Original Article

Genetic Characterization of Fusion Protein of Human Respiratory Syncytial Virus: Beijing

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Objective Fusion protein is a subunit of the human respiratory syncytial virus (HRSV) and a potential vaccine candidate. Thus, a study on the genetic characteristics of F protein was considered important for further investigations in this field. The aim of this study was to determine the prevalence and genetic diversity of the F gene of HRSV infections in hospitalized pediatric patients in Beijing with acute lower respiratory tract infections and to compare the circulating genotypes that are currently found worldwide.

Methods HRSV particles were amplified by RT-PCR and the PCR products were purified for sequencing. Further analysis was carried out by Bioedit and MEGA 3.0 biological software programs.

Results Seventy-six samples (23.1%) were positive for HRSV. The percentage of cases in patients younger than 1 year was 84.21%. Among the six Beijing isolates, four belonged to subgroup A, whose respective F genes shared 97.0%-97.4% nucleotide sequence identity and 92.1%-93.0% amino acid sequence identity. The other two isolates belonged to subgroup B. Here, 97.3% and 98.2% sequence identity were found at nucleotide and amino acid levels, respectively.

Conclusions Phylogenetic analysis of nucleotide sequences revealed that those four isolates within subgroup A were monophyletic and closely related to each other, but those two within subgroup B distributed in two distinct clusters. Subgroup A and B strains co-circulated, indicating that two different transmission chains occurred in Beijing from 2003-2004.

Key words: Human respiratory syncytial virus; F protein; Nucleotide sequence; Amino acid sequence

Human respiratory syncytial virus (HRSV) is a member of the genus pneumovirus in the paramyxoviridae family.¹ It is the most common viral pathogen that causes lower respiratory tract infection in infants and young children.^{2,3} In the United States, at least 50% children are infected during their first HRSV season, and virtually all children have been infected by two years of age.⁴ Among children, HRSV infection causes 50%–90% of hospitalizations for bronchiolitis, 5%–40% of hospitalizations for pneumonia, and 10%–30% of hospitalizations for tracheobronchitis.⁵ In Beijing, HRSV infection caused 48% of viral pneumonia and 58% of bronchiolitis between 1980 and 1984.⁶ HRSV is also an important respiratory disease agent in the elderly and in post-transplantation patients.⁷⁻⁹ Understanding of the pathogenic mechanisms and molecular characteristics of HRSV is still incomplete, which has hindered the

development of a successful treatment or an effective vaccine. Moreover, most of the research on molecular epidemiology has focused on the attachment of the G glycoprotein (G protein), while the fusion protein (F protein) has rarely been studied (<http://www.ncbi.nlm.nih.gov/GenBank/>).

In this novel study, we analyzed the complete nucleotide sequence of the F gene from HRSV strains isolated between 2003 and 2004 in Beijing and compared this sequence with other F genes from HRSV isolates available in GenBank database.

MATERIALS AND METHODS

Viruses

Three-hundred and twenty-nine respiratory specimens (nasopharyngeal swabs or aspirates) were collected from each child (< 3 years old) with acute lower respiratory tract infections (ALRTIs) at the Beijing Children's Hospital during the period from 2003 to 2004. ALRTI was defined as the presence of bronchitis, bronchiolitis and pneumonia according to the World Health Organization criteria. Collected samples

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Figure 1. Neighbor Joining (NJ) trees of Beijing HRSV subgroup A nucleotide sequences.

were stored at -80°C until processed. Seventy-six strains of HRSV were isolated from nasopharyngeal aspirates or nasal swabs and kept frozen at -80°C until propagated in Hep-2 cell culture. A total of 73 strains were successfully recovered from Hep-2 cell cultures. Nucleotide sequencing of the gene for G protein found that 71 strains belonged to subgroup A and 2 strains belonged to subgroup B. The four most distant strains in subgroup A were chosen on the basis of their 71 G gene sequences (Figure 1). Six strains were chosen to determine the entire nucleotide sequence of the F protein gene.

