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ORIGINAL ARTICLE

Community acquired bacterial pneumonia: aetiology, laboratory detection and antibiotic susceptibility pattern

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Abstract

This cross sectional study was conducted to identify the common bacterial causes of community acquired pneumonia (CAP) from sputum and blood by culture and polymerase chain reaction (PCR) and to evaluate the effectiveness of these tests. A total of 105 sputum and blood samples were collected from patients with pneumonia on clinical suspicion. Common causative bacterial agents of pneumonia were detected by Gram staining, cultures, biochemical tests and PCR. Among 55 sputum culture positive cases, a majority (61.82%) of the patients were in the age group between 21-50 years and the ratio between male and female was 2.5:1. Most (61.90%) of the cases were from the lower socio-economic group. Out of 105 samples, 23 (37.12%) were positive by Gram stain, 29 (27.62%) yielded growth in culture media and 37 (35.24%) were positive by PCR for *Streptococcus pneumoniae* and *Haemophilus influenzae*. *Streptococcus pneumoniae* was the most common aetiological agent (19.05%) followed by *Klebsiella pneumoniae* (13.33%), *Haemophilus influenzae* (8.57%) and *Pseudomonas aeruginosa* (5.71%). Multiplex PCR is a useful technique for rapid diagnosis of bacterial causes of pneumonia directly from sputum and blood. Considering culture as a gold standard, the sensitivity of PCR was 96.55% and specificity was 88.15%. More than 80% of *Streptococcus pneumoniae* isolates were found to be sensitive to ampicillin, amoxycillin-clavulanate, and ceftriaxone. Susceptibilities to other antimicrobials ranged from 65% for azithromycin to 70% for levofloxacin. On the other hand, the Gram negative organisms were more sensitive to meropenem, ceftriaxone, amoxycillin-clavulanate and amikacin.

Keywords: Pneumonia, sputum, blood, PCR, *Streptococcus pneumoniae*, *Haemophilus influenzae*, bacterial aetiology, antibiotic susceptibility, Bangladesh.

INTRODUCTION

Pneumonia is defined as an acute respiratory illness associated with recently developed radiological pulmonary shadowing which may be segmental, lobar or mutilobar.[1] It occurs about five times more frequently in the developing world than the developed world.[2] The incidence of community acquired pneumonia (CAP) range from 4 million to 5 million cases per year, with about 25% requiring hospitalization.[3] CAP is commonly defined as an acute infection of the pulmonary parenchyma that is associated with at least some symptoms of acute infection and is accompanied by the presence of an acute infiltrate on a chest radiograph or auscultatory findings consistent with pneumonia (such as altered breath sounds and/or localized rales) and occurs in a patient who is not hospitalized or residing in a long-term-care facility for ≥14 days before the onset of symptoms.[4] Diagnosis depends on isolation of the infective organism from sputum and blood. Knowledge of predominant microbial patterns in CAP constitutes the basis for initial decisions about empirical antimicrobial treatment.[5]

The bacterial causes of CAP varies between countries and changes with time within the same country, which is probably due to frequent use of antibiotics, changes in environmental pollution, increased awareness of the disease and changes in life expectancy.[6] For instance, *Streptococcus pneumoniae* remains the most common organism leading to community acquired pneumonia in most parts of Europe,[7] United States[8] and Iraq.[9] In Singapore, *Klebsiella pneumoniae* is the most common pathogen leading to admission

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to a medical intensive care unit amongst the more severe cases of CAP.[10] The problem is much greater in the developing countries where pneumonia is the most common cause of hospital attendance in adults.[11] Although an aetiological diagnosis is optimal in the management of CAP, the responsible pathogens are not identified in 50% of the patients even when extensive diagnostic test are performed.[12,13] Polymerase chain reaction (PCR) is considered as a novel diagnostic method for *H. influenzae* and pneumococcal pneumonia.[14] PCR applied to sputum can be used to obtain presumptive diagnoses of the aetiology of CAP in adult populations[15] that can guide antibiotic therapy and support treatment with narrow-spectrum antibiotics.[16] The main objective of this study was to determine the common causative bacterial agents of CAP and their antimicrobial susceptibility pattern among patients in Dhaka Medical College Hospital of Bangladesh. The study also aims to evaluate the use of multiplex PCR in identifying *Streptococcus pneumoniae* and *Haemophilus influenzae* from sputum and blood from these patients.

