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Diagnosis and epidemiological studies of human metapneumovirus using real-time PCR

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Abstract

Background: Human metapneumovirus (hMPV) is prevalent in children, the elderly and immunocompromised individuals, but available epidemiological data is limited.
Objectives: (1) To develop and validate a real-time PCR method for hMPV diagnosis. (2) To determine the percentage of hMPV in respiratory specimens from the community and its association with outbreaks in our geographic area. (3) To provide epidemiological data in terms of age distribution, seasonality and co-infections.
Study design: A real-time PCR assay was designed for detection of hMPV lineages A and B. Prospective testing for hMPV over a 22-month period was then undertaken.
Results: The real-time PCR was sensitive and specific for detection of both lineages of hMPV. hMPV was detected in 9.5% ($n = 8239$) of the specimens and 25% of the outbreaks ($n = 100$) tested. The hMPV-positive patients ranged in age from 18 days to 99 years with a median age of 24 months. The number of positive samples peaked during the winter months of December, January and February. A high rate of co-infections was noted in the samples tested.
Conclusions: hMPV is common in the community and is associated with outbreaks. Including hMPV in routine testing improves etiological diagnosis of acute respiratory infections.

Keywords: Human metapneumovirus; Epidemiology; Co-infections; Age distribution; Real-time PCR; Hydrolysis probes

1. Introduction

Human metapneumovirus (hMPV) is common, has a world-wide distribution including Canada (Bastien et al., 2003; Hamelin et al., 2005; Robinson et al., 2005) and plays a significant role in respiratory infection and disease. Detection and analysis of hMPV by culture or antigen testing has proved difficult and may lack sensitivity compared with nucleic acid amplification tests (NATs) (Boivin et al., 2002; Gerna et al., 2006; van den Hoogen et al., 2001).

There are two major lineages of hMPV and each of the lineages is represented by two sub-lineages (Boivin et al., 2004; van den Hoogen et al., 2004a,b). Limited sequence diversity has been observed in the fusion (F) gene making it a good target for detection by NATs (Agapov et al., 2006; Galiano et al., 2006; Kuypers et al., 2005; van den Hoogen et al., 2004a,b; Winther et al., 2005). We have developed a real-time PCR amplification and detection assay for hMPV with primers and hydrolysis probes targeting the F gene. Here we report the development, validation and performance of the hMPV assay as well as our observations of hMPV seasonality,

Abbreviations: hMPV, human metapneumovirus; NATs, nucleic acid amplification tests; NP, nasopharyngeal; BAL, bronchoalveolar lavage; F, fusion gene; DFA, direct fluorescent antigen

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Table 1
Samples types analyzed for this study

Specimen type[a]	Number tested (% of all samples)	Number positive (% of all samples)
Upper respiratory	5921 (71.8)	697 (8.5)
Lower respiratory	2228 (27.0)	81 (1.0)
Tissue/fluid	90 (1.1)	0 (0.0)
Total numbers	8239 (100.0)	778 (9.4)

[a] Upper respiratory specimens include nasopharyngeal samples (including swabs and fluids), throat swabs and other nasal specimens. Lower respiratory specimens include bronchial and tracheal specimens, tissue and fluid include biopsy samples, pleural, chest, and pericardial fluid. Percentages for samples tested and positives were calculated based on the total ($n = 8239$).

affected populations, co-infection rate and association with outbreaks.

2. Methods

2.1. Clinical specimens

Specimens submitted to the Provincial Laboratory for Public Health (ProvLab) for respiratory virus testing from 1 January 2005 to 31 March 2006 were included in the main analysis for this study. The numbers and types of specimens tested are listed in Table 1. NP samples were screened initially by direct fluorescent antigen (DFA) for influenza A and B, parainfluenza 1–3 and respiratory syncytial virus (RSV). Prior to 1 November 2005 only lower respiratory tract specimens were tested for hMPV. After 1 November 2005, DFA-negative NP samples and all other specimen types were tested for hMPV.

