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Short Communication

Respiratory Viruses in Hospitalized Children with Acute Lower Respiratory Tract Infections in Harbin, China

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SUMMARY: This study investigated the prevalence of respiratory viruses, including respiratory syncytial virus (RSV), influenza virus types A and B (Flu A/B), parainfluenza virus (Para) 1-3, and adenovirus (Ad), in hospitalized children with acute lower respiratory tract infections (ALRIs). Immunofluorescence assays identified viral etiology in 412 patients younger than 16 years old. The overall viral isolation rate was 63.1% (260/412). The RSV was detected in 25.0%, Flu A/B in 19.4%, Para 1-3 in 14.6%, and Ad in 4.1% of the total sample. Multiple viruses were detected in 6.6% of the study population. Most viral infections occurred in the first 5 years of life, and the incidence of viral infection peaked during early spring and winter. Infection with Ad often resulted in the development of severe pneumonia in older children, and during the summer. The sequences of the isolated Ad hexons belonged to species B, and were closely related to the Gomen strain isolated in the United States in the 1950s. The study results will help determine the etiologic agents of ALRI in children and establish prevention and treatment programs.

Viruses are the most frequent cause of acute lower respiratory tract infections (ALRIs), and are responsible for a considerable percentage of childhood deaths (1). The most important viruses are respiratory syncytial virus (RSV), influenza virus types A and B (Flu A/B), parainfluenza virus (Para) 1-3, and adenovirus (Ad) (2,3). The RSV is the primary cause of viral lower respiratory tract illness in children, particularly in those younger than 6 months (4,5). In addition, RSV infections are responsible for most cases of severe symptoms such as bronchiolitis with recurrent wheezing and pneumonia, which lead to a high rate of hospitalization (3,6). Flu A/B and Para 1-3 are serious public health problems worldwide, with children constituting the most-affected age group (7). Moreover, Ad is a well-known cause of respiratory illness in children, and can produce upper and lower respiratory tract infections, including bronchiolitis and pneumonia. The hexon, which is the major capsid protein of the Ad particle, carries group-specific antigenic determinants (8,9).

Our study cohort resided in Harbin, a city located in northeast China that is cold during the winter, and hot during the summer (July - August). The temperature in Harbin varies greatly during early spring and winter, during which the rate of viral infections is higher in children than in general population. However, few studies have focused on the viral origin of this peak. The purpose of this study was to investigate the etiologic agents, age distribution, and seasonal occurrence of ALRI in hospitalized children residing in Harbin, China (10).

Nasopharyngeal aspirates were collected from 412 children under 16 years of age who were hospitalized with ALRIs from January 2008 to December 2008 at the first clinical college of Harbin Medical University. Patients were divided into four age groups: 0-1 years, 2-4 years, 5-11 years, and >11 years. The following were considered as ALRIs: tracheobronchitis (cough and rhonchi, no laryngeal obstruction or wheezing), bronchiolitis (expiratory wheezing with or without tachypnea, air trapping, and substernal retractions, with hyperinflation and reticulogranular on radiography), and pneumonia (fever, cough, moaning, tachypnea, rales, or evidence of perihilar and parenchyma infiltrates on radiograph) (11). All samples were collected after informed consent had been obtained from the patients' caretakers. The study was carried out in compliance with the Declaration of Helsinki and was approved by the ethics committee of Harbin Medical University.

Indirect immunofluorescence assays were used to detect the following respiratory viruses: RSV, Flu A/B, Para 1-3, and Ad. The Ads isolated by these assays were seeded onto a confluent monolayer of HeLa cells. Viral DNA extraction and purification were performed according to the manufacturer's instructions (Watson Biotechnologies, Shanghai, China). The partial sequence of the Ad hexon gene was amplified using the following primer pairs: forward, 5′-GAGTTGCTTTCA AGATGGCC; and reverse, 5′-GCTTGTCAAAGGTTCCC AGGAAATAG. A total of 50 μl of fluid containing 5 μl of 10× reaction buffer, 4 μl of 2 mM dNTP, 4 μl of forward and reverse primers at 10 μM, and 0.5 μl of 2.5 U Taq polymerase (Promega, Madison, Wis., USA). The reaction was induced by preheating the mixture at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. At the end of the amplification, the samples were separated by electrophoresis on 0.7% agarose gels and were stained with ethidium bromide. The amplified hexon fragments were sequenced using the ABI PRISM BigDye Terminator (version 3.1) system and were analyzed using the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). The nucleotide sequences of the hexon were com-