MATERIALS AND METHODS

A cross sectional study was conducted in the Department of Microbiology of Dhaka Medical College, Dhaka during July 1, 2011 to June 30, 2012. This research protocol was approved by research review committee (RRC) and ethical review committee (ERC) of Dhaka Medical College, Dhaka, Bangladesh. Informed written consent was obtained from each patient before collecting sputum and blood samples. 105 consecutive samples of blood and sputum from patients over 18 years of age with the diagnosis of CAP seen at Dhaka Medical College, were included in the study. CAP was defined as new or progressive pulmonary infiltrates on chest radiograph with fever plus one or more of the followings: cough, sputum, pleuritic chest pain, signs of consolidation or dyspnoea. Patients suffering from tuberculosis, bronchial asthma, congenital heart diseases, renal failure, foreign body aspiration and patients who were on antibiotic therapy or had completed antibiotic therapy within the last three days were excluded from the study.

Sputum and blood samples were studied to detect the bacterial aetiology of pneumonia. Sputum containing more than 25 poly-morphonuclear cells and less than 10 epithelial cells per low power field was subjected to Gram staining and culture. The samples were processed according to standard microbiological practices. All organisms that were isolated were identified by the conventional methods and biochemical tests.

The final identification of *Streptococcus pneumoniae* and *Haemophilus influenzae* was done by PCR using species specific primers. In addition, Multiplex PCR technique was applied for simultaneous detection of *Haemophilus influenzae* and *Streptococcus pneumoniae* from the sputum and blood samples (Fig. 1).

Antimicrobial susceptibility testing was performed according to the CLSI guidelines by the disk diffusion technique using commercial antibiotic disks (Oxoid Ltd. UK). E*scherichia coli* ATCC 25922 strain was used for quality control.

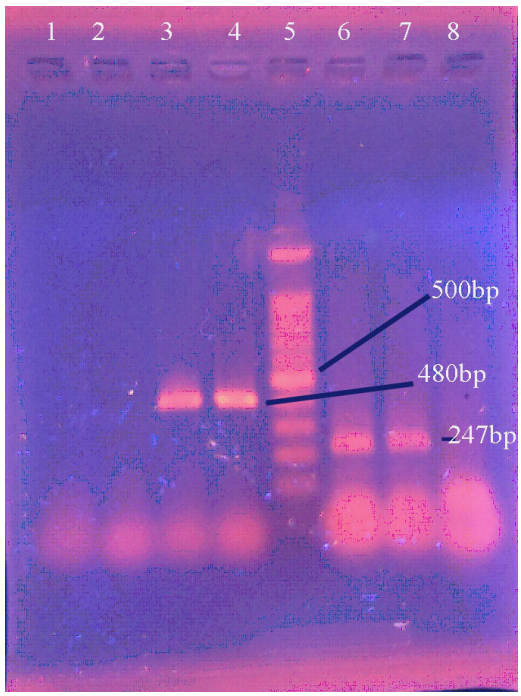

FIG. 1: Gel electrophoresis of multiplex PCR product for detection of *Streptococcus pneumonia* and *Haemophilus influenza* from sputum samples. Lane 1: *Klebsiella pneumoniae* (negative control); Lane 2: negative sample; Lane 3: positive sample *(Streptococcus pneumoniae)*; Lane 4: Positive control *(Streptococcus pneumonie)*. Lane 5: 100bp DNA ladder; Lane 6: positive sample *(Haemophilus influenzae)*; Lane 7: positive control *(Haemophilus influenzae)*; Lane 8: negative sample

Multiplex PCR technique: DNA extraction from isolated bacterial strains: DNA was extracted by boiling method. Bacterial pellet was suspended in 300 µl of sterile deionized water in a microcentrifuge tube, boiled in a heat block for 15 minutes, centrifuged at 4°C at 10000 rpm (or 12000xg) for 10 minutes. The supernatant was used as a DNA template for amplification of DNA by PCR.

