Impact of bacterial colonization on the severity, and accompanying airway inflammation, of virus-induced wheezing in children

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Abstract

It is reported that bacterial colonization of the airway in neonates affects the likelihood and severity of subsequent wheezing in childhood. This study aimed to explore the impact of bacterial colonization on the severity of virus-induced wheezing, and accompanying airway inflammation. Nasopharyngeal aspirates (NPAs) from 68 hospitalized children with bronchiolitis and 85 children with recurrent wheezing were obtained. Eleven common respiratory viruses were sought by PCR and/or direct fluorescence assay. Bacteria were isolated from NPAs by routine culture methods. Cell numbers and concentrations of cytokines/chemokines in the NPAs were measured, and nucleated cells were characterized. The frequency of bacterial colonization in children with recurrent wheezing was significantly higher than in children with an initial attack of bronchiolitis. Bacterial colonization accompanying virus infection had no effect on clinical manifestations, duration of hospitalization, concentrations of cytokines/chemokines (except interleukin-10 (IL-10)) or cellularity in the children with bronchiolitis; however, among the children with recurrent wheezing, those who had coexistent non-invasive bacterial colonization and virus infection presented more frequent cyanosis, longer duration of hospitalization, a higher concentration of IL-10 and a higher percentage of neutrophils in NPAs than those with virus infection but without bacterial colonization. Bacterial colonization was common in children with virus-induced wheezing, particularly in the situation of recurrent wheezing. To some extent, bacterial colonization accompanying virus infection may contribute to the severity of the wheezing because of its impact on airway inflammation.

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Introduction

The nasopharynx may be densely colonized by a broad variety of microorganisms, including commensal bacteria and potential pathogens such as *Streptococcus pneumoniae*, *Haemophilus influenzae* (essentially non-typeable strains) and *Moraxella catarrhalis*. In most cases, these organisms are carried without causing clinical symptoms.

It has been reported that up to 88% of cases of wheezing in children are associated with respiratory virus infections [1]. A previous investigation showed that acute viral wheezing in children was often associated with bacterial upper respiratory tract infections, such as otitis media and sinusitis, but blood leukocyte counts and C-reactive protein (CRP) levels were generally not raised. [2]. It has also been suggested that, in synergy with respiratory viral infection, bacterial colonization may contribute to respiratory inflammation. Bisgard *et al.* [3] reported that neonates who had *S. pneumoniae*, *H. influenzae* or *M. catarrhalis*, or a combination of these organisms, colonizing the hypopharyngeal region were at increased risk of recurrent wheezing and asthma early in life [4]. This result might indicate that such bacterial colonization in neonates is not a neutral matter but plays a critical role in infantile wheezing.

In this study, we aimed to assess the relationships among bacterial colonization, virus infection and infantile wheezing. To achieve this, the clinical characteristics and also the cellularity and cytokine/chemokine levels of nasopharyngeal aspirates were compared between two groups of children who had virus-induced wheezing, and between those in each group with bacterial colonization and those without.

Materials and Methods

Research subjects and sample collection

From November 2006 to March 2008, 68 children with bronchiolitis and 85 with recurrent wheezing, who were hospitalized in the Department of Respiratory Medicine, Children's Hospital, Chongqing Medical University, were enrolled in this study. The study protocol was approved by the Institutional Review Boards of the hospital, and written informed consent of a parent or legal guardian was obtained before the infants were enrolled. Bronchiolitis was defined as the first episode of acute expiratory wheezing in a child <3 years of age. Recurrent wheezing was characterized as daily symptoms for at least four consecutive weeks: all of the children in this group were taking inhaled bronchodilators and corticosteroids without relief.

Immediately following hospital admission, nasopharyngeal aspirates (NPAs) were prospectively collected from all subjects, and venous blood was taken for determination of total leukocyte count and CRP level. Blood culture was performed when NPA culture was positive. Positive blood culture in patients with a CRP concentration of >80 mg/L or a white blood cell count of $>15.0 \times 10^9$/L was considered to represent true bacterial infection, and these patients were excluded. All children were examined by one of two study physicians twice daily during hospitalization. Signs and symptoms were recorded on a standard form used in our hospital. Children were discharged from hospital after the wheezing improved or was cured. Severity was determined on the basis of clinical symptoms and the duration of hospitalization.

