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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 study describes the development of a novel real-time PCR duplex assay to detect *M. pneumoniae*, which incorporates an internal control for real-time PCR [2,5]. Molecular techniques offer advantages, such as improved sensitivity and faster microbiological diagnosis.

Pneumonia is characterised by inflammation of the alveolar spaces, usually caused by infection. It can be classified according to the aetiology (bacterial, viral, or fungal), the setting in which the infection was acquired (community acquired, hospital acquired or healthcare-associated), or pathophysiology (e.g., aspiration or immunosuppression related) [6,7]. *Mycoplasma pneumoniae* is responsible for 6 to 20% of Community-Acquired Pneumonia (CAP) and Lower Respiratory Tract Infections (LRTI), particularly in older children and adults. The incidence of *M. pneumoniae* in adults can reach 30%, depending on the population studied and the diagnostic

methods used. The diagnosis traditionally relies on serology, but molecular techniques have become more prominent in recent years. Laboratory confirmation of *M. pneumoniae* is crucial, as its clinical presentation is not significantly different from that of other pathogens that cause pneumonia. Since *M. pneumoniae* is resistant to ß-lactam antibiotics, which are commonly used for the empirical treatment of LRTI, rapid diagnostic methods are essential to prescribe effective antibiotics [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 remains a global health challenge with high rates of morbidity and mortality. Identifying causative pathogens is critical to optimal clinical management of pneumonia patients, but poses a significant challenge for conventional microbiological methods. The development and application of molecular diagnostic tests have marked a major advance in the detection of respiratory pathogens in recent years. However, emerging knowledge about the lung microbiome, which reveals the lungs as a dynamic microbial ecosystem, challenges our current understanding of pneumonia. This new perspective presents both microbiologists and clinicians with the challenge of integrating advanced diagnostic technologies into clinical practice [1,14,15]. Therefore, the current study was carried out to develop a novel duplex real-time PCR assay to detect *M. pneumoniae* that includes a built-in internal real-time PCR control. The study investigates the implementation of real-time nucleic acid sequence-based amplification (NASBA) using an iCycler instrument as the second detection method.

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, this study was approved by the Research Ethics Ethics Committee of the Hafr Al Batin University of Medical Sciences in Saudi Arabia on 10 February 2022 (Approval No. H-2022-034). 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 reveals the data of the bacterial strains used to test the specificity of the real-time PCR. A suspension of colonies of all bacterial isolates was made in 0.9% NaCl before nucleic acid extraction. This strain was presented in a proficiency panel on behalf of quality control of a *M. pneumoniae* PCR used in the first national external quality assessment for laboratories. The investigator took a cotton-tipped swab sample in the clinic or at home from each patient at the first visit.

The investigator takes a cotton-tipped swab sample from patients in the hospital clinic managed in the laboratory directly on the day of collection and stored at 70°C before nucleic acid isolation for molecular assays. Blood samples were taken from each patient at the first hospital visit and another blood sample was taken after 10 to 14 days. The sera were stored at 20°C and used for serological tests. The sputum was collected if the patient produced it.

Nucleic Acid Isolation

A QiaAmp DNA kit was used to extract nucleic acid isolation from *M. Pneumonia*-positive material, clinical specimens and other bacterial isolates. All samples were extracted as instructed by the manufacturer, resulting in 200 µL of purified nucleic acids stored at 20°C. In each run, negative controls were included. Instead of a specimen, sterile distilled water was added for control. Conventional PCR. Using primers, PCR amplification was performed. Briefly, 10 µL of isolated DNA was amplified for 40 cycles with P1-specific gene primers. The cycling conditions were 3 min at 94°C, followed by 40 of 30 s cycles at 94°C, 30 s at 65°C and 45 s at 72°C, followed by a hold at 72°C for 10 min. The product was detected by an enzymatic reaction with a specific probe for product P1; digoxigenin was labelled as the probe. Spiking of samples with *M. pneumoniae* was evaluated for inhibition and control in a separate amplification.

Table 2 demonstrates the primers and probes for *Mycoplasma* real-time PCR. The primer and molecular beacon sequences were selected from the P1 cyt adhesion *Pneumoniae* gene sequence of M, using the criteria required for the design of molecular beacon assays. Using the primer 3 programme, the PCR primers were designed to ensure the absence of secondary structures. To verify the specificity of the DNA sequences of the primers and probes, an ABLAST search was carried out. 6-carboxy-fluorescein (FAM) was the fluorescent reporter on the 5th end of the probe and Dabcyl was the quencher on the 3rd end. The molecular beacons and primers were prepared by Biolegio. Chosen primers and probes. Regarding primers and probes for internal control real-time PCR, also in Table 2, a real-time PCR assay for phocine herpes virus (PhHV) was used to display inhibition of real-time PCR; therefore, the assay was redesigned for the use of molecular beacons. The design of

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

the PhHV assay was carried out under conditions that represented those of the *Mycoplasma* real-time PCR assay to simplify multiplexing of the two assays.

Regarding real-time PCR was performed in 50 µL of a reaction mixture entailing 25 µL platinum Supermix (Invitrogen), 3.5 mM MgCl2, 0.4 µM of the primary *Mycoplasma* separately, 0.2 µM of each PhHV primary concentration, 0.34 µM of the *Mycoplasma* molecular beacon concentration, 0.2 µM of the PhHV molecular beacon concentration and 10 µL of the template primary concentration. With an iCycler IQ real-time detection system, amplification, detection and data analysis were carried out. With 103 copies of PhHV that were co-extracted with the sample, every sample was also spiked and the assay was implemented as a duplex PCR.

Variability of Inter- and Intraassay

From M, DNA was extracted. ATCC 15492 *Pneumoniae* (Mycobacterium avium complex) and stored in an AE buffer (50 mMNa acetate [pH 5.3], 10 mM EDTA [pH 8.0]). DNA was diluted at a concentration equal to 50 CCU/100 µL and stored at 20°C in small aliquots. An aliquot was used to determine inter- and intra-assay variation.

NASBA is an Isothermal Amplification of RNA

An RNA polymerase drags a promoter site that had been devoted to the target RNA by specific primers. The molecular beacon and primers were planned in harmony with the features of the 16S rRNA gene (16S rDNA) of *M. pneumoniae*. In addition to using a NucliSens basic kit for NASBA. In addition, double-stranded DNA was added by adding 5 µL of the model RNA to a 10 µL reaction mixture that contained primers. This mixture was heated for two minutes at 65°C and then cooled to 41°C for 2 minutes. Subsequently, 5 µl of the enzyme combination that includes T7 polymerase was further at 41°C. The last concentrations were100 mM KCl and 0.2 µM for the molecular beacon and, respectively, for the primers. All recognition and information examination were performed on an iCycler IQ real-time discovery system.

Validity and Reliability

The validity of the questionnaire sheet was tested by a panel of five experts in the field of microbiology, three professors from the Clinical Microbiology Department of Hafr Al Batin University. Two professors from the Department of Clinical Microbiology at Hail University aimed to evaluate the efficiency and content validity of the tool and to find possible obstacles and problems that could be faced during data collection.

Statistical Analysis

Data and results were collected, reviewed and prepared for computer entry. They were then coded, analysed and tabulated. Data entry and statistical analysis were performed using the SPSS 23 software package and Microsoft Excel.

Appropriate statistical tests were applied to assess whether there were statistically significant differences between the study variables. A p-value ≤0.001 was considered the statistical significance threshold. The result was classified as a true positive for *M. pneumoniae* according to the conventional PCR findings. Sensitivity and specificity were calculated for each assay according to the results of the study.

RESULTS

The results shown in Table 1 showed that *M. pneumonia* type 1 & 2 by real-time PCR, real-time nucleic acid sequence-based amplification and conventional PCR. It was observed that analysis sensitivity dilutions of DNA extracted from an *M. Pneumoniae*-positive strain of ATCC. 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 to 0.7).

Evaluation of PCR Inhibition

Table 3 shows that PhHV coamplification leads to *M. pneumoniae* amplification Ct values akin to those from amplifications without spikes. Proper controls are essential to apply PCR amplification in the microbiological diagnosis of clinical specimens. It is also crucial to monitor both the DNA isolation procedure and any potential inhibitors alongside specific PCR controls. The real-time PCR assay for *M. pneumoniae* involved duplexing with a PhHV spike amplification reaction, which served as an internal control. A PhHV dilution series was coamplified with *M. pneumoniae*; however, the efficiency and sensitivity of the reaction remained largely unaffected by the *Pneumonia* targets. A fixed amount of PhHV dilution was added to the lysis buffer immediately following nucleic acid extraction.

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 findings were confirmed by conventional PCR and NASBA, which overwhelmed a diverse part of the genome.

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. Comparing the clinical profiles of *M. pneumoniae* positive patients (n = 14) with their negative counterparts (n = 96) showed that a younger average age, chills, elevated Erythrocyte Sedimentation Rate (ESR) and C-reactive protein levels (>50 mg/L) suggested a possible *Mycoplasma* infection. Rhinitis was significantly more prevalent in *Mycoplasma*-negative patients (p<0.001). Furthermore, patients with *Mycoplasma* infection did not have pulmonary disease, nausea, vomiting, fever, or chills. No significant differences were statistically observed between patients with negative and positive *Mycoplasma* on clinical charactaristics.

Table 3: Dilution series of Ct values for *M. pneumoniae* after real-time duplex PCR amplification with and without 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)

DISCUSSION

M. pneumonia is a common cause of CAP and, during close contact, is transmitted from person to person via respiratory droplets. As the infection can be subclinical or cause milder diseases that do not require hospitalisation, the incidence may be much higher. According to the study by (Abdulhadi and Kiel 2017), who stated that only 5-10% of people are infected with *M. pneumonia* also in all age groups, it causes upper and lower respiratory tract infections, particularly in those >5 years and <40 years of age.

The existing study revealed that by analysing sensitivity dilutions of DNA extracted from an M. The lowest detection level was determined to be 5 CCU/100µl from serial 10-fold dilution tests of DNA in Tris buffer. The variation of the standard sample was 0.7, as determined from the threshold cycle (Ct) values obtained from five consecutive runs. The mean variation of the intra-assay was 0.28 (range 0.1 to 0.7). Also, the current study, an internally controlled real-time PCR assay for M diagnosis, targets the P1 adhesion gene. *Pneumoniae* infections have been developed. Fluorescence tests based on real-time PCR have advantages over conventional. Without the requirement of post-PCR processing, the fluorescent probes provide extra specificity for PCR. This reduces the potential risk of carrying over of the product. More specifically, real-time PCR leads to a significant decrease in the time to outcomes and therefore improved patient management [16-18].

The study by Ferwerda *et al.* [19], Loens [20], Medjo *et al.* [21] and Dash *et al.* [22] found that the laboratory investigation technique for the finding of *M. pneumoniae* as an etiological agent has been culture or serology PCR is a better diagnostic test than conventional techniques [23,24] and recently showed that NASBA is also a good alternative. The real-time PCR assay for *M. pneumoniae*, which was shown to be specific and to have good analytical sensitivity, was clinically evaluated using a group of patients with LRTI. In a group of 110 patients, 14 (12.73%) positive results were detected and this finding is in agreement with the study by Lieberman *et al.* [25], Ieven *et al.* [26] on the rate of *M. pneumoniae* infection in adult populations. Furthermore, other studies in Ohio and Spain have been described for CAP patients, rates of (15 to 30%) or as low as (1 or 3%). Real-time PCR positive results for *M. pneumoniae* were confirmed by conventional PCR [27-29]. This PCR targets the same gene; therefore, additional approval was sought in the NASBA. The NASBA reaction is directed at 16s of rRNA and similar positives were noticed more than once, consequently in (100%) agreement with the results obtained by the molecular methods. An additional feature of this NASBA is that the improved products are noticed using a fluorescent probe in real time and that this isothermal NASBA amplification and discovery can be performed on an iCycler IQ real-time finding system. The NASBA reaction notices not only *M. pneumoniae* types 1 and 2, but also M. genitalium, which has the similar 16_s rRNA sequence. Although reports show that M. genitalium is initiated in the respiratory tract, no evidence of its existence was noticed in patients in our study group, as all positive NASBA outcomes were also real-time PCR positive [30-32].

The diagnosis of *M. Pneumoniae* appear to be superior to serology by molecular methods. Sensitivity increased to 67% by adding the convalescent phase sample. In the PPA assay, the cutoff point for a positive result was established at a titer of ≥320. There was no increase in titer in convalescent-phase serum in a few samples with an acute-phase titer of 160. Obviously, a titer of 320 is a better indication of *M. Pneumoniae* infection, particularly since convalescent phase serum is not always obtained in the diagnostic laboratory. One sample was negative by serological procedures together, but positive by molecular techniques. In this the case, the serum samples had been collected only 8 days apart, which could have affected the sensitivity of the second sample; These findings are in agreement with Dorigo-Zetsma *et al.* [33], Herrera *et al.* [34] and Yan *et al.* [35], reported that a larger number of positive

results have been obtained by serology than by PCR methods in the current. All serologically positive results were also positive by PCR and NASBA. The current finding is relatively similar to the meta-analysis study of Cai *et al.* [36]. The collective SEN and SPE to diagnose *M. pneumoniae* were 0.90 and 0.98, respectively. The PLR was 31.25 and NLR 0.10, DOR 399.32 and AUC 0.9892 [37]. Moreover, Huang *et al.* [37] observed in the meta-analysis that the results of the combined statistics for the diagnosis of *M. pneumoniae* infection by NASBA were 0.77 SEN; 0.98 SPE; 0.22 LR; 50.38 LR, 292.72 DOR; and 0.9875 SROC.

CONCLUSIONS

Our main findings are that (i) The efficiency and sensitivity of the reaction were not significantly affected by the pneumonia targets, (ii) The inclusion of a fixed amount of PhHV dilution in the lysis buffer prior to nucleic acid extraction was effective, (iii) Rhinitis was significantly more abundant in patients negative for *Mycoplasma* ($p<0.001$), (iv) None of the *mycoplasma-positive* patients exhibited pulmonary disease, nausea, vomiting, fever, or chills, (v) No statistically significant differences were observed in the clinical characteristics of patients with positive and negative *mycoplasma*. It is also strongly recommended that further implementation is necessary to discuss with the microbiology department and the treating specialist which molecular tests must be used and which pathogens should be targeted. More studies are needed to evaluate their performance features and determine how these new technologies will improve diagnostic testing for respiratory pathogens. A protocol that addresses these issues should be developed and evaluated in clinical practice.

Ethical Statement

A formal approval was obtained from the Research Ethics Committee of the XX University of X Sciences in X on 10 February, 2022, Registered number H-2022-034, to carry out the study. Then a copy of the approval letter was sent to the hospitals selected previously to obtain approval to meet the patients. In addition, written informed consent was obtained from each of the participants after a full explanation of the purpose of the study from those who agreed to participate in the study were ensured confidentiality, privacy and the right to withdraw from the study at any time.

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