2.2. Sample preparation

Respiratory samples were pre-treated with 25 μl of 0.01 mAU/μl protease (Qiagen, Mississauga, Ontario) in a thermomixer (Eppendorf, Westbury, New York) at 56 °C and 1000 rpm for 10 min or until the specimen cleared. If the sample was viscous (likely indicating high cell/protein content) 50 μl of protease was used. Viral RNA was extracted from the treated samples using the nucliSENS® extractor (bioMérieux, Durham, NC, USA) and subsequently the easyMAG® automated extractor (bioMérieux) according to the manufacturer's instructions. The extracted nucleic acid was eluted in 110 μl from a sample input volume of 200 μl. Tissue samples were extracted using the total nucleic acid extraction protocol for tissue specimens using the QIAamp® kit (Qiagen) according to manufacturer's instructions.

2.3. Design of primers and probes

Primers and probes were designed for this study using Primer Express 2.0 [Applied Biosystems (ABI), Foster City, CA, USA] and are listed in Table 2. All available F gene sequences from GenBank (as of July 2004) were aligned to design primers to amplify a 114–117 bp region of the F gene of hMPV lineages A and B viruses. The forward and reverse primers for lineage A were hMPV-For1-taqman and hMPV-Rev1-taqman, respectively. Two forward primers (hMPV-For2-taqman and hMPV-For2a-taqman) and one reverse primer (hMPV-Rev2-taqman) were designed for the amplification of lineage B strains. A universal minor groove binding probe hMPV-Uni-Fam was designed to simultaneously detect both lineages of hMPV. Two lineage-specific probes hMPV1-Probe and hMPV2-Probe labeled with FAM and VIC, respectively were also designed and used for detection and differentiation between lineages A and B viruses in a real-time multiplex format. The primer and probe sets were compared to all four predicted prototype sequences A1, A2, B1, and B2 (van den Hoogen et al., 2004a,b) (GenBank accession nos.: AF371337, AY304360, AY304361, and AY304362) to ensure that all lineages would be amplified and detected using this assay. The primers were synthesized at the University core DNA services (University of Calgary, Alberta, Canada) and the probes were synthesized by ABI.

2.4. Real-time RT-PCR assay

A two-step RT-PCR method was used for the amplification and detection of hMPV. The reverse transcription step was performed using Superscript II™ and RNaseOUT™ (InVitrogen, CA, USA). The master mix was composed of DTT at

Table 2
Primers and probes used for detection of hMPV lineage A and hMPV lineage B viruses

Target	Primer/probe name	Nucleotide location related to GenBank[a]	Primer/probe sequence (5′–3′)
hMPV lineage A	hMPV-For1-taqman	601–623	CTAAATGTTGTGCGGCAATTTTC
hMPV lineage A	hMPV-Rev1-taqman	601–623	ACATGCCAACATCTGCAGGAC
hMPV lineage B	hMPV-For2-taqman	602–626	CTAAATGTTGTGCGGCAGTTTTC
hMPV lineage B	hMPV-For2a-taqman	640–653	TAAATGTTGTGAGGCAGTTTTCAGA
hMPV lineage B	hMPV-Rev2-taqman	698–718	ACATGCCGACATCTGCAGG
hMPV lineage A	hMPV1-Probe	662–679	TAATGACAGATGCTGAAC (FAM)
hMPV lineage B	hMPV2-Probe	662–679	TAATGACTGATGCTGAGC (VIC)
hMPV lineages A and B	hMPV-Uni-Fam	640–653	ACACCAGCAATATC-FAM
hMPV lineages A and B	hMPVClonFor	286–302	GAAAATCCCAGACAATC
hMPV lineages A and B	hMPVClonRev	794–811	CCATGTAAATTACGGAGC

[a] Nucleotide positions for lineage A and B viruses are according to GenBank AF371337 and GenBank AY304361.1 respectively.

a final concentration of 5 μM, total dNTPs at 375 nM, 600 ng of random hexamer, 20 U of RNaseOUT™ and 100 U of Superscript™ II in a final volume of 20 μl. Five microlitres of the extracted RNA was combined with 15 μl of the master mix and the RT step was performed at 42 °C for 60 min followed by an enzyme inactivation step at 70 °C for 15 min using a GeneAmp PCR System 2700 (ABI). After cDNA synthesis, PCR was performed in an ABI PRISM 7000 or 7500 sequence detection system in optical tubes from ABI using 5 μl of the cDNA, 0.9 μM each of sense and antisense primers and 0.2 μM each of the lineages A- and B-specific probes or 0.2 μM of the universal probe. PCR was performed using the TaqMan® universal PCR master mix (ABI), according to manufacturer's instructions and the TaqMan universal amplification protocol.

