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Awareness of diverse bacterial flora distribution causing pneumonia in Dir, Khyber Pakhtunkhwa, Pakistan

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Abstract
Background: Pneumonia is a serious illness and one of the main causes of mortality and morbidity. Knowledge of etiological agents causing pneumonia is necessary for selection of antibiotics therapy. The aim of this study was to determine the diverse bacterial contribution causing pneumonia in Dir, Khyber Pakhtunkhwa, Pakistan.
Methods: In the undertaken study sputum samples of 159 patients with a clinical presentation suggestive of pneumonia were analyzed for detection of the typical and atypical bacterial pathogens using conventional microscopy, culturing and multiplex PCR.
Results: Among 159 patients, mostly were male (56.6%) outpatients with age between 41 to 60 years (29.6%). Typical bacterial pathogens were detected in 102 (64.2%) patients from sputum culturing whereas atypical bacterial pathogens were detected in 6 (2.8%) patients using multiplex PCR. S. *pneumoniae*, *H. influenza* and Gram-negative enteric bacteria (*GNEB*) most frequently caused typical pneumonia among male and female patients of all age groups. Particularly, *GNEB* predominately caused typical pneumonia among younger patients (age < 21 years) and H. *influenza* frequently caused typical pneumonia among female patients. *Mycoplasma pneumoniae*, *Legionella pneumophila* and *Chlamydia pneumoniae* caused atypical pneumonia among older age (61-80 years) patients.
Conclusion: Findings of the current study may be helpful to improve diagnosis of pneumonia and to provide significant information for management of patients with pneumonia in Khyber Pakhtunkhwa region of Pakistan.

Keywords: Pneumonia, bacterial pathogens, sputum culture, multiplex PCR, Pakistan

Introduction
Pneumonia is the infection of lower respiratory tract, caused by different pathogens including viruses, bacteria, and fungi [1]. It is associated with high morbidity and mortality; with approximately 30% patients need admission in the hospital [2]. Globally, every year an estimated 2 million children die of age less than 5 years due to pneumonia [3]. However, the death rate is also high in older individuals with age more than 75 years [4]. According to a report, approximately ten million pneumonia cases appear every year and more than 91, 000 children die of this disease in Pakistan [5].
Streptococcus pneumoniae is the primary pathogen in causing typical pneumonia and accounted for two-third of all diagnosed cases of bacterial pneumonia [2]. Besides this *Haemophilus influenzae*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and Gram-negative bacilli also cause atypical pneumonia. *L. pneumophila*, *M. pneumoniae* and *C. pneumoniae* are the bacterial pathogens which cause atypical pneumonia [6, 7].
In the routine diagnosis of pneumonia, sputum culture may assist in etiology identification [8]. Culture assays may be useful for detection of the common etiologic pathogens of pneumonia. However, atypical bacteria grow either very slowly in culture or fail to grow which may delay diagnosis of the disease. Furthermore, serological tests are not useful for detecting the pathogens in an early phase of infection due to the low level of antibodies in serum [9]. Currently, the PCR based diagnostic assays have great potential for the rapid, specific and sensitive detection of causative pathogens in clinical samples of patients with infectious diseases, particularly pneumonia [10]. The successful amplification of nucleic acid of etiological agents by PCR based assays is particularly advantageous for the detection of fastidious

pathogens. Detection of a single pathogen by using PCR is limited particularly for patients with pneumonia. However, for simultaneous detection of various atypical bacterial pathogens in clinical samples, multiplex PCR is an efficient diagnostic tool [11].

As pneumonia is an infectious disease with a high mortality and the etiologic pathogens of pneumonia may vary depending on the hospital, geographical region, and country. If information on the etiologic pathogens of pneumonia is available, may increase the chance of appropriate antimicrobial therapy, thereby reducing the mortality and improving the prognosis [12]. Therefore, this study was conducted to investigate the bacterial etiologic pathogens of pneumonia among patients in a tertiary care hospital in Khyber Pakhtunkhwa, Pakistan.