NPA analysis

After NPA was collected, it was gently aspirated with a Pasteur pipette and then expelled repeatedly until mixed uniformly: 0.5 mL of the specimen was then transferred to another tube for cellular identification and cytokine estimation. To this sample, about four times its volume of 0.1% dithiothreitol was added, and the whole was mixed with a Pasteur pipette, vortexed for 15 s, and rocked on a bench rocker for 15 min. The suspension was subsequently filtered through 48-μm nylon gauze to remove mucus and debris without removing any of the cells, and then centrifuged at 1500 r.p.m. for 10 min. The cell-free supernatant was stored at −80°C. The cell pellet was resuspended, and the total cell count was determined with a Neubauer haemocytometer. The Trypan blue exclusion method was used to determine cell viability, blue cells being considered to be non-viable. Differential cell counts were obtained using a modified version of Wright staining (Liu A and Liu B solution, Baso, Zhuhai, China) and cytocentrifuged smears of the NPA cell suspensions. At least 400 cells were examined by the same observer in each specimen. Cell counts are expressed as multiples of 10^5/mL; results are presented as means and standard deviations.

Viral antigen detection, culture for bacteria and fungi and cellular identification were performed immediately, and the samples prepared for PCR assays and cytokine estimation were stored in tubes at −80°C before processing.

Detection of bacteria and yeasts

Qualitative and semiquantitative cultures for bacteria and yeasts were performed using standard microbiological methods [5,6]. For all samples, macroscopically distinct colonies were isolated in pure culture, and standard methods for identification, typing and establishing antibiotic sensitivity patterns were used.

Respiratory virus detection

RNA was extracted from 140 μL of the NPA using the QIAamp Virus RNA Mini kit (Qiagen, MD, USA), and eluted in 60 μL of RNase-free water. DNA was extracted from 200 μL of NPA using the QIAamp DNA mini kit (Qiagen) and was eluted in 200 μL of AE buffer. Viral antigens were detected for adenovirus, influenza A virus, influenza B virus, parainfluenza virus 1, 2 and 3 and respiratory syncytial virus (RSV) by direct fluorescent assay (D^3 DFA Respiratory Viruses Screening & ID Kit; Diagnostic Hybrids, Hannover, Germany). PCR was used for the detection of adenovirus and human bocavirus. RT-PCR was used for the detection of rhinoviruses, RSV, human coronaviruse OC43, influenza A virus, influenza B virus, parainfluenza virus 1–3 and human metapneumovirus. Reverse transcription of 0.5 mg of each RNA was performed in a final volume of 10 μL containing 25 pmol of Oligo dT primer and 50 pmol of random 6 mers, 5 × PrimeScript Buffer, and 0.5 μL of PrimeScript RT Enzyme Mix (TaKaRa, Dalian, China). Amplification was performed with a Bio-Rad thermal cycler, using commercially available master mixes (Promega, Madison, WI, USA) and standard protocols. All PCR assays were performed using 2 μL of cDNA and 1 mM each primer. The primer sequences, PCR protocols and other viral detection methods are shown in Table 1. The unclassified rhinoviruses were not identified further. A specimen was regarded as positive if either one or two methods gave positive results.

Cytokine/chemokine analysis

According to the manufacturer's instructions, NPAs were tested by ELISA (BD Pharmingen, San Diego, CA, USA) for interleukin-6 (IL-6), interleukin-8, interleukin-10 (IL-10) and

TABLE 1. Primers used in PCR

Virus	Primer	Sequence	References
HBoV	NP-1 188F	5′-GAGCTCTGTAAGTACTATTAC-3′	[7]
	542R	5′-CTCTGTGTTGACTGAATACAG-3′	
	NS1HBoV01	5′-TATGGCCAAGGCAATCGTCCAAG-3′	[8]
	2 HBoV02.2	5′-GCCGCGTGAACATGAGAAACAGA-3′	
hMPV	F-gene (forward)	5′-GCAACAATTGAACTGATCTTCAGGAAAC-3′	[9]
	F-gene (reverse)	5′-GCAACATTGAACTGATCTTCAGGAAAC-3′	
	F-gene (forward)	5′-ACATGCCAACATCTGCAGGACAAATAAAAC-3′	
	F-gene (reverse)	5′-ACATGCTGTTCACCTTCAACTTTGC-3′	
RV	5′-Noncoding	5′-CGGACACCCAAAGTAG-3′	[10]
		5′-GCACTTCTGTTTCCCC-3′	
HCoV OC43	Nucleoprotein	5′-TGCAAAGATGGGGAACTGTGGG-3′	[10]
		5′-AGGAAGGTCTGCTCCTAATTCC-3′	
Flu A	Matrix	5′-CAGAGACTTGAAGATGTCTTTGC-3′	[10]
		5′-GCTCTGTCCATGTTATTTGGATC-3′	
Flu B	Matrix	5′-GAAAAATTACACTGTTGGTTCGGTG-3′	[10]
		5′-AGCGTTCCTAGTTTTACTTGCATTGA-3′	
PIV 1	HN gene	5′-ATTTCTGGAGATGTCCCGTAGGAGAA C-3′	[11]
		5′-CACATCCTTGAGTGATTAAGTTTGATG-3′	
PIV 3	HN gene	5′-CTCGAGGTTGTCAGGATATAG-3′	[12]
		5′-CTTTGGGAGTTGAACACAGTT-3′	
RSV	Nucleoprotein	5′-TGGGACACTCTTAATCAT-3′	[13]
		5′-TGATTCCAAGCTGAGGAT-3′	
		5′-GTTGTAGGTGTGTTTC-3′	

