Human Respiratory Syncytial Virus in Children with Acute Respiratory Tract Infections in China[▽]

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There are limited data on the prevalence and clinical and molecular characterization of human respiratory syncytial virus (HRSV) in children with acute respiratory tract infections (ARTIs) in China. From December 2006 to March 2009, 894 nasopharyngeal aspirates (NPAs) were collected from children under 14 years of age with ARTIs. Samples were screened for HRSV and genotyped by reverse transcription-PCR (RT-PCR) and sequencing. Demographic and clinical information was recorded. A total of 38.14% (341/894) of samples were positive for HRSV. Phylogenetic analysis revealed that 60.4% of the selected 227 RSV strains were GA2, 34.4% were BA, 4.8% were GB2, and 0.4% were GB3. A total of 40.47% of all of the RSV-positive samples were coinfecting with other respiratory viruses, and adenovirus was the most common additional respiratory virus. No statistical differences were found in the frequency of diagnosis and symptoms between the coinfection group and monoinfection group. Additionally, no statistical differences were found in epidemiological characterizations or disease severity between genotype BA- and GA2-positive patients, except for a greater frequency of lower respiratory tract infections (LRTIs) (mostly bronchitis) with BA. HRSV is the most important viral pathogen in Chinese children with ARTIs. Four genotypes (i.e., GA2, BA, GB2, and GB3) circulate locally, and the predominant genotype may shift between seasons. Coinfection with other viruses does not affect disease severity. HRSV genotypes were not associated with different epidemiological characterizations or disease severity.

Human respiratory syncytial virus (HRSV) is a major cause of lower respiratory tract infections (LRTIs) among infants and young children worldwide and is also a major cause of morbidity in children under 1 year of age (2). Bronchiolitis and pneumonia, which are attributed to HRSV infection, are observed most frequently during the first few months of life (7). Almost all children are infected with HRSV by 2 years of age, and half of all children experience two infections (8, 13).

Based on genetic and antigenic variations in structural proteins, HRSV isolates have been subdivided into two major antigenic groups (i.e., A and B), and both subgroups are associated with different severities of infection (15, 18, 22–24). The nucleotide and deduced amino acid sequence similarities are 67% and 53% between group A and group B strains, respectively (11, 19). Antigenic variability is thought to contribute to the capacity of the virus to infect people repeatedly and cause yearly outbreaks. These characteristics may pose a challenge for vaccine design and development (27). The G protein, a surface-expressed glycoprotein that is associated with attachment of the virus, shows the largest antigenic and genetic

differences between the two antigenic HRSV subgroups and is one of the targets for neutralization and protective antibody responses (1, 19). The G protein contains two hypervariable regions; the second variable region, which corresponds to the C-terminal region of the G protein (HVR2), reflects overall G protein gene variability and has been analyzed in molecular epidemiological studies (9, 25–28).

Although HRSV has been recognized as an important agent, no effective vaccine is currently available for prophylaxis and there is no effective antiviral treatment against current HRSV infections. The importance of strain differences in relation to clinical features and vaccine development has not been fully elucidated (8, 22). In the present study, 894 children with acute respiratory tract infections (ARTIs) were examined over three consecutive seasons for the presence of HRSV, and HRSV strains were genotyped by sequencing HVR2. Demographic and clinical information was collected from all patients. The prevalence and clinical and molecular characterization of HRSV genotypes were further analyzed.

MATERIALS AND METHODS

Patients and specimens. From December 2006 to March 2009, 894 nasopharyngeal aspirates (NPAs) were collected from children with ARTIs on Tuesday every week in the First Hospital of Lanzhou University, China. ARTIs were classified according to WHO definitions (38). Informed consent was obtained from the parents of all children who provided specimens. The study protocol was approved by the hospital ethics committee. All NPA specimens were collected and transported immediately to the laboratory at the National Institute for Viral

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TABLE 1. Subgroup distribution of HRSV A and RSV B infections in children with ARTIs from 2006 to 2009

Epidemic season	No. of samples	No. (%) of samples RSV positive	No. (%) of typed samples	No. (%) of RSV infections with subgroup:	
				RSV A	RSV B
2006-2007	283	80 (28.3)	48 (60.0)	48 (100)	0
2007-2008	320	138 (43.1)	87 (63.0)	85 (97.7)	2 (2.3)
2008-2009	291	123 (42.3)	92 (74.8)	4 (4.3)	88 (95.7)
Total	894	341 (38.1)	227 (66.7)	137 (60.4)	90 (39.6)

Disease Control and Prevention, China CDC, and stored at -80°C until further analysis. Demographic and clinical data were recorded.

