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Original article

Molecular detection of bacterial agents of atypical pneumonia among patients from six hospitals in Suez Canal region

Heba Ahmed El-Sayed [1], Mohamed Aly El-Sweify [2], Waheed Fawzy Hessam [2], Eman Riad Hamed [3], Marwa Mohamed Fouad [*2]

1- Microbiology, and Immunology Department ,Faculty of Medicine, Port-Said University.
2- Microbiology and Immunology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt
3- Internal Medicine Department ,Chest Unit, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.

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ABSTRACT

Background: Reliable diagnosis of atypical pneumonia microbial agents is crucial since they do not respond to beta-lactams but to other groups of antibiotics. Therefore, empiric treatment with beta lactam groups will be ineffective for their eradication if no additional antibiotics as macrolides are administered to the management plan. This study aimed to determine prevalence of *Mycoplasma pneumoniae (M. pneumoniae)*, *Chlamydophila pneumoniae (C. pneumoniae)* and *Legionella pneumophila* (*L. pneumophila*) among patients with atypical pneumonia, and drawing physicians' attention to the role of these pathogens as etiologic agents of atypical pneumonia in the Suez Canal region. **Methods:** Eighty-four atypical pneumonia cases were enrolled in this study. Respiratory samples were collected. Part of each specimen was inoculated onto blood, MacConkey, and chocolate agar plates, another part of specimens was processed for DNA extraction and multiplex PCR assay for detection of *M. pneumoniae* p1adhesion gene, *C. pneumoniae* outer membrane protein (ompA) gene, and *L. pneumophila* macrophage infectivity potentiator (mip) gene. **Results:** Out of the 84 atypical pneumonia cases, atypical bacteria were detected by multiplex conventional PCR in 12 (14%) cases and they all were *L. pneumophila*, 3 cases (4%) were mixed with *Staphylococcus aureus*, and 2 (2%) cases were mixed with *Streptococcus pyogens*. *Mycoplasma pneumoniae* and *C. pneumoniae* were not detected by PCR in our samples. **Conclusions:** *Legionella pneumophila* incidence is not low in our geographical region in patients with atypical pneumonia; so it is of pivotal importance to recruit sensitive and reliable molecular based techniques to detect and control this infection in healthcare environments.

Introduction

Lower respiratory tract infections have become the third leading cause of death worldwide. Pneumonia is the most severe of these infections. One-fifth of community-acquired pneumonia cases are atypical [1].

Reliable diagnosis of microbial agents causing atypical pneumonia is crucial since they do not respond to beta-lactams but to other groups of antibiotics [2]. Therefore, empiric treatment with penicillin derivatives and other beta lactam groups will be ineffective for their eradication if no additional antibiotics as macrolides are administered to the management plan [3].

The most common bacterial agents of atypical pneumonia are *Mycoplasma pneumoniae*

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* *Corresponding author:* Marwa Mohamed Fouad
E-mail address: marwafouad80@gmail.com

(M. pneumoniae), Chlamydophila pneumoniae (C. pneumoniae), and Legionella pneumophila (L. pneumophila). Other less common agents are *Mycobacterium tuberculosis, Bordetella pertussis, Chlamydophila psittaci, and Coxiella burneti, respiratory syncytial virus, influenza virus, human rhinovirus human adenovirus, and human parainfluenza virus* [4]**.**

Although *Streptococcus pneumonia*e is the most isolated bacteria in community acquired pneumonia, the atypical respiratory pathogens *M. pneumoniae, L. pneumophila* and *C. pneumoniae* are being isolated with increasing frequency [5].

Few studies were conducted in Egypt to detect the etiological agents of acute respiratory infections and concluded that atypical pneumonia pathogens which have a significant role in the etiology were *M. pneumoniae and C. pneumoniae* with the need for further studies [6].

Conventional diagnostic methods are often insufficient for etiological diagnosis, since each atypical pathogens need special media for culture and / or special stain procedure for microscopic examination, and in many cases the causative pathogen cannot be determined.

