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RT-PCR–Based Molecular Approach for Screening Atypical Respiratory Pathogens in Bronchoalveolar Lavage Samples at A Tertiary Care Center

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ABSTRACT
Background: Lower respiratory tract infections (LRTIs) caused by atypical pathogens such as *Pneumocystis jirovecii*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae* contribute substantially to the global health burden, particularly among immunocompromised populations. These infections are frequently underdiagnosed due to limitations of conventional microscopy and culture methods. Bronchoalveolar lavage (BAL) provides direct access to the lower respiratory tract, and when coupled with molecular diagnostics, improves pathogen detection in severe or atypical presentations. **Methodology:** A periodic cross-sectional study was conducted at a 2200-bed tertiary care hospital in South India. A total of 151 BAL samples were analysed using smear microscopy (Gomori Methenamine Silver [GMS], Toluidine Blue O [TBO], and Giemsa stains) and real-time polymerase chain reaction (RT-PCR) targeting specific genes of *P. jirovecii*, *M. pneumoniae*, and *C. pneumoniae*. Clinical data of positive patients were reviewed and findings compared with existing literature. **Results:** Of the 151 samples, *P. jirovecii* was detected in 3 cases (2.0%) and *M. pneumoniae* in 4 cases (2.6%) by RT-PCR; *C. pneumoniae* was not detected. Two *P. jirovecii* cases were positive by both PCR and smear, whereas one was detected only by PCR, underscoring the higher sensitivity of molecular methods. All *P. jirovecii* cases occurred in immunocompromised patients: adults responded to cotrimoxazole, while one paediatric case was fatal. All *M. pneumoniae* cases, in both paediatric and adult patients, responded favourably to therapy. **Conclusion:** Molecular diagnostic techniques, particularly RT-PCR, enhance the detection of atypical respiratory pathogens in BAL samples compared to smear microscopy. Early and accurate identification facilitates timely targeted therapy, reduces unnecessary empirical treatment, and improves clinical outcomes in LRTI patients.

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INTRODUCTION:

Respiratory tract infections remain a major cause of morbidity and mortality worldwide, particularly in resource-limited settings where diagnostic and therapeutic facilities are constrained[1,2,3]. Among the wide range of pathogens, atypical organisms such as *Pneumocystis jirovecii*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae* are of growing clinical concern. These pathogens often do not grow on routine culture media and may be resistant to commonly used antibiotics, making early and accurate diagnosis a challenge[4,5].

Pneumocystis jirovecii continues to be a leading opportunistic infection among immunocompromised individuals, including those with HIV, malignancies, or transplant recipients, despite advances in antiretroviral therapy and prophylaxis[3,6,7,8]. *Mycoplasma pneumoniae* is an underreported but important cause of atypical pneumonia in children and young adults, with studies from Indian tertiary centres highlighting its prevalence in acute respiratory infections[2,9]. Similarly, *Chlamydia pneumoniae* contributes to community-acquired pneumonia and exacerbations of chronic respiratory conditions, though its true burden in India is underestimated due to diagnostic limitations[1,4]. Molecular techniques such as PCR and bronchoalveolar lavage (BAL) sampling have improved diagnostic yield, but their limited availability restricts widespread clinical application[10,11].

AIMS:

To screen for the presence of atypical respiratory pathogens in the bronchoalveolar lavage samples.
OBJECTIVES:

- To do smear microscopical analysis for the presences of *P. jirovecii* by special staining - GMS, Giemsa, and OTB stain.
- To perform molecular method, Real Time PCR for the detection using the target genes mt LSU r RNA for P. jirovecii, P1 for M. pneumoniae and ompA for C. pneumoniae.
- To compare efficacy between microscopy and Real Time PCR for P. jirovecii.

MATERIALS AND METHODS:

Study Setting:
A periodic cross-sectional study was conducted between September 2024 and February 2025 in South India 2200-bedded tertiary care center. A total of 151 bronchoalveolar lavage (BAL) samples from patients with severe lower respiratory tract infections (LRTI) were included. The study was approved by the Institutional Ethics Committee (CSP-MED/24/JAN/97/02).

Inclusion and Exclusion Criteria:
Inclusion: All non-repetitive BAL samples across all age groups sent for routine bacterial culture with LRTI symptoms.
Exclusion: Repetitive samples from the same patient and inadequate samples.

Sample Processing:
All BAL samples were screened for *Pneumocystis jirovecii*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae* using microscopy and real-time PCR.
Microscopy (Three staining methods were used for P. jirovecii)
Gomori Methenamine Silver (GMS): Cyst walls appear black against a green background (gold standard).
Toluidine Blue O (OTB): Cysts appear blue-purple and round to oval.
Giemsa: Trophozoites show a central dark nucleus with pale cytoplasm.

Molecular Detection (PCR):
DNA was extracted from BAL samples using the HELINI Purefast Bacterial DNA Mini Spin Prep Kit (Cat. No. 2004) following the manufacturer's instructions. Real-time PCR for *P. jirovecii* and *M. pneumoniae* was performed using commercial kits (Cat. No. 8265 for *P. jirovecii* and Cat. No. 8222 for *M. pneumoniae*, HELINI Biomolecules). The assays targeted the *mtLSU* gene for *P. jirovecii* and the *P1* gene for *M. pneumoniae*, employing TaqMan-based technology with FAM as the probe reporter dye and HEX as the internal control. Quality control measures included positive controls provided by the manufacturer, a no-template control, and internal controls to detect inhibition. All procedures were performed in Class II biosafety cabinets with unidirectional workflow. The thermal cycling conditions were: initial denaturation at 95°C for 15 minutes (1 cycle), followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 56°C for 20 seconds, and extension at 72°C for 20 seconds. Results were interpreted based on amplification in the FAM channel (Ct 15–35 considered positive) with HEX channel amplification (Ct 11–31) confirming internal control stability.

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For *C. pneumoniae*, real-time PCR was performed using a commercial kit (Cat. No. 8225, HELINI Biomolecules) targeting the *ompA* gene with TaqMan-based technology. The thermal cycling profile consisted of an initial denaturation at 95°C for 15 minutes (1 cycle), followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 20 seconds. Interpretation criteria were similar, with amplification in the FAM channel (Ct 15–35) indicating positivity, and internal control amplification in the HEX channel (Ct 17–31) confirming validity of the run.

RESULTS:

A total of 235 BAL samples with clinical suspicion of LRTI were initially screened for *Pneumocystis jirovecii*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*. Of these, 151 samples fulfilled the inclusion criteria and were enrolled in the study.

Demographic and Clinical Characteristics:

Among the 151 non-repetitive BAL samples, 110 (72.8%) were from male patients and 41 (27.2%) from females. The age of patients ranged from 10 months to 88 years, with the highest representation in those aged >60 years (35%; n=53), followed by 41–50 years (19.9%; n=30), 51–60 years (16.6%; n=25), 31–40 years (11.3%; n=17), 21–30 years (8.6%; n=13), and 1–20 years (8.6%; n=13).

Comorbid conditions were documented in 69 (45.7%) of the enrolled patients, which included Type 2 Diabetes Mellitus, Systemic Hypertension, chronic kidney disease, Pulmonary Tuberculosis, HIV infection, and malignancy. The most common comorbidity was Diabetes (35.7%), followed by Hypertension (25.2%), Pulmonary Tuberculosis (13.9%), malignancy (8.6%), chronic kidney disease (6.6%), and HIV (1.3%) (Table 1).

Table 1. Distribution of comorbid conditions in patients with severe LRTI.

Co-Morbidity	Percentage (%)
Type-2 Diabetes	35.7
Hypertension	25.2
Pulmonary Tuberculosis	13.9
Malignancy	13
Chronic Kidney Disease	6.6
HIV	1.3

Screening for P. jirovecii:

All samples were subjected to special staining with GMS, Giemsa, and OTB stains. Of the 151 BAL samples, 2 (1.3%) were positive for *P. jirovecii* by microscopy (Fig: 1&2). Real-time PCR targeting the *mtLSU* gene detected *P. jirovecii* in 3 (2%) samples (Fig. 3). Of these, two were also microscopy-positive, while one was smear-negative but PCR-positive; notably, this sample was an endotracheal aspirate from a 10-month-old child.

