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Molecular Diagnosis of *Mycoplasma Pneumonia* Isolated from Hospitals in Hafer Al Batin, Saudi Arabia

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Abstract **Background:** *Mycoplasma pneumoniae* is often responsible for community-acquired pneumonia and Lower Respiratory Tract Infections (LRTI). **Objective:** This study aimed to develop a novel duplex real-time PCR assay to detect *M. pneumoniae*, which includes a built-in internal real-time PCR control and to assess real-time nucleic acid sequence-based amplification (NASBA) compared to conventional PCR. **Methods:** A total of 103 samples from 110 clinical patients with LRTI were analyzed using duplex real-time PCR, NASBA and conventional PCR. Clinical symptoms and laboratory findings, e.g., Erythrocyte Sedimentation Rate (ESR), C-Reactive Protein (CRP) were compared between *M. pneumoniae*-positive patients and those with other LRTIs. **Results:** Evaluation of 103 test samples revealed *Mycoplasma pneumoniae* infection in 14 (13.59%) of 110 patients with LRTI using a combination of molecular tests. A comparison of clinical symptoms existed between patients with *M. pneumoniae*-positive and those with different lower respiratory tract infections. The clinical presentation between *M. pneumoniae* infections showed various characteristics, including a lower patient age combined with chill symptoms and a higher level of ESR and CRP, levels that produced statistically significant results ($p<0.05$). Molecular diagnostic approaches performed better than other methods for the identification of *M. pneumoniae*, providing faster and more accurate results. **Conclusion:** The study concluded that PCR along with loop-mediated isothermal amplification (LAMP) provides a highly sensitive and specific diagnostic evaluation that serves as a beneficial diagnostic alternative for the clinical diagnosis of *Mycoplasma pneumoniae*.

Key Words Community-Acquired Pneumonia, Isothermal Nucleic Acid Amplification Techniques, Lower Respiratory Tract Infection, *Mycoplasma pneumonia*, PCR

INTRODUCTION

Pneumonia, a type of acute respiratory tract infection (ARTI), affects the lungs by filling the alveoli with pus and fluid, leading to painful breathing and reduced oxygen intake [1-3]. The most common causes of pneumonia are bacterial and viral infections, *Streptococcus pneumoniae, Haemophilus influenzae* type b (Hib) and Respiratory Syncytial Virus (RSV) being the main pathogens [4].

Pneumonia can affect people of any age and global data indicate that approximately 30% of pneumonia patients require hospitalisation. In 2014, pneumonia was the most common contributing cause of death in patients with Chronic Obstructive Pulmonary Disease (COPD) and accounted for a third of deaths related to dementia. With an ageing population, the burden of pneumonia is expected to increase. Traditionally, pneumonia has been diagnosed using serological methods, but molecular techniques are increasingly being used. However, there are a limited number of studies that compare these diagnostic approaches. This research outlines the establishment of a new duplex real-time PCR assay for the detection of *Mycoplasma pneumoniae*, incorporating an internal amplification control to ensure result accuracy and assay reliability [2,5]. Molecular techniques offer advantages, such as improved sensitivity and faster microbiological diagnosis.

Pneumonia refers to an inflammatory condition of the alveoli, typically arising from infectious causes. The disease may be classified by its etiology such as bacterial, viral, or fungal origin by the context in which it develops, including community-acquired, hospital-acquired, or healthcare-related, or by its underlying pathophysiological mechanism, such as aspiration or immune suppression [6,7]. *Mycoplasma pneumoniae* accounts for 6-20% of cases of Community-Acquired Pneumonia (CAP) and Lower Respiratory Tract Infections (LRTIs), especially among adolescents and adults. In certain adult populations, the prevalence can be as high as 30%, depending on the diagnostic techniques employed. While serology has been the conventional diagnostic tool, molecular-based methods are

increasingly adopted in current practice. Laboratory identification of *M. pneumoniae* remains essential because its clinical features often mimic those of other pneumonia-causing pathogens. Moreover, as this organism is inherently resistant to β-lactam antibiotics, which are widely used for empirical LRTI treatment, rapid and accurate diagnostic approaches are necessary to guide effective antibiotic selection [8,9].

Mycoplasma species are unique in that they lack cell walls, making them invisible on Gram staining. They are among the smallest free-living organisms in nature. Of the more than 120 known *Mycoplasma* species, only 13 have been isolated from humans and only 4 are known to cause disease in humans. *M. pneumoniae* is the most common pathogenic species in humans. Although it can be cultured in serum-supplemented media, this process is challenging and time-consuming, making it impractical for routine clinical use. Furthermore, *M. pneumoniae* can persist in the respiratory tract for weeks after acute infection, which means that its isolation does not necessarily indicate acute infection [10-13].

