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Direct Detection of Atypical Bacteria in Clinical Samples from Patients with Chronic Obstructive Pulmonary Disease (COPD) by PCR and RT-PCR

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A B S T R A C T

Chronic obstructive pulmonary disease (COPD) represents an important public health challenge and is a major cause of morbidity and mortality throughout the world. The chronic course of COPD is often accompanied by acute exacerbations of COPD (AECOPD) where 70% of exacerbations are caused by infection due to aerobic bacteria, 30% due to viruses and 5-10 % by atypical bacteria, most commonly *Mycoplasma pneumoniae (M. pneumoniae)*, *Chlamydia pneumoniae (C. pneumoniae)* and *Legionella pneumophila (L. pneumophila)*. Identification of these atypical microorganism is challenging, however, with the availability of newer molecular diagnostic techniques such as PCR and Real time PCR (RT-PCR) the sensitivity in detecting these pathogens has improved and can be tested simultaneously. So, the present prospective study was undertaken to determine the atypical pathogens in patients with COPD by PCR and RT-PCR in the clinical samples. 196 hospitalized patients were included in the study. They were categorized into different stages of COPD and AECOPD using GOLD and Anthonisen criteria. Clinical samples such as throat swab, nasopharyngeal swab, sputum, were collected. Atypical bacteria were identified by using PCR and RT-PCR methods. All these patients showed varying degree of exacerbation. Majority, 78/196 (39.79%) of the patients belonged to severe COPD category. Whereas, 96/193(49.74%) patients had only mild exacerbation. PCR was found to be positive in 11 and 22 numbers for *M. pneumoniae* and *L.pneumophila*. None of the samples were positive for *Chlamydia pneumophila.* Presence of pathogens, atypical bacteria did not show any correlation with the staging of AECOPD. The results were not statistically significant. No one method is suitable for the detection of atypical bacteria. Hence, a combination of tests for identification of Atypical pathogens is required for better sensitivity.

Introduction

Chronic obstructive pulmonary disease (COPD) is a prevalent condition characterized by persistent respiratory symptoms and occasional acute exacerbations, often resulting in hospitalization and a more rapid decline in lung function (Criner *et al.,* 2015; Global Initiative for Chronic Obstructive Lung Disease,

2021). Numerous studies, such as the observational Acute Exacerbation and Respiratory Infections in COPD (AERIS) study, have demonstrated a connection between exacerbation events and an increased prevalence of airway bacteria, with atypical bacterial infection.

Chronic obstructive pulmonary disease (COPD) presently ranks as the fourth leading cause of global mortality, but projections indicate that it will ascend to the third position by 2020. The persistent nature of COPD is frequently marked by acute exacerbations (AECOPD), primarily attributed to heightened inflammation. Existing data indicate that the cause of exacerbations remains unclear in about 30% of cases, while the remainder is attributed to respiratory tract infections (50–60%) or environmental factors (10%) (Global Initiative for Chronic Obstructive Lung Disease, 2021) (WHO, 2007).

The origin of acute exacerbations of chronic obstructive pulmonary disease (AECOPD) is diverse and remains a topic of ongoing debate. There has been longstanding controversy over whether bacteria contribute to AECOPD and, consequently, whether antibiotics have a role in managing the disease (WHO, 2007; Sethi, 2000). Numerous studies have indicated a connection between the presence of specific bacterial species, such as *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Haemophilus influenzae*, and AECOPD (WHO, 2007).

Approximately 70% of COPD exacerbations are attributed to aerobic bacterial infections, 30% to viral infections, and 5-10% to atypical bacteria (Lieberman *et al.,* 2002). The term "atypical pathogens" commonly refers to *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella pneumophila*. However, the specific role of these bacteria in acute exacerbations of COPD (AECOPD) remains uncertain.

Serological investigations propose a potential significant involvement of atypical pathogens in acute exacerbations of chronic obstructive pulmonary disease (AECOPD) (Lieberman *et al.,* 2002; Lieberman *et al.,* 2002; Lieberman *et al.,* 2001; Ewig, 2002; Lieberman *et al.,* 2001; Hahn, 1999; Beaty *et al.,* 1991).

Nevertheless, comprehending the role of these atypical pathogens in AECOPD poses challenges due to their challenging cultivation from respiratory tract specimens. Additionally, there is variability among authors in terms of the reliability and interpretation of serological assay results.

