PREVALENCE OF ATYPICAL BACTERIAL PNEUMONIA IN PATIENTS PRESENTING WITH LOWER RESPIRATORY TRACT INFECTIONS AT A TERTIARY CARE CENTRE

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ABSTRACT

BACKGROUND

A number of different viral, bacterial, fungal and protozoan organisms can cause atypical pneumonia; the three most common are Chlamydia pneumoniae, Legionella pneumophila, and Mycoplasma pneumoniae. Atypical pneumonias are by far the most underdiagnosed and underreported clinical entities, and very few studies have been reported in India. This study was conducted from March 2015 to September 2015 to determine the seroprevalence of M. pneumoniae, Chlamydia pneumoniae and Legionella pneumophila antibodies in patients with lower respiratory tract infections, and to compare IgM ELISA with that of the Sanger sequencing. Sequencing proved to be one of the rapid, sensitive and specific method of diagnosis in these cases.

MATERIALS AND METHODS

This cross sectional hospital based study was done at Government General and Chest Hospital, Erragadda, Hyderabad. The study population included 90 patients attending outpatient department and admitted as inpatients between March 2015 to September 2015 with a high probability of atypical pneumonia based on clinical symptoms and signs like fever, cough, dyspnoea, headache. Crepitations and presence of new pulmonary infiltrates on chest x-ray, in cases like COPD with acute exacerbation, bronchial asthma with acute exacerbation and Community-acquired pneumonia cases. Sputum and serum samples collected from each patient. 90 blood samples collected were subjected to ELISA to detect IgM antibody (EUROIMMUN) for Chlamydia pneumoniae, Legionella pneumophila, and Mycoplasma pneumoniae. The sputum samples showing no pathogenic organisms (NPO) were subjected to PCR for amplification of V3 region of 16 SMA followed by Sanger sequencing.

RESULTS

17 cases were positive for IgM antibodies for atypical bacteria and 8 cases were positive by Sanger sequencing. The common presenting symptoms were cough, fever, chest pain, dyspnoea and headache. More number of cases were detected in Community-acquired pneumonia patients followed by COPD and bronchial asthma with acute exacerbation.

CONCLUSION

IgM ELISA having correlated clinically with reasonable sensitivity could be a useful tool in diagnosing atypical pneumonia caused by L. pneumophila, M. pneumoniae and C pneumoniae. Sanger sequencing in comparisons of low sensitivity although highly specific can be recommended for application in discriminating species and not for diagnosis from sputum samples.

KEYWORDS

Atypical Bacteria, Sanger Sequence, ELISA.

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BACKGROUND

Community-acquired pneumonias (CAP) are caused by an infection with Streptococcus pneumonia. In 30 to 40 percent of cases, so-called "atypical pathogens" are responsible for the disease.¹ Although a number of different viral, bacterial, fungal and protozoan organisms can cause atypical pneumonia, the three most common are Chlamydia pneumoniae, Legionella pneumophila, and Mycoplasma pneumoniae.²

Mycoplasma pneumoniae are a common cause of community-acquired, respiratory tract infections, especially in children and young adults. Approximately, 10% of the cases of CAP that occur endemically, and up to 50% of the cases that occur in epidemic periods are caused by M. pneumoniae³ groups. Pneumonia due to M. pneumoniae is typically characterised by a violent and dry cough producing only sparse whitish mucus. Other symptoms include headache, malaise and sore throat.⁴ Legionnaires' pneumonia is caused by Legionella species which are responsible for 2-55% of CAP.⁵ Legionella pneumophila (92%) is the most common cause of Legionnaires' disease. Majority of clinical strains are serogroup 1 isolates of L. Pneumophila.⁶ Chlamydia pneumoniae, common cause of community-acquired respiratory infections, including bronchitis and upper respiratory tract infections, are responsible for 6-20% of all community-acquired pneumonias.⁷

