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ORIGINAL ARTICLE

Viral etiology of bronchiolitis among pediatric inpatients in northern Taiwan with emphasis on newly identified respiratory viruses



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Purpose: Viral etiology of bronchiolitis in children in Taiwan has been fragmentary. We conducted a prospective study to figure out the viral epidemiology of bronchiolitis in Taiwan.

Materials and methods: From January 2009 to March 2011, a total of 113 children with bronchiolitis, aged <2 years, hospitalized in Chang Gung Children's Hospital were randomly selected for viral etiology investigation. Nasopharyngeal aspirates were obtained from each case and sent for viral detection by tissue culture, antigen test, and polymerase chain reaction.

Results: A total of 120 viruses were detected from 113 children. Positive viral etiology was identified in 86 (76%) children. Mixed viral pathogens were found in 28 cases (25%). Respiratory syncytial virus (RSV) was the most common pathogen and was identified in 43.4% of the cases. Human bocavirus (hBoV) was the second most common identified virus (in 19.5%), followed by human metapneumovirus (hMPV), rhinovirus, influenza viruses, and coronavirus OC43. In terms of clinical characteristics, no significant difference was found among the children with bronchiolitis either caused by different single or mixed viral infection.

Conclusion: RSV was the most common etiologic agent for children with bronchiolitis in Taiwan. Newly identified viruses, including hMPV and hBoV, were also among the common causative agents. Clinical characteristics were not significantly different among the children with bronchiolitis caused by different viruses.

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Introduction

Bronchiolitis is the most common lower respiratory tract infection and a major cause of hospitalization in infants around the world. It is characterized by acute inflammation, edema, and necrosis of epithelial cells lining small airways; increased mucus production; and bronchospasm. Clinical symptoms and signs of bronchiolitis typically include rhinitis, tachypnea, wheezing, cough, crackles, use of accessory muscles, and/or nasal flaring.^{1,2}

With the development of molecular techniques and the availability of monoclonal antibodies for numerous viral species, detection of viral respiratory agents has been markedly improved in the past decades. These advances allowed re-evaluation of the role of various respiratory viruses in the pathogenesis of acute bronchiolitis. However, it is not infrequently seen that no causative agent can be identified in patients with bronchiolitis.

Respiratory syncytial virus (RSV) is the most frequently identified agent responsible for bronchiolitis worldwide.^{3,4} However, many other respiratory viruses may also cause bronchiolitis. Until now, viral etiology of bronchiolitis in children in Taiwan has been limited, particularly the newly identified viruses, including human metapneumovirus (hMPV), human bocavirus (hBoV), and human coronavirus (hCoV) NL-63.^{5,6} Therefore, we conducted a prospective study to figure out the viral epidemiology of bronchiolitis among pediatric inpatients in Taiwan.

Materials and methods

Patients and respiratory specimens

The study was approved by the Institutional Review Board of Chang Gung Memorial Hospital, and informed consent was obtained from parents or legal guardians of the children. From January 2009 to March 2011, children (aged <24 months) with the initial diagnosis of acute bronchiolitis, hospitalized in Chang Gung Children's Hospital situated in northern Taiwan, were eligible for this study, and up to three cases per week were randomly selected for viral etiology investigation. Nasopharyngeal aspirates were collected prospectively from all patients within 1–3 days after hospital admission.

The diagnosis of acute bronchiolitis was made by an acute onset of respiratory distress with cough, tachypnea, retraction, and expiratory wheezes, often accompanied by rales. Patients with underlying chronic diseases, including cerebral palsy with bedridden status, tracheostomy, congenital heart disease, chronic pulmonary disease, and immunodeficiency, were excluded.

Medical records were reviewed for detailed demographic, clinical, and laboratory data; radiographic images; and underlying conditions of the patients. All the clinical symptoms and signs were recorded on a standardized form while the patients were hospitalized.

