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Genetic Variability of Human Metapneumovirus Isolated From Chilean Children, 2003–2004

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Human metapneumovirus (hMPV) is a significant cause of acute lower respiratory tract infection in all age groups, particularly in children. Two genetic groups and four subgroups of hMPV have been described. They co-circulate during an epidemic in variable proportions. The aims were to characterize the genotypes of hMPV recovered from children hospitalized for acute lower respiratory tract infection and to establish the molecular epidemiology of strains circulating in Santiago of Chile during a 2-year period. The detection of the N gene by reverse-transcription polymerase chain reaction was carried out for screening 545 infants hospitalized for acute lower respiratory tract infection in Santiago during 2003–2004. The genetic typing of hMPV was performed by analyzing the fusion gene sequences. hMPV was detected in 10.2% (56/545 cases). Phylogenetic analysis of F gene sequences from 39 Chilean hMPV strains identified the two groups and four subgroups previously described. Strains clustered into group A were split further into the sub lineages A1, A2, and A3. Most Chilean strains clustered into the proposed novel A3 sub lineage (59%). A3 viruses were present in both years, while A1 and A2 circulated just in 1 year. In conclusion, hMPV is a relevant cause of acute lower respiratory infection in Chilean children and the potential novel cluster of group A emphasize the need for further regional genetic variability studies. ***J. Med. Virol. 81:340–344, 2009.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: human metapneumovirus; Chile; genotypes

INTRODUCTION

Human metapneumovirus (hMPV) is a member of the family Paramyxoviridae, genus *Metapneumovirus* [Mackay et al., 2003]. It is an important etiological agent of acute lower respiratory tract infection in all age groups. hMPV was first identified in the Netherlands [van den Hoogen et al., 2001] and thereafter the virus has been detected worldwide [Peret et al., 2002; Stockton et al., 2002; Bastien et al., 2003; Osterhaus et al., 2003] at different rates depending upon age, gender, season and the diagnostic method used. In South America, hMPV has been reported in children at Brazil [Cuevas et al., 2003], Argentina [Galiano et al., 2004], Chile [Luchsinger et al., 2005] and Peru [Gray et al., 2006]. Likewise, there is serologic evidence of hMPV spread in Uruguay [Mirazo et al., 2005]. Many viral genes have been used to perform phylogenetic analyses, notably the F gene has been used widely for these purposes [Huck et al., 2006; Banerje et al., 2007; da Silva et al., 2008; Boivin et al., 2004]. Two groups and four subgroups (A1, A2, B1, and B2) have been identified. [van den Hoogen et al., 2004; Ludewick et al., 2005]; furthermore, new genetic clusters have been proposed in subgroup A2, designated A2a and A2b [Huck et al., 2006].

The F glycoprotein is highly conserved among hMPV strains, showing 95% identity at the amino acid level between the A and B groups; it is a major neutralization antigen that confers cross reactive protection among different lineages [Skiadopulos et al., 2004]. The aims of this report were to determine the frequency of hMPV in a Chilean population from 2003 through 2004 and to identify the subgroups circulating currently by analysis of F gene sequences.

Abbreviations: hMPV, human metapneumovirus; IFA, indirect immunofluorescence assay; RSV, respiratory syncytial virus; PCR, polymerase chain reaction; N gene, nucleocapsid gene; $MgCl_2$, magnesium chloride; F-gene, fusion protein gene; AMPV, avian metapneumovirus C.

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MATERIALS AND METHODS

Collection of Clinical Samples

Children less than 2 years old hospitalized for acute lower respiratory tract infection (bronchiolitis and/or pneumonia) at the Roberto del Río Children's Hospital from January 2003 to December 2004 were enrolled for respiratory viruses surveillance [Avendaño et al., 2003]. Exclusion criteria were: prematurity; recurrent wheezing or asthma; and underlying chronic pulmonary, cardiac or neurological diseases. Nasopharyngeal aspirates obtained within the first 48 hr after admission were split for RNA extraction, viral isolation and indirect immunofluorescence assay (IFA) for human respiratory syncytial virus (HRSV), adenovirus, influenza A and B, and parainfluenza viruses 1–3 as described previously [Avendaño et al., 2003]. For IFA, monoclonal antibodies (HRSV A + B: 18B2, Dr. Pierre Pothier, Dijon France; Adenovirus: mouse antiadenovirus (blend) MAB 805, Chemicon Int®; parainfluenza virus 1, 2, 3: MAB 819, Chemicon Int®; influenza A virus: MAB 8251, Chemicon Int®; influenza B virus: MAB 8661, Chemicon Int®), and a fluorescein-labeled anti mouse IgG were used.

