Human metapneumovirus infection in a cohort of young asymptomatic subjects

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SUMMARY

Human metapneumovirus (HMPV) is a recently described cause of respiratory infection. The purpose of this study was to to further the available information on the circulation of HMPV among young asymptomatic subjects. The epidemiology of HMPV was examined in a cohort of 73 university students during a winter season by using polymerase chain reaction. HPMV was detected in 3/73 (4.1%) asymptomatic subjects. Phylogenetic analysis has shown that circulating viruses belonged to different HMPV sublineages. Our findings indicate that asymptomatic carriage of HMPV might be a neglected source of viral transmission in the community.

KEY WORDS: Human metapneumovirus; molecular epidemiology

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Human metapneumovirus (HMPV) was identified in 2001 in the Netherlands from nasopharyngeal aspirate samples (Van den Hoogen *et al.,* 2001) of children with acute respiratory infections (ARI). HMPV has been classified into the subfamily *pneumovirinae* within the family *paramyxoviridae* (Van den Hoogen *et al.,* 2001; Galiano *et al.,* 2006) genus *metapneumovirus*, species *human metapneumovirus*. HMPV has been identified worldwide (Kahn *et al.,* 2006) and longitudinal surveys have indicated that HMPV has a seasonal distribution, mainly occurring during winter to early spring (Van den Hoogen *et al.,* 2001; Bastien *et al.,* 2003; Williams *et al.,* 2006; Caracciolo *et al.,* 2008). Incidence of HMPV infection can substantially vary from year to year, being sometimes infrequent or rivaling respiratory syncitial virus (RSV) (Maggi *et al.,* 2003; Caracciolo *et al.,* 2008).

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Based on genomic sequencing and phylogenetic analysis, there are two major genotypes of HMPV, designated A and B (Biacchesi *et al.,* 2003; Kahn, 2006) and each genotype appears to have at least two distinct subgroups (MacKay *et al.,* 2004). It has been shown that concurrent annual circulation of all four subgroups in the same geographical area is common, with a single, usually different, HMPV subgroup predominating each year (Kahn 2006; Caracciolo *et al.,* 2008). Seroprevalence studies in the Netherlands (Van den Hoogen *et al.*, 2001), Israel (Wolf *et al.,* 2003) and Japan (Ebihara *et al.,* 2003) have shown that by the age of 5 to 10 years, seropositivity reaches virtually 100%. Moreover, reinfections have been observed in all age groups.

Since its initial description, HMPV has been considered a leading cause of serious lower respiratory diseases in infants and young children (Van den Hoogen *et al.,* 2001; Hamelin *et al.,* 2004). Belonging to the same subfamily, HMPV is compared frequently with RSV because they share many biologic properties and cause a similar, but not identical, disease (Regev *et al.*, 2006; Wolf *et al.,* 2006; Caracciolo *et al.,* 2008).

The importance of HMPV infection in high risk adults, especially among the elderly and im-

munocompromised hosts, has also been increasingly recognized (Falsey *et al.,* 2006) even if the real pathogenic role of HMPV in some of these categories of patients remains uncertain (Debiaggi *et al.,* 2007). In symptomatic adults the clinical characteristics of HMPV do not appear to be distinctive from those of illness due to other winter respiratory viruses even if HMPV infection may be somewhat less severe than RSV infection and influenza. Colds, asthma exacerbations, and influenza-like illness have been documented in older children and young adults (Falsey *et al.,* 2003; Hamelin *et al.,* 2004). On the other hand, HMPV infections in younger healthy adults generally have not been appreciated being often an asymptomatic infection. Cases of reinfection in adult subjects suggest that, despite universal infection in childhood, new infections can occur throughout life due to incompletely protective immune responses and/or acquisition of new genotype.

At present, consistent data on the presence of HMPV in asymptomatic persons in the community are still not available.

The purpose of this study was to determine the prevalence of HMPV in specimens obtained from young students at Calabria University that were randomly sampled. Students who were in good general health were eligible for the study. Exclusion criteria included current lower respiratory symptoms, cardiovascular or other serious disease (i.e., immunodeficiencies, cystic fibrosis) or a history of chronic respiratory diseases.