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The atypical pathogens do not respond to β -lactam antimicrobial therapy. Therefore, appropriate treatment of CAP requires the identification of the infecting pathogens⁸ with rapid and sensitive diagnostic test which is essential not only for treatment but also for the implementation of preventive measures⁹ as CAP are clinically not distinguishable from other pneumonias. Timely and appropriate treatment improves the prognosis and can be achieved by rapid diagnosis.¹⁰ Thus, for better management a laboratory diagnosis is important to detect or to rule out *M. pneumoniae* infection from other respiratory infection.¹¹

Atypical pneumonias are by far the most underdiagnosed and underreported clinical entities, and very few studies have been reported in India. This study was conducted to determine the seroprevalence of *M. pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila* antibodies in patients with lower respiratory tract infections and to compare IgM ELISA with that of the Sanger sequencing.

Aims and Objectives

- To study the prevalence of infection with *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Legionella* species in patients presenting with signs and symptoms of lower respiratory tract infection.
- To diagnose atypical pneumonia with IgM ELISA [*L. pneumophila*, *M. pneumoniae*, *C. pneumoniae*] of serum samples.
  - To subject sputum samples to Sanger sequencing and to compare sensitivity of the two methods.
  - To evaluate use of these two methods in support of clinico-radiological diagnosis in diagnosis and timely treatment of cases of atypical pneumonia.

MATERIALS AND METHODS

The study was conducted during the period March 2015 to September 2015. The study group consisted of 90 patients attending medical outpatient department and admitted in Government General and Chest Hospital, Erragadda, Hyderabad.

Inclusion Criteria

- Patients with Community-acquired pneumonia.
- Acute exacerbation of bronchial asthma.
- Chronic Obstructive Pulmonary Disease.
- Age 16 to 65 years.
- Patients presenting with symptoms and signs of fever, cough, dyspnoea and headache, presence of new pulmonary infiltrates and features of interstitial consolidation on chest x-ray.
- All patients were admitted in medical wards during the period of evaluation.

Exclusion Criteria

- Hospital-acquired pneumonia.
- Bronchiectasis.
- Interstitial lung disease.
- Pulmonary tuberculosis.
- Age <16 years and >65 years.
- ICU patients and patients with cardiac symptoms.

Sputum and serum samples were collected from patients with a high probability of atypical pneumonia based on clinical symptoms and signs like fever, cough, dyspnoea, headache. Crepitations and presence of new pulmonary infiltrates on chest x-ray, in cases like COPD with acute exacerbation, bronchial asthma with acute exacerbation and community-acquired pneumonia cases. 90 blood samples collected were subjected to ELISA to detect IgM antibody (EUROIMMUN) for *Chlamydia pneumoniae*, *Legionella pneumophila*, and *Mycoplasma pneumoniae* was performed according to manufacturer's instructions.

EUROIMMUN Recommends Interpreting Results as follows-

Ratio: <0.8 negative.

Ratio: R>0.8 to 1.1 borderline.

Ratio: >1.1 positive.

Sputum Collection

Sputum samples were collected in 2 containers before the patient was started on antibiotics.

Aerosol-induced sputum collected by allowing the patient to breathe aerosolised droplets, using an ultrasonic nebuliser containing 0.85% NaCl or until a strong cough reflex is initiated. Sputum is collected from the patients in separate sterile container under aseptic conditions and transported to laboratory immediately for macroscopic examination, Gram stain and culture sensitivity. The samples which are showing no pathogenic organisms (NPO) were subjected to PCR for amplification of V3 region of 16 SMA followed by Sanger sequencing.

RESULTS

90 patients were included in the study.