Pneumonia continues to pose a significant global health burden, with substantial morbidity and mortality rates. Effective clinical management requires precise identification of the causative pathogens, yet conventional microbiological methods often prove inadequate in this regard. In recent years, the adoption of molecular diagnostic assays has greatly enhanced the detection of respiratory pathogens. Meanwhile, growing evidence regarding the lung microbiome now understood as a dynamic microbial community has reshaped perspectives on pneumonia pathogenesis. This evolving knowledge compels microbiologists and clinicians to adapt by incorporating advanced diagnostic techniques into everyday practice [1,14,15]. In light of these considerations, the current study aimed to develop an innovative duplex real-time PCR assay for the detection of *Mycoplasma pneumoniae*, incorporating an internal amplification control to ensure assay reliability. Furthermore, this study investigated the application of real-time nucleic acid sequence-based amplification (NASBA) using the iCycler system as an alternative molecular approach for pathogen detection.

METHODS

Study Design, Period and Setting

The study was carried out in the laboratory of the Hospital affiliated with health affairs in the Hafr Al Batin Governorate, Saudi Arabia. These hospitals were chosen because patient turnover was satisfactory for the study. The total sample size is 110 males and females from the previously mentioned setting. Provided informed consent was given, patients attended previous hospitals for the general practitioner. Over 24 months, from February 2022 to February 2024. They were selected according to the following inclusion criteria: Age ranging from 18-60 years, patients have pulmonary auscultation abnormalities. Patients have clinical data such as fever or dyspnea or cough, nausea and vomiting, malaise, shaking, or chills. A chest radiograph was performed on each patient. Willing to participate in the study.

Table 1 provides an overview of the bacterial strains employed to assess the analytical specificity of the developed real-time PCR assay. Prior to nucleic acid extraction, all bacterial isolates were suspended in a 0.9% sodium chloride solution. These isolates were also included in a proficiency testing panel as part of the quality control measures for the M. pneumoniae PCR assay, which was conducted during the initial phase of the national external quality assessment for clinical laboratories. During the first patient encounter, cotton-tipped swab samples were collected by the investigator, either at the healthcare facility or directly from patients at their residences.

Cotton-tipped swab samples were obtained from patients visiting the hospital clinic, immediately processed in the laboratory on the day of collection, and stored at -70°C prior to nucleic acid extraction for molecular testing. Blood samples were obtained during the patients' initial hospital visit, followed by another collection after an interval of 10–14 days. The separated serum was stored at –20°C for subsequent serological testing. Furthermore, sputum samples were gathered whenever patients were capable of producing them.

Nucleic Acid Isolation

Genomic nucleic acids were extracted from *Mycoplasma pneumoniae*–positive samples, clinical specimens, and other bacterial isolates using the QIAamp DNA extraction kit in accordance with standard procedures. The procedure was carried out in accordance with the manufacturer's guidelines, yielding 200 µL of purified nucleic acids that were stored at –20°C until further use. Each amplification run included a negative control in which sterile distilled water was substituted for the clinical specimen. Conventional PCR amplification was conducted with specific primers; briefly, 10 µL of extracted DNA was amplified through 40 cycles using primers targeting the *P1* gene. The thermal cycling program began with an initial denaturation at 94°C for 3 minutes, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 45 seconds at 72°C, and concluded with a final extension at 72°C for 10 minutes. Amplified products were identified via an enzymatic reaction employing a *P1*-specific probe labeled with digoxigenin. To monitor potential inhibition and ensure internal control accuracy, *M. pneumoniae*–spiked samples were amplified separately under the same conditions.

Table 2 outlines the primer and probe sequences developed for the *Mycoplasma pneumoniae* real-time PCR assay. The molecular beacon and primer designs were derived from the *P1* cytadhesion gene sequence of *M. pneumoniae*, following the standard parameters recommended for molecular beacon assay development. The Primer3 software was used to generate primers while avoiding secondary structures, and sequence specificity was confirmed using BLAST searches. Each probe was labeled with 6-carboxy-fluorescein (FAM) at the 5′ end as a reporter and Dabcyl at the 3′ end as the quencher. Primers and molecular beacons were synthesized by Biolegio. For internal control, the assay incorporated primers and probes targeting phocine herpesvirus (PhHV), previously used to detect PCR inhibition, and redesigned in a molecular beacon