Clinical severity score. Based on variables reported in previous studies (15, 22), a severity index was defined a priori by assigning 1 point to each of the following: use of supplemental oxygen, duration of hospital stay >5 days, and admission to an intensive care unit (ICU).

DNA/RNA extraction. Viral DNA and RNA were extracted from 140 μl of each NPA specimen, using the QIAamp viral DNA and QIAamp viral RNA minikits (Qiagen, Shanghai, China) according to the manufacturer's instructions. cDNA was synthesized by using random hexamer primers with Superscript II RT⁺ reverse transcriptase (Invitrogen, Carlsbad, CA).

Viral detection. For HRSV screening, reverse transcription (RT)-PCR was performed with primers rsv1 and rsv2 to amplify a 278-bp fragment of the N gene (28). Primer sets RSVF/RSVA and RSVF/RSVB were used, respectively, to amplify a 270-nucleotide region of HVR2 for group A and B isolates, as described elsewhere (28). All specimens were screened for human metapneumovirus (HMPV), influenza virus A and B (IFVA and IFVB), parainfluenza virus types 1 to 3 (PIV1 to -3), human rhinoviruses (HRVs), and human coronaviruses (HCoVs: strains 229E, OC43, NL63, and HKU1), using RT-PCR (4, 5, 36, 37). Additionally, patients were screened for adenovirus and human bocavirus by traditional PCR methods (17).

Nucleotide sequence analysis. All positive PCR products were sequenced by the HUADA Gene Company. Sequences were determined and analyzed with the DNAMAN software package. A neighbor-joining tree was constructed by using the MEGA software 4.0 (34). The deduced amino acid sequences were analyzed with BioEdit software. A 270-nucleotide segment of HVR2 was compared to those available from GenBank.

Statistical analysis. Significant differences in rates between various groups were tested with a chi-square test, Student's *t* test, and a Mann-Whitney U test. A *P* value of <0.05 was considered to be statistically significant. The index of severity was tested by logistic regression. Analyses were performed with SPSS13.0 software.

Nucleotide sequence accession numbers. The HVR2 sequences have been deposited in GenBank under accession no. GU357503 to GU357548.

RESULTS

Patient characteristics. During the three epidemic seasons, 283 (31.65%) patients from December 2006 to July 2007 (2006-2007 season), 320 (35.79%) patients from August 2007 to July 2008 (2007-2008 season), and 291 (32.55%) patients from August 2008 to March 2009 (2008-2009 season) were enrolled in this study. The ages of children with ARTIs ranged from 1 day to 168 months. The majority of patients (90.69%) were under 5 years old. The ratio of boys to girls was 1.6:1, and the ratio of outpatients to inpatients was 1:3.

Detection of HRSV and other viral agents. We found that 38.14% (341/894) of samples were positive for HRSV, and 227 (67%) specimens were selected randomly from every month for subgroup analysis based on HVR2 sequencing (Table 1). Among these samples, 137 were RSV A (60%) and 90 were RSV B (40%). At least one respiratory virus was detected in 683 out of 894 samples (76.40%). HRSV accounted for 33.14% (341/1029) of the total viral agents. Additionally, 40.47% (138/

341) of all RSV-positive children were found to be coinfecting with other respiratory viruses, including 37 with adenovirus, 34 with HRV, 21 with IFVA, 12 with HMPV, 16 with human parainfluenza virus type 3 (HPIV3), 9 with coronavirus HKU1, 4 with coronavirus NL63, and 26 with bocavirus.