Sample	No
Sputum	90
Serum	90

Table 1. Total Samples Collected (n=90)

Age	No	%
16-26	12	13.3
27-36	14	15.5
37-46	4	4.4
47-56	26	28.8
57-66	10	11.1%
67-76	4	4.4%

Table 2. Age Wise Distribution of Cases (n=90)

Sex	No	%
Males	59	59
Females	31	31

Table 3. Sex Wise Distribution of Cases (n=90)

Patients	No	%
Outpatients	54	48.6
In Patients	36	40

Table 4. Outpatient Inpatient Ratio (n=90)

Symptom	No	%
Cough	80	88.8
Fever	65	72.2
Chest Pain	18	20
Dyspnoea	42	46.6
Headache	8	8.8

Table 5. Common Symptoms (n=90)

Pathogen	No	%
Chlamydia pneumoniae	2	2.2%
Mycoplasma pneumoniae	4	4.4%
Legionella pneumophila	11	12.2%

Table 6. IgM Antibodies Detection of Atypical Pathogens by ELISA (n=90)

Pathogen	No	%
Chlamydia pneumoniae	1	1.1%
Mycoplasma pneumoniae	2	2.2%
Legionella pneumophila	5	5.5%

Table 7. Detection of Atypical Pathogens by Sanger Sequencing (n=90)

Pathogen	No	%
Streptococcus spp.	20	22.2
Streptococcus pneumoniae	16	17.7
Pseudomonas aeruginosa	8	8.8
Stenotrophomonas maltophilia	4	4.4
Prevotella spp.	2	2.2
Chryseobacterium spp.	2	2.2
Uncultured bacteria	30	33.3

Table 8. Other Bacteria Detected by Sanger Sequencing (n=90)

DISCUSSION

The study includes 90 samples of sputum and serum from each patient. Among the 90 samples collected, 31 were female patients, 59 were male patients. A slight male preponderance was seen in the present study. According to a study by Maria A. Marwetez et al, 2008, Chile,¹² there were 192 (53.8%) males and 165 (46.2%) females.

The patients' age ranged from 18 to 94 years (median 63 years). The patients included in our study group ranged from 17-76 years. The most commonly affected age group was 47-66 years followed by 27-36 years. Seroprevalence was detected by IgM antibody test using ELISA (EUROIMMUN) microplate wells coated with antigens (Detergent extract of Mycoplasma pneumoniae, strain MAC ATCC 15531, MOMP of CWI-029 strain of Chlamydia pneumoniae, LPS Legionella pneumophila strain 1-7) for IgM detection of Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila. Out of 90 serum samples, 17 samples positive for IgM antibodies for atypical bacteria identified by ELISA Mycoplasma pneumoniae 4 (4.4%), Chlamydia pneumoniae 2 (2.2%), Legionella pneumophila 11 (12.2%).

According to a study by Levent Erkan et al 2008,¹³ pathogens most commonly demonstrated in COPD were: Haemophilus influenzae (30%), Chlamydia pneumoniae (17%), and Mycoplasma pneumoniae (9%).

Out of 17 positive cases, 12 (70.5%) were male subjects and 5 (29.4%) were female subjects. The present study

detected IgM antibodies for Mycoplasma pneumoniae in 4.4% patients.

Two other recent studies have found C. pneumoniae to be associated with exacerbations in 24% (Mogulkoc et al 1999; Karnak et al 2001).

Author	Place	Year	Mycoplasma pneumoniae %
Seungioon et al ¹⁴	Korea	2002	8.6
Bansal S et al ¹⁵	India	2004	15
Oberola et al ¹⁶	India	2006	16.5
Youninglui et al ¹⁷	China	2010	20.7
EI sayedzaki M ¹⁸	Egypt	2009	5
Fang Chingliu et al	Japan	2008	3.7%
Ramachandary et al ¹¹	India	2013	4.47
Present study	India	2015	4.4%

Table 9. Studies showing Detection of Mycoplasma pneumoniae IgM Antibodies

According to a study by Rama Chaudhry et al, 2013, India,¹¹ IgM antibodies for Mycoplasma pneumoniae were detected in 4.47% of patients of Community-acquired pneumonia.

In 2004, 3.7% cases of Community-Acquired Pneumonia were detected due to Mycoplasma pneumoniae and 2.9% in year 2005, this was seen in adult population.