DNA extraction from clinical samples: Sputum and serum samples (200 µl) from suspected cases of pneumonia were mixed with equal volume of lytic buffer (200 µl) and was incubated at 60°C for 3 hours, then boiled in heat block (DAIHA Scientific, Seoul, Korea) at 100°C for 10 minutes, centrifuged at 4°C at 14000 rpm for 10 minutes. Finally supernatant was taken using micropipette and used as template DNA for PCR and was kept at -20°C for future use.[14,17]

The following pairs of previously used primers were used to yield PCR products: for Streptococcus pneumoniae ATC-GAA-ATT-AAT-GTG-AGT-A (forward), AGC-TCT-CAG-CAT-TCC-A (reverse), for Haemophilus influenzae, GCG-AAA-GTG-ACC-TCT-TAT-CTC-TC (forward), GCT-TAC-GCT-TCT-ATC-TCG-GTG-AA (reverse).[17]

The following cycling parameters were used: initial denaturation at 95°C for 10 minutes, then 35 cycles of denaturation at 95°C for one minute, annealing at 55°C for 45 seconds and extension at 72°C for 30 seconds and final extension at 72°C for 10 minutes. The amplified DNA were loaded into a 1.5% agarose gel, electrophoresed at 100 voltages for 35 minutes, stained with 1% ethidium bromide and visualized under UV light.

RESULTS

Patients' demography

The ages of the study population ranged from 18 to 81 years. The mean age was 30.9 ±9.3 years with a male: female ratio of 2.5:1. Among 55 culture positive cases, 34 (61.82%) were between 21-50 years of age with maximum proportion (34.55%) in 21-30 years. Of the 105 sputum samples, 55 (52.38%) yielded growth. Among the 55 growths, 23 (21.91%) were Gram positive cocci, 22 (20.94%) were Gram negative bacilli and 10 (9.53%) were Gram negative coccobacilli (Table 1). *Streptococcus pneumoniae* was the predominant Gram positive cocci and *Klebsiella pneumoniae* was the predominant Gram negative bacilli (Table 1). Among the bacteria isolated from sputum, *Streptococcus pneumoniae* was isolated in 20 (19.05%), *Klebsiella pneumoniae* in 14 (13.33%), *Haemophilus influenzae* in 9 (8.57%), *Pseudomonas aeruginosa* in 6 (5.71%), *E. coli* in 3 (1.90%), *Staphylococcus aureus* in 3 (2.86%) and *Acinetobacter baumannii* in one (0.96%) sputum samples. Out of 105 patients, 2 (1.90%) cases were positive by blood culture; one of them was *Streptococcus pneumoniae* and another was *Pseudomonas aeruginosa* (Table 1).

TABLE 1: Bacteria isolated from sputum and blood culture (n=105).

Name of the bacterial isolates	Sputum n (%)	Blood n (%)
A. Gram positive cocci		
Streptococcus pneumoniae	20 (19.05)	1 (0.95)
Staphylococcus aureus	3 (2.86)	0 (0.00)
B. Gram negative bacilli		
Klebsiella pneumoniae	14 (13.33)	0 (0.00)
Pseudomonas aeruginosa	6 (5.71)	1 (0.95)
Escherichia coli	2 (1.90)	0 (0.00)
C. Gram negative cocobacilli		
Haemophilus influenzae	9 (8.57)	0 (0.00)
Acinetobacter baumani	1 (0.96)	0 (0.00)
Total	55 (52.38)	2 (1.90)