Reverse transcription–polymerase chain reaction analysis

The stock viruses were propagated in Hep-2 cells. When an extensive cytopathic effect was evident, the cells were harvested, lysed with Trizol in chloroform. RNA was reverse-transcribed using specific primers: P1 (5'-GGC AAA TAA CmA TGG AG-3') and P3 (5'-CAT CmC CTC TAT GyA CmA-3'). In order to amplify F genes from different strains, primers from both ends of the full sequence (one primer from G gene and the other from M2 gene) were designed. RT-PCR was performed to amplify the entire sequence of the

F gene using a Taq DNA polymerase AMV (Biolab, Beijing, China). The primers were: P1, P2 (5'-GTT GGT kGT rCA TAG AG-3'), P3 and P4 (5'-CTT AAA TAr TGT AAG TGA GAT GG-3'). To amplify F genes from different strains, primers from both ends of the full sequence (one primer from G gene and the other from M2 gene) were designed. The size of the PCR products was determined by 1.7% agarose gel electrophoresis. Note: m, y, k and r in primers above represent as m = A or C, y = C or T, k = G or T, r = A or G.

Detection and genotyping

The PCR products were purified using a QIAquick PCR purification kit and labeled using the Bigdye Terminator Cycle Sequencing Reaction kit (Qiagen, Valencia, CA, USA) and the following primers: P5 (5'-TTA TCA ATC AAG ATG CAG TG-3'), P6 (5'-CTT TTC CTT TTC TTG CT-3'), P7 (5'-AGC AAG AAA AGG AAA AG-3'), P8 (5'-CGG AGC TGC TTA CAT CT-3'), P9 (5'-TGC TGG TAA ATC AAC CAC AAA T-3') and P10 (5'-ATT TGT GGT TGA TTT ACC AGC-3'). The final PCR products were sequenced using fluorescent dye-labeled terminators on an ABI3100 sequencer (Certified Genetool Inc, USA). Nucleotide sequences were analysed using SequencerTM 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA),

BioEdit Sequence Alignment Editor (V.7.0.9.0)¹⁰ and BLAST-N¹¹ sequences were aligned with reference F sequences from GenBank. After manual adjustment of the alignment, phylogenetic analysis was performed with MEGA 3.0 using neighbor-joining methods.

RESULTS

Clinical characteristics

Seventy-six samples (23.1%) were positive for HRSV (97.3% genotype A and 2.75% genotype B, respectively). The percentage of cases in patients younger than 1 year was 84.21% (64/76), 11.84% (9/76) in patients between 1 and 2 years, and 3.95% (3/76) were accounted to children aged between 2 and 3 years. Among these, 51 (67.1%) samples from boys

and 25 (32.9%) from girls were positive for HRSV, giving a ratio of 2.04 : 1. Bronchiolitis and pneumonia were diagnosed in 69 out of 76 patients (90.8%), while bronchitis and pharyngitis were found in seven cases (9.2%).

RT-PCR

Two pairs of primers were used to amplify the entire sequence of the F gene. The expected size of each PCR product was 800–1000 base pairs (bp), which was confirmed through agarose gel electrophoresis.

Phylogenetic analysis

To investigate the genetic relationship between HRSV Beijing isolates and other isolates available from GenBank, a phylogenetic analysis was conducted

Figure 2. Neighbor Joining (NJ) trees of Beijing HRSV subgroup A and B nucleotide sequences.

Table 1. Homology analysis of nucleotides and amino acids of the four Beijing HRSV A group isolates and sequences obtained from GenBank

