ORIGINAL ARTICLE

Detection of human coronavirus NL63, human metapneumovirus and respiratory syncytial virus in children with respiratory tract infections in south-west Sweden

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ABSTRACT

Two recently detected viruses, human metapneumovirus (hMPV) and coronavirus NL63 (HCoV-NL63), have been associated with acute respiratory tract infections, particularly in young children. This study investigated the frequency of hMPV and HCoV-NL63 infections in Swedish children by screening 221 nasopharyngeal aspirates, collected between November 2003 and May 2005, from 212 children attending the paediatric department of a county hospital in Sweden or submitted from local general practitioners. The samples were originally submitted to be tested for respiratory syncytial virus (RSV), and were examined retrospectively for hMPV and HCoV-NL63 by RT-PCR. Of the 212 patients, 101 were positive for RSV (48%), 22 (10%) were positive for hMPV, and 12 (6%) were positive for HCoV-NL63. The frequency of HCoV-NL63 infection increased from 1% in 2003–2004 to 10% in 2004–2005. Sequence analysis of parts of the coronavirus genomes showed considerable similarity to the HCoV-NL63 prototype sequence. The study demonstrated that HCoV-NL63 and hMPV occur in south-west Sweden with essentially the same frequency, seasonal distribution and clinical characteristics as have been reported in other countries.

Keywords Bronchiolitis, children, human coronavirus NL63, human metapneumovirus, respiratory syncytial virus

INTRODUCTION

Respiratory syncytial virus (RSV) is the most common viral cause of lower respiratory tract infection in young children, followed by parainfluenza virus, influenza virus and other viruses, but no aetiological cause is identified for many cases of infection [1–5]. The number of unidentified causes has decreased following the discovery of human metapneumovirus (hMPV) in 2001 [6], and may be reduced still further following the discovery of human coronavirus NL63 (HCoV-NL63) in 2004 [7,8].

Infections with hMPV have been reported in many countries, suggesting a worldwide distribution [9–17]. hMPV seems to cause a range of symptoms and respiratory tract diseases similar to those caused by RSV, mainly in infants and elderly individuals. The epidemiology of hMPV infections appears to be seasonal, with a peak in late winter and early spring. In the case of HCoV-NL63, there have been a number of worldwide reports that describe an association with both upper and lower respiratory tract infections, and specifically with clinical croup [18–26].

The present study presents data obtained with 221 nasopharyngeal aspirates collected between November 2003 and May 2005 from 212 children with symptoms of upper and lower respiratory tract infections in Halmstad, Sweden. The samples were analysed for the presence of RSV, hMPV and HCoV-NL63. To simplify the diagnostic procedures on bronchiolitis samples, the RSV, hMPV and HCoV-NL63 real-time RT-PCRs were performed simultaneously on the same platform.

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MATERIALS AND METHODS

Clinical specimens and patient data

Between 1 November 2003 and 31 May 2005, nasopharyngeal aspirates submitted for RSV testing were examined consecutively. RNA was separated from these samples (see below) and stored immediately at −70°C. More than 95% of the specimens originated from the outpatient clinic and inpatient wards of the Paediatric Department of Halmstad Hospital, and the remainder (<5%) from general practitioners in the county of Halland, Sweden. All specimens were obtained from patients aged <10 years with symptoms of respiratory tract infection. Clinical and demographical patient data were obtained from the medical records of the patients and recorded on a standard form. Fig. 1 shows the age distribution of the patients; >90% of the samples were taken from patients aged <1 year.

RNA extraction

RNA was extracted from *c.* 140 μL of each nasopharyngeal aspirate with a QiaAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. RNA extraction and storage at −70°C were completed on the day that the sample arrived at the laboratory. The dates of sample collection and arrival at the laboratory were identical for 80% of the samples, while the remaining 20% had been stored at 4°C overnight and were delivered the following day.