Present study shows similarity in prevalence when compared to these two studies. In the present study, IgM antibodies for M. pneumoniae have been noted in 2 cases of CAP and 2 cases of bronchial asthma with acute exacerbations.

Blasi et al, 2004, Europe reported a role for C. pneumoniae and M. pneumoniae infection as a trigger for 5-30% episodes of wheezing or acute asthma exacerbation.²⁰

Paraskevi Xepapadaki et al, 2008²¹ found that M. pneumoniae were associated with hospitalisation for asthma exacerbation in 18%.

Rama Chaudhry et al¹¹ found that males are more commonly affected than females, male to female ratio in 6:1.

The present study showed 4 cases positive for M. pneumoniae of which 3 (75%) cases were male and 1 (25%) female.

In our study, the patients presenting with cough were 80 (88.8%), fever 65 (72.2%), dyspnoea 42 (46.6%).

Rama Chaudhry et al 2013 found that¹¹ most common clinical symptoms are cough (91%), fever (74.5%), dyspnoea (63%), diarrhoea 19%.

Our study detected IgM antibodies for Legionella pneumophila in 12.2% of patients. S Ewing et al, 2002^{22,23} study in Europe on a large population, hospitalised with acute exacerbation of COPD, provides evidence for the first time for Legionella spp. infection as a potential underlying pathogen in as many as 16.7% of cases detected by serology.

Author	Place	Year	Mycoplasma pneumoniae %
Almudenarajas et al ²⁴	Spain	2005	29.7
Sabah Javed et al ²⁵	India	2010	15.92
Present study	India	2015	12.2

Table 10. Studies showing Detection of Legionella Pneumophila IgM Antibodies

According to a study by Sabah Javed et al, 2010,²⁶ IgM antibodies for *Legionella pneumophila* were detected in 15.92% of cases. Most common presenting symptoms are fever 80.6%, cough 96.7%, dyspnoea 58%, headache 16.1% our study showed fever (76.6%) 69 cases, cough (87.7%) 72 cases, dyspnoea (38.8%) 35 cases, headache (8.8%) 8 cases.

Almudenarojas et al, 2005²⁷ reported 29.7% positive for IgM *L. pneumophila* and that most cases are caused by serogroup-1.

In this study, ELISA kit that has been used contained antigen of *Legionella pneumophila* serogroup 1-7. Hence in cases detected by this method, disease could be caused by any of these serogroups, further correlation has been attempted with Sanger sequencing.

Direct method of diagnosis include culturing, direct fluorescent staining, and antigen detection in urine. While the first two methods display low and variable sensitivities, the latter has become a reference technique in most laboratories, enabling easy and early diagnosis of legionellosis. Indirect immune fluorescence is the most common method for serological diagnosis although serology yields good sensitivity and specificity data. The enzyme-linked immunosorbent assay (ELISA) technique generally shows higher sensitivity and better characteristics in terms of both automation and objective measurement than immunofluorescence.

In a study by Lt. Col. Dr. Agarwal et al, 2008, India *Chlamydia pneumoniae* has been discussed as a possible co-factor causing chronic obstructive pulmonary disease (COPD) and asthma. They detected IgM antibodies for *Chlamydia pneumoniae* in 18.3% of patients. In contrast to above study, our study detected only 2.2% of *Chlamydia pneumoniae* by ELISA.

According to Miyashita N et al, 1998 and Gencay M et al, 2001,^{28,29} the rate of 3% compares favourably to serological studies of *Chlamydia pneumoniae* in COPD and asthma.

