Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Clinical Virology 33 (2005) 306–311

www.elsevier.com/locate/jcv

Diagnosis of human metapneumovirus and rhinovirus in patients with respiratory tract infections by an internally controlled multiplex real-time RNA PCR

S.A. Scheltinga, K.E. Templeton, M.F.C. Beersma, E.C.J. Claas *

Department of Medical Microbiology, Center Infectious Diseases, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands

Received 3 February 2004; received in revised form 17 August 2004; accepted 17 August 2004

Abstract

Background: Adequate laboratory diagnosis of human rhinoviruses (hRV) and human metapneumoviruses (hMPV) requires molecular methods as viral culture lacks sensitivity. However, setting up individual PCRs for all respiratory viruses is not practical so preferentially multiplex PCRs are used.
Objectives: To develop for routine diagnosis a rapid real-time PCR assay for detection of hRV and hMPV including an internal control in a single tube multiplex reaction using probes carrying different fluorophores to discriminate targets.
Study design: The multiplex real-time RNA PCR was optimized to include the internal control virus and a total of 358 respiratory samples from 239 patients taken over a one-year period were analyzed by the multiplex assay.
Results: The multiplex assay with co-amplification of the internal control was as sensitive and specific as the individual assays. Application of this assay on clinical samples from 239 patients in a one-year period resulted in an incidence of hRV and hMPV of 41/239 (17.1%) and 6/239 (2.5%), respectively. Inhibition, defined as poor internal control amplification, was detected in 8 (2.2%) samples. Culture was performed on these samples and only four hRV were detected.
Conclusions: This real-time PCR method enables sensitive diagnosis of these two respiratory pathogens with the potential to expand the assay as part of a full molecular respiratory viral screen.

Keywords: Real-time RNA PCR; Respiratory viruses; Rhinoviruses; Metapneumoviruses; Multiplex PCR; Internal control

1. Introduction

Acute upper respiratory tract infection (URTI) is the most widespread type of acute infection. Two common causes of URTI are human rhinovirus (hRV) and human metapneumovirus (hMPV), accounting for approximately 48% and 10% of URTIs, respectively (Steininger et al., 2001; Kahn, 2003). However, hRV and hMPV can also cause lower respiratory tract (LRT) symptoms such as severe bronchiolitis and pneumonia (Van den Hoogen et al., 2001; Falsey et al., 2002).

Detection of both viruses is non-optimal by conventional cell culture and the slow-growth of hMPV was one of the reasons for the late discovery of the virus (Osterhaus and Fouchier, 2003). Accurate diagnosis of hRV and hMPV requires molecular methods (Arruda et al., 1997; Van den Hoogen et al., 2001; Blomqvist et al., 2002; Boivin et al., 2002; Peret et al., 2002; Stockton et al., 2002; Vicente et al., 2003). Most of the PCRs described for detection of hMPV and hRV are designed to identify the virus with individual PCRs and are either time consuming as they require post PCR analysis or costly as they require two PCR reactions. One of the benefits of real-time PCR is that amplifications can be multiplexed as probes carrying different fluorophores enable

Abbreviations: hRV, human rhinoviruses; hMPV, human metapneumoviruses; URTI, upper respiratory tract infection; LRT, lower respiratory tract; EAV, equine arteritis virus; $TCID_{50}$%, tissue culture infective dose; RSV, respiratory syncytial virus; PIV, parainfluenza virus

* Corresponding author. Tel.: +31 71 526 3650; fax: +31 71 524 8148.
E-mail address: E.Claas@lumc.nl (E.C.J. Claas).

1386-6532/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jcv.2004.08.021

differentiation of the amplified products. This also results in savings of time and money. To date no hMPV multiplex applications have been described.

When using PCR as a diagnostic tool, adequate controls should be included to ensure the integrity of the assay. Monitoring RNA isolation and inhibition of the samples can be achieved by spiking the clinical samples with a known amount of a non-human virus (Niesters, 2002). This internal control is co-extracted and co-amplified in the reaction and results in a fixed cycle threshold (Ct) value in the real-time PCR for a properly isolated and un-inhibited sample. Obviously the spike reaction has been designed in a way that the other PCRs in the multiplex are not affected. In this way negative results are generated with greater confidence.