2. Materials and Methods

2. 1 Patients and Sampling

This study was approved by Advance Studies and Research Board, Kohat University of Science and Technology (KUST), Khyber Pakhtunkhwa, Pakistan. Pre-informed consent for participation in this study was obtained from each patient. The study was carried out in accordance with the ethical principles as stated by the Declaration of Helsinki. The study population, where patients of different age groups and gender, seeking treatment at a tertiary care hospital Timergara, Dir, Khyber Pakhtunkhwa between October 2015 to April 2016. A total of 159 patients from districts Upper and Lower Dir visited this hospital for treatment during the study period. The inclusion criteria consisted of patients with new pulmonary infiltrate on a chest radiograph within 24 hours of presentation; the presence of one of the following clinical major characteristics: a cough, temperature > 37.8 °C, sputum production or minor symptom s such as pleuritic chest pain, dyspnea, altered mental status, pulmonary consolidation on examination, and white blood cell count of > 12,000 cells/µL. Patients with clinical evidence of tuberculosis were excluded from this study. The sputum samples were collected aseptically in a sterile container from inpatients and outpatients with pneumonia and were then processed within 24 hours for microbiological examination.

2.2 Detection of Bacterial Pathogens Causing Typical Pneumonia

Conventional microscopy and culture were performed in the diagnostic laboratory at Timergara Dir, Khyber Pakhtunkhwa hospital to determine the precise cause of typical pneumonia infection. Briefly only viscous, mucoid or purulent sputum specimens were considered for analysis. A gram-stained smear of each specimen was examined microscopically. The bacterial pathogens were isolated using suitable bacteriological media such as Blood Agar, MacConkey Agar, and Chocolate Agar. The plates were then incubated at 37 ºC for 24-48 hours. Identification and confirmation of bacterial isolates were completed using a variety of biochemical tests, as described by Mac Faddin [13].

2.3 Detection of Bacterial Pathogens Causing Atypical Pneumonia

Multiplex PCR was performed for detection of atypical bacterial pathogens in clinical samples of patients in which cause of pneumonia infection could not be determined by using conventional culture and serological tests. Total DNA from sputum samples was extracted by the boiling method as reported previously by Akter *et al*. [14]. In sputum samples of patients with pneumonia, the DNA of atypical bacterial pathogens was detected by using primers to amplify 360 bp *P1* gene for *M. pneumoniae*, 487 bp *mip* gene for *L. pneumophilia*, and 283 bp *Pst1* gene for *C. pneumoniae* reported previously by McDonough *et al*. [12] in multiplex PCR reactions. Amplification was performed in a thermal cycler (Eppendorf Mastercycler Gradient, Germany) with a reaction volume of 25 µl reaction containing 250 ng of DNA, 600 nM of each primer, 200 µM of each dNTPs, 1U of Taq DNA polymerase, 2 mM $MgCl_2$ in 1 x PCR buffer. Amplification was performed with an initial denaturation for 14 minutes at 94 ^{0}C, followed by 35 cycles of denaturation at 94 ^{0}C for 30 seconds, annealing at 59 ^{0}C for 90 seconds, extension at 72 ^{0}C for 30 seconds and a final extension at 72 ^{0}C for 7 minutes.

A positive control ladder was generated from positive samples for *M. pneumonia, C. pneumoniae* and *L. pneumophila*, which displayed bands from all targets in the multiplex PCR as reported previously by McDonough *et al*. [15]. Genomic DNA of *E. coli* was used as negative control. The PCR products were electrophoresed on 1. 2% agarose gel stained with ethidium bromide and visualized under UV transilluminator (BioTop gel documentation system, China).

3. Results

In this study in total 159 patients with pneumonia were investigated to determine bacterial cause of pneumonia. Among 159 patients, 90 (56.6%) were male and 69 (43.4%) were female. The participated patients were both outpatients (n=109; 68.6%) and inpatients (n=50; 31.4%) of different age groups. The greater number of patients (n=47; 29.6%) belonged to the age group 41-60 followed by age groups 61-80 years (n=39; 24.5%), 21-40 years (n=36; 22.6%) and 0-20 years (n= 34; 21.4%). Lowest frequency (n=3; 1.9%) was observed in old patients with age 81 years or above (Figure 1).