2.5. Preparation of RNA transcripts for sensitivity studies

Primers hMPVClonFor and hMPVClonRev (Table 2) were designed to amplify a 526 bp region of the F gene including the region used for diagnostic amplification and detection of hMPV lineages A and B. Separate clones were generated from lineages A and B control viruses. Control viruses used as templates for cloning were provided by Dr. Yan Li (National Microbiology Laboratory, Winnipeg, Mannitoba, Canada). The PCR products were cleaned using the QIAquick® PCR purification kit (Qiagen), cloned into pCR®2.1-TOPO vector using TOPO TA cloning Kit (InVitrogen) and transformed into One Shot TOP-10 F' competent *Escherichia coli* cells (InVitrogen) by electroporation. The presence of a cloned insert was detected by PCR using the M13 forward (−20) primer and M13 reverse primer provided in the TA cloning kit. The presence and orientation of the insert was confirmed by sequencing using the ABI PRISM® BigDye® terminator v3.1 cycle sequencing kit in the ABI PRISM® 3100 Avant genetic analyzer with data collection software v2.0. The sequences were analyzed using the sequence analysis software BioEdit v7.1.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The plasmid DNA was linearized using restriction enzymes KpnI or XhoI. The DNA was then transcribed using the T7 RiboMAX™ express (Promega, Madison, WI, USA) to synthesize hMPV lineage A and B RNA *in vitro*. The transcribed RNA was spectrophotometrically quantified by measuring the absorbance at 260 nm. This absorbance value was used to calculate the copies/ml of transcribed RNA present.

2.6. Sensitivity, specificity, and reproducibility of RT-PCR

A series of eight 10-fold dilutions was set up starting with 1.5×10^6 copies-reaction of pre-quantified *in vitro* transcribed RNA for hMPV lineages A and B. Testing was carried out on three different days on eight replicates to assess end point sensitivity.

The specificity of the assay was determined by testing high copy number samples in duplicate of common respiratory pathogens including influenza virus A and B, parainfluenza virus 1, 2, 3, 4A and 4B, RSV A and B, human coronavirus 229E and OC43, rhinovirus type 1B, adenovirus types 2 and 4, *Chlamydophila pneumoniae, Legionella pneumophila*, and *Mycoplasma pneumoniae*.

The reproducibility of the hMPV RT-PCR was evaluated in two consecutive runs of two positive clinical specimens tested in triplicate on each run.

2.7. Sequence analysis of hMPV-positive samples

The primers hMPVClonFor and hMPVClonRev were used to amplify 526 bases of the F gene from randomly selected positive samples. The PCR products were sequenced as described in Section 2.5 and analyzed using BioEdit v7.1.1. The phylogenetic tree was constructed using the MegAlign module from Lasergene v6 (DNAstar, Madison, WI, USA). The multiple sequence alignment for the tree was generated using ClustalW with the default alignment parameters. The sequences for hMPV lineages A1 (GenBank AF371337), A2 (GenBank AY304360.1), B1 (GenBank AY304361.1), and B2 (GenBank AY304362.1) (van den Hoogen et al., 2004a,b) were used as references for comparison.

2.8. Statistical analysis

The SPSS software v14.0 was used for statistical analysis of data. The association of age with the detection of hMPV was analyzed using Pearson Chi-squared (χ^2) analysis. Probit analysis was undertaken to determine assay limit of detection (95% confidence interval).