The use of multiplex polymerase chain reaction (PCR), which is reported to be a reliable molecular method for diagnosing lower respiratory tract infections, has been used increasingly in recent years. The prominent advantage of PCR method compared to culture is that, since PCR is based on replicating the DNA or RNA of very small amount of microorganisms, it does not require living organisms and therefore is not affected by the prior use of antibiotics. In addition, PCR is more sensitive for detection of multiple microorganisms in mixed infections and delivers fast results [7]**.**

This study aimed to determine prevalence of *M. pneumoniae, C. pneumoniae* and *L. pneumophila* among patients with atypical pneumonia, and drawing physicians' attention to the role of these pathogens as etiologic agents of atypical pneumonia in the Suez Canal region.

Subjects and Methods

This is a cross-sectional descriptive study that was conducted on outpatients and admitted patients in pediatric, chest and ICU wards in Suez Canal University hospitals in Ismailia, Ismailia Chest hospital, and General hospital, Port-Said Chest hospital, El-Nasr pediatric hospital and Port-Said General hospital. It was carried out over the period of 28 months, from October 2018 to April 2020.

Subjects

Eighty-four patients were enrolled in the study with the following criteria:

Inclusion criteria

1. Patients presented to the Emergency Department or admitted to hospital, diagnosed as having atypical pneumonia by the presence of diffuse pulmonary infiltrate on chest radiograph, together with fever (>38.5 ^{0}C), cough, and leukocytosis over 10,000/mm^3.
2. Both genders
3. All age groups

Exclusion criteria

1. Patients who were diagnosed to have tuberculous pneumonia.
2. Patients who received non-beta lactam antimicrobial therapy,
3. Patients who were diagnosed by reliable specific radiological methods to have other noninfectious causes such as pulmonary infarction, sarcoidosis, or bronchogenic carcinoma.

Complete blood count, kidney and liver function tests, serum electrolytes, and C-reactive protein were recorded.

Information regarding date of sample collection, gender, age, residence, smoking, clinical symptoms and signs, presumed clinical diagnosis, lab results, radiological reports, current therapy, date of admission and hospital stay duration, accompanying chronic co-morbidities as diabetes mellitus, hypertension, bronchial asthma, congestive heart failure, chronic kidney or liver diseases were registered for each patient for further analysis.

Collection of specimens

During the acute phase of the illness, sputum specimens, pediatric nasopharyngeal suctioning and endotracheal tube aspirate samples were collected. Specimens were placed in a sterile bottle and transported on ice bags to the diagnostic lab, Microbiology Department, Faculty of Medicine, Suez Canal University within one hour for processing under complete aseptic condition.

Specimen culture and bacterial identification

Part of each specimen was inoculated onto blood (LAB M Limited, UK), MacConkey (LAB M Limited, UK), and chocolate agar plates to detect causes of pneumonia (such as *Streptococcus pneumonia, Staphylococcus aureus and*

Haemophilus influenza) as potential bacterial pathogens or accompanying atypical bacterial microorganisms. Plates were incubated in 3-5% CO_2 at 37°C. If there was no growth after 48 h, plates were discarded. Bacterial isolates were identified by their colonial morphology, gram staining and biochemical characteristics to detect significant bacterial growth [8].

Molecular identification of specimens

Part of specimens was stored at - 80°C for DNA extraction and multiplex PCR assay.

DNA extraction

DNA from clinical specimens was extracted using the Qiamp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. To ensure good DNA extraction from our samples, DNA concentration was measured by nanodrop spectrophotometer (NanoDrop ND-1000 spectrophotometer "NanoDrop Tech., Inc. Wilmington, DE, USA") in sample volume of one microliter [9]. Nucleic acids and proteins have absorbance maximum at 260 and 280 nm, respectively. Historically, the ratio of absorbances at these wavelengths has been used as a measure of purity in both nucleic acid and protein extractions. A ratio of ~1.8 is generally accepted as pure" for DNA; a ratio of ~2.0 is generally accepted as " pure" for RNA.

