

Original Article

SP-D, KL-6, and HTI-56 levels in children with *Mycoplasma pneumoniae* pneumonia

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Abstract: The study was aimed to evaluate the potential biomarkers from pulmonary surfactant protein D (SP-D), Krebs von den Lungen-6 (KL-6), and 56-kD a human type I protein (HTI-56) in serum and bronchoalveolar lavage fluid samples of children with *Mycoplasma pneumoniae* pneumonia. This retrospective study, self-controlled study enrolled 34 Chinese children with *M. pneumoniae* pneumonia. The levels of SP-D, KL-6, and HTI-56 in bronchoalveolar lavage fluid samples were assessed and compared between patients with unilateral lung infection and contralateral lungs without any abnormal findings. Significant differences in the levels of SP-D, KL-6, and HTI-56 were observed in infected bronchoalveolar lavage fluid samples compared with uninfected samples (all $P < 0.05$); however, there was no correlation between the serum level of SP-D, KL-6, and HTI-56 and their levels in infected and uninfected bronchoalveolar lavage fluid samples ($P > 0.05$). **Conclusion:** The high levels of SP-D, KL-6, and HTI-56 in infected bronchoalveolar lavage fluid samples may reflect the injury of alveolar epithelium caused by *M. pneumoniae*. Instead of SP-D in uninfected bronchoalveolar lavage fluid samples obtained by invasive bronchoscopy, serum SP-D may serve as a convenient medium to distinguish lung infection caused by *M. pneumoniae*.

Keywords: *Mycoplasma pneumoniae* pneumonia, bronchoalveolar lavage fluid, pulmonary surfactant protein D, Krebs von den Lungen-6

Introduction

In patients with community acquired pneumonia (CAP), radiologic imaging could only reveal the location, shape, and density of lesions in an infected lung. Imaging cannot determine the injury of pulmonary alveolar cells by pathogens such as viruses, *Mycoplasma pneumoniae*, or other bacteria. Therefore, it is important to identify biomarkers that might be clinically useful for evaluating parenchymal injury and recovery of lung tissue and predicting response to treatment.

The integrity of alveolar epithelium is believed to be of major importance to the pathogenesis of and recovery from lung infection. Alveolar epithelial type II cells (AEC-II), cuboidal alveolar type cells, cover 5% of the alveolar surface; and they are the source of pulmonary surfactant. Krebs von den Lungen-6 (KL-6) and pulmonary

surfactant protein D (SP-D) are synthesized and secreted from AEC-II [1]. SP-D plays important roles in local innate immunity [2] which includes inhibiting the growth of gram-negative bacilli and *M. pneumoniae* and promoting macrophages to eliminate pathogens [3].

The levels of plasma surfactant proteins SP-A, -B, and -D have been shown to increase in children with acute lung injury (ALI), but only the levels of SP-D markedly increase in bronchoalveolar lavage fluid (BALF) [4, 5]. This finding indicates that there is a direct association between the levels of plasma SP-D and BALF SP-D after damage to AEC-II during lung infection. SP-D may also be a more sensitive and specific marker for lung cell injury caused by infection than SP-A and -B. Leth-Larsen *et al.* [6] reported that serum SP-D levels in 61 patients with CAP-suspected bacterial infection

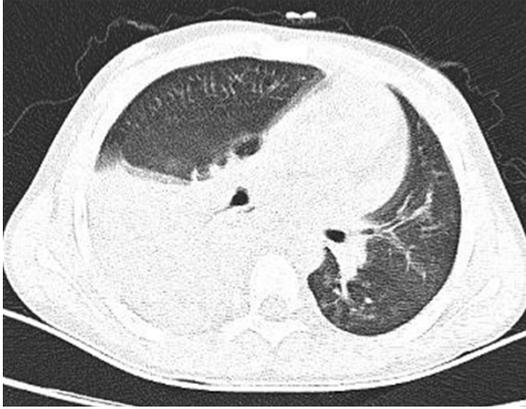


Figure 1. Representative high-resolution computed tomography images from one of 32 children with *Mycoplasma pneumoniae* pneumonia. Imaging of all the study participants showed unilateral consolidation of lung. For study enrollment, the area of consolidation was required to be greater than 1/3 the area of the unilateral lung with no contralateral consolidation at the same level.