Phylogenetic analysis of HRSV. HVR2 obtained in the present study was aligned with the HVR2 sequences of other reported strains downloaded from GenBank (Fig. 1). Phylogenetic analysis revealed that all 137 strains clustered as the GA2 genotype, and the nucleotide and amino acid homologies among the strains were 92.2 to 100% and 81.8 to 100%, respectively. Ninety subgroup B strains clustered into three groups: 78 sequences with a 60-nucleotide duplication were placed into the newly identified BA genotype, 1 was placed in the GB3 genotype, and 11 were placed into the GB2 genotype. All BA genotype strains were further clustered into two groups: BA-II and BA-IV. In addition, the nucleotide and amino acid homologies among these group B viruses were between 78.5 and 100% and between 65.9 and 100%, respectively. The BA strains shared 92.73 to 100% nucleotide identity and 83.3 to 100% amino acid identity.

Epidemiology of HRSV. HRSV was detected in samples obtained every month, except for July, August, and September 2008. The majority of the cases (79%) were reported in the winter to spring months (November to March) (Fig. 2). HRSV A was detected in all 3 years and was dominant in 2006 to 2007 and 2007 to 2008. In contrast, HRSV B was detected in 2007 to 2008 and prevailed in 2008 to 2009 (Table 1). During 2007 to 2008, two BA strains were detected in December 2007. Among HRSV-positive patients, only 4.4% (15/341) were older than 5 years, and the age range was 1 day to 144 months. HRSV-infected children were significantly younger than HRSV-negative children (median of 10 versus 16 months) (Mann-Whitney U test; *P* = 0.000). The male/female ratio in the HRSV-infected group was 1.9:1, whereas in the RSV-negative group, the ratio was 1.6:1; however, this difference was not significant (chi-square test; *P* = 0.398).

Amino acid analysis. Deduced HVR2 amino acid (aa) sequences of 18 group A (GA2) and 29 group B isolates were aligned with the strains Long and CH18537 as references, respectively. Sixteen (16) group A strains encoded 89 amino acids with 1 deficient at the C-terminal third, whereas the remaining two encoded 90 amino acids, as well as the Long strain. Specific amino acid substitutions for the GA2 genotype were identified. Thr²⁶⁹ and Ser²⁸⁹ were conserved among all GA2 genotypes in this study. As compared with CH18537, the deduced amino acid sequences of Chinese group B strains

FIG. 1. Phylogenetic tree for Chinese HRSV group A and group B nucleotide sequences, based on the second variable region of G protein (270 bp). Phylogenetic trees were constructed by the neighbor-joining method, using MEGA 4.0. Bootstrap values were determined for 1,000 replicates and are shown at the branch nodes. Reference strains for each genotype obtained from GenBank are marked. The genotype assignment is indicated to the right by brackets.

exhibited five different lengths (83, 87, 100, 103, and 107 aa, with predicted G protein lengths of 295, 299, 312, 315, and 319 aa, respectively). The deduced amino acid sequences of BA strains observed in this study had three predicted lengths (312, 315, and 319 aa) due to a 60-nucleotide duplication.

Clinical characteristics of HRSV infection. Information on clinical characteristics was available for 327 (327/341) RSV-positive patients. Of the 327 subjects analyzed, 131 (40%) had mixed viral infections, and the most frequent clinical findings among RSV-positive patients were cough (95%), fever (57%), and wheezing (50%). Chest radiographs were obtained from 101 RSV-positive patients, and 93 showed abnormal findings: 53 patients had intrapulmonary punctate and patchy shadows, 39 displayed coarse lung markings, and 1 had lobar infiltrations.

Among the RSV monoinfection and RSV coinfection groups, no significant differences were observed in the frequency of acute upper respiratory infections (AURIs) and LRTIs between the two groups. The monoinfection and coinfection groups were also not significantly different with respect

to the clinical presentations observed in RSV-positive children, including cough, fever, crackles, wheezing, heart failure, cyanosis, and duration of hospitalization (Table 2). The rates of coinfection with other respiratory viruses were similar between the older group (>12 months old) and the younger group (≤ 12 months old) ($P = 0.349$).