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Table 1. IFA for detection of co-infection viruses in ALRI

Co-infected virus	No. of cases
RSV, Flu B	3
RSV, Flu A	5
RSV, Flu B, Flu A	2
Flu A, Para 3	5
Flu A, Para 1	3
Flu A, Ad	1
Flu A, Para 1, Para 3	1
Flu A, Para 3, Ad	2
Flu B, Para 2	1
Flu B, Para 3	2
Para 1, Para 3	2

IFA, immunofluorescence assay.

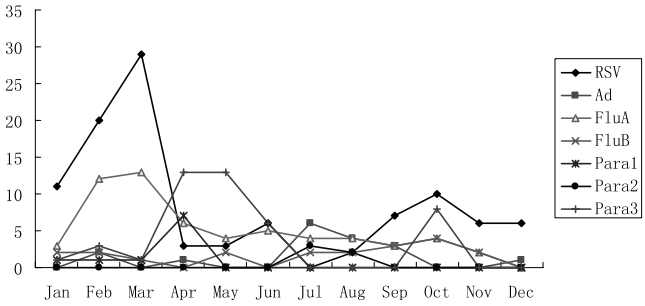

Fig. 1. Monthly incidence of the respiratory viruses among children aged from 1 month to 16 years by IFA from January to December 2008, Harbin.

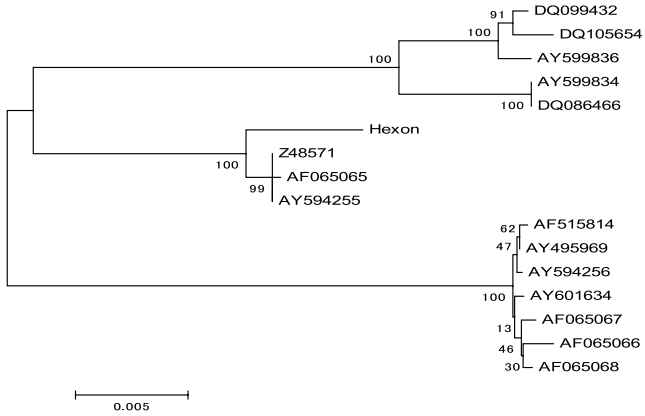

Fig. 2. Phylogenetic tree of 16 adenovirus strains constructed by the neighbor-joining method based on the nucleotide sequence of the hexon gene. Bootstrap support values, given as a percentage of 1,000 replicates, are indicated at each branch.

pared to those of the following Ad hexon sequences with >95% homology, as previously reported in GenBank using the NCBI BLAST program: nos. AY594256 (Ad7 vaccine USA), AY495969 (Ad7 vaccine China), AF515814 (Ad7 hexon China), AY601634 (Ad7 NHRC1315 USA), AF065068 (Ad7 T96-0620 USA), AF065066 (Ad7 S-1058 USA), AF065067 (Ad7 55142 USA), AF065065 (Ad7 hexon USA), AY594255 (Ad7 Gomen USA), Z48571 (Ad7 hexon Sweden), AY599834 (Ad3 GB USA), DQ086466 (Ad3 Switzerland), AY599836 (Ad3 NHRC1276 USA), DQ099432 (Guangzhou01, China), and DQ105654 (Guangzhou02, China). Multiple sequence alignments and phylogenetic analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) software (version 4.0) (12). The phylogenetic tree was constructed by the neighbor-joining method (13), with a bootstrap analysis of 1,000 replicates. Partitioning by the chi-square test was applied in order to compare the rates in different patient groups using SPSS (version 13.0) software. Probability values of $P < 0.05$ were regarded as statistically significant.