Seq->	A2	226524	U39662	AY330616	M22643	AY330612	AY330613	AY330614	AY330611	D00151	AY198176	AY198177
A2		0.978	0.972	0.979	0.979	0.979	0.979	0.979	0.979	0.970	0.955	0.952
Z16524	0.945		0.968	0.991	0.991	0.990	0.990	0.990	0.990	0.956	0.953	0.953
U39662	0.927	0.916		0.970	0.970	0.969	0.969	0.969	0.969	0.998	0.969	0.966
AY330616	0.944	0.981	0.916		0.999	0.999	0.999	0.999	0.999	0.968	0.956	0.955
M22543	0.945	0.983	0.918	0.998		0.998	0.998	0.998	0.998	0.968	0.956	0.955
AY330612	0.944	0.981	0.916	1.000	0.998		0.998	0.998	0.998	0.968	0.955	0.954
AY330613	0.942	0.980	0.914	0.998	0.996	0.998		0.998	0.998	0.968	0.955	0.954
AY330614	0.942	0.980	0.914	0.998	0.996	0.998	0.996		0.998	0.968	0.955	0.954
AY330611	0.942	0.980	0.914	0.998	0.996	0.998	0.996	0.996		0.968	0.955	0.954
D00151	0.921	0.910	0.994	0.911	0.912	0.911	0.909	0.909	0.909		0.963	0.965
AY198176	0.882	0.878	0.920	0.882	0.882	0.882	0.880	0.880	0.880	0.918		0.981
AY198177	0.887	0.872	0.911	0.876	0.876	0.876	0.875	0.875	0.875	0.909	0.958	
AY198175	0.872	0.868	0.913	0.875	0.875	0.875	0.873	0.873	0.873	0.911	0.958	0.953
AY063315	0.944	0.981	0.916	1.000	0.998	1.000	0.998	0.998	0.998	0.911	0.882	0.876
U50363	0.966	0.944	0.928	0.944	0.945	0.944	0.942	0.942	0.942	0.923	0.884	0.877
U50362	0.966	0.944	0.928	0.944	0.946	0.944	0.942	0.942	0.942	0.923	0.884	0.877
U63644	0.966	0.944	0.928	0.944	0.946	0.944	0.942	0.942	0.942	0.923	0.884	0.877
AF035006	0.985	0.933	0.916	0.935	0.935	0.935	0.933	0.933	0.933	0.911	0.896	0.889
L15351	0.946	1.000	0.916	0.981	0.983	0.981	0.980	0.980	0.980	0.910	0.878	0.872
BJ01-10	0.821	0.820	0.854	0.818	0.820	0.818	0.817	0.817	0.817	0.848	0.916	0.921
BJ04-12	0.882	0.882	0.921	0.880	0.882	0.880	0.879	0.879	0.879	0.919	0.918	0.925
BJ04-9	0.887	0.811	0.916	0.879	0.881	0.879	0.878	0.878	0.878	0.915	0.930	0.937
BJ04-13	0.856	0.858	0.902	0.857	0.857	0.857	0.865	0.855	0.855	0.896	0.912	0.919

Seq->	AY198175	AY330615	U50363	U50362	U63644	AF035006	L25361	BJ01-10	BJ04-12	BJ04-9	BJ04-13
A2	0.953	0.979	0.997	0.997	0.979	0.993	0.978	0.954	0.957	0.954	0.950
Z16524	0.952	0.990	0.977	0.977	0.977	0.973	1.000	0.956	0.956	0.956	0.950
U39662	0.969	0.969	0.972	0.972	0.972	0.968	0.968	0.967	0.968	0.969	0.964
AY330616	0.956	0.999	0.979	0.979	0.979	0.975	0.991	0.956	0.957	0.957	0.951
M22543	0.956	0.993	0.979	0.979	0.979	0.975	0.991	0.956	0.957	0.957	0.951
AY330612	0.955	0.993	0.979	0.979	0.979	0.974	0.990	0.956	0.957	0.957	0.951
AY330613	0.955	0.993	0.979	0.979	0.979	0.974	0.990	0.956	0.957	0.957	0.951
AY330614	0.955	0.993	0.979	0.979	0.979	0.974	0.990	0.956	0.957	0.957	0.951
AY330611	0.955	0.993	0.979	0.979	0.979	0.974	0.990	0.956	0.957	0.957	0.951
D00151	0.963	0.963	0.970	0.970	0.970	0.967	0.966	0.965	0.968	0.969	0.963
AY198176	0.984	0.955	0.957	0.957	0.957	0.961	0.953	0.966	0.968	0.973	0.967
AY198177	0.983	0.954	0.954	0.954	0.954	0.958	0.953	0.971	0.973	0.976	0.970
AY198175		0.955	0.956	0.956	0.956	0.959	0.952	0.967	0.969	0.973	0.968
AY063315	0.875		0.979	0.979	0.979	0.974	0.990	0.956	0.957	0.957	0.951
U50363	0.874	0.944		1.000	1.000	0.995	0.977	0.954	0.956	0.953	0.949
U50362	0.874	0.944	1.000		1.000	0.995	0.977	0.954	0.956	0.953	0.949
U63644	0.874	0.944	1.000	1.000		0.995	0.977	0.954	0.956	0.953	0.949
AF035006	0.886	0.935	0.985	0.985	0.985		0.973	0.950	0.952	0.948	0.945
L15351	0.868	0.981	0.944	0.944	0.944	0.933		0.956	0.956	0.956	0.950
BJ01-10	0.907	0.879	0.885	0.885	0.885	0.872	0.820		0.989	0.975	0.970
BJ04-12	0.909	0.879	0.885	0.885	0.885	0.873	0.881	0.973		0.979	0.971
BJ04-9	0.923	0.880	0.879	0.879	0.879	0.868	0.832	0.939	0.994		0.974
BJ04-13	0.903	0.857	0.865	0.865	0.865	0.852	0.858	0.923	0.921	0.930	