3. Results

3.1. Assessment of RT-PCR assay performance

All studies for assessing the assay limit of detection were performed using *in vitro* transcribed RNA prepared from cloned target template of lineage A and B viruses. Results of probit analysis for limit of detection [(95% CI) are given in Fig. 1]. Limit of detection was 17.38 copies for hMPV lineage A *in vitro* transcribed RNA and 13.34 copies for hMPV lineage B *in vitro* transcribed RNA when tested with the universal probe. Limit of detection was 56.23 copies for hMPV lineage A RNA and 75.86 copies for the lineage B RNA when the lineage-specific probes were used. The hMPV assays did not amplify other viral and bacterial respiratory pathogens, showing 100% specificity.

The two clinical specimens tested in triplicate and repeated on different runs gave a mean crossing threshold (C_t) value of 32.2 ± 0.3 and 31.0 ± 0.6, respectively, for a total coefficient of variation (CV) of 0.9% and 1.9% suggesting good overall precision for the assay. The intra-assay variability for the two

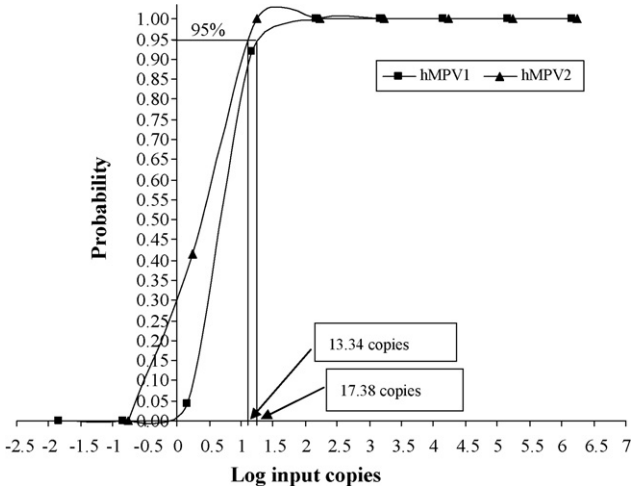

Fig. 1. Limit of detection analysis (probit) hMPV lineages A and B.

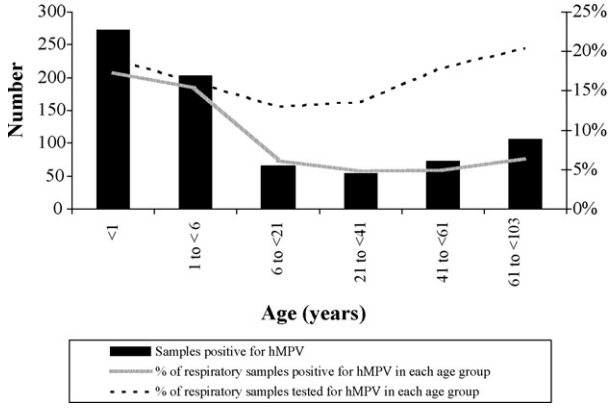

Fig. 2. Age range of patients positive for hMPV. Samples for this analysis were collected from January 2005 to March 2006 (inclusive) with $n = 8213$.

specimens was 0.2% and 0.7%. The inter-assay variability was 0.9% and 2.0%.

3.2. Prospective analysis of hMPV results for respiratory specimens

During the study period for this analysis (1 January 2005 to 31 March 2006), 8239 respiratory specimens from individual patients and respiratory outbreaks were tested using the universal probe designed to detect lineages A and B of hMPV as described in Section 2. Analysis of the data shows that 778 specimens were positive for hMPV giving an overall positive rate of 9.4% during this period. The C_t values for positive samples ranged from 18.3 to 44.9 representing a wide distribution in viral load, the mean C_t value was 30.2 with a standard deviation of ±2.74. Specimen types for the 778 positive samples were diverse and included 697 upper respiratory specimens (89.6% of all positive specimens) and 81 lower respiratory specimens (10.4% of all positive specimens) as shown in Table 1.

Age-specific data was available for 8213 samples. The positive patients ranged in age from 18 days to 99 years with the majority of positive specimens from patients less than 6 years as shown in Fig. 2. A total of 1566 (19%) and 856 (10%) of the samples tested were from patients <12 months and 12–24 months of age, respectively. The positive rate for hMPV was significantly higher in the younger age groups as compared to those >24 months of age (χ^2, $p < 0.001$).

