Jpn. J. Infect. Dis., 61, 154-156, 2008

Short Communication

Comparison of Human Metapneumovirus Genotypes from the Province of Bolzano in Northern Italy with Strains from Surrounding Regions in Italy and Austria

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(Received August 20, 2007. Accepted January 30, 2008)

SUMMARY: The epidemiology of the genetic sublineages of human metapneumovirus (hMPV) and their clinical relevance are not fully understood. We compared hMPV genotypes isolated in the province of Bolzano in Northern Italy with strains from nearby Italian and Austrian regions by sequencing of NP- and L-gene fragments. Our results suggest that similar strains cycle through adjacent geographic areas, with the respective subtypes replacing each other on a seasonal basis.

Human metapneumovirus (hMPV) is a major respiratory pathogen found in humans worldwide. In our previous study, hMPV was surprisingly the most frequently detected viral pathogen associated with acute respiratory tract infection in hospitalized children in Western Austria (1), outnumbering even the human respiratory syncytial virus (RSV). Similar findings have been reported from other developed countries (2). There are four major subtypes of hMPV (A1, A2, B1, B2), and based on neutralization assays, the two hMPV genotypes have been also suggested to represent serotypes (3), although animal data suggest a high antigenetic relationship (4). The clinical relevance in terms of different physicochemical properties or infectivity is unclear, as is the question of whether certain genetic sublineages are associated with more severe forms of the disease (5,6). The epidemiology and regional or seasonal distribution of strains are still poorly understood. It appears that the patterns of detection can change within a short time (2,6), and periodicity of recurrence of smaller hMPV "epidemics" in different communities may exist.

During the winter season 2005/06, 164 respiratory samples were collected by bronchoalveolar lavage or nasopharyngeal aspiration in the regional hospital of Bolzano, Northern Italy. Three quarters of the samples (120/164) originated from adult hematological patients, with the rest from pediatric patients. Samples were tested by direct immunofluorescence (DFA) using monoclonal antibodies (mAb) from Chemicon International (Harrow, UK) including pools for RSV, adenovirus, parainfluenzavirus (PIV) types 1, 2, 3, and influenza A and B. hMPV was stained by a mAb cocktail as described earlier (7). Where possible, virus isolation by "shell vial" cultures was also applied, incubating the samples for 48 h and 7 days, respectively, with a mixture of human lung adenocarcinoma A549 cells and mink lung epithelial Mv1Lu cells (both from Vircell S.L., Adaltis, Italy); cells were stained with mAb. Approximately 20% of the samples were infected with RSV, 5.5% with hMPV, 1% with adenovirus, 1% with PIV2, and 2% with PIV3. No double infections were observed. Most hMPV-positive specimens were diagnosed in December and January, whereas RSV peaked in February and March (Figure 1).

Samples positive for hMPV by DFA were also confirmed by reverse transcriptase-PCR. Viral RNA was extracted from 200-μl respiratory samples using the viral RNA mini kit (Qiagen, Hilden, Germany) and reverse-transcribed using AMV-reverse transcriptase (Promega, Madison, Wis., USA). The hMPV nucleoprotein (NP) gene sequences were amplified using two published primer sets (8,9). For hMPV L-gene sequences, the same primers were chosen as in a recent study of isolates from nearby Italian regions (10). Amplification was performed using RedTaq^R Ready-Mix (Sigma-Aldrich, Taufkirchen, Germany) and a 60 - 50°C "touch-down" protocol in the RapidCycler thin capillary PCR system (Idaho Tech-