A nasal swab specimen was collected from each subject between January 15th and January 26th 2007 by gently rubbing the nasal turbinates with a moistened cotton tip swab and placed in 2 ml of transport medium (ViralPack, Biomedics s.r.l., Madrid, Spain). Specimens collected were maintained at 4°C and transferred within 4 hours after sampling to the Microbiology Laboratory at the University of Calabria where they were immediately frozen at -70°C. Specimens were screened for the presence of HMPV, RSV, influenza A and B viruses, adenoviruses, and parainfluenza viruses (serotypes 1-3) by PCR assays as previously described (Caracciolo *et al.*, 2008). Briefly, nucleic acids were extracted from a 200 µl aliquot of samples with the RNeasy Kit (Qiagen, Milan, Italy), according to the manufacturer's instructions and cDNAs were synthesized from 20 µl of eluted RNA using the cDNA Archive mini kit (Applied Biosystems, Monza, Italy). Primers used for the amplification of the HMPV fusion (F) gene were as follows: F-f (5′-GTY AGC TTC AGT CAA TTC AAC AGA AG-3′); F-r (5′-CCT GTG CTG ACT TTG CAT GGG-3′) (Huck *et al.,* 2006). To confirm HMPV infection samples were also amplified with HMPV N gene primers (N-f: 5′-CCY TCA GCA CCA GAC ACA CC-3′; N-r: 5′-AGA TTC AGG RCC CAT TTC TC-3′). Finally, the amplified products were analyzed by electrophoresis on agarose gel after ethidium bromide staining, and the size of the amplicons were compared with standard molecular weight markers. To validate the amplification process and to exclude carryover contamination, positive and negative controls were run in each PCR. The PCR products of the F gene of HMPV (nt 3,624-4,130) were purified with Wizard SV Gel and PCR Clean-Up System (Promega, Milan, Italy) and sequences were performed at the CRIBI BMR Genomics Sequence Facility (Padua, Italy).

Sequences were aligned with prototype HMPV strains that are representative of all the known HMPV different subgroups [NL/1/00 (GenBank accession number AF371337) for A1,1 JPS 03-240 (GenBank accession number AY530095) for A2b, CAN97/83 (GenBank accession number AY145296) for A2a, NL/1/99 (GenBank accession number AY304361) for B1 and CAN98/79 (GenBank accession number AY145293) for B2] by using the CLUSTAL W algorithm of the BioEdit software (http://www.mbio.ncsu.edu/bioEdit/bioedit.htlm). Phylogenetic trees were constructed by FastME algorythm of the DAMBE program (http://dambe.bio.uottawa.ca/dambe.asp). The F gene of the avian pneumovirus subgroup C (GenBank accession number AF187152) was chosen as outgroup to root this phylogenetic tree. The significance of the grouping was evaluated by bootstrap parsimony analysis.

Nasal swabs were obtained from asymtomatic young volunteers referring to the Calabria University Health Centre for routine medical examinations. All the volunteers gave their informed consent to the proposed study. There were 53 females and 20 males with a median age of 24.3 years (range 19 to 29 years). At least one identifiable respiratory virus was detected in 10 (13.7%) cases, whereas none was detected in 63 (86.3%). HMPV was detected in specimens obtained from 3 (30%) of the total virus-infected

young adults, all females. RSV was detected in 5 (50%) subjects, 4 (80%) males and 1 (20%) female. The occurrence of HMPV and RSV was 4.1% and 6.8% respectively, when virus detection was reported to the total number of cases (n =73) examined. Distribution and characteristics of the cohort taken into consideration are summarized in Table 1. None of the HMPV-infected subjects was referred for respiratory symptoms, nor had a background respiratory disease.