Molecular diagnostic methods, including PCR, have proven to be valuable tools for identifying the etiology of lower respiratory tract infections (Schoonbroodt *et al.,* 2023). PCR is capable of detecting small amounts of nucleic acids from a wide range of potential pathogens. Unlike culture-based methods, it is not dependent on the viability of the target microorganism and may be less influenced by prior antimicrobial treatment. Moreover, molecular techniques, particularly PCR, offer rapid results and distinct advantages over traditional tests in the identification of microorganisms such as *Mycoplasma pneumoniae, Chlamydia pneumoniae*, and *Legionella spp*. in pneumonia patients (Murdoch, 2004; Murdoch, 2003). However, with the availability of newer molecular diagnostic techniques such as PCR and Real time PCR (RT-PCR) the sensitivity in detecting these pathogens has improved and can be tested simultaneously.

So, the present prospective study was designed to determine the atypical pathogens in patients with COPD by PCR and RT-PCR from the clinical samples.

Materials and Methods

Participants

This is a prospective study.196 adult patients attending Viswanathan Chest Hospital with COPD were included in the study. Institutional Human ethics clearance was taken for this study. Written informed consent was taken from all patients. Confidentiality of data has been maintained and there is no conflicts of interest. Staging of COPD was done using GOLD criteria (GOLD, 2018).

Patient with exacerbations was also noted using the Anthonisen criteria (Anthonisen *et al.,* 1987). Clinical details regarding cough with or without production of sputum, dyspnoea, history of smoking patient, risk factors for infection, exposure to antibiotics, fever, history of prior hospitalization was assessed. Spirometry findings were also assessed for staging of COPD and AECOPD.

Subjects not willing to participate in the study were excluded.

Sample Collection

Clinical samples such as sputum, nasopharyngeal swabs, throat swabs were collected from each patients included

in the study. The specimens were subjected to appropriate laboratory investigations for atypical bacterial pathogens (*Mycoplasma, Legionella* and *Chlamydia*).

DNA isolation from clinical samples

DNA was extracted from sputum, nasopharyngeal swabs and throat swab. DNA extraction was carried out following the QIAamp DNA blood extraction kit protocol (Qiagen). Briefly, 20 µl, of QIAGEN proteinase K was pipetted into the bottom of a 1.5 µl microcentrifuge tube and 200 µl of sample was added into the microcentrifuge tube, then 200 µl AL buffer and 2 µl of internal control was added to the sample and was mixed by pulse vortexing for 15sec. The mixture was then incubated at 56° C for 10 minutes. After that 200 µl of ethanol was added to the sample and mixed again by pulse vortexing for 15 sec. The mixture was briefly centrifuged and then transferred to the QIAGEN mini spin column kept in a 2 ml centrifuge tube without wetting the rim and centrifuged at 8000 rpm for 1 minute. The spin column was then placed in another 2ml collection tube and 500 µl of washing buffer AW1 was added and centrifuged at 8000rpm for 1min. The spin column was again placed in a 2ml collection tube and 500 µl washing buffer AW2 was added and centrifuged at 13000 rpm for 3 minutes. Then the spin column was placed in a clean 1.5 ml microcentrifuge. DNA was eluted in a final volume of 55 µl, and stored at -20 °C for further use.

PCR assay targeting *M. pneumoniae P1* gene and *L. pneumophila mip* gene

Multiplex PCR assay targeting *M. pneumoniae P1* gene and *L. pneumophila mip* gene were carried out using the published protocols (Williamson *et al.,* 1992; Welti *et al.,* 2003). Two sets of primers each specific for *P1 adhesin* gene of *M. pneumoniae and* macrophage infectivity potentiator (*mip*) gene of *L. pneumophila* were used.

For *M. pneumoniae* both forward primer CAAGCC AAACACGAGCTCCGGCC and reverse primer CCAG TGTCAGCTGTTTGTCCTTCCCC and for *L. pneumophila* forward primer- GACAAGGATAA GTTGTCTTATAGC and reverse primer- ACGACCAG TGTATTCCACAG were used targeting 543 bp segment of *M. pneumoniae* and 375 bp segment of *L. pneumophila* (Fig 1,) PCR standardization was done by using *M. pneumoniae* ATCC 15531 strain and *L. pneumophila* ATCC 33152 strain. Reaction mixture was prepared in a final volume of 25 µl containing 2.5 µl of 10x PCR buffer, 0.5µl of dNTPs, 0.5 µl of Taq polymerase, 2 µl of DNA sample, 1 µl of 5 pmol/µl of each forward and reverse primers of each gene and nuclease free water to achieve desired final volume. The reaction was performed in a thermocycler (Biorad) under the following conditions. 94°C for 5 minutes, followed by 35 cycles of amplification each at 94°C for 1 minute denaturation, 55°C for 1 minute annealing and 72°C for 2 minutes extension, and a final elongation step of 72°C for 10 minutes (Table 1, 2).