The purpose of this study was to develop a multiplex real-time PCR for hMPV, hRV with an internal control, which would provide a rapid, sensitive and specific test with sufficient controls for use in the diagnosis of these viruses. Additionally, the prevalence and clinical significance of these viruses was assessed in 358 respiratory samples taken from 239 patients in December 2001 until December 2002.

2. Materials and methods

2.1. Viruses and viral stocks

hMPV (Ned/00/01) was cultured on rhesus monkey kidney (LLC-MK2) cells and hRV (hRV type 16 – laboratory isolate) in human epidermoid larynx carcinoma (HEL) cells. Ten-fold dilution series of the virus stocks were cultured in duplicate on 24-well plates, centrifuged for 30 min at $1500 \times g$ and incubated in CO_2 (hMPV at 36 °C and hRV 32 °C) for 10 days. The 50% tissue culture infective dose ($TCID_{50}$) with the presence of the cytopathic effect (CPE) was determined and aliquots were stored for PCR optimisation. Some additional subtypes of hRV were cultured as well: hRV1a, hRV1b, hRV15, hRV39, hRV51, hRV59, hRV85, hRV86, hRV91.

Equine arteritis virus (EAV) (Bucyrus strain) was used as the internal control and was cultured in baby hamster kidney cells (BHK-21) at 39.5 °C. The stock contained 10^{10} plaque forming units/ml.

2.2. Clinical specimens

The respiratory specimens consisted of all samples that were submitted for conventional routine diagnosis of respiratory viruses from December 2001 to December 2002. In total 358 samples were collected from 239 patients of whom 146 were immunocompromised and 93 immunocompetent. The specimens collected were 178 nasal swabs, 146 throat washes, 21 bronchial alveolar lavages (BALs) and 13 sputa. Aliquots of all specimens were stored at −80 °C prior to nucleic acid isolation and PCR.

2.3. Respiratory viral culture

Cultures were performed by inoculating HEp-2, epidermoid larynx carcinoma (HEL) and LLC-MK2 cells with each of the clinical samples. Approximately 100 μl of the sample was inoculated onto the cell-lines in shell vials. Shell vials were centrifuged at $1500 \times g$ for 30 min. One HEL shell vial was incubated at 32 °C and the other at 37 °C. The LLC-MK2 and HEp-2 were incubated at 35 °C. All shell vials were incubated for 14 days and examined daily for cytopathic effect. Immunofluorescence with commercial monoclonal antibodies for influenza A, influenza B, respiratory syncytial virus (RSV), parainfluenza virus (PIV) 1–3 and adenoviruses confirmed positive cytopathic effects. (Dako Diagnostics, Glostrup, Denmark). Rhinoviruses were distinguished from enteroviruses by means of acid-lability testing.

2.4. Specificity of multiplex PCR

A panel of respiratory viruses and bacteria commonly found in the respiratory tract was used to determine the specificity of the multiplex RNA-PCR. Nucleic acids were extracted from influenza A, influenza B, RSV, PIV1–4, adenovirus (type 5), coronavirus (229E), echovirus 7, coxsackievirus B4, human parechovirus type 1 (formerly echovirus 22), mumps virus, measles virus, *Bordetella bronchiseptica*; *Bordetella parapertussis*; *Bordetella pertussis*; *Burkholderia cepacia*; *Chlamydia pneumoniae*; *Haemophilus influenzae*; *Klebsiella pneumoniae*; *Legionella pneumophila*; *Mycoplasma pneumoniae*; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; and *Streptococcus pneumoniae* and tested by the multiplex PCR.

2.5. RNA isolation

For the development and optimisation of the multiplex RNA-PCR, RNA of hMPV and hRV positive material was extracted from cell culture supernatants by using the QiaAmp® Viral RNA Mini kit (Qiagen, Hilden, Germany). The MagNA Pure LC System (Roche Applied Science, Penzberg, Germany) was used to extract the nucleic acids from clinical specimens to assess automated extraction for routine diagnosis.

