Serologic evidence of human metapneumovirus circulation in Uruguay

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First identified in 2001, the human metapneumovirus (hMPV), is a respiratory tract pathogen that affects young children, elderly, and immunocompromised patients. The present work represents the first serologic study carried out in Uruguay. It was performed with the purpose of obtaining serological evidence of hMPV circulation in Uruguay and to contribute to the few serologic reports described until now. Sixty nine serum samples collected between 1998 and 2001 by vein puncture from patients without respiratory symptoms or underlying pathology aged 6 days to 60 years were examined using an indirect immunofluorescence assay (IFA). The global seropositivity rate of the samples was 80% (55/69). Rates of 60% (15/25) and 91% (40/44) were observed for the pediatric and adult cohorts, respectively. Results obtained from a longitudinal analysis of 6 children aged 6 days to 18 months are discussed. These results are a clear evidence of hMPV circulation in Uruguay, at least since 1998, and reinforce the previous data on worldwide circulation of this virus.

Key words: human metapneumovirus - serological evidence - immunofluorescence assay - Uruguay

Human metapneumovirus (hMPV) was first isolated in the Netherlands, in June 2001, from nasopharyngeal aspirates of 28 children with respiratory tract infections (van den Hoogen et al. 2001). The clinical symptoms of these children were largely similar to those caused by human respiratory syncytial virus (hRSV), ranging from upper respiratory tract disease to bronquiolitis and pneumonia (van den Hoogen et al. 2001). Several studies have shown that hMPV is also an important etiologic agent of respiratory tract infections in adults (Boivin et al. 2002, Falsey et al. 2003).

On the basis of virological features and detailed molecular studies, this newly discovered virus was classified as a member of the genus *Metapneumovirus* of the *Pneumovirinae* subfamily, within the *Paramyxoviridae* family. Several studies showed that it has a high percentage of nucleotide sequence identity with the, until now, sole member of the *Metapneumovirus* genus: the avian pneumovirus (APV) also known as turkey rhinotracheitis virus (TRTV). However, van den Hoogen et al. (2001) showed that these two viruses do not cross infect, and hMPV has a restricted host range. Until now, no serological cross reactivity between APV and hMPV was reported. Phylogenetically, the most closely related human virus is hRSV, that belongs to the genus *Pneumovirus,* which together with *Metapneumovirus* constitute the subfamily *Pneumovirinae* (van den Hoogen et al. 2001, Crowe 2004).

Since the initial report in 2001, hMPV has been studied, basically by reverse transcription and polymerase chain reaction (RT-PCR) all over the world. At present it has been reported in all the continents (Hamelin et al. 2004, Crowe 2004). On the contrary, there are very few reports about hMPV seroprevalence studies. The first one, carried out by Dutch researchers using IFA (van den Hoogen et al. 2001), revealed that between 6 and 12 months of age, 25% of the children that presented respiratory tract infections had been infected with hMPV, and that by the age of 5 virtually all the children had become seropositive. They also found a 100% seroprevalence in serum samples obtained from individuals 8 to 99 years old that were collected in 1958, suggesting that hMPV had circulated in humans for at least 50 years. Similarly in Japan, a study conducted in human serum samples from people without respiratory symptoms between ages of one month to 35 years, revealed that by the age of 10 years, all children had hMPV antibodies (Ebihara et al. 2003). In Israel, Wolf et al. (2003), reported a seroprevalence of 52% among healthy children at the age of 24 months and found a 100% seroprevalence for hMPV among twenty five 8-year-old children that had recently emigrated from Ethiopia. Both studies were performed by IFA. More recently in the United States, a seroepidemiology study revealed a rate bigger than 95% in individuals older than 11 years (Leung et al. 2005).

In the present study, we investigated the presence of hMPV antibodies in serum samples from children and adults by IFA. Our purpose was to obtain serological evidence of the hMPV presence in Uruguay and to contribute to the few serologic reports described until now. To date, no similar study has been reported in our country.

MATERIALS AND METHODS

Serum samples - Serum samples were collected from patients who visited the National Service of Blood and the Pediatric Hospital Pereira Rossell of Montevideo, Uruguay, between 1998 and 2001. A total of 69 serum samples, 44 randomly obtained from donor adults (aged 18 to 60 years) and 25 coming from 16 children (aged 6 days to 18 months) without any underlying pathology**,** were extracted by vein puncture and kept at –20ºC until their processing (Table). To facilitate discussion, serum samples were di-

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vided into four groups, according to age of sampling, as follows: group A: 0-4 months; group B: > 4-9 months; group C: > 9-18 months, and group D: samples corresponding to adults (18-60 years).

