PCR technique for detecting *M. pneumoniae* was performed with the Primus 96 Advanced Thermocycler (Peqlab

TABLE 1. Primers used for organism detection

Organism	Target gene	Oligonucleotide sequence (5'–3')	Product size (bp)	Reference
<i>Mycoplasma pneumoniae</i>	<i>PI</i>	F: AAAGGAAGCTGACTCCGACA R: TGGCCTTGCGCTACTAAGTT	450	[21]
<i>Legionella pneumophila</i>	<i>Mip</i>	F: CAATGGCTGCAACCGATGC R: GGGATAACTTGTGAAACCTG	487	[8]
<i>Chlamydia</i> spp.	16S rRNA	F: GCCTACCGGTTACCAAC R: GGCGCAATGATTCTCGAT	230	[22]

Biotechnology, Erlangen, Germany) as follows: the 25 μ L reaction mixture contained 3 μ L DNA, 0.5 μ L (10 pmol) of each primer and 12.5 μ L Master Mix (Ampliqon, Odense, Denmark), composed of 1.5 mM MgCl₂, 0.2% Tween 20, 0.4 mM dNTPs, 0.05 U/ μ L Ampliqon Taq DNA polymerase and 8.5 μ L distilled water. Cycling conditions were as follows: predenaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at 58°C for 40 seconds and extension at 72°C for 45 seconds, then final extension at 72°C for 5 minutes.

PCR technique for detection of *L. pneumophila* was performed as follows: the 25 μ L reaction mixture contained 3 μ L DNA, 2 μ L (10 pmol) of each primer, 12.5 μ L Master Mix (Ampliqon) and 5.5 μ L distilled water. Cycling conditions were as follows: predenaturation at 95°C for 7 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 60 seconds and extension at 72°C for 60 seconds, with final extension at 72°C for 7 minutes.

PCR technique for detection of *Chlamydia* spp. was performed as follows: the 25 μ L reaction mixture contained 3 μ L DNA, 2.5 μ L (10 pmol) of forward primer and 1.25 μ L of reverse primer (10 pmol), 12.5 μ L Master Mix (Ampliqon) and 5.75 μ L distilled water. Cycling conditions were as follows: predenaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds and extension at 72°C for 45 seconds, then final extension at 72°C for 3 minutes.

M. pneumoniae ATCC 29342 and *L. pneumophila* ATCC 33152 were used as positive controls. PCR was performed on one known *C. pneumoniae* isolate using 16S rRNA primers (Table 1). The PCR product was sequenced and BLASTed via NCBI (National Center for Biotechnology Information Basic Local Alignment Search Tool; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and used as positive control for detection of *Chlamydia* spp.

Agarose gel electrophoresis was performed as follows: 5 μ L of PCR product was thoroughly mixed with 1 μ L of FluoroDye DNA Fluorescent Loading Dye (green, 6 ×), then electrophoresed for 90 minutes at 120 V on a 1.5% agarose gel in 0.5 × Tris–borate–EDTA buffer.

Statistical analysis

Analysis of demographic data was carried out by SPSS 24 software (IBM, Armonk, NY, USA). Categorical variables were compared by the Pearson or Fisher exact tests, as appropriate. Univariate analysis was performed to determine any association between variables and positivity of the three agents. Crude odds ratio and 95% confidence interval were calculated. Variables with $p \leq 0.2$ in univariate analysis were included in a multivariate logistic regression model. Adjusted odds ratio and

its 95% confidence interval were determined, and values of $p \leq 0.05$ were considered to be statistically significant.

Results

A total of 150 patients were enrolled onto the study, consisting of 86 women (57.3%) and 64 men (42.3%), with a median age of 50 years old (interquartile range, 35–65 years). Demographic information and clinical manifestations of all the participants are listed in Table 2.