Based on conventional microscopy and culturing, the precise cause of typical pneumonia infection was determined among 102 (64.2%) patients while 57 (35.8%) patients were recognized as patients of unspecified pneumonia as a cause of infection could not be determined using these tests. The typical bacterial pathogens detected were *Streptococcus pneumoniae* (30.8%), *Haemophilus influenza* (18.9%), *GNEB* (8.8%) and *Staphylococcus aureus* (5.7%) shown in Table 1. Among 57 patients with unspecified pneumonia, typical bacterial pathogens were detected in only 6 (2.8%) patients using multiplex PCR and 51(23.3%) patients remained unspecified as neither the conventional tests nor the multiplex PCR assay detected bacterial pathogens in them. On amplification of target gene sequences, multiplex PCR produced a band of 360 bp *P1* gene for *M. pneumoniae*, 487 bp *mip* gene for *L. pneumophila*, and 283 bp *Pst1* gene for C. pneumoniae on agarose gel electrophoresis. The results of multiplex PCR are shown in Figure 2.

When multiplex PCR was performed for *M. pneumoniae, L. pneumophila* and *C. pneumoniae*, a single bacterial pathogen was detected in 5 patients, while co-infection of two pathogens was detected in one patient. In total of six pneumonia patients, etiology of three bacterial pathogens found was *M. pneumoniae* in 3 (50%) patients, *L. pneumophila* in 1 (16.67%) samples, both *M. pneumoniae* and *L. pneumophila* in 1 (16.67%) sample, *C. pneumoniae* in 1 (16.67%) sample as shown in Table 2.

Bacterial etiology according to gender, age and status of pneumonia patients are shown in Table 2. Overall, typical

bacterial pathogens most frequently caused pneumonia among outpatients (77.1%) of all age groups. S. *pneumoniae* and *H. influenza* most commonly caused pneumonia among patients with age between 21 to 80 years while *GNEB* caused pneumonia among patients with age < 21 years. Typical bacterial pathogens most frequently caused pneumonia among older age (61-80 years) patients. Overall, the rate of detection of pathogenic bacteria was high in male (83.3%) as compared to female (47.8%). *S. pneumoniae* was the most common cause of was pneumonia (49.3%) among male patients, whereas H. *influenza* was the most common cause of pneumonia (51.5%) among female patients.

4. Discussion

The clinical signs and symptoms of pneumonia are not the sufficiently specific to the type of pneumonia and pathogens. Therefore, precise detection of causative pathogens of pneumonia in clinical specimens is critical in diagnostic laboratories. However, diagnosis of etiological pathogens of pneumonia has always been difficult, with the causative organism is often determined in only 15% of cases with routine microbiological tests, especially when infection is caused by atypical respiratory pathogens [16]. However, PCR based diagnostic assays are more sensitive for detection of multiple microorganisms particularly atypical respiratory pathogens and thus increased the diagnostic yield. Despite the sensitivity and the availability of PCR based diagnostic assays, a variety of diagnostic laboratory tests for pneumonia include serology and culture-based methods in routine in low-income countries. Moreover, for the establishment of the etiology of pneumonia, sputum is a sample of choice, as it is well studied for the detection of both typical and atypical bacteria due to significant organisms yield [17, 18].

In this study, approximately in 64.2% sputum samples precise cause of typical pneumonia infection was determined by conventional microscopy and culturing. Among typical bacterial pathogens *S. pneumoniae* (30.8%), *H. influenza* (18.9%), Gram-negative enteric bacilli (8.8%) and *S. aureus* (5.7%) were detected in sputum of the pneumonia cases in this region of Pakistan. These bacterial pathogens with variable frequencies were also identified as the leading cause of pneumonia in a population of Trinidad, West Indies by Nagalingam and his colleagues in a previous study [19]. Similarly, Akter and coworkers identified *S. pneumoniae* (19.05%), *K. pneumoniae* (13.33%), *H. influenzae* (8.57%) and *P. aeruginosa* (5.71%) from clinical samples of patients with pneumonia in Bangladesh (14). In contrast to our study, Chi *et al*. [12] in a study isolated *S. aureus* (44%), *Acinetobacter baumanii* (30%), *P. aeruginosa* (12%), *Stenotrophomonas maltophilia* (7%), *K. pneumoniae* (6%), and *Serratia marcescens* (2%) from clinical samples of patients with ventilator-associated pneumonia. Differences in the microbiota of pneumonia reported in this study and in previous studies could be attributed to a well-known fact that the frequencies and composition of the microbiota of pneumonia may be variable depending on the location and studied population.