2.6. Primers and probes

The primers and probe sequences were selected from the L Gene sequence of hMPV (Van den Hoogen et al., 2001), the 5′ non-coding region of hRV, and the replicase ORF1a polyprotein gene of EAV. The first step in the design of the PCR was to perform an alignment to ensure selection of conserved regions within a target. The primer and probe interactions were checked using Beacon Designer 2.0 using Accession no AF371337 of hMPV, D00239 for hRV and X53549 for EAV (Premier Biosoft International, Palo Alto, USA). A BLAST search was performed to check specificity

Table 1
Primers and probes used in the multiplex assay

Target	Primer	Sequence and label	PCR product size (bp)
hMPV	342MPVs	CATGCCCACTATAAAAGGTCAG	170
	343MPVas	CACCCCAGTCTTTCTTGAAA	
	542MPVMBHEX	HEX-*GCTGCGG*CATGYCAYTGGTGTGGGATATT*CGCAGC*-BHQ-1	
HRV	235HRVs	GACARGGTGTGAAGSYC	142
	236HRVas	CAAAGTAGTYGGTCCCATCC	
	522HRV-TQ-FAM	FAM-TCCTCCGGCCCCTGAATG*YGGCTAA*-BHQ-1	
EAV	417EAVs	CATCTCTTGCTTTGCTCCTTAG	134
	418EAVAS	AGCCGCACCTTCACATTG	
	562EAVMBCY5	CY5-*CGCGCT*CGCTGTCAGAACAACATTATTGCCCAC*AGCGCG*-BHQ-2	

The stem structure of the molecular beacon is italicised. Reporter dyes: hexachlorofluorescein (HEX); carboxy-fluorescein (FAM); and indodicarbocyanine (Cy5). Quenchers: black hole quencher 1 (BHQ1); dabcyl; and BHQ-2. Probes: molecular beacon (MB); Taqman probe (TQ).

of the sequences of the primers and probes. Primers Taqman and molecular beacons are described in Table 1. The primers were prepared by Eurogentec (Eurogentec, Seraing, Belgium) and the probes were synthesized by Biolegio (Biolegio, Malden, The Netherlands).

2.7. Multiplex real-time RNA PCR

Before the multiplex PCR was set up, the individual assays were optimised by determining optimum Mg^{2+}, annealing temperatures, and primer and probe concentrations. For the multiplex RNA-PCR the Qiagen® one-step RT-PCR kit (Qiagen, Hilden, Germany) was used. The reaction was performed in 50 μl of reaction mixture consisting of 10 μl of 5X Qiagen one-step RT-PCR buffer, 10 mM dNTPs, 4.5 mM $MgCl_2$, 0.6 μM of each primer for hMPV, 0.4 μM of each primer for hRV and EAV, 0.34 μM of molecular beacon for hMPV and of Taqman probe for hRV and 0.25 μM of the molecular beacon for EAV. After addition of 5 μl template. The PCR thermal profile consisted of an initial cDNA step of 30 min at 50 °C followed by 15 min at 95 °C and 50 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. Amplification, detection and data analysis were performed with an iCycler IQ real-time detection system (BioRad, Veenendaal, The Netherlands).

2.8. Nucleotide sequence analysis of hMPV positive patients

For all hMPV positive patients, the PCR products with a size of approximately 170 bp were subjected to nucleotide sequence analysis to determine the hMPV subtypes. The analysis was carried out on an ABI Prism 310 genetic analyser using the Big Dye terminator kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

3. Results

3.1. Multiplex RNA-PCR for hMPV, hRV and EAV

The analytical sensitivity of the multiplex PCR was determined at 0.01 $TCID_{50}$ for both hMPV and hRV. The cycle threshold values obtained in the multiplex analyses were compared to the individual PCRs and no difference in sensitivity was achieved as shown in Table 2.

Specificity testing of the multiplex PCR showed that no other respiratory pathogens were amplified in this assay except for enteroviruses. A high titre enterovirus stock of 100 $TCID_{50}$ resulted in weak amplification (Ct 42) in the hRV assay.

When analysing the PCR products of the hMPV individual RNA-PCR on a 2%-agarose gel, non-specific bands were amplified as well as shown in Fig. 1. Thus, the specificity of the reaction was conferred by hybridisation of the molecular beacon. All PCR-products with an approximate size of 170 bp were analysed by sequence analysis. Samples positive for hMPV in real-time PCR showed a complete match to the sequence of the hMPV isolate with Genbank number AF371337. The non-specific bands, negative by real-time PCR, appeared to be human DNA of a variety of chromosomes after sequencing.