Flu A, influenza A virus; Flu B, influenza B virus; HBoV, human bocavirus; HCoV, human coronavirus; hMPV, human metapneumovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus; RV, rhinovirus.

monocyte chemoattractant protein-1. Each sample was measured in duplicate by Varioskan Flash (Thermo), according to the kit's directions. The concentration of each cytokine in NPA was determined by interpolation from the corresponding standard curve. When values were below the detection threshold, the minimum detectable level was assigned.

Statistical analysis

Differences in continuous variables or proportions were tested using *t*-test, one-way ANOVA or chi-square test, or Fisher's exact, where appropriate. Non-parametric tests and median values were used for those variables, such as cell count and cytokine/chemokine concentrations, that were not normally distributed. Correlations were made with Spearman's two-tailed rank correlation in addition to Pearson's linear regression two-tailed analysis. Two-tailed $p < 0.05$ was considered to be statistically significant. SPSS (version 13.0, Chicago, IL, USA) was used for all analyses.

Results

Microbiological results

Participating in this study were 68 children in the bronchiolitis group, aged from 4 to 30 months, with a median age of 7.15 ± 0.5 months, 49 of whom (72.06%) were male, and 85 children with recurrent wheezing, aged from 3 to 30 months, with a median age of 8.34 ± 0.54 months, 60 of whom (70.58%) were male (Table 2).

TABLE 2. Demographic and epidemiological characteristics of children with bronchiolitis and with recurrent wheezing

Variable	Bronchiolitis (*n* = 68)	Recurrent wheezing (*n* = 85)	p
Age (mean ± SEM) (months)	7.15 ± 0.50 (range: 4–30)	8.34 ± 0.54 (range:2–30)	0.11
Male sex	49/68	60/85	0.86
Family history of atopy	47/68	49/85	0.18
Duration of hospitalization (days)	7.81 ± 0.36	14.58 ± 0.83	<0.0001

SEM, standard error of the mean.

Table 3 shows the detection rate for viruses and bacterial colonization in the two groups. Among the children with bronchiolitis, one virus was detected in 29 samples (42.65%) and two or more viruses in 27 samples (39.71%). RSV was the most frequenly detected virus. One species of bacterium was found in 29 samples (42.65%), and more than one species in nine samples (13.24%). *H. influenzae*, *Haemophilus parainfluenzae* and *Klebsiella pneumoniae* were the most frequently detected bacteria. Among the children with recurrent wheezing, one virus was found in 32 samples (37.65%), and more than one was detected in 31 samples (36.47%). Human bocavirus was the most frequently detected virus. One bacterial species was found in 49 samples (57.65%), and more than one in 21 samples (24.71%). In this group, *S. pneumoniae*, *H. influenzae* and *K. pneumoniae* were most frequently detected. The frequency of bacterial colonization in

TABLE 3. Microorganism detection in children with bronchiolitis and children with recurrent wheezing