Multiplex PCR

The reaction mixture was prepared in a total volume of 25 µl of reaction mixture containing 10mM Tris–HCl, 50mM KCl, 1.5mM $MgCl_2$, 5% dimethylsufoxide, 200 mM dNTPs, 5 U AmpliTaq DNA polymerase. One µl (0.2–0.4 mM) of each of the three primer pairs were added. Three µl of DNA template were added. The volume was completed with distilled water up to 25µl. Reaction mixtures without a DNA template served as negative controls. Amplification was carried out in a thermal cycler **(Peltier Thermal cycler, MJ Research, USA).** The PCR conditions were as follows:[10]

1- Initial denaturation phase at 95ºC for 10 minutes.
2- Forty cycles of amplification, each consists of:
   a) Denaturation at 94^0C for 30 seconds.
   b) Annealing at 60^0C for 30 seconds.
   c) Extension at 72^0C for 60 seconds.
3- Final elongation was held at 72^0C for 10 minutes.

Amplicons obtained from PCR reactions were analyzed by gel electrophoresis **(Major Science, Taiwan)** using 1 % agarose gel in 1 x Tris-Borate-EDTA (TBE) buffer containing 5 µl/mL ethidium bromide at 100 volts for 45 minutes [10]. Bands were visualized with ultraviolet light. Amplicon size of the target genes was identified by comparing to a100 bp molecular size standard DNA ladder **(Sigma-aldrich).** The appearance of 236, 157 or 88 base pair amplification products corresponding to *C. pneumoniae, L. pneumophila and M. pneumoniae*, respectively, was a positive reaction.

Regarding the small band size of *M. pneumoniae* (88bp) rather than be detected; repeated monoplex conventional PCR was developed based on different specific primers for *M. pneumonia*e *P1adhesion* gene to detect a larger band (225 bp) on gel electrophoresis.

In each experiment, negative and internal kit positive controls for each pathogen were used. Positive controls were made with the PCR-TOPO 2.1 cloning kit (Invitrogen).

Table 1. Primers sequence and amplicon size for detection of target genes [10]

Bacterial target gene	Primer sequence	Amplicon size
M. pneumoniae p1adhesion gene	F: 5'ATT GCC TTG GTA GGC CGTTAC CCC AC3'	88 bp
	R: 5'CAA AGT TGA AAG GAC CTGCAA G3'	
	F: TCACCGATCTGTTTGATCCGG	225bp [11]
	R: GTAAGAAGTCACCGTTATTCGG	
C. pneumoniae outer membrane protein (*ompA)* gene	F: 5'CTC GTT GGT TTA TTC GGA GTT AAA G-3'	236 bp
	R: 5′GAG AAT TGC GAT ACG TTA CAG ATC A 3′	
L. pneumophila macrophage infectivity potentiator (*mip*) gene	F: 5′-AGT GCTTTG TTT GCA GGT ACG-3′)	157 bp
	R: 5′-CAC CAA CATCAG TAA AAC CAT TAT AGC-3′	

Ethical considerations

- The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.
- This cross-sectional study was conducted according to STRBE guidelines.
- The study was approved by the Research Ethics committee, Faculty of Medicine, Suez Canal University. *Reference: Research# 3590. Date: 10/9/2018.*
- An informed consent was obtained from all participants enrolled in this study.

Data management and statistical analysis

Statistical analysis was done using IBM Statistical Package of Social Sciences (SPSS) software version 22 for Windows ® software.

Descriptive statistics .numerical presentation of data was done using frequency distribution tables. According to Kolmogorov-Smirnov normality testing, the data was non-parametric. Thus non-parametric data analysis was recommended; here Chi-squared test was used for study variables. *p* value was significant at *< 0.05*.

Results

Eighty-four patients from all age groups and both genders were selected. Seventy-one percent of our patients were males and 29% were females and, the mean age of the patients was 41±27 years old.

Among the 84 atypical pneumonia cases enrolled in this study, multiplex PCR detected *L. pneumophila* in 12 cases (14%). Three cases (4%) were mixed with *S. aureus*, and 2 cases (2%) were mixed with *S. pyogens*.

Mycoplasma pneumoniae and C. pneumoniae were not detected by PCR in our samples. Other bacterial causes were detected by conventional cultural methods on blood, MacConkey and chocolate agar as *K. pneumoniae* were detected in 2%, S. pyogens in 5%, E. coli in 8%, and S. pneumoniae in 2% of samples as shown in **table (3)**.