In the present study, 57 (35.8%) sputum samples not yielded a particular pathogen growth by conventional culturing. The negative results of sputum cultures among the pneumonia patients could be due to previous treatment with antibiotics or infection by other etiological agents such as virus and atypical bacteria which are routinely not cultured in the laboratory. It is a common practice in Pakistan that the patients use antibiotics themselves without prescription of clinicians before coming to a hospital. One of the limitations of this study was that serological tests for detection of causative pathogens including viruses and atypical bacteria were not performed as used in published studies [5-7, 20] Alternatively, Multiplex PCR was carried out to detect DNA of the three atypical bacteria in culture negative samples.

In the present study, atypical bacterial pathogens were detected in 2.8% sputum samples of patients with pneumonia. Previously, a high prevalence (31.8%) of atypical pathogens was reported among patients with pneumonia [21]. The precise cause of pneumonia in 23.3% patients could not be determined due to the fact that multiplex PCR was optimized only for three atypical bacterial pathogens. Moreover, a wide variety of other pathogens that also cause pneumonia were not considered, and that is another limitation of our study. Among atypical pathogenic bacteria, *M. pneumoniae, L. pneumophila* and *C. pneumonia* were recognized as important causes of pneumonia in the present study and in studies from Bangkok, Saudi Arabia by Prapphal *et al*. [20] and Al-ssum and Al-Malki [22] respectively. However, McDonough *et al.* [15] also detected Bordetella pertussis along with *M. pneumoniae, L. pneumophila* and *C. Pneumoniae* in clinical samples of patients with pneumonia. *M. pneumoniae, L. pneumophila* and *C. pneumoniae* are key bacterial pathogens causing atypical pneumonia. The reported incidence of *M. pneumoniae* is 10-30% [23] and of *C. pneumonia* is 10% of all cases of pneumonia [24], whereas *L. pneumophila* account to 5-10% of all cases of pneumonia [25]. Therefore, in the last few years various PCR based diagnostic assays have been optimized for the detection of these atypical bacterial pathogens in clinical specimens of patients with pneumonia [15, 26-27]. PCR amplification of the *P1, mip* and *PstI* genes fragments was shown to be highly specific and sensitive for detection of pneumonia-causing strains of *M. pneumoniae, L. pneumophila* and *C. pneumoniae* respectively in various studies [7, 15, 27-30].

Gender and age were found powerful determinants of pneumonia in some studies [12, 19, 31]. In current study *S. pneumoniae*, *H. influenza* and *GNEB* were detected as most common etiologies of pneumonia among outpatients of both genders and of all age groups. Some gender and age differences were also observed among studied patients with pneumonia in this study. Most of the diagnosed patients of pneumonia were between age 21 to 60 years and older patients aged 61-80 years were at risk for an "atypical" bacterial etiology. Similarly, in Dutch cohorts, males aged <60 years significantly more frequently had pneumonia caused by atypical pathogens [32]. In contrast, Lim *et al*., [33] and Gutiérrez *et al.* [34] determined the high prevalence of atypical pathogens among younger patients and observed a declining trend with age. Moreover, we detected *GNEB* as common causative pathogens of pneumonia among younger patients (age <21 years), however, *GNEB* were identified as causative agents of pneumonia in elderly patients in various studies [19, 35-36]. Previously, Nagalingam *et al*., [19] and Bénet *et al*. [37] reported that the prevalence of pneumonia, by gender of patients and each of the bacteria did not differ significantly. However, in this study, *H. influenza* was detected as a common causative pathogen of pneumonia among female patients whereas *S. pneumoniae* was detected as a causative pathogen of pneumonia among male patients. Poor socio-economic status, gender preferences for medical care, compromised health care services due to overcrowding, race and genetic makeup of individuals may contribute to differences in bacterial etiology of pneumonia according to

age and gender of patients in our country.

In conclusion, besides providing data on the bacterial etiology of pneumonia Khyber Pakhtunkhwa region of Pakistan, our results may be used to improve diagnosis of pneumonia in diagnostic laboratories, to guide clinicians for patient management and to understand etiopathology of pneumonia. Ultimately, this study will help to reduce the burden of pneumonia in Pakistan.

Competing interests

The author (s) declare that they have no competing interests.