Virus propagation and antigen preparation - LLC-MK_2 cells (rhesus monkey kidney, *Macaca mulata*) were grown in 25 cm^2 culture dishes containing 5 ml of Eagle's minimum essential medium (E-MEM-Gibco), 10% fetal calf serum (ICN Biomedicals Inc.), gentamicin (50 µg/ml), and glutamine (30 mg/ml) and incubated at 37°C. After semi confluent monolayer establishment, the cells were inoculated with a hMPV natural isolate kindly provided by Dr Mónica Galiano from "Centro de Educación Médica e Investigación Clínica" (CEMIC, Buenos Aires, Argentina) and cultured in a fetal calf serum-free E-MEM supplemented with penicillin (100 µg/ml) and trypsin (TPCK-Sigma) (2 mg/ml) until a cytophatic effect (CPE), characterized by focal rounding and subsequent cell detachment from the monolayer, was reached. These hMPV-infected cells were used as antigen-positive for an IFA. Mock-infected cell culture, grown in parallel, was used as antigen-negative control. The cell smears (a suspension of 3600 cells/ml was used in each case) were fixed on glass slides with methanol-acetone (methanol at –20°C for 5 min followed by acetone at –20°C for 10 min).

TABLE

Detailed information of samples corresponding to 16 children

Patient	Sample [a]	Age [b]	IFA
I	1-4	11 d	+
	2-4	1 mo	+
	3-4	2 mo	+
	4-4	9 mo	-
II	1-3	10 d	+
	2-3	6 mo	-
	3-3	13 mo	+
III	1-2	2 mo	+
	2-2	7 mo	+
IV	1-2	6 d	+
	2-2	4 mo	+
V	1-2	10 d	-
	2-2	18 mo	-
VI	1-2	20 d	+
	2-2	13 mo	-
VII	1-1	27 d	+
VIII	1-1	2 mo	-
IX	1-1	27 d	+
X	1-1	9 mo	-
XI	1-1	14 mo	+
XII	1-1	5 mo	-
XIII	1-1	4 mo	-
XIV	1-1	6 mo	-
XV	1-1	9 mo	+
XVI	1-1	1 mo	+

a: specimen-total of specimens analyzed for each child; *b*: age of patients at the time of taking the sample (d: days, mo: months).

RT-PCR - In order to confirm hMPV antigen-positive cells, we performed a RT-PCR using primers (N1: 5'-ATGGGACAAGTGAAAATGTC-3'; N2: 5'-GAGTCTC AGTACACAATAA-3' and N3 5'-GCATTTCCGAGAA CAACAC-3') designed from a sequence of the hMPV nucleoprotein (N) gene (modified from Coté et al. 2003).

Total RNA was extracted from infected cultures by Trizol® method (Trizol® Reagent, Gibco BRL) according to the manufacturer's instructions and diluted in 40 µl of distilled water. For the cDNA synthesis, first we incubated 5 µl of the extracted RNA, 1 µl of 10 mM dNTPs, and 10 pmol of primer N-1 for 5 min at 65ºC. This was added to a mixture containing 4 µl of First Strand 5× buffer (Invitrogen), 2 µl of 0.1 M DTT (Invitrogen), 5U/ml of ribonuclease inhibitor (Promega, Madison, WI) and distilled water to 19 µl of final volume and incubated for 2 min at 37ºC. Finally we added 200 U of *Superscript II*®- Reverse transcriptase (Invitrogen) and incubated for 50 min at 42ºC, then the enzyme was inactivated at 70ºC for 5 min.

Five microliters of cDNA were amplified by PCR in a 50 µl reaction containing the following: 1 µl of 10mM dNTPs, 5 µl of 10× PCR buffer minus Mg (Invitrogen), 1.5 µl of 50 mM $MgCl_2$ (Invitrogen), 10 pmol of N2 and N3 primers, 1.25 U of Taq Polymerase (Invitrogen) and completed with DNAse and RNAse-free distilled water (Gibco). Cycling conditions were as follows: an initial denaturation step of 3 min at 94°C, followed by 50 cycles of 45 s at 94°C, 30 s at 50°C, and 30 s at 72°C and a final extension of 10 min at 72°C. The expected size of the N gene PCR product was 928 base pairs.