Positive PCR results were reported in 66 patients (44%), of whom seven (4.7%) had a coinfection (six patients by *M. pneumoniae* and *L. pneumophila* and one by *L. pneumophila* and *Chlamydia* spp.). *M. pneumoniae*, *L. pneumophila* and *Chlamydia* spp. were detected in 37 (24.7%), 25 (16.7%) and 11 (7.3%) patients respectively.

There was no statistically significant difference in the incidence of any of the above infections in both male and female subjects (Tables 3–5). The prevalence of *M. pneumoniae* and *Chlamydia* spp. infections was statistically insignificant with respect to age groups (Tables 3 and 5). Overall, the prevalence of atypical pneumonia was higher among people over the age of 60 (Table 2). Of note is a significant difference in the frequency of *L. pneumophila* among people over 60 years old compared to other age groups (Table 6).

Clinical signs and symptoms of patients were statistically analysed. Dyspnoea was the most common sign in our studied population and consequently was the most prevalent among infected patients. Chest pain, sputum production and non-productive cough were other common signs among patients with positive PCR test results (Tables 3–5). None of the patients infected with *Chlamydia* spp. complained of vomiting (Table 5).

TABLE 2. Demographic and clinical characteristics of 150 patients

Characteristic	N (%)
Age group	
0–18 years	13 (8.7)
19–44 years	43 (28.7)
45–59 years	40 (26.7)
>60 years	54 (36)
Total	150 (100)
Sex	
Female	86 (57.3)
Male	64 (42.7)
Symptom	
Fever	31 (20.7)
Headache	33 (22)
Vomit	14 (9.3)
Sore throat	16 (10.7)
Dyspnoea	99 (66)
Chest pain	71 (47.3)
Sputum production	77 (51.3)
Nonproductive cough	67 (44.7)
Fatigue	53 (35.3)

TABLE 3. Demographic and clinical characteristics of 37 patients infected with *Mycoplasma pneumoniae*

Characteristic	N (%)	95% CI	p
Age group			0.56
0–18 years	4 (10.8)	Ref.	
19–44 years	10 (27)	(0.17–2.69)	
45–59 years	7 (18.9)	(0.11–2.00)	
>60 years	16 (43.2)	(0.25–3.53)	
Total	37 (100)	—	
Sex		0.36–1.64	0.57
Female	23 (62.2)	—	
Male	14 (37.8)	—	
Symptom			
Fever	13 (35.1)	1.23–6.63	0.02*
Headache	7 (18.9)	0.31–1.98	0.66
Vomit	3 (8.1)	0.21–3.11	1
Sore throat	2 (5.4)	0.09–1.87	0.36
Dyspnoea	28 (75.7)	0.79–4.27	0.17**
Chest pain	19 (51.4)	0.59–2.61	0.7
Sputum production	16 (43.2)	0.31–1.37	0.34
Nonproductive cough	17 (45.9)	0.51–2.26	1
Fatigue	12 (32.4)	0.38–1.85	0.7

*p < 0.05; **p ≤ 0.2.

Significant associations were observed between fever and *M. pneumoniae* (p 0.01) and *L. pneumophila* (p 0.02) infection (Table 6).

Discussion

We evaluated the frequency of *M. pneumoniae*, *L. pneumophila* and *Chlamydia* spp. in patients with atypical CAP. Our results demonstrated that 44% of the patients in our studied population were infected by at least one of these atypical pathogens. There are significant regional variations in the atypical aetiology of CAP. In China it has been reported that the atypical agents are responsible for 20% to 40% of CAP cases; however, in this review article, serologic methods are mainly used, which has

TABLE 4. Demographic and clinical characteristics of 25 patients infected with *Legionella pneumophila*

Characteristic	N (%)	95% CI	p
Age group			0.003*
0–18 years	1 (4)	Ref.	
19–44 years	2 (8)	0.05–7.03	
45–59 years	5 (20)	0.18–16.18	
>60 years	17 (68)	0.66–45.9	
Total	25 (100)	—	
Sex		0.45–2.54	1
Female	14 (56)	—	
Male	11 (44)	—	
Symptom			
Fever	8 (32)	0.81–5.42	0.17**
Headache	4 (16)	0.2–1.99	0.6
Vomit	3 (12)	0.36–5.48	0.7
Sore throat	1 (4)	0.04–2.43	0.47
Dyspnoea	18 (72)	0.54–3.6	0.65
Chest pain	11 (44)	0.36–2.02	0.83
Sputum production	13 (52)	0.44–2.44	1
Nonproductive cough	12 (48)	0.5–2.77	0.83
Fatigue	10 (40)	0.53–3.07	0.65

*p < 0.05; **p ≤ 0.2.