Microorganism detection	Bronchiolitis (*n* = 68), *n* (%)	Recurrent wheezing (*n* = 85), *n* (%)	p
Virus detection	56 (82.35)	63 (74.12)	0.246
Single virus-positive	29 (42.65)	32 (37.65)	0.619
RSV	22 (32.35)	5 (5.88)	<0.01
PIV 3	4 (5.88)	7 (8.24)	0.756
RV	2 (2.94)	3 (3.53)	1
PIV 1	1 (1.47)	0 (0)	–
HBoV	0 (0)	11 (12.94)	–
Flu A	0 (0)	6 (7.06)	–
More than one virus-positive	27 (39.71)	31 (36.47)	0.739
Bacterial colonization	38 (55.88)	70 (82.35)	0.001
Single species present	29 (42.65)	49 (57.65)	0.075
Streptococcus pneumoniae	2 (2.94)	7 (8.24)	0.3
Haemophilus influenzae	7 (10.29)	6 (7.06)	0.565
Haemophilus parainfluenzae	5 (7.35)	8 (9.41)	0.774
Klebsiella pneumoniae	3 (4.41)	13 (15.29)	0.034
Moraxella catarrhalis	2 (2.94)	2 (2.35)	1
Other bacteria	10 (14.71)	13 (15.29)	1
More than one species	9 (13.24)	21 (24.71)	0.101
Virus-positive with bacterial colonization	30 (44.11)	50 (58.82)	0.076

Flu A, influenza A virus; HBoV, human bocavirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus; RV, rhinovirus.

children with recurrent wheezing was significantly higher than in children with bronchiolitis. However, the frequency of simultaneous bacterial colonization and virus infection in the two groups was not significantly different.

Clinical characteristics of children with virus infection, with or without bacterial colonization (Table 4)

Among the children with bronchiolitis, the two groups (with and without bacterial colonization) were similar in all clinical manifestations and duration of hospitalization. Among the children with recurrent wheezing, the two groups were similar in all clinical manifestations except cyanosis, but children who had virus infection with bacterial colonization had a significantly longer duration of hospitalization.

Cell counts and cytokine/chemokine concentrations (Table 5)

Among the children with bronchiolitis, concentrations of cytokines/chemokines and cellularity in NPA were not significantly different between the two groups, except in the case of IL-10, the level of which was higher in the bacteria-negative group. In the children with recurrent wheezing, a higher concentration of IL-10 and a higher percentage of neutrophils were found in those who had virus infection with bacterial colonization than in children who had virus infection without bacterial colonization, whereas the percentage of macrophages was lower in the former group.

Discussion

Previous studies have shown that bacterial colonization of the upper respiratory tract is common throughout infancy [14–18]. In one investigation of monthly nasopharyngeal cultures taken from healthy infants from the time of birth, 78% were found to have bacterial colonization during the first 2 years of life [15]. The clinical significance of bacterial colonization has been disputed in the various reports. It is widely accepted that viral infections [19] and bacterial upper respiratory tract infections [20] can exacerbate symptoms of asthma. However, observations in chronic obstructive pulmonary disease patients have suggested that isolation of bacteria from sputum during exacerbations represents chronic

TABLE 4. Clinical characteristics of children who had virus infection with or without bacterial colonization

Variable	Bronchiolitis with viral detection		Recurrent wheezing with viral detection	
	Bacteria-positive (*n* = 30)	Bacteria-negative (*n* = 26)	Bacteria-positive (*n* = 50)	Bacteria-negative (*n* = 13)
Clinical symptoms				
Cough	30 (100)	26 (100)	50 (100)	13 (100)
Fever	6 (20)	5 (19.23)	20 (40)	4 (30.77)
Rhinorrhoea	4 (13.33)	4 (15.38)	14 (28)	4 (30.77)
Vomiting	1 (3.33)	2 (7.69)	7 (14)	1 (7.69)
Shortness of breath	3 (10)	0	11 (22)	3 (23.08)
Diarrhoea	15 (50)	11 (42.31)	12 (24)	1 (7.69)
Rapid breathing	9 (30)	4 (15.38)	17 (34)	3 (23.08)
Cyanosis	14 (46.67)	6 (23.08)	19 (38)[a]	1 (7.69)[a]
Atopy	21 (70)	18 (69.23)	34 (68)	7 (53.85)
Duration of hospitalization (days)[b]	7.57 ± 0.57	7.92 ± 0.57	18.18 ± 2.03[c]	14.32 ± 1.00[c]
White blood cell count[b] (10^1 cell/mL)	9.52 ± 0.57	9.90 ± 1.16	10.26 ± 0.55	9.50 ± 0.69

Note: Data are no. (%) of children.
[a]Calculated using Fisher's exact test. p 0.047 (considered to indicate statistical significance).
[b]Duration of hospitalization is presented as mean ± standard deviation.
[c]Calculated using the Mann–Whitney *U*-test. p 0.0379 (considered to indicate statistical significance).