Real time PCR for identification of *M. pneumoniae, L. pneumophila and C. pneumoniae*

Multiplex PCR for detection of *M. pneumoniae*, *L. pneumophila, C. pneumoniae* from the clinical samples and the positive culture were carried out by using FTD Atypical CAP kit. (FTD Bacterial pneumonia CAP (RUO)) on a CFX96, Biorad thermocycler. The assay was performed according to the manufacturer instructions.

Statistical Analysis

Statistical analysis was carried out using Stata 12.0 (College Station, Texas, USA). Data were presented as number (percentage) or mean + SD as appropriate. Chi-square/ Fishers exact test was used to test the association between severity of COPD and AECOPD with sex, AECOPD and Pathogens, AECOPD and atypical bacteria and AECOPD and comorbid conditions, Atypical bacteria with comorbid condition. The p value less than 0.05 was considered statistically significant.

Results and Discussion

A total of 196 patients admitted to Vishwanathan chest hospital with COPD were recruited into the prospective study. Clinical samples collected from these patients included 196 nasopharyngeal swabs, 196 throat swabs and 164 sputum samples.

Out of the total 196 COPD patients 44 (Mild), 38 (Moderate), 78 (Severe) and 36 (very severe) were observed). All these patients showed varying degree of exacerbation**.** Majority, 78/196 (39.79%) of the patients belonged to severe COPD category. Whereas 96/193(49.74%) patients had only mild exacerbation.

A multiplex PCR and Real time PCR for *M. pneumoniae* and *Legionella* was carried out on the DNA isolated from clinical samples. 11 and 22 were positive for *Mycoplasma* and *Legionella* respectively. Among the total number of *M. pneumoniae* isolates 11 isolates were positive by PCR. Out of these 3 were positive by RT - PCR only. (Table 3, Fig 2, 3). Out of these 12 were positive by RT PCR only. This could be because of the degradation of the DNA or could be non specific positive by PCR which couldn't be further confirmed by sequencing method.

None of the samples were positive for *Chlamydia pneumoniae* in RT- PCR and PCR.

It was observed that throat swab was a better sample than Nasopharyngeal swab in isolation of *M. pneumoniae*. This observation is concordant with other studies (Gnarpe *et al.,* 1997; Honda *et al.,* 2000). This could be because the Nasopharyngeal swabs cannot be manipulated much unlike Throat swab and the clinical material in the NP may be inadequate.

In the current study, a total of 196 COPD patients were included, with 193 patients exhibiting varying degrees of exacerbations. PCR analysis identified only 11 (5.6%) isolates as positive for *M. pneumoniae*. The potential reasons for false-negative PCR results include a bacterial load below the detection limit, sample dilution in the transport medium, or the presence of interfering DNA from human cells or other respiratory tract microorganisms that could impact amplification. Legionella was detected in 22 cases through PCR, and Real-Time PCR identified *M. pneumoniae* in three additional samples and *Legionella* in 12 more samples compared to conventional PCR. Real-Time PCR's higher sensitivity is well-established, allowing the detection of low copy numbers present in clinical samples (Gullsby *et al.,* 2008).

The study by Diederen *et al.,* (2007) aimed to explore the involvement of atypical pathogens, including *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella pneumophila*, in acute exacerbations of chronic obstructive pulmonary disease (COPD).

Real-time PCR was utilized to analyze 248 sputum samples from 104 COPD patients, comprising 122 stable-state samples and 126 samples during acute exacerbations. The results indicated no presence of *M. pneumoniae* or *C. pneumoniae* DNA in stable-state or exacerbation samples. One exacerbation sample showed positive results for *Legionella* non-pneumophila DNA. The findings suggested no clear association between these atypical pathogens and the etiology of acute exacerbations in COPD (Diederen *et al.,* 2007).

