Original Article Role of preceding viral or *Mycoplasma pneumoniae* infection in invasive bacterial diseases in Chinese children

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Abstract: Background: Invasive bacterial disease, which could result from bacterial colonization, continues to be a major cause of morbidity and mortality in children worldwide. The aim of this study was to analyze correlations between preceding viral or Mycoplasma pneumoniae infections and bacterial colonization or invasive bacterial diseases in China. Methods: From January 2006 to December 2013, nasopharyngeal aspirate samples were obtained from children admitted to Children's Hospital of Soochow University. Common pathogens and bacterial colonization were detected using direct immunofluorescence, polymerase chain reaction, and bacterial culture. During the same period, cases with culture-confirmed invasive bacterial diseases were collected. Correlations between preceding viral or Mycoplasma pneumoniae infections, bacterial colonization, and invasive bacterial disease were analyzed. Results: Mycoplasma pneumoniae (24.9%) was the most common respiratory pathogen, followed by respiratory syncytial virus (15.7%) and human bocavirus (7.1%). Meanwhile, the most common bacterial colonizations were Streptococcus pneumoniae (12.7%), Haemophilus influenzae (4.9%), and Moraxella catarrhalis (3.7%). Septicemia was one of the most common invasive bacterial diseases. Human bocavirus was correlated with Streptococcus pneumoniae whereas influenza virus B, parainfluenza virus 3, and adenovirus were correlated with Haemophilus influenza. Mycoplasma pneumoniae was correlated with Moraxella catarrhalis. However, only respiratory syncytial virus and Streptococcus pneumonia were correlated with invasive bacterial disease. There were strong correlations between preceding viral or Mycoplasma pneumoniae infection, bacterial colonization, and invasive bacterial disease. Conclusion: Prevention of virus or Mycoplasma pneumonia infection may ultimately be an important strategy to control invasive bacterial disease.

Keywords: Respiratory virus, Mycoplasma pneumoniae, bacterial colonization, invasive bacterial infection

Introduction

Respiratory tract infections are common and frequently occur in childhood. According to the 2013 United Nations newsletter, about 260,000 children in China die due to community-acquired pneumonia every year, and it is the leading cause of death in children [1]. Most children with pneumonia die of severe invasive primary or secondary bacterial infections such as empyema, sepsis, peritonitis, and purulent meningitis. This phenomenon is particularly significant in developing countries where vaccines are not included in the national immunization plan, inflicting enormous medical burdens. Invasive bacterial disease (IBD) is a major cause of morbidity and mortality in children worldwide and is the most common is invasive pneumococcal disease [2].

The incidence of IBD varies seasonally and is related to preceding respiratory viral infections, environmental factors such as pollution, and climate factors such as temperature, humidity, and rainfall [3-7]. Bacterial infection in the lower respiratory tract often develops from bacterial nasopharyngeal colonization under circumstances such as immunodeficiency and previous viral infection. Consequently, nasopharyngeal colonization is a source of lower respiratory tract infection to some extent [8], and there are associations between viral or Mycoplasma pneumoniae (M. pneumoniae) infections and nasopharyngeal colonization and IBD. Nasopharyngeal colonization of bacteria such as Streptococcus pneumoniae (S. pneumoniae), Haemophilus influenzae (H. influenzae), and Moraxella catarrhalis (M. catarrhalis) may become pathogenic and cause IBD after previous viral or M. pneumoniae infections. A recent study has shown the correlation of preceding respiratory syncytial virus (RSV), influenza virus (IV), and human metapneumovirus (hMPV) infections with invasive pneumococcal disease, with a lag effect [4].

Up until now, no reported studies have examined the relationship between preceding viral infections and bacterial colonization or invasive IBD in China. Therefore, the aim of this study was to analyze the correlations between preceding viral or *M. pneumoniae* infections and bacterial colonization or IBD, as well as to provide strategies for early warning of the occurring disease, early prevention, and appropriate treatment of children with serious bacterial infections without the protection of vaccines in Suzhou, China.

Materials and methods

Study design

Children's Hospital of Soochow University providing 800 beds is the only tertiary teaching hospital in the Suzhou area of China, and cares for about 80% of hospitalized children in that area. This Children's Hospital has 1,350,000 visitors in outpatient and emergency departments, and discharges 40,000 patients every year. This study was conducted from January 2006 to December 2013 for 8 consecutive years in the Children's Hospital of Soochow University. The study consisted of 14,043 patients admitted to Department of Respiratory Disease, of whom 8649 were male (61.6%) and 5394 were female (38.4%), with a 1.60:1 maleto-female ratio, aged 1 month to 14 years old. All the admitted patients met the diagnosis criteria of acute lower respiratory tract infection including acute bronchiolitis, bronchitis, and pneumonia.

All the patients who were admitted to the Department of Respiratory Disease underwent multiple pathogens and bacterial colonization testing. This study analyzed the associations between preceding viral or *M. pneumoniae* infection and bacterial colonization or IBD. The Ethics Committee of Children's Hospital of Soochow University reviewed this study and all parents of children signed a declaration of informed consent.

Nasopharyngeal secretion collection and viruses or M. pneumoniae detection

From January 2006 to December 2013, collection of nasopharyngeal aspirate samples, detection of seven common viruses by direct immunofluorescence, and detection for hMPV and M. pneumoniae by polymerase chain reactions (PCR) were conducted as previously described [9-11]. In brief, nasopharyngeal aspirate samples were collected within 24 hours of admission to the hospital using disposable suction tubes inserted 5-10 cm deep into the nasal cavity, using negative pressure to draw out 1-2 mL of secretion, and centrifuged at 500 × g for 10 minutes. The samples were then resuspended in 2 mL saline and divided equally into three portions. Using direct immunofluorescence [9], RSV, IV-A, IV-B, parainfluenza viruses (PIV-1, PIV-2, PIV-3), and adenovirus (ADV) were detected. Meanwhile, hMPV was detected using reverse transcription PCR [10] and M. pneumoniae using quantitative PCR [11]. In order to exclude *M. pneumoniae* colonization, nasopharyngeal secretions containing more than 1×10^3 copies/mL of DNA were considered as true positives.

From January 2009 to December 2013, to test for human bocavirus (HBoV), a final 200 µL of DNA was eluted for each sample. Primers were designed using sequence information from the NP1 gene sequence available from the GenBank database. Primers and probe were synthesized using the following sequences: HBoV-forward: 5'-TGACATTCAACTACCAACAAC-CTG-3'; HBoV-reverse: 5'-CAGATCCTTTTCCTC-CTCCAATAC-3'; and HBoV-probe: AGCACCACA-AAACACCTCAGGGG-TAMRA (Sangon Biotech, Shanghai, China). PCR was performed in a reaction volume of 25 µL using an iQ5[™] BIO-RAD iCycler (BIO-RAD, Carlsbad, CA, USA). The 25-µL amplification reaction contained 3 µL sample DNA, 0.25 µL TaqMan (Promgea, Madison, WI, USA), 14.75 µL diethylpyrocarbonate treated water, 2.5 µL buffer solution, 2 µL 25 mM MgSO₄, 1 μ L dNTP, and 0.5 μ L each of forward and reverse primers and probe. Amplification was performed with the following settings: 40

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Figure 1. Monthly distribution of total tested samples and positive samples or rate in hospitalized children from January 2006 to December 2013.

cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. Positive controls, containing the target genes for all four HBoV subtypes (Sangon Biotech, Shanghai, China), and no-template controls were included in each run. The concentration of each detected sample was calculated automatically according to a standard curve.

Bacterial colonization of nasopharyngeal secretion

The nasopharyngeal aspirate specimens were made into suspension, and inoculated for 18-24 hours in as elective medium (both enriched chocolate agar and sheep blood agar for each sample) in an incubator at 35°C. All samples were cultured in trypticase soy broth containing 5 mg/mL gentamicin on enriched chocolate agar plates and on selective sheep blood agar plates containing 5 mg/mL gentamicin. Isolates were identified as S. pneumoniae, H. influenzae, and M. catarrhalis according to the laboratory's standard operating procedure. Determination of bacterial colonization was based on the positive bacterial culture of nasopharyngeal secretions (the amount of bacteria < +++), the duration of fever after admission less than 3 days, no significant increase in white blood cells (less than $15 \times 10^9/L$), C-reactive protein (CRP), and negative blood culture.

Identification of culture-confirmed IBD

All samples of IBD cases were collected from hospitalized children in all departments of

Soochow University Affiliated Children's Hospital, including the Respiratory Disease Department, if they were identified by the presence of original infectious diseases combined with positive bacterial culture in pleural effusion, peripheral blood, peritoneal fluid, cerebrospinal fluid, and other sterile body fluids.

Statistical analysis

The unit of time for colonization or various pathogen detection methods in this study was one month. Statistical data analysis was performed using the SPSS17.0 software. Correlations between virus or *M. pneumoniae* infection, bacterial colonization, and IBD were analyzed using Pearson correlation coefficient or Spearman non-parametric tests if the data did not meet the normal distribution. All *P* values less than 0.05 were considered statistically significant.

Results

Seasonal distribution of respiratory pathogens in hospitalized children

Of the 14,043 patient samples that were tested for pathogens from 2006 to 2013, 49.2% were positive for pathogens, and at least one pathogen was detected in 11.2% of all cases. Monthly distribution of total tested samples and positive samples or rate are shown in **Figure 1**. At 24.9%, *M. pneumoniae* had the highest detection rate, followed by RSV at 15.7%, HBoV at 7.1%, hMPV at 6.3%, PIV-3 at 4.5%, IV-A at 2.0%, ADV at 1.2% PIV-1 at 0.7%, IV-B at 0.6%, and PIV-2 at 0.1%.

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Figure 2. Seasonal and monthly distribution of common respiratory viruses and *M. pneumoniae* in hospitalized children from January 2006 to December 2013.



Figure 3. Monthly distribution of nasopharyngeal bacterial colonization in hospitalized children from January 2006 to December 2013.

M. pneumoniae was detected from samples collected year round and the highest monthly detection rate was 73.2% (41/56 cases, November 2008). The rate peaked in the summer and autumn every year from June to July and October to November, termed bimodal phenomenon. RSV and IV-A were detected mostly in the winter (December to February), and the highest monthly detection rates were 58.16% (82/141) and 14.58% (14/96), respectively. The peak activities of RSV and IV-A overlapped completely. HBoV was detected mostly in the summer and autumn seasons, and the highest monthly detection rate was 18.9% (17/90 cases). Meanwhile hMPV was detected mostly in the spring season (March to May) with the highest monthly detection rate at 31.6% (31/98 cases). PIV-3 and ADV were detected in the spring and summer (March to August), and the

highest monthly detection rates were, 21.5% (39/181) and 6.6% (8/121), respectively. IV-B, PIV-1, and PIV-2 detection rates had no significant seasonal trends, as shown in **Figure 2**.

Seasonal distribution of nasopharyngeal colonization in hospitalized children

From 2006 to 2013, 14,043 nasopharyngeal specimens were cultured for bacteria and the total detection rate for three common types of bacterial colonies was 19.2%. The S. *pneumoniae* detection rate was 12.7%, *H. influenzaewas* 4.9%, and *M. catarrhalis* was 3.7%. S. *pneumoniae* was detected year round and the detection rate was highest during winter months with the monthly detection rate up to 28.0% (28/100 cases). The *H. influenzae* detection rate was higher during spring months and

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Figure 4. Monthly distribution of invasive bacterial disease and correlations with *M. pneumoniae*, respiratory syncytial virus, human metapneumonia, and adenovirus in hospitalized children from January 2006 to December 2013.

Table 1. Clinical Syndrome Associated	With
Culture-Confirmed IBD in Children	

Clinical Syndrome	n (%)
Septicemia	1476 (94.6)
Empyema	60 (3.8)
Meningitis	8 (0.5)
Peritonitis	6 (0.4)
Empyema + Septicemia	6 (0.4)
Meningitis + Septicemia	3 (0.2)
Peritonitis + Septicemia	2 (0.1)
Total IBD	1561 (100)

IBD: invasive bacterial disease.

the highest monthly detection rate was 21.1% (30/142 cases). The *M. catarrhalis* detection rate was high in the winter months, the highest monthly detection rate was up to 13.2% (9/68 cases), as shown in **Figure 3**.

Monthly distribution of IBD in hospitalized children

From 2006 to 2013, a total of 1561 cases of children were diagnosed with IBD, and the monthly distribution of IBD is shown in **Figure 4**. Septicemia was one of the most common diseases (1,476 cases), followed by empyema (60 cases), purulent meningitis (8), peritonitis (6), and there were 11 cases of children with two diseases simultaneously (**Table 1**).

Correlation between viruses or M. pneumoniaeactivity, nasopharyngeal colonization, and IBD

Over the same period (no time lag), there was a positive correlation between HBoV and S.

pneumoniae only. HBoV, IV-B, PIV-3, ADV, and *M. pneumoniae* were found to be correlated *H. influenzae* was found. HBoV and *M. pneumoniae* were negatively correlated, while IV-B, PIV-3, and ADV were positively correlated with *H. influenzae*. As for *M. catarrhalis*, the only positive correlation was found with *M. pneumoniae*. With a one-month lag, IV-A and PIV-1 showed a negative correlation with *S. pneumoniae*. RSV and hMPV showed a positive correlation with *H. influenza*. *M. catarrhalis* showed a negative correlation with PIV-1. Compared to the same period (no time lag), the negative correlations of HBoV, *M. pneumoniae*, and *H. influenzae* had an increasing trend (**Table 2**).

Over the same period (no time lag), RSV, hMPV, ADV, and *M. pneumoniae* had correlation with IBD. However, it was only positively correlated with RSV. With a one-month lag, the correlation of hMPV, ADV, and *M. pneumoniae* with IBD still occurred (**Table 2**).

With regard to correlations between bacterial colonization and IBD, only *S. pneumoniae* colonization and IBD showed positive correlation over the same period (no time lag). With a one-month lag, correlations of *S. pneumoniae* colonization with IBD showed an increasing trend. However, *H. influenzae* and IBD showed a negative correlation (**Table 3**).

Discussion

Preceding viral or *M. pneumoniae* infection could affect nasopharyngeal colonization of bacteria in children [12] and it is closely related to invasive bacterial infection diseases [3, 4, 6,

	Bacterial colonization					IBD		
Respiratory	S. pneumoniae		H. influenzae		M. catarrhalis			
pathogens	0 month	1 month	0 month	1 month	0 month	1 month	0 month	1 month
	lag	lag	lag	lag	lag	lag	lag	lag
RSV	0.143	0.074	-0.047	0.336**	0.135	0.130	0.323**	0.151
hMPV	-0.161	-0.145	0.140	0.261*	0.100	0.048	-0.396**	-0.510**
HBoV	0.322*	0.285*	-0.304*	-0.406	0.053	-0.018	0.018	0.091
IV-A	-0.197	-0.290**	-0.073	0.128	-0.104	0.001	0.014	-0.092
IV-B	-0.032	-0.061	0.304**	0.263**	-0.055	-0.054	-0.040	-0.106
PIV-1	0.130	-0.037	0.133	-0.015	-0.137	-0.214*	-0.021	0.050
PIV-2	-0.033	-0.061	0.001	-0.161	-0.130	-0.043	-0.028	0.031
PIV-3	-0.027	0.064	0.221*	-0.044	-0.155	-0.122	-0.109	0.027
ADV	-0.134	-0.204*	0.320**	0.181	-0.056	-0.128	-0.378**	-0.327**
M. pneumonae	-0.051	-0.045	-0.208	-0.295**	0.219	0.205*	-0.319**	-0.254**

 Table 2. Correlation Coefficient between respiratory pathogen detection and bacterial colonization or IBD

*P < 0.05; **P < 0.01. IBD: invasive bacterial disease; RSV: respiratory syncytial virus; hMPV: human metapneumovirus; HBoV: human bocavirus; IV-A or B: influenza virus type A or B; PIV-1, 2, 3: parainfluenza virus type 1, 2, 3; ADV: adenovirus; *M. pneumonaie*: *Mycoplasma pneumonia*; S. *pneumonia*: Streptococcus pneumonia; *H. influenzae*: Haemophilus influenzae; *M. catarrhalis*.

Table 3. Correlation Coefficient between bacterial colonization and IBD

Bacterial	IBD			
colonization	0 month lag	1 month lag		
S. pneumoniae	0.220*	0.273**		
H. influenzae	-0.113	-0.217*		
M. catarrhalis	-0.011	-0.139		

*P < 0.05; **P < 0.01. IBD: invasive bacterial disease; S. pneumonia: Streptococcus pneumonia; H. influenzae: Haemophilus influenzae; M. catarrhalis: Moraxella catarrhalis.

8, 13, 14]. This study monitored the main pathogens of respiratory infection for eight consecutive years and analyzed the correlations between preceding virus or *M. pneumoniae* infection and nasopharyngeal colonization or IBD. The present study confirmed the correlations among respiratory infection with different pathogens and bacterial colonization or IBD and presumed that preceding viral infections could enhance the prevalence of bacterial colonization, and high prevalence of bacterial colonization increased the probability of IBD. However, not only positive correlations but also negative correlations were found in this singlecenter study.

Nasopharyngeal bacterial colonization often precedes serious invasive bacterial infections, and such phenomenon is more significant in

children with viral respiratory infections. The present study also found that RSV, hMPV, and ADV could enhance *H. influenzae* colonization, and that a lag effect was found with RSV and hMPV infection; these observations are consistent with a recent study of the association between RSV infection and H. influenzae colonization [15]. In vitro, respiratory viruses (RSV and PIV-3) promoted bacterial adhesion to respiratory epithelial cells as well as the expression of several known receptors for pathogenic bacteria, and this process increased bacterial colonization and contributed to disease [16]. In vivo, studies have shown that RSV infection of airway epithelial cells could induce an imbalance in the expression of epithelial cell antimicrobial peptide β-defensin-1, and allow for easier colonization of H. influenzae in the nasopharynx [12] and the M. catarrhalis colonization rate in the nasopharynx increased to 100% after one week in an advanced RSV infected animal model [17].

In terms of correlations between pathogen infection and IBD, the present study showed that preceding RSV infection was strongly positively correlated to IBD, which is consistent with a previous study [4]. Numerous previous studies have suggested that preceding respiratory viral infections are involved in the pathogenesis of invasive S. pneumoniae and invasive H. influenzae infections [18, 19]. However, only a few of them had large sample sizes to study preceding respiratory viral infections and IBD. To our interest, hMPV, ADV, and *M. pneumoniae* showed negative correlations with IBD. This may be a consequence of different seasonal distributions of hMPV, ADV, and *M. pneumoniae* in the spring or summer compared to RSV in the winter.

With regard to correlations between bacterial colonization and IBD, longitudinal studies have demonstrated that nasopharyngeal colonization is a necessary step in the pathogenesis of IBD, especially for S. Pneumoniae [20, 21]. In the present study, only S. pneumoniae colonization correlated with IBD, because S. pneumoniae was the most common pathogen in children with culture-confirmed IBD (data not shown). The mechanisms behind the transitions from asymptomatic colonization to dissemination and disease in otherwise sterile sites remain poorly understood but are epidemiologically strongly linked to infection with respiratory viruses. Preceding influenza virus infection significantly increased the nasopharyngeal colonization of S. pneumoniae, thereby increasing the risk of invasive pneumococcal disease [22] through host-derived inter-kingdom signals [23].

One limitation of this study is that environmental factors and climate factors were not included. Second, this study only comprised of patients admitted to Children's Hospital of Suzhou University and to a certain extent, could not be representative of the entire Suzhou area. Moreover, there was an overlap in the season between the occurrences of RSV, hMPV, and IV-A, so we could not determine the effect of a single bacterial colonization or IBD. Finally, this study did not include healthy children, which might have biased the results.

Conclusion

In summary, there was a correlation between viral or *M. pneumoniae* activity, nasopharyngeal colonization, and invasive bacterial infection in the present study and this study analyzed the lag effect, confirming that the lag effect included positive and negative correlations between preceding respiratory infections with different pathogens, nasopharyngeal colonization, and invasive bacterial diseases. By

analyzing the prevalence of pathogen causing respiratory tract infection in hospitalized children, the epidemiological profiles of bacterial colonization and secondary IBD could be predicted to some extent. We could further predict the time that IBD may occur and provide useful information for clinicians in the diagnosis and treatment of IBD.

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Disclosure of conflict of interest

None.

Abbreviations

IBD, invasive bacterial disease; M. pneumoniae, Mycoplasma pneumoniae; S. pneumoniae, Streptococcus pneumoniae; H. influenzae, Haemophilus influenzae; M. catarrhalis, Moraxella catarrhalis; RSV, respiratory syncytial irus; IV, influenza virus; hMPV, human metapneumovirus; PCR, polymerase chain reaction; PIV, parainfluenza virus; ADV, adenovirus; HboV, human bocavirus; CRP, C-reactive protein.

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