TABLE 2: Results of culture and PCR for *Streptococcus pneumoniae* and *Haemophilus influenzae* in sputum samples

PCR	Culture for *Streptococcus pneumoniae*			Culture for *Haemophilus influenzae*		
	Positive n (%)	Negative n (%)	Total n (%)	Positive n (%)	Negative n (%)	Total n (%)
Positive	19 (95)	7 (8.24)	26 (24.76)	9 (100)	2 (2.08)	11 (10.48)
Negative	1 (5)	78 (91.76)	79 (75.24)	0 (0)	94 (97.92)	94 (89.52)
Total	20 (100)	85 (100)	105 (100)	9 (100)	96 (100)	105 (100)

For *Streptococcus pneumoniae* X2 =65.4, df=1, p<0.001. The difference in positivity between culture and PCR was statistically significant. For *Haemophilus influenzae* X2 =84.1, df=1, p<0.001. The difference in positivity between culture and PCR was statistically significant.

Aetiological agents

A total of 29 (27.61%) of the 105 sputum were culture positive and 37 (35.23%) were PCR positive for *Streptococcus pneumoniae* and *Haemophilus influenzae* DNA (Tables 2 & 3). Twenty-eight (96.55%) were positive by both culture and PCR and 9 (11.86%) of the 76 culture negative sputum were positive by PCR and one (3.44%) was negative by PCR but positive by culture (p<0.01)(Table 2). Of the 105 sputum samples, 22 (59.46%) were positive by both Gram stain and PCR, 15 (40.54%) of the 67 gram stain negative samples were PCR positive, and one (1.47%) was negative by PCR but positive by Gram stain (p<0.01)(Table 3). Considering culture as a gold standard, Sensitivity of PCR was 96.55%, specificity was 88.15%, positive predictive value was 75.67%, negative predictive value was 88.15% and accuracy was 90.47%.

Antimicrobial susceptibility pattern

Among the Gram positive bacteria 20 isolated *Streptococcus pneumoniae*, 19 (95%) were sensitive to amoxyclav followed by 17 (85%) to ampicillin, 16 (80%) to ceftriaxone, 14 (70%) to levofloxacin and 12 (60%) to doxycycline. The resistance rates to azithromycin and cefixime were 13 (65%) and 10 (50%) respectively. All (100%) the *Staphylococcus aureus* were sensitive to amoxyclav, ampicillin and doxycycline and one (33.33%) was resistant to levofloxacin, azithromycin, ceftriaxone and cefixime. All (100%) the gram negative bacteria were sensitive to meropenem with variable sensitivity to different antimicrobial agents (Table 4). Ceftriaxone was an effective drug against *Klebsiella pneumoniae* (92.85%) and *E. coli* (100%), and not at all effective against *Acinetobacter baumannii*. Amikacin was found moderately effective against *Pseudomonas aeruginosa* (83.34%).

TABLE 3: Results of PCR and Gram stain for *Streptococcus pneumoniae* and *Haemophilus influenzae* in sputum samples.

Gram stain	PCR for *Streptococcus pneumoniae*			PCR for *Haemophilus influenzae*		
	Positive n (%)	Negative n (%)	Total n (%)	Positive n (%)	Negative n (%)	Total n (%)
Positive	15 (57.69)	1 (1.27)	16 (15.24)	7 (63.64)	0 (0)	7 (6.67)
Negative	11 (42.31)	78 (98.73)	89 (84.76)	4 (36.36)	94 (100)	98 (93.33)
Total	26 (100)	79 (100)	105 (100)	11 (100.00)	94 (100)	105 (100)

For *Streptococcus pneumoniae* X2 =48.2, df=1, p<0.001. The difference in positivity between PCR and Gram stain was statistically significant. For *Haemophilus influenzae* X2 =64.1, df=1, p<0.001. The difference in positivity between PCR and Gram stain was statistically significant.

