

## Etiological classification and clinical research on community-acquired pneumonia in Yantai, China.

Maomao Zhao<sup>#</sup>, Bo Song<sup>#</sup>, Yan Liu, Zenghui Pu, Hongxia Yu<sup>\*</sup>

Department of Infectious Diseases, Yantai Yuhuangding Hospital, Yantai, PR China

<sup>#</sup>These authors contributed equally to this work

### Abstract

This study aimed to determine the distribution of pathogens and drug-resistance of *Mycoplasma pneumoniae* in cases of Community-Acquired Pneumonia (CAP). A total of 128 CAP patients were selected for pathogen detection. Testing was performed for *Streptococcus pneumoniae* and *Legionella* urinary antigens, and bacterial sputum culture was performed. Throat swab samples were taken from all patients for culture and Polymerase Chain Reaction (PCR) testing for *M. pneumoniae* and viruses. Paired sera were used to test for *M. pneumoniae* antibodies. Of 128 cases, 79 tested positive for pathogens. *M. pneumoniae* was the most common pathogen, with a positive rate of 35.16% (45 cases), followed by viruses, with a positive rate of 30.16% (38 cases); bacteria accounted for a small proportion, with a positive rate of 13.28% (17 cases). *M. pneumoniae* infection played an important role in CAP, but the proportion of viral pneumonia was also significant. The rate of *M. pneumoniae* resistance to macrolides was 100% and should be considered when preparing the treatment plan.

**Keywords:** Pneumonia, Community-acquired, Pathogen, Epidemiology, Drug resistance.

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### Introduction

Community-Acquired Pneumonia (CAP) is one of the most common respiratory diseases; CAP is a significant cause of morbidity and mortality and is often misdiagnosed [1]. In the USA, a co-diagnosis of acute asthma is common in children with CAP; at the same time, misdiagnosis of CAP could lead to inappropriate treatment [2]. Accordingly, the etiology and treatment remain a focus of attention. Various pathogenic microorganisms can cause CAP, and their distribution varies with time, environment, population structure, and antibiotic usage. Several Chinese medical institutions performed an etiological classification of CAP, and found that the main pathogens included bacteria, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*. Early diagnosis of pneumococcal pneumonia facilitates appropriate antibiotic therapy [3]. Because of differences in diagnostic methods, the incidence rates of CAP caused by *M. pneumoniae* and *C. pneumoniae* (which are atypical pathogens) varied greatly in reports, while there were few comprehensive studies on viruses. Viruses are increasingly recognized as major causes of CAP. Few studies have investigated the clinical predictors of viral pneumonia, and the results have been inconsistent [4]. One recent study in India showed that viruses and gram-negative bacilli are the dominant causes of CAP [5]. The ESCAPED study enrolled 254 patients, 28% of whom had viruses; intracellular bacteria were found in 8 (3%) patients [6]. In this study, we aimed to summarize the clinical features of various pathogenic

microorganisms that cause CAP, thus providing a scientific basis for empiric treatment of CAP in this region, to reduce the inappropriate use of antibiotics.

### Materials and Methods

#### Study participants

This study included 128 CAP outpatient cases treated in our hospital from December 2010 to March 2012.

**Inclusion criteria for CAP cases were:** 1) met diagnostic criteria for CAP; 2) age > 14 years old, regardless of sex; and 3) volunteered to participate this survey.

**Exclusion criteria:** 1) pregnancy or breast-feeding status; 2) bronchiectasis; 3) active pulmonary tuberculosis; 4) aspiration or obstructive pneumonia; 5) hospitalization within 2 weeks of CAP onset, and inability to rule-out community-acquired infection; and 6) HIV-positive status. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Yantai Yuhuangding Hospital. Written informed consent was obtained from all participants' guardians.

