



Medicine Updates Faculty of medicine
July 2021, volume 6, issue 6

https://muj.journals.ekb.egdean@med.psu.edu.vice_dean_postgraduate@med.psu.edu.eg
DOI: 10.21608/ muj.2021.75578.1051

ISSN : 2682-2741

Submitted: 7/5/2021

Accepted: 29/6/2021

Pages: 90- 115

**" Molecular Detection of Bacterial Agents of Atypical
Pneumonia: *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae*, and
Legionella pneumophila in Suez Canal region**

Authors:

**Heba Elsayed: Medical Microbiology and Immunology,
Faculty of Medicine, Port Said University, Egypt.**

كلية الطب
Faculty of Medicine

Abstract

Background: Atypical bacterial infections played an important role in community-acquired pneumonia (CAP). It is difficult to detect atypical pathogens by conventional microbiological diagnostic methods. Atypical bacteria do not respond to beta-lactam antibiotics. The use of multiplex polymerase chain reaction (PCR) methods enables rapid and simultaneous detection of many pathogens in a single analysis. **Aim:** detecting the prevalence of atypical bacterial pathogens as etiologic agents of atypical pneumonia, in the Suez Canal region.

Materials and Methods: This cross-sectional descriptive study was conducted throughout 18 months, from October 2018 to April 2020. It included 84 Egyptians suffered from CA atypical pneumonic patients of all age groups from Suez-Canal region, Egypt. Sputum samples were collected for identification of *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae*, and *Legionella pneumophila* by using multiplex PCR.

Results: Among the 84 atypical pneumonia patients, *L. pneumophila* were detected in 12 (14%) patients. *M. pneumoniae* and *C. pneumoniae* were not detected in our samples. Compared with *L. pneumophila* -negative cases, *L. pneumophila* -positive cases were more prevalent in middle aged males, smokers, COPD, diabetic and asthmatic patients (P values = 0.048). Persistent cough, elevated levels of C-reactive protein (C-RP), bilateral pulmonary infiltration are significant clues for predicting *L. pneumophila pneumonia*.

Conclusions: *L. pneumophila* incidence is not low in our geographical region in atypical pneumonia patients. Clinicians should consider atypical bacterial pathogens while prescribing antimicrobial management plan.

Keywords: Atypical pneumonia, *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae*, *Legionella pneumophila*, multiplex-PCR.

Introduction

Community-acquired pneumonia (CAP) has become the third leading cause of death worldwide. One-fifth of CAP cases are atypical (Wijesooriya et al., 2018).

Atypical pneumonia is a respiratory infection confined to alveolar septa and pulmonary interstitial radiologically characterized by pulmonary patchy inflammation (Zare et al., 2017).

Atypical pneumonia is caused by atypical microorganisms include special bacteria, viruses, and fungi (Wagner et al., 2018).

Pathogens of atypical pneumonia are not diagnosed by conventional microbiological techniques. They do not respond to beta-lactams (Sharma et al., 2017).

In Egypt, few studies have been done to detect the prevalence and role of atypical bacteria causing atypical pneumonia. Most of them used less sensitive serological methods for atypical bacterial detection, and all included only one age group in each study (El Seify et al., 2016).

Multiplex PCR is a widespread molecular biology technique for amplification of multiple targets in a single PCR experiment (Lee et al., 2018).

A desire to correct the overuse of antibiotic therapies is recommended by The American Thoracic Society so, clinicians need to be aware of the spectrum of local pathogens, by more research of detecting the cause of pneumonia to decrease rate of treatment failures with empiric beta-lactam antibiotics (Metlay et al., 2019).

Molecular detection of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila* in sputum samples of atypical pneumonia patients to determine their prevalence. Conclude the possible risk factors for infection with these atypical bacteria by relating the infection with these atypical bacteria and different demographic, clinical, laboratory and radiological findings.

Materials and Methods

Study Design:

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 General hospital over the period of 18 months, from October 2018 to April 2020.