Conventional RT-PCR and sequencing for HCoV-NL63

To detect HCoV-NL63 RNA, two separate nested PCRs were performed, following one combined RT step. The PCRs amplified sequences in the replicase and N genes [7]. Reverse transcription was performed using 20 μL of each of the RNA preparations. The 40-μL RT-PCR mixes contained MMLV-reverse transcriptase (Invitrogen, Carlsbad, CA, USA) 5 U/μL, 500 μM dNTPs, 10 mM Tris-HCl, pH 8.3, Triton-X 0.1% v/v, 5.5 mM $MgCl_2$, 50 mM KCl and the two RT primers repSZ-RT and N3-RT (Table S1; see Supplementary material) at 250 pg/μL. The reaction mix was incubated at 37°C for 90 min.

In the first-round PCR, 20 μL of the cDNA was added to final reaction volumes of 50 μL containing Ampli*Taq*Gold 0.025 U/μL, 50 μM dNTPs, 12 mM Tris-HCl, pH 8.3, bovine serum albumin 60 ng/μL, 1 mM $MgCl_2$, 30 mM KCl and each

primer (Table S1) at 1 ng/μL. Nested PCRs were performed with 2.5 μL of the product from the first-round PCRs. Final concentrations in the 50-μL reaction volumes were Ampli*Taq*-Gold 0.02 U/μL, 100 μM dNTPs, 20 mM Tris-HCL, pH 8.3, bovine serum albumin 100 ng/μL, 2 mM $MgCl_2$, 50 mM KCl and each primer at 1 ng/μL (Table S1).

Thermocycling conditions for the replicase gene PCR and the N gene PCR were identical. The first- and the second-round PCRs differed only in the number of cycles, and comprised 95°C for 5 min, followed by 35 cycles (first-round PCR) or 25 cycles (second-round PCR) of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, and finally 72°C for 10 min. PCR products were separated by electrophoresis on agarose gels and visualised by staining with ethidium bromide. Positive and negative controls were used in each RT-PCR and in each PCR. The positive control consisted of RNA isolated from passage of HCoV-NL63 in LLC-MK2 cells [7], using an amount of RNA equivalent to 10^5–10^6 RNA copies for the RT-PCR and subsequent PCRs; the negative control was sterile water.

Real-time RT-PCR assay for HCoV-NL63

A 98-bp segment of the N gene was amplified using a one-step real-time RT-PCR and primers, as described by Fouchier *et al.* [8]. Briefly, the final volume of 25 μL contained 3 μL of RNA preparation, 800 nM each primer, 300 nM probe, 1× *Taq*Man Universal PCR Master Mix and 1× MultiScribe and RNAse Inhibitor (*Taq*Man One-Step RT-PCR Master Mix Reagents; Roche, Branchburg, NJ, USA). Cycling parameters comprised 30 min at 48°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C and 1 min at 60°C. Reverse transcription of RNA, amplification and detection of cDNA were performed using an ABI Prism 7000 *Taq*Man instrument (Applied Biosystems, Foster City, CA, USA). The positive control was the same material used for the conventional PCR, but at a concentration of 30–300 RNA copies/PCR. Part of the 5'-region of the spike insert gene was sequenced if a PCR product for this gene was generated with the primers listed in Table S1; if not, the PCR product from the real-time RT-PCR assay for HCoV-NL63 was used as the template for sequencing. The PCR products were sequenced directly using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) with an ABI 3100 Genetic Analyser (Applied Biosystems) at the BioMedical Laboratory, Furulund, Sweden. Both sense and anti-sense strands of the PCR products were sequenced.

Real-time RT-PCR assay for hMPV

A segment of the F gene was amplified with a one-step real-time RT-PCR based on the method described by Maertzdorf *et al.* [27], which detects all known hMPV genotypes. In brief, the final 25-μL volume contained 3 μL of RNA preparation, 300 nM both primers (Table S1), 200 nM probe, 1× *Taq*Man Universal PCR Master Mix and 1× MultiScribe and RNAse Inhibitor. As this assay was performed in combination with the HCoV-NL63 and the RSV assays, the cycling parameters and the instrument were the same as those described for HCoV-NL63.