TABLE 5. Cellular content and cytokine/chemokine concentrations of nasopharyngeal aspirates (NPAs) in children who had virus infection with or without bacterial colonization

Variable	Bronchiolitis with viral detection		Recurrent wheezing with viral detection	
	Bacteria-positive (n = 30)	Bacteria-negative (n = 26)	Bacteria-positive (n = 50)	Bacteria-negative (n = 13)
Cytokine/chemokine (pg/mL)				
IL-8	1682 ± 462.5	1726 ± 427.7	2757 ± 1203	2213 ± 809.4
IL-6	14.9 ± 6.1	56.1 ± 42.3	44.5 ± 25.7	44.2 ± 10.6
IL-10	30.7 ± 8.0[a]	62.5 ± 24.1[a]	133.6 ± 55.4[b]	47.9 ± 10.8[b]
MCP-1	14.5 ± 1.6	9.6 ± 2.0	13.5 ± 11.1	3.5 ± 1.1
Absolute number of cells in NPA ($\times 10^5$/mL)				
Total number	20.60 ± 7.03	29.72 ± 6.50	68.57 ± 19.92	38.10 ± 19.60
Percentages of cell types in nucleated cells of NPA				
Macrophage	23.61 ± 5.40	19.91 ± 4.68	26.73 ± 6.86[c]	40.71 ± 3.92[c]
Lymphocyte	1.15 ± 0.37	1.35 ± 0.75	2.96 ± 0.90	8.04 ± 1.94
Neutrophil	64.43 ± 5.70	68.04 ± 6.19	60.73 ± 7.81[d]	43.66 ± 4.67[d]

Note: All data are presented as mean ± standard deviation.
IL, interleukin; MCP, monocyte chemoattractant protein.
[a]Calculated using the Mann–Whitney *U*-test. p 0.0452 (considered to indicate statistical significance).
[b]Calculated using the Mann–Whitney *U*-test. p 0.0161 (considered to indicate statistical significance).
[c]Calculated using the Mann–Whitney *U*-test. p 0.0408 (considered to indicate statistical significance).
[d]Calculated using the Mann–Whitney *U*-test. p 0.0106 (considered to indicate statistical significance).

colonization, which is an innocent bystander role for the bacteria [21–23]. In most cases, colonization persists for weeks to months without symptoms [24,25], but the bacteria may spread contiguously to produce local respiratory tract disease. Even during colonization, bacteria in these airways are in a constant state of turnover, releasing extracellular products, and undergoing lysis with release of a variety of proteins, lipo-oligosaccharides and peptidoglycans [26]. Bacterial products in the airways may be potent stimuli for neutrophil migration thither, and elastase released from these neutrophils can act synergistically with bacterial products and cause further inhibition of tracheobronchial ciliary function.

In this study, we found that many of the children with virus-induced wheezing had nasopharyngeal bacterial colonization, and this proportion was higher among children with recurrent wheezing. In the children with bronchiolitis, although concentrations of IL-10 were different in the two groups of children, all clinical manifestations and duration of hospitalization were similar. The predominant cells in the airway in RSV bronchiolitis are neutrophils. Activation of innate immunity against the virus by the promotion of proinflammatory mediators, such as IL-6, IL-10 and monocyte chemoattractant protein-1, and cytokines such as IL-6 and interleukin-8, correlates with disease severity [27–29]. A previous study indicated that bacterial colonization did not add specific symptoms to the clinical signs already present in RSV bronchiolitis, except that, in patients with severe disease, those with bacterial colonization required ventilation for a longer period [30]. Our data indicate that coexisting non-invasive bacterial colonization has little impact on the severity of an attack of virus-induced bronchiolitis.

However, in the children with recurrent wheezing, a higher frequency of cyanosis and a longer duration of hospitalization were found in children in whom bacterial colonization accompanied virus infection. Moreover, the concentration of IL-10 and percentage of neutrophils were similarly found to differ between these two groups. Previous studies have demonstrated increased numbers of macrophages and neutrophils in bronchoalveolar lavage from young children with severe recurrent wheezing [31]. The concentration of IL-10 was found to be four times that of CXCL-8 and 140 times that of CCL-3 in the airways of infants with severe wheezing disease. IL-10 acts to recruit monocytes into the lung to help the phagocytosis of apoptotic neutrophils [32]. These data suggest that coincident non-invasive bacterial colonization and virus infection might have contributed to the severity and persistence of wheezing in the children with recurrent wheezing.

In conclusion, this study provides further evidence that coexisting non-invasive bacterial colonization in children with virus-induced wheezing is common, in particular in those with recurrent wheezing. To some extent, coexisting non-invasive bacterial colonization with virus infection might contribute to the severity of recurrent wheezing because of its impact on airway inflammation.

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Transparency Declaration

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