On comparing between *L. pneumophila* positive cases and *L. pneumophila* negative cases in this study, there was a statistically significant association between smoking and detection of *L. pneumophila* (p <0.05). However, no statistically significant association was found between age, gender, and residence with *L. pneumophila* positive cases (p > 0.05).

On comparing *L. pneumophila* positive cases versus *L. pneumophila* negative cases in this study regarding co-morbidities, there was a statistically significant association of COPD, bronchial asthma, and DM with *L. pneumophila* positive cases (p <0.05). Whereas hypertension, and cardiac diseases were not significantly related to *L. pneumophila* positive cases (p > 0.05).

Table 2. Clinical, laboratory, radiological and co-morbidity data of 84 studied patients

Characters	**No (%)**
• Inpatients	47 (56%)
• Outpatients	37 (44%)
Complaint	
• Cough	76 (91%)
• Fever	74 (88%)
Lab work	*Mean ± SD*
• C-reactive protein (mg/L)	16±14.35
• WBCs count (cell/ µl)	13350±1840
X-ray findings	
• Unilateral infiltrate	29 (34%)
• Bilateral infiltrate	55 (65%)
Comorbidity	
• Diabetes mellitus	42 (50%)
• Hypertension	27 (32%)
• COPD*	25 (29%)
• Bronchial asthma	23 (27%)
• Cardiac diseases	16 (18%)

*COPD: Chronic Obstructive Lung Disease

Table 3. Pathogens detected from the respiratory specimens (n=84).

Pathogen		No	Frequency%	Detection method culture or PCR	
Single bacterial pathogen	*S. aureus*	5	6%	Culture	-
	K. pneumoniae	2	2%	Culture	-
	S. pyogens	5	6%	Culture	-
	E. coli	8	10%	Culture	-
	S. pneumoniae	2	2%	Culture	-
	L. pneumophila	12	14%	-	PCR
	M. pneumoniae	0	0%	-	PCR
	C. pneumoniae	0	0%	-	PCR
Mixed bacterial pathogens	*L. pneumophila+ S. aureus*	3	4%	PCR / Culture	
	L. pneumophila + S. pyogens	2	2%	PCR / Culture	

Table 4. Patient's demographic data in relation to presence of *L. pneumophila* (n=84).

General characteristics	*Legionella* + ve cases (n=12)	*Legionella* –ve cases (n=72)	*P-value*
Male	10 (83.3%)	50 (69.4%)	*0.495*
Female	2 (16.7%)	22 (30.6%)	
Age Mean± SD	36±27 y	42±27 y	*0.447*
Children (< 18)	4 (33.3%)	20 (27.8%)	*0.732*
Adults (18-59)	5 (41.7%)	24 (33.3%)	
Old age (> 59)	3 (25%)	28 (38.9%)	
Ismailia residence	7 (58.3%)	34 (47.2%)	*0.544*
Port-Said residence	5 (41.7%)	38 (52.8%)	
Smokers	11 (92%)	41 (57%)	*0.025**

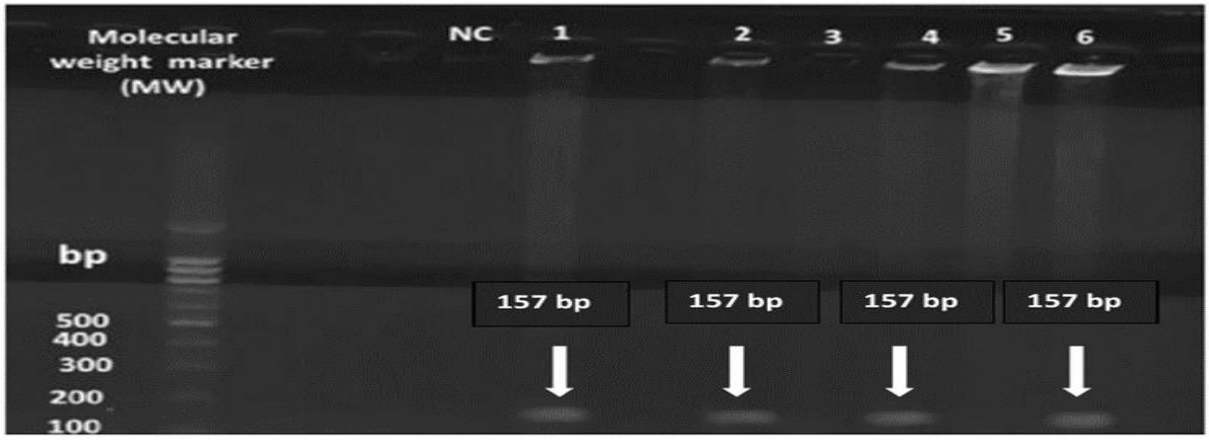
Table 5. Relationship between *L. pneumophila* detection and presence of co-morbidities among the studied patients (n=84).