RNA Extraction and Reverse-Transcription Polymerase Chain Reaction

All samples positive for HRSV and those negative for other common viral agents were subject to total RNA extraction by the guanidinium thiocyanate–phenol–chloroform method [Chomczynski and Sacchi, 1987]. Reverse transcription was performed in a 20-μl reaction mix containing 10 μl of RNA, random hexamer primers (0.52 μM) (Amersham Bioscience, NJ), 20 U of Recombinant Rnasin Ribonuclease Inhibitor (Promega, Madison, WI), deoxynucleotide triphosphates (0.25 mM each), 200 U of M-MLV reverse transcriptase (Promega), and M-MLV buffer 5×. cDNA was synthesized at 37°C for 60 min and 95°C for 5 min in a Perkin Elmer Gene Amp® PCR System 2400. Real time PCR was performed with 0.5 μM of specific primers for hMPV N gene (N2–N3) [Cote et al., 2003], $MgCl_2$ (4 mM), FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany) (2 μl) and 2 μl of cDNA (total volume 20 μl) in a Light Cycler 1.5 instrument (Roche). Cycling conditions included a denaturation step of 10 min at 94°C, followed by 40 cycles of 10 sec at 94°C, 5 sec at 50°C, 30 sec at 72°C. hMPV amplicons were analyzed by melting curve analysis; the expected melting temperature of the amplicon (928 bp) was 84°C. A negative (water) and two positive controls with RNA from viral culture in LLC-MK2 cells were included in each assay. The positive culture was kindly provided by Dr. Mónica Galiano (Argentina).

Nucleotide Sequence Analysis

All positive samples for the N gene were further amplified with F1 and F2-gene primers [Falsey et al., 2003] for sequencing of the F gene. The 347-pb products were purified with the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and the nucleotide sequences corresponding to positions 3341 to 3687 of the hMPV genome were determined by automated DNA sequencing (ABI Prism 377; Applied BioSystems, Foster City, CA) with Big Dye Terminator Kit v3.1 (Applied BioSystems). Sequences were aligned with all unique sequences corresponding to the amplified fragment of the F gene published in GenBank, including prototypic sequences of different types (A and B) and subtypes (A1, A2, B1, and B2) from the Netherlands and Canada, and sequences of the avian metapneumovirus C (AMPV), using ClustalW software [Thompson et al., 1994]; distances were calculated with the Kimura 2 parameter substitution model. Phylogenetic trees were generated by the neighbor-joining method with MEGA version 4 [Tamura et al., 2007] and by maximum parsimony and likelihood analysis with PAUP 4.0 ß version [Swofford, 2000]. Bootstrap values were obtained with 500 replicas. In the case of clusters with inner distances ≤ 0.02 only one sequence is shown in the phylogenetic tree.

Nucleotide Sequence Accession Numbers

The sequences analyzed in this study were submitted to the GenBank under accession numbers EU273923–EU273937.

RESULTS

hMPV was studied in 545 from 613 cases enrolled in the respiratory viral surveillance. The exclusion criteria were detection of adenovirus, influenza or parainfluenza viruses. A total of 56/545 (10.2%) cases were positive for hMPV. From 146 samples positive for HRSV, only one was positive for hMPV (0.6%); 55 out of 399 samples negative for any other virus were positive for hMPV (13.78%). The detection rates were 11.1% (28/251) in 2003 and 9.5% (28/294) in 2004. Fifty-two percent of patients admitted during spring months (September–December) were positive (Fig. 1). The median age of positive cases was 5 months (range 0–21 months).

F-gene sequences were determined for 39 of the 56 Chilean strains. Regardless of the analysis strategy, phylogenetic trees of the F-gene fragment of 15 unique sequences of these strains and 109 representative sequences of hMPV did not show important differences.

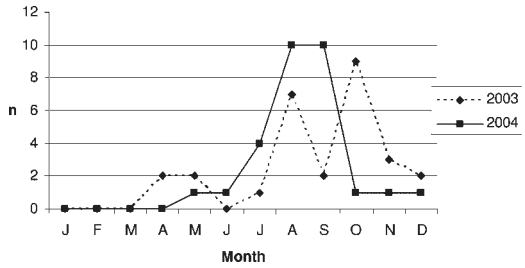

Fig. 1. Monthly hMPV detection in infants hospitalized for acute lower respiratory tract infection in a Children's Hospital. Santiago of Chile, January 2003 to December 2004. Months are sequential from left to right, starting with January (J).