The inter-run variation for hMPV and hRV in the multiplex PCR was tested amplifying a control (100 $TCID_{50}$) in 10 separate runs. The mean Ct value was 26.0 ± 0.5 and 19.7 ± 0.9 for hRV and hMPV, respectively.

Table 2
Comparison of Ct values after amplification of dilution series of titrated virus in mono-specific and multiplex real-time PCR

	Ct value (individual PCR)	Ct value (multiplex)	Ct value (multiplex with EAV spike)
Rhinovirus			
100 $TCID_{50}$	26.2	26.5	26.3
10 $TCID_{50}$	29.4	29.3	29.7
1 $TCID_{50}$	33.1	33.1	33.4
0.1 $TCID_{50}$	36.5	36.3	37.1
0.01 $TCID_{50}$	40.2	40.5	40.9
0.001 $TCID_{50}$	0	0	0
Metapneumovirus			
100 $TCID_{50}$	19.2	19.1	19.7
10 $TCID_{50}$	23.1	23.3	23.5
1 $TCID_{50}$	27.3	27.1	27.9
0.1 $TCID_{50}$	31.2	31.2	32.0
0.01 $TCID_{50}$	36.4	38.1	38.2
0.001 $TCID_{50}$	0	0	0

For each virus a 10-fold dilution series is shown with Ct values. For each virus the lowest level of detection is shown.

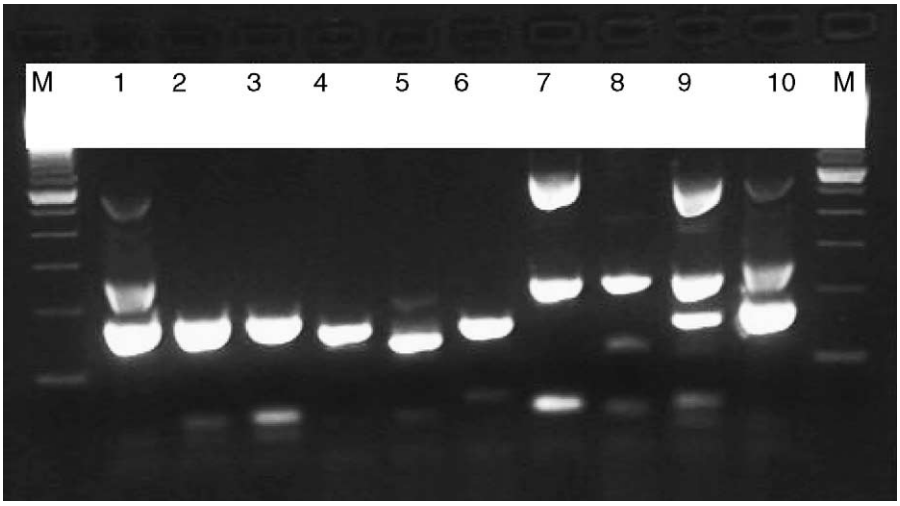

Fig. 1. PCR products generated by the hMPVprimers on a 2%-agarose gel. *M* = 100 bp ladder. Lane 1 is the hMPV positive control and lane 2–3 are sequence analysis confirmed hMPV positives. Lane 4–10 are aspecific products amplified by the primers.

3.2. Testing inhibition using an EAV internal control.

In this real-time assay an EAV spike was used as an internal control. The EAV dilution used for the spike was 10^4 plaque forming units/ml with an input of 10 μl per extraction which resulted in a positive result at a Ct value of 33–34. The efficiency of the amplification of dilution series of hRV and hMPV was not significantly affected by co-amplification of EAV in the multiplex reaction (Table 2). Of the 358 clinical samples tested, 8 (2.2%) were inhibitory and those were five throat washes and three nasal washes.

3.3. Clinical application of the multiplex RNA-PCR

All 358 specimens collected from 239 patients were cultured to detect common respiratory viruses. None of the specimens were cultured positive for hMPV, probably because the cultures were terminated after 14 days. However, the multiplex PCR revealed that six patients (2.5%) were positive for hMPV. Five patients were children (≤18 years old) and one patient was an adult. Infection with hMPV occurred in five patients in December and in one patient in January. Two patients showed a co-infection of hMPV with RSV. All patients with an hMPV infection were hospitalised with fever and URTI.