The positive control consisted of RNA transcribed *in vitro* with nucleotide sequences derived from an hMPV isolate of lineage A (kindly provided by L. P. Nielsen, University Hospital, Odense, Denmark). The PCR product generated by the two diagnostic primers was cloned into pGEM vector (Promega, Madison, WI, USA) under the control of a T7
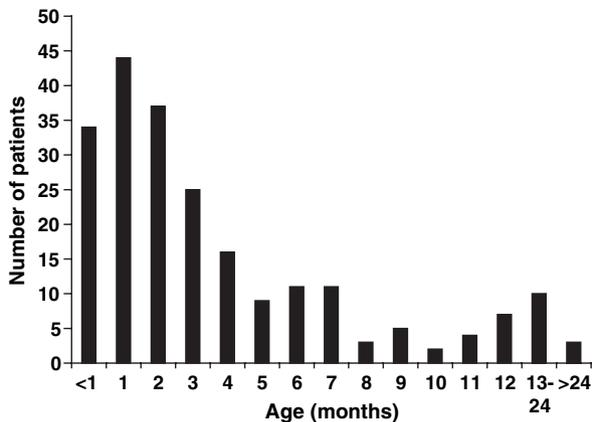

Fig. 1. Age distribution of patients included in the study.

promoter. Run-off transcripts were generated using the Ribo-probe in-vitro transcription system (Promega) and were quantified by measuring the absorption at A_{260} in a spectrophotometer. The RNA transcripts were then diluted for use as a positive control, with 90 copies/RT-PCR. This corresponds to a concentration ten-fold higher than the detection limit. This concentration was also used as a control for inhibitors in specimens that tested negative in all PCRs.

Real-time RT-PCR assay for RSV

A multiplex assay for RSV A and RSV B used primers and probes from the respective N genes, both of which are highly conserved. The separate RT-PCR and PCR steps, as described by van Elden *et al.* [28], were combined in a one-step reaction. In brief, the final 25-μL volumes contained 3 μL of RNA preparation, 900 nM each primer (Table S1), 200 nM each probe, 1× *Taq*Man Universal PCR Master Mix and 1× Multi-Scribe and RNAse Inhibitor. As this assay was performed in combination with the HCoV-NL63 and the hMPV assays, the cycling parameters and the instrument used were identical to those described for HCoV-NL63.

Run-off transcripts for RSV A and RSV B were used as positive controls. The nucleotide sequences originated from routine samples and had identical sequences for all primer and probe binding sites, as determined by sequencing the PCR products that were used for cloning. The cloning and in-vitro transcription procedures were the same as described for hMPV. The RNA transcripts were then diluted for use at a concentration ten-fold higher than the detection limit.

RESULTS

Evidence of RSV, hMPV and HCoV-NL63 in nasopharyngeal aspirates

RSV was detected most frequently, with 101 (48%) of 212 patients yielding positive results. hMPV was detected in 22 (10%) patients, three of whom showed positive results for both RSV and hMPV. HCoV-NL63 RNA was detected in 12 (6%) patients, including one co-infected with hMPV. Inhibition was observed for one of the 221 samples. The RNA preparation from this sample was diluted ten-fold and re-tested in both PCRs. Following dilution, no inhibition was observed, but the sample continued to yield negative results.

Evidence of HCoV-NL63 infection

Of the 12 samples from 12 individuals positive for HCoV-NL63 RNA, one sample was taken in the winter season 2003–2004, while the other 11 were collected between December 2004 and February 2005 (Fig. 2). Eight samples were also positive according to the two conventional HCoV-NL63 PCRs. The remaining four samples generated positive results only with the real-time PCR.

The two full genome sequences of HCoV-NL63 in GenBank showed substantial differences in the 5′ region of the spike gene. It was possible to generate and sequence a PCR product from this gene for ten of the 12 positive samples (GenBank accession numbers: AY902242 and DQ231158–DQ231166). These spike gene sequences showed 0–7 substitutions compared with the NC005831 prototype sequence, which represents the sequence of the first HCoV-NL63 strain to be detected, and 62–65 substitutions compared with the prototype of the other genetic lineage AY518894 (Fig. 3). PCR products from the remaining two samples that tested positive only in the real-time assay were also sequenced and were shown to be HCoV-NL63 sequences.