They confirmed the existence of two main groups, A and B, clearly identified with a bootstrap value of 100%. Nucleotide identity between members of groups A and B was in the range 78.4–83.7%. Within group A it ranged between 93.4% and 100% and within group B between 91.0% and 100%. Samples classified in group B corresponded to the two formerly reported subgroups, B1 and B2. In group A, we observed three subclusters (Fig. 2):

	Mean	Range
Intracluster distances		
A1	0.012	0.000 – 0.027
A2	0.013	0.003 – 0.024
A3	0.013	0.003 – 0.020
Intercluster distances		
A1 vs A2	0.040	0.017 – 0.059
A1 vs A3	0.050	0.034 – 0.070
A2 vs A3	0.051	0.031 – 0.067

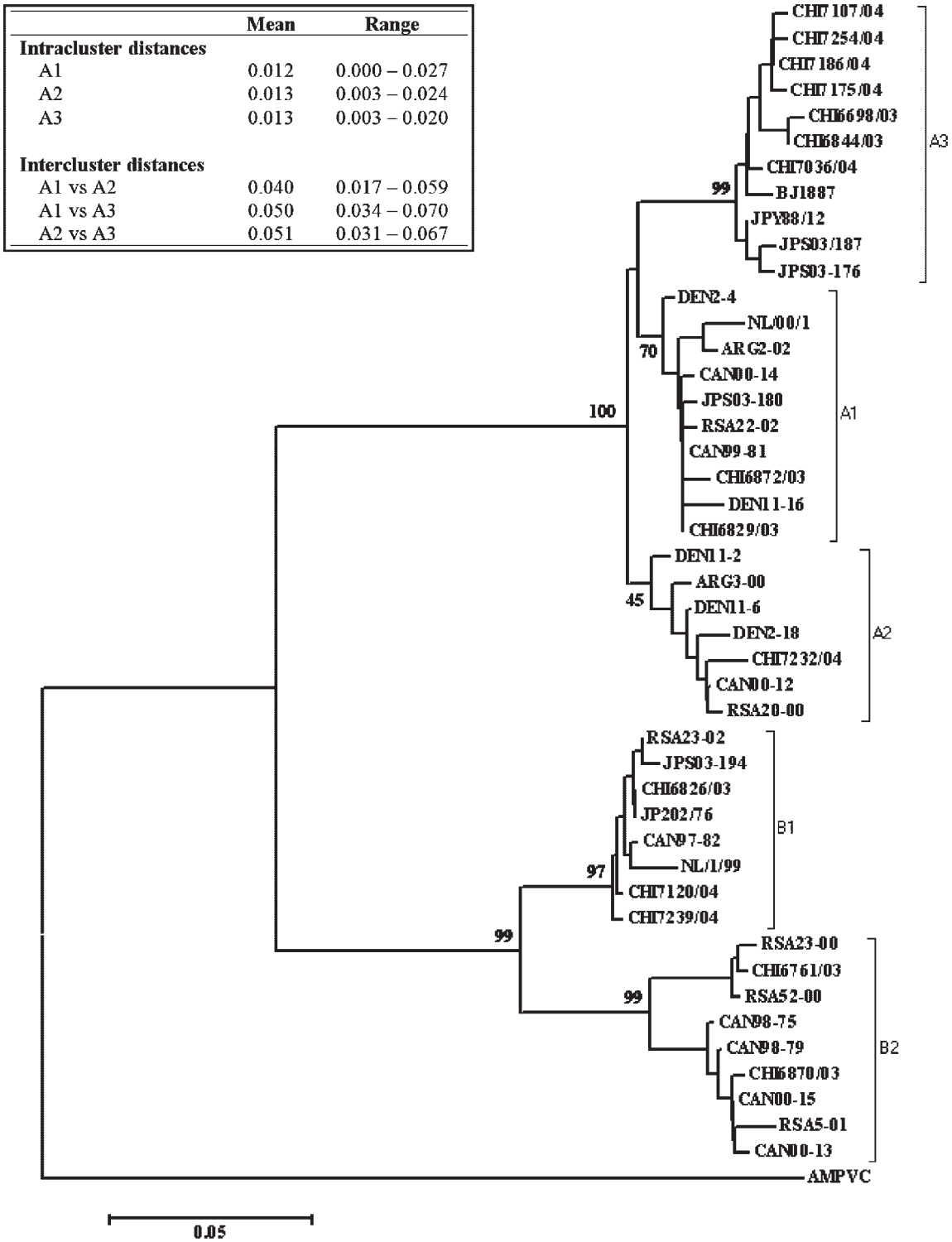

Fig. 2. Neighbor-joining tree of a fragment of F gene of Chilean strains of hMPV (named with the prefix CHI followed by the sample number and the year it was obtained). Inset: calculated *P*-distances among the A group.

the previously described A1 and A2, and a third one, tentatively named A3. The mean distances within groups were: A1 = 0.012, A2 = 0.013, and A3 = 0.013 (ranges: 0.000–0.027; 0.003–0.024, and 0.003–0.020, respectively). The mean and range of intergroup distances were: A1 versus A3: 0.050 (0.034–0.070); A2 versus A3: 0.051 (0.031–0.067), and A1 versus A2: 0.040 (0.017–0.059). According to the neighbor-joining analysis, the bootstrap values for the A1, A2, and A3 groups were 70%, 45%, and 99%. No insertions/deletions were observed in our complete alignment.