To assess the relationship between clinical severity and HRSV genotypes, subjects with missing clinical data were excluded. Additionally, GB2 and GB3 were not included because there were too few cases, and 128 cases with GA2 and 77 with BA were included. Among these, 10 (10/128 + 77) children had an important underlying medical condition (9 with congenital heart disease and 1 with chronic pulmonary conditions), 79 (79/205) had mixed viral infections, and 155 were hospitalized.

Gender differences were not statistically significant between the GA2 and BA cases (chi-square test; $P = 0.42$). The mean age of patients with GA2 infections was greater than that of patients with BA infections; however, this difference was not statistically significant (Student's t test; $P = 0.24$), which is

FIG. 2. Monthly distribution of HRSV infections.

consistent with data by Rodica Gilca et al. (15). In this study, 41.74% of the GA2 patients were <6 months old (4 cases <1 month old), while 36.36% of the BA patients were <6 months old (6 cases <1 month old). The distributions of GA2 and BA infections were similar for the four age groups as defined in this study (chi-square; $P = 0.77$) (Table 3). Infections with both subgroups were more prevalent in children younger than 6 months.

In a univariate analysis, no significant differences were found with respect to the presence of cough, fever, wheezing, crack-

TABLE 2. Comparison of clinical manifestations in the HRSV monoinfection group and coinfection group

Characteristic	No. of patients in group with:		<i>P</i> value
	Monoinfection (<i>n</i> = 196)	Coinfection (<i>n</i> = 131)	
Diagnosis			
URTI	10 (5.1)	13 (9.9)	0.095 ^a
LRTI	186 (94.9)	118 (90.1)	
Age (mo)			
≤12	115 (58.7)	70 (53.4)	0.349
>12	81 (41.3)	61 (46.6)	
Type of patient			
Inpatient ^b	144 (73.5)	104 (79.4)	0.220 ^a
Outpatient	52 (26.5)	27 (20.6)	
Symptoms			
Cough	188 (95.9)	124 (94.7)	0.593 ^a
Fever	112 (57.1)	76 (58.0)	0.876 ^a
Crackles	153 (78.1)	98 (74.8)	0.495 ^a
Wheezing	103 (52.6)	62 (47.3)	0.355 ^a
Cyanosis	28 (14.3)	19 (14.5)	0.956 ^a
Heart failure	15 (7.7)	9 (6.9)	0.790 ^a
Underlying condition	15 (7.7)	11 (8.4)	0.807 ^a

^a Chi-square test.

^b The median durations of hospitalization for the monoinfected and coinfecting groups were 9.918 ± 4.895 days (range, 1 to 36 days) and 9.039 ± 3.366 days (range, 3 to 23 days), respectively ($P = 0.160$ by Student's *t* test).

TABLE 3. Comparison of clinical and demographic characteristics between the HRSV GA2 and BA groups in children with ARTIs

Variable	No. (%) of patients in HRSV group ^c :		<i>P</i> value
	GA2 (<i>n</i> = 128)	BA (<i>n</i> = 77)	
Age group (mo) ^b			
<6	50 (39.1)	28 (36.4)	0.77 ^a
7–12	24 (18.8)	17 (22.1)	
13–24	17 (13.3)	13 (16.9)	
>24	37 (28.9)	19 (24.7)	
Mixed infections	59 (46.1)	20 (26.0)	0.004 ^a
Underlying conditions	4 (3.1)	6 (7.8)	0.13 ^a
Clinical manifestations			
Cough	123 (96.1)	73 (94.8)	0.66 ^a
Wheezing	65 (50.8)	38 (49.4)	0.84 ^a
Fever	70 (54.7)	42 (54.5)	0.98 ^a
Crackles	94 (73.4)	50 (64.9)	0.19 ^a
Heart failure	11 (8.6)	8 (10.4)	0.66 ^a
Type of patient			0.29 ^a
Inpatient	101 (78.9)	54 (70.1)	
Outpatient	27 (21.1)	23 (29.9)	
Final diagnosis			
URTI	16 (12.5)	2 (2.6)	0.015 ^{a,d}
Bronchiolitis	43 (33.6)	25 (32.5)	
Pneumonia	60 (46.9)	39 (50.6)	
Bronchitis	9 (7.0)	11 (14.3)	
Severity index			
0	46 (35.9)	10 (13)	>0.05 ^c
1	48 (37.5)	46 (59.7)	
2	34 (26.6)	21 (27.3)	

^a Chi-square test.