TABLE 5. Demographic and clinical characteristics of 11 patients infected with *Chlamydia* spp.

Characteristic	N (%)	95% CI	p
Age group			0.97
0–18 years	1 (9.1)	Ref.	
19–44 years	3 (27.3)	0.08–9.47	
45–59 years	2 (18.2)	0.05–7.6	
>60 years	5 (45.5)	0.13–11.48	
Total	11 (100)	—	
Sex		0.49–5.75	0.53
Female	5 (45.5)	—	
Male	6 (54.5)	—	
Symptom			
Fever	0	0.71–0.85	0.12**
Headache	5 (45.5)	0.94–11.61	0.65
Vomit	0	0.85–0.95	0.6
Sore throat	2 (18.2)	0.39–10.11	0.33
Dyspnoea	8 (72.7)	0.36–5.55	0.75
Chest pain	7 (63.6)	0.57–7.32	0.35
Sputum production	7 (63.6)	0.48–6.16	0.53
Nonproductive cough	5 (45.5)	0.3–3.55	1
Fatigue	4 (36.4)	0.29–3.76	1

*p < 0.05; **p ≤ 0.2.

lower sensitivity and specificity [23]. Meanwhile, in Egypt, among 400 patients with CAP, only 12 patients (3%) were infected with atypical pathogens [24]. Although the progression of atypical pneumonia may be mild, in some cases it can lead to hospitalization. For example, in Turkey only 6% of patients hospitalized for CAP were infected with atypical agents, while in the Netherlands these pathogens were detected in 20.7% of the patients [25,26]. To our knowledge, only limited comprehensive studies have been conducted to evaluate the role of atypical agents in CAP patients in Iran. Our research is thus one of the first studies in this field.

Among bacterial pathogens, *M. pneumoniae* is known to be the most prevalent atypical agent of pneumonia. In the present study the frequency of *M. pneumoniae* was highest among other bacteria, a finding that corresponded to the findings of two recent studies in Tehran. The first one was conducted by Arfaatabar et al. [27], who reported a high frequency (26.4%) of this agent in their studied population. The second one was done by our own group and found the rate of *M. pneumoniae* to be 25.2% [6]. The prevalence of *M. pneumoniae* varies in different studies of Iran [28], which may be influenced by seasonal differences, target group, outbreaks and diagnostic methods. The prevalence of *M. pneumoniae* in some Asian countries is much higher than in Europe and the United States. Among US CAP patients only 4% were positive for *M. pneumoniae*, while in China this pathogen was the most common agent among adults with CAP [1,23]. Also, in other Asian countries such as Saudi Arabia and India high frequencies of *M. pneumoniae* were previously reported [29,30].

L. pneumophila was detected in 16.7% of patients in our studied population. The water supply system provides crucial potential sources of infection. It has been reported that the

TABLE 6. Factors associated with positivity of *Mycoplasma pneumoniae* and *Legionella pneumophila*

Factor	Univariate analysis			Multivariate analysis		
	COR	95% CI	p	AOR	95% CI	p
Factors associated with positivity of <i>M. pneumoniae</i>						
Fever	2.86	1.23–6.63	0.01	3.04	1.29–7.17	0.01*
Dyspnoea	0.21	0.79–4.27	0.16	2.00	0.84–4.77	0.12
Factors associated with positivity of <i>L. pneumophila</i>						
Fever	2.09	0.8–5.42	0.13	4.07	1.27–13.04	0.02
Age group						
0–18 years (Ref)						
19–44 years	0.58	0.05–7.02	0.67	1.08	0.08–13.76	0.95
45–59 years	1.71	0.18–16.18	0.64	4.25	1.27–13.03	0.24
>60 years	5.51	0.66–45.9	0.11	13.35	1.35–132.5	0.03