Moreover, within the HMPV-infected population, this virus was the solely pathogen found among the microorganisms routinely tested. On the other hand, a significant percentage of RSV-infected subjects (60%) were symptomatic. In this study we never observed the concomitant presence of multiple viruses in the same subject. Co-infections are instead frequent events in infected children with severe respiratory diseases (Caracciolo *et al.,* 2008). If the lack of co-infection was due to the development of more severe symptoms, falling into one of the exclusion criteria or not, is a question that remains to be addressed. It is worth noting that during the season of observation (winter 2006/2007) influenza virus circulation was rare (Caracciolo *et al.,* 2008). In this study we did not observe the presence of influenza virus either in symptomatic or asymptomatic subjects. The lack of circulation of important pathogens has been hypothesized as an event favoring the circulation of other respiratory pathogens. The relatively high rate of circulation of HMPV and RSV observed in this study seems to strengthen this hypothesis.

The circulation incidence of HMPV observed in this study is in agreement with other studies where 4.1% of asymptomatic young persons were found infected with HMPV infection (Falsey *et al.,* 2003) whereas RSV asymptomatic infection was a relatively uncommon finding (Hall *et al.,* 2001).

Asymptomatic respiratory infections both community- and nosocomially-acquired are recognized as an important source of virus spreading to risk population. In fact, even asymptomatic infections may be accompanied by the release of appreciable quantity of virus, as already demonstrated for RSV (Hall *et al.,* 1979). In this context, it is relevant that HMPV infection is more frequent in young healthy persons that in elderly people (Falsey *et al.,* 2003). This is likely explained by their extensive contact with children (Hashem *et al.,* 2003) but they may, in turn, become a source of infection. Moreover, it is worth stressing that infections in adult individuals, even if subclinical, may also represent an unrecognized risk, since the virus population is expanding in the face of a previous immune response. This situation might increase the probability of antigenic variants being selected from the expanded viral population.

As shown in Figure 1A, sequencing of a portion of the conserved F gene showed that two of the three HMPV isolates were genetically related (Cs01/06-7 and Cs01/06-73) whereas the third (Cs01/06-14) showed consistent mutations. This result suggests that more than one HMPV strain may co-circulate in a restricted and crowded environment such as the university community and that HMPV circulation within this population may be not exclusively due to a spread within this environment.

TABLE 1 - *Characteristics of the cohort examined.*

	Total	*HMPV*	*RSV*	*Other viruses*
Subjects	73 (100)	3 (4.1)	5 (6.8)	2 (2.7)
Females (%)[a]	53 (72.6)	3 (100)	4 (80)	1 (50)
Males (%)[a]	20 (27.4)	0 (0)	1 (20)	1 (50)
Age (Range)	23.3 (19-29)	23.3 (21-27)	22.2 (19-25)	25.5 (23-28)
Upper respiratory symptoms (%)	13 (17.8)	0 (0)	3 (60)	1 (50)

[a]Calculated within the virus-positive population.

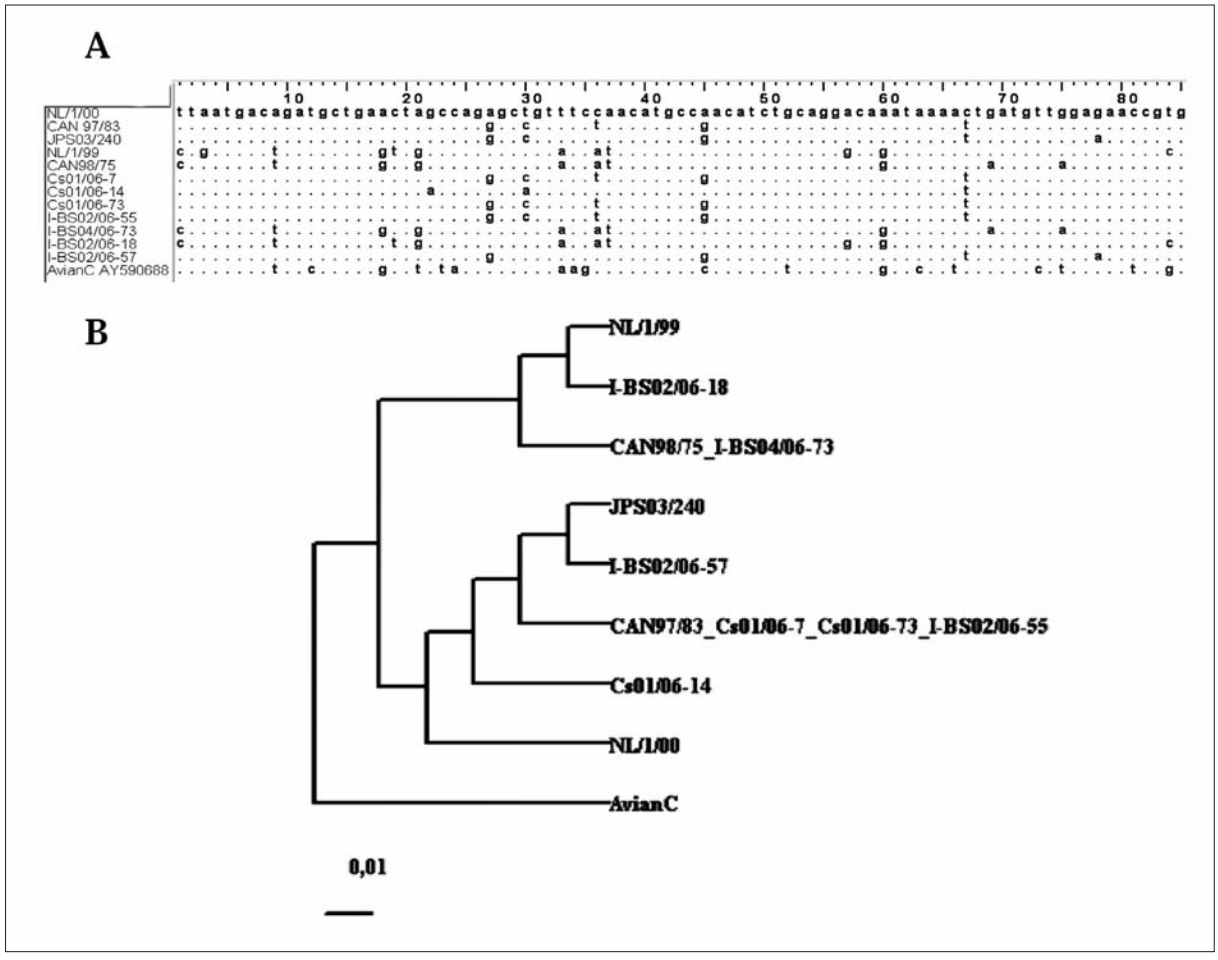

FIGURE 1 - *(A) Nucleotide sequence alignment of the HMPV isolates from a region of gene F of HMPV. Viruses identified among the asymptomatic university population were compared with the reference viruses NL-001 (A1 strain), CAN97/83 (A2a strain), JPS03-240 (A2b strain), NL/01/99 (B1 strain) and CAN99/75 (B2 strain) representing the prototype HMPV strains and with primary HMPV isolates representative of each strain derived from a symptomatic pediatric population (Caracciolo et al., 2008). Viruses detected in the present study have the prefix Cs. Dots indicate identical residues. Alignment was performed with the Clustal W program. (B) Phylogenetic analysis of HMPV strains circulating during the winter season 2006-2007 was performed using the same F gene fragment. The divergence bar is shown at the bottom of the figure.*

This was confirmed by phylogenetic analysis. In fact, Cs01/06-7 and Cs01/06-73 were found to belong to the genotype A2a while Cs01/06-14 was related to the HMPV subgroup A2b (Figure 1B). No HMPV subgroup A1, B1 and B2 were identified during this study period. This result is in agreement with other epidemiological studies showing that a concurrent annual circulation of different HMPV subtypes is common, with a single HMPV subtype predominating in a given year (Kahn *et al.,* 2006). We recently demonstrated that during the winter 2006 circulation of HMPV was indeed frequent in Italy with the subtype A2a predominating over the others (Caracciolo *et al.,* 2008).

In summary, our data demonstrated that asymptomatic young adults can harbor HMPV and might represent a neglected source of transmission of HMPV to risk populations.

Additional studies will be needed to fully determine the rate of HMPV circulation in different symptomatic and asymptomatic populations as well as their potential role in the spread of infection.

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