#### Data collection

Data on demographic factors (sex, age, smoking, coexisting disease, and antibiotic pre-treatment), clinical symptoms and

signs (fever, heart rate, respiratory rate, cough, sputum production, dyspnoea, chest pain, dizziness, headache, moist rales and dry rales), and laboratory test results (White Blood Cell (WBC) count, neutrophils, lymphocytes, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Lactic Dehydrogenase (LDH), and Creatine Kinase (CK)) were collected using data abstraction forms for patients meeting the inclusion criteria.

### **Microbiological laboratory tests**

Two pharyngeal swabs were taken: one was used for viral Polymerase Chain Reaction (PCR) testing (influenza A virus, influenza B virus, parainfluenza virus types 1, 2, 3, and 4, respiratory syncytial virus types A and B, adenovirus, human coronavirus 229E/NL63 and OC43, human rhinovirus A/B/C, human bocavirus, human metapneumovirus, and enterovirus), and the other was used for *M. pneumoniae* culture and PCR assay. One urine sample was used for antigenic testing for *S. pneumoniae* and *Legionella*. Acute and convalescent sera were sampled for *M. pneumoniae* antibody. One sputum sample was used for routine bacterial smears, Gram staining, acid-fast staining, and bacterial culture, and another was used for *M. pneumoniae* culture, viral PCR testing, and *M. pneumoniae* and *Legionella* assays. In addition, all patients underwent routine blood biochemical testing.

### **Urinary antigen test**

The Binax NOW<sup>®</sup> pneumococcal antigen detection kit (colloidal gold method) and the *Legionella* pneumonia antigen detection kit (colloidal gold method) were used to test urine for *S. pneumoniae* and *Legionella* in patient urine. All kits were products of Inverness Medical Innovation (USA).

### **Bacterial test**

Purulent sputum was smeared for Gram stain, and microscopically-qualified specimens (squamous cells <10/low-magnification field, polymorphonuclear leukocytes >25/low-magnification field, or a ratio of the above two <1:2.5) were then submitted for bacterial culture; conventional methods were used to separate and identify the bacteria.

### **Respiratory virus nucleic acid test**

A multiplex viral nucleic acid PCR (MP-PCR) detection kit (RV 15 ACE Detection, Seegene, Inc., Seoul, Korea) was used to screen pharyngeal swab specimens for 15 kinds of respiroviruses, including influenza virus A, adenovirus, metapneumovirus, rhinovirus, respiratory syncytial virus A, respiratory syncytial virus B, coronavirus OC43 HKU1, coronavirus 229E/NL63, parainfluenza virus 1, parainfluenza virus 2, parainfluenza virus 3, parainfluenza virus 4, influenza virus B, bocavirus, and enterovirus. Applied Biosystems<sup>®</sup> 9700 PCR amplifier (Foster City, CA, USA) was used for analysis.

### ***M. pneumoniae* nucleic acid test**

A Fluorescence Quantitative PCR method (FQ-PCR) was used to detect *M. pneumoniae* nucleic acid, with sequences of primers and probes as follows: F: 5'AAGGGTrCAn mG-3; R: 5 a CGCCTGCGCTrGCTII-AC-3; probe i: 5-AGGTAATGGCTAGAG, GACTG. 3FQ. The PCR amplification parameters were: 93°C for 2 m; 93°C for 45 s, 55°C for 60 s, for 10 cycles; 93°C for 30 s, 55°C for 45 s, for 30 cycles.

### ***M. pneumoniae* culture and susceptibility testing**

A classic color-changing liquid culture medium was used. The basic broth medium and medium additives were purchased from OXIOD to self-prepare the liquid culture medium. The strains with positive culture results underwent MP-PCR testing. SP4 liquid medium (Remel Inc.) was used to detect Minimum Inhibitory Concentrations (MICs) for 9 antibiotics (erythromycin, clarithromycin, azithromycin, tetracycline, minocycline, moxifloxacin, gatifloxacin, levofloxacin, and ciprofloxacin) against *M. pneumoniae* using a micro-dilution assay (-). A standard strain of *M. pneumoniae* MPFH (ATCC 15531) was used for quality control; macrolide-resistant strains had an MIC for erythromycin of  $\geq 32 \mu\text{g/ml}$ , and sensitive strains had an MIC of  $\leq 0.008 \mu\text{g/ml}$ .