In subgroup A3 only Japanese, Chinese and Chileans strains were clustered and only one change in the deduced amino acid sequences of this new cluster was observed.

Alignment of deduced amino acid sequences showed a 90–95% identity between groups A and B and 94–100% within A, and 97–100% within B.

In summary, 13% of the samples belonged to subgroup A1 (n = 5), 2.5% were A2 (n = 1), 58.9% were A3 (n = 23); 7.6% were B1 (n = 3), and 18% were B2 (n = 7). Some subgroups were detected in samples from both years (Fig. 3).

DISCUSSION

This is the first study on the genetic diversity of hMPV strains isolated in Chile. The F gene was chosen for this analysis because it has allowed classification of hMPV isolates in subgroups [van den Hoogen et al., 2004]. Phylogenetic analyses of Chilean hMPV strains identified three clusters in group A, tentatively named A1, A2, and A3, unlike Huck et al. [2006] who proposed subclusters "a" and "b" in subgroup A2. This because having incorporated Chilean strains, the distance among any sample of the A3 cluster and any sample from either A1 or A2 clusters (range 0.031–0.070) is larger than the maximum distance between pairs in the same cluster (range 0.000–0.027). The analysis shows that the bootstrap value for the A3 cluster is 99%, which is higher than the value obtained for both A1 and A2 clustering (70% and 45%, respectively). The apparent disagreement with the Huck model may be explained because the region of the F gene used in that analysis showed lower variability. Furthermore, the attempt to include the A3 cluster into A1 or A2 subgroups resulted in an increased intragroup mean distance until 0.032, which is much higher than the discriminative distance established using the three subgroups model (see inset in Fig. 2).

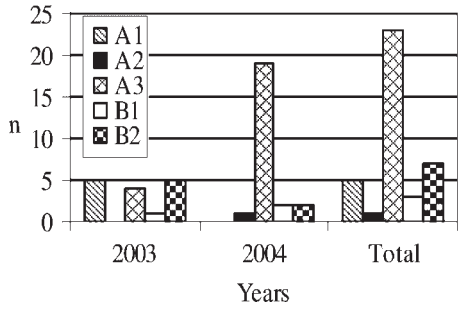

Fig. 3. Distribution of human metapneumovirus (hMPV) subtypes over two consecutive years in Santiago, Chile.

It is interesting that only Japanese and Chinese strains clustered with Chilean strains in the A3 subgroup. Since apart from this study only Argentinean hMPV strains [Galiano et al., 2006] have been studied, it is necessary to evaluate the genetic diversity of this virus in other locations of South America.

The hMPV incidence (10.2%) reported above was higher than the 5.4% previously shown [Luchsinger et al., 2005], difference explained by the better sensitivity of the real time PCR as compared with the conventional PCR used previously. This incidence confirms the assumption that hMPV is the second most frequent cause of hospitalization for viral lower respiratory infections in children and therefore it is necessary to implement its routine detection. Immunodiagnostic tests for hMPV antigens may be the rapid, sensitive and inexpensive diagnostic method required [Ingram et al., 2006], for an expanded use.

The detection of hMPV in Chilean young children hospitalized for acute lower respiratory tract infection during spring in Santiago is in agreement with reports from Argentina [Galiano et al., 2004]. Nevertheless, in other countries the seasonal distribution is different, the greatest incidence being during the cold season [Nissen et al., 2002; Freymuth et al., 2003; Osterhaus and Fouchier, 2003]. Further epidemiological studies are necessary, taking advantage of our respiratory virus surveillance system set up in 1989 [Avendaño et al., 2003], to understand the seasonal distribution in the previous years, as well as the frequency of mixed respiratory viral infections [Palomino et al., 2004]. Further addition of new sequences may allow the consolidation or even the generation of new genetic groups.

In summary, phylogenetic analyses of F gene of Chilean hMPV strains confirmed the presence of subgroups A1 and A2 in the A lineage and two subgroups, B1 and B2 in the B lineage, and showed the existence of the new subgroup A3, which co-circulated in different proportions. hMPV is emerging as the second cause of acute lower respiratory tract infection in hospitalized children in Chile.

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