Table 2. Homology analysis of nucleotides and amino acids of the two Beijing HRSV B group isolates (BJ04-66 and BJ04-11) and further sequences from GenBank

Seq->	AF013255	AF013254	NC001781	D00334	AY353550	BJ04-66	BJ04-11
AF013255		0.998	0.998	0.982	0.981	0.975	0.971
AF013254	0.998		1	0.983	0.982	0.976	0.972
NC001781	0.998	1		0.983	0.982	0.976	0.972
D00334	0.989	0.991	0.991		0.985	0.972	0.968
AY353550	0.991	0.992	0.992	0.991		0.971	0.967
BJ04-66	0.982	0.984	0.984	0.982	0.984		0.973
BJ04-11	0.985	0.987	0.987	0.985	0.987	0.982	

Note: Percentages on the right upper part denote nucleotide identities and on the left bottom part show amino acid identities.

on the complete F nucleotide sequences of six Beijing isolates. A bootstrap analysis of the HRSV sequences found two distinct subgroups: subgroup A and subgroup B, similar to that found in a previous study.¹² Four Beijing isolates belonging to subgroup A were monophyletic and closely related to each other, which suggested a common origin (Figure 2). The highest level of homology of the F gene nucleotide sequence was found between four Beijing isolates and AY198175, AY198176, and AY198177 (Hebei, China 1992). Isolates BJ04-66 and BJ04-11 on the other hand fell into another group, with BJ04-66 forming the sister clade to AFO13255, NC001781 and AF013254, and BJ04-11 as a sister taxon to this cluster. The highest level of nucleotide homology was found among BJ04-66, BJ04-11, and D00334, AY353550 (Figure 2), and a full comparison of sequence and amino acid homologies are shown in Table 1 and Table 2.

Nucleotide sequence and deduced amino acid sequence analysis

The complete F gene sequences of Beijing isolates were compared with the corresponding sequences of HRSVs in GenBank database. The percentage identity was calculated by pairwise comparison of aligned nucleotide and amino acid sequences. The four Beijing isolates belonging to subgroup A had 97.0%–97.4% identity with F gene sequence at nucleotide level and 92.1%–93% sequence identity at amino acid level. The nucleotide and amino acid sequences of F genes from the four Beijing isolates showed the highest identities with the corresponding sequences of AY198175, AY198176, and AY198177 (Hebei, China 1992) (96.6%–97.6% and 90.3%–93.7%, respectively; Table 1). For BJ04-11 and BJ04-66 which belong to subgroup B, the F gene sequence identity was 97.3% and 98.2% at nucleotide and amino acid levels, respectively. F genes from BJ04-11 and BJ04-66 showed the highest nucleotide sequence identity with those from NC001781 and F013254 (97.2% and 97.6%, respectively) and the highest amino acid identity with NC001781, AF013254 and AY353550 (98.7% and 98.4%, respectively; Table 2). The variation in amino acid sequence of F protein was higher in subgroup A than that in subgroup B. The most divergent region of F protein was F2 for subgroup A and F1 for subgroup B. Differences of the proteins were mainly due to amino acid substitution.

The F gene signal peptide region of four Beijing subgroup A isolates had 99.36%–99.73% nucleotide homology and 99.13%–99.65% amino acid homology with HRSV A2; the F protein coding region had 95.99%–96.53% and 97.39%–97.74% homology in nucleotide and amino acid sequence, respectively. The nucleotide homology for F1 and F2 regions were 98.93%–99.31% and 96.69%–97.33%, and

98.78%–99.48%, 98.61%–99.30% for amino acid homology, respectively.