### **Detection of macrolide resistance associated mutations**

The *M. pneumoniae* clinical isolates were identified by PCR. The total length of the 23S ribosomal RNA (rRNA) gene of each *M. pneumoniae* strain was amplified and sequenced. The mutations of 23S rRNA were determined by alignment with sequences of MPFH (ATCC 15531).

### ***M. pneumoniae* antibody detection**

Acute and convalescent venous blood (2 ml each) was collected, and *M. pneumoniae* IgG antibodies were quantitatively determined with ESR127G kit (Virion-Serion, Germany).

### **Positive diagnostic criteria for pathogen confirmation**

1) One or more strains of bacteria were cultured from the qualified sputum samples; 2) the PCR assay detected *M. pneumoniae* or a virus from a pharyngeal swab specimen; 3) *M. pneumoniae* was cultured from pharyngeal swab specimens or sputum samples; 4) serum *M. pneumoniae* antibody increased 4-fold or more between acute and convalescent periods; 5) *S. pneumoniae* or *Legionella* antigen was detected in the urine sample.

### **Statistical analysis**

All statistical analyses were performed with SPSS statistical software (version 16.0; SPSS Inc., Chicago, IL, USA). Continuous data and categorical variables were expressed as ( $\bar{x} \pm s$ ) and frequency, respectively. We used logistic regression

analysis to identify variables capable of identifying *M. pneumoniae* and viral CAP. A two-tailed  $P < 0.05$  was considered statistically significant.

**Results**

**Characteristics of the study participants**

The 128 CAP patients included 66 males and 62 females, with a mean age of  $(49.92 \pm 18.67)$  years. The frequencies of smoking, coexisting disease, fever, cough, expectoration, dyspnoea, chest pain, dizziness, headache, and moist and dry rales were 14.8%, 7.0%, 78.1%, 89.8%, 67.8%, 10.2%, 12.5%, 7.1%, 20.3% and 2.3%, respectively. The WBC counts, neutrophil and lymphocyte percentages, and ALT and AST levels were  $(8.22 \pm 3.84) \times 10^9/L$ ,  $(66.40 \pm 12.79\%)$ ,  $(23.60 \pm 10.94\%)$ ,  $(29.24 \pm 21.36)$  U/L and  $(26.56 \pm 11.77)$  U/L, respectively (Table 1).

**Pathogen test**

Double pharyngeal swab specimens were sampled in all 128 patients for *M. pneumoniae* and 15 kinds of viruses; urine was tested for *S. pneumoniae* and *Legionella* antigens; acute and convalescent serum specimens were tested in 103 patients using a *Mycoplasma* antibody test; 60 cases underwent sputum culture; and 2 cases underwent blood culture. Among the 128 patients, 79 had positive pathogen test results (61.72%). A total of 45 cases had positive results for *M. pneumoniae* (35.16%), among whom 27 cases were positive on the pharyngeal swab PCR assay (21.09%); 22 cases had a 2-fold or greater increase in *M. pneumoniae* antibody in the recovery period (17.19%), and 20 cases had positive results on *Mycoplasma* culture (15.63%); the pharyngeal swab PCR assay revealed 38 cases positive for virus detection (30.16%), including 11 cases of adenovirus (8.59%), 11 cases of influenza virus A (8.59%), 8 cases of parainfluenza virus (6.25%), 7 cases of rhinovirus (5.47%), 4 cases of metapneumovirus (3.13%), 1 case of respiratory syncytial virus (0.78%), 1 case of enterovirus (0.78%); 17 cases exhibited positive sputum bacterial culture (13.28%), including 9 cases of *S. pneumoniae* (7.03%); 6 cases of *Haemophilus influenzae* (4.69%); 1 case of *Staphylococcus aureus* and *Klebsiella pneumoniae* each (0.78%). Nineteen cases showed mixed infection (14.83%) (Table 2).