Table 1: Bacterial species and their strains and source

Species	Strain or type	Source*
Mycoplasma pneumoniae	ATCC 29085 (PI 1428)	ATCC
Mycoplasma genitalium	ATCC 33530 (G-37)	ATCC
Mycoplasma buccale	NC10136	NCTC
Mycoplasma hominis	NC10111	NCTC
Mycoplasma fermentans	NC10117	NCTC
Mycoplasma orale	NC10112	NCTC
Legionella pneumophila	ATCC 33152	ATCC
Mycoplasma salivarium	NC10113	NCTC
Streptococcus pneumoniae	ATCC 49150	ATCC
Streptococcus pyogenes	ATCC 12344	ATCC
Moraxella catarrhalis	ATCC 25238	ATCC
Haemophilus influenzae	ATCC 43065	ATCC
Staphylococcus aureus	ATCC 12600	ATCC
Klebsiella pneumoniae	ATCC 13883	ATCC
Enterococcus faecalis	ATCC 12984	ATCC
Escherichia coli	ATCC 11775	ATCC
Enterobacter aerogenes	ATCC 13048	ATCC
Pseudomonas aeruginosa	ATCC 10145	ATCC
Neisseria meningitidis	ATCC 13090	ATCC

*ATCC: American Type Culture Collection, Antwerp, Belgium, NCTC: National Collection of Type Cultures (Central Public Health Laboratory, London, England)

Table 2: PCR primers and probes used in the study

Real-time PCR	Target	Probe or Primer	Sequence
	Mpn P1	ATTCGCGAACATAATAATGA	Upstream primer
	Mpn P1	CTTTGACATAGTCCGTGAAG	Downstream primer
	Mpn P1	CGTGCCCCAAAGCCACCCTGATCACCCGGCAGC-Dabcyl	Molecular beacon
	PhHVgB	TTGCATTTAAAACCCTCAAA	Upstream primer
	PhHVgB	GACGCCCCTGGTTTTTAACGTACGGGAACTGGCGAC-BHQ2	Molecular beacon

format. To facilitate multiplexing, the PhHV assay was optimized under experimental conditions similar to those used for the *Mycoplasma* real-time PCR assay.

Real-time PCR amplification was conducted in a total volume of 50 µL comprising 25 µL of Platinum Supermix (Invitrogen), 3.5 mM $MgCl_2$, 0.4 µM of each *Mycoplasma* primer, 0.2 µM of each PhHV primer, 0.34 µM of the *Mycoplasma* molecular beacon, 0.2 µM of the PhHV molecular beacon, and 10 µL of template DNA. Amplification, fluorescence detection, and subsequent data interpretation were carried out using the iCycler iQ real-time detection system. To validate the duplex PCR assay, each clinical specimen was supplemented with 10^3 copies of PhHV, which were co-extracted and processed concurrently with the sample.

Variability of Inter- and Intraassay

Genomic DNA was extracted from the *Mycoplasma pneumoniae* strain ATCC 15492 (*Mycobacterium avium* complex) and stored in AE buffer containing 50 mM sodium acetate (pH 5.3) and 10 mM EDTA (pH 8.0). The extracted DNA was then diluted to a final concentration of 50 CCU per 100 µL and aliquoted into small portions for preservation at –20°C. One aliquot was later utilized to evaluate both inter-assay and intra-assay variability.

NASBA as an Isothermal RNA Amplification Technique

RNA polymerase initiates transcription at the promoter site specified for the target RNA through the use of sequence-specific primers. The primers and molecular beacons were designed based on conserved regions within the 16S rRNA gene of *Mycoplasma pneumoniae*. NASBA reactions were conducted using the NucliSens Basic Kit according to standard protocols. To synthesize double-stranded DNA, 5 µL of template RNA was mixed with 10 µL of a primer-containing reaction solution, followed by incubation at 65°C for 2 minutes and subsequent cooling to 41°C for another 2 minutes. Afterward, 5 µL of an enzyme mixture containing T7 RNA polymerase was added, and the reaction was maintained at 41°C. The final concentrations in the mixture included 100 mM KCl, 0.2 µM of the molecular beacon, and 0.2 µM of each primer. Amplification monitoring and data acquisition were carried out using the iCycler iQ real-time detection system.

Validity and Reliability

The validity of the questionnaire was examined by a panel of five microbiology experts, which included three professors from the Clinical Microbiology Department of Hafr Al Batin University. In addition, two professors from the Department of Clinical Microbiology at Hail University reviewed the tool to assess its efficiency and content validity, as well as to highlight possible obstacles and issues that could occur during the data collection process.