3.3. Co-infections with hMPV

Co-infection with other respiratory viruses was relatively common; 15.1% ($n = 118$) of the 778 hMPV-positive samples analyzed had another detectable virus and 1.2% ($n = 9$) were positive for two additional viral targets by nucleic acid testing. Of the 118 specimens where a co-infection was identified, parainfluenza virus and adenovirus were the most common viruses identified at 36.2% and 35.4%, respectively, followed by influenza B (10.2%), RSV (7.9%) and influenza A (3.1%). Nine samples were positive for three viral targets including hMPV: three with adenovirus and influenza B, three with adenovirus and parainfluenza, one sample each with influenza B and parainfluenza, parainfluenza and RSV, and adenovirus and RSV. There was a tendency towards more co-infections in younger individuals (<2 years of age) but this did not reach statistical significance (χ^2, $p = 0.056$). Dual infections were detected in 127 specimens, of these 113 were upper and 14 were lower respiratory specimens.

3.4. hMPV in respiratory outbreaks

An outbreak is defined when there are epidemiologically linked cases of respiratory illness identified by the Medical Officer of Health and Communicable Disease Units within the respective health region. During the study, a total of 397 specimens from 100 outbreaks at long-term assisted care centers (mean patient age = 82 years) and schools (mean patient age = 13 years) were tested for hMPV, 46 (11.6%) of these samples were positive and 25 (25.0%) of the outbreaks were associated with hMPV infection. In nine outbreaks, hMPV was the only etiological agent found, influenza B and hMPV were identified in four outbreaks, influenza A and hMPV were identified in four outbreaks, parainfluenza and hMPV were found in three outbreaks and adenovirus was isolated as an additional etiologic agent in one outbreak. Four outbreaks were associated with more than two respiratory viruses including hMPV, presumably representing the diverse co-circulating viruses in the community. Eight of the hMPV-positive outbreaks were in schools and 17 were in long term and assisted care centers although numbers in these categories were not high enough to assess statistical significance.

3.5. Assessment of hMPV seasonality

To study the seasonal distribution of hMPV in our geographic area, the percentage of hMPV infections in res-

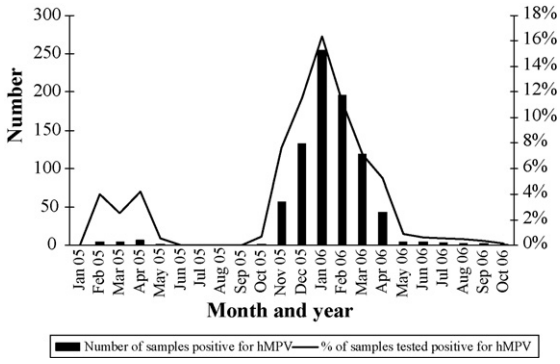

Fig. 3. Seasonality of hMPV. Distribution of positives over the course of the year, percent of respiratory samples tested and the percent positives for hMPV from January 2005 to October 2006 (inclusive) with $n = 12{,}445$. Prior to 1 November 2005 only lower respiratory tract specimens were tested for hMPV. After 1 November 2005 DFA-negative NP samples and all other specimen types were tested for hMPV.

piratory specimens collected during the months of 1 January 2005 to 31 October 2006 are included in Fig. 3. These results illustrate an increase in hMPV activity during the winter months of December, January, and February.

3.6. Sequence analysis of hMPV-positive samples

Amplification and sequencing of a 526 base pair fragment of the F gene from 17 randomly selected hMPV positives showed that five of the viruses belonged to the B2 lineage and 12 were of the A2 lineage. All five F gene sequences from the B2 lineage were identical to each other (submitted sequence given GenBank accession no. EF121384) and 10 bases different from the reference sequence (AY304362) resulting in 98.1% sequence identity. Four of the hMPV lineage B2 viruses were from samples obtained during December 2005 from the same geographic location and one of the viruses was isolated from a patient in a different city in March 2006. The sequences belonging to the A2 lineage were more divergent than the B2 lineage with 8–23 base pairs variation (98.3–95.4% percent identity) compared with the reference sequence (AY304360). GenBank accession numbers for these sequences are listed here: EF121377, EF121378, EF121379, EF121380, EF121381, EF121382, EF121383, and EF121384. The phylogenetic tree illustrating the relationship between 17 hMPV partial F gene sequences is shown in Fig. 4.