Target population:

Inclusion criteria:

1. Patients presented to the Emergency Department or admitted to hospital, diagnosed as having community acquired atypical pneumonia according to American Thoracic Society guidelines (Metlay et al., 2019) by the presence of diffuse pulmonary infiltrate on chest radiograph, together with fever ($>38.5^{\circ}\text{C}$), cough, and leukocytosis over $10,000/\text{mm}^3$.
2. Both genders
3. All age groups that were divided into three categories:
 - Children < 18 years
 - Adults 18-59 years
 - Old age > 59 years

Exclusion criteria:

We excluded patients who were diagnosed to have tuberculous pneumonia, pulmonary infarction, sarcoidosis, or bronchogenic carcinoma. Patients who suffered atypical pneumonia for more than 7 days or received non-beta lactam antimicrobial therapy were also excluded. Hospital acquired atypical pneumonia were excluded as our patients' sample were diagnosed as having atypical CAP on admission not 72 hours after admission (Gerald et al., 2005).

Complete blood count, kidney and liver function tests, serum electrolytes, C-reactive protein were recorded. All patients have done a plain chest X-ray at admission.

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.

Specimens were collected during the acute phase of the illness. Respiratory samples including sputum specimens, pediatric nasopharyngeal suctioning, and endotracheal tube aspirate samples were collected. In pediatric patients, sputum induction was undertaken by nebulization with salbutamol (100 µg in <1 year old, 200 µg in >1 year old) mixed with 2 cc plain normal saline solution, and oxygen. Sputum was obtained either by expectoration or by suctioning through the nasopharynx.

Sputum samples are superior to nasopharyngeal swabs and throat swabs for multiplex PCR to detect bacterial and viral causes of pneumonia (Raty et al., 2005), (Cho et al., 2012) described 69% sensitivity for sputum samples, 50% and 37.5% for throat swabs and nasopharyngeal swabs, respectively.

The specimens were placed in a sterile bottle and transported to the diagnostic lab, Microbiology Department, Faculty of Medicine, Suez Canal University within one hour for processing under complete aseptic condition .

Part of each specimen was inoculated onto blood, MacConkey, and chocolate agar plates to detect causes of pneumonia (such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and fungi) as potential pathogens or accompanying atypical microorganisms.

Plates were incubated in 3-5% CO₂ at 37°C. If there was no growth after 48 h, plates were discarded.

Bacterial isolates were identified by their colonial morphology, microscopic examination by gram staining and biochemical characteristics to detect significant bacterial growth from the respiratory specimens.

Molecular identification of specimens

Three millimeters of sputum specimen was processed by adding equal volume of Dithiothreitol (DTT) to liquify viscous sputum, followed by 1 ml of phosphate buffer saline (PBS), vortexed for 15 seconds, then was frozen at

–80°C for DNA extraction and multiplex PCR assay (Saraswathy et al., 2015).

DNA from clinical specimens was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

- Two hundred µl of the thawed, vortexed sample were added to 1.5 ml microcentrifuge tube with 20µl of proteinase K.
- Two hundred µl of lysis buffer (AL) were added to the tube and vortexed.
- Incubation of the mixture at 56 °C for 10 minutes.
- Sample was mixed with 200 µl ethanol (96–100%) then added the mixture to the QIAamp Mini spin column, centrifuged at 6000 g (8000 rpm) for 1 min and the filtrate was discarded.
- Then we added 500 µl of AW1 washing buffer, then centrifuged at 6000 g (8000 rpm) for 1 min, then the filtrate was discarded.
- We added 500 µl of AW2 buffer, centrifuged at 20,000 g (14,000 rpm) for 3 mins and the filtrate was discarded.
- DNA trapped within the spin column was eluted in a final volume of 150 µl of AE buffer, incubated at room temperature for 1 min then centrifuged at 6000 g (8000 rpm) for 1 min to elaborate the extracted pure DNA from the spin column, the column was then removed from the tube, then keep DNA solution stored at –20°C until performance of the PCR.

To ensure good DNA extraction from our samples, we measured DNA concentration by nanodrop spectrophotometer in sample volume of one microliter.

Primers: (Miyashita et al., 2004).