Virus detection

All the specimens were processed, and then nucleic acids (including DNA and RNA) were extracted by commercial

kits and kept in a refrigerator at -70°C for further analysis. All specimens were sent for viral detection by conventional viral culture, immunofluorescent antigen detection for RSV, and multiplex reverse transcription-polymerase chain reaction (RT-PCR) for six viruses, including rhinovirus (RV), hMPV, hCoV-229E, hCoV-OC43, NL-63, and hBoV.

Sample preparation for virus culture and immunofluorescence

Nasopharyngeal aspirates were mixed with sterilized phosphate-buffered saline (PBS) followed by vigorous vortex. Then the cell suspension was centrifuged at 3000 rpm for 10 minutes to get the cell pellet. Finally, the supernatant was discarded and PBS was added to resuspend the cells again. The procedures were repeated for three times. After the third time wash, the cell pellet was resuspended in viral transport medium and treated with antibiotics for 30 minutes.

Virus isolation

Specimens of cell suspension prepared as described above were inoculated into MK2, MRC-5, and MDCK cells and incubated at 35°C for 2 weeks. Cytopathic effect (CPE) of all culture tubes was checked every 2 days. For CPE-positive tubes, a screening kit of immunofluorescence assay for respiratory virus (Chemicon Inc., Temecula, California, USA) was used for further examination of respiratory virus infection. Only the respiratory viruses identified, including RSV, parainfluenza (PIV)-1, PIV-2, PIV-3, adenovirus, influenza viruses A and B, were regarded as pathogens.

Immunofluorescent assay for the detection of RSV antigen

The cell suspension (50 μL) was added to a slide and fixed in acetone for 10 minutes. Monoclonal antibody that conjugated Fluorescein isothiocyanate (FITC) to RSV was used to detect RSV antigen.

RT-PCR assay

It is designed to amplify conserved region of each virus target. Sequence of primer for each virus target is given in [Supplementary Table 1](#). Viral RNAs were extracted from 200 μL respiratory specimens using QIAmp Viral RNA kit (Qiagen, Chatsworth, CA, USA), and reverse transcription reactions were performed for complementary DNA synthesis using SuperScriptTM III One-Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA). Each reaction included the following components: 0.2 M probe, 0.4 M primer, 12.5 μL of $2\times$ ABI Master mixture (containing 0.4 mmol/L deoxyribonucleotide triphosphate (dNTP) and 2.4 mmol/L MgSO_4), and 5 μL of specimen RNA extract or control. The final reaction volume was adjusted to 25 μL with PCR-grade water, and RT-PCR amplification was performed using the following conditions: an initial cDNA step at 50°C for 30 minutes, followed by at 95°C for 15 minutes, and 50 cycles at 95°C for 30 seconds, at 55°C for 30 seconds, and at 72°C for 30 seconds. RT-PCR products of hCoV-NL63 and hBoV for positive control were kindly provided by Professor Patrick C.Y. Woo (Hong Kong University).

Statistical analysis

One-way analysis of variance and Student *t* test were used for the comparison of continuous variables. The χ^2 test was used to analyze categorical independent variables. Results for continuous data were expressed as mean \pm SD. A *p* value of <0.05 was considered statistically significant. All analyses were performed using the SPSS version 17.0 for Windows.

Results

A total of 113 children were included in this study. The median age of the children was 9 months, ranging from 1.5 to 24 months, and 77 (68%) were male. Clinical characteristics of the patients are described in Table 1. In our study, 24/113 (21.2%) children received antibiotic treatment during hospitalization mostly due to secondary bacterial infection such as acute sinusitis, acute otitis media, or pneumonia.

A total of 132 viruses were detected from 113 children, but 12 viruses, including cytomegalovirus in seven and enterovirus in five, were regarded as nonpathogenic agents and excluded from analysis in this study. At least one viral pathogen was identified in 86 children (76%), of whom single viral pathogen was detected in 58 (67%) and at least two viruses were detected in 28 (33%). RSV was the most common virus identified and accounted for 49 (43.4%) cases, followed by hBoV in 22 (19.5%), hMPV in 19 (16.8%), and RV in 14 (12.4%) (Fig. 1). CPE was observed in one case, but the virus species could not be identified. All 113 specimens were negative for CoV-229E or influenza virus B. Of the 49 RSV-positive children, 18 (36.7%) had coinfection with other viruses.