False-positive reactions in the HCoV-NL63 replicase gene PCR

All samples were run on gels after the second round of PCRs. Two of the 221 samples, from two different individuals, generated amplicons in the replicase gene PCR that had the same size as the positive control amplicon. No amplicons were generated by the N gene PCR from these two samples, but gel electrophoresis of the first-round replicase gene PCR showed products of the same size as the positive control after the second-round PCR (169 bp). These products were still detectable after the second-round replicase gene PCR. Sequencing of the two products revealed that they comprised part of the human X-chromosome. Alignment of the two first-round primers to this sequence revealed two probable target sites with good 3′-attachment (10-bp identity for the repSZ1 primer and 9-bp identity for the repSZ3 primer). The predicted size of the resulting amplification product was 174 bp, which is close to the size of the second-round product of the HCoV-NL63 assay (169 bp).

Comparison between the conventional and real-time RT-PCRs

The two conventional RT-PCRs were unable to detect <100–1000 RNA copies of HCoV-NL63/PCR, while the real-time assay yielded positive results from 0.3 — 3 RNA copies/PCR. This difference in sensitivity is consistent with the finding that four samples that were positive in the real-time assay, and had sequences matching with
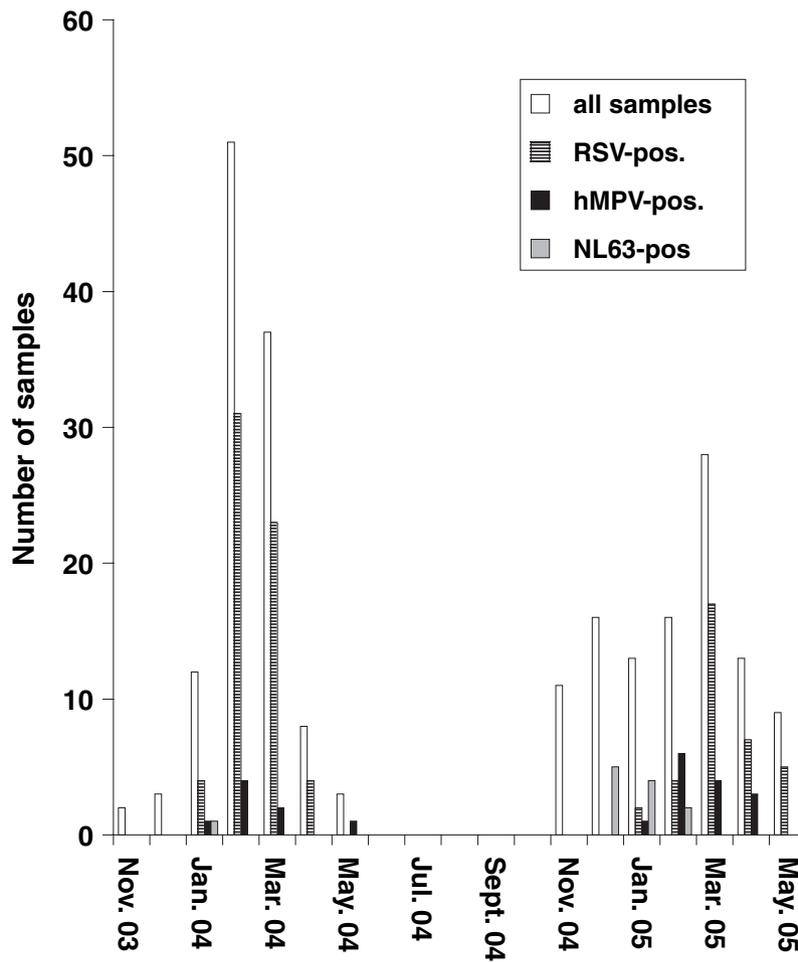
Fig. 2. Monthly distribution of all samples (white bars), RSV-positive samples (striped bars), hMPV-positive samples (black bars) and HCoV-NL63-positive samples (grey bars).

HCoV-NL63, did not yield positive results in the two conventional PCRs. The two false-positive results in the conventional replicase gene PCR also resulted in a decreased specificity compared with the two other methods.