Author	Place	Year	%
N Sopena et al ²⁸	Spain	1999	13.5
WS Lim et al ²⁹	UK	2001	13
Obero A et al ¹⁶	India	2006	17.6
Youninglui et al ¹⁷	China	2010	6.6
Present	India	2011	2.2

Table 11. Studies showing detection of *Chlamydia pneumoniae* IgM antibodies

According to a study by J. Nagesh, 2004, UK,³⁰ found that Serological testing is considered the most useful means of determining the prevalence of *C. pneumoniae* infection. MIF is currently the standard in *C. pneumoniae* Serology, but is subjective and requires an expert microscopist to interpret the result. Inter-laboratory variation of MIF shows an overall agreement with reference standard titres of c.80%. ELISA is more objective, can be automated. ELISA is therefore easier to standardise and is the preferred diagnostic method. ELISA may become a preferred objective test in the sero-epidemiological study of *C. pneumoniae* infection and its link with atherosclerotic vascular disease.

According to a study by Surinder Kumar et al, 2011,³¹ India, tachypnoea was documented in 9 (75%) cases; cough and coryza in 12 (100%) cases; fever in 8 (66.67%), while 4 (33.33%) cases were afebrile.

Five (41.67%) cases documented audible/auscultable wheezing and 3 (25%) crepitations. The presence of *C. pneumoniae* antibody was higher in males [10 (7.87%)] males than in females [2 (2.74%)].

Our study detected *Chlamydia pneumoniae* antibodies in 2 male patients and none in the female patients.

Urinary antigen detection has been treated as the most specific reference test for diagnosis of legionellosis, but expensive.

In view of these facts further correlation has been attempted with help of Sanger sequencing to establish diagnosis and to compare sensitivity and specificity due to the following reasons-

1. IgM antibodies to the three organisms appear in 2 week in most cases.
2. IgM antibodies cross react with other gram negative bacteria.

Hence in order to diagnose atypical pneumonia is acute stage IgM detection could be unreliable.

Above lines correlated with the study conducted by M Socan al²¹ 1999- In our study cases have been selected based on high suspicion of atypical pneumonia and patients showing positive IgM for atypical bacteria were treated with macrolides to which they responded and showed clearing of lung fields in follow up therefore clinical correlation helped to clinch clinical diagnosis and proved IgM detection as a useful tool in diagnosis. All patients received Tab Azithromycin for a period of seven days and had good recovery of symptoms.

Out of 90 sputum samples, 8 samples were positive for atypical bacteria by Sanger sequencing (PCR of 16S rRNA followed by sequencing)- *Mycoplasma pneumoniae* 2 (2.2%), *Chlamydia pneumoniae* 1 (1.1%) *Legionella pneumophila* 5 (5.4%).

Maria A. Martinez et al 2008 detected *M. pneumoniae* in 6.4% of patients by PCR (targeting 16S rRNA gene) study by Charlotte Gaydos et al 1992 showed *Chlamydia pneumoniae* positive in 5.1% of patients by PCR (targeting 16S rRNA gene).

Tsutomu Yamazaki et al 2006 Japan³² detected 19.5% of *Chlamydia pneumoniae* cases positive by PCR.

A study by Bernard la Scola et al 1997 France³³ found polymerase chain reaction (PCR) analysis with use of 16S rRNA gene primers with a broad specificity detected bacterial DNA in pus samples. Subsequent nucleotide base determination of the amplified DNA demonstrated that the detected DNA was derived from *Mycoplasma pneumoniae*. When the sequence was aligned and compared with 16S rRNA gene sequences available in GenBank, it demonstrated a 100% similarity to the 16S rRNA gene sequence.

In a M.W. Carter et al 1991 UK Computer taxonomic study, using the nucleotide and inferred amino acid sequence of the MOMP of *C. pneumoniae* IOL-207 and all known *Chlamydia* MOMP sequences supported the designation of *C. pneumoniae* as a new species. Comparative studies of the nucleotide sequences of key *C. pneumoniae* antigens with other *Chlamydia* species showed that there was 67.2-67.9% homology between the *C. pneumoniae* MOMP nucleotide sequence and *C. trachomatis* sequences and 71.5-72.4% homology with the *C. psittaci* sequences.

A study by Bertil Pettersson et al 1997 Atlanta³⁴ found that comparison of RNA sequences of the ribosomal small subunit (16S) has proved very useful. Pneumonia strains and 4 *C. pecorum* strains were obtained by semiautomated solid-phase DNA sequencing.