^b The mean ± SD ages for the GA2 and BA patients were 19.81 ± 21.86 months and 16.51 ± 17.43 months, respectively ($P = 0.24$ by Student's *t* test).

^c Logistic regression.

^d Comparison of upper respiratory tract infections versus lower respiratory tract infections (bronchiolitis, pneumonia, and bronchitis).

^e The male/female ratios for the GA2 and BA groups were 81:47 and 53:24, respectively ($P = 0.42$ by chi-square analysis).

les, and heart failure between the two groups. Bronchiolitis and pneumonia (80.46% in the GA2 group and 83.11% in the BA group) were the most frequent clinical diagnosis in HRSV-infected children; however, LRTIs (mostly bronchitis) were more frequently observed in BA-infected children (chi-square test; $P = 0.015$). In the present study, a severity index with scores ranging from 0 to 2, with no case admitted to the ICU, was used to assess the severity of the disease. The multivariate logistic regression showed that there was no relationship between disease severity and HRSV genotypes (BA and GA2), which takes into account gender, age, mixed infections, and underlying conditions.

DISCUSSION

In the present study, 894 specimens were collected from children with ARTIs over three epidemic seasons and a significant rate of RSV infection (38.14%) was found. A similar incidence rate was reported in Japan (31). Furthermore, RSV is the most often detected pathogen out of the common respiratory-associated viral agents. Our results show that at least one-third of children with ARTIs have associated HRSV infection, and HRSV is the most important viral pathogen in Lanzhou City, China.

Previous studies (24, 30) suggest that male children are more susceptible to severe disease than females. In our study, the majority of HRSV-infected children (223/341) were male, but further statistical analysis found no differences in hospitalization rates and clinical presentation between male and female patients (data not shown). In addition, most of the HRSV-infected patients were younger than 6 months. These data are consistent with the earlier reports (29, 43). HRSV infections reached a peak in November to April in our study. In the 2006-2007 season; however, HRSV infections peaked in May, which may have been caused by the relatively limited number of clinical samples.

Phylogenetic analysis revealed that viruses of the group A (A2) and group B (BA, GB3, and GB2) genotypes circulated locally over three consecutive seasons. Group A strains were detected at higher rates than group B strains during December 2006 to November 2008. Interestingly, a rapid change in the HRSV predominant strains occurred, group B strains appeared to be more prevalent during November 2008 to March 2009, and the new BA genotype constituted most of the RSVB isolates. This result is consistent with previous studies, which showed that the predominant genotype can shift among seasons (3, 13, 28, 33, 43). Interestingly, a study from Chongqing (40), a city in southwestern China that is distant from Lanzhou City, demonstrated the same circulation pattern as ours during the same period. This may be explained by frequently increasing population movements. However, this pattern is not consistent with those in other studies, which suggest the replacement of the predominating HRSV genotype occurs in each epidemic (28). In addition, A2 strains (LZ68, LZ394, LZ360, LZ195, and LZ268) from the 2006-2007 epidemic season were identical to A2 isolates (LZY103, LZY142, and LZY145) from 2008 to 2009, which indicates that the RSV A2 strain can remain stable for more than one epidemic season.

The BA genotype was first detected in Buenos Aires, Argentina, during 1999 and was subsequently found in other

countries (21, 26, 33, 41). In China, this strain was first detected in 2005 (14). In the present study, the BA strain became predominant in the 2008-2009 season. The BA genotype has been classified into six clusters: BA-I to -VI (35). All BA viruses in this study were further clustered into BA-II and BA-IV and were found to be closely related to NG/153/03 (isolated in 2003 from Japan) and BA/100/04 (isolated in 2004 from Spain), respectively. These findings indicate that this novel BA strain has spread globally. All sequences with 60-nucleotide duplications were clustered in the same genotype (BA). No other group B sequences showed duplications, which confirms a previous hypothesis that the BA virus was generated from common ancestry (35).