DISCUSSION

The F protein gene is one of the most conserved genes of HRSV. The active paramyxovirus F protein consists of two disulfide-linked units, F1 and F2, which are generated from F0 (an inactive precursor). F0 is cleaved by the endoprotease furin into an N-terminal F2 subunit and a C-terminal, membrane-anchored F1 subunit carrying the fusion peptide.^{13,14} The F1 unit is more conservative and so might be potential as an anti-HRSV agent. HRSV strains have been classified into antigenic subgroups A and B on the basis of the reactivity with monoclonal antibodies.^{15,16}

Belonging to the same subfamily, HRSV is compared frequently with human metapneumovirus (hMPV) because they share many biologic properties, including their natural host. Phylogenetic trees based on the nucleotide sequences of the complete F protein can be used to differentiate HRSV strains into subgroups A and B. More males than females had combined hMPV and HRSV infections and more males than females had HRSV infections, as shown by Hussey.¹⁷ But whether there are difference in T cell immunity and balance of Th1/Th2 between male and female remains to be further investigated. HRSV was detected in about 3.95% of children between two and three years whereas hMPV was detected in about 33.6% of children between two and five years. This indicates that patients with HRSV infection are significantly younger than patients infected with hMPV.¹⁷ More infants (younger than 1 year old) than children (older than 1 year) had HRSV infections and more patients were diagnosed with bronchiolitis and pneumonia, which is consistent with the findings of others¹⁹⁻²¹ and may be related to fewer CD3⁺CD4⁺CD8⁺ cells in the peripheral blood of infants.

The percentage of identity was calculated by pairwise comparisons of aligned nucleotide and amino acid sequences. F gene nucleotide and amino acid sequence of the four Beijing isolates in subgroup A had the highest identities with the Hebei (1992) isolates. Although the four Beijing subgroup A isolates and Hebei isolates stem from the same virus ancestor, their distribution in different areas of China differs. Phylogenetic analysis of the F gene from subgroup B strains (BJ04-66 and BJ04-11) found they were in two distinct clusters, indicating that viruses from different clusters were co-circulating in Beijing during the same epidemic season. The nucleotide diversity resulted in a higher amino acid diversity in subgroup A than in subgroup B, suggesting that more of the mutations in subgroup B were neutral and translated into the same amino acid. The level of nucleotide homology was

higher than that of amino acid homology in subgroup A, which was opposite in subgroup B. The levels of nucleotide and amino acid homology were higher in subgroup B than in subgroup A. HRSV is capable of repeated infection, but the amino acid sequences vary. Antibodies produced in response to the last infection may not protect against a current infection. Thus, the greater amino acid sequence variability of subgroup A may confer greater persistence with a higher level of infectivity. The most divergent region of the F protein sequence was F2 for subgroup A, as described by Plows,²² and F1 for subgroup B. Anti-HRSV antibodies to the AA200-225 epitope of F1 subunit can react with and neutralize HRSV. Since the amino acid sequence in this region of the protein is conserved, this peptide represents a promising candidate for an HRSV vaccine. Epidemiological studies found subgroup A and B strains co-circulating in two communities during annual epidemics.²³ Subgroup A strains occurred more commonly than subgroup B strains²⁴ due to the following reasons:

- a. The most common HRSV strains are from subgroup A.
- b. Subgroup A strains have stronger pathogenicity.
- c. Subgroup B strains infections elicit stronger protective immunity than subgroup A strains infections.
- d. Subgroup B strains is more difficult to isolate.

In summary, this is the first description of the genetic characteristics of the F gene in human HRSV isolated during an epidemic period from children in Beijing, China. These data improve our understanding of the patterns of HRSV circulation in Beijing. Elucidation of the genetic diversity of epidemic HRSV will aid the development of diagnostic reagents and vaccines.

Conflict of interest

The authors who have taken part in this study declared that they have no conflict of interest with respect to this manuscript

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Ethical approval

By Human Ethics Committee of Beijing Children's Hospital, Capital Medical University.

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