IFA - For the detection of hMPV-specific human inmunoglobulins A, G, and M, an IFA was conducted in the following way (modified from van den Hoogen et al. 2001). Briefly, slides were blocked with fat-less milk diluted to 5% in phosphate-buffered saline (PBS-Biocientífica S.A.) for 45 min at 37°C. After the blocking agent was removed, the cell smears were incubated for 30 min at 37°C with 30 µl of sera diluted at 1:10 with bovine serum albumin 1% in PBS and then washed three times in PBS. Once the slides were dried, they were incubated for 45 min at 37°C with 25 µl of fluorescein isothiocyanate-conjugated rabbit anti-human immunoglobulin G, A, and M (FITC-Ig G, A, M) (Biocientífica) at 1:400 in PBS. The slides were washed three times in PBS for 10 min each time and air-dried. Then, the cell smears were stained with DAPI ('-6-Diamidino-2-Phenylindole, Sigma) and mounted with glycerol and PBS (1:10), and finally examined under fluorescence microscopy.

Serum belonging to a hMPV-infected patient was used as positive control and each serum sample corresponding to adults and children was tested in duplicate. Assays in which the infected cells presented an apple-green appearance were considered as positive. Contrarily, in negative assays the cells did not presented this typical pattern or displayed no signal at all.

RESULTS

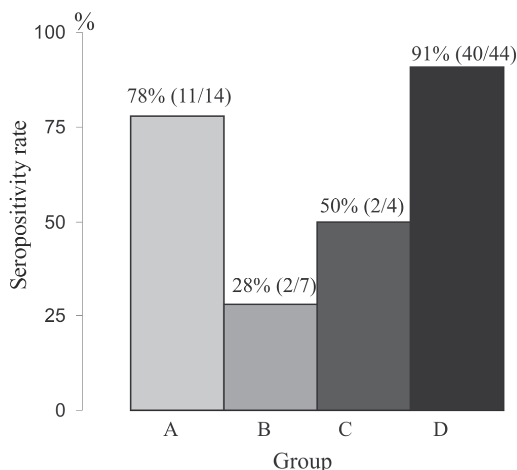
LLC-MK_2-expressed hMPV proteins recognized by human Ig G, A, M inmunoglobulins were typically present in the cytoplasm and on cell surface with an apple-green appearance and no positive signal was observed in the nucleus.

hMPV overall seropositivity rate of the studied samples from patients aged 6 days to 60 years was 80% (55/69). Within the pediatric cohort, 15 of 25 (60%) samples presented anti-hMPV antibodies, while these was found in 91% (40/44) of the adult individuals (Figure). The highest antibody prevalence (78%) of the pediatric specimens was detected in group A, and the lowest (28%) was observed in group B. In group C (> 9-18 months), the seropositive rate increased to 50%, respect to group B.

As seen in the Table, longitudinal serological analyses conducted in several children evidenced the following results. Child I (Table) presented anti-hMPV antibodies during the first two months of life, but no reactivity was detected at the 9th month. The study of specimens belonging to child II showed that he had anti-hMPV antibodies at the age of 10 days and 13 months**,** but on the contrary, at the 6th month of life he was seronegative. Child III presented specific hMPV antibodies in the first sample (at 2 months of age), remaining seropositive at 7 months. Samples corresponding to child V did not present hMPV antibodies. This was the oldest seronegative child.

Among the rest of the children, most aged 0-9 months were seronegative and three were seropositive, while child aged 14 months was seropositive.

Ninety one per cent (40/44) of the adults aged 18-60 years were seropositive.



Graphic showing specimens positive percentages ordered in four groups. A: 0-4 months; B: > 4-9 months; C: > 9-18 months; D: 18-60 years.

DISCUSSION

In this study we examined 69 serum samples from adults and children for anti-hMPV antibodies using an IFA. The highest seropositive rate (78%) observed among children's samples corresponded to group A (0 to 4 months), while the lowest rate (28%) was represented by group B specimens (> 4-9 months). In group C, a rate of 50% was detected (Figure). Since it has been shown that maternally derived anti-hMPV antibodies decline at 4 to 5 months of age (Ebihara et al. 2004) the difference in rates among group A and the other two groups may be due to their presence in samples from children aged 0-4 months. Furthermore, our results are largely similar to those described previously for other regions (Ebihara et al. 2003, 2004). The fact that only 28% of group B samples presented anti-hMPV antibodies, against 78% of the first group, clearly correlates to the process of immunologic system maturation and the production of characteristic antibodies of the child, and the decrease of maternally derived anti-hMPV antibodies.