4. Discussion

Human metapneumovirus is an emerging pathogen which has been associated with symptoms ranging from mild upper respiratory tract infections to severe pneumonia, exacerbation of asthma and chronic obstructive pulmonary disease (Alto, 2004; Esper et al., 2003; Fouchier et al., 2005; Kahn, 2003; van den Hoogen et al., 2004a,b; Williams et al., 2004). Serological studies show that the virus has been circulating undetected in humans for at least 50 years (van den Hoogen et al., 2001). It is thus an important pathogen and it is essential to obtain a better understanding of the associated disease and risk factors. There are two major lineages of hMPV and each of the lineages is represented by two sub lineages (Boivin et al., 2004; van den Hoogen et al., 2004a,b). The two genetic lineages are antigenically highly related but not serotypically distinct (MacPhail et al., 2004; Skiadopoulos et al., 2004; van den Hoogen et al., 2004a,b) hindering serological detection and differentiation based on antibody assays. The lineages can, however, be differentiated using molecular methods. We have developed and applied a real-time PCR test for the detection of hMPV in respiratory samples. We have used this assay to determine the percentage and seasonal distribution of hMPV infections in respiratory specimens from our community and have assessed its association with community respiratory outbreaks.

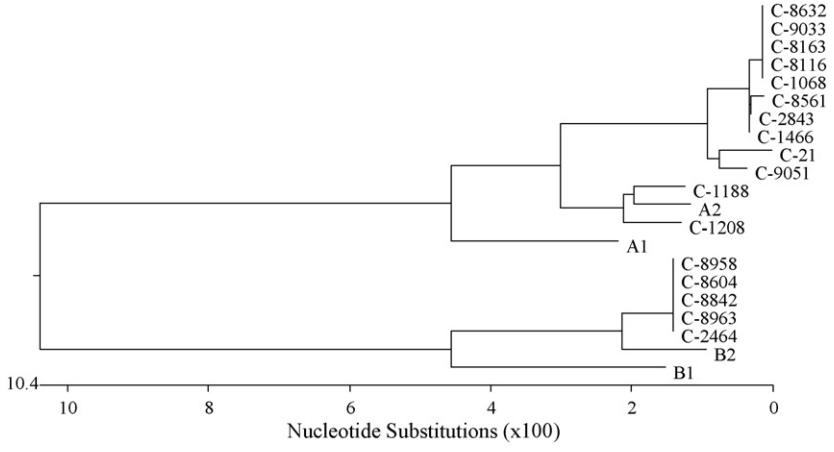

Fig. 4. Phylogenetic tree showing the relationship between hMPV viruses based on partial F gene sequence. Reference sequences belonging to lineages A1, A2, B1, and B2 (van den Hoogen et al., 2004a,b) are given. The length of each pair of branches represents the distance between sequence pairs. The scale below the tree indicates the number of nucleotide substitutions and the units show the number of substitution events. See Section 3.5 for GenBank accession numbers of sequences generated in this study and reference sequences.

Nucleic acid amplification-based protocols for the detection of hMPV have been published based on the F, nucleoprotein (N), phosphoprotein (P) and polymerase (L) genes (Agapov et al., 2006; Boivin et al., 2003; Chan et al., 2003; Cote et al., 2003; Ebihara et al., 2004; Esper et al., 2003; Falsey et al., 2003; Gerna et al., 2006; Kuypers et al., 2005; Mackay et al., 2003; Maertzdorf et al., 2004; Stockton et al., 2002; van den Hoogen et al., 2003). Studies have shown that the sequence diversity in the F gene from patient samples is limited (Agapov et al., 2006; Galiano et al., 2006; Kuypers et al., 2005; van den Hoogen et al., 2004a,b; Winther et al., 2005). Our assay is highly sensitive for detection of hMPV and the sensitivity is comparable when the assay is performed using the lineage A, lineage B or universal probes. Although we have not compared our newly developed assay with those previously reported, the limit-of-detection studies suggest that the assay is comparable in sensitivity to other reported nucleic acid detection methods for hMPV.