Monthly distribution of the viruses identified during the study period is illustrated in Fig. 2. The incidence of RSV infection peaked in April 2009, March 2010, and November 2010. The incidence of hMPV detection peaked in April 2009, while that of hBoV peaked in August 2010. We could

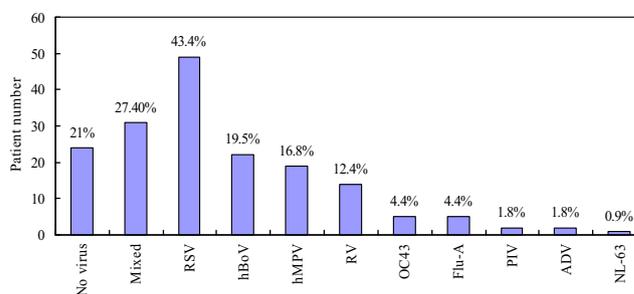


Figure 1. Distribution of viruses identified from 113 children with bronchiolitis (calculated as virus number/total patient number). ADV = adenovirus; CPE = cytopathic effect; Flu-A = influenza A virus; hBoV = human bocavirus; hMPV = human metapneumovirus; NL-63 = coronavirus NL-63; OC43 = coronavirus OC43; PIV = parainfluenza virus; RSV = respiratory syncytial virus; RV = rhinovirus.

not evaluate the differences in incidence of other viruses due to the small case numbers.

Bronchiolitis due to single viral infection was identified in 58 patients, of whom RSV was detected in 31 (53%), hBoV in six (10%), hMPV in six (10%), and RV in seven (12%). Clinical characteristics of these patients are given in Table 2. No significant differences were observed in terms of sex, age, radiographic findings, duration of fever, length of hospital stay, antibiotic treatment, leukocyte count, and serum C-reactive protein level.

Multiple viral infections were found in 28 cases (25%). RSV was the most common virus encountered in mixed infections, followed by hBoV, hMPV, and RV. The detailed incidence rate of mixed viral infection is listed in Table 3. In addition, clinical characteristics of the children with RSV-mixed infection are described in Table 2. Clinical manifestations of children with RSV-mixed infection were similar to those with single viral infection, and no significant differences were found among the different groups.

Discussion

To our knowledge, this is the first comprehensive viral etiology study of hospitalized pediatric patients with bronchiolitis, including newly identified respiratory viruses, in Taiwan. Results from the present study showed that RSV, hMPV, and hBoV were the most common viral pathogens for acute lower respiratory tract infection among hospitalized children, less than 2 years of age, in northern Taiwan. We demonstrated again that RSV remained the major pathogen in infants with bronchiolitis.^{5,7} By the combination of virus isolation and antigen test, RSV was detected in 43% of the patients in the current study. RSV bronchiolitis followed the biennial seasonal pattern of Taiwan, with peaks during the spring and the fall (April 2009, March 2010, and November 2010), which were consistent with those previously reported from Taiwan.⁸

This study was strengthened by the use of RT-PCR assays in combination with virus culture and immunofluorescent assay to detect respiratory viruses, including the newly identified viruses (hMPV, hBoV, and hCoV-NL63) and

Table 1 Clinical characteristics of 113 children hospitalized with bronchiolitis

Variable	No. (%)
Male sex	77 (68.1)
Age (mean \pm standard deviation, mo)	10.1 \pm 5.6
Radiographic findings ^a	
Normal	49 (45.4)
Diffused air trapping	40 (37.0)
Patchy infiltrates	19 (17.6)
Fever duration (mean \pm standard deviation, d)	3.0 \pm 2.5
Hospital stay (mean \pm standard deviation, d)	6.4 \pm 5.0
Antibiotic treatment	24 (21.2)
Leukocytes (1000 cells/mm ³)	11.7 \pm 5.5
Serum C-reactive protein (mean \pm standard deviation, mg/L)	14.4 \pm 20.1

^a Five children did not take chest X-ray during hospitalization.