TABLE 4: Sensitivity pattern of Gram negative bacteria to different antimicrobial agents.

		Bacterial isolates				
Antimicrobial agents	Sensivity pattern	*K.pneumoniae* (n = 14)	*H.influenzae* (n = 9)	*Pseudomonas* (n = 6)	*E. coli* (n = 2)	*Acinetobacter* (n = 1)
Meropenem	S	14 (100)	9 (100)	6 (100)	2 (100)	1 (100)
	R	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Ceftriaxone	S	13 (92.85)	7 (77.78)	1 (16.66)	2 (100)	0 (0.00)
	R	1 (7.15)	2 (22.22)	5 (83.34)	0 (0.00)	1 (100)
Clarithromycin	S	6 (42.85)	5 (55.56)	0 (0.00)	0 (0.00)	0 (0.00)
	R	8 (57.14)	4 (44.44)	6 (100)	2 (100)	1 (100)
Amoxyclav	S	12 (85.71)	7 (77.78)	0 (0.00)	1 (50)	1 (100)
	R	2 (14.28)	2 (22.22)	6 (100)	1 (50)	0 (0.00)
Ciprofloxacin	S	8 (57.14)	1 (11.11)	3 (50)	1 (50)	1 (100)
	R	6 (42.85)	8 (88.89)	3 (50)	1 (50)	0 (0.00)
Cefixime	S	7 (50)	2 (22.22)	1 (16.66)	0 (0.00)	0 (0.00)
	R	7 (50)	7 (77.78)	5 (83.34)	2 (100)	1 (100)
Amikacin	S	10 (71.42)	6 (66.67)	5 (83.34)	2 (100)	1 (100)
	R	4 (28.57)	3 (33.33)	1 (16.66)	0 (0.00)	0 (0.00)
Gentamicin	S	9 (64.28)	6 (66.67)	2 (33.33)	1 (50)	1 (100)
	R	5 (35.71)	3 (33.33)	4 (66.67)	1 (50)	0 (0.00)

Numbers within parentheses indicate percentage. S= Sensitive, R= Resistant

DISCUSSION

In the study, by Gutiérrez F *et al*. in Spain, 41.82% of culture positive cases were in the 21-40 years of age. The majority (32.7%) of the cases reported was in the age group of 15 to 44 years and 22.1% of cases were in the age group of 45 to 64 years of age.[18] Those findings are in agreement with the finding of the present study. However, the mean age of the present study (30.9±9.3 years), was lower than the mean age reported from previous studies which ranged from 54.6 to 68.9 years.[19-21] This higher mean age among CAP patients in those studies might be due to higher life expectancies in their population. It is also due to the exclusion factors in this study – congestive cardiac failure and renal failure would exclude many in the older aged group.

In the present study, 50 (47.62%) out of 105 sputum samples yielded no growth. The negative results of sputum cultures among the CAP patients could be due to the fact that these patients might had been infected by other aetiological agents such as virus, *Legionella pneumophilia*, *Chlamydophilia pneumoniae*, or *Mycoplasma pneumoniae* which are routinely not cultured in the laboratory. Another possibility could be due to previous treatment with antibiotics. It was reported that one fifth of the patients used antibiotics in rural Bangladesh before coming to a hospital.[22] The main limitation of the study was that serological tests for *Legionella pneumophilia*, *Mycoplasma pneumoniae*, *Chlamydophilia pneumoniae* and common respiratory viruses were not performed and thus, these organisms which are common causative agents in atypical pneumonia would just remained as possible diagnoses in the culture negative cases in this study. Alternatively, DNA of these organisms can be detected by PCR.