Statistical Analysis

Data and results were compiled, verified, and organized for computer processing. Following coding, the data were analyzed and summarized in tabular form. Statistical evaluation and data entry were performed using SPSS version 23 and Microsoft Excel software. Appropriate statistical tests were applied to determine the presence of

significant differences among study variables, with a p-value ≤0.001 considered statistically significant. The results were classified as true positives for *Mycoplasma pneumoniae* based on conventional PCR outcomes. Sensitivity and specificity for each assay were calculated in accordance with the analytical results obtained during the study.

RESULTS

The results illustrated in Table 1 confirm the identification of *Mycoplasma pneumoniae* types 1 and 2 through three distinct molecular approaches: real-time PCR, real-time nucleic acid sequence-based amplification (NASBA), and conventional PCR analysis. Sensitivity analysis was performed using serial dilutions of DNA extracted from the *M. pneumoniae* ATCC reference strain, confirming the assay's reliability and detection efficiency. Tests using Tris buffer solutions showed a minimum detection threshold of 5 CCU/100 µL through 10-fold serial dilutions. During the Belgium proficiency panel, real-time PCR accurately identified the result in 19 out of 20 samples, with only one low positive sample yielding an inaccurate result. The multiplex assay's reproducibility was evaluated by analyzing samples containing 50 CCU/100 µL in five repetitions, assessing both interassay and intraassay consistency. The standard sample variation was 0.7, based on threshold cycle (Ct) values in five successive runs. The average intra-assay variation was recorded at 0.28, a range from 0.1-0.7.

Evaluation of PCR Inhibition

Table 3 demonstrates that co-amplification with PhHV yielded Ct values for *M. pneumoniae* similar to those obtained without spiking. Implementing proper controls is essential for reliable PCR-based diagnosis of clinical samples. In addition, careful monitoring of the DNA extraction procedure and identification of potential inhibitors are necessary alongside specific PCR controls. In this assay, duplex real-time PCR was performed with PhHV spike amplification serving as an internal control. A serial dilution of PhHV was amplified concurrently with *Mycoplasma pneumoniae*, and the reaction's efficiency as well as sensitivity showed no notable variation in the presence of pneumonia target sequences. To maintain assay consistency, a fixed volume of PhHV dilution was added to the lysis buffer immediately after nucleic acid extraction as part of the standardization process.

Clinical Evaluation of *M. pneumoniae* Real-Time PCR

As shown in Table 4, this illustrates that 130 adult patients were examined and diagnosed by X-ray for lower respiratory tract infections. Full sets of samples were obtained from 103 of these adults, allowing the execution of serological and three molecular assays. From the 103 clinical samples, 14 (13.59%) showed a positive result in real-time PCR. These results were further validated using conventional PCR and NASBA assays, both of which targeted distinct genomic regions to confirm the accuracy of detection.

Table 5 provides clinical data, revealing that even with a limited number of positive samples, evaluation was conducted *M. pneumoniae* diagnoses were based on PCR results. A comparison between *Mycoplasma pneumoniae*–positive patients (n = 14) and those who tested negative (n = 96) indicated that younger age, the presence of chills, and elevated inflammatory markers—specifically an increased erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels exceeding 50 mg/L—were potential indicators of *Mycoplasma* infection. Conversely, rhinitis was found to be significantly more common among *Mycoplasma*-negative individuals ($p<0.001$). Furthermore, patients with *Mycoplasma* infection did not have pulmonary disease, nausea, vomiting, fever, or chills. No significant differences

Table 3: Serial dilution series showing the Ct values of *Mycoplasma pneumoniae* obtained from real-time duplex PCR amplification performed with and without the addition of the PhHV spike

CCU of *M. pneumoniae*/ mL	Ct value	
	Without spike	With spike
10,000	27.1	37.1
1,000	21.4	20.2
100	32.2	27.1
10	33.4	29.6

Table 4: Laboratory assay findings with positive results for each (n = 14)