Co-morbidities *Freq. (%)*	Legionella +ve (n=12)	Legionella -ve (n=72)	*P-value*
COPD	9 (75%)	19 (26%)	*0.002**
Hypertension	4 (33.3%)	23 (31.9%)	*0.583*
Bronchial asthma	7 (58%)	18 (25%)	*0.04**
Cardiac diseases	3 (25%)	13 (18.1%)	*0.41*
Diabetes mellitus	10 (83%)	31 (43%)	*0.01**

**P- value* is statistically significant if *< 0.05.*

Figure1. Agarose gel electrophoresis showing positive PCR products for *L. pneumophila* (Lanes 1, 2 and 4).

- Molecular weight marker: 100 bp.
- NC: negative control.
- Lane 6 is the positive control.
- Bands marked with arrows correspond to the amplicons (157 bp) of *L. pneumophila macrophage infectivity potentiator (mip) gene*.



Discussion

Atypical pneumonia constitutes a considerable percentage of community-acquired pneumonia in both adults and children [12]. In Egypt, few studies have been done to detect the prevalence and role of atypical bacteria causing atypical pneumonia.

This study was performed to detect the prevalence and role of atypical bacterial pathogens in causing atypical pneumonia in Suez Canal area. Unlike many other studies, our study aimed to detect prevalence of atypical bacterial pathogens among hospitalized patients as well as community acquired pneumonia patients, including all age groups.

The mean age of our atypical pneumonia patients in this study was 41 years which indicated high prevalence of atypical pneumonia among middle age patients, and this was in contrast with **Rivero-Call et al.** [13] who reported that community acquired pneumonia prevalence increased with age > 65 years. Higher rate of exposure of middle age group patients to air conditioning at work and homes than elderly group patients might increases the prevalence of atypical pneumonia among this group [14] which supports our results.

Co-morbid conditions were present in 36 patients (43%). Diabetes mellitus was the most common comorbidity (50%) followed by hypertension (32%) and COPD (29%). This might be explained by adverse effects of smoking and COPD on the respiratory epithelium and the clearance of bacteria from the respiratory tract. Moreover, diabetes mellitus has been associated with defects in innate and adaptive immunity which increases the risk of infections including pneumonia [15].

We collected sputum samples in our study as recommended by previous studies that stated that sputum samples are superior to nasopharyngeal swabs and throat swabs for multiplex PCR to detect bacterial and viral causes of pneumonia [16]. **Error! Reference source not found.**

Atypical bacterial etiology was identified in 12 cases (14%) out of our 84 samples. All positive PCR cases were *L. pneumophila* (detected as 157 bp bands on agarose gel electrophoresis).

Other microbial causes that were detected by conventional cultural methods included *K. pneumoniae* in (2%), *S. pyogens* in (5 %), *E. coli* in (8%), and *S. pneumoniae* in (2%) of samples. Co-infections were detected in 5 samples out of 12 samples of *L. pneumophila* positive, 3 samples were co-infected with *S. aureus* and *L. pneumophila*, and 2 samples were co-infected with *L. pneumophila* and *S. pyogens.*

Legionella pneumophila was detected in previous studies in respiratory specimens of CAP patients by molecular methods with variable prevalence rates ranging from 0.7%-5.6%, but in our study, the prevalence of *L. pneumophila* in those with suspected atypical pneumonia was much higher (14%). This higher frequency could be explained by the difference in inclusion criteria between those

studies and ours, since they included CAP patients but we included only atypical CAP patients as evidenced by clinical picture and chest X-ray, and they included only one age group either children or adults only but our study included all age groups.