Figure 2. Standard of bronchi of lobar *Mycoplasma pneumoniae* pneumonia (MPP) with bronchial normal appearance and structure.

were lower than the SP-D levels in control patients on the first day of hospitalization, and the levels increased on the fifth hospitalized day. These findings indicate that serum SP-D is not only associated with lung infection, but also that concentrations are directly proportional to the course and severity of lung infection.

Previous studies [7, 8] found that increased levels of KL-6, a high-molecular-weight glycoprotein, in the plasma and epithelial lining fluid in patients with acute respiratory distress syndrome were predictive of poor outcomes. Kubota et al. [9] reported that measurement of the serum KL-6 level was useful for the management of common pediatric respiratory infections. However, the role of KL-6 and the relationship of its levels in the serum and BALF in patients with common pediatric respiratory infections are largely unknown.

Thin cytoplasmic extensions of AEC-I cover more than 95% of the internal surface area of the lungs. Dobbs et al. [10] have developed an antibody that specifically reacts with human type I cell 56-kDa protein (HTI-56), which is localized to the AEC-I apical plasma membrane. The HTI-56 levels in alveolar edema and plasma of patients with ALI were much higher than HTI-56 levels in patients with alveolar edema and plasma of hydrostatic pulmonary edema.

To date, there are no reports available on the correlation of levels of SP-D, KL-6, and HTI-56

in the serum and BALF samples of children with *M. pneumoniae* pneumonia (MPP). Hence, the present study aimed to measure the levels of SP-D, KL-6, and HTI-56 in the serum and BALF samples of children with MPP.

Materials and methods

Study setting and ethical consideration

This was a prospective self-controlled study conducted at the Pediatric department of Shengjing Hospital of China Medical University, Shenyang, China from January 2012 to August 2013. The study was performed in accordance with the Declaration of Helsinki and local legislation. The child assent form was obtained from parents. The ethical committee of Shengjing Hospital approved the research.

Patients and selection criteria

Thirty-four children (boys: 18; girls: 16) with MPP, who were hospitalized during the study period were enrolled. Study participants were selected according to the following inclusion criteria: [1] presentation with clinical symptoms and signs such as fever, cough, dyspnea, rales, and attenuated breath sounds [2]; ≥ 4 -fold increase in *M. pneumoniae* antibody (MP-Ab) or titer of mycoplasma antibody-Immunoglobulin M (MP-IgM) was positive or positive polymerase chain reaction (PCR) assay for *M. pneumoniae* [3]; high-resolution chest computed tomogra-