Of the 239 patients tested, 41 (17.1%) were positive for hRV in the multiplex RT-PCR and 4 (1.6%) of these were also positive in viral culture. From the 41 patients positive for hRV, 95.2% of the infections were present in children younger than 18 years of age and 4.8% were found in adults. The episodes of hRV positivity detected by PCR per month of the year are shown in Fig. 2. Infections with hRV were particularly

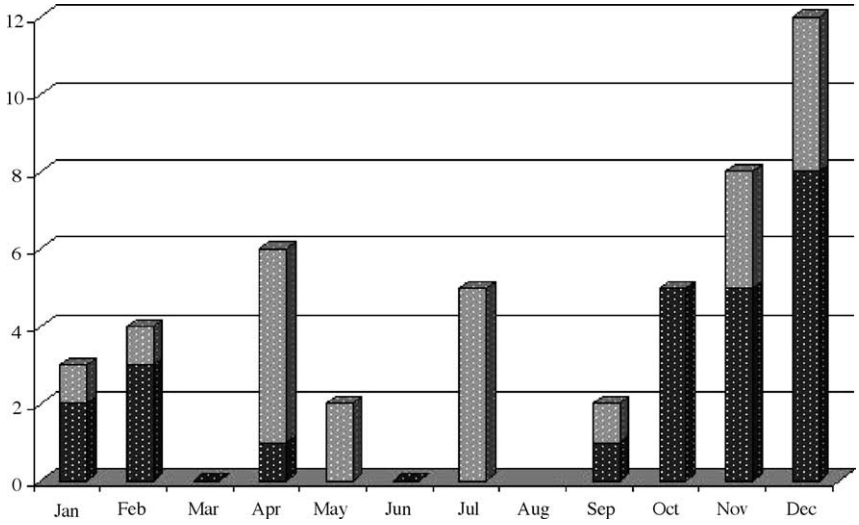

Fig. 2. Cumulative number of episodes of hRV positivity detected by PCR in immunocompromised and immunocompetent patients per month of the year. Immunocompetent patients = ■ Immunocompromised patients = ▩ .

Table 3
Clinical symptoms in the hRV positive paediatric patients

Symptom	Immunocompromised ($n = 146$)	Immunocompetent ($n = 93$)
URTI	10	16
LRTI	1[a]	4[b]
Pneumonia	0	0
Asthma Exacerbation	0	2
No symptoms	8	0
Total	19	22

URTI was defined as coryza, or rhinorrhea or cough with or without fever but without hypoxia or changes on the chest radiograph. LRTI was defined as signs or symptoms of pulmonary infection such as cough, rales, wheezes, dyspnea or shortness of breath or low oxygen saturation. Pneumonia was defined when in addition to this also abnormalities on chest radiograph (such as infiltrates) were observed.

[a] Patient co-infected with CMV.

[b] Three patients co-infected with RSV.

present in the winter months, but also prevalent in summer in immunocompromised patients. Seven patients showed a co-infection with RSV, one with adenovirus and one with PIV1. In two immunocompromised patients, initially an infection with hRV was detected, and at a later time-point an infection with another respiratory virus, RSV and PIV3, respectively. In both cases hRV was detectable for over 4 weeks by the multiplex PCR. The clinical symptoms observed in the hRV positive patients are summarised in Table 3. Respiratory symptoms were observed in all immunocompetent patients but in the 19 immunocompromised patients 8 had no respiratory symptoms.

4. Discussion

In order to provide a technique for rapid detection of hRV and hMPV a multiplex RNA PCR was developed with an internal control. These viruses cause a significant number of acute respiratory viral infections in children and are found in more severe respiratory disease as well. The assay was proven to be sensitive with no negative effects by multiplexing. As different probes were used for the two viruses and the internal control, no post PCR analysis was required. The multiplex RT-PCR is specific for hMPV, but the primers and probe designed for hRV also amplify high input concentrations of some enterovirus subtypes with a weak signal. Differentiation between hRV and enteroviruses using hybridisation probes has been described (Lönnrot et al., 1999; Kares et al., 2004). Adding a specific EV PCR in the multiplex or sequencing the whole PCR product would solve the problem of differentiation. Unfortunately, no appropriate sequence suitable for real-time PCR and discriminating between hRV and enterovirus subtypes is available (Nijhuis et al., 2002). However, in clinical diagnosis the necessity of further discrimination of hRV and enteroviruses is questionable, as management and therapy issues are not affected by this identification.