*Mycoplasma pneumonia*e and *C. pneumoniae* were not detected in our study samples by multiplex PCR; although, we repeated our work for *M. pneumoniae* by using monoplex PCR with other primers. This might be explained by several factors related to our patients, sampling technique and processing or the technique of PCR.

The time of sampling affects the accuracy of PCR, which might decrease at ≥ 7 days after onset of disease and thus might increase the rate of false negative PCR results [17]. Also, the presence of PCR inhibitors in samples, coming from human cells or colonizing microorganisms may lead to false negative results [18].

Bacterial load in the specimen might be below the detection limit of the PCRs, which could be caused by dilution of samples during processing, degradation of significant amounts of DNA during the sample storage process, or the tendency of *M. pneumoniae* cells to form conglomerates, which would affect amplification, so affect the sensitivity of PCR [19].

Compounds as phosphates when phosphate-buffered saline buffer is used to collect samples, glove powder, dust, and laboratory plasticware including some micro-centrifuge tubes may cause complete reaction failure or reduced sensitivity for *C. pneumoniae* detection [20]**.**

Other diagnostic methods were widely used to diagnose *M. pneumoniae, C. pneumoniae and L. pneumophila* pneumonia such as serological diagnostic methods. **Zaki and Goda** [21] in Mansoura- Egypt diagnosed *L. pneumophila* in 5% of adult CAP patients by serological detection of specific IgM, while, **Hussein et al.** [22] diagnosed *L. pneumophila* by detection of specific IgM in 33.3% of infants and preschool children CAP patients. **Error! Reference source not found.**

Serological diagnostic method was not done in our study since it is more expensive, and not commercially available, than PCR. Diagnosis of atypical pneumonia caused by *M. pneumoniae, C. pneumoniae and L. pneumophila* by serological methods was controversial as they show high seroprevalence of IgG antibodies in the general healthy population (up to 60%, 70%, 2.6%, respectively) [17].

A major disadvantage of IgM-based diagnosis of *M. pneumoniae* is that these antibodies are not constantly produced in adults, most likely as a result of multiple previous infections, so depending on IgM is not accurate especially in adults [23]. *Legionella pneumophila* IgM detection by ELISA presented a low sensitivity (30%) especially with old age as IgM response is affected by immune status [24].

According to multiplex-PCR performed, we classified the studied group into 2 groups; the PCR positive *L. pneumophila* group (12 patients) and the PCR negative *L. pneumophila* group (62 patients) and each group was related to clinical, laboratory and radiological parameters of the patients.

Several risk factors for acquisition of *L. pneumophila* infection have been identified in our study. These factors include the conditions with local impairment of the muco-ciliary clearance, including cigarette smoking, bronchial asthma, chronic lung disease, or that causing systemic immunosuppression as diabetes mellitus. Also, several predictive clues for *L. pneumophila* pneumonia have been identified in our study such as persistent cough, high inflammatory markers as CRP, chest X-ray with bilateral patchy or fluffy cotton appearance.

Smoking and COPD (Chronic obstructive pulmonary disese) are considered risk factors for *L. pneumophila* atypical pneumonia as detected in 92% and 75% respectively, of positive *L. pneumophila* cases. In addition to the role of smoking and COPD in increased susceptibility to bacterial infections, tobacco smoking impairs neutrophil and monocyte antibacterial phagocytosis, reactive oxygen species generation, and specific bacterial killing [25].

Bronchial asthma is considered another risk factor for *L. pneumophila* infection as 58% of *L. pneumophila* positive cases were asthmatic. Bronchial asthma predisposes to several respiratory infections by intracellular pathogens. This is due to certain immunological consequences such as: (1) a T-helper 2 cell predominance with increased levels of IL-4 and IL-13, (2) blocking of T-helper 1 cell cytokines such as IL-12 and (3) impaired production of antimicrobial peptides such as human β-defensin. Impaired Toll-like receptor 2 (TLR-2) mediated signal transduction was established in asthmatic patients [26,27]. TLR-2 was an important molecule

for host resistance against the intracellular growth of *L. pneumophila*. TLR-2 dysfunction in macrophages and dendritic cells of asthmatic patients showed impaired response to PAMP recognition of *L. pneumophila* LPS and subsequent decreased resistance to intracellular *L. pneumophila* growth, and thus bronchial asthma is considered a risk factor for *L. pneumophila* infection.