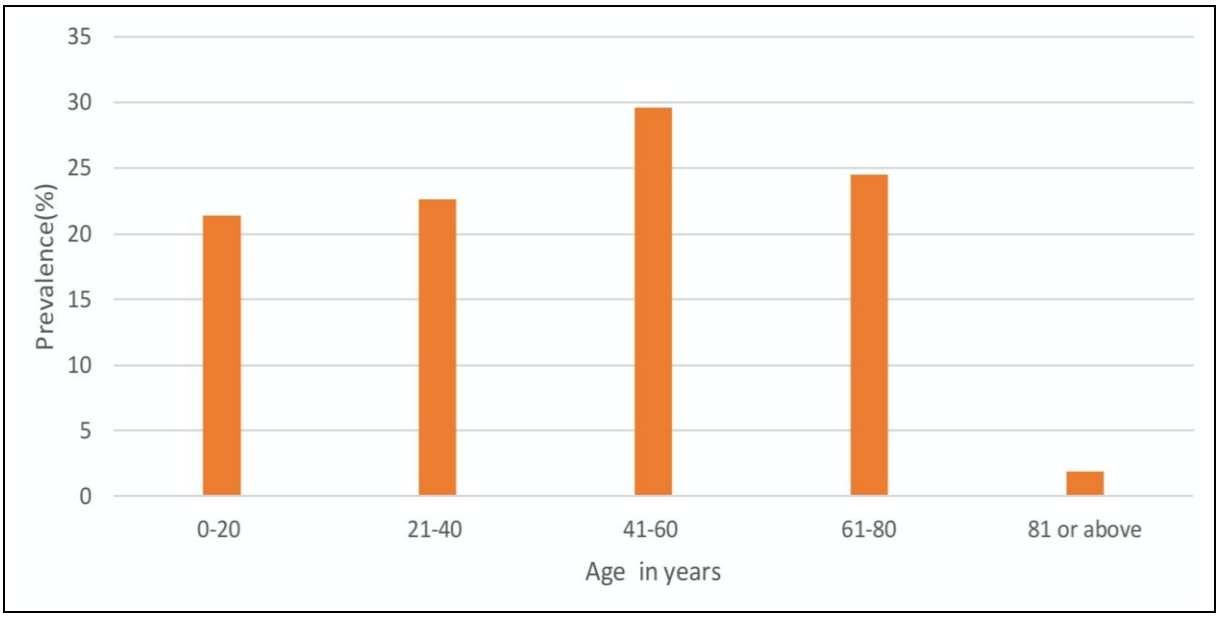

Fig 1: age –wise distribution of patients with pneumonia

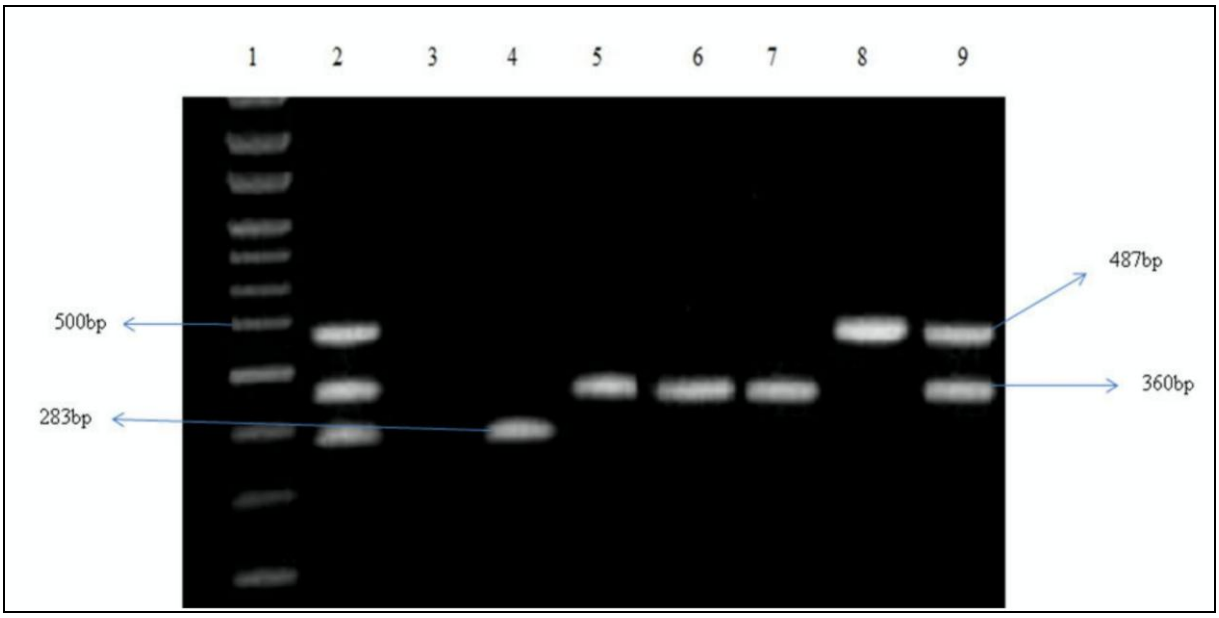

Fig 2: multiplex PCR detection of atypical bacterial pathogens: Bands in lane l shows ladder, lane 2 positive control, lane 3 negative control, lane 4 (283 bp) *C. pneumoniae*, lane 5,6, and 7 (360 bp) *M. pneumoniae*, lane 8 (487 bp) *L. pneumophila* and lane 9 co-infection of *M. pneumoniae* and *L. pneumophila*.

Table 1: Frequencies of bacteria species isolated from 159 sputum samples of patients with pneumonia

Bacterial isolates	Number (%)
Streptococcus pneumoniae	49 (30.8)
Haemophilus influenzae	30 (18.9)
Gram-negative enteric bacilli	14(8.8)
Staphylococcus aureus	9(5.7)
M. pneumoniae	3(1.9)
C. pneumoniae	1 (0.6)
L. pneumophila	1(0.6)
M. pneumoniae + *L. pneumophila* co-infection	1(0.6)

Table 2: Bacterial etiology of pneumonia according to gender, age group and status of patients

Parameters	Total cases of pneumonia (N=159)	Causative Bacteria of pneumonia No. (%)	Prevalence of pathogenic bacteria
Gender			
Male	90	75(83.3)	*S. pneumoniae* (n=37; 49.3%), *H. influenzae* (n=13; 17.3%), *GNEB* (n=11; 14.8%), *S. aureus* (n=9; 12.0%), *M. pneumoniae* (n=3; 4.0%), *L. pneumophila* (n=1;1.3%), *M. pneumoniae* + *L. pneumophila* co-infection (n=1;1.3%)