Clinical data

Of the 22 positive samples from 22 individuals detected by the real-time RT-PCR assay for hMPV, eight were collected between January and May 2004, and the remaining 14 between January and April 2005, with February being the peak month during both periods (Fig. 2). Three of the 22 hMPV-positive samples also contained RSV RNA, and one sample also contained HCoV-NL63 RNA.

The medical files of 203 patients were available (100 from 2003–2004 and 103 from 2004–2005). The patients were divided into four groups: 85 who were only RSV-positive (RSV group); 18 who were only hMPV-positive (hMPV group); 11 who were only HCoV-NL63-positive (HCoV-NL63 group); and 85 who were negative for all three viruses (negative group). The clinical data for the patients in these groups are summarised in Table 1. Three patients who were positive for both RSV and hMPV, together with one patient who was positive for both hMPV and HCoV-NL63, were not included in this analysis. The clinical features of the 12 patients who were positive for HCoV-NL63 are shown in Table 2.

DISCUSSION

This study investigated the frequency of HCoV-NL63 and hMPV infections among a group of children with symptoms of respiratory tract infection in Sweden during two consecutive winter seasons. HCoV-NL63 was detected at an overall frequency of 6% (1% during 2003–2004 and 10% during 2004–2005). This observation of annually changing frequencies is

Fig.3. Phylogenetic analysis of the HCoV-NL63 isolates described in this study. Phylogenetic analysis was performed using the neighbour-joining method of the MEGA program. The nucleotide distance matrix was generated by Kimura's two-parameter estimation [39]. Bootstrap re-sampling (1000 replicates) was used to provide approximate CIs on individual branches. Bootstrap values >70 are indicated. Sequences from several HCoV-NL63 strains that have been submitted previously to GenBank from previous studies (AY46451–AY46458, AY675541–AY675553 and AY66005–AY66010) have been included in the analysis.
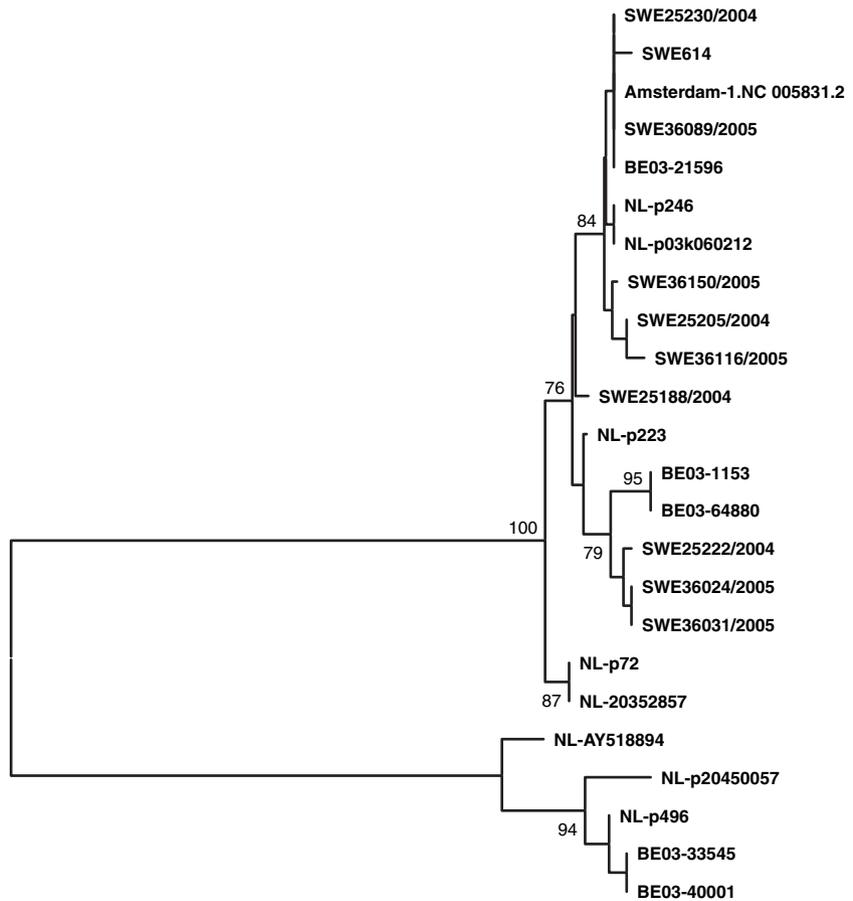
Table 1. Clinical data for patients included in the study[a]