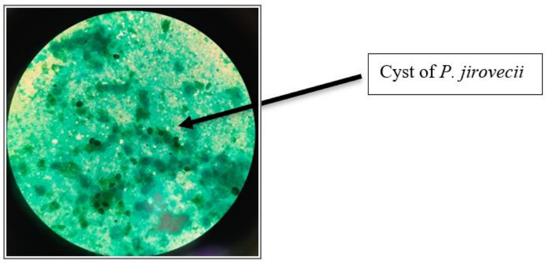


Fig 1: Microscopical analysis of GMS stain, showing Cyst of P. jirovecii.

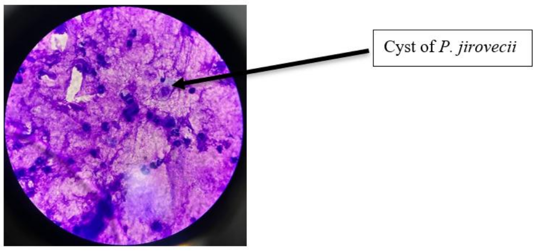
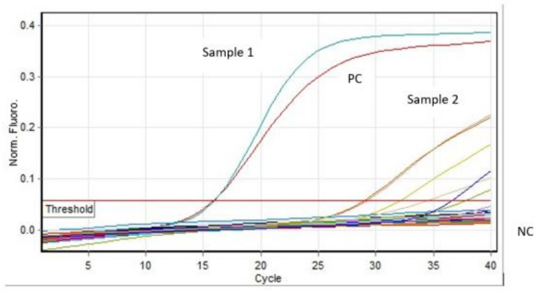


Fig 2: Microscopical analysis of OTB stain, showing Cyst of *P. jirovecii.*

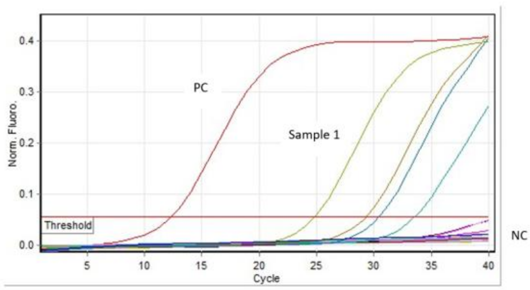


PC – Positive control, NC – Negative control.

Fig 3: Graphical amplification results of Real time qualitative PCR (TaqMan) showing Positive results of P. jirovecii.

Screening for M. pneumoniae:

Real-time PCR targeting the *P1* gene identified *M. pneumoniae* in 4 (2.6%) of the 151 BAL samples (Fig. 4).

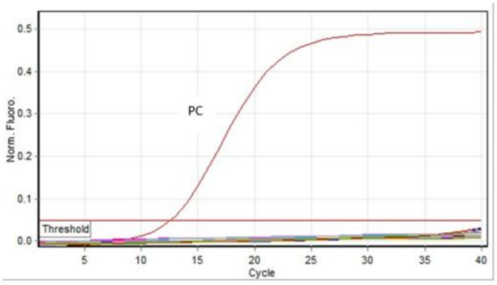


PC – Positive control, NC – Negative control.

Fig 4: Graphical amplification results of Real time qualitative PCR (TaqMan) showing Positive results of M. pneumoniae.

Screening for C. pneumoniae:

Real-time PCR targeting the *ompA* gene revealed no positive cases among the 151 samples tested (Fig.5).



PC – Positive control, NC – Negative control.

Fig 5: Graphical amplification results of Real time qualitative PCR (TaqMan) showing results of C. pneumoniae.

Incidence of Atypical Pathogens and Association with Comorbidities:

Overall, 7 of 151 samples (4.6%) were positive for atypical respiratory pathogens (*P. jirovecii* = 3; *M. pneumoniae* = 4; *C. pneumoniae* = 0). Among these, three patients (43%) had co-infections with HIV, *Burkholderia pseudomallei*, or *Cryptococcus neoformans*.