There have been conflicting data about whether the evolution of HRSV is related temporally or geographically (12, 20, 21, 27). In our study, LZ171 and LZ226 strains isolated in the 2006-2007 season were most closely related to strains GQ259155 from Karaj, Iran, isolated in 2008. However, these strains were distinct from LZ189, isolated during the same period, which is most closely related to Beijing/A/01/10 strains isolated in 2001 in Beijing, China. Likewise, LZY42 from the 2008-2009 season is most closely related to NG/153/03 from Japan, isolated in 2003. These results confirm a previous study that strains isolated in the same place during the same epidemic season may be distantly related (10, 12, 39). No apparent temporal or geographic relationship was found, however, between China strains and other foreign strains. These results indicate that HRSV may have evolved via a complex pattern of transmission and circulation determined by viral strain, host, and local characteristics (27, 42).

In the present study, the G protein of HRSV subgroup A, as well as the other HRSV A reference strain, was predicted to be 297 or 298 amino acids in length. The deduced amino acid homologies of the GA2 genotype in HRV2 were 81.8 to 100%. Previous studies have speculated that the global prevalence of HRSV A is due to a higher degree of divergence (4). Among the group B strains, the deduced amino acid homologies in HRV2 were 65.9 to 100% and six different G protein lengths were observed. The usage of an alternative termination codon is one of the major mechanisms for generating additional diversity among subgroup B viruses. These results demonstrated that subgroup B viruses display more mechanisms of variation in the G gene than RSV A viruses and also have a greater potential for divergence, which coincides with recent other studies (28, 29). Different evolutionary patterns were also indicated between the two subtypes.

Bronchiolitis and pneumonia were the most frequent diagnoses in our study, as reported by T. F. M. Oliveira et al. (24). Between the monoinfection group and the coinfection group of RSV-positive patients, no differences were found in the frequency of cough, fever, crackles, wheezing and cyanosis, heart failure, and duration of hospitalization. Although Greensill et al. (16) reported that RSV coinfection with HMPV may be a determination of disease severity, our results indicated that coinfection of HRSV with other respiratory viruses has no additional effects on disease severity (Table 2).

There is limited information regarding the relationship between HRSV genotypes and the severity of disease, especially with respect to the BA virus. In our study, no significant differences in clinical symptoms and signs between the GA2 and

BA groups were found, based on a univariate analysis. In addition, no new clinical features were found to be associated with the emerging BA strain, except for the greater frequency of LRTIs (mostly bronchitis) in the BA group, as compared with the GA2 group viruses. Although we lack blood gas analysis in our clinical data, a more frequent requirement for supplemental oxygen and a longer duration of hospitalization could be related to a greater disease severity. Using a composite severity index and controlling for multiple confounding factors that would affect the assessment of disease severity, no relationship was found between disease severity and HRSV genotypes based on a multivariate analysis. This relationship is not sufficiently understood (15, 22), and contradictory results were found in previous studies (6, 15, 22, 24, 32). These inconsistencies may have been caused by the study design, the clinical methods for assessing illness severity, the type of patients enrolled, the distribution of HRSV genotypes, ethnic differences, etc.

In summary, HRSV is a very important pathogen in children with ARTIs in China. Multiple genotypes circulate locally, and the predominant genotype may shift between seasons. The present study showed that HRSV genotypes (GA2 and BA) were not associated with different epidemiological characterizations or disease severities, except for a greater frequency of LRTIs (mostly bronchitis) in BA. Additionally, no differences in clinical presentation and disease severity between the HRSV mono-infection group and coinfection group were observed. This is the first report to describe the relationship between RSV genotype and disease severity, especially BA type from China. However, the present study was limited to three winter seasons, and additional consecutive investigations should be conducted in order to provide useful information for HRSV disease treatment.

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