The primers and probes designed in this study allow for differential as well as simultaneous detection of both the major lineages A and B. Lineage-specific detection can facilitate molecular epidemiological studies for hMPV. Design characteristics for the assay would allow detection of all four lineages. Preliminary sequencing analysis of positive samples reveals that both lineages A and B are represented in our population. Further sequencing of isolates will determine the occurrence of the different lineages in our geographic area.

Incidence of hMPV reported in different parts of the world using both conventional and real-time RT-PCR methods varies between 4% and 18% (Boivin et al., 2003; Ebihara et al., 2004; Kuypers et al., 2005; Mullins et al., 2004; Peiris et al., 2003; Viazov et al., 2003). The results of our study are consistent with reported literature; we observed the frequency of hMPV infections in respiratory specimens to be 9.4% overall in patients ranging in age from 18 days to 99 years, but was as high as 16.3% of all tested specimens in January 2006. It should be noted that our positive rate could be underestimated as DFA-positive samples would not be tested for hMPV by NAT according to our current diagnostic testing algorithm. The median age for hMPV infections has been reported to vary between 6 and 12 months (Kuypers et al., 2005; Mullins et al., 2004; Williams et al., 2004) however two of these studies investigated a pediatric population and one included adults only up to the age of 20 years. Our data shows a median age of 24 months and indicates a higher incidence of hMPV infection in the young (less than 24 months of age).

Our data shows a higher activity of hMPV in the winter months of December, January and February 2005–2006; fewer samples were tested in early 2005 and thus the peak in hMPV activity was not evident in this season. Previous reports have shown a peak in hMPV infections during the months of February to April in various Canadian provinces (Bastien et al., 2003; Boivin et al., 2004) and between the months of January to May in the United States (Agapov et al., 2006; Boivin et al., 2003; Esper et al., 2003; Kuypers et al., 2005; Mullins et al., 2004; Williams et al., 2004). Human metapneumovirus activity has been shown to peak during the months of June to January in Argentina (Galiano et al., 2004), April in Hong Kong (Peiris et al., 2003), February in Italy (Sarasini et al., 2006) and April in Japan (Kaida et al., 2006). The seasonality of hMPV may thus vary geographically and continued surveillance over a number of years will help in understanding these patterns. It has been suggested that hMPV circulates predominantly in late winter and spring in the temperate climates; however, it has been detected throughout the year at lower levels (Kahn, 2003).

Co-infecting respiratory viruses were found in a high proportion of community acquired cases of hMPV. The most common co-infecting viruses were parainfluenza virus, adenoviruses, influenza B virus, RSV and influenza A in the order of prevalence with a tendency towards higher co-infection rate in patients less than 2 years of age. Other reports have suggested that RSV and influenza A are the most prevalent co-infecting agents (Boivin et al., 2003; Kuypers et al., 2005; Viazov et al., 2003), but based on our data, co-infections seem to depend on other common viruses circulating in the community. A higher proportion of co-infections was noted for upper respiratory specimens. One of the reasons for this could be that nasopharyngeal samples are the most common type collected from children. The patients for whom lower respiratory samples were submitted are unselected and may include individuals with disease processes other than viral infection thus diluting the rate of identifiable pathogens in these specimen types. In addition, collection of BAL specimens includes a saline instillation and suction process that could have diluted the viral load in a sample. Increased use of sensitive NATs for respiratory viruses that are poorly detected by traditional methods facilitates identification of multiple viruses from a single sample.

Real-time PCR assays are useful for resolving respiratory infections of previously unknown etiology, both presenting as an acute infection and in the context of an outbreak. Performing ongoing surveillance for hMPV and analysis of data over multiple respiratory seasons will prove valuable in our understanding of hMPV epidemiology.

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