Longitudinal analysis conducted with child I evidenced that he lost maternally derived antibodies (becoming seronegative) at a given time between 2 and 9 months, since during the first 2 months of life he had anti-hMPV antibodies. Similarly, child II became seronegative by 6 months of age being then infected with hMPV presenting his own specific antibodies by age 13 months. When we analyzed child V samples, we observed that none had anti-hMPV specific antibodies; surprisingly, this child was seronegative at 10 days of age. Taking into account that no anti-hMPV specific immune response was detected at 18 months, it can be assumed that child V had not been exposed to the virus, thus being the oldest seronegative child in this study.

The fact that children X, XII, XIII, and XIV resulted seronegative likely indicates that they lost maternally derived antibodies, becoming seronegative, and were never be in contact with the virus or their mothers, as seen in child V, were seronegative. However, the information obtained from child V strongly suggests us a seronegative condition of his mother. The situation for child VIII could be compared with child V since his seronegative condition may be probably explained by an absence of maternally derived protective antibodies and not by a lost of them. So, the mother of this child was seronegative, contrarily to that observed for children VII and XVI.

In comparison with previous data which showed a 100% seroprevalence in adults (van den Hoogen et al. 2001, Ebihara et al. 2003, Wolf et al. 2003), the seropositivity rate observed in our study (91%) is smaller. However it is quite similar to that recently reported in United States (95.5%) by Leung et al. (2005) for the adult population. Differences might probably be explained, at least partially, by the influence of many variables, among them the period of the year in which the specimens were obtained and the number of samples considered.

Our results suggest that hMPV has been circulating in Uruguay at least since 1998, confirming the previous reports that indicate a worldwide distribution of this virus. In spite of the limited number of samples analyzed in this study, there is a great similarity among our results for the pediatric cohort and those described previously (Ebihara et al. 2003). It is thus necessary to develop a more exhaustive and wider investigation to corroborate these primary results, to determine the prevalence and to learn about the epidemiology of hMPV in our country.

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REFERENCES

Boivin G, Abed Y, Pelletier G, Ruel L, Moisan D, Coté S, Peret T, Erdman D, Anderson LJ 2002. Virological features and clinical manifestations associated with human metapneumovirus: a new paramyxovirus responsible for acute respiratory-tract infections in all age groups. *J Infect Dis 186*: 1330-1334.

Coté S, Abed Y, Boivin G 2003. Comparative evaluation of real-time PCR assays for detection of the human metapneumovirus. *J Clin Microbiol 41*: 3631-3635.

Crowe MD 2004. Human metapneumovirus as a major cause of human respiratory tract disease. *Ped Infect Dis J 23*: S215-221.

Ebihara T, Endo R, Kikuta H, Ishiguro N, Yoshioka M, Ma X, Kobayashi K 2003. Seroprevalence of human metapneumovirus in Japan. *J Med Virol 70*: 281-283.

Ebihara T, Endo R, Kikuta H, Ishiguro N, Ishiko H, Kobayashi K 2004. Comparison of the seroprevalence of human metapneumovirus and human respiratory syncytial virus. *J Med Virol 72*: 304-306.

Falsey AR, Erdmann D, Anderson L, Walsh EE 2003. Human metapneumovirus infections in young and elderly adults. *J Infect Dis 187*: 692-698.

Hamelin ME, Abed Y, Boivin G 2004. Human metapneumovirus: a new player among respiratory viruses. *Emerging Infections 38*: 983-990.

Leung J, Esper F, Weibel C, Kahn J 2005. Seroepidemiology of human metapneumovirus (hMPV) on the basis of a novel enzyme-linked immunosorbent assay utilizing hMPV fusion protein expressed in recombinant vesicular stomatitis virus. *J Clin Microbiol 43*: 1213-1219.

van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, Osterhaus AD 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat Med 7*: 719-724.

Wolf D, Zakay-Ronos Z, Fadeela A, Greenberg D, Dagan R 2003. High seroprevalence of human metapneumovirus among young children in Israel. *J Infect Dis 188*: 1865-1867.