	RSV group (*n* = 85)	hMPV group (*n* = 18)	HCoV-NL63 group (*n* = 11)	Negative group (*n* = 85)
Mean age (median) in months	6 (3)	7 (5)	2 (2)	4 (3)
Gender				
Female	40	6	3	35
Male	45	12	8	50
Fever (at least once >38°C)	51%	39%	18%	32%
Chest X-rays taken (irrespective of results)	26%	22%	9%	24%
Antibiotic treatment given	17%	22%	9%	18%
Duration of hospital stay in days	3.7	1.6	1.5	1.9

[a]Patients who were positive for more than one virus are not included in this analysis.

consistent with the findings from the studies of Moes *et al.* [22] and Arden *et al.* [19]. In agreement with other reports, there was a seasonal distribution of HCoV-NL63 infections [18,22,25,26,29], with most HCoV-NL63 infections occurring before RSV and hMPV infections during 2004–2005 (Fig. 2). However, it was not possible to compare the seasonal differences with other studies because of the limited data

available and the different climates at the various study sites.

All of the HCoV-NL63 isolates belonged to the same genetic lineage. This may reflect the homogeneity of the circulating viruses within one season. Other studies have shown co-circulation of genetically more diverse variants of the virus within a single season [19,20,22,25,29]. As little is known concerning the genetic heterogeneity of the virus, it is possible that the RT-PCR protocols used were not optimal for all HCoV-NL63 variants, and that both the frequency and the variability may be greater than observed. However, since three PCR protocols that targeted two presumably well-conserved genes were used, false-negative samples should be rare.

The mean age (2 months) of patients in the HCoV-NL63 group was low compared with most previous studies, but the overall age of the patient groups was low (Fig. 1), and might reflect the method of selecting patients at the paediatric department. Children aged <1 year with symptoms of respiratory tract infection are usually

Table 2. Clinical features of the 12 patients who were positive for human coronavirus NL63

Sample number	Date of collection	Gender	Age	Underlying conditions	Symptoms	Physical examination findings	Other information	Discharge diagnosis
10614	040127	M	3 weeks	None	Rhinitis, dyspnoea	No focus of infection		Upper respiratory tract infection
25188	041222	M	3 weeks	None	Rhinitis, dyspnoea, vomiting	Crepitations, rhonchi	Adrenaline inhalations	Bronchiolitis
25190	041222	M	1 month	None	Rhinitis, dyspnoea	Tachypnoea, crepitations, pharyngitis	Adrenaline inhalations	Bronchiolitis
25205	041226	M	2 months	None	Dyspnoea, fever, apathy	Crepitations, rhonchi, rash	Adrenaline inhalations	Viral infection
25222	041228	F	3 months	None	Rhinitis, fever	Fever		Upper respiratory tract infection
25230	041230	M	3 months	None	Rhinitis, cough, secretion from right eye	Bell's palsy, right side	Onset of palsy probably weeks before rhinitis	Upper respiratory tract infection, Bell's palsy
36024	050105	F	4 months	None	Dyspnoea, vomiting	Crepitations		Upper respiratory tract infection
36031	050110	M	2 months	Pre-term week 28, RDS	Apathy, cough, dyspnoea	Pale, dehydrated	CXR: bilateral apical infiltrates, CPAP ventilation	Upper respiratory tract infection
36089	050120	F	4 months	None	Cough, dyspnoea	Tachypnoea, crepitations		Upper respiratory tract infection
36116	050131	M	1 month	None	Vomiting, cough	No focus of infection	Hospitalisation for pylorostenosis	Pylorostenosis, upper respiratory tract infection
36150	050207	M	4 weeks	None	Rhinitis, dyspnoea	Pharyngitis, fever		Upper respiratory tract infection
36164	050210	F	2 years	Pre-term week 24, RDS, repeated asthmatic bronchiolitis	Dyspnoea, insufficient effect of asthma medication	Crepitations, rhonchi	CXR: unilateral basal infiltrate, co-infection with hMPV	Asthma, infection triggered

RDS, respiratory distress syndrome; CXR, chest X-ray; CPAP, continuous positive airways pressure; hMPV, human metapneumovirus.

treated in hospital, while older children are treated at home by general practitioners. Most previous studies have contained a larger proportion of older children, who account for a substantial proportion of HCoV-NL63 infections. The present study contained few samples from children aged >1 year, and only 11 patients were positive for HCoV-NL63 alone, so that comparisons with previous studies are not valid.