Titer for:					Result of:		
	Sample				Real-time		
Patient No.	Serodia sample 1	Serodia sample 2	CFT sample 1	CFT sample 2	PCR	PCR	NASBA
1	332	1.241	<8	65	+	+	+
2	80	>2.561	35	>129	+	+	+
3	80	80	8	129	+	+	+
4	321	>2.561	33	>129	+	+	+
5	321	641	33	>129	+	+	+
6	80	80	<8	<8	+	+	+
7	80	80	<8	<8	+	+	+
8	321	641	<8	<8	+	+	+
9	>2.561	>2.561	>129	>129	+	+	+
10	80	>2,560	9	129	+	+	+
11	80	80	9	129	+	+	+
12	321	641	<8	<8	+	+	+
13	321	641	<8	<8	+	+	+
14	80	80	<8	<8	+	+	+

For Serodia assays, a titer of = 321 represented a positive outcome; for CFT, a titer of = 129 or a four-fold increase in titer represented a positive result

Table 5: Clinical results for the Mycoplasma positive and negative groups (n = 103)

Characteristics	No. of patients with: Positive Mycoplasma interval) PCR results (n =14)	Negative Mycoplasma PCR results (n = 89)	P	Odds ratio confidenceinterva
Sex (female)	5 (36%)	53(59.6%)	0.16	0.5 (0.1-1.5)
Fever	0	52(58.43%)	0.012	-
shaking or chills	1(10%)	20 (22.27%)	0.22	-
Sputum	0	75(84.26%)	0.057	3.6 (1.0-12.5)
No sputum	7(50%)	19(21.34%)	0.22	0.1 (0.1-12.4)
Rapid heartbeat	-	59(66.29%)	0.057	0.4 (0.1-0.5)
Rhinitis	6(50%)	53(59.55%)	<0.001	0.5 (0.0-0.4)
Cough	0(0%)	19(21.34%)	0.14	3.5 (1.0-12.4)
Vomiting, nausea	0(0%)	19(21.34%)	0.22	-
Painful lymph nodes	0(0%)	19(21.34%)	0.60	-
Chest pain	0(0%)	19(21.34%)	0.04	-
Difficulty or Shortness of breath	0(0%)	17(19.10%)	0.26	-
Infiltrate on chest X-ray (n = 100)	5(35)	17(19.10%)	0.26	-
CRP[a] ≥50 mg/liter (n = 101)	12(90(100%)	49(55.05%)	0.04	3.2(0.7-8.4)
ESR[b] (n = 102)	14 (100%)	53(59.55%)	0.004	5.1(1.1-25.7)

[a]CRP: C-reactive protein, [b]Normal ESR levels were adjusted for age and sex as follows: for women age 19-52 years = (0-25) mm/h, for women age 52-60 years = (0-30), for men age 19-52 years = (0-15), for men age 52-60 years = (0-20), for men and women over 60 years of age = (0-40)

were statistically observed between patients with negative and positive *Mycoplasma* on clinical characteristics.

DISCUSSION

Mycoplasma pneumoniae is recognized as a frequent etiological agent of community-acquired pneumonia (CAP) and is transmitted between individuals through respiratory droplets during close contact. Because the infection may present subclinically or with mild symptoms that do not require hospitalization, its actual incidence is likely underestimated. As reported by Abdulhadi and Kiel (2017), approximately 5–10% of the population is infected with *M. pneumoniae*, which can affect individuals across all age groups, predominantly causing upper and lower respiratory tract infections in those aged between 5 and 40 years.

The findings of this study indicated that sensitivity assessment using DNA dilutions of *Mycoplasma pneumoniae* established a detection threshold of 5 CCU per 100 µL, determined through serial tenfold dilutions prepared in Tris buffer. The standard sample exhibited a variability of 0.7, as calculated from cycle threshold (Ct) values obtained over five successive runs, while the mean intra-assay variation was 0.28, ranging from 0.1 to 0.7. In addition, a real-time PCR assay incorporating an internal control and designed to target the *P1* adhesion gene was established to improve the accuracy and reliability of *Mycoplasma pneumoniae* detection. Compared to conventional PCR, fluorescence-based real-time assays offer clear advantages. The use of fluorescent probes eliminates the need for post-PCR processing, thereby increasing assay specificity and reducing the risk of carry-over contamination. Furthermore, real-time PCR markedly reduces the time to results, which translates into improved clinical management of pneumonia patients [16-18].