Diabetes mellitus (DM) was detected in 83% of our cases. **Wickramasekaran and colleagues** [28] had reported that DM is a strong risk factor for *L. pneumophila* infection. Hyperglycemia negatively affects all immune system components especially the neutrophil functions, decreasing their phagocytosis and degranulation ability [29,30]. Neutrophils and their secreted inflammatory mediators as tumor necrosis factor (TNF) and reactive oxygen species (ROS) are key components of innate immunity against *L. pneumophila* [31]**.**

As regarding the radiological findings in our results of *L. pneumophila* positive cases, chest X-rays showed segmental distribution of broncho-pulmonary infiltration of fluffy cotton appearance bilaterally in 58% of cases. **Error! Reference source not found.** It was demonstrated that segmental distribution resulted more frequent than non-segmental distribution in *L. pneumophila* pneumonia; the incidence of bilateral or unilateral involvement was quite similar [32].

As regarding clinical and laboratory findings, *L. pneumophila* infection was associated with fever, cough, and high levels of inflammatory markers such as CRP. In PCR-positive group for *L. pneumophila*, fever > 38.50C was found in 82% of cases, and cough in 81% of patients. These findings are strongly correlated with measures of the severity of airway damage such as mucous necrosis in proximity to pulmonary circulation and subsequent airway remodeling, which produces an immunological stimulus to the liver and production of different pattern of cytokines and acute-phase proteins such as CRP. Our study confirmed this association as there was significant statistical difference between *L. pneumophila* positive and negative cases regarding elevated CRP level. These results were in agreement with **Bellmann-Weiler and colleagues** [33] who demonstrated the clinical potential of *L. pneumophila* infection with high CRP level. Due to this different inflammatory host response of *L. pneumophila*, CRP might aid physicians to rule out *L. pneumophila* pneumonia.

The prevalence of *L. pneumophila* in our study was 14%. This must draw the attention of our physicians to the role of *L. pneumophila* in causing atypical pneumonia. Environmental decontamination of sources of infection is difficult.

Nowadays, during COVID-19 pandemic, and due to the activity lockdown, the restaurants, offices, schools, colleges, and factories had been closed. This may allow *Legionella* to flourish in water pipes and air-conditioning systems or spa pools/tubs if they are not managed adequately. The implementation of a suitable flushing regime, or draining, and the monitoring water and air conditioning systems are needed to reduce the risk of Legionella overgrowth. Emergency clinicians need to consider *L. pneumophila* among other differential diagnoses after the end of the lockdown due to the COVID-19 pandemic [34].

Recently, SARS-CoV-2 co-infection with other atypical pneumonia pathogens especially with *M. pneumoniae*, *C. pneumoniae and L. pneumophila* had been reported widely in Europe and USA [35].

In conclusion, *L. pneumophila* incidence is not low in our geographical region in patients with atypical pneumonia. The overall prevalence of *L. pneumophila* in our study was 14% so, the study recommends physicians to highly consider *L. pneumophila* in the differential diagnosis of atypical pneumonia cases admitted to hospitals especially among COPD, asthmatic and diabetic patients. They should be under coverage of empiric treatment with macrolides or fluoroquinolones. Additionally, it is of pivotal importance to recruit sensitive and reliable molecular based techniques to detect and control this infection in healthcare environments.

Limitations of the study included the inability to detect *M. pneumonia*e and *C. pneumoniae* in gel electrophoresis in our specimens. This might have been overcome by trying different primer sets, or using other detection methods such as serological diagnostic tests or Real-Time PCR. However this was not done for financial issues. Therefore, we recommend further attempts to explore the prevalence of *L. pneumophila, M. pneumoniae and C. pneumoniae* using different detection methods, that may include, for instance: serological tests, Real-Time PCR, different primer sets.

Conflict of interest

The authors have no conflict of interest to declare.

Financial disclosures

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