The study by Schoonbroodt *et al.,* (2023) compared real-time PCR with culture-based methods for identifying bacteria in sputum samples from chronic obstructive pulmonary disease (COPD) patients across three studies. Real-time PCR exhibited higher positivity rates for *H. influenzae and Moraxella catarrhalis* compared to culture. *Streptococcus pneumoniae* showed higher positivity rates with PCR in two studies but lower in one due to misidentification. Concordance analysis revealed lower sensitivity of culture-based methods, particularly for *H. influenzae*. Real-time PCR on frozen samples demonstrated enhanced sensitivity and specificity, supporting its use for identifying respiratory bacteria in COPD patients (Schoonbroodt *et al.,* 2023).

Culturing, especially for *M. pneumoniae*, is time-consuming and relatively insensitive, requiring 2-5 weeks for visible colonies. Serology, while an alternative, relies on the timing of sample collection and the availability of paired serum. In adults, the production of IgM may be absent, and direct IgG formation may occur early in infection or due to reinfection, making interpretation challenging. Nucleic acid amplification techniques offer rapid, specific, and sensitive results, but their increased sensitivity may lead to false positives. In the absence of a reliable gold standard method, an expanded gold standard approach, incorporating multiple tests, is recommended.

It is widely acknowledged that atypical bacteria contribute to 5-10% of AECOPD, either as primary pathogens or more commonly as co-pathogens. However, various serological studies have reported considerably higher proportions of exacerbated patients showing recent infections with *C. pneumoniae, M. pneumoniae*, or *Legionella spp*. with rates of up to 34%, 14%, and 17%, respectively. In the current study, a single patient was identified with both *Mycoplasm*a and *Legionella*.

It was anticipated that patients experiencing a severe stage of exacerbation would exhibit higher rates of pathogen isolation. However, in the current study, atypical bacteria did not demonstrate any correlation with the staging of AECOPD, and the results were not statistically significant.

Table.1 Target gene and primer sequence of *M.pneumoniae* and *L.pneumophila.*

Organism	Target gene	Forward primer	Reverse primer
Mycoplasma pneumoniae	*P1* adhesin gene	CAAGCCAAACACGAGCTCCGGCC	CCAGTGTCAGCTGTTTGTCCTTCCCC
Legionella pneumophila	*mip* gene	GACAAGGATAAGTTGTCTTATAGC	ACGACCAGTGTATTCCACAG

Table.2 PCR reaction mixture of a total volume of 25µl for a single reaction.

10x buffer	dNTPs	Taq polymerase	*P1* forward primer	*P1* reverse primer	*mip* forward primer	*mip* reverse	DNA sample	Nuclease free water
2.5µl	0.5µl	0.5µl	1µl	1µl	1µl	1µl	2µl	17.5µl

Table.3 PCR results of *Mycoplasma pneumoniae*, *Legionella pneumophila* and *Chlamydia pneumophila* according to expanded gold standard

Organism	**PCR positive(=n)**	**RTPCR positive(=n)**
Mycoplasma pneumoniae	8	3
Legionella pneumophila	10	12
Chlamydia pneumophila	**0**	**0**

Figure.1 PCR band pattern of *Mycoplasma pneumoniae* and *Legionella pneumophila A*TCC . Lane 1- 100 bp ladder marker, lane 4- p1 DNA product of *Mycoplasma pneumoniae* (543bp), lane 5- mip DNA product of *Legionella pneumophila* (375bp)