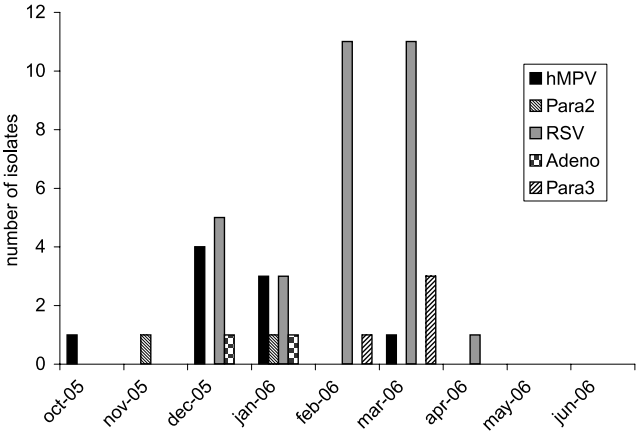

Fig. 1. Local distribution patterns of studied respiratory pathogens by month of isolation. Human metapneumovirus (hMPV), respiratory syncytial virus (RSV), adenovirus and parainfluenzavirus types 2 and 3 were detected.

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nology, Salt Lake City, Utah, USA).

Seven of the newly isolated hMPV viruses obtained in the 2005/06 season in Bolzano as well as several hMPV strains that had been isolated earlier in nearby Western Austria (1,11) were forwarded to automated DNA sequencing (ABI-3130 Genetic Analyzer), and compared to available sequences of isolates from nearby Italian regions. PCR fragments of both the NP-gene (516 bp) and the L-gene (170 bp) were sequenced and aligned with reference strains representative of the four accepted major subtypes (3). Phylogenetic trees were generated by the neighbour-joining method using ProSeq v2.91, and the Jukes and Cantor differences were expressed on the diagrams as percentages of nucleotide difference at the main internal branches.

The hMPV NP-gene sequences were more suitable for assigning the strains to the four major subgroups than the L-gene. As shown in Figure 2A, L-gene types of most of our newly isolated hMPV strains (IT/BZ05, 06) were quite different from the strains sequenced in 2002 by a group in Pisa, Italy (10). Except for isolate IT/BZ06-1, our local isolates were rather homogenous with regard to the hMPV L-gene. Although the short sequences obtained did not allow for further discrimination into major subgroups, the two A-type strains (as assigned by NP sequencing) from our region differed surprisingly with respect to the L-gene, with the "A2a" subtype isolate IT/BZ06-4 being more distant from the sequences obtained by the group in Pisa than the "A2b" isolate IT/BZ06-1.

Alignments of 450 bp of the amplified 516-bp NP-gene fragments were more discriminative, and the sequenced strains from Bolzano in 2005/06 could be assigned to three of the four accepted major subgroups A2, B1, and B2 (Figure 2B). Interestingly, our strains from winter 2005 were all of the B genotype, whereas in spring 2006 both B strains and

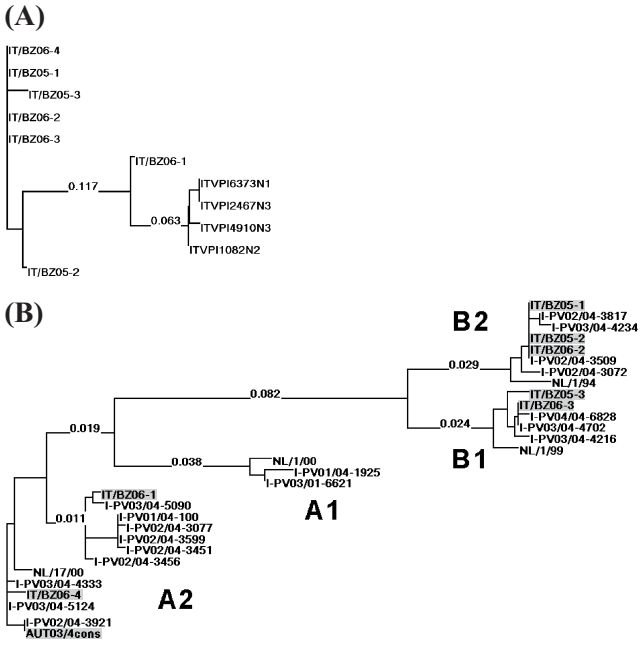

Fig. 2 (A) Comparison of amplified L-gene sequences of the hMPV strains from Bolzano 2005/06 with strains from Pisa, Italy. (B) Comparison of 450-bp NP-gene sequences of the hMPV isolates from Bolzano 2005/06 (shaded) with Dutch reference strains and strains from Pavia, Italy. As a comparison the consensus sequence of the Austrian A2 strains from the season 2003/04 (AUT03/4cons) is also shown. Percentage of nucleotide difference is depicted at the main internal branches.

the A2 strain were detected, with the latter containing two distinct subpopulations which have recently been proposed as new subtypes A2a and A2b (12). Moreover, the local A2 isolates (IT/BZ06-1 and 4) also seemed to differ from the A2 strains characterized in the Italian Pavia region in previous seasons, as no absolute sequence identity was detected. This is in contrast to the B-genotype strains from Bolzano, where NP-sequences identical with the Pavia strains were found in most cases (IT/BZ05-1,2,3 and IT/BZ06-3). The majority of strains tested in Bolzano in the 2005/06 season belonged to the B-genotype and were highly homologous to the B-strains from surrounding Italian regions of the previous seasons. This suggests that a minority viral subpopulation, such as the B-type viruses found in 2003/04 in Pavia (9), became the dominant subgroup in the province of Bolzano within 2 years. Thus, the A-type strains, which clearly dominated in the winter of 2003/04 in the Italian Pavia region (9) and also in Western Austria, seem to have been widely replaced by B genotype strains by the winter of 2005/06, as we only detected A2 strains in this study (AUT03/4cons).

The two hMPV isolates originating from bone marrow transplant patients (IT/BZ06-2 and IT/BZ06-4) were of different genotypes and appear to have originated from within the community, as similar sequences were detected in samples from children, and identical B1 sequences were found in one isolate. This finding seems to exclude nosocomial spread, which has recently been suspected in lung transplant patients (11). In these patients, an association with graft rejection and a higher overall mortality rate was observed in the hMPV-positive group, along with prolonged viral shedding. This can be most likely attributed to the compromised lung function (11).

As the NP-gene consensus sequence of the A2 strains dominating Western Austria in 2003/04 (AUT03/4cons) was also found in Pavia, Italy in the same season and we also obtained a very similar strain from Freiburg, Germany (11), it is likely that closely related hMPV subtypes may occur in wider geographical areas of Central Europe.

A periodical change of viral subtypes seems reasonable in light of the rather rapidly occurring "herd immunity" against hMPV, with about 50% seropositivity in 2-year-old and nearly 100% in older children (13,14). If one assumes that, similar to other respiratory viruses, the immune response against hMPV may be type- rather than group-specific, one would expect that such a relative "immunity" against selected subtypes of the population would limit the spread of the prevalent hMPV strains within time and enforce a change of genotype. This hypothesis has not been proven, however, and there is no data about the duration of "immunity" or partial immunity to reinfection with the same isolate or cross protection against closely related hMPV strains.

Nevertheless, in other parts of the world different isolates were dominant during the studied time periods, as was shown for Australia, where mainly B1 isolates were detected (2). It would therefore be interesting to determine whether there is also a global exchange of hMPV isolates with the introduction of new isolates into different areas. Whether there are quantitative differences in the extent of community "outbreaks" of hMPV associated with certain genotypes showing enhanced virulence or infectivity remains to be clarified. Our empirical observations argue for "stronger" and "weaker" hMPV seasons following each other.

In summary, the present data suggest the existence of a cyclic pattern in the occurrence of hMPV, with varying

subtypes and similar strains arising in different seasons and/or geographical locations. However, a complex interplay of changing viral variants with the immune system and additional influences of demographic and/or climatic factors will make it extremely difficult to predict seasonal communicable disease morbidity.

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