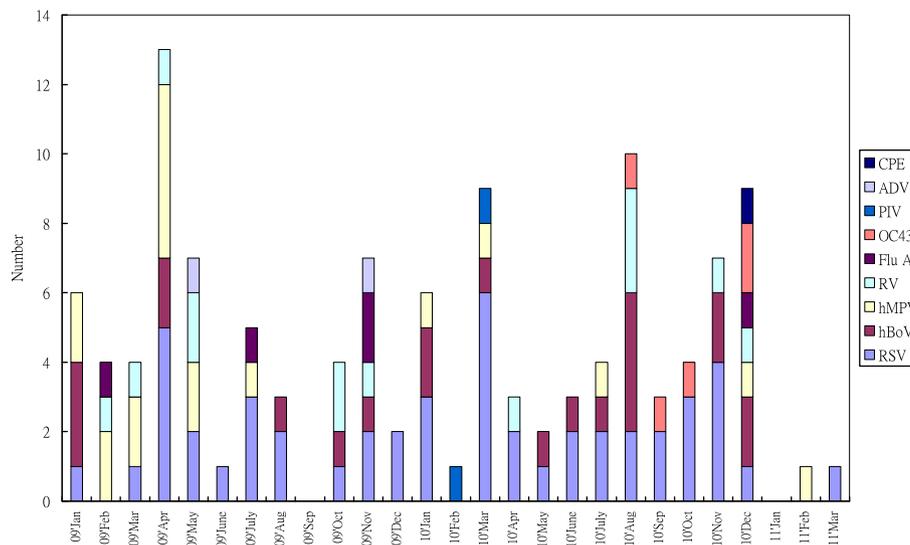


Figure 2. Monthly distribution according to the viral pathogen. ADV = adenovirus; CPE = cytopathic effect; Flu-A = influenza A virus; hBoV = human bocavirus; hMPV = human metapneumovirus; NL-63 = coronavirus NL-63; OC43 = coronavirus OC43; PIV = parainfluenza virus; RSV = respiratory syncytial virus; RV = rhinovirus.

other viruses (RV, hCoV-OC43, and hCoV-229E), which may be responsible for bronchiolitis but not identified by routine virus tissue culture. With these technologies, we were allowed to detect up to 15 different viruses (groups) and could also explain a higher viral detection rate (79%) in the present study.

In 57% of the patients, viruses other than RSV were identified; after RSV, the most frequently detected viruses included hBoV, hMPV, and RV. The prevalence rate for hBoV (16.7%) was the highest so far reported among infants hospitalized for bronchiolitis.^{6,9,10} Calvo et al reported an incidence rate of 11.4% for hBoV detection and Midulla et al a rate of 12.2%. The role of these respiratory agents in causing single or multiple viral infections in bronchiolitis has not yet been thoroughly studied. These findings suggest that many other respiratory viruses, in addition to RSV, may also cause acute bronchiolitis.

In the present study, clinical characteristics of children with bronchiolitis due to different viral etiologies seemed similar, regardless of whether the infection was caused by single or multiple viruses. In our study, we also observed no statistical significance in clinical characteristics between children with bronchiolitis caused by different single viral infection. However, a trend related to older age in hBoV-infected children than in RSV-infected was observed in previous studies.^{6,10,11} The proportion of RV infections (12.4%) in Taiwan is slightly lower than that reported from the USA (16%) and Spain (17.4%),^{6,12} but higher than that from Italy (8.8%).¹⁰ The prevalence rate of hMPV (14.4%) in this study was higher than in most previous reports (1.7–9%),^{6,10,12} except in the report by Xepapadaki et al¹³ (16%).