Studies by Ferwerda *et al.* [19], Loens [20], Medjo *et al.* [21], and Dash *et al.* [22] reported that culture and serology have traditionally been the main laboratory techniques for identifying *M. pneumoniae* as a causative pathogen. However, PCR has been shown to be superior to conventional approaches [23,24], and more recently, NASBA has also proven to be a reliable alternative. The real-time PCR assay for *Mycoplasma pneumoniae*, which demonstrated high specificity and analytical sensitivity, was clinically evaluated in patients with LRTI. Out of 110 individuals, 14 (12.73%) were found positive, aligning with the observations of Lieberman *et al.* [25] and Ieven *et al.* [26], who also described similar prevalence in adult populations. Other investigations from Ohio and Spain further highlighted the variability in CAP cases, with infection rates ranging from 15-30% in some studies to as low as 1-3% in others. All *Mycoplasma pneumoniae*–positive results obtained through real-time PCR were further verified using conventional PCR assays [27–29]. Because both assays target the same gene, additional confirmation was performed using the NASBA technique. The NASBA reaction, which amplifies the 16S rRNA region, consistently yielded positive outcomes that showed 100% concordance with those obtained from other molecular detection methods. A major advantage of NASBA lies in its capacity for real-time detection of amplified products through fluorescent probe technology, with the entire isothermal amplification and detection process conducted on the iCycler iQ system. Additionally, NASBA is capable of identifying both *M. pneumoniae* types 1 and 2, as well as *M. genitalium*, due to similarities within their 16S rRNA sequences. Nevertheless, although previous reports have suggested the presence of *M. genitalium* in the respiratory tract, no such evidence was found in the present study, as all NASBA-positive samples were also confirmed by real-time PCR [30–32].

Molecular diagnostic techniques for *Mycoplasma pneumoniae* demonstrated superior accuracy compared to conventional serological testing. The inclusion of convalescent-phase samples enhanced the overall assay sensitivity to 67%. In the PPA assay, a titer value of ≥320 was defined as the cutoff for a positive diagnosis. However, in several cases with an initial acute-phase titer of 160, no subsequent rise in antibody levels was detected in the convalescent-phase sera. Consequently, a titer of 320 serves as a more dependable marker for *M. pneumoniae* infection,

especially since convalescent-phase samples are not always available in diagnostic laboratories. Notably, one specimen that tested negative by serology was found positive through molecular testing. In this case, the two serum samples were obtained merely eight days apart, a short interval that may have contributed to the reduced sensitivity observed in the second measurement. These findings align with those of Dorigo-Zetsma *et al.* [33], Herrera *et al.* [34], and Yan *et al.* [35], who reported that in certain contexts, serology has yielded more positive results than PCR techniques. All serology-positive samples were also confirmed positive by PCR and NASBA assays. These findings align closely with the meta-analysis of Cai *et al.* [36], which reported pooled sensitivity and specificity values of 0.90 and 0.98, respectively. The analysis further indicated a PLR of 31.25, an NLR of 0.10, a DOR of 399.32, and an AUC of 0.9892 [37]. Similarly, Huang *et al.* [37], in another meta-analysis, demonstrated that NASBA-based detection of *M. pneumoniae* infection achieved a sensitivity of 0.77, specificity of 0.98, NLR of 0.22, PLR of 50.38, DOR of 292.72, and an SROC of 0.9875.

CONCLUSIONS

The key findings of this research can be summarized as follows: (i) the assay's efficiency and sensitivity were not notably affected by the presence of pneumonia target sequences; (ii) incorporation of a defined concentration of PhHV dilution into the lysis buffer before nucleic acid extraction proved to be an effective internal control measure; (iii) rhinitis was found to be significantly more frequent among *Mycoplasma*-negative individuals ($p<0.001$); (iv) none of the *Mycoplasma*-positive participants exhibited symptoms such as pulmonary complications, nausea, vomiting, fever, or chills; and (v) no statistically significant variations were detected in the overall clinical characteristics between *Mycoplasma*-positive and *Mycoplasma*-negative patient groups. It is strongly advised that microbiologists and treating clinicians collaborate to determine which molecular diagnostic assays should be employed and which pathogens should be prioritized. Further studies are needed to evaluate the performance of these methods and to explore how novel molecular technologies can advance diagnostic testing for respiratory pathogens. A structured protocol addressing these issues should be designed and validated in clinical settings.

Ethical Statement

A facilitation letter for the researcher was issued by the Dean of the College of Applied Medical Sciences and sent to the hospital administration to ensure permission and support for collecting the required samples for the research study. Informed consent was obtained verbally from all participants after a thorough explanation of the study's objectives, significance, and procedures. Participants were assured of the confidentiality and privacy of their information, and that their data would not be used for purposes outside the scope of the study. Participants were also informed of their right to withdraw from the study at any time without any impact on their services or relationship with the hospital. All established ethical principles in scientific research were strictly followed to safeguard the rights and well-being of the participants.

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