The hMPV assays described by van Hoogen et al. have been used in several other studies. (Boivin et al., 2002; Peret et al., 2002; Stockton et al., 2002; Vicente et al., 2003). All these studies have performed RNA-PCR with detection by agarose gel electrophoresis followed by sequencing or hybridisation to confirm an hMPV positive sample. Clearly, the primers for hMPV in the L Gene results in non-specific amplification products on clinical samples (Fig. 1) but specificity could be obtained using the molecular beacon. Other real-time RT-PCRs have been described for hMPV using the Lightcycler instrument (Boivin et al., 2002; Mackay et al., 2003) but none in an internally controlled multiplex PCR format.

So far, hRV PCR methods have required post-PCR analysis like hybridisation and agarose gel-electrophoresis (Arruda et al., 1997; Pitkaranta et al., 1997; Steininger et al., 2001; Blomqvist et al., 2002; Falsey et al., 2002; Billaud et al., 2003) and no real-time PCR methods have been described. Only two multiplex formats have been described with hRV, both of which are nested PCR assays with detection by gel electrophoresis (Ishibashi, 2003).

For a real clinical impact for diagnosis of respiratory viruses, other pathogens than hMPV and hRV would have to be included in the assay as well. Previously, a two tube multiplex for influenza A, influenza B, RSV, PIV1–4 was described (Templeton et al., 2004). A screening assay for all major respiratory RNA viruses, except for coronaviruses, with results available within one day is possible by combining these assays.

Addition of an internal control reaction is an important requisite for any routine diagnostic PCR assay as each sample is checked for the quality of the nucleic acid extraction and for inhibition. Inhibition was detected in 2.2% of the nasal swabs and throat washes. Although, BALs and sputa are more likely to be inhibitory in this study no inhibition was detected, probably because the limited number of these specimens.

Infection rates for hMPV have ranged from 2% to 35.1% in children (Van den Hoogen et al., 2001; Boivin et al., 2002; Stockton et al., 2002; Vicente et al., 2003). In this study, the infection in children was 2.2%, which is comparable to the study by Stockton et al. (Stockton et al., 2002). One of the reasons for these diverse percentages of hMPV infections is that different clinical criteria were used to select the patients in different studies. In the current study, samples of all patients coming in the hospital with request for respiratory testing were collected, including those of immunocompromised patients, which undergo screening regardless of symptoms. All hMPV infections occurred in the winter-months, which is comparable to what has been found in other studies (Boivin et al., 2002; Peret et al., 2002; Stockton et al., 2002; Vicente et al., 2003). Although all samples were inoculated onto the appropriate cells no hMPV was isolated.

An infection rate of 17.1% hRV was detected in this study and 95% of the hRV infections occurred in children younger than 18 years of age. Only 4 patients were positive for hRV by viral culture, while the multiplex RNA-PCR revealed 41

positive patients. Therefore, viral culture for diagnosis of hRV is very insensitive in this study, but PCR has been shown to be superior for detection of hRV in other studies as well. Molecular methods have demonstrated infection rates as high as 50% in URTI (Steininger et al., 2001; Blomqvist et al., 2002). In this study, the incidence of hRV infections peaked during the winter, but was also present in autumn and summer in immunocompromised patients.

Co-infections were detected as well; twice hMPV was found together with RSV and six times hRV and RSV were detected. It has been suggested that more severe RSV infection is observed upon co-infection with hMPV (Greensill et al., 2003) but with the limited numbers obtained the significance of the co-infection is uncertain. However, in the future these viruses could be routinely detected by using the multiplex assays resulting in further insight into respiratory viral co-infections.

In conclusion, this real-time RNA PCR method enables sensitive diagnosis of hMPV and hRV infections, which are poorly detected by cell culture. This assay can be part of a molecular full respiratory viral screen, thus increasing the understanding of the clinical relevance and epidemiology of these pathogens in respiratory infections.

Acknowledgements

This study was supported by European Commission (Framework V) Grant No QLK2-CT-2000-00294. We thank Prof. Dr. Albert D.M.E. Osterhaus of Erasmus University Rotterdam, The Netherlands for the hMPV and Dr. Eric. J. Snijder of Leiden University Medical Center, Leiden, The Netherlands for the EAV stocks.