The median age of the 412 children was 3 years and 7 months old (range, 1 month to 16 years), and the gender ratio was 1.77:1 (263 males, 149 females). Viruses were found in 260 of the 412 patients (63.1%): RSV in 25.0% ($n = 103$), Flu A in 14.6% ($n = 60$), Para 3 in 10.9% ($n = 45$), Flu B in 4.9% ($n = 20$), Ad in 4.1% ($n = 17$), Para 1 in 2.4% ($n = 10$), and Para 2 in 1.2% ($n = 5$). More than 1 virus was found in 27 patients (6.6%) (Table 1). Of the 260 virus positive samples, pneumonia was detected in 53.1% ($n = 138$), bronchiolitis in 25.8% ($n = 67$), and tracheobronchitis in 21.2% ($n = 55$) of patients. The seasonal distribution of viruses is shown in Figure 1.

We also examined differences in virus types among the different age groups. Most viral infections occurred during the first 5 years of life: the infection rate was highest in children aged <6 months, with a secondary peak at 2-4 years. The rate of RSV infection was significantly higher in infants during the first 6 months of life than in any other age group ($P < 0.05$). Flu A/B and Para 1-3 were the most frequent antigens in children aged 2-4 years, whereas Ad infection was detected primarily in children aged 11-16 years.

Tracheobronchitis was detected in most age groups (primarily in those aged 2-4 years), and was associated with Flu A. Bronchiolitis was detected frequently in children younger than 2 years, and rarely in those older than 5 years of age. The most frequent cause of bronchiolitis was RSV infection. Pneumonia was caused by most of the viral agents. The RSV and Para 1-3 were the most common antigens in children younger than 6 months and in those 2-4 years old, respectively. Interestingly, Ad developed into severe pneumonia in 70% (12/17) of older children (age 11-16 years).

We isolated 17 Ad strains, of which 12 strains were from severe pneumonia samples obtained between June and August. Amplification of the hexons of these 12 Ad strains revealed that the lengths of the PCR products were identical to the expected hexon length (2,985 bp). The sequencing results demonstrated that the hexons had very high homology (99.5%). Figure 2 shows the results of the phylogenetic analysis.

In this study, respiratory viruses were detected in 63.1% of children hospitalized with ALRIs, which is consistent with previously reported rates of 30-90% (14,15). The RSV predominated in infants younger than 6 months old, which may be due to a rapid loss of RSV-neutralizing maternal antibodies in this population. The secondary peak in viral infection occurred at 2-4 years, which we attributed to the children attending kindergarten and therefore being in daily contact with other children; even if children of this age possess antibodies, these antibodies may be insufficient to provide protection against reinfection(s) with different strains of virus. It was of note that Ad was detected most frequently during the summer, and that it caused severe pneumonia in older children. This hot and dry season overlaps with the examination preparation period for many students, and the stress associated with a considerable amount of homework from school, irregular recess time, crowded school conditions, and endocrine disturbances could all contribute to reducing immunity. As regards seasonality, viral infections in general

occurred primarily during the months with the greatest temperature changes in Harbin (during early winter, and especially in early spring).

The hexon sequence analysis demonstrated that high homology among isolated strains belonging to the species B genome type. This analysis also revealed similarities between the sequence of the amplicons and those of the Gomen strain (AY594255) of Ad7, which was isolated from an American patient in the 1950s. The desparate times and geographic settings of detection of these strains indicate that the Gomen strain has not disappeared, and may have been consistently involved in respiratory infections for many years. One possible reason for the spread of the species B virus is that the city of Harbin hosts many international conferences and events, which attracts many visitors from Western countries. The phylogenetic tree indicated that Ad7 and Ad3 have high homology, confirming that the hexon is conserved between Ad7 and Ad3.

Our study was limited in that it only considered lower respiratory tract infections that required hospitalization and was conducted in one city (Harbin) for only a single year. Therefore, an annual nationwide survey will still be necessary to characterize the viral epidemiology associated with respiratory illnesses in China, which in turn would help pediatricians to better manage children with ALRIs.

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