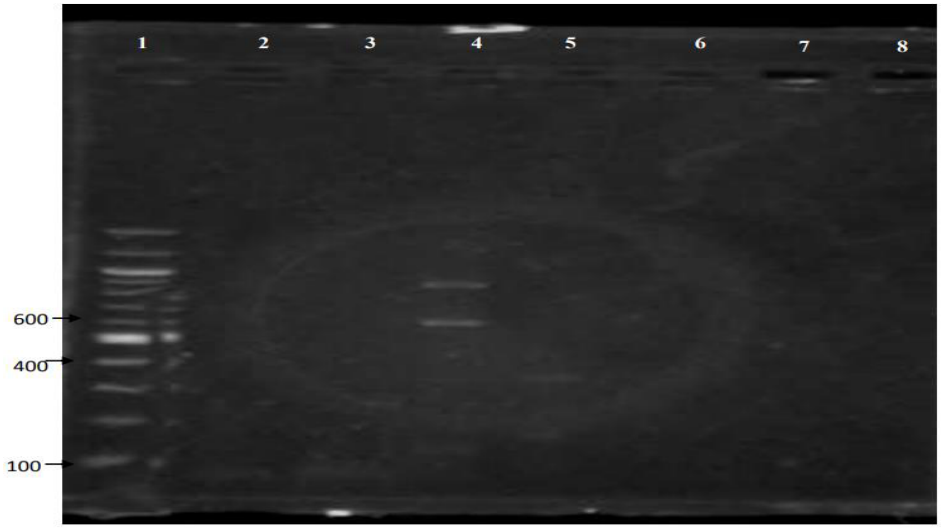


Figure.2 Identification of *Legionella pneumophila* by PCR. lane 1- 100bp ladder marker, lane 3, 7, 12 showing mip gene of *Legionella*(375bp)

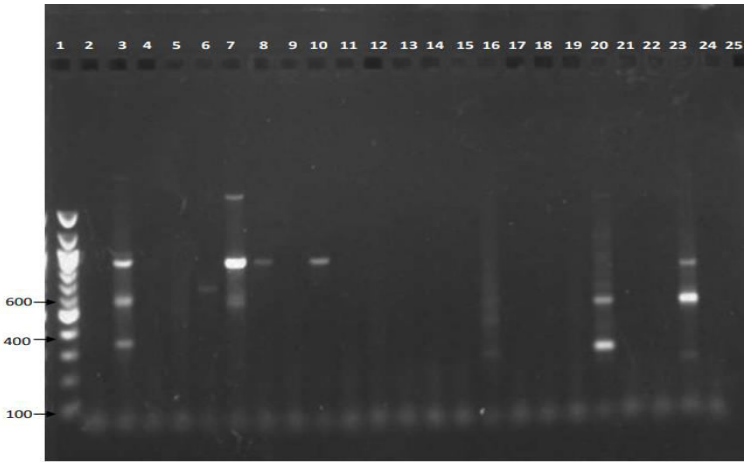
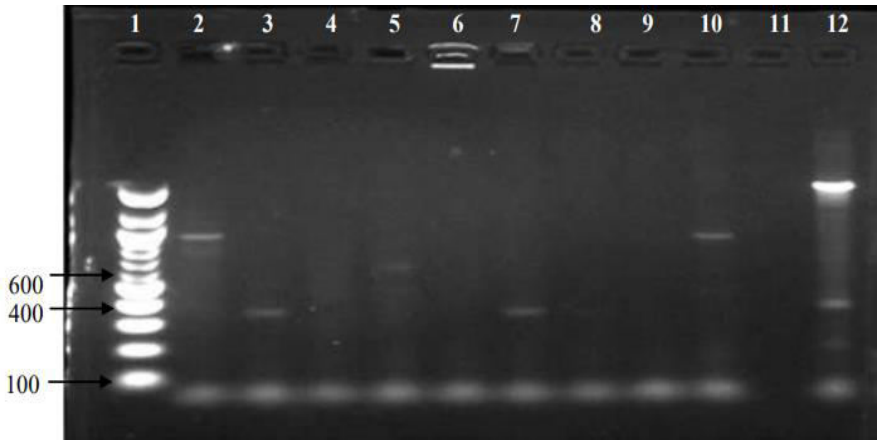


Figure.3 Identification of *Mycoplasma pneumoniae* and *Legionella pneumophila* by multiplex PCR. Lane 1-100 bp ladder marker, lane - 3, 21, 24 shows p1 gene and mip gene product of *Mycoplasma pneumoniae* (543 bp) and *Legionella pneumophila* (375 bp)



These findings align with similar studies, suggesting that the lack of correlation may be attributed to compromised lung function in severe patients, making them more susceptible to non-infectious factors such as air pollutants and heart failure, leading to quicker and exacerbated symptoms. Additionally, the study found no statistically significant correlation between staging and *M. pneumoniae*.

In conclusion, the detection of atypical bacteria necessitates a combination of tests, as no single method is sufficient, aiming to enhance sensitivity and accuracy in identification. This study provided insights into diagnostic challenges and the intricate relationship between atypical bacteria and AECOPD, emphasizing the ongoing need for research to refine diagnostic methods and unravel the complexities of COPD exacerbations.

Author Contribution

T. Sonowal: Investigation, formal analysis, writing—original draft. M. Shariff: Validation, methodology, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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