AOR, adjusted odds ratio; CI, confidence interval; COR, crude odds ratio.
*p < 0.05.

prevalence of *Legionella* spp. are high in water resources in Iran, and that the most prevalent is *L. pneumophila* [31]. It has been estimated that the pooled prevalence of this pathogen in clinical samples in Iran is 9.6%, ranging from 0.4% to 22.1% [5]. The numbers show that this agent can play an important role in patients with respiratory issues in Iran. Therefore, an effective diagnostic method we can use to treat patients with compatible symptoms would be helpful. Unlike *M. pneumoniae*, our study showed a much higher frequency of *L. pneumophila* than other Asian countries. For comparison, only 3.65% of patients with CAP were infected by this bacterium in China [23], and the rate of this pathogen was only 2.4% in Korea, which is not in line with our findings [32]. Our assumption is that the contaminated water source and inefficient diagnostic and controlling strategies might have resulted in this high prevalence.

Few studies have investigated the frequency of *Chlamydia* spp. in patients with CAP in Iran. However, the role of this pathogen in patients with pneumonia is still unclear in our country. In our research 11 patients (7.3%) were infected with *Chlamydia* spp. According to the questionnaires they filled out, no patient had a history of close contact with birds. Considering the site of the sample collection, as well as considering the fact that respiratory psittacosis is a rare disease, we assume that all of our positive cases were caused by *C. pneumoniae*. Previous studies that investigated the frequency of this bacterium in Iran are either too old or the methodology is inadequate (such as serology). Javadi Nia et al. [33] determined the prevalence of *C. pneumoniae* in children with adenoid hypertrophy concomitant with rhinosinusitis. In their study the pathogen was detected in 13.5% of patients using PCR, but their target group is completely different from ours. One recent study in Ahvaz, south of Iran, showed that the prevalence of *C. pneumoniae* was approximately 6% in their region, which corresponded to our findings in Tehran [34]. The prevalence of *C. pneumoniae* in countries neighbouring Iran is not different from our findings. In

Jordan and Turkey this pathogen was found in 5% and 8.8% of CAP patients respectively [35,36]. The prevalence of this agent is much lower in developed countries, such as in Norway (3%) and Germany (0.9%) [37,38].

The major clinical manifestations of atypical pneumonia include cough, fever and dyspnoea. We found a significant association between fever and the presence of *M. pneumoniae* and *L. pneumophila*; this is not surprising as fever is the common sign in patients infected by these pathogens [39]. The fact that Legionnaires disease poses a high risk to the elderly has long been known [40]. In our study the frequency of *L. pneumophila* in patients older than 60 was significant. Proper diagnostic and therapeutic approaches in the elderly with typical manifestations could be helpful to reduce this infection's complications.

Some limitations regarding our study were taken into consideration during the planning and interpretation of the results. Lower respiratory secretions are preferred for detecting Legionnaires disease, while in the present study we used throat swabs.

In this study we focused on the three most important atypical agents of pneumonia in a short period of time. Further long-term studies with larger sample sizes and detection of other bacterial and viral pathogens are needed to reach a better understanding of the status of CAP in Iran.

Conclusion

We found *M. pneumoniae* to be the most prevalent agent of atypical pneumonia, accounting for 24.7% of the study population. However, *L. pneumophila* was significantly more prevalent among elderly people (>60 years old) compared to other age groups. A more comprehensive study is recommended to assess the prevalence of CAP and its aetiologic agents in order to improve the diagnosis and treatment of CAP patients.

Conflict of interest

None declared.

Acknowledgement

Supported by Tehran University of Medical Sciences, Tehran, Iran (grant 39778).

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