Multiple viral infections were present in one-fourth of the patients with positive respiratory viral detection. In

Table 2 Comparison of clinical characteristics among children with bronchiolitis due to single RSV, hBoV, hMPV, and RV infection, and RSV-mixed viral infection

Variable	RSV (n = 31)	hBoV (n = 6)	hMPV (n = 6)	RV (n = 7)	RSV-multiple (n = 18)
Male sex	22 (75.9)	4 (66.7)	5 (83.3)	7 (100)	13 (65)
Age (mean ± standard deviation, mo)	9.5 ± 6.3	15.0 ± 5.4	7.6 ± 4.4	7.5 ± 2.9	9 ± 5.9
Radiographic findings					(n = 16)
Normal	17 (54.8)	3 (50)	2 (33.3)	3 (42.9)	4 (25)
Diffused air trapping	10 (32.3)	0	4 (66.7)	3 (42.9)	9 (56.3)
Patchy infiltrates	4 (12.9)	3 (50)	0	1 (14.3)	3 (18.8)
Fever duration (mean ± standard deviation, d)	2.7 ± 2.3	4.8 ± 2.5	3.3 ± 2.7	2.3 ± 2.2	3.0 ± 2.9
Hospital stay (mean ± standard deviation, d)	7.6 ± 9.3	5.8 ± 2.6	6.5 ± 1.9	7.7 ± 2.4	6.0 ± 2.0
Antibiotic treatment	8 (27.6)	1 (16.7)	3 (50)	1 (14.3)	3 (15)
Leukocytes (mean ± standard deviation, ×1000 cells/mm ³)	11.7 ± 6.9	11.7 ± 3.6	11.0 ± 4.1	14.0 ± 5.7	10.6 ± 5.6
Serum C-reactive protein (mean ± standard deviation, mg/dL)	13.1 ± 17.7	10.5 ± 8.8	13.5 ± 18.4	9.0 ± 6.8	13.2 ± 22.1

hBoV = human bocavirus; hMPV = human metapneumovirus; RSV = respiratory syncytial virus; RV = rhinovirus.

Table 3 Frequency of mixed viral infections among children infected with different viruses

Mixed viral infection	No.	%
Respiratory syncytial virus	18/49	36.7
Human bocavirus	16/22	72.7
Human metapneumovirus	12/19	63.2
Rhinovirus	7/14	50
Influenza A virus	4/5	80
Coronavirus OC43	3/5	60
Parainfluenza viruses	0/2	0
Adenovirus	1/2	50
Coronavirus NL-63	1/1	100
Cytopathic effect only	1/1	100

agreement with previous studies, coinfection of hBoV and hMPV with other viruses was very common.^{6,9,10,14} The role of viral coinfection is still unclear, and the controversies regarding their severities were found in the literature.^{15–19} In this study, clinical manifestations of children with RSV-mixed infection were similar to those with single viral infections.

Although the combination of PCR method with other technologies was used, the causative viral agents remained undetected for one-fifth of the patients, which was in agreement with previous studies.^{5,8,9} The negative results may be due to technical problems related to sample collection, processing, and storage, or due to that bronchiolitis may be caused by unidentified viruses. In this study, we did find a specimen with CPE in the virus culture, but the viral pathogen could not be identified.

There were several limitations in the present study. First, a relatively small sample size made it difficult to compare among children with different viral etiologies. Second, the severity score of bronchiolitis could not be calculated to evaluate the disease severity of patients because oxygen saturation data were not detected for most patients and oxygen supplement such as O₂ hood was routinely provided for children hospitalized with bronchiolitis in our hospital. Third, we included only those inpatients who may affect the true prevalence rate of the viral etiology of bronchiolitis, since not all patients with bronchiolitis needed hospitalization.

In conclusion, although RSV remains the major viral pathogen for bronchiolitis, more than half of bronchiolitis are associated with other respiratory viruses, including newly identified viruses such as hMPV and hBoV. Molecular diagnostic methods such as RT-PCR provide a rapid, sensitive, and specific approach to improve the etiological diagnosis for bronchiolitis, which enables to prompt a more appropriate and rapid management. Further studies are needed to identify new viruses implicated in childhood bronchiolitis.

Conflicts of interest

All authors declare that they have no conflicts of interest related to the material discussed in this article.

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Appendix A. Supplementary material

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.jmii.2012.08.012>.

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