Female	69	33(47.8)	*S. pneumoniae* (n=12; 36.4%), *H. influenzae* (n=17; 51.5%), *GNEB* (n=3; 9.1%), *C. pneumoniae* (n=1; 3.0%)
Age (years)			
0-20	34	12(35.3)	*S. pneumoniae* (n= 4; 33.3%), *GNEB* (n=8; 66.7%)
21-40	36	31(86.1)	*S. pneumoniae* (n=17; 54.8%), *H. influenzae* (n=6; 19.4%), *S. aureus* (n=; 4; 12.9%), *GNEB* (n=3; 9.7%), *M. pneumoniae* (n=1;3.2%)
41-60	47	38(80.9)	*S. pneumoniae* (n=19; 50%), *H. influenzae* (n=14; 36.8%), *GNEB* (n=2; 5.3%), *S. aureus* (n=3; 7.9%),
61-80	39	26(66.7)	*S. pneumoniae* (n=9; 33.3%), *H. influenzae* (n=10; 37.1%), *S. aureus* (n=2; 7.4%), *L. pneumophila* (n=1;3.7%), *M. pneumoniae* (n=2;7.4%), *M. pneumoniae* + *L. pneumophila* co-infection (n=1; 3.7%), *C. pneumoniae* (n=1; 3.7%)
81 & above	3	1(33.3)	*GNEB* (n=1; 100%)
Patient Status			
Out patients	109	84(77.1)	*S. pneumoniae* (n=49; 58.3%), *H. influenzae* (n=26; 30.9%), *GNEB* (n=4; 4.8%), *M. pneumoniae* (n=3;3.6%), *C. pneumoniae* (n=1; 1.2%), *L. pneumophila* (n=1;1.2%)
Inpatients	50	24(48)	*GNEB* (n=10; 41.7%), *S. aureus* (n=9; 37.5%), *H. influenzae* (n=4; 16.7%), *M. pneumoniae* + *L. pneumophila* co-infection (n=1; 4.1%)

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