References

Arruda E, Pitkaranta A, Witek Jr TJ, Doyle CA, Hayden FG. Frequency and natural history of rhinovirus infections in adults during autumn. J Clin Microbiol 1997;35:2864–8.

Billaud G, Peny S, Legay V, Lina B, Valette M. Detection of rhinovirus and enterovirus in upper respiratory tract samples using a multiplex nested PCR. J Virol Methods 2003;108:223–8.

Blomqvist S, Roivainen M, Puhakka T, Kleemola M, Hovi T. Virological and serological analysis of rhinovirus infections during the first two years of life in a cohort of children. J Med Virol 2002;66:263–8.

Boivin G, Abed Y, Pelletier G, Ruel L, Moisan D, et al. 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 2002;186:1330–4.

Falsey AR, Walsh EE, Hayden FG. Rhinovirus and coronavirus infection-associated hospitalisations among older adults. J Infect Dis 2002;185:1338–41.

Greensill J, McNamara PS, Dove W, Flanagan B, Smyth RL, et al. Human metapneumovirus in severe respiratory syncytial virus bronchiolitis. Emerg Infect Dis 2003;9:372–5.

Ishibashi T, Monobe H, Nomura Y, Shinogami M, Yano J. Multiplex nested reverse transcription-polymerase chain reaction for respiratory viruses in acute otitis media. Ann Otol Rhinol Laryngol 2003;112:252–7.

Kahn JS. Human metapneumovirus, a newly emerging respiratory virus. Pediatr Infect Dis J 2003;22:923–4.

Kares S, Lönnrot M, Vuorinen P, Oikarinen S, Taurianen S, Hyöty H. Real-time PCR for rapid diagnosis of entero- and rhinovirus infections using LightCycler. J Clin Virol 2004;29:99–104.

Lönnrot M, Sjoroos M, Salminen K, Maaronen M, Hyypia T, et al. Diagnosis of enterovirus and rhinovirus infections by RT-PCR and time-resolved fluorometry with lanthanide chelate labeled probes. J Med Virol 1999;59:378–84.

Mackay IM, Jacob KC, Woolhouse D, Waller K, Syrmis MW, et al. Molecular assays for detection of human metapneumovirus. J Clin Microbiol 2003;41:100–5.

Niesters HGM. Clinical virology in real time. J Clin Virol 2002;25(Suppl. 3):S3–12.

Nijhuis M, van Maarseveen N, Schuurman R, Verkuijlen S, de Vos M, et al. Rapid and sensitive routine detection of all members of the genus enterovirus in different clinical specimens by real-time PCR. J Clin Microbiol 2002;40:3666–70.

Osterhaus A, Fouchier R. Human metapneumovirus in the community. The Lancet 2003;361:890–1.

Peret TC, Boivin G, Li Y, Couillard M, Humphrey C, et al. Characterization of human metapneumoviruses isolated from patients in North America. J Infect Dis 2002;185:1660–3.

Pitkaranta A, Arruda E, Malmberg H, Hayden FG. Detection of rhinovirus in sinus brushings of patients with acute community-acquired sinusitis by reverse transcription-PCR. J Clin Microbiol 1997;35:1791–3.

Steininger C, Aberle SW, Popow-Kraupp T. Early detection of acute rhinovirus infections by a rapid reverse transcription-PCR assay. J Clin Microbiol 2001;39:129–33.

Stockton J, Stephenson I, Fleming D, Zambon M. Human Metapneumovirus as a cause of community-acquired respiratory illness. Emerg Infect Dis 2002;8:879–901.

Templeton KE, Scheltinga SA, Beersma MFC, Kroes ACM, Claas ECJ. Design and clinical evaluation of multiplex real-time RNA PCR for diagnosis of influenza A, influenza B, respiratory syncytial virus and parainfluenza viruses 1, 2, 3 and 4. J Clin Microbiol 2004;42:1564–9.

Van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med 2001;7:719–24.

Vicente D, Cilla G, Montes M, Perez-Trallero E. Human metapneumovirus and community-acquired respiratory illness in children. Emerg Infect Dis 2003;9:602–3.