The clinical symptoms associated with NL63 infections have yet to be established. Some studies have found that HCoV-NL63 infections are predominantly severe lower respiratory tract infections, mainly in patients with underlying diseases [8,22,29], while other studies have reported mild respiratory tract infections in previously healthy patients [7,19–21,26]. The clinical features in the HCoV-NL63 group in the present study (Table 1 and Table 2) were indicative of a lower disease severity than for RSV and hMPV infections. However, only nasopharyngeal aspirates of children aged <10 years were studied. The causative role of HCoV-NL63 in different clinical manifestations and age groups has yet to be established. Therefore, it is important to conduct a wide range of retrospective and prospective studies in order to obtain a better estimate of the incidence and clinical effects of HCoV-NL63 in different populations.

Two false-positive PCR results were observed with the replicase gene PCR. Both products were sequenced and showed the same degree of homology with part of the human X-chromosome. The PCR signal generated was quite weak and was not observed with any of the other samples. Considering that all the samples will have contained X-chromosome DNA, the fact that only two of 221 samples tested positive could reflect sequence differences at the primer binding sites of the X-chromosome, or simply different amounts of X-chromosome DNA in the samples. The replicase gene PCR could be used as a screening tool, but all positive findings should be confirmed by sequencing. The higher sensitivity of real-time RT-PCR in comparison with the two conventional RT-PCRs, together with a more convenient protocol, supports the use of this method as a screening tool.

In addition to HCoV-NL63, this is the first report of hMPV infections in Sweden. The finding that 10% of the samples contained hMPV RNA is comparable with the results of previous studies that detected hMPV in 2.9–17.5% of respiratory samples from non-selected groups of children [10,15–17,30–36]. Two reports from Finland (positivity rates of 4% and 8%), one from Denmark (positivity rate of 2.9%) and two from Norway show that the virus has been circulating in Scandinavia [11–13,37,38].

The present study indicated that hMPV infections have the same seasonal distribution as RSV infections (Fig. 2). All the aforementioned studies documented hMPV during the winter and spring months, and some investigators have observed

differences in the age distribution between RSV-infected and hMPV-infected children [10,36]. The present study observed a similar median age in the group of hMPV-infected children to that in the group of RSV-infected children, as reported also by van den Hoogen *et al.* [9].

The pathogenicity of hMPV compared with RSV is also a matter for debate. Williams *et al.* [10] did not observe differences in chest X-ray and hospitalisation rates. Other studies have found lower hospitalisation rates, a shorter duration of symptoms and lower illness scores for hMPV-positive patients [9,35]. The present study also found evidence of shorter hospitalisation periods with hMPV infection, while the similar rate at which chest X-rays were taken and the low frequency of antibiotic treatment strengthen the observations made by Williams *et al.* [10]. To determine the incidence and clinical features of HCoV-NL63 and hMPV infection, testing was limited to these two viruses and RSV. Thus, the overall burden of infection caused by respiratory tract pathogens was not investigated, and it is possible that some co-infections with other viruses or bacteria were not detected.

The availability of simple and quick methods suggests that combined testing of RSV and hMPV should be considered for routine diagnostic use. Both viruses are present in the same patient groups and cannot be differentiated clinically. Cohorting strategies for both viruses to prevent nosocomial spread can be based on this technique. The combined real-time assay can be complemented with the real-time assay for HCoV-NL63 so that additional diagnostic and clinical knowledge of its role in different clinical manifestations can be gained.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available online from http://www.blackwell-synergy.com: **Table S1.** Sequences of primers and probes used in the study.

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