**Drug resistance of *M. pneumoniae***

Sensitivity experiments were performed for the 20 cases that were positive for *M. pneumoniae*, and the results showed they were all highly resistant to macrolides; the resistance mechanism was the mutation from A to G at locus 2063 of the

23s rRNA gene; sensitivity to quinolones and tetracyclines was also noted (Table 2).

**Factors affecting *M. pneumoniae* and viral CAP in the logistic stepwise regression model**

We performed a logistic regression analysis to determine the association of *M. pneumoniae* and viral CAP with other factors (Table 3). The independent variables were sex, age, smoking status, coexisting disease, fever, cough, sputum production, dyspnoea, dizziness and headache, chest pain, WBC counts, LDH, CK, frequency of neutrophils and lymphocytes. Age was independently and inversely associated with *M. pneumoniae* infection. In addition, age, cough, and dyspnoea were positively correlated with viral CAP, but sex and smoking status were negatively correlated.

**Table 1.** Characteristics of the study participants.

Variable	Total population
Gender (male/female)	66/62
Age (year)	49.92 ± 18.67
Smoking status (%)	19 (14.8)
Coexisting disease (%)	9 (7.0)
Fever (%)	100 (78.1)
Heart rate (beats/minute)	82.21 ± 11.28
Respiratory rate (breaths/minute)	18.51 ± 3.44
Cough (%)	115 (89.8)
Expectoration (%)	74 (57.8)
Dyspnoea (%)	13 (10.2)
Chest pain (%)	16 (12.5)
Dizziness and headache (%)	9 (7.1)
Moist rales (%)	26 (20.3)
Dry rales (%)	3 (2.3)
Antibiotic pretreatment (%)	41 (32.0)
WBC counts ( $\times 10^9/L$ )	8.22 ± 3.84
Neutrophil (%)	66.40 ± 12.79
Lymphocyte (%)	23.60 ± 10.94
ALT (U/L)	29.24 ± 21.36
AST (U/L)	26.56 ± 11.77

**Table 2.** Pathogen classifications of CAP in Yantai.

Positive pathogen n=79	<i>Mycoplasma</i> cases (%)	Virus cases (%)	Bacteria cases (%)	Mixed infection cases (%)
	PCR assay	Adenovirus	<i>S. pneumoniae</i>	Bacteria and viruses
	27 (21.09)	11 (8.59)	9 (7.03)	2 (1.56)

Serum antibody assay	Influenza virus	<i>H. influenzae</i>	Bacteria and <i>mycoplasma</i>
22 (17.19)	11 (8.59)	6 (4.69)	8 (6.25)
Positive culture	Parainfluenza virus	<i>S. aureus</i>	<i>Mycoplasma</i> and virus
20 (15.63)	8 (6.25)	1 (0.78)	7 (5.47)
	Rhinovirus	<i>K. pneumonia</i>	3 kinds of pathogens
	7 (5.47)	1 (0.78)	2 (1.56)
	Metapneumovirus		
	4 (3.13)		
	Respiratory syncytial virus		
	1 (0.78)		
	Enteroviruses		
Sum	45 (35.16)	38 (30.16)	19 (14.84)

**Table 3.** Factors affecting *Mycoplasma pneumonia* and viral CAP in the logistic stepwise regression model.

Independent variable	$\beta$	S.E.	P
<i>Mycoplasma pneumonia</i>			
Age	-0.047	0.018	0.009
Viral CAP			
Gender	-1.69	0.765	0.027
Age	0.04	0.018	0.028
Smoking status	-2.705	1.127	0.016
Cough	3.039	1.47	0.039
Dyspnoea	3.621	1.781	0.042

The following variables were included in the model: gender, age, smoking status, coexisting disease, fever, cough, expectoration, dyspnoea, dizziness and headache, chest pain, WBC counts, LDH, CK, frequency of neutrophil and lymphocyte.