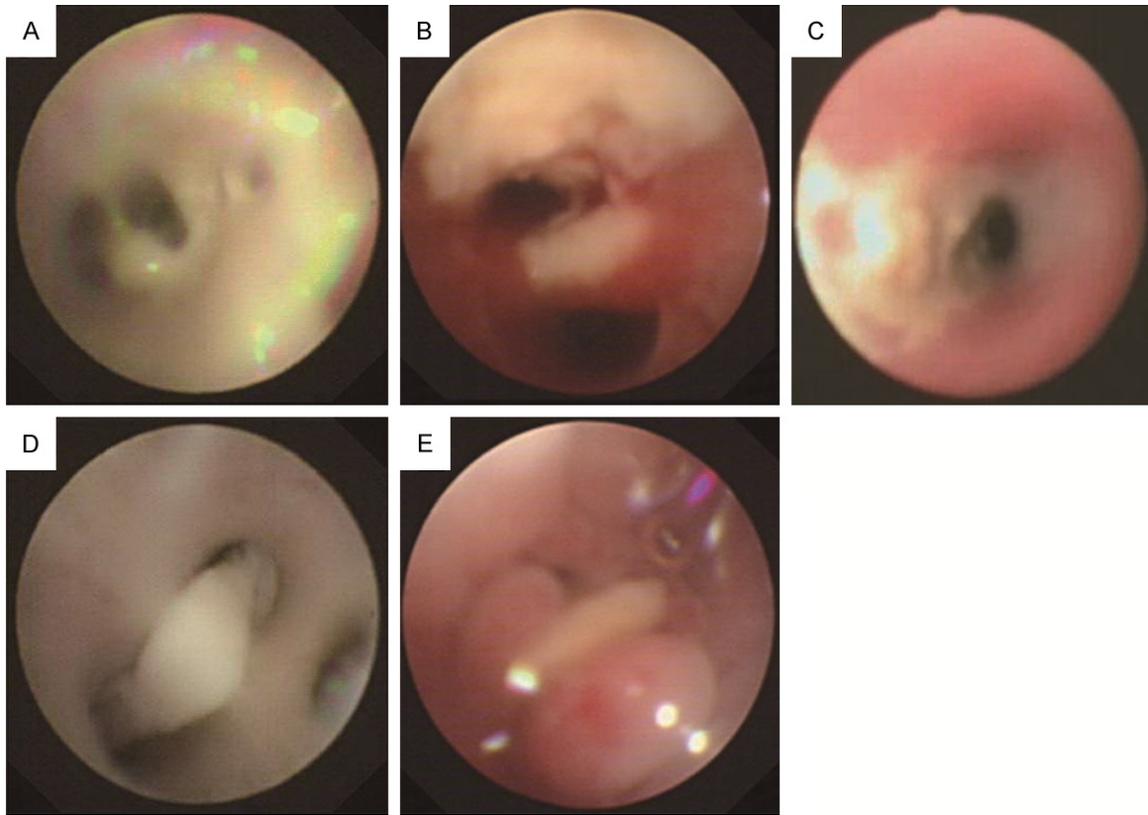


Figure 3. Cases were excluded. A. Cases with mixed infection of *Mycoplasma pneumoniae* (*M.p*) and *Streptococcus pneumoniae* (*S.p*) in the right lower lobe were excluded. B. Cases with *Tubercle bacilli* infection in the right lower lobe were excluded. C. Cases with *Aspergillus fumigatus* infection in the primary bronchi were excluded. D. Cases infected by *Mycoplasma pneumoniae* (*M.p*) with obstruction of bronchi were excluded. E. Cases infected by *Mycoplasma pneumoniae* (*M.p*) with the obstruction of bronchus by foreign body and polypus were excluded.

phy (HRCT) imaging showing healthy lung without any infiltration and abnormalities on one side of the chest and the contralateral infected lung with 1/3 or more segmental consolidation and/or atelectasis at the same HRCT level with no pleural effusion (**Figure 1**); and [4] flexible bronchoscopy was performed for microbiological diagnosis, to obtain BALF samples, and for resolution of atelectasis [11, 12]. Patients were excluded based on the following criteria: [1] BALF was obtained from the bronchi of *M. pneumoniae*-infected lobe with bronchial normal appearance and structure (**Figure 2**); and other pulmonary infections such as bacterial (**Figure 3A**) and viral pneumonia, pulmonary tuberculosis (**Figure 3B**), and fungal (**Figure 3C**) and human immunodeficiency virus infections [2]; underlying condition predisposing to pneumonia such as asthma, congenital heart disease, and airway abnormality [3]; extrapulmonary diseases including immunosuppression (cancer treatment or use of systemic steroids);

and [4] atelectasis caused by airway obstruction (**Figure 3D**), foreign body and polypus (**Figure 3E**), or other abnormality.

Blood sample collection

On the second day of hospitalization, peripheral blood samples were taken for routine laboratory examinations, C-reactive protein (CRP) levels, and bacterial cultures. A PCR assay was used to detect *M. pneumoniae* in nasopharyngeal secretions. Acute serum were collected and measured using a particle agglutination test per manufacturer's instructions for MP-IgM (*Mycoplasma pneumoniae* IgM Kit, Cat. no. EI 2202-9601 M, EUROIMMUN Medizinische labordiagnostika AG, Germany) and MP-Ab (*Mycoplasma pneumoniae* Ab Kit, Cat. no. YZB/JAP 1885-2009, FUJIREBIO INC., Japan). On the day of bronchoscopy, 2 mL of whole blood was collected in vacutainer tubes before the procedure. Serum samples were centrifuged