Among the *P. jirovecii*-positive patients, two (66.6%) were HIV-positive and also had T2DM, while one patient had systemic hypertension. One HIV-positive individual also had *C. neoformans* co-infection. Of the four patients positive for *M. pneumoniae*, one had both T2DM and malignancy (chronic lymphocytic leukemia), whereas no associations were found with S.HTN, CKD, PTB, or HIV. No comorbidity analysis was possible for *C. pneumoniae* as all samples were negative. Comparison of Real-Time PCR and Smear Microscopy in Detection is shown in Table 2.

Table 2. Diagnostic yield of Real-Time PCR versus microscopy.

Pathogens	No of Samples Tested	PCR Positive (%)	Smear Microscopy Positive (%)
P. jirovecii	151	3(2%)	2(1.3%)
M. pneumoniae	151	4(2.6%)	-
C. pneumoniae	151	0	-

DISCUSSION:

This study aimed to determine the prevalence and clinical outcomes of *P. jirovecii*, *M. pneumoniae*, and *C. pneumoniae* in BAL samples from patients with suspected severe LRTI. Both smear microscopy and real-time qualitative PCR were employed, which is particularly relevant as *P. jirovecii* and *C. pneumoniae* are non-cultivable, while *M. pneumoniae* requires prolonged incubation of more than 7 days)[12].

Pneumocystis jirovecii:

The prevalence of *P. jirovecii* was 2%, comparable to Pates et al. (2.2%) and lower than Matouri et al. (5.3%) [13,14]. Higher prevalence rates (up to 44%) have been reported among HIV-positive populations[3]. In our study, three patients tested positive by PCR, with two also detected by microscopy, reaffirming that PCR provides superior sensitivity [14,15]. The *mtLSU* gene target used here has shown high reliability in prior studies [16,17,18]. Two of the three cases were HIV-positive, consistent with previous reports linking *P. jirovecii* to immunocompromised states[6,19,20,21]. Detection in a non-HIV patient aligns with Orsini *et al.* (2020), who highlighted the role of immunosuppressive therapy as a risk factor[22]. Co-trimoxazole remains the first-line therapy, with corticosteroids and second-line agents reserved for refractory cases [23,24,25]. Favourable outcomes in two adults underscore the importance of early diagnosis, while the paediatric mortality observed emphasizes the need for timely intervention[26].

Mycoplasma Pneumoniae:

The prevalence of *M. pneumoniae* was 2.6%, lower than earlier studies in paediatric populations (12–14%)[27]. Our inclusion of both adult and paediatric patients may explain this difference, though the pathogen remains clinically relevant across all age groups[28]. All four positive patients responded well to azithromycin, supporting macrolides as first-line therapy in regions with low resistance[29,30]. However, rising resistance has been reported globally[31], underscoring the need for antimicrobial stewardship. A co-infection with *Burkholderia pseudomallei* in one case reflects the complexity of severe LRTIs, consistent with recent reports of polymicrobial infections[32].

Chlamydia pneumoniae

No cases of *C. pneumoniae* were detected in our study, in contrast to earlier Indian studies reporting 5–6% prevalence [33,34]. The exclusive use of BAL samples, which are less frequently obtained from Pediatric patients, may explain the lower detection rate compared to studies using sputum or nasopharyngeal swabs[27,35]. The *ompA* gene target used here remains a reliable marker for detection[36,37].

CONCLUSION:

In conclusion, our findings highlight the importance of incorporating molecular diagnostics into the routine workup of suspected atypical pneumonia, as these techniques provide greater sensitivity compared to conventional microscopy. The detection of *P. jirovecii* and *M. pneumoniae* among patients with comorbidities underscores the need for heightened clinical vigilance, particularly in immunocompromised individuals. Future studies should include large multicentre cohorts to better define the epidemiology of these pathogens in diverse Indian populations. Continuous monitoring of antimicrobial resistance in *M. pneumoniae* is essential, and integration of advanced molecular methods, including metagenomic sequencing, may further improve detection and guide targeted therapy.

CONFLICT OF INTEREST: Nil

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