## Discussion

The Chinese and foreign guidelines on CAP suggested that bacteria, atypical pathogens, and viruses are the main pathogenic microorganisms of CAP [7,8]. Before the antibiotic era, more than 95% pneumonia cases were caused by *S. pneumoniae*. Although *S. pneumoniae* remains the major pathogen causing CAP, the incidence rate has dropped significantly than previously; in the USA, only 10-15% hospitalized CAP patients were infected with *S. pneumoniae* [9-11]. Limited by such reasons as inspection techniques, domestic studies on CAP pathogens mainly focused on bacteria and atypical pathogens. Since the appearance of SARS and Human Highly Pathogenic Avian Influenza (HPAI), as well as the worldwide pandemic of influenza in 2009, worldwide concern over viral pneumonia has increased [12-14]; however, most studies focused on a single virus, and observational studies on the proportion of viral pneumonia in CAP are very few. Recently, European Respiratory Society and European

Society of Clinical Microbiology and Infectious Diseases co-revised the treatment guidelines on adult community-acquired respiratory tract infections. Diagnostic tools for common lower respiratory tract infection causing pathogens were evaluated in details; nucleic acid amplification detection was recommended for clinical examination, emphasizing that for patients with lower respiratory tract infections, the diagnosis and differentiation of bacterial, viral, and atypical pathogens is very important [8,15]. Recent foreign and domestic studies also showed that the impacts of virus towards respiratory tract infections in adults were underestimated [16,17]. A recent domestic study found that adenovirus 55 is an important pathogen causing adult CAP [18]. This study showed that among the CAP cases in Yantai, 38 (30.16%) were caused by viral infection and 23 (17.97%) were caused by simplex virus infection, indicating that the proportion of viral infections in CAP cannot be ignored.

Because of the different diagnostic methods, the incidence rates of CAP caused by *M. pneumoniae* and *C. pneumoniae* (atypical pathogens that could cause CAP) differ largely between studies. The newest PCR technique could help clarify the true incidence rate. The results of epidemiological surveys from domestic and the Asia-Pacific region show that *M. pneumoniae* is the main pathogen causing CAP [19,20]. The macrolide antibacterial drugs are the traditional treatment of *M. pneumoniae*; however, in recent years, drug resistance of *M. pneumoniae* to macrolides has increased. According to the recent statistical data, the resistance rate of *M. pneumoniae* to macrolides in France is 9.8% [21], in Germany 3% [22], in the USA 27% [23], and in Japan 30.6% [24]. In China, the drug resistance surveillance revealed it to be 83% and 92% that towards children in 2009 [25,26], and 69% in one adult drug resistance surveillance in 2010 [27]. In our study, 20 cases of *M. pneumoniae* infection exhibited drug resistance to macrolides, and the drug resistance rate was 100%. Although the resistance of *M. pneumoniae* to macrolides would not lead to increased treatment failure and mortality, it might prolong the duration of fever and disease in the patients [25,27]. One

study showed that doxycycline can safely and effectively replace macrolides and can be combined with lactams to treat adult CAP [28]. This study suggested that the health workers in this region should avoid using macrolides as much as possible for treating *M. pneumoniae*, and should use quinolones or tetracycline drugs.

Mixed infection in CAP has always been attention research interest. Our study shows that in Yantai, 19 CAP cases (14.84%) were caused by mixed infections; *M. pneumoniae* was the common pathogen in mixed infections. This result is consistent with previous studies. Among the cases of mixed infection, rates of mixed infection with *M. pneumoniae* and other bacteria and with *M. pneumoniae* and virus were both high. Lepow might be able to generalize the roles of mixed infections in CAP; however, the role of mixed infections remains to be studied. Whether the infection of a pathogen facilitates infection with another pathogen or simultaneous infection with multiple pathogens cause's respiratory infections is not very clear.

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### Conflicts of Interest

All of the authors declare that they have no conflicts of interest regarding this paper.

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#### \*Correspondence to

Hongxia Yu

Department of Infectious Diseases

Yantai Yuhuangding Hospital

PR China