In the present study, among 105 patients, 20 (19.05%) were positive for *Streptococcus pneumoniae* and 9 (8.57%) were positive for *Haemophilus influenzae*. The findings were similar with a nother study whereby the same organisms were isolated in which *Streptococcus pneumoniae* and *Haemophilus influenzae* constituted 15.3% and 10.9% respectively.[23] The isolation rates of *Streptococcus pneumoniae* were 16.8% in Spain,18 26% in Jordan,[24] 23% in Japan[25] and 24% in England[26] among the CAP

patients, which are in agreement with the present findings. In Australia, 42% of *Streptococcus pneumoniae* were reported[27] which was higher than the present study. The isolation rate of *Haemophilus influenzae* were 3-10% in England,[3] 7.4% in Japan[25] and 9% in Australia[27] which is similar to the present study. In contrast, 17% *Haemophilus influenzae* were reported in Jordan among the study population which was higher than the present study.[24]

In the present study, among 105 patients, 14 (13.33%) were positive for *Klebsiella pneumoniae*. The isolation rates of *Klebsiella pneumoniae* among CAP patients were 3% in India,[6] 4.3% in Japan,[25] 3% in England[26] and 6% in Jordan[24] which were lower than the present study. Gram negative bacteria were reported in 8 - 10% and *Staphylococcus aureus* in 3 - 5 % cases.[3,27] *Pseudomonas* species were isolated in 3.2% cases of CAP patients.[18] These findings are in agreement with the findings of the present study. On the other hand, higher isolation rate of *Staphylococcus* (8%)[26] and *Pseudomonas aeruginosa* (10% - 11%) were reported.[6,28] In India, it was reported that Gram negative organisms were the commonest cause of CAP (19%) because patients had COPD (57%), structural lung disease (21%), so this could be the reason for the high isolation of *Pseudomonas* (10/29).[6]

Of the 105 sputum samples, a total 37 were multiplex PCR positive for *Streptococcus pneumoniae* and *Haemophilus influenzae*, 26 of them were positive for *Streptococcus pneumoniae* and 11 were for *Haemophilus influenzae.* For *Streptococcus pneumoniae*, 19 (95%) were positive by both culture and PCR, 7 (8.24%) of the 85 culture negative samples were positive by PCR and one (3.44%) was negative by PCR but positive by culture. Findings of the present study coincide with the findings of other studies. These 7 (8.24%) PCR positive culture negative samples might be due to presence of dead bacteria or presence of inhibitory substances in sputum that hampers growth. Considering culture as gold standard, the sensitivity of PCR was 96.55% and specificity was 88.15%, positive predictive value was 75.67%, negative predictive value was 88.15% and accuracy was 90.47% The difference in positivity between culture and PCR was statistically significant ($p<0.001$). Sensitivity of PCR was reported as 90% - 95%[14,15,29] and reported specificity was 80%.[1] However, 42% - 75% specificity was reported by other authors[15,29] which are lower than the present study.

In this study, the isolated *Streptococcus pneumoniae* were found to be sensitive to commonly used antibiotics such as amoxycillin-clavulanate (95%), ampicillin (85%) and ceftriaxone (80%). Susceptibilities to quinolones ranged from 65% for azithromycin to 70% for levofloxacin. On the other hand, the Gram negative organisms were more sensitive to meropenem and ceftriaxone. Regarding resistance pattern to ciprofloxacin, 88% *Haemophilus influenzae*, 42.85% *Klebsiella pneumoniae*, 50% *Pseudomonas aeruginosa* and 50% *E. coli* were found resistant to it and the resistance rates of these organisms to clarithromycin ranges from 44.44% to 100% and to cefixime from 50 to 100%. It was observed that isolated Gram negative bacteria were resistant to commonly used antibiotics is alarming and resistant bacteria is emerging.

Even a single DNA can be amplified to millions of copies by PCR that can help in diagnosis. Along with culture PCR can be used for diagnosis of bacterial causes of CAP. It is especially helpful in culture negative cases. Multiplex PCR is a helpful technique for rapid diagnosis of bacterial causes of pneumonia directly from sputum and blood.

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