In a study by J.L. Cloud et al 2000,³⁵ the *Legionella*-specific PCR assay, the 16S rRNA gene was very sensitive, (100%). Their studies indicate that sequencing of all PCR-positive samples for confirmation of results provide a higher degree of specificity without a loss of sensitivity.

According to a study by M. Socan et al 1999,³⁶ selection of 16S rRNA gene sequence harbours many advantages. A large database of 16S rRNA genes is available, facilitating selection of appropriate sequences, large amplification products are generated that can easily be identified by agarose gel electrophoresis even after digestion with restriction endonucleases.

A pilot study conducted by Sandra Reuter et al³⁷ demonstrates the feasibility of using rapid whole genome sequencing (WGS) to discriminate outbreak from non-outbreak isolates of *L. pneumophila* compared with uncut typing method. It also provides high degree of discrimination for management of other bacteria such as MRSA, *Klebsiella*.

A study by Kwok-Huggchan et al³⁸ compared two sequencing methods to assess macrolide resistant *Mycoplasma pneumoniae* mutant genotypes and showed that pyrosequencing and Sanger sequencing can identify mutations.

A comparative genome analysis of *M. Pneumoniae* was done by Lixao et al³⁹ in their study by using whole genome sequencing, which showed that all 15 strains show high degree of sequencing similarity (>99%).

Taking above studies in to consideration, present study has adopted Sanger sequencing in identifying and discriminating a range of bacteria in seroprevalence samples taken from patients suspected of suffering from atypical pneumonia, although this study has been designed to identify and report prevalence of the 3 organisms *L. pneumonia*, *M. Pneumoniae* and *C. Pneumoniae*; 16S rRNA has been targeted by Sanger sequencing in order to identify other bacteria implicated in atypical pneumonia.

Sanger sequencing in available data has been employed mainly as a research tool to identify strains in *Legionella* spp., *Mycoplasma* spp., *Chlamydia* species and has reported as highly specific.

Low sensitivity of Sanger sequencing in comparison with IgM detection could be due to low bacterial load at the time of collection of the samples. As a result, this method has identified the predominant bacteria in the samples.

SUMMARY AND CONCLUSION

- Out of 90 serum samples, 17 cases were positive for IgM antibodies for atypical bacteria.
- Out of 90 sputum samples, 8 cases were positive by Sanger sequencing.
- The common presenting symptoms were cough, fever, chest pain, dyspnoea and headache.
- More number of cases were detected in Community-acquired pneumonia patients followed by COPD and bronchial asthma with acute exacerbations.
- Atypical bacterial pneumonias presenting as Community-acquired pneumonia need specific diagnosis and respond better to Macrolide antibiotics.

Serological diagnosis correlated with clinical symptoms & signs using IgM ELISA (EUROIMMUN) and has been found to be more sensitive (74%) for three organisms.

Cases from which samples showed IgM negative clinically proved to be atypical pneumonia since they were treated with macrolides discharged and on followup showed resolution of lung fields in x-ray. IgM ELISA in serum has been found to be more sensitive than Sanger sequencing in sputum samples. The reasons being low bacterial load at the time of collection which might have resulted in missing the 16S rRNA of the pathogens in question. The uncultured bacteria reported in Sanger sequencing might have potential in being implicated in aetiology of atypical pneumonia is 30%. We here by conclude that IgM ELISA having correlated clinically with reasonable sensitivity could be a useful tool in diagnosing atypical pneumonia caused by *L. pneumophila*.

M. pneumoniae and *C. pneumoniae* Sanger sequencing in comparisons of low sensitivity although highly specific can be recommended for application in discriminating species and not for diagnosis from sputum samples.

We further conclude that clinicoradiological diagnosis supported by IgM ELISA is a reliable tool in identifying cases of atypical pneumonia caused by the three organisms *L. pneumonia*, *M. Pneumoniae* and *C. Pneumoniae*.

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