Bacterial target gene	Primer sequence	Amplicon size
<i>M. pneumoniae</i> <i>p1adhesion</i> gene	F: 5'ATT GCC TTG GTA GGC CGTTAC CCC AC3'	88 bp
	R: 5'CAA AGT TGA AAG GAC CTGCAA G3'	
	F: TCACCGATCTGTTTGATCCGG	225 bp (Geertsens et al., 2007)
	R: GTAAGAAGTCACCGTTATTCGG	
<i>C. pneumoniae</i> outer membrane protein (<i>ompA</i>) 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'	
<i>L. pneumophila</i> macrophage infectivity potentiator (<i>mip</i>) gene	F: 5'-AGT GCTTTG TTT GCA GGT ACG-3')	157 bp
	R: 5'-CAC CAA CATCAG TAA AAC CAT TAT AGC-3'	

Multiplex PCR

A- The preparation of PCR reactions.

The reaction mixture was prepared in a total volume of 25 µl of reaction mixture containing 10mM Tris-HCl, 50mM KCl, 1.5mM MgCl₂, 5% dimethylsulfoxide, 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

25 μ l. Reaction mixtures without a DNA template served as negative controls.

B- The amplification of PCR products.

Amplification was carried out in a thermal cycler (**Peltier Thermal cycler, MJ Research, U.S.A**) with the following thermal cycling:

- 1- Initial denaturation phase at 95 $^{\circ}$ C for 10 minutes.
- 2- Amplification cycle consists of:
 - a) Denaturation at 94 $^{\circ}$ C for 30 seconds.
 - b) Annealing at 60 $^{\circ}$ C for 30 seconds.
 - c) Extension at 72 $^{\circ}$ C for 60 seconds.

Forty cycles of amplification were performed.

- 3- Final elongation was held at 72 $^{\circ}$ C for 10 minutes.

C- Detection of the Amplicons by gel electrophoresis.

Amplicons obtained from PCR reactions were analyzed by gel electrophoresis (**Major Science, Taiwan**) in 1 % agarose gel in 1 x Tris-Borate-EDTA (TBE) buffer containing 5 μ l/mL ethidium bromide at 100 volts for 45 minutes. Bands were visualized with ultraviolet light. Amplicon size of the target genes was identified by comparing to a 100 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. pneumoniae* *pladhesion* 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).

Data Management and Statistical Analysis

- The data were coded, checked, entered, and analyzed using Statistical Package for Social Science program (SPSS version 22 for windows).

- Continuous variables are expressed as mean \pm SD while categorical variables are expressed as frequencies and percent.
- Chi square, Fischer test were used to identify factors and clinical signs, or symptoms related to atypical bacterial infection; the level of significance was 95%, p-value is statistically significant if < 0.05 .

Ethical considerations

- The study approved by Local Research and Studies Committees.
- The study approved by Local Suez Canal University Research Ethics Committee.
- An informed written consent was obtained from all participants enrolled in this study.

The consent included:

- Explanation of the study in a simple manner understood by the common people.
- That procedure of obtaining sample was of mild risk.
- That all data was considered confidential and was not used outside this study without patient's approval.
- That all samples were used in the research only and were discarded after completing the data collection.
- Researcher phone number and all possible communicating methods were identified to the participants to return at any time for any explanation.
- That all participants were announced by the result of the study.
- That the patients had the right to refuse participating; and they could withdraw at any time without any loss of benefit or penalty, they received their usual care.
- Signature or fingerprints of the participants.

Results

Eighty-four atypical pneumonia patients from all age groups and both genders were selected. 71% (60) of patients were males and 29% (24) were females and, the mean age of our sample was 41 ± 27 years old **Table (1)**.

Atypical bacterial etiology was identified in 12 cases (14%) out of our 84 samples. All positive PCR cases were *L. pneumophila*. Positive cases were diagnosed by agarose gel electrophoresis of 157 bp bands. We did not detect *M. pneumoniae* (88 bp) bands or *C. pneumoniae* (236 bp) bands in gel electrophoresis in our specimens. Regarding the small band size of *M. pneumoniae* (88bp) rather than be detected; repeated multiplex conventional PCR was developed based on different specific primers for *M. pneumoniae p1adhesion* gene to detect a larger band (225 bp) on gel electrophoresis, still no specimen was positive for *M. pneumoniae* tested by the other primer **Figure (1)**.

On comparing between *L. pneumophila* positive cases and *L. pneumophila* negative cases in this study regarding general data, there was a statistically significant association between smoking and *L. pneumophila* positive cases ($p = 0.048$). However, no statistically significant association was found between age, gender, and residence with *L. pneumophila* positive cases **Table (4)**.

No statistically significant association was found between different age groups and *L. pneumophila* positive cases.

On comparing between *L. pneumophila* positive cases versus *L. pneumophila* negative cases in this study, there was not statistically sig difference between inpatients and outpatients regarding the presence of *L. pneumophila positive cases* ($p > 0.05$).

Regarding symptoms, there was a statistically significant association between cough and *L. pneumophila* positive cases ($p = 0.048$), while fever was not significantly related to *L. pneumophila* positive cases **Table (4)**.

Regarding laboratory data, the percentage of C-reactive protein was significantly associated with *L. pneumophila* positive cases ($p = 0.048$), However, WBCs count was not significantly related to *L. pneumophila* positive cases.

Regarding chest X-ray, the presence of bilateral lung involvement with interstitial pulmonary infiltration was significantly related to *L. pneumophila* positive cases ($p = 0.048$) **Table (4)**.

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 ($p = 0.048$), whereas hypertension, and cardiac diseases were not significantly related to *L. pneumophila* positive cases **Table (4)**.

Discussion

Atypical pneumonia constitutes a considerable percentage of community-acquired pneumonia in both adults and children [Elkolaly et al.,2019]. 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-aged patients, and this was in contrast with Rivero-Call et al. 2019 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 increase the prevalence of atypical pneumonia among this group (Masiá et al., 2007) 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 (Torres et al.,2013). 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 (Cho et al.,2012).

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. pyogenes* 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. pyogenes*.

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 pneumoniae 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 (Jung et al.,2018).

Also, the presence of PCR inhibitors in samples, coming from human cells or colonizing microorganisms may lead to false negative results (Zhang et al.,2011).

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 (Herrera et al.,2016). 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 (Hvidsten et al.,2009).

Other diagnostic methods were widely used to diagnose *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila* pneumonia such as serological diagnostic methods. Zaki and Goda, 2009 in Mansoura- Egypt diagnosed *L. pneumophila* in 5% of adult CAP patients by serological detection of specific IgM, while Hussein et al. 2019 diagnosed *L. pneumophila* by detection of specific IgM in 33.3% of infants and preschool children CAP patients.

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) (Jung et al.,2018). A major disadvantage of IgM-based diagnosis of *M. pneumoniae* is that these antibodies are not constantly produced in adults, most likely because of multiple previous infections, so depending on IgM is not accurate especially in adults (Kumar et al.,2018).

Legionella pneumophila IgM detection by ELISA presented a low sensitivity (30%) especially with old age as IgM response is affected by immune status (Marimuthu et al.,2018). 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 disease) 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 (Bagaitkar et al., 2008).

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 (Habibzay et al., 2012), (Juhn et al., 2014). 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 (Wickramasekaran et al., 2015) 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 (Jafar et al., 2016), (Akash et al., 2020). 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* (Ziltener et al., 2016).

As regarding the radiological findings in our results of *L. pneumophila* positive cases, chest X-rays showed segmental distribution of bronchopulmonary infiltration of fluffy cotton appearance bilaterally in 58% of cases. 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 (Vinciguerra et al., 2012).

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 agreed with Bellmann-Weiler and colleagues (Bellmann-Weiler et al., 2010) 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 (Palazzolo et al., 2020). 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 (Richardson et al., 2020).

Conclusion

L. pneumophila prevalence 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. pneumoniae* 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.

Summary

- a) Atypical pneumonia is an acute febrile respiratory infection which is radiologically characterized by patchy inflammatory changes in the lungs.
- b) The most common bacterial agents of atypical pneumonia are *Mycoplasma pneumoniae*, *Chlamydothila pneumoniae*, and *Legionella pneumophila*.
- c) This work represents an advance in biomedical science as it confirms that multiplex PCR provide a rapid, reliable, and sensitive diagnostic method.
- d) This work determines the prevalence of atypical bacteria among atypical pneumonia patients and by correlating the demographic data, clinical presentation, comorbidities, laboratory findings and radiological data of included patients, the probable risk factors and predicting signs for atypical bacterial infection were determined.

References

- Akash M. Rehman K, Fiayyaz F, Sabir S, Khurshid M. Diabetes-associated infections: development of antimicrobial resistance and possible treatment strategies. Archives of Microbiology. 2020; 1-13.
- Babaei Z, Pouladi I, Ashtari A, Azimi G, Niakan M. The Prevalence of Atypical Pneumonia Caused by *Mycoplasma pneumoniae* (p1 gene) in Patients with Respiratory Infections by Culture and Molecular PCR Methods in Tehran, Iran. Jundishapur Journal of Microbiology. 2019; 12.(11)
- Bagaitkar J, Donald R, Avid A. Tobacco use increases susceptibility to bacterial infection. Tobacco induced diseases. 2008; 12-22.
- Bellmann-Weiler R, Ausserwinkler M, Kurz K, Theurl I, Weiss G. "Clinical potential of Creactive protein and procalcitonin serum concentrations to guide differential diagnosis and clinical management of pneumococcal and *Legionella pneumophila*. Journal of Clinical Microbiology 2010; 48(5): 1915-1917.
- 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. *American Journal of Tropical Medicine Hygiene*. 2017; 97(6):1710-1716 .

- Cho M, Kim H, An D, Lee M, Noh S, Kim M, Chong Y, Woo J. Comparison of sputum and nasopharyngeal swab specimens for molecular diagnosis of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella pneumophila*. *Annals of Laboratory Medicine*. 2012; 32(2):133-8.
- Daxboeck F, Krause C, Wenisch R. Laboratory diagnosis of *Mycoplasma pneumoniae* infection. *Clinical Microbiology and Infection*. 2003; 9(4): 263-273.
- El Baroudy R, El Refay S, Hamid A, Hassan M, et al. Respiratory viruses, and atypical bacteria co-infection in children with acute respiratory infection. *Open access Macedonian journal of medical sciences*. 2018; 6(9), 1588.
- El Seify M, Fouda M, Ibrahim H, Fathy M, Al Hussein A. Microbial etiology of community-acquired pneumonia among infants and children admitted to the pediatric hospital, Ain Shams University. *European Journal of Microbiology and Immunology*. 2016; 3.(6)
- Elkolaly R, Shams Eldeen M. "Easy and rapid diagnosis of *Mycoplasma pneumoniae*: is it possible?" *Egyptian Journal of Bronchology* 2019; 13(3): 394.
- Farnham A, Alleyne L, Cimini D, Balter S. Legionnaires' disease incidence and risk factors, New York, USA, 2002-2011. *Emerging Infectious Diseases*. 2014; 20(11):1795-1802.
- Geertsen R, Kaeppli F, Sterk-Kuzmanovic N, Andrasevic S, et al. A multiplex PCR assay for the detection of respiratory bacteria in nasopharyngeal smears from children with acute respiratory disease. *Scandinavian Journal of Infectious Diseases*. 2007; 39(9):769-74.
- Gerald L. Mandell, John E. Bennett, Raphael Dolin (eds): *Principles and Practice of Infectious Diseases*, 6th edition: Elsevier, Philadelphia, 2005;765–767.
- Gullsby K, Olsen B, Bondeson K. Molecular Typing of *Mycoplasma pneumoniae* Strains in Sweden from 1996 to 2017 and the Emergence of a New p1 Cytadhesin Gene, Variant 2e. *Journal of Clinical Microbiology*. 2019; 57(6): 49-19 .

- Habibzay M, Saldana J, Goulding J, Lloyd C, Hussell T. Altered regulation of Toll-like receptor responses impairs antibacterial immunity in the allergic lung. *Mucosal Immunology*. 2012; 5(5): 524-534.
- Herrera M, Aguilar Y, Rueda Z, Muskus C, Vélez L. Comparison of serological methods with PCR-based methods for the diagnosis of community-acquired pneumonia caused by atypical bacteria. *Journal of Negative Results in Biomedicine*. 2016;15(1): 3.
- Huong P, Hien T, Lan N, Binh T, Tuan D, Anh D. First report on prevalence and risk factors of severe atypical pneumonia in Vietnamese children aged 1–15 years. *BMC public health*. 2014; 14(1): 1-8 .
- Hvidsten D, Halvorsen D, Berdal B, Gutteberg T. *Chlamydomydia pneumoniae* diagnostics: importance of methodology in relation to timing of sampling. *Clinical microbiology and infection*. 2009; 15(1): 42-49.
- Izumikawa K. Clinical Features of Severe or Fatal *Mycoplasma pneumoniae* Pneumonia. *Frontiers in Microbiology*. 2016; 7:800 .
- Jafar N, Edriss H, Nugent K. The effect of short-term hyperglycemia on the innate immune system. *The American journal of the medical sciences* 2016; 351(2): 201-211.
- Juhn YJ. Risks for infection in patients with asthma (or other atopic conditions): is asthma more than a chronic airway disease? *Journal of Allergy and Clinical Immunology*. 2014; 134(2): 247-257.
- Jung C, Choe Y, Lee S, Kim W, Lee J, Ra S, Choi E, et al. Use of serology and polymerase chain reaction to detect atypical respiratory pathogens during acute exacerbation of chronic obstructive pulmonary disease. *The Korean Journal of Internal Medicine*. 2018; 33(5):941.
- Kumar S, Garg I, Sethi G, Kumar S, Saigal S. Detection of immunoglobulin M and immunoglobulin G antibodies to *Mycoplasma pneumoniae* in children with community-acquired lower respiratory tract infections. *Indian Journal of Pathology and Microbiology*. 2018;61(2):214.
- Lee J, Lee H, Ahn Y, Eun B, et al. Clonal Expansion of Macrolide-Resistant Sequence Type 3 *Mycoplasma pneumoniae*, South Korea. *Emerging Infectious Diseases*. 2018; 24(8):1465-1471 .

- Luchsinger V, Ruiz M, Zunino E, Martínez M, Machado C, et al. Community-acquired pneumonia in Chile: the clinical relevance in the detection of viruses and atypical bacteria. *Thorax*. 2013; 68(11):1000-6.
- Marimuthu S, Wolf L, Summersgill J. Real-Time PCR Detection of *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae* and *Legionella pneumophila* in Respiratory Specimens Using the ARIES® System. *The University of Louisville Journal of Respiratory Infections*. 2018; 2(1):3.
- Masiá M, Gutiérrez F, Padilla S, Soldán B, Mirete C, et al. Clinical characterization of pneumonia caused by atypical pathogens combining classic and novel predictors. *Clinical Microbiology and Infection*. 2007; 13(2):153-61.
- Metlay J, Waterer G, Long A, Anzueto A, Brozek J, et al. Diagnosis and treatment of adults with community-acquired pneumonia. An official clinical practice guideline of the American Thoracic Society and Infectious Diseases Society of America. *American Journal of Respiratory and Critical Care Medicine*. 2019; 200(7): 45-67.
- Miyashita N, Akaike H, Teranishi H, Kawai Y, Ouchi K, et al. *Chlamydomphila pneumoniae* serology: cross-reaction with *Mycoplasma pneumoniae* infection. *Journal of Infection and Chemotherapy*. 2013; 19(2):256-60 .
- Miyashita N, Saito A, Kohno S, Yamaguchi K, Watanabe A, et al. Multiplex PCR for the simultaneous detection of *Chlamydomphila pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumophila* in community-acquired pneumonia. *Respiratory Medicine*. 2004; 98(6): 542-550.
- Palazzolo C, Maffongelli G, D'Abramo A, Lepore L, Mariano A, Vulcano A, et al. *Legionella pneumophila*: increased risk after COVID-19 lockdown? Italy. *Eurosurveillance: European Communicable Disease Bulletin* 2020; 25(30): 200-1372.
- Raty R, Ronkko E, Kleemola M. Sample type is crucial to the diagnosis of *Mycoplasma pneumoniae* pneumonia by PCR. *Journal of Medical Microbiology*. 2005; 54(3): 287–291.

- Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, et al. Presenting characteristics, comorbidities, and outcomes among 5700 patients hospitalized with COVID-19 in the New York City area. *Journal of the American Medical Association* 2020; 323:2052–2059.
- Rivero-Calle I, Cebey-López M, Pardo-Seco J, Yuste J, et al. Lifestyle and comorbid conditions as risk factors for community-acquired pneumonia in outpatient adults (NEUMO-ES-RISK project). *British Medical Journal*. 2019; 6(1): 359.
- Romay-Lema E, Corredoira-Sánchez J, Ventura-Valcárcel P, et al. Community acquired pneumonia by *Legionella pneumophila*: Study of 136 cases. *Medicina Clinica Barcelona*. 2018; 151(7):265-269.
- Saraswathy V, Sara G, Jayasree K, Sujathan K. Comparative analysis of cell morphology in sputum samples homogenized with dithiothreitol, N-acetyl-l cysteine, cytorich® red preservative and in cellblock preparations to enhance the sensitivity of sputum cytology for the diagnosis of lung cancer. *Diagnostic Cytopathology*. 2015; 43(7): 551-558.
- Sharma L, Losier A, Tolbert T, Cruz CS and Marion CR. Pneumonia updates on *Legionella*, *Chlamydia*, and *Mycoplasma pneumoniae*. *Clinics in Chest Medicine*. 2017; 38(1):45.
- Shen Y, Chen Y, Huang Z, Huang J, Li X, Tian Z, Li J. "Associations between untraditional risk factors, pneumonia/lung cancer, and hospital fatality among hypertensive men in Guangzhou downtown." *Scientific Reports*. 2020; 10 (1): 1-9.
- Shi W, Wei M, Wang Q, Wang H, Ma C, Shi C. Rapid diagnosis of *Mycoplasma pneumoniae* infection by denaturation bubble-mediated strand exchange amplification: comparison with LAMP and real-time PCR. *Scientific Reports*. 2019; 9(1): 896 .
- Soriano J, Visick G, Muellerova H, Payvandi N, Hansell A. Patterns of comorbidities in newly diagnosed COPD and asthma in primary care. *Chest*. 2005;128: 2099–2107.
- Torres A, Peetermans W, Viegi G, Blasi F. Risk factors for community-acquired pneumonia in adults in Europe: a literature review. *Thorax*. 2013; 68(11): 1057-65.

- Vinciguerra M, Stefanetti L, Teti E, Argentieri G, Vismara LG, De Dominicis C. "Radiological features of Legionella Pneumophila Pneumonia." European Society of Radiology 2012.
- Wagner K, Springer B, Imkamp F, Opota O, Greub G, et al. Detection of respiratory bacterial pathogens causing atypical pneumonia by multiplex Lightmix® RT-PCR. International Journal of Medical Microbiology. 2018; 308(3): 317-323.
- Waites K, Xiao L, Liu Y, Balish M, Atkinson T. Mycoplasma pneumoniae from the Respiratory Tract and Beyond. Clinical Microbiology Review. 2017; 30 (3):747-809.
- Wickramasekaran R, Sorvillo F, Kuo T. Legionnaires' disease and associated comorbid conditions as causes of death in the U.S., 2000-2010. Public Health Reports. 2015; 130(3): 222-229 .
- Wijesooriya L, Kok T, Perera J, Tilakaratne Y, Sunil-Chandra N. Mycoplasma pneumoniae DNA detection and specific antibody class response in patients from two tertiary care hospitals in tropical Sri Lanka. Journal of Medical Microbiology.2018; 67(9): 1232-1242.
- Zare M, Shahram K, Mihan P. Radio-pathological correlation of organizing pneumonia (OP): a pictorial review. International Journal of Radiology. 2017; (90): 1071.
- Zhang L, Zong Z, Liu Y, Ye H, Lv X. PCR versus serology for diagnosing Mycoplasma pneumoniae infection: a systematic review & meta-analysis. Indian Journal of Medical Research. 2011;134(3):270-280.
- Ziltener P, Reinheckel T, Oxenius A. Neutrophil and alveolar macrophage-mediated innate immune control of Legionella pneumophila lung infection via TNF and ROS. Plos Pathogens 2016; 12(4): e1005591.

Tables

Table (1): Description of patients' age group data (n=84).

Data	Patients' age groups		
	Children < 18 y	Adults 18-59 y	Old age > 59 y
• Number	24	29	31
• Age Range	0.5-18 years	20-58 years	60-78 years
• Mean ± SD			
• Smoking (no-freq. %)	5.08±5.16	45.80±10.14	67.19±4.27
• COPD (no-freq. %)	0 (0%)	25 (86%)	26 (84%)
	0 (0%)	20 (69%)	15 (48%)

Table (2): Clinical, laboratory, radiological and co-morbidity data of the studied patients (n=84).

Characters	No (%)
• Inpatients	47 (56%)
• Outpatients	37 (44%)
Complaint	
• Cough	76 (91%)
• Fever	74 (88%)
Lab work	<i>Mean ± SD</i>
• 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%)

Table (3): Detected significant bacterial growth from the respiratory specimens (n=84).

Pathogen		No	frequen cy%	Cultur e	PCR
Single bacterial pathogen	<i>S. aureus</i>	5	6%	+	-
	<i>K. pneumoniae</i>	2	2%	+	-
	<i>S. pyogens</i>	5	6%	+	-
	<i>E. coli</i>	8	10%	+	-
	<i>S. pneumoniae</i>	2	2%	+	-
	<i>L. pneumophila</i>	12	14%	-	+
	<i>M. pneumoniae</i>	0	0%	-	+
	<i>C. pneumoniae</i>	0	0%	-	+
Mixed bacterial pathogens	<i>L. pneumophila</i> + <i>S. aureus</i>	3	4%	+	+
	<i>L. pneumophila</i> + <i>S. pyogens</i>	2	2%	+	+

Data are described in number of cases and frequency (%).

Table (4): Patient's demographic data in relation to presence of *L. pneumophila* (n=48).

Characteristics	Legionella + ve cases (n=12)	Legionella -ve cases (n=72)	<i>P</i>- 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*
Cough <i>Freq. (%)</i>	12 (100%)	41 (56.9%)	0.004*
Fever <i>Freq. (%)</i>	11 (91.7%)	63 (87.5%)	0.564
Hospital Inpatient <i>Freq. (%)</i>	6 (50%)	41 (56.9%)	0.443
Hospital Outpatient <i>Freq. (%)</i>	6 (50%)	31 (43.1%)	
Unilateral infiltration <i>Freq. (%)</i>	5(41.7)	52(72.2)	0.042*

Bilateral infiltration <i>Freq. (%)</i>	7(58.3)	20(27.8)	
C-reactive protein	12 (100%)	17 (23.6%)	<0.000 1*
WBCs count <i>Mean± SD</i>	13.1±2	13.3±2	0.753
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*

Figures

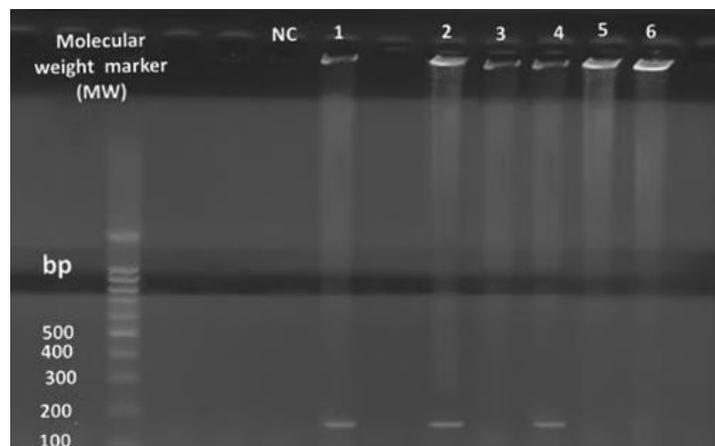


Fig. (1): Agarose gel electrophoresis showing positive PCR products for *L. pneumophila* (Lanes 1, 2 and 4).

- Molecular Weight Marker: 100 bp molecular weight markers.
- NC: negative control.
- Lane 6 is the positive control.
- Lines marked with arrows correspond to the amplicons from 157 bp copies of *L. pneumophila* macrophage infectivity potentiator (*mip*) gene.