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Table 1. Patient's characteristics

Number of patients	34
Age (mean \pm SD, year)	5.08 \pm 3.31 (0.30~14)
Male gender	18 (52.94%)
Right lung/left lung	17/17
Course of disease (day)	11.23 \pm 6.52
Hospitalization duration (day)	15.70 \pm 4.99
Complete Blood Count	
WBC ($\times 10^9$)	9.11 \pm 3.57
Ne%	52.48 \pm 18.3
Lc%	33.55 \pm 16.86
CRP (mg/L)	44.34 \pm 58.62
Prealbumin (g/L)	0.15 \pm 0.17
Albumin (g/L)	36.43 \pm 5.07

CRP = C-reactive protein, Ne = neutrophils, Lc = leucocytes, WBC = white blood cells.

(1000 g at 4°C for 15 min), and the supernatant was stored at -80°C until analysis.

Bronchoscopy

After the patient was anesthetized, bronchoscopy was performed using a flexible bronchoscope (Olympus Company, Japan. Olympus BF-XP260F, BF-P260F, BF-260, external diameter: 2.8 mm, 4.0 mm, and 4.9 mm). The bronchoscope was first inserted into the healthy lung and placed in the wedge position at the level of the third- to fourth-order bronchi, and sterile isotonic saline (0.9% NaCl, (1 ml/Kg) was slowly instilled through the biopsy channel by syringe and repeated 3 times in 2 or 3 different regions. Fluid was aspirated using constant, gentle suction to minimize bronchial collapse. The volume of recovery was usually 40%-70% of the instilled volume [13]. The flexible bronchoscope was then withdrawn to the tracheal carina and inserted into the contralateral lung into the segments identified as infected by HRCT. Collected BALF samples were centrifuged at 4°C for 3000 rpm for 10 minutes, and the supernatant liquid stored at -80°C until analysis.

High resolution (HRCT) chest imaging

HRCT chest imaging was performed for each patient after admission. Based on the HRCT findings, flexible bronchoscopy was used to investigate airway abnormalities and to obtain BALF samples for analysis.

Measurement of SP-D, KL-6, and HTI-56 levels

Samples of serum and infected and uninfected BALF were assayed using ELISA kits as follows: SP-D, the Human SP-D ELISA Kit, (Cat. No. MBS720637; MyBioSource Co., Ltd., USA); KL-6, the Human KL-6 ELISA Kit (BioVendor Inc., USA); and HTI-56, the Human HTI-56 ELISA Kit (Cat. No. ABIN942599; Antibodies-Online, USA).

Statistical analysis

All analyses were performed using the statistical package SAS, Version 9.0 (SAS Institute Inc., United States of America). Data were tested for normality (Shapiro-Wilks *W*-test) and homogeneity (Bartlett's test for unequal variances). For each group, the values of mean \pm standard deviation (SD) were calculated for continuous variables, and relative frequencies were calculated for categorical variables. Chi-square test was used to calculate the association between categorical variables. Comparisons were made using the paired samples *t* test, and Pearson's correlation statistic was calculated to determine the relationship between 2 variables. All tests were 2-tailed, and a *P*-value less than 0.05 were considered statistically significant.

Results

Demographic characteristics

Of the 34 patients, 18 were male (52.94%). The mean age of patients was 5.08 \pm 3.31 years. There were 17 patients with infections in the right lungs, and the other patients had infections in the left lungs. Their mean hospitalization was 15.70 \pm 4.99. All other patient characteristics are mentioned in **Table 1**.

Levels of SP-D, KL-6, and HTI-56 in serum and BALF samples

The levels of SP-D, KL-6, and HTI-56 were measured in infected BALF, uninfected BALF, and serum samples. The levels of SP-D were 336.43 \pm 508.90, 107.24 \pm 144.77, and 73.23 \pm 71.85 in infected BALF, uninfected BALF, and serum samples, respectively. The levels of KL-6 were 261.63 \pm 318.06, 95.06 \pm 113.68, and 45.79 \pm 36.71 in infected BALF, uninfected BALF, and serum samples, respectively. The

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Table 2. Levels of KL-6, SP-D, and HTI-56 in serum and BALFs (ng/mL; Mean \pm SD)

Samples	SP-D	KL-6	HTI-56
Infected BALF	336.43 \pm 508.90 ^b	261.63 \pm 318.06 ^b	168.69 \pm 294.79
Uninfected BALF	107.24 \pm 144.77 ^a	95.06 \pm 113.68 ^{a,b}	47.16 \pm 92.93 ^a
Serum	73.23 \pm 71.85	45.79 \pm 36.71 ^a	31.98 \pm 46.48

^aCompared with infected BALF, $P < 0.05$; ^bCompared with serum BALF, $P < 0.05$.

Table 3. Correlation between serum levels of KL-6, SP-D, and HTI-6 and levels in BALF samples

Serum		Infected BALF	Uninfected BALF
SP-D	Correlation Co.	0.247	0.043
	<i>P</i> Value	0.180	0.819
KL-6	Correlation Co.	0.287	0.015
	<i>P</i> Value	0.111	0.935
HTI-56	Correlation Co.	-0.091	-0.204
	<i>P</i> Value	0.622	0.262

Co.: Correlation coefficient.

levels of HTI-56 were 168.69 \pm 294.79, 47.16 \pm 92.93, and 31.98 \pm 46.48 in infected BALF, uninfected BALF, and serum samples, respectively. SP-D, KL-6, and HTI-56 levels in infected BALF were significantly higher than the levels in uninfected BALF ($P < 0.05$).

To KL-6, there were great significant differences between serum levels and infected/uninfected BALF levels ($P < 0.05$). To HTI-56, there were no great significant differences between serum levels and infected/uninfected BALF levels ($P > 0.05$). To SP-D, there was significant difference between serum levels and infected BALF levels ($P < 0.05$), but no significant difference between serum levels and uninfected BALF levels ($P > 0.05$) (**Table 2**).

Correlation between serum levels of SP-D, KL-6, and HTI-56 and levels in infected and uninfected BALF samples

Rank correlation analysis was performed to explore the correlation between serum levels of SP-D, KL-6, and HTI-56 and levels in infected and uninfected BALF samples. The results revealed that no correlation between serum SP-D, KL-6, and HTI-56 and their corresponding levels in infected and uninfected BALF samples ($P > 0.05$) (**Table 3**).

Discussion

The present study involved the measurement of SP-D, KL-6, and HTI-56 levels in serum and BALF samples of children with MPP. The study results provided clinically relevant information to guide decisions in treatment and management of MPP.

CAP is one of the most common infectious diseases of children. In recent years, MPP has become a common cause of CAP in southeastern China. In 2008, pneumonia was considered as one of the leading causes of death in Chinese children fewer than 5 years of age [14]. To date, there are no clinically useful biomarkers to identify the infection and healing of alveolar epithelial cells and to predict response to treatment.

The results of previous studies indicate that the serum levels of SP-D, KL-6, and HTI-56 are elevated in a variety of lung diseases that are characterized by alveolar epithelial cell and air-blood barrier damage. Because the serum levels of SP-D, KL-6, and HTI-56 have been shown to be correlated with alveolar-capillary permeability, elevated levels of circulating SP-D, KL-6, and HTI-56 are believed to be associated with increased leakage from the alveolar space into the circulation [15]. Before the serum levels of SP-D, KL-6, and HTI-56 increase, they must first increase in BALF, which means increased concentrations of SP-D, KL-6, and HTI-56 in infected BALF is associated with damaged alveolar epithelial cells and air-blood barrier. Elevated levels of SP-D, KL-6, and HTI-56 in infected BALF samples of children with lung disease may reflect the presence of alveolar epithelial cell damage, followed by regeneration of type II pneumocytes (AEC-II) [16]. It can be speculated that AEC-II can initially proliferate during the early stages of MPP, accelerate the synthesis and secretion of SP-D and KL-6, leading to increased levels of SP-D and KL-6 in infected BALF; however, after development of severe AEC-II damage, AEC-II death or disorganized epithelial repair occurs, leading to decreases in the levels of SP-D and KL-6 in infected BALF [16].

The current study found that the levels of SP-D, KL-6, and HTI-56 in infected BALF were significantly higher than the corresponding levels in uninfected BALF. These levels were all low in

serum samples. In the normal lung, SP-D and KL-6 can be found in ACE-II, and their expression is greatly increased in proliferating, regenerating, and injured ACE-II. The study results suggest that both ACE-II and ACE-I were injured during *M. pneumoniae* infection in the study population. The rate of synthesis and secretion of SP-D and KL-6 in ACE-II and HTI-56 in ACE-I was increased along with their release into the infected alveoli. After cleavage of the S-S bond near the surface of the epithelial cell membrane, SP-D, KL-6 and HTI-56 can diffuse into the fluid of the pulmonary epithelial lining [14] and can be detected in BALF and serum.

An increased level of SP-D, KL-6 and HTI-56 in infected BALF compared with uninfected BALF is thought to be due at least partly to destruction of alveolar epithelial cell membranes and release into the alveoli. In the acute stage of *M. pneumoniae* infection, damage to the alveolar capillary network and pulmonary air-blood barrier were not severe enough to lead to increased capillary permeability and subsequent increased leakage of SP-D, KL-6, and HTI-56 from infected BALF into the circulation. Therefore, SP-D, KL-6 and HTI-56 levels in serum and uninfected BALF samples were significantly lower than the levels in infected BALF samples. If capillary permeability is enhanced and destruction of the air-blood barrier occurs as a result of the infection, the subsequent leakage of SP-D, KL-6 and HTI-56 into the circulation leads to increased serum levels and decreased infected BALF levels. If ACE-II and ACE-I are severely damaged, synthesis of SP-D, KL-6, and HTI-56 will be greatly reduced, leading to a marked reduction in their concentration in infected BALF. In addition, the study data suggest that ACE-II as part of the innate immune defense system, is more susceptible to injury than ACE-I. KL-6 is synthesized and secreted from ACE-II, and during infection, appears to be more sensitive than SP-D and HTI-56. The serum level of KL-6 cannot represent the injury degree of infected and uninfected lungs because there were significant differences both with infected and uninfected BALF samples. The serum level of HTI-56 also cannot indicate the injury of infected and uninfected lungs because there were no significant differences between both with infected and uninfected BALF samples. There was significant difference between serum SP-D/uninfected BALF SP-D and infected BALF SP-D, and no significant difference between serum SP-D and unin-

ected BALF SP-D. That meant the serum level of SP-D maybe represent the level of SP-D in uninfected BALF. It can be speculated that serum SP-D perhaps plays an important role to distinguish infected and uninfected lung caused by *M.p* and other pathogens. There was no correlation between the serum level of SP-D, KL-6 and HTI-56 and their levels in infected and uninfected BALF samples.

The current study had some limitations. Because of ethical considerations, there were no healthy subjects, who underwent bronchoscopy. Therefore, there were no data showing the normal levels of SP-D, KL-6 and HTI-56 in serum and BALF samples. They were compensated by only enrolling pediatric patients with unilateral infections of *M. pneumoniae* as seen on chest HRCT imaging. In addition, this was a small study; a larger number of children would be preferable. Also, little is known about the levels of SP-D, KL-6 and HTI-56 in children with CAP caused by other pathogens, in addition to the temporal changes of SP-D, KL-6 and HTI-56 during acute and convalescent stages of infection.

In conclusion, flexible bronchoscopy enabled to observe that the high levels of SP-D, KL-6 and HTI-56 in infected BALF samples which maybe reflect the degree of alveolar epithelial injury caused by *M. pneumoniae* infection. Instead of SP-D in uninfected BALF obtained by invasive bronchoscopy, serum SP-D maybe better to distinguish infected and uninfected lungs in children with MPP.

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Disclosure of conflict of interest

None.

Abbreviations

MPP, *Mycoplasma pneumoniae* pneumonia; SP-D, pulmonary surfactant protein D; KL-6,

Krebs von den Lungen-6; BALF, bronchoalveolar lavage fluid; AEC-II, alveolar epithelial type II cells; AEC-I, alveolar epithelial type I cells.

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