Multiplex RT-PCR in the Diagnosis of Human Picornaviruses and Human Respiratory Viruses

Pia Jokela

Department of Virology
Haartman Institute
Faculty of Medicine
University of Helsinki

and

Department of Virology and Immunology
Section of Clinical Microbiology
HUSLAB
Helsinki University Central Hospital

and

Division of General Microbiology
Department of Biosciences
Faculty of Biological and Environmental Sciences
University of Helsinki

ACADEMIC DISSERTATION

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Supervisors: Docent Maija Lappalainen
Department of Virology and Immunology
Section of Clinical Microbiology
HUSLAB
Helsinki University Central Hospital
Helsinki, Finland

Docent Heli Piiparinen
Department of Virology
Haartman Institute
University of Helsinki
Helsinki, Finland

Reviewers: Docent Leena Maunula
Department of Food Hygiene and Environmental Health
University of Helsinki
Helsinki, Finland

Docent Merja Roivainen
Intestinal Viruses Unit
National Institute for Healts and Welfare
Helsinki, Finland

Opponent: Docent Janne-Juhana Aittoniemi
Fimlab Laboratories
Tampere, Finland

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5`UTR</td>
<td>5’-untranslated region</td>
</tr>
<tr>
<td>AdV</td>
<td>Adenovirus</td>
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<tr>
<td>AV</td>
<td>Aichi virus</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CVA</td>
<td>Coxsackie virus A</td>
</tr>
<tr>
<td>CVB</td>
<td>Coxsackie virus B</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DFA</td>
<td>Direct fluorescent assay</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotidetriphosphate</td>
</tr>
<tr>
<td>E11</td>
<td>Echovirus 11</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>FRET</td>
<td>Fluorescent resonance energy transfer</td>
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<tr>
<td>HAV</td>
<td>Hepatitis A virus</td>
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<tr>
<td>hBoV</td>
<td>Human bocavirus</td>
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<tr>
<td>hCoV</td>
<td>Human coronavirus</td>
</tr>
<tr>
<td>HEV</td>
<td>Human enterovirus</td>
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<tr>
<td>hMPV</td>
<td>Human metapneumovirus</td>
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<tr>
<td>hPEV</td>
<td>Human parechovirus</td>
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<tr>
<td>HRV</td>
<td>Human rhinovirus</td>
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<tr>
<td>IAV</td>
<td>Influenza A virus</td>
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<td>IBV</td>
<td>Influenza B virus</td>
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<tr>
<td>IFA</td>
<td>Indirect fluorescent assay</td>
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<tr>
<td>LFIC</td>
<td>Lateral flow immunochromatography</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>NPA</td>
<td>Nasopharyngeal aspirate</td>
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<tr>
<td>NPS</td>
<td>Nasopharyngeal sample</td>
</tr>
<tr>
<td>NTR</td>
<td>Non-translated region</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleotidetriphosphate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PIV</td>
<td>Parainfluenza virus</td>
</tr>
<tr>
<td>PV</td>
<td>Poliovirus</td>
</tr>
<tr>
<td>QCMD</td>
<td>Quality Control for Molecular Diagnostics</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative luminescence units</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>SARS-CoV</td>
<td>Severe acute respiratory syndrome-associated coronavirus</td>
</tr>
<tr>
<td>T&lt;sub&gt;M&lt;/sub&gt;</td>
<td>Melting temperature</td>
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<tr>
<td>UNG</td>
<td>Uracil N-glycosylase</td>
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SUMMARY

The family *Picornaviridae* includes many human pathogens. Human enteroviruses (HEVs) exhibit a variety of clinical manifestations ranging from poliomyelitis and encephalomyelitis to respiratory infections and rashes. Human rhinoviruses (HRVs) are the major causes of the common cold. Human parechoviruses (HPeVs) and Aichi virus (AV) are mostly detected in cases of gastroenteritis, and hepatitis A virus (HAV) causes hepatitis with favourable prognosis. In addition to HEVs and HRVs, a large number of viruses are recognized as respiratory pathogens. The conventional respiratory pathogens include influenza A and B viruses, human respiratory syncytial virus (RSV), adenoviruses (AdVs), parainfluenza viruses (PIVs) and the human coronaviruses (hCoVs) OC43 and 229E. Moreover, several new respiratory pathogens, such as human metapneumovirus (hMPV), severe acute respiratory syndrome coronavirus (SARS-CoV), and the hCoVs HKU1 and NL63 have been found during the 2000s. Human bocavirus (hBoV) is also increasingly being recognized as a true pathogen of humans.

Since many clinical illnesses may be caused by several different viruses, multiplex assays for simultaneous detection of several viruses are increasingly being applied. Real-time multiplex polymerase chain reaction (PCR) assays for detection of viral nucleic acids offer remarkable benefits, such as short turnaround time and the non-necessity for handling amplified products. Since multiplexing, utilizing real-time PCR, is limited by reduction in amplification efficiency due to multiple primer and probe sets, separate amplification and hybridization reactions have re-emerged in attempts to develop tests with broad diagnostic range. With this approach microarrays, which have the potential for resolving complex mixtures of amplification products, may be applied.

In this study, a multiplex reverse transcription-PCR (RT-PCR) and liquid hybridization assay for sensitive detection of HEV, HRV, HPeV and AV were developed and a single RT-PCR and liquid hybridization assay for detection of HAV was optimized. In analysis of clinical samples, the results obtained by the multiplex assay were consistent with those obtained by routine
diagnostic assays. When 68 stool samples were analysed for the presence of HPeV and AV, one sample positive for HPeV was detected. This finding is in line with the current knowledge of neither of these viruses being very common enteric pathogens.

More rapid detection of HEV and HRV in respiratory samples was achieved when a real-time duplex RT-PCR assay for detection of these viruses was developed. The same approach was used to develop another assay for more sensitive detection of RSV than with the direct fluorescent assay (DFA) and simultaneous identification of hMPV. Both multiplex real-time RT-PCR assays provided reliable and sensitive detection of their targets, except for detection of HRV, since doubts were raised on the ability of the assay to detect all rhinoviruses. Moreover, two commercial hMPV antibodies were found applicable for detection of the virus in respiratory samples by DFA. Results from analysis of respiratory samples using the duplex real-time RT-PCR assays were compared with those obtained with DFA and the Respiratory Viral Panel (RVP) Fast test, a bead-based suspension microarray test evaluated for routine diagnosis. The RVP Fast assay and PCR showed similar detection rates, except for HEV/HRV, for which a higher detection rate by RVP was observed. All PCR-based assays presented more findings of their target viruses than DFA. The broad detection range of the RVP Fast assay resulted in a nearly threefold overall detection rate, compared with that by DFA. Moreover, analysis of clinical samples resulted in a notable prevalence of hMPV and non-SARS-hCoVs, which emphasizes the role of these viruses as respiratory pathogens. Although the RVP Fast assay demonstrated adequate overall performance, doubts were raised on the ability of the test to detect the H1N1 2009 influenza A virus and all AdV serotypes.

Evaluation of the RVP Fast assay demonstrated the remarkable increase in overall viral detection rate that results from adapting a PCR-based multiplex assay to virus diagnostics. The sensitive detection of all the viruses of clinical relevance facilitates efficient infection control measures and appropriate patient management and enables systematic studies on the clinical importance of coinfections. Moreover, collection of data on occurrence of all the viruses of clinical relevance will enable a better understanding of the seasonality, geographical distribution and risk groups of the viral pathogens.
Tiivistelmä (Summary in Finnish)

Picornavirusten suku käsittää monia ihmiselle patogeeneja viruksia. Ihmisen enterovirukset (HEV) aiheuttavat lukuisia tautitiloja, kuten poliomyelitiä, enkefalomyelitiä, hengitystieinfektiota ja ihottumia. Ihmisen rinovirukset (HRV) ovat yleisin flunssan aiheuttaja. Ihmisen parechovirusta (HPeV) ja Aichi virusta (AV) tavanaan lähinnä gastroenteritiessa ja hepatiti A virus (HAV) aiheuttaa hyväennusteista hepatitiä. Enteronja rinovirusten lisäksi suuri joukko viruksia aiheuttaa hengitystieinfektiota. Perinteisiä hengitystieviruksia ovat influenssavirukset, respiratory syncytial virus (RSV), adenovirus (AdV), parainfluenssavirukset (PIV) ja ihmisen koronavirukset (hCoV) OC43 ja 229E. Lisäksi 2000-luvulla on löydetty joukko uusia hengitystiepatogeeneja viruksia, kuten ihmisen metapneumovirus (hMPV), severe acute respiratory syndrome -koronavirus (SARS-CoV), ja ihmisen koronavirukset (hCoV) HKU1 ja NL63. Myös ihmisen bokaviruksen (hBoV) rooli todellisena hengitystiepatogeenina on saanut vahvistusta.

Koska monet tautitilat voivat olla usean eri viruksen aiheuttamia, on usean viruksen nukleinihappojen osoittaminen näytteestä samanaikaisesti multiplex testien avulla yleistynyt. Virusten nukleinihappojen osoittaminen reaaliaikaisilla multiplex polymeraasiketjureaktioon (PCR) perustuvilla testeillä mahdollistaa testitulosten nopean saatavuuden ilman monistustuotteiden käyttöä. Koska monistustehokkuuden aleneminen käytettäessä useita alukepareja ja koettimia rajoittaa reaaliaikaisen PCR:n käyttöä usean viruksen osoittamiseen, on erillisen monistus- ja hybridisaatioreaktion käyttö uudelleen yleistynyt laajennettaessa testien kattavuutta. Käytettäessä tällaista lähestymistapaa voidaan monistustuotteiden spesifiin osoittamiseen hyödyntää mikrosirutestejä, joilla on suuri kapasiteetti eri monistustuotteiden spesiifiin osoittamiseen PCR-reactioseoksesta.

Tässä tutkimuksessa kehitettiin multiplex RT-PCR -monistukseen ja liuoshybridisaatioon perustuvaa testi HEV, HRV, HPeV ja AV osoittamiseksi. Lisäksi optimoitiin vastaava erillinen testi HAV osoittamiseksi. Ulostenätteiden analysissä multiplex RT-PCR testillä löydettiin yksi HPeV-positiivinen näyte. Tulos vastaa hyvin nykykäsitystä, jonka mukaan HPeV ja AV


REVIEW OF THE LITERATURE

1 HUMAN PICORNAVIRUSES

Picornaviruses are small nonenveloped viruses with a single-stranded RNA genome of positive polarity. Four genera of the family *Picornaviridae* include viruses that cause disease in humans (Table 1). The majority of human picornaviruses are classified in the genus *Enterovirus*. Human parechoviruses (hPeVs) are currently recognized as members of their own genus, *Parechovirus*. The other two genera, *Hepatovirus* and *Kobuvirus*, both include one human pathogen, hepatitis A virus (HAV) and Aichi virus (AV), respectively. In addition to human pathogens, the family *Picornaviridae* includes veterinary viruses of great importance, such as foot-and-mouth disease virus.

1.1 Human enteroviruses

Human enteroviruses (HEVs) belong to the large genus *Enterovirus* and include polioviruses (PVs), Coxsackie viruses A and B (CVA and CVB), echoviruses and the chronologically numbered enteroviruses discovered more recently. Based on relationships of the viral genomes, current taxonomy classifies the HEVs into four species: HEV A, -B, -C and -D (Brown et al. 2003; Hyytiä et al. 1997; Knowles et al. 2011; Pöyry et al. 1996). Currently, more than 100 serotypes of HEVs are recognized and the number is increasing as the new sequence-based typing approach unveils new serotypes among HEV isolates untypeable by classical identification methods (Oberste et al. 1999).

HEVs are ubiquitous worldwide and they circulate throughout the year, with a summer-fall seasonality of infections in the temperate regions (Cabrerizo et al. 2008; Lee et al. 2005) and the predominant strains changing over time (Thoelen et al. 2003). The major mode of transmission is the faecal-oral route, although for some HEV serotypes, transmission via the
respiratory route is important. HEVs cause significant morbidity and mortality, particularly in neonates (Tebruegge & Curtis 2009), although the majority of infections are clinically inapparent or appear as self-limiting gastroenteritis when the virus replicates in its normal replication site, the intestinal tract. However, spreading of the virus to other organs causes diverse clinical syndromes, such as rash, conjunctivitis and myocarditis. HEVs are also neurovirulent, with the target region in the central nervous system (CNS) differing among the viruses. The majority of aseptic meningitis cases are of enteroviral aetiology (Lee & Davies 2007), while other neurological manifestations, such as encephalitis and acute flaccid paralysis, are also associated with these viruses (Rhoades et al. 2011). Several HEVs, particularly CVA, CVB and the echoviruses, are commonly detected in upper and lower respiratory tract infections (Bourgeois et al. 2006; Jacques et al. 2008) and are also currently known to be associated with acute expiratory wheezing (Jartti et al. 2004b). However, interpretation of a positive result must take into account the possibility of a coincidental infection, since enteroviral RNA can be detected in respiratory samples from healthy subjects and in cases of previous infection (Jartti et al. 2004a; Nokso-Koivisto et al. 2002). Increasing evidence suggests that a variety of HEVs, particularly CVBs, also play roles in the onset of type I diabetes (Roivainen & Klingel 2010; Tauriainen et al. 2011).

1.2 Human rhinoviruses

Human rhinoviruses (HRVs) are closely related to HEVs and the genus Rhinovirus is no longer a valid taxon (Knowles et al. 2011). HRVs are classified in the genus Enterovirus as three species, HRV A, -B and -C (Simmonds et al. 2010), and they comprise more than 100 serotypes. These viruses circulate throughout the year, with occurrence typically peaking in spring and autumn in the temperate regions (Jartti et al. 2004b; Winther et al. 2006).

Transmission of HRVs involves both direct hand-to-hand contact (Ansari et al. 1991) and inhalation of aerosols (Dick et al. 1987). HRVs infect the upper and lower respiratory tract (Arruda et al. 1995; Jakiela et al. 2008) and are known as the major causes of the common
Table 1. Classification of human picornaviruses (Knowles et al. 2011).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Serotypes</th>
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<tbody>
<tr>
<td><strong>Enterovirus</strong></td>
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<tr>
<td><em>Human enterovirus A</em></td>
<td>Human coxsackieviruses A2-A8, A10, A12, A14, A16 Human enteroviruses A71, A76, A89-A91</td>
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<tr>
<td><em>Human enterovirus B</em></td>
<td>Human coxsackieviruses B1-B6, A9 Human echoviruses 1-7, 9, 11-21, 24-27, 29-33 Human enteroviruses 69, 73-75, 77-88, 93, 97, 98, 100, 101, 106, 107, 110</td>
<td></td>
</tr>
<tr>
<td><em>Human enterovirus C</em></td>
<td>Human polioviruses 1-3 Human coxsackieviruses A1, A11, A13, A17, A19-A22, A24 Human enteroviruses 95, 96, 99, 102, 104, 105, 109, 113, 116</td>
<td></td>
</tr>
<tr>
<td><em>Human enterovirus D</em></td>
<td>Human enteroviruses 68, 70, 94, 111</td>
<td></td>
</tr>
<tr>
<td><strong>Human rhinovirus A</strong></td>
<td>Human rhinoviruses 1, 2, 7-13, 15, 18-25, 28-34, 36, 38-41, 43-47, 49-41, 53-68, 71, 73-78, 80-82, 85, 90, 94, 95, 96, 98, 100-103</td>
<td></td>
</tr>
<tr>
<td><strong>Human rhinovirus B</strong></td>
<td>Human rhinoviruses 3-6, 14, 17, 26, 27, 35, 37, 42, 48, 52, 69, 70, 72, 79, 83, 84, 86, 91-93, 97, 99</td>
<td></td>
</tr>
<tr>
<td><strong>Human rhinovirus C</strong></td>
<td>Human rhinoviruses C1-C49&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td><strong>Parechovirus</strong></td>
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<tr>
<td><em>Human parechovirus</em></td>
<td>Human parechoviruses 1-16</td>
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<tr>
<td><strong>Ljungan virus</strong></td>
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<td><strong>Hepatovirus</strong></td>
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<tr>
<td><em>Hepatitis A virus</em></td>
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<tr>
<td><strong>Kobuvirus</strong></td>
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</tr>
<tr>
<td><em>Aichi virus</em></td>
<td>Aichi virus 1</td>
<td></td>
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</table>

<sup>a</sup> Based on Simmonds et al. 2010
cold. HRV infections are also associated with wheezing (Jackson et al. 2008; Kusel et al. 2006; Pitkäranta & Hayden 1998), low lung function, and exacerbations and onset of asthma, although the causality for the last-mentioned is unknown (Denlinger et al. 2011; Guilbert et al. 2011; Jackson et al. 2008). Some studies suggest that the more recently found HRV-C strains are associated with higher viral loads and more severe clinical outcomes than the HRV-A and -B strains (McErlean et al. 2008; Piralla et al. 2009), whereas others show no difference in clinical outcome among HRV species (Piotrowska et al. 2009). Persistent HRV infections are rare and prolonged illnesses rather result from a series of infections (Jartti et al. 2008). For these reasons, polymerase chain reaction (PCR)-positive results are considered to reflect a true infection, although persistence of HRV RNA for several weeks after the onset of symptoms in nasal mucus has been described and viral RNA is occasionally also detected in asymptomatic subjects (Jartti et al. 2004a; Nokso-Koivisto et al. 2002; Winther et al. 2006).

1.3  Human parechoviruses

HPeVs, initially included in the genus Enterovirus, are currently classified as their own genus, Parechovirus (Hyypiä et al. 1992; Knowles et al. 2011). Sixteen HPeV types known to infect human have been identified, the most prevalent genotypes being HPeV1 and HPeV3 (Bennett et al. 2011; Chieochansin et al. 2011; Harvala et al. 2011). The seasonal distribution of HPeV varies among virus types. For HPeV3 a biennial pattern of circulation, with spring-summer occurrence has been observed, whereas HPeV1 circulates in small numbers throughout every year (Harvala et al. 2011; van der Sanden et al. 2008).

Transmission of HPeVs occurs mainly through the faecal-oral route. HPeVs are detected in subjects of all ages, but the incidence of infections is highest in children under 3 years of age, with the majority of infections occurring in infants (Benschop et al. 2010; Chieochansin et al. 2011, Harvala et al. 2011). Similar to HEVs, HPeVs are associated with a wide array of clinical illnesses, the most common manifestations of these viruses being...
gastroenteritis and respiratory tract illness (Harvala et al. 2008). Moreover, HPeVs are increasingly recognized as pathogens of the CNS, particularly HPeV3, which is often detected as a causative agent of febrile neonatal illness (Harvala et al. 2009) and CNS diseases (Walters et al. 2011; Wolthers et al. 2008).

1.4 Hepatitis A virus

HAV is classified in its own genus, Hepatovirus, in the family Picornaviridae. The HAV particle retains infectivity in the environment, allowing transmission of the virus through the human faecal-oral route. Unlike the other picornaviruses, HAV is characterized by its tropism for the liver. The clinical spectrum of infection ranges from asymptomatic infection to fulminant hepatitis, with disease severity increasing with age. The highest burden of symptomatic HAV infection occurs in populations with lack of immunity, due to decreased incidence of childhood infection resulting from improvement in socioeconomic conditions (Moon et al. 2010). Although prolonged or relapsing disease is occasionally observed, persistent infection is not associated with HAV, but patients with clinical illness and laboratory abnormalities recover within a few months from the onset of symptoms (Cuthbert 2001; Sjogren et al. 1987).

1.5 Aichi virus

AV is the prototype virus of the quite recently established genus Kobuvirus and is further divided into three genotypes: A, B and C (Ambert-Balay et al. 2008; Knowles et al. 2011; Yamashita et al. 2000). Presumably, transmission of AV occurs via the faecal-oral route, and the virus has been identified in cases of gastroenteritis in all continents (Reuter et al. 2011), with most of the outbreaks associated with oysters or seafood (Ambert-Balay et al. 2008; Yamashita et al. 1991, 2000, 2001). The high seroprevalence of AV together with a low detection rate in gastroenteritis suggest a mild clinical illness related to the virus (Reuter et al. 2011). Indeed, in symptomatic cases the virus is often present together with
other pathogens in mixed infections (Ambert-Balay et al. 2008; Räsänen et al. 2010). Occasional detection of AV has also been reported in cases of respiratory tract illness (Reuter et al. 2009; Yamashita et al. 1993).

2 HUMAN RESPIRATORY VIRUSES

Respiratory viruses are the most important causes of morbidity and mortality throughout the world (Fauci & Morens 2012). They create numerous health problems in the developed world, but a greater burden is placed on people in the developing countries, and the majority of disease burdens fall on children and the elderly. Several viruses from different taxonomic families have traditionally been suspected as the causes of respiratory illnesses and are routinely sought in clinical samples. These viruses include influenza A and B viruses (IAV and IBV), parainfluenza viruses 1, 2 and 3 (PIV1–3), respiratory syncytial virus (RSV) and adenovirus (AdV). Moreover, HRVs, HEVs, and human coronaviruses (hCoVs) 229E and OC43 also infect the respiratory tract, but their impact has only recently been recognized as having clinical importance greater than being the causative agents of the common cold. Since 2000, newly discovered viruses including human metapneumovirus (hMPV), severe acute respiratory syndrome CoV (SARS-CoV), hCoVs NL63 and HKU1, parainfluenza virus 4 (PIV4) and human bocavirus (hBoV) have emerged. Although the clinical importance of hBoV is currently not clear, the other viruses mentioned above cause both upper and lower respiratory tract infections with overlapping clinical presentations. Furthermore, several of the viruses may circulate simultaneously in the population and coexist in infected subjects, making differentiation of the causative agent without a laboratory diagnosis impossible (Honkinen et al. 2011; Ruuskanen et al. 2011).
2.1 Influenza viruses

Influenza viruses, classified in the family *Orthomyxoviridae*, are enveloped viruses with segmented RNA genome of negative polarity. These viruses are divided into three genera: *Influenzavirus A*, -B and –C. Influenza viruses are transmitted from person to person via droplets, and through direct or indirect contact with contaminated secretions. IAVs and IBVs cause annual midwinter epidemics of respiratory illness in the temperate regions. The most severe epidemics are caused by IAVs that infect a wide spectrum of birds and mammals, including humans. Aquatic birds are the reservoir of IAV (Shinya et al. 2010) and pigs are thought to be an intermediate host for the generation of reassortants between human and avian viruses (Ito et al. 1998; Nelli et al. 2010). IAVs are further subtyped, based on the antigenic properties of their surface proteins, haemagglutinin (H) and neuraminidase (N). The most recent pandemics have been caused by IAV strains H1N1 and H3N2, which have been cocirculating in humans since 1977 (Medina & García-Sastre 2011).

IAVs and IBVs cause a serious human illness called influenza. The characteristics of influenza are an abrupt onset of febrile respiratory infection accompanied by other systemic symptoms, such as headache, malaise and myalgia. In uncomplicated influenza, systemic symptoms may persist for up to 1 week and respiratory symptoms often remain after fever has subsided. Typically, the highest attack rate of influenza is among young people that possess no immunity to the circulating virus. The mortality associated with influenza infections, due to primary or secondary pneumonia, is highest in the elderly with underlying cardiopulmonary diseases (Fiore et al. 2010). However, during the recent 2009 H1N1 pandemic, young individuals with no underlying medical conditions also suffered from this complicated influenza and comprised a substantial portion of fatal cases (Charu et al. 2011; Mytton et al. 2011).
Table 2. Classification of human respiratory viruses.

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Orthomyxoviridae</strong></td>
<td><strong>Influenza virus A</strong></td>
<td>Influenza A virus</td>
</tr>
<tr>
<td></td>
<td><strong>Influenza virus B</strong></td>
<td>Influenza B virus</td>
</tr>
<tr>
<td></td>
<td><strong>Influenza virus C</strong></td>
<td>Influenza C virus</td>
</tr>
<tr>
<td><strong>Adenoviridae</strong></td>
<td>Mastadenovirus</td>
<td>Adenovirus A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenovirus B1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenovirus B2</td>
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<tr>
<td></td>
<td></td>
<td>Adenovirus C</td>
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<td>Adenovirus D</td>
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<tr>
<td></td>
<td></td>
<td>Adenovirus E</td>
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<tr>
<td></td>
<td></td>
<td>Adenovirus F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenovirus G</td>
</tr>
<tr>
<td><strong>Paramyxoviridae,</strong></td>
<td><strong>Respirovirus</strong></td>
<td>Parainfluenza virus 1</td>
</tr>
<tr>
<td>Subfamily <strong>Paramyxovirinae</strong></td>
<td></td>
<td>Parainfluenza virus 3</td>
</tr>
<tr>
<td></td>
<td><strong>Rubulavirus</strong></td>
<td>Parainfluenza virus 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Parainfluenza virus 4</td>
</tr>
<tr>
<td><strong>Subfamily Pneumovirinae</strong></td>
<td><strong>Pneumovirus</strong></td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metapneumovirus</td>
</tr>
<tr>
<td><strong>Coronaviridae</strong></td>
<td><strong>Coronavirus</strong></td>
<td>Human coronavirus OC43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human coronavirus 229E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human coronavirus NL63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human coronavirus HKU1</td>
</tr>
<tr>
<td><strong>Parvoviridae</strong></td>
<td><strong>Human bocavirus</strong></td>
<td>Human bocavirus 1</td>
</tr>
<tr>
<td>Subfamily <strong>Parvovirinae</strong></td>
<td></td>
<td>Human bocavirus 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human bocavirus 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human bocavirus 4</td>
</tr>
</tbody>
</table>
2.2 Adenoviruses

AdVs are nonenveloped double-stranded DNA viruses belonging to the genus *Mastadenovirus* of the family *Adenoviridae*. There are currently 52 serotypes described and they comprise the seven AdV species designated A–G (Jones et al. 2007). Species B is further divided into B1 and B2, based on haemagglutination properties. AdVs remain infectious for prolonged periods in the environment, enabling efficient transmission of the virus via water and fomites. The virus is stable at low pH and resistant to gastric and biliary secretion, and is therefore capable of replication in the gut (Echavarria 2008).

AdVs occur worldwide and throughout the year. Although the majority of infections of immunocompetent subjects are subclinical, a wide range of clinical illnesses, such as respiratory infections, gastroenteritis, epidemic conjunctivitis and haemorrhagic cystitis, occur in association with these viruses. Diseases are typically mild and resolve without sequelae (Lenaerts et al. 2008). However, in individuals with compromised immunity, AdV infections may be persistent or acute and often result in disseminated and possibly life-threatening disease (Echavarria 2008). The tissue tropism of AdV to some extent coincides with the virus species. Respiratory symptoms are commonly associated with these viruses and AdV serotypes of species B1, C and E are commonly detected in cases of respiratory tract infections (Lenaerts et al. 2008; Selvaraju et al. 2011; Wong et al. 2008).

2.3 Respiratory syncytial virus

RSV is an enveloped virus with a single-stranded RNA of negative polarity. RSV belongs to the genus *Pneumovirus* of the family *Paramyxoviridae* and, as its name indicates, the virus replicates solely in the epithelial cells of the respiratory tract. There is only one serotype of the virus, but based on antigenic properties two subgroups, designated A and B, are distinguished, both of which are generally present in outbreaks (Mufson et al. 1985). Transmission of RSV requires close contact via large-particle aerosols or via fomites (Hall
et al. 1983). RSV activity follows a seasonal pattern in temperate regions, with outbreaks occurring during the winter months throughout the world, although onset, peak and duration of the season may vary from one year to the next.

RSV is a very common respiratory pathogen of children under 1 year of age and virtually all children are infected by the age of 3 years (Choi et al. 2006). RSV is the most frequent cause of paediatric bronchiolitis and pneumonia (Shay et al. 1999). Reinfections occur in older children and adults, but clinical manifestations usually include mild upper respiratory tract illnesses. Infants, the immunocompromised and the elderly are at risk of evolving serious lower respiratory tract disease (Murata & Falsey 2007; Nair et al. 2010). It remains controversial whether subtype A virus is more strongly associated with severe disease and more frequently results in the need for intensive care (Do et al. 2011; Gerna et al. 2008; Perkins et al. 2005; Walsh et al. 1997).

2.4 Parainfluenza viruses

PIVs are enveloped viruses that have single-stranded RNA with negative polarity. They are members of the family *Paramyxoviridae* and are further classified in the Paravirinae subfamily. There are four serotypes of the virus, designated as PIV1–4. Of these, PIV1 and -3 are classified in the genus *Respirovirus* and PIV2 and -4 in *Rubulavirus*. PIV4 is further subdivided into PIV4-A and -B. PIVs are transmitted by aerosolization and contact with contaminated surfaces. The seasonal epidemiology of these respiratory pathogens is dependent on the virus type. PIV1 and -2 occur biennially during winter and early spring, PIV3 circulates throughout the year, with incidence peaking in April or May, and PIV4 is seldom found (Weigl et al. 2007).

PIV1–3 are significant pathogens in subjects of all ages, with predilection for small children. Most PIVs are classically associated with croup (Denny et al. 1983), but they cause a spectrum of respiratory infections and are also associated with otitis media (Marx et
Infections of immunocompetent subjects caused by PIVs are generally mild and only seldom require hospitalization (Reed et al. 1997). Due to the infrequent occurrence of PIV4, the clinical manifestations associated with this serotype are poorly known.

2.5 Human metapneumovirus

hMPV, first described in 2001, is the first human virus in the genus *Metapneumovirus* of the family *Paramyxoviridae* (van den Hoogen et al. 2001). hMPV is an enveloped virus possessing a negative-sense RNA genome. Two distinct genetic groups (A and B) with four distinct genetic subgroups (A1, A2, B1 and B2) of hMPV have been designated, based on the sequence of the fusion protein gene (Biacchesi et al. 2003; van den Hoogen et al. 2004a). Transmission of the virus is thought to occur by contact with contaminated secretions involving aerosols, droplets, or contaminated surfaces. hMPV has a seasonal pattern of circulation, with the virus predominantly occurring in springtime (Choi et al. 2006; García-García et al. 2007).

Although hMPV is also currently recognized as an infectious agent of adults (Walsh et al. 2008), the virus causes both upper and lower airway symptoms, mainly in young children. Children with hMPV infections most commonly exhibit upper respiratory symptoms such as rhinorrhoea, cough or fever (van den Hoogen et al. 2004b). Lower respiratory tract infection occurs more commonly in children less than 1 year of age (Williams et al. 2004), and in infants the virus is often second only to RSV as a causative agent of bronchiolitis (García-García et al. 2007; Williams et al. 2004). The virus has also been described in cases of pneumonia (Nascimento-Carvalho et al. 2011) and in association with asthma exacerbations and wheezing in the paediatric population (García-García et al. 2007; Jartti et al. 2002; Williams et al. 2004, 2005). Other clinical manifestations, such as otitis media (Schildgen et al. 2005a; Williams et al. 2006a), conjunctivitis (van den Hoogen et al. 2004b) and encephalitis (Arnold et al. 2009; Kaida et al. 2006; Schildgen et al. 2005b) have also been described as being in association with hMPV.
2.6 Human coronaviruses

The hCoVs are a large group of viruses in the family *Coronaviridae*. They all are enveloped viruses with a positive-strand RNA-genome. Five hCoV species have been identified. hCoVs 229E and OC43 were already isolated in the 1960s (Hamre & Procknow 1966; McIntosh et al. 1967), and hCoVs were generally considered as benign pathogens responsible for common colds (Bradburne et al. 1967). The mild nature of respiratory disease associated with hCoVs was revisited in 2003 with the isolation of SARS-CoV, the most pathogenic hCoV known today (Drosten et al. 2003; Ksiazek et al. 2003; Peiris et al. 2003). Since SARS-CoV, an additional two hCoVs, NL63 and HKU1, have been identified (van der Hoek et al. 2004; Woo et al. 2005). Phylogenetic analyses have proven these viruses to be old newly found pathogens rather than new emerging viruses (Pyrc et al. 2006). The major routes of hCoV transmission in humans are droplet infection, aerosolization and fomites. All the non-SARS-hCoVs circulate worldwide with large yearly variations in occurrence and incidence, peaking in the winter and spring months (Gerna et al. 2006a; van der Hoek et al. 2010).

Currently, all the non-SARS hCoVs are known to be associated with upper and lower respiratory tract infections, mainly in children (Dominguez et al. 2009; Heugel et al. 2007; Kuyper et al. 2007), with severe lower respiratory tract illnesses occurring in the first years of life (Gerna et al. 2006a). Common diagnoses associated with these viruses include rhinitis, croup, bronchitis, bronchiolitis, wheezing and pneumonia (Gerna et al. 2006a; van der Hoek et al. 2005, 2010). The higher proportion of hCoVs OC43 and NL63 detected in respiratory illnesses may be due to neutralizing antibodies cross-protective against the hCoVs HKU1 and 229E (Dijkman et al. 2012).
2.7 Human bocavirus

hBoV belongs to the subfamily Parvovirinae of the family Parvoviridae. It is a nonenveloped virus with a single-stranded DNA genome. The first of the currently known four hBoV species, hBoV1, was discovered in 2005 (Allander et al. 2005; Arnold et al. 2009; Kapoor et al. 2009, 2010) and circulates year-round, but predominantly during winter and spring (Allander et al. 2005; Choi et al. 2006; Christensen et al. 2010; Fry et al. 2007). The seroprevalence of hBoV is high, with immunity increasing until a 100% prevalence is reached at the age of 7 years (Söderlund-Venermo et al. 2009). The transmission routes of hBoV are not known.

Although hBoV DNA is common in healthy children, increasing evidence suggests a causative role for hBoV1 in upper and lower respiratory tract infections of small children (Christensen et al. 2010; Fry et al. 2007; Kesebir et al. 2006). The virus is also associated with acute otitis media (Lehtoranta et al. 2011) and gastroenteritis (Campe et al. 2008; Yu et al. 2008). Although hBoV2 is occasionally detected in nasopharyngeal samples (NPS) (Han et al. 2009; Song et al. 2010), hBoV2–4 occur predominantly in stool (Arnold et al. 2009; Chieochansin et al. 2009; Chow et al. 2010; Kapoor et al. 2009, 2010). Whether these species are true enteric pathogens is not known.

3 DIAGNOSTIC APPROACHES FOR HUMAN PICORNAVIRUSES AND HUMAN RESPIRATORY VIRUSES

Respiratory tract infections have a variety of aetiological agents and distinguishing them based on clinical presentation is impossible. The same holds for many other illnesses, such as gastroenteritis, a clinical manifestation of several human picornaviruses. Timely diagnosis of the pathogenic agent causing the symptoms is required to facilitate infection control measures and appropriate patient management, including early administration of
antiviral therapy and reduction of unnecessary antibiotics. The wide range of pathogens and requirement for rapid results poses a challenge for laboratory diagnosis. To come up to expectations of specific, sensitive, rapid and cost-effective diagnosis, virology laboratories have four methodological approaches to utilize: virus culture, antigen detection, nucleic acid detection and serology. The test approaches available for virus identification all have their advantages and limitations and choosing a test may require compromising short turnaround time, sensitivity or specificity of detection. Table 3 describes the methods most commonly utilized for diagnosis of human picornaviruses and human respiratory viruses.

3.1 Virus culture

Infectious viruses may be isolated from various human secretions in cell monolayers that support the growth of viruses and then develop cytopathic effect (CPE), virus induced morphological alterations of the host cells. Inoculation of a sample into two or three cell lines enables detection of the majority of cultivable viruses of clinical importance. Since orthomyxoviruses and paramyxoviruses may not produce a visible CPE, a haemadsorption test is used to detect these viruses in cell culture. A viral haemagglutinin, expressed on the plasma membrane of virus-infected cells, mediates attachment to erythrocytes, thereby causing clumping of guinea pig red blood cells when introduced into the culture (Minnich & Ray 1987). Development of CPE usually requires an incubation period of several days, resulting in a long turnaround time of the method. To hasten the detection of viruses, shell vial culture, which utilizes centrifugation of a sample onto the cell monolayer and identification of early viral antigens with fluorescent antibodies, may be used (Espy et al. 1986; Olsen et al. 1993; Rabalais et al. 1992; Van Doornum & Jong 1998).

Virus culture was primarily the way to identify many viruses, and until recently it was the gold standard for detection of respiratory viruses. Currently, however, virus culture is of limited value in diagnosis of human respiratory viruses and picornaviruses, and
Table 3. Methods commonly used for diagnosis of human picornaviruses and human respiratory viruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Methods used for diagnosis&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdV</td>
<td>DFA and virus culture are used for detection of the virus in respiratory samples. Electron microscopy is utilized in analysis of stool samples. PCR is also used for detection of the viral DNA, but sequence heterogeneity makes detection of all serotypes challenging.</td>
</tr>
<tr>
<td>AV</td>
<td>RT-PCR is favored over antigen detection. Serology and analysis of PCR products is performed for identification of prevalent strains.</td>
</tr>
<tr>
<td>HAV</td>
<td>Diagnosis is commonly established by serology. Detection of the viral RNA by RT-PCR may be utilized.</td>
</tr>
<tr>
<td>HEVs</td>
<td>RT-PCR is the current standard for analysis of CSF samples. Virus culture is utilized for analysis of respiratory and stool samples.</td>
</tr>
<tr>
<td>hBoV</td>
<td>Although PCR is widely used for analysis of respiratory samples, mere presence of the viral DNA is insufficient proof of an acute infection, but serology or PCR of serum is required for diagnosis.</td>
</tr>
<tr>
<td>hCoVs</td>
<td>Diagnosis is based on RT-PCR. Serology may be used for diagnosis of SARS-CoV.</td>
</tr>
<tr>
<td>HPeV</td>
<td>RT-PCR is commonly used for diagnosis and virus culture may also be utilized.</td>
</tr>
<tr>
<td>hMPV</td>
<td>Diagnosis relies on detection of the viral genome using RT-PCR. Diagnosis through DFA and virus culture is also available.</td>
</tr>
<tr>
<td>HRVs</td>
<td>RT-PCR is commonly favoured for diagnosis and traditional detection by virus culture is seldom used.</td>
</tr>
<tr>
<td>IAV, IBV</td>
<td>Virus culture is feasible, but is currently downgraded in favour of RT-PCR, which also enables rapid subtyping of the virus. Also DFA, ELISA and rapid LFIC tests for detection of the viral antigens are commonly utilized.</td>
</tr>
<tr>
<td>PIVs</td>
<td>DFA is commonly used for detection of PIV1–3 antigens, although RT-PCR, covering also PIV4, is increasingly applied. Virus culture is also feasible. Serologic differentiation of the PIV types is unreliable.</td>
</tr>
<tr>
<td>RSV</td>
<td>Detection of RSV antigens by DFA or rapid LFIC tests is used for rapid diagnosis. RT-PCR assays are also commonly used and many of them are capable of differentiating subtypes A and B. Lability of the virus limits the use of virus culture.</td>
</tr>
</tbody>
</table>

<sup>a</sup> DFA, direct fluorescent assay; PCR, polymerase chain reaction; RT, reverse transcription; ELISA, enzyme linked immunosorbent assay; LFIC, lateral flow immunochromatography
distinguishing between PVs and other HEVs can be achieved by molecular methods as well as by neutralization typing of a cultured virus. Even if virus isolation methods are currently downgraded in favour of antigen and genome detection for rapid diagnosis, still there is a role for it in diagnostics. Important benefits of the method include the great sensitivity, which allows detection of even one infective virus, and the ability to grow a wide variety of viruses, including unknown viruses. A positive result of virus culture demonstrates the presence of viable virus in the sample, which simplifies interpretation of test results. Additionally, viruses detected in cell culture can be isolated for epidemiological purposes, drug susceptibility assays or typing and assessment of pathogenesis. This is essential for guidance of infection control measures and for annual characterization of influenza virus isolates to direct vaccine development.

3.2 Antigen detection

Viral infections may be demonstrated by visualizing viral proteins in infected cells of clinical samples. In the test, either a specific fluorescein-labelled antibody in direct fluorescent assay (DFA), or a specific primary antibody and a labelled antispecies antibody in indirect fluorescent assay (IFA) are applied to the sample fixed on a microscope slide. The indirect method may be more sensitive, because more label is bound to an infected cell. However, DFA is commonly favoured due to smaller amounts of nonspecific staining and simpler, more rapid performance, enabling turnaround time of 2 hours from sample receipt. DFA is commonly used as a first-line test for diagnosis of respiratory tract infections, whereby a panel of antibodies is utilized to detect RSV, PIV1–3, IAV, IBV and AdV. The limitations of DFA include the labour-intensive nature of the assay, subjective interpretation of test results and poor sensitivity in comparison to viral culture and nucleic acid amplification techniques (Rahman et al. 2008). A particular advantage of the method is that microscopic examination can be used to determine the presence of adequate cell numbers for reliable analysis.
Viral antigens may also be detected in solid-phase systems by enzyme-linked immunosorbent assay (ELISA), in which an antibody bound to a solid phase captures the antigens in the sample. Subsequent enzyme-labelled antibody enables detection and quantification of the antigens via a visible colour reaction. Unlike DFA described above, ELISA tests can also detect cell-free viral antigens, possibly enhancing diagnosis of infection, since most of the infecting virus in a sample may be cell-free (Hendry et al. 1986; Semple et al. 2007). Additional advantages include automation and an objective read-out. ELISA tests were first applied in detection of RSV, AdV and IAV (Harmon & Pawlik 1982; McIntosh et al. 1982) and were commonly applied to detection of respiratory viruses in a microwell format. Currently, simple rapid tests, such as membrane ELISA and lateral flow immunochromatography (LFIC) assays are available for rapid point-of-care testing of RSV and IAV. Both membrane ELISA and LFIC have poor sensitivity, compared with DFA and viral culture (Grijalva et al. 2007; Hurt et al. 2007; Rahman et al. 2008; Uyeki et al. 2009). Therefore, rapid tests are more successful in paediatric populations than in adults, who shed lower titres of virus (Casiano-Colón et al. 2003; Landry & Ferguson 2003; Ohm-Smith et al. 2004; Rahman et al. 2008). Samples with negative rapid test results should be confirmed by culture or PCR. In addition to sensitivity, specificity of the rapid tests is also of concern, especially at times of low disease prevalence when false-positives can exceed true positives. Thus, rapid tests should be delineated for diagnosis of patients with compatible symptoms during seasons of high prevalence.

3.3 Serology

A humoral response generated in all viral infections is an indication of infection and can therefore be used for diagnosis. Serum is the sample type used for analysis, although saliva may also be used for detection of different antibodies (Parry et al. 1987). In cases of suspected CNS infections, a cerebrospinal fluid (CSF) sample is analysed and the antibody ratio is compared with that of serum to confirm the presence of intrathecal antibody synthesis. Serologic diagnosis of a primary infection is made by demonstrating
seroconversion from a negative sample to a positive virus-specific IgG antibody response, or by detecting virus-specific IgM. A fourfold rise in IgG antibody titre between acute and convalescent phase sera is also indicative of a primary infection. In situations where production of IgM is prolonged or is produced in response to reinfection, recent and past infections are distinguished by an antibody avidity test. This is performed using a chaotrophic agent, such as urea, during the ELISA washing state, which causes low-affinity antibodies to preferentially dissociate from the antigen, in contrast to higher-affinity antibodies, that predominate at a later stage of infection. Solid-phase ELISA tests are commonly used for serologic analysis, but haemagglutination inhibition and latex agglutination may also be used.

Serological diagnosis is dependent on the ability of the subject to mount an appropriate immune response to infection, leading to a limited role of serology in diagnosis of immunocompromised individuals, neonates and the elderly. The requirement for paired serum samples to detect the rise in IgG antibody also makes serological diagnosis impractical in acute infections. For these reasons, serology is of limited clinical value. Indeed, detection of the virus itself should be of priority and serological diagnosis be limited to situations in which detection of the virus itself is difficult, time-consuming or where virus excretion is likely to have ceased by the time of investigation. Diagnosis of HAV has largely been established on serology, due to the poor growth of the virus, and even though molecular detection currently is widely available, diagnosis of the virus is still largely based on detection of virus-specific antibodies. In diagnosis of hBoV, for which virus culture is not available and presence of the viral DNA in NPS and stool samples does not constitute proof of current infection, serologic analysis is also central (Lindner et al. 2008; Söderlund-Venermo et al. 2009).
3.4 Nucleic acid detection

Nucleic acid amplification testing is emerging as the preferred approach of diagnostic testing. Real-time technology and the ability to perform multiplex testing have facilitated this emergence, in which multiplex PCR combined with suspension microarrays or DNA chips are the most recent diagnostic advancement. Currently, several methods for amplification of viral genomes, such as nucleic acid sequence-based amplification, transcription-mediated amplification, loop-mediated isothermal amplification and ligase chain reaction, are available, but PCR is still the method most frequently utilized. For all amplification methods, samples are extracted to dissociate the viral genome from the viral capsid and to remove the potential inhibitors of amplification. Extraction may be done by traditional phenol/chloroform extraction or by use of a spin column containing a nucleic acid-binding silica gel-based membrane. Currently, these manual methods are being increasingly replaced by automated extraction devices utilizing nucleic acid-binding magnetic beads.

Nucleic acid amplification techniques have sensitivity superior to that of traditional virus detection methods (Sanghavi et al. 2012) and have enabled detection of pathogens that conventional methods did not reveal. Currently, diagnosis is being increasingly established by PCR, which permits the most sensitive detection of viruses. This has led to situations, in which clinicians need to interpret the results carefully to differentiate between a real pathogen causing the symptoms and an innocent bystander present in the sample.

3.4.1 Conventional PCR

In PCR amplification, annealing of a pair of oligonucleotide primers complementary to the viral DNA is followed by extension of the primers, utilizing a thermostable DNA polymerase (Mullis & Faloona 1987; Saiki et al. 1988). Consecutive temperature cycles of denaturation, annealing and extension phases result in an exponential accumulation of DNA
copies of the target DNA. Viral RNA genomes require transcription to complementary DNA (cDNA) prior to amplification. A reverse transcription (RT) reaction and subsequent amplification may be performed in separate tubes or as a single reaction using a thermostable DNA polymerase with RT activity. The thermal stability of the polymerase permits the RT reaction to be carried out at high temperatures, which improves specificity of the primer-template interaction and increases the efficiency of the RT reaction, due to greater destabilization of the secondary structures of an RNA template. The approach to performing amplification as a single-tube reaction also reduces the risk of cross-contamination.

Although a specific PCR amplification produces an amplicon of known size that can be detected on an agarose gel, this does not verify amplicon authenticity. For this reason and to increase sensitivity (Freymuth et al. 1995; Johnston et al. 1993), nucleic acid hybridization of the amplicon to a labelled oligonucleotide probe, targeted to a conserved sequence of the amplicon, is a better approach. Hybridization may be accomplished through liquid-phase techniques, that can be undertaken within a streptavidin-coated microtitre plate, in which the PCR product binds via a biotinylated primer. Thereafter, addition of a specific enzyme-conjugated probe and a substrate for the enzyme leads to formation of a reaction product that can be measured with a spectrophotometer or a luminometer. This method is labour-intensive, but provides good sensitivity and specificity and is utilized in many commercial PCR assays.

Conventional PCR is inherently a qualitative assay. However, utilizing internal standards for a known number of control genomes, a quantitative competitive assay can generate quantitative value for a clinical sample. The linearity of the reaction determines the dynamic range of an assay and the extremes of the dynamic range are likely to show the greatest variability. Many quantitative competitive assays encounter a problem of quantifying high viral loads while maintaining sensitivity at the lower end of the assay.
Conventional PCR, discussed above is an end-point reaction and detection of the amplification product requires postamplification manipulations. In contrast, detection in real-time PCR occurs within a closed system and without further post-PCR detection proceeds through measuring the increase in fluorescence during amplification. Accumulation of amplicons is detected, using either nonspecific DNA-binding fluorophores, such as SYBR green, or specific fluorophore-labelled oligonucleotide probes that exploit fluorescent resonance energy transfer (FRET) technology. Nonspecific fluorophores bind to any double-stranded DNA in the amplification reaction, but in the subsequent melting curve analysis, a decrease of fluorescence at a specific melting temperature (T\text{M}) verifies amplicon authenticity. In ‘Taqman’ technology, a specific dual-labelled hydrolysis probe binds to the amplicon produced in the reaction. The 5’-exonuclease activity of the DNA polymerase subsequently hydrolys the probe, releasing the fluorophore from its quencher, an event that results in an increase in fluorescence (Morris et al. 1996). In another commonly used system, the ‘LightCycler’, a pair of adjacent fluorogenic hybridization probes is utilized (Wittwer et al. 1997). One of the probes is labelled with a 3’ donor fluorophore, and the other probe is labelled with an acceptor fluorophore at the 5’-terminus. During the amplification reaction, hybridization of the probes to the target sequence results in an increase in fluorescence proportional to the amount of amplicon synthesized.

Although not pivotal in diagnosis of human picornaviruses and respiratory viruses, quantification is essential in detection of many other viruses for which determination of the replication kinetics is of clinical relevance. Real-time PCR is inherently suitable for quantitative PCR. Quantification is based on the number of temperature cycles required for a threshold fluorescent signal to be reached (Threshold cycle, C\text{T}) in comparison to that with an external standard curve. The problem of intervessel variation, related to this approach, may be overcome by including capacity to detect and correct for variation in the emission of an internal reference fluorophore. Moreover, by using internal controls, both extraction and
amplification steps may be monitored. Real-time PCR assays have a wide dynamic range that overcomes the limitation to quantitate both high and low viral loads, a problem encountered by many quantitative competitive reactions. In addition, intra- and interassay variability is reduced in comparison to that in quantitative competitive reactions (Locatelli et al. 2000). Moreover, in real-time PCR results are provided rapidly, because postamplification manipulation of the sample is avoided. Real-time PCR may be coupled with an extraction phase to form a completely automated assay, such as the Jaguar system (BD Diagnostics-GeneOhm; Becton, Dickinson and Co., Franklin Lakes, NJ, USA) (Beck et al. 2010).

3.4.3 Multiplex PCR

Since more than one virus is often sought in a sample, amplification of several viral target sequences in one multiplex PCR assay, using multiple sets of primers, is convenient and cost-efficient. Such an assay requires careful design and optimization to ensure that efficient amplification of any one target is not compromised. The amplicons generated in a multiplex PCR must be identified to differentiate which of the pathogens sought is present in the sample. Although agarose gel electrophoresis may be applied for identification of amplicons with different lengths, the low sensitivity of detection and inability to verify amplicon authenticity require that other approaches be used. Differential detection of the amplicons may be achieved in liquid hybridization when the multiplex amplification reaction is split among several wells of a microtitre plate and a different virus-specific labelled probe is added to each well to determine the virus possibly present in the sample.

Real-time thermocyclers that detect fluorescence at several different wavelengths simultaneously enable identification of amplicons, based on probes that are labelled with different fluorescent molecules. This strategy limits the number of pathogens that can be detected in a single reaction to the number of fluorescent filters on the camera. Moreover, FRET-based assays are limited by the spectral overlap of labels and, in general, multiplex
real-time amplification techniques are limited to differential detection of five targets. Alternatively, probes to different viruses can be designed to have different melting temperatures. Using this approach, the probes of a multiplex assay may be labelled with one dye, and the offending virus identified by the $T_M$ of the probe. To further increase the number of pathogens detected by a single multiplex real-time PCR assay, both multiple dyes and differing $T_M$ values can be incorporated (Beck et al. 2010; Bose et al. 2009).

The tendency for multiple primer and probe sets to reduce amplification efficiency limits multiplexing that employs real-time technology. In attempts to broaden the diagnostic range, one approach is to abandon real-time detection and perform separate amplification and hybridization reactions. With this approach, microarrays that have the potential to resolve complex amplicon mixtures may be utilized, e.g. in the detection of respiratory viruses. In addition to conventional hybridization (Quan et al. 2007), flow-through (Kessler et al. 2004) and resequencing procedures (Lin et al. 2007; Malanoski et al. 2006; Metzgar et al. 2010) are also being applied for species- and strain-level identification of respiratory pathogens on solid-phase microarrays. Recently developed systems for identification of respiratory viruses include the fully automated Infinity (AutoGenomics Inc., Vista, CA, USA) (Raymond et al. 2009) and FilmArray technologies (Idaho Technology, Inc., Salt Lake City, UT, USA), the electronic microarray-based NanoChip system (Nanogen Inc., San Diego, CA, USA) (Li et al. 2007; Takahashi et al. 2008) and TaqMan Low Density Array cards utilizing real-time PCR assays (Kodani et al. 2011).

Suspension microarrays that entail rapid hybridization kinetics and flexibility in formatting the assay for detection of new sequences (Dunbar 2006) may prove useful in contending with evolving viral genomes and implementing the detection of new emerging viruses to daily virus diagnostics. Suspension microarrays employ an array technology known as Luminex® xMAP™ (Luminex Molecular Diagnostics Inc., Toronto, Ontario, Canada), which enables multiplexing of up to 100 analytes based on fluorescent detection of amplicons and identification of bead sets. The PCR products are bound to different beads, either through template-specific probes (Li et al. 2007) or by performing an extension and
labelling reaction to incorporate unique capture sequences used for detection (Lee et al. 2007; Mahony et al. 2007). The Luminex suspension array is the detection platform for common respiratory viruses in the ResPlex II assay from Qiagen (Venlo, the Netherlands) (Brunstein et al. 2008; Li et al. 2007), the MultiCode-PLx respiratory viral panel (RVP) assay (EraGen Biosciences Inc., Madison, WI, USA) (Lee et al. 2007; Nolte et al. 2007), and the xTAG RVP from Luminex Molecular Diagnostics (Mahony et al. 2007; Merante et al. 2007). In the RVP Fast assay, the latest version of the xTAG RVP, the sequential PCR, an exonuclease-phosphatase reaction and target-specific primer extension steps of the original RVP Classic assay (Merante et al. 2007) have been replaced by a PCR amplification using biotin-labelled primers, followed by hybridization of the amplicons to fluorescent beads with specific anti-tag sequences. The biotin label of the amplicons enables attachment of a reporter molecule, streptavidin-R-phycoerythrin. During the detection phase, the beads are identified and the signal from the phycoerythrin is measured as an indicator of the specific amplification product present.
AIMS OF THE STUDY

The overall aim of the study was to develop multiplex RT-PCR assays to facilitate efficient detection of human picornaviruses and human respiratory viruses and to assess the performance of the assays in analysis of clinical samples. To achieve this, the specific aims described below were set.

The aim was to develop an assay based on multiplex RT-PCR and liquid hybridization for simultaneous detection of human picornaviruses and to test the assay performance in the analysis of clinical samples.

For more rapid detection of HEVs and HRVs, the aim was to optimize a real-time duplex RT-PCR assay for detection of HEVs and HRVs and to evaluate the assay for detection of the viruses in respiratory samples.

To obtain rapid and sensitive detection of RSV and hMPV, the aim was to develop a real-time duplex RT-PCR assay for detection of these viruses and to evaluate the assay for detection of the viruses in respiratory samples, with special reference to occurrence of hMPV.

The study aimed at setting up DFA detection of hMPV, utilizing commercial antibodies for detection of the virus.

The aim was to evaluate the performance of the RVP Fast assay in analysis of respiratory samples in comparison to the DFA and real-time RT-PCR assays developed for detection of HEVs, HRVs, hMPV and RSV in the present study.
MATERIALS AND METHODS

1 CLINICAL SAMPLES (I–III)

A set of stool samples was collected at the Department of Virology at the Turku University between January and December 2001 (I). All other clinical samples in the study were sent for daily virus diagnostics to the Department of Virology and Immunology, HUSLAB, Helsinki University Central Hospital. Two sets of respiratory samples were collected during the periods of November 2007 to June 2008 (2007–2008 sample set) (II) and December 2009 to April 2010 (2009–2010 sample set) (III). Nasopharyngeal aspirate (NPA) and bronchoalveolar lavage (BAL) samples were collected from paediatric and adult patients with respiratory symptoms. An additional 42 respiratory samples that were positive for IAV by real-time RT-PCR (Rönkkö et al. 2011; Ward et al. 2004) and 34 samples positive for either IBV or PIV3 by DFA were analysed in evaluation of the RVP Fast assay (III). These samples were collected in 2010 and 2011. Moreover, four isolates of AdV, two strains of AdV3, one AdV5 strain and one AdV7 strain from patient samples were analysed with the RVP Fast assay. All clinical samples analysed in the study are described in Table 4 and further information on the sample sets is also provided in the Results and Discussion.

2 QUALITY ASSURANCE SAMPLES (II, III)

Eight panels of quality assurance samples provided by Quality Control for Molecular Diagnostics (QCMD; Glasgow, Scotland, UK) and a Nucleic Acid Test Controls and Calibrators NATrol Respiratory Validation Panel 2 (NATRVP-2 Global Panel) by Zeptometrix Co. (Buffalo, NY, USA) were available to the present study. A detailed description of the viruses included in the quality assurance panels analysed is presented in Table 5. Many of the viruses were present at several dilutions in the panels. Analysis of the samples was performed to assess the sensitivity and specificity of the duplex real-time RSV/hMPV RT-PCR (II) and the duplex real-time HEV/HRV RT-PCR (III).
### Table 4. Clinical samples analysed in the study.

<table>
<thead>
<tr>
<th>Year</th>
<th>Type (and number) of samples</th>
<th>Viruses tested for</th>
<th>Method used in analysis (Original publication)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>CSF (14)</td>
<td>AV, HEV, HPeV, HRV</td>
<td>multiplex RT-PCR and liquid hybridization&lt;sup&gt;a&lt;/sup&gt; (I)</td>
</tr>
<tr>
<td></td>
<td>NPS (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>serum (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>stool (68)</td>
<td>AV, HPeV</td>
<td>single RT-PCR and liquid hybridization&lt;sup&gt;a&lt;/sup&gt; (I)</td>
</tr>
<tr>
<td>2007-2008</td>
<td>BAL (24)</td>
<td>AdV, IAV, IBV, PIV1-3, RSV</td>
<td>DFA in routine diagnostics (II)</td>
</tr>
<tr>
<td></td>
<td>NPA (326)</td>
<td>hMPV</td>
<td>DFA set up in the present study (II)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hMPV, RSV</td>
<td>duplex real-time RT-PCR&lt;sup&gt;a&lt;/sup&gt; (II)</td>
</tr>
<tr>
<td>2009-2010</td>
<td>BAL (13)</td>
<td>AdV, IAV, IBV, PIV1-3, RSV</td>
<td>DFA in routine diagnostics (III)</td>
</tr>
<tr>
<td></td>
<td>NPA (284)</td>
<td>HEV/HRV, hMPV, RSV,</td>
<td>duplex real-time RT-PCR&lt;sup&gt;a&lt;/sup&gt; (III)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AdV, IAV, IBV, PIV1-4,</td>
<td>RVP Fast (III)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RSV, HEV/HRV, hMPV, hBoV,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>hCoV 229E, OC43, NL63, HKU1</td>
<td></td>
</tr>
<tr>
<td>2009-2010</td>
<td>NPA (42)</td>
<td>IAV (H1N1)2009</td>
<td>real-time RT-PCR&lt;sup&gt;b&lt;/sup&gt; (III)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AdV, IAV, IBV, PIV1-4,</td>
<td>RVP Fast (III)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RSV, HEV/HRV, hMPV, hBoV,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>hCoV 229E, OC43, NL63, HKU1</td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>NPA (34)</td>
<td>AdV, IAV, IBV, PIV1-3, RSV</td>
<td>DFA in routine diagnostics (III)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AdV, IAV, IBV, PIV1-4,</td>
<td>RVP Fast (III)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RSV, HEV/HRV, hMPV, hBoV,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>hCoV 229E, OC43, NL63, HKU1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The assay developed in the present study

<sup>b</sup> The assay described by Ward et al. 2004 applied for analysis

<sup>c</sup> The assay described by Rönkkö et al. 2011 applied for analysis
Table 5. Quality assurance samples analysed in the study.

<table>
<thead>
<tr>
<th>Quality program/panel, Supplier</th>
<th>Year</th>
<th>Viruses present in the panel</th>
<th>Assay used in analysis, (Original publication)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdV program, QCMD</td>
<td>2006</td>
<td>AdV4, AdV5, AdV7</td>
<td>duplex real-time RSV/hMPV RT-PCR&lt;sup&gt;a&lt;/sup&gt; (II)</td>
</tr>
<tr>
<td>HEV program, QCMD</td>
<td>2006</td>
<td>Echoviruses 16 and 30, CVB3</td>
<td>duplex real-time RSV/hMPV RT-PCR&lt;sup&gt;a&lt;/sup&gt; (II)</td>
</tr>
<tr>
<td>hMPV &amp; RSV program, QCMD</td>
<td>2006</td>
<td>hMPV-B1, hMPV-A1, RSV-A, RSV-B</td>
<td>duplex real-time RSV/hMPV RT-PCR&lt;sup&gt;a&lt;/sup&gt; (II)</td>
</tr>
<tr>
<td>hMPV &amp; RSV program, QCMD</td>
<td>2007</td>
<td>hMPV-I, hMPV-II, RSV-A, RSV-B</td>
<td>duplex real-time RSV/hMPV RT-PCR&lt;sup&gt;a&lt;/sup&gt; (II)</td>
</tr>
<tr>
<td>HEV &amp; HPeV program, QCMD</td>
<td>2009</td>
<td>Echoviruses 11 and 16, HEV71, CVA16, CVB3, PV3</td>
<td>duplex real-time HEV/HRV RT-PCR&lt;sup&gt;b&lt;/sup&gt; (III)</td>
</tr>
<tr>
<td>HRV &amp; hCoV program, QCMD</td>
<td>2009</td>
<td>HRVs 16, 72 and 90, hCoV NL63 and OC43</td>
<td>duplex real-time HEV/HRV RT-PCR&lt;sup&gt;b&lt;/sup&gt; (III)</td>
</tr>
<tr>
<td>HEV &amp; HPeV program, QCMD</td>
<td>2010</td>
<td>Echoviruses 11 and 30, HEV71, CVA9, CVB3</td>
<td>duplex real-time HEV/HRV RT-PCR&lt;sup&gt;b&lt;/sup&gt; (III)</td>
</tr>
<tr>
<td>HRV &amp; hCoV program, QCMD</td>
<td>2010</td>
<td>HRVs 8, 16, 42, 72 and 90, CVA21, hCoVs 229E, OC43 and NL63</td>
<td>duplex real-time HEV/HRV RT-PCR&lt;sup&gt;b&lt;/sup&gt; (III)</td>
</tr>
<tr>
<td>NATRVP-2 Global Panel, Zeptometrix</td>
<td>2010</td>
<td>IAV H1N1, IAV H3N2, IBV, RSV-A, RSV-B, PIV1-3, AdV7A, HEV/HRV, hCoVs 229E and OC43, SARS-CoV</td>
<td>RVP Fast (III)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The assay developed in the present study

3 NUCLEIC ACID EXTRACTION (I–III)

Nucleic acids from the samples collected during 2001 were isolated by phenol/chloroform extraction from 200 µl of sample. The resulting precipitate of CSF samples was resuspended in 22 µl of sterile water. For other sample materials, an elution volume of 100 µl was used.
For the 2007–2008 sample set and the quality assurance samples of the 2006 and 2007 panels, nucleic acid isolation from 200 µl of sample was performed, using a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer’s instructions. The resulting extracts were immediately subjected to the duplex real-time RSV/hMPV RT-PCR assay.

For the 2009–2010 sample set, the additional 76 respiratory samples from 2009 to 2011 and the four AdV isolates, total nucleic acid isolation was performed by a MagNA Pure LC instrument (Roche Diagnostics, Basel, Switzerland). A schematic presentation of the work flow in the 2009–2010 sample set is shown in Figure 1. Briefly, 200 µl of sample was subjected to external lysis for 45 min by addition of 200 µl of lysis buffer. Preceding the external lysis, 20 µl of internal positive control (Escherichia coli phage MS2) provided by the manufacturer (Luminex) and 5 µg carrier RNA (Qiagen) per 200 µl of sample were added to the lysis buffer. Finally, the purified nucleic acids were eluted in 55 µl of low-salt elution buffer. The extracts were immediately subjected to the RVP Fast assay (Luminex) and the duplex real-time RSV/hMPV RT-PCR assay. An aliquot of the extracts was stored at -70 °C to be later subjected to the duplex real-time HEV/HRV RT-PCR assay. Similar isolation was performed for the additional 76 respiratory samples and the four AdV isolates analysed.

Figure 1. Schematic presentation of the work flow in the 2009–2010 sample set (III).
4 PRODUCTION OF VIRAL RNAs (I–III)

Viral RNA transcripts were used for optimization of the multiplex RT-PCR assays and determination of the detection limits of the assays. These transcripts were produced by *in vitro* transcription of the viral cDNA clones and PCR products of the quality assurance samples.

4.1 RNA transcripts of the picornaviruses (I, III)

RNA transcripts of the picornaviruses were produced from recombinant plasmids containing cDNA copies of the viral genomes. The AV cDNA (strain A844/88) was provided by Dr. T. Yamashita (Aichi Prefectural Institute of Public Health, Aichi, Japan), the cDNA clone of HAV (strain HM-175/7) was from Dr. R. H. Purcell (Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, MD, USA), HPeV1 (Harris strain) and HRV1B cDNA clones were from Dr. G. Stanway (Department of Biological Sciences, University of Essex, Colchester, United Kingdom), while the echovirus 11 (Gregory strain) cDNA was constructed by Dahllund et al. (1995).

The recombinant plasmids were propagated in *Echerichia coli* strain DH5α. The purified plasmids (Qiagen Plasmid Maxi Kit) were linearized with restriction endonucleases (New England Biolabs, Ipswich, MA, USA) and transcribed with T7 RNA polymerase (Promega Corp., Fitchburg, WI, USA), with the exception of the SP6 RNA polymerase used for the HAV template, according to the manufacturer’s instructions. The template plasmids were degraded in DNase (Promega) incubation for 15 min at 37 °C, the RNAs were purified with a Qiagen RNeasy Mini Kit (Qiagen) and analysed by RNA agarose gel electrophoresis. The RNA concentrations were determined by measuring the optical density at 260 nm.
4.2 RNA transcripts of RSV and hMPV (II)

An RNA transcript of RSV was produced from RSV strain B/Wash/18537/’62 (CH18537) (ATCC No. VR-1401) obtained from the American Type Culture Collection. For transcription of hMPV RNA, hMPV type B1 (strain NL/1/99) and type A1 (strain NL/1/00), both obtained as quality control samples of the 2006 Metapneumovirus and Respiratory Syncytial virus RNA Proficiency Programme (QCMD), were used.

For production of viral RNAs, the viral templates were amplified in RT-PCR, using the primers RSV-CF, RSV-CR, MPV-CF and MPV-CR targeted at conserved regions of the viral genomes (Table 6). Following RT-PCR, a transcription reaction using T3 RNA polymerase (AmpliScribe™ T3-Flash Transcription Kit; Epicentre Biotechnologies, Madison, WI, USA) was performed according to the manufacturer’s instructions. The template DNAs were degraded with DNase (Turbo DNA-free™ Kit; Ambion Inc., Austin, TX, USA) and the RNAs were purified with an RNeasy Mini Kit (Qiagen) and analysed in agarose gel electrophoresis. The concentrations of the RNA transcripts were determined by measuring the optical density at 260 nm.

Table 6. Primers used for production of RSV and hMPV transcripts.

<table>
<thead>
<tr>
<th>Target virus</th>
<th>Oligonucleotide designation</th>
<th>Sequence (5’→ 3’), orientationa</th>
<th>Original publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV</td>
<td>Primer RSV-CFb</td>
<td>GATGGGGCAATATGGAACAA, +</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Primer RSV-CR</td>
<td>GATTGCAAATCGTGTAGCTGT, -</td>
<td>II</td>
</tr>
<tr>
<td>hMPV</td>
<td>Primer MPV-CFb</td>
<td>AATGTCTCTTCAAGGGATTCAC, +</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Primer MPV-CR</td>
<td>GTTCTGCAGCTTTTTTTTCCTTC, -</td>
<td>II</td>
</tr>
</tbody>
</table>

a +, sense; -, antisense
b T3 RNA polymerase promoter sequence AATTAACCCCTCCTAAAGGGAGA before the viral sequence
Optimization of the multiplex RT-PCR involved resolving the optimal concentrations of the reaction components as well as the best thermal cycling conditions for amplification of the viral targets to occur.

5.1 Multiplex RT-PCR and liquid hybridization for human picornaviruses (I)

For detection of human picornaviruses, an assay consisting of a multiplex RT-PCR followed by differential detection of the amplicons by liquid hybridization, was developed. The primers for amplification of HEV, HRV, AV, HPeV and HAV were all designed from the conserved 5’untranslated region (5’UTR) of the viral genomes. The sequences of the primers are provided in Table 7 and in Table 1 (I).

5.1.1 RT reactions (I)

For RT reactions of single viral targets, the reaction mixture (total volume of 20 µl) contained the following components: 1.25 µM of the antisense primer (primer 4-, Hpev1-, Aichi 1- or Hav-1), 1 mM deoxyribonucleotide triphosphates (dNTPs) (Amersham Biosciences, Amersham, UK; now GE Healthcare, Little Chalfont, Buckinghamshire, England), 50 mM Tris-HCl; pH 8.4, 40 mM KCl, 5 mM MgCl₂, 0.5 % Tween, 20 U RNasin® Ribonuclease Inhibitor (Promega) and 50 U Expand Reverse Transcriptase (Roche). Prior to the reaction, template RNA, dNTPs and the negative strand primer were denatured at 85 ºC for 3 min, cooled on ice, and the other reaction components were added. RT reactions were carried out at 42 ºC for 60 min.

The reaction mixture for simultaneous RT reaction of the viral targets consisted of 1.25 µM of the antisense primers, 1 mM dNTPs (Amersham Biosciences), 50 mM Tris-Ac; pH 8.4, 75 mM KAc, 8 mM MgAc₂, 10 mM dithiothreitol (DTT), 20 U RNase OUT (Invitrogen
Corp. (now Life Technologies), Carlsbad, CA, USA) and 15 U ThermoScript RT (Invitrogen) in a total volume of 20 µl. Prior to the RT reaction, template RNA, dNTPs and the primer were denatured at 85 ºC for 3 min, cooled on ice, and the other reaction components were added. The RT reaction occurred at 65 ºC for 60 min, followed by a 5-min termination step at 85 ºC.

Table 7. Primers used in RT-PCR.

<table>
<thead>
<tr>
<th>Target virus</th>
<th>Oligonucleotide designation</th>
<th>Oligonucleotide Sequence (5’→ 3’), orientation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Original publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEV/HRV</td>
<td>Primer 3B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Biotin-CGGCCCCTGAATGCAGCTAA, +</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Primer 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GAAACACGGACACCCAAAGTA, -</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Primer EV-F1</td>
<td>GACATGTTGTYGAAGAGTCTATTGAG, +</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Primer EV-F2</td>
<td>GACATGTTGTYGAAGAGTCTATTGAGCT, +</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Primer RV-F1</td>
<td>AGGTGTGAAGAGCCCCGTGT, +</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Primer RV-F2</td>
<td>AAGGTTGAAGAGGCCCGTGTT, +</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Primer picorna-R</td>
<td>GAAACACGGACACCCAAAGTAGT, -</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Probe EV</td>
<td>Fam-CGGCCCCTGAATGCAGCTAAATCC, +</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Probe RV</td>
<td>Hex-CGGCCCCTGAATGYYGGCTAATCC, +</td>
<td>III</td>
</tr>
<tr>
<td>HPeV</td>
<td>Hpev 1B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Biotin-TGCCTCTTGGGGCAAAGAAG, +</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Hpev 1-</td>
<td>CAGGGATCCCCCCTGGGT, -</td>
<td>I</td>
</tr>
<tr>
<td>AV</td>
<td>Aichi 1B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Biotin-GAGTGCTGTTCCTCCCAAGCC, +</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Aichi 1-</td>
<td>TTAGCCAGGTCCCCGTCATCC, -</td>
<td>I</td>
</tr>
<tr>
<td>HAV</td>
<td>Hav 1B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Biotin-ACGGGTGAAACCTCTTAGGC, +</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Hav 1-</td>
<td>TGCCCTAAGACAGAGAGG, -</td>
<td>I</td>
</tr>
<tr>
<td>RSV</td>
<td>RSV-F</td>
<td>TGGAACACATACGTGAAACAARCTTCA, +</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>RSV-R</td>
<td>GCACCCATATGTGTWAGTGATGC, -</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Probe RSV</td>
<td>CGAAGGCTCCACACACACAGCGGTGCTGT, +</td>
<td>II</td>
</tr>
<tr>
<td>hMPV</td>
<td>Primer MPV-F&lt;sup&gt;c&lt;/sup&gt;</td>
<td>TCATATAAGCATGCTATTAAAAAGAGTCTCA, +</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Primer MPV-R&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CCTATYCTGCAGCATATTGTGTAATCAG, -</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Probe MPV-A</td>
<td>ACAAAACACTGCAGTCACCCCTCTACATT, +</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Probe MPV-B</td>
<td>ACCAAACACTGCAGTCACCCCTCTACATT, +</td>
<td>II</td>
</tr>
</tbody>
</table>

<sup>a</sup> +, sense; -, antisense

<sup>b</sup> Lönnrot et al. 1999

<sup>c</sup> Adapted and modified from Maertzdorf et al. 2004
5.1.2 PCR (I)

For PCR amplification, 10 µl of the RT reaction product was added to the PCR mixture containing the primer pairs at 0.5 µM concentrations, 200 µM dNTPs (Amersham Biosciences), 10 mM Tris-HCl; pH 8.3, 50 mM KCl, 1.5 mM MgCl₂ and 7.5 U AmpliTaq Gold polymerase (Roche). The PCR was performed in a total volume of 100 µl using touch-down amplification. After primary denaturation at 95 ºC for 7 min, the cycling conditions were the following: denaturation at 94 ºC for 40 s, annealing for 40 s starting from 63 ºC, followed by 1 ºC decrease per cycle and elongation at 72 ºC for 40 s. The steps were repeated eight times, and thereafter 45 additional cycles were conducted, using the same cycling conditions except for annealing, in which a temperature of 54 ºC was used. The amplification products were analysed in agarose gel electrophoresis prior to detection in liquid hybridization. Analysis of CSF and NPS was repeated one to two times in separate RT-PCR assays depending on the volume of the sample.

5.1.3 Liquid hybridization (I)

The liquid hybridization step was performed in streptavidin-coated wells (Thermo Labsystems Ltd., Vantaa, Finland) and the multiplex amplification reaction was split among several wells to achieve separate detection reactions with each probe in duplicate. The probes used in the assay are described here in Table 8 and in Table 1 (I).

For detection of the biotinylated amplicons, 10 µl of the amplification reaction was added to streptavidin-coated wells containing 40 µl of binding buffer (25 mM Tris-HCl, pH 7.5, 125 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 % Tween 20 and 0.5 × Denhardt’s solution). The plates were incubated at 22 ºC for 30 min with agitation (650 rpm), 50 µl of elution buffer (100 mM NaOH, 300 mM NaCl) was added and incubation was continued for 1 min. After washing the plate with Buffer 1 (0.25 M Tris-
Table 8. Probes used in liquid hybridization.

<table>
<thead>
<tr>
<th>Target virus</th>
<th>Oligonucleotide designation</th>
<th>Sequence (5’→ 3’), orientation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Original publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEV</td>
<td>Probe HEV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>TAITCGGTTCCGCTGC, -</td>
<td>I</td>
</tr>
<tr>
<td>HRV</td>
<td>Probe HRV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>TAGTTGGTCCCCITCCCG, -</td>
<td>I</td>
</tr>
<tr>
<td>HPeV</td>
<td>Probe HPEV</td>
<td>GCCCCAGATCAGATCCA, -</td>
<td>I</td>
</tr>
<tr>
<td>AV</td>
<td>Probe AV</td>
<td>ATCACTACGGTCGGGAG, -</td>
<td>I</td>
</tr>
<tr>
<td>HAV</td>
<td>Probe HAV</td>
<td>CACTCAATGATCCACTG, -</td>
<td>I</td>
</tr>
</tbody>
</table>

<sup>a</sup> +, sense; -, antisense  
<sup>b</sup> Reported by Lönnrot et al. 1999

HCl, pH 7.5, 1.25 M NaCl, 20 mM MgCl₂, 3% Tween 20), 50 µl of hybridization buffer (0.1% sodium dodecyl sulphate (SDS), 5 × saline sodium citrate (SSC), 1 × Denhardt’s solution) containing 6.7 fmol of appropriate probe was added and incubated at 42 °C for 30 min with agitation (650 rpm). Unbound probe was removed by washing six times with Buffer 2 (0.05 × SSC, 0.3% Tween 20). After this, 50 µl of conjugation buffer (25 mM Tris-HCl, pH 7.5, 125 mM NaCl, 2 mM MgCl₂, 0.3% Tween 20, 1% BSA) containing 5 mU of antidigoxigenin-alkaline phosphatase conjugate (Roche) was added and incubated at 22 °C for 30 min, and the wells were washed six times with Buffer 1. A 50-µl volume of Lumiphos 538 substrate (Lumigen Inc., Southfield, MI, USA) was added to the wells and incubated for 35 min at room temperature protected from light, and the resulting luminescence was measured (Luminoskan RS Microplate reader; Thermo Labsystems). A schematic presentation of the liquid hybridization reaction is shown in Figure 2.
5.2 Real-time duplex RT-PCR for detection of HEVs and HRVs (III)

For the real-time detection of HEVs and HRVs, three primers and two probes, described in Table 7 and III, were designed. Furthermore, two primers, EV-F2 and RV-F2, were used for reanalysis of clinical samples with discordant results. The reaction mixture for single RT-PCR included 300 µM dNTPs (600 µM for deoxyuridine triphosphate (dUTP)), 50 mM Bicine, 115 mM KAc, 0.01 mM EDTA, 60 nM Rox, 3.0 mM MnAc, 5 U *Tth* DNA polymerase and 0.5 U uracil N-glycosylase (UNG) in a total volume of 50 µl (TaqMan® EZ RT-PCR Kit; Applied Biosystems). For the duplex RT-PCR, an elevated concentration of 4.0 mM MnAc and 2 U of Ampli Taq Gold polymerase (Roche) were used. A concentration of 300 nM was used for primer EV-F1, 400 nm for RV-F1 and 700 nm for antisense primer picorna-R. The optimal concentrations for the EV probe and RV probe were 150 nM and 200 nM, respectively.

Amplification was performed on a Stratagene MXP3000 (Stratagene Corp., La Jolla, CA, USA) in duplicate reactions, except in the analysis of clinical samples, in which three reactions were performed on each sample: one for HEV RT-PCR, one for HRV RT-PCR and one for duplex RT-PCR. A UNG treatment of 2 min at 50 ºC was performed to remove uracil from the deoxyuridine monophosphates incorporated into any contaminating molecules, followed by an RT reaction at 60 ºC for 40 min and inactivation of UNG at 95 ºC for 5 min. The cycling conditions were the following: denaturation at 94 ºC for 20 s and annealing and extension at 60 ºC for 60 s. These steps were repeated 45 times.

The precision of the assay was studied in analysis of dilution series of the RNA transcripts of E11 and HRV1B, corresponding to $1 \times 10^3$, $1 \times 10^4$, $1 \times 10^5$, $1 \times 10^7$ and $1 \times 10^9$ genome equivalents per reaction. Intra-assay variability was evaluated by running five parallel reactions of the dilution series on one plate. For determination of interassay variability, one dilution series was tested on 4 consecutive days.
5.3 Real-time duplex RT-PCR for detection of RSV and hMPV (II)

For real-time detection, two primer sets for amplification of RSV (primers RSV-F and RSV-R) and hMPV (primers MPV-F and MPV-R) genome regions were designed (Table 7). The reaction conditions and cycling conditions of the real-time duplex RSV/hMPV RT-PCR were as described for the duplex HEV/HRV RT-PCR above (section 3.2), except that 4.5 mM of manganese was used. A concentration of 500 nM was used for primers RSV-F, RSV-R and MPV-R, and 700 nM concentration for MPV-F. The optimal concentrations for the combined MPV-A and MPV-B probes and the RSV-probe were 100 nM and 150 nM respectively. A 10-µl volume of the viral template was used in a total volume of 50 µl reaction mixture.

6 DIRECT FLUORESCENT ASSAY (II, III)

Cells of the clinical samples were pelleted by centrifugation, placed on two slides, dried and fixed in cold acetone for 10 min. One of the slides was tested for AdV, IAV, IBV, RSV and PIV1-3 as primarily requested, using a Light Diagnostics™ Respiratory DFA Viral Screening and Identification Kit (Millipore Corp., Billerica, MA, USA). The other slide was stored at -70 ºC and later tested for the presence of hMPV antigen using four antibodies: anti-hMPV (Argene, Massapequa, NY, USA; now BioMérieux, Marcy l’Etoile, France); D³ DFA Metapneumovirus Identification Kit (Diagnostic Hybrids, Inc., Athens, OH, USA); Imagen™ hMPV (Oxoid Ltd, Basingstoke, Hampshire, UK) and hMPV DFA Reagent (Light Diagnostics™). Of these the D³ DFA is a mixture of three hMPV-specific murine monoclonal antibodies (Percivalle et al. 2005).

7 RVP FAST ASSAY (III)

The RVP Fast assay was performed according to the manufacturer’s instructions. A schematic presentation of the assay is shown in Figure 3. Briefly, a multiplex RT-PCR was performed. To monitor the assay performance, a positive run control (bacteriophage lambda
DNA) provided by the manufacturer (Luminex) was amplified in each run. After applying a bead mix (provided), the amplification products and a reporter solution (provided) to a 96-well plate, a bead hybridization reaction was conducted for 20 min at 45 °C. Thereafter, the median fluorescence intensities (MFIs) were analysed by the Luminex 100 IS system and Tag Data Analysis Software (TDAS) RVP Fast version 2.00 (Luminex). The RVP Fast assay detects IAV, IBV, RSV, PIV1–4, AdV, hMPV, HEV/HRV, hBoV, and hCoVs 229E, OC43, NL63, and HKU1. The assay also allows subtyping of IAV to prepandemic seasonal H1 or H3 virus.

Figure 3. Illustration of the procedure of the RVP Fast assay. Tagged amplification products are captured onto beads by complementary anti-tags. The biotin label incorporated into the amplicons enables attachment of a reporter molecule streptavidin-phycoerythrin (SA-PE). During the detection phase, beads (and hence anti-tags) are identified and the signal from phycoerythrin is measured as an indicator of the specific amplification product present.

8 STATISTICS (III)

Concordance of the results obtained for the clinical samples by the RVP Fast assay and DFA, as well as RVP Fast and the real-time multiplex RT-PCR assays, was examined by McNemar’s test using the SPSS/PASW statistical program package (version 18; IBM SPSS Inc., Chicago, IL, USA). Statistical significance was set at P < 0.05 and the kappa value was computed to assess the agreement between the methods.
RESULTS AND DISCUSSION

1 MULTIPLEX RT-PCR ASSAYS FOR DETECTION OF HUMAN PICORNAVIRUSES (I, III)

The aim was to set up an assay for detection of human picornaviruses (I) in CFS, NPS, serum and stool samples, using laboratory facilities suitable for routine daily diagnostics at the Virology Laboratory of HUSLAB, Helsinki University Central Hospital. Later, since the daily diagnostics started to shift towards real-time detection of pathogens, the aim was set to optimize a real-time multiplex assay for detection of HEVs and HRVs in NPS (publication III).

1.1 Performance of the multiplex RT-PCR and liquid hybridization assay (I)

In the present study, a multiplex RT-PCR and liquid hybridization assay for detection of HEVs, HRVs, HPeVs and AV was accomplished. In vitro RNA transcripts of E11, HRV1B, HPeV1, AV and HAV described in section 4.1 were produced for optimization of reaction conditions and assessment of the sensitivity and precision of the assay. Combining the single reactions to achieve a multiplex assay required optimization of the RT reaction, and simultaneous amplification of all other viral target RNAs, except HAV RNA, was achieved by raising the temperature of the RT reaction to 65 ºC instead of the 42 ºC used in the single RT-PCRs. Although successful performance of the multiplex RT reaction required exclusion of the HAV primer, a separate assay for detection of HAV was achieved. In contrast to the RT reaction, the conditions of the single amplification reactions also worked well in a multiplex format and no further optimization was performed.
1.1.1 Analytical sensitivity and precision of the multiplex RT-PCR and liquid hybridization assay (I)

For detection of HEVs and HRVs, primers previously described by Lönnrot et al. (1999) were used. Several primers for amplification of HPeVs, AV and HAV genomes were designed from the conserved 5’UTR region of the viral genomes, which are commonly used for detection of picornavirus species (Chapman et al. 1990; Rotbart et al. 1994, 1997). The primers described in Table 7 achieved the most sensitive amplification of the viral targets in the corresponding single amplification reactions. The multiplex RT-PCR produced amplification products visible on agarose gel when a 100 genome equivalents of HEV, HRV, HPeV or AV RNA transcript per reaction was used as a template in repeated experiments (Fig. 1B in I). In the corresponding single amplification reactions, a sensitivity of 10 genome equivalents was achieved for HEV and HRV. For HPeV and AV, more efficient amplification in single reactions was also observed as an increase of amplicon intensity on agarose gel, although 100 genome equivalents produced visible amplicons both in single and multiplex reactions. The drop in sensitivity observed for the multiplex amplification reaction was not particularly a drawback for the present study, but rather a well-known phenomenon associated with multiplex PCRs in general (Beck & Henrickson 2010). The single RT-PCR for detection of HAV also exhibited high sensitivity, since 10 genome equivalents of RNA transcript per reaction produced an amplicon perceptible to the eye in agarose gel electrophoresis and also detectable in liquid hybridization.

Several probes for detection of amplification products in liquid hybridization were designed and the ones described in Table 8 were the most sensitive, with minimal signal from the other amplicons produced in the multiplex RT-PCR. In the liquid hybridization step of the assay, HEV and HRV amplicons from the multiplex RT-PCR, corresponding to 10 genome equivalents of viral RNA transcripts, were detected and the sensitivity of HPeV and AV detection varied from 10 to 100 genome equivalents (Fig. 3A I). Thus, for the detection of HEV and HRV, the hybridization step of the assay provided a tenfold increase in sensitivity,
as described in literature (Freymuth et al. 1995). When the fluorescent signals for the multiplex PCR amplification products were scrutinized, a decreased level of luminescence was observed compared with the amplicons of the corresponding single PCR assays. This decrease in luminescence probably resulted from the lower amplification efficiency of the target sequences in the multiplex RT-PCR, which was demonstrated in agarose gel electrophoresis (Fig. 1 in I). However, the effect of purification of the amplicons to remove the excess biotinylated primers was not examined and this could have improved the performance of the liquid hybridization in terms of increased luminescent signal. Concerning the precision of the assay, a variations of 0.94%, 4.83%, 4.44% and 5.87% of the mean relative luminescence unit (RLU) value were observed when amplicons corresponding to 10–1000 genome equivalents of RNA transcripts of E11, HRV1B, HPeV1 and AV, respectively, were analysed in parallel liquid hybridization reactions. In detection of amplicons corresponding to 10 genome equivalents of RNA per amplification reaction, the lowest detection limit of the liquid hybridization assay, variations of 1.75–4.95% of the mean RLU were observed. The values described above may be considered to reflect a favourable intra-assay precision of the assay, since reasonable variability was also shown in the lowest extreme of detection. Variation in RLUs from amplification products of parallel RT-PCR was not determined.

Since coinfections are increasingly recognized as a common event, the effect of simultaneous amplification of several viral targets on the sensitivity of the assay was assessed. When the multiplex RT-PCR followed by liquid hybridization was used for simultaneous detection of picornaviruses possibly present in respiratory samples, HEVs, HRVs and HPeVs, the overall sensitivity of the assay decreased, allowing detection of 1000, 10 and 1000 genome equivalents of viral RNA, respectively (Fig. 3B in I). Here again, an increase in sensitivity was provided by the liquid hybridization assay, since amplicons coinciding with 100–1000 genome copies per multiplex RT-PCR were perceptible in agarose gel electrophoresis. Simultaneous amplification of enteric picornaviruses, HEVs, HPeVs and AV also resulted in decreased sensitivities of 1000, 1000 and 10 genome equivalents of RNA in the liquid hybridization assay, respectively.
1.1.2 Clinical sensitivity and specificity of the assay (I)

To assess the clinical sensitivity and specificity of the multiplex RT-PCR and liquid hybridization, 91 clinical samples described in detail in Table 4 were analysed. In the analysis, samples positive for HEV or HRV in routine diagnostics at the Virology Laboratory of HUSLAB, Helsinki University Central Hospital were also positive by the multiplex RT-PCR and liquid hybridization assay (Table 2 in I). These findings included five CSF samples and one serum sample positive for HEV and five HRV-positive NPS. Furthermore, the three sera and nine CSF samples, tested negative for HEV and HRV in daily diagnostics, remained negative when analysed with the multiplex RT-PCR and liquid hybridization assay (Table 2 in I). Of the 68 stool samples submitted for gastroenteritis investigations and screened for the presence of HPeV and AV, one sample from a 1-month-old baby was positive for HPeV, using the single RT-PCR. A similar result was obtained by the multiplex RT-PCR and liquid hybridization. The detection rate of 1.5% obtained for HPeV may seem low, but incidences over the same range have been reported by others (Han et al. 2011) and detection rates above 5.0% are often reported in paediatric populations or in sample sets screened negative for other viruses causing gastroenteritis (Pham et al. 2011; Wolffs et al. 2011). No AV was found in the stool samples, a finding in accordance with the low incidence of the virus described. A recent study also found a 0.5% detection rate for AV in stool samples from 1063 infant outpatients and hospitalized children with acute gastroenteritis in Finland (Kaikkonen et al. 2010).

Analysis of clinical specimens showed favourable clinical specificity of the multiplex RT-PCR, since nonspecific amplification was detected in agarose gel electrophoresis only in the case of stool samples. Furthermore, luminescent signals from liquid hybridization were also specific, with the mean background signal of all probes ranging from 1.0 to 9.7 RLU. Exceptionally, however, luminescent signals between 45 and 63 RLU with the HPEV probe were observed for one HEV-positive CSF sample and two HRV-positive NPS. Existence of two coinfecting viruses in these three samples was thought unlikely, since the luminescence signals of the positive samples in the multiplex assay were as high as 630–2350 RLU,
whereas values less than 4 RLU were obtained for samples that were negative for HEV and HRV in daily diagnostics. For this reason, the luminescence observed with the HPEV probe for the three samples was considered a background signal, although no cutoff values for the probes of the assay were determined. The signals of the clinical samples showed intra-assay variations of 0.2–23.6 % of the mean RLU when the amplification product from one multiplex RT-PCR was tested in two parallel hybridization reactions. Variation in RLU from the amplification products of the parallel RT-PCRs were not determined.

1.2 Performance of the real-time duplex HEV/HRV RT-PCR (III)

A real-time duplex RT-PCR was designed to achieve rapid detection of HEVs and HRVs from respiratory samples. *In vitro* RNA transcripts of E11 and HRV1B, described in section 4.1, were used for optimization of the reaction conditions and assessment of the sensitivity and precision of the assay. Amplification was performed in a one-tube RT-PCR with *Tth* polymerase capable of cDNA synthesis at 60 °C. To enable simultaneous detection of four respiratory viruses, reagents and cycling conditions similar to those described for the real-time duplex RSV/hMPV RT-PCR were utilized. Both the quality assurance samples described in Table 5 and the clinical samples described in Table 4 were analysed with the assay, results for which are presented in sections 1.2.2 and 4, respectively.

1.2.1 Analytical sensitivity of the real-time duplex HEV/HRV RT-PCR (III)

For amplification of the HEV and HRV sequences, primers from the conserved 5’UTR region of the viral genomes were designed (Table 7). To distinguish between HEVs and HRVs, two probes, one for detection of each enterovirus species, were designed (Table 8). In the single amplification reactions, detection of 10 and 50 RNA transcripts was achieved for E11 and HRV1B, respectively. In the multiplex assay, a decrease in sensitivity was observed, since 50 RNA transcripts of E11 and 100 transcripts of HRV1B per reaction, corresponding to $10^3$ copies per ml of sample, were detected. The sensitivity of the real-time
multiplex RT-PCR assay corresponded to that observed for the detection of HEVs and HRVs by the conventional multiplex RT-PCR assay described in section 1.1. In the precision study, the mean intra-assay coefficients of variation (CV) of the cycle threshold ($C_T$) values for E11 and HRV1B were 1.52% and 1.00% and the mean interassay CV% values were 1.48% and 3.60%, respectively.

1.2.2 Analytical specificity of the real-time duplex HEV/HRV RT-PCR (III)

Of the 44 quality assurance samples analysed, all HEVs and HRVs present in the QCMD panels, except HRV72, were detected. When the sequences of HRV72 available in GenBank were scrutinized, four mismatching nