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Genotypic Detection of Bacterial Pathogens from Sputum among Patients with Community Acquired Pneumonia by Multiplex PCR

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Abstract

Background: Community-acquired pneumonia (CAP) is an acute respiratory illness responsible for significant morbidity and mortality globally. Objective: The objective of this study was to detect the common causative bacterial agents of CAP and their antimicrobial sensitivity pattern and also to detect the DNA of *Streptococcus pneumoniae, Haemophilus influenzae, Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophila, Staphylococcus aureus* and *Moraxella catarrhalis* from sputum sample by multiplex real-time PCR. Methodology: This cross-sectional observational study was carried out in the Department of Microbiology, Chittagong Medical College, Chattogram from July 2019 to June 2020. A total of 87 sputum samples were collected from CAP patients. Common causative bacterial agents of pneumonia were detected by gram staining, culture, biochemical tests, and multiplex real-time PCR of sputum. Results: In this study, among 87 CAP patients' age was in the range of 12 years to 85 years with the mean age being 55.09 (±18.74) years. Bacterial isolation was determined in 33 (37.9%) of all patients with the culture method, this number increased up to 48(55.2%) with multiplex real-time PCR. The bacteria most commonly identified by multiplex real-time PCR were Streptococcus pneumoniae 20(41.7%), *Haemophilus influenzae* 12(25.0%) and *Staphylococcus aureus* 11(22.9%). The most frequent bacteria found in the culture was *Pseudomonas species* 11(33.3%), followed by *Staphylococcus aureus* 10(30.3%), *Klebsiella species* 7(21.2%), *Streptococcus pneumoniae* 4(12.1%) and *Escherichia coli* 1(3.1%). Regarding antibiotic sensitivity patterns Meropenem and Clarithromycin were the most sensitive drug both in Gram-positive and Gram-negative organisms. Cefixime and azithromycin were the most resistant drug in both groups. Considering culture as the gold standard the sensitivity of PCR was 100%, the specificity was 80.72%, positive predictive value (PPV) was 20.0% and negative predictive value (NPV) was 100.0 % and the accuracy was 81.61%. Out of 87 patients, 51(58.3%) sputum samples were positive by Gram staining. In Gram-stain-positive cases, the most frequently detected bacteria were Gram-negative bacilli 24(27.1%), followed by Gram-positive cocci 12 (13.72%), Gram-negative coccobacillus 7(8.0%), Gram-positive diplococci 4(4.5%) and mixed type 4 (4.5%). Conclusion: In conclusion multiplex real-time PCR was highly sensitive, specific, and superior to other conventional methods for the detection of bacterial pathogens in the sputum of CAP patients.

Keywords: CAP; Bacterial pathogens; sputum; multiplex Real-time PCR; Culture

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Introduction

Pneumonia is an infection of the pulmonary parenchyma[1]. It is usually classified as community-acquired pneumonia (CAP), Hospital-acquired pneumonia (HAP), pneumonia in the immune-compromised host, and aspiration pneumonia[2]. CAP is

a lower respiratory tract infection acquired outside the hospital or long-term health care facility. It is diagnosed in the community or within 48 hours of admission to the hospital[3]. It is mainly caused by several infectious agents including viruses, bacteria, fungi, and parasites[4,1]. Common etiological agents causing CAP include *Streptococcus pneumoniae* (20.0% to 60.0%), *Haemophilus influenzae* (3.0% to 10.0%), *Gram-negative bacilli* (3.0% to 5.0%), *Chlamydia pneumoniae* (4.0% to 6.0%), *Mycoplasma pneumoniae* (1.0% to 6.0%), *Legionella species* (2.0% to 8.0%), *Staphylococcus aureus* (3.0% to 5.0%), and virus (2 to 13%)[5-6]. CAP is a frequent and deadly infectious disease and is responsible globally for 3 million death annually [7].

In Bangladesh, pneumonia accounts for 15.0% of the 119,000 total deaths of children, and mortality from pneumonia in adults was 7.3% in Asia and 3.3% to 11.0% in India[8-10]. Chest radiographs (CXRs) are the primary diagnostic tool for CAP but cannot distinguish between viral and bacterial infections[11]. Conventional methods for the identification of the bacteria are Gram staining, culture, and serology. The diagnostic value of Gram staining and culture of expectorated sputum has limitations in the identification of organisms. Moreover, when a patient is treated with antibiotics before hospitalization, the culture may give a false negative result. Also, atypical organisms cannot be cultured on standard media or seen on Gram's stain and serology is slow and lack of sensitivity1. Serology usually requires documentation of a rise in antibody concentration from an acute phase serum sample to a convalescent serum sample[12].

In this context, molecular diagnostic methods such as the Polymerase Chain Reaction (PCR) tests are highly sensitive techniques for the rapid detection of nucleic acid sequences from viruses and bacteria in clinical specimens. It is also advantageous for the detection of fastidious or difficult-to-culture organisms such as atypical pathogens[13]. Multiplex real-time PCR can identify many pathogens simultaneously in CAP, with high sensitivity and specificity. It reduces analysis time and improves cost performance, for the rapid and precise detection of causative agents[11]. Furthermore, multiplex real-time PCR decreases the false positive result and possible to detect microorganisms, even if bacteria have been damaged by antibiotic pre-treatment[14].

With the above background, the present study was carried out to find common bacterial agents of CAP such as *Streptococcus pneumoniae, Haemophilus influenzae, Chlamydia pneumoniae, Mycoplasma pneumoniae, Staphylococcus aureus* and *Moraxella catarrhalis* from sputum by multiplex real-time PCR and culture.

Methodology

Study Settings and Population: This cross-sectional study was carried out in the Department of Microbiology at Chittagong Medical College, Chattogram, Bangladesh from July 2019 to June 2020 for a period of one year on clinically diagnosed adult and children patients of community acquired pneumonia (CAP) admitted to Chittagong Medical College Hospital (CMCH), Chattogram, Bangladesh and also from the outpatient department of CMCH during the study period. Patients were excluded from the study if patients getting antibiotics for more than 48 hours and refuse to participate in this study. Chittagong Medical College is situated in the center of Chittagong City which is located south-east corner of Bangladesh. It is a tertiary care teaching hospital in Bangladesh.

Study Procedure: A questionnaire was used for each case. All the relevant information and data were systematically recorded in a pre-designed case record form. The sputum sample was collected as per the standard recommended protocol, then a thin smear was made for Gram staining and microscopy. To obtain a pure culture, it is necessary to reduce the number of commensals inoculated. Ways of reducing commensal numbers include washing the sputum free from saliva or liquefying and it was done by using normal saline. Then sputum was inoculated in Blood agar media, Chocolate agar media, and Mac Conkey's agar media. Isolation and Identification of bacteria were done by Colony morphology, Gram stain, and Biochemical test. Antimicrobial susceptibility test was determined by disc diffusion method using the Modified Kirby Bauer technique using Blood agar media (for Streptococcus pneumoniae) and Mueller Hinton Agar media (for Escherichia coli, Pseudomonas species) as per recommendations of the CLSI guideline 2017.

Antimicrobial Agents Use (CLSI 2017): Following antimicrobials and their concentration per disc were used for susceptibility tests as a) for Gram-positive cocci and diplococci: meropenem (10µg), ceftriaxone (30µg), co-amoxiclav (30µg), levofloxacin (5µg), azithromycin (1µg), cefixime (30 µg), clarithromycin and vancomycin (30µg). b) for Gram negative bacilli and coccobacilli: meropenem (10µg), ceftriaxone (30µg), amikacin (10µg), azithromycin (15µg),

levofloxacin (5µg). co-amoxiclav (30µg), clarithromycin and cefixime (30µg). The antibiotic sensitivity testing discs were procured from the local market and manufactured by Oxoid Ltd, UK.

Procedure of Multiplex Real-time PCR: Major steps of real-time PCR include DNA extraction, amplification in a thermal cycler, and analysis of the result. Bacterial DNA extraction is done according to the manufacturer's instruction supplied with the PCR kit. DNA Amplification was started at 500C for 15 minutes as the first step followed by 40 cycles of PCR at 940 C for 1 minute, at 600C for 30 s, and at 720 C for 10s successively. If the cut value is less than 33 indicates the organism was detected but if the cut-off value is more than 33 it indicates the organism was not detected.

Statistical Analysis: Statistical analysis was performed by Windows based software named as Statistical Package for Social Science (SPSS), versions 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Categorical data were summarized in terms of frequency counts and percentages. Continuous data were expressed as mean, standard deviation, minimum and maximum.

Ethical issue: All procedures of the present study were carried out in accordance with the principles for human investigations (i.e., Helsinki Declaration 2013) and also with the ethical guidelines of the Institutional research ethics. Formal ethics approval was granted by the local ethics committee. Participants in the study were informed about the procedure and purpose of the study and confidentiality of information provided. All participants consented willingly to be a part of the study during the data collection periods. All data were collected anonymously and were analyzed using the coding system.

Results

A total of 87 patients of CAP were included in the study from Chittagong Medical College Hospital (CMCH) during the period from July 2019 to June 2020. Gram stain, culture, and multiplex real-time PCR tests were done with sputum to detect bacterial pathogens in CAP patients. Antibiotic sensitivity tests were done with isolated bacteria.

Among 87 patients of CAP, 59 (67.8%) were male & 28 (32.2%) were female. The highest 27 (31.0%) of CAP occur in the 61–70 years of age group. The age range was 12 to 85 years with mean age of 55.09 (±18.7) years and the male and female ratio was 2.11:1 (Table 1).

Table 1: Distribution of Age and Gender among Community-Acquired Pneumonia Patients

Age Group	Male	Female	Total
≤20 Years	4(6.8%)	1(3.6%)	5 (5.7%)
21 to 30 Years	5(8.5%)	1(3.6%)	6 (6.9%)
31 to 40 Years	6(10.2%)	5(17.9%)	11(12.6)
41 to 50 Years	4(6.8%)	7(25.0%)	11(12.6)
51 to 60 Years	7(11.9%)	3(10.7%)	10(11.5)
61 to 70 Years	20(33.9%)	7(25.0%)	27(31.0)
71 to 80 Years	10(16.9%)	4(25.0%)	14(16.1)
More Than 80 Years	3(3.4%)	0(0%)	3 (3.4)
Total	**59(67.8%)**	**28(32.2%)**	**87(100.0)**
Mean ± SD	56.2 (±19.9)	52.9 ±16.2)	55.09 (±18.7)
Range	12-85	20-75	12-85

Among 87 sputum samples of CAP cases, 48 (55.2%) were positive by multiplex real-time PCR, 33 (37.9%) by conventional culture, and 51 (58.3%) by Gram stain (Table 2).

Table 2: Detection of bacteria among CAP patients among the Study Population (n=87)

Name of Tests	Frequency	Percent
Multiplex real time PCR	48	55.2
Culture positive	33	37.9
Gram staining	51	58.3

In 51 smear-positive sputum samples most frequently detected bacteria were Gram-negative bacilli 24(27.1%), followed by Gram-positive cocci 12(13.7%), Gram-negative coccobacili 7(8.0%), Gram-positive diplococci 4(4.5%) and mixed type 4 (4.5%) (Table 3).

Table 3: Distribution of Bacteria in Gram Staining (n=87)

Gram Staining Smear	Frequency	Percent
Gram negative bacilli	24	27.1
Gram positive cocci	12	13.7
Gram negative coccobacilli	7	8.0
Gram positive diplococci	4	4.5
Mixed Gram Positive & Negative	4	4.5
Total	51	58.3
Gram smear negative	36	41.7
Grand Total	**87**	**100.0**

Out of 87 sputum samples 33(37.9%) culture positive. In 33 culture-positive cases, the most frequently detected bacteria were Pseudomonas species 11(33.3%), followed by Staphylococcus aureus 10(30.3%), Klebsiella species 7(21.2%), Streptococcus pneumoniae 4(12.1%) and Escherichia coli 1(3.1%) (Table- 4).

Table 4: Bacteria Isolated from Sputum by Culture (n=33)

Name of Bacteria	Frequency	Percent
Pseudomonas spp.	11	33.3
Staphylococcus aureus	10	30.3
Klebsiella spp.	7	21.2
Streptococcus pneumoniae	4	12.1
Escherichia coli	1	3.1
Total	**33**	**100.0**

Out of 87 sputum samples, 48 yielded positive results in multiplex real-time PCR. Most frequent bacteria were Streptococcus pneumoniae 20(41.7%) followed by Haemophilus influenzae 12(25.0%), Staphylococcus aureus 11(22.9%), Moraxella catarrhalis 4(8.3%) and Chlamydia pneumoniae 1(1.9%) (Table 5).

Table 5: Bacteria Detected from Study Population from Sputum by Multiplex Real Time PCR (n=48)

Type of Organism	Frequency	Percent
Streptococcus pneumoniae	20	41.7
Haemophilus influenzae	12	25.0
Staphylococcus aureus	11	22.9
Moraxella catarrhalis	04	8.3
Chlamydia pneumoniae	01	1.9
Legionella pneumophilia	0	0.0
Mycoplasma pneumoniae	0	0.0
Total	**48**	**100.0**

Table 6: Comparison of Multiplex Real Time PCR and Culture of Sputum for detection of *Streptococcus Pneumoniae* (n=87)

Multiplex RT-PCR	Culture		Total
	Positive	Negative	
Positive	4(4.59%)	16(18.39%)	20(22.9%)
Negative	0(0.0%)	67(77.02%)	67(77.1%)
Total	**4(4.59)**	**83(95.41)**	**87(100%)**

The comparison of the results of culture and multiplex real-time PCR for Streptococcus Pneumoniae among the CAP patients had been recorded. Among 20 Streptococcus Pneumoniae positive cases by multiplex real-time PCR, where 4 were positive by culture. Considering culture as the gold standard the sensitivity of PCR was 100.0%, the specificity was 80.72%, Positive Predictive Value was 20.0% and Negative Predictive Value was 100.0 % and Accuracy was 81.6% (Table 6).

Antibiotic sensitivity pattern of Gram-negative bacteria to different antibiotics had been recorded. Pseudomonas spp. were highly sensitive to Meropenem (100%), followed by Clarithromycin (90.9%), Amikacin (63.6%), Ceftriaxone (63.6%) and Levofloxacin (63.6%), but resistance to Amoxicillin-Clavulanate (90.9%) followed by Cefixime (81.8%) and Azithromycin (54.5%). Klebsiella spp. were 100% sensitive to Meropenem and Clarithromycin followed by Amikacin (85.7%) and Ceftriaxone (71.4%) but resistant to Amoxicillin-Clavulanate, (57.1%) and Cefixime (57.1%) (Table-7).

Table 7: Sensitivity Pattern of Gram-Negative Bacteria to Different Antibiotics

Antimicrobial Agents	Sensitivity Pattern	Bacterial agents		
		Pseudomonas Spp. **(n=11)**	***Klebsiella Spp.*** **(n=7)**	***Escherichia coli*** **(n=1)**
Amoxicillin-	S	1(9.1%)	3(42.9%)	0 (0%)
Clavulanate	R	10(90.9%)	4(57.1%)	1(100%)
	S	5(45.5%)	4(57.1%)	0 (0%)
Azythromycin	R	6(54.5%)	3(42.9%)	1(100%)
	S	11(100%)	7(100%)	1(100%)
Meropenem	R	0 (0%)	0 (0%)	0 (0%)
	S	7(63.6%)	5(71.4%)	1(100%)
Ceftriaxone	R	4(36.4%)	2(28.5%)	0 (0%)
	S	2(18.2%)	3(42.9%)	0 (0%)
Cefixime	R	9(81.8%)	4(57.1%)	1(100%)
	S	7(63.6%)	4(57.1%)	1(100%)
Levofloxacin	R	4(36.4%)	3(42.9%)	0(0%)
	S	10(90.9%)	7(100%)	1(100%)
Clarithromycin	R	1(9.9%)	0 (0%)	0 (0%)
	S	7(63.6%)	6(85.7%)	0 (0%)
Amikacin	R	4 (36.4%)	1(14.3%)	1 (100%)

Table 7: Sensitivity Pattern of Gram-Negative Bacteria to Different Antibiotics

Antimicrobial Agents	Sensitivity Pattern	Bacterial agents	
		Staphylococcus aureus **(n=10)**	***Streptococcus pneumonia*** **(n=4)**
Amoxicillin-Clavulanate	S	8(80.0%)	0(0.0%)
	R	2(20.0%)	4(100.0%)
Azythromycin	S	4(40.0%)	2(50.0%)
	R	6(60.0%)	2(50.0%)
Vancomycin	S	9(90.0%)	4(100%)
	R	1(10.0%)	0(0%)
Meropenem	S	8(80.0%)	4(100%)
	R	2(20.0%)	0(0%)
Ceftriaxone	S	7(70.0%)	2(50.0%)
	R	3(30.0%)	2(50.0%)
Cefixime	S	3(30.0%)	1(25.0%)
	R	7(70.0%)	3(75.0%)
Levofloxacin	S	8(80.0%)	1(25.0%)
	R	2(20.0%)	3(75.0%)
Clarithromycin	S	10(100%)	4(100%)
	R	0 (0%)	0(0%)

The sensitivity pattern of Gram-positive bacteria to different antibiotics was recorded. *Staphylococcus aureus* was highly sensitive to Clarithromycin (100.0%) followed by Vancomycin (90.0%), meropenem, levofloxacin, and amoxicillin-clavulanate 80.0% each, whereas resistance to cefixime (70.0%) and azithromycin (60.0%). *Streptococcus pneumoniae* was 100% sensitive to meropenem clarithromycin and vancomycin but resistant to amoxicillin-clavulanate (100.0%) followed by cefixime (75.0%), levofloxacin (75.0%) (Table 8).

Discussion

Community-acquired pneumonia (CAP) is still a major cause of morbidity and mortality worldwide. Knowledge of pathogens causing CAP constitutes the basis for the selection of antimicrobial treatment[12]. In the present study, the maximum 27(31.0%) cases of CAP were in the age group of 61 to 70 years, with a mean age of around 55 years. Naik et al[15] in India and Jain et al[16] in the USA showed that the average age was around 53 and 57 years respectively. However, it differs from the study of Salam et al[17] in Bangladesh; where the corresponding figure was comparatively lower (39 years).

In this study, male 59 (67.8%) patients were more than female 28(32.2%) patients and the male-female ratio was 2.11:1. This male predominance was also observed in a study conducted by Liapikou et al[18] where male to female ratio was 2.1:1.

In this study, 51 (58.3%) were Gram stain positive and 36 (41.7%) were Gram stain negative. A study done in Bangladesh by Kausar et al[19] showed that sputum Gram stain positive in 63.2% of cases. In Gram-stain-positive cases, more frequently detected bacteria were Gram-negative bacilli 24 (27.1%), followed by Gram-positive cocci 12 (13.7%) cases. Akter et al[20] found 19.04% Gram-negative organisms and 15.23% Gram-positive organisms in their study.

In this study, the most common isolated bacteria from sputum culture were *Pseudomonas spp.* 11(33.3%), followed by *Staphylococcus aureus* 10 (30.3%), *Klebsiella species* 7(21.2%), *Streptococcus pneumoniae* 4(12.1%) and *Escherichia coli* 1(3.1%). Naik et al[15] showed that *Pseudomonas species* (34.48%) as the most common pathogen followed by *Staphylococcus aureus* (24.14%) in their study.

A study was done by Kausar et al[19] in Bangladesh where the most common bacteria were *Klebsiella pneumoniae* (39.1%) followed by *Pseudomonas aeruginosa* (10.3%), *Staphylococcus aureus* and *Escherichia coli* (5.7%). But it differs from Salam et al[17] study in Dhaka Medical College Hospital (DMCH), Dhaka, Bangladesh, where they found *Streptococcus pneumoniae* (53.8%) followed by *Klebsiella species* (26.9%) from sputum culture of CAP patients.

In the current study, 48 (55.2%) cases were positive by

multiplex real-time PCR. Mustafa et al[12] found that, 39.1% of patients by culture and 65.2% by multiplex real-time PCR method in their study. Templeton et al21 reported that 49.5% of patients were positive by conventional methods and 76.0% of patients were positive by multiplex real-time PCR.

In the present study, the most frequently detected bacteria were *Streptococcus pneumoniae* 20(41.7%) followed by *Haemophilus influenza* 12(25.0%), *Staphylococcus aureus* 11(22.9%), *Moraxella catarrhalis* 4(8.3%) and *Chlamydiae pneumoniae* 1(1.9%) by multiplex real-time PCR. In a study done by Aydemir et al[22] where the most commonly identified bacteria by multiplex real-time PCR were Community-acquired pneumonia (CAP) is still a major cause of morbidity and mortality worldwide. Knowledge of pathogens causing CAP constitutes the basis for the selection of antimicrobial treatment[12]. In the present study, the maximum 27(31.0%) cases of CAP were in the age group of 61 to 70 years, with a mean age of around 55 years. Naik et al[15] in India and Jain et al[16] in the USA showed that the average age was around 53 and 57 years respectively. However, it differs from the study of Salam et al[17] in Bangladesh; where the corresponding figure was comparatively lower (39 years).

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In the current study, 48 (55.2%) cases were positive by multiplex real-time PCR. Mustafa et al[12] found that, 39.1% of patients by culture and 65.2% by multiplex real-time PCR method in their study. Templeton et al[21] reported that 49.5% of patients were positive by conventional methods and 76.0% of patients were positive by multiplex real-time PCR.

In the present study, the most frequently detected bacteria were *Streptococcus pneumoniae* 20(41.7%) followed by *Haemophilus influenza* 12(25.0%), *Staphylococcus aureus* 11(22.9%), *Moraxella catarrhalis* 4(8.3%) and *Chlamydiae pneumoniae* 1(1.9%) by multiplex real-time PCR. In a study done by Aydemir et al[22] where the most commonly identified bacteria by multiplex real-time PCR were *Streptococcus pneumoniae* (32%) and *Haemophilus influenzae* (31%). Considering culture as the gold standard the sensitivity of multiplex real-time PCR for *Streptococcus pneumoniae* was 100%, specificity of 80.72%.

Yang et al[23] reported 90.0% sensitivity and 80.0% specificity for *Streptococcus pneumoniae*. Abdeldaim et al[24] also reported that sensitivity was 90.0% and specificity 75.0% in the detection of *Streptococcus pneumoniae* by multiplex real-time PCR. *Pseudomonas species* was 100% sensitive to meropenem, followed by clarithromycin (90.9%), ceftriaxone, levofloxacin, and amikacin (63.6%) each. In Shaibal et al[25] study, it was 100.0% sensitive to meropenem and Akter et al[20] and Ahmed et al[26] showed that it was 81.0% sensitive to amikacin.

It was resistant to amoxicillin-clavulanate, cefixine, and azithromycin. Shaibal et al[25] and Hossain et al[27] reported that, it was resistant to amoxicillin-clavulanate, cefixime and azithromycin. In this study, *Klebsiella spp.* was 100% sensitive to meropenem and clarithromycin followed by amikacin (85.7%) and ceftriaxone (71.4%). Jitendranath and Koshy[28] in India showed that *Klebsiella species* was 100.0% sensitive to meropenem. Salam et al[29] showed that *Klebsiella spp.* was 73.3% sensitive to

ceftriaxone and Ahmed et al[26] reported that 74% sensitive to amikacin. In the present study, *Klebsiella species* was high resistance to amoxicillin-clavulanate (57.1%), cefixime (57.1%), and azithromycin (42.9%). Akter et al20 and Salam et al[17] in Bangladesh showed that *klebsiella species* were highly resistant to commonly used antibiotics for CAP.

Kausar et al[19] reported that *Klebsiella species* was 81.8% resistance to amoxicillin-clauvulanate, and 69.7% resistance to cefixime. *Staphylococcus aureus* was sensitive to clarithromycin (100.0%) followed by vancomycin (90.0%) meropenem (80.0%), amoxtcillin-clavulanate (80%), and ceftriaxone (70.0%). Akter et al[20] and Shaibal et al[25] showed that *Staphylococcus aureus* was 100.0% sensitive to ceftriaxone and meropenem. *Staphylococcus aureus* was resistant to cefixime (70.0%) and to azithromycin (60%) which is similar to the Hossain et al[27] study.

Streptococcus pneumoniae were 100.0% sensitive to meropenem, clarithromycin, and vancomycin whereas it was resistant to amoxicillin-clavulanate, cefixime, and levofloxacin. Shaibal et al[25] reported that *Streptococcus pneumoniae* were 100.0% sensitive to meropenem, but 77.8% sensitive to clarithromycin. Moreover, Akter et al[20] showed that *Streptococcus pneumoniae* was 80.0% sensitive to ceftriaxone.

There are some limitations of this study. Small sample size and thus the number of positive specimens were unfortunately too small to perform elaborate statistical analysis. There was difficulty in obtaining representative respiratory samples from some patients. Primers for Gram-negative bacteria were not used in the PCR panel.

Conclusion

The multiplex real-time PCR method is superior to conventional methods in the detection of multiple pathogens simultaneously and is a highly specific, sensitive, and rapid method. Moreover, in multiplex real-time PCR method, the organism does not need to be viable and can detect even when the microbial concentration is very low or damaged bacteria with antibiotic therapy. Based on the study observation, widespread use of multiplex real-time PCR methods could be recommended during the treatment of CAP patients. However, Gram-negative organisms should be included in the multiplex real-time PCR panel. Further multi-Centre, larger scale, prospective study is necessary.

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Conflict of Interest
The authors declare no conflict of interest.

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Authors' contributions
Amin US, Uddin AMS, conceived and designed the study, analyzed the data, interpreted the results, and wrote up the draft manuscript. Rahman H, Fatema K, contributed to the analysis of the data, interpretation of the results and critically reviewing the manuscript. Akter N, Abbasi MA, involved in the manuscript review and editing. All authors read and approved the final manuscript.

Data Availability
Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate
Ethical approval for the study was obtained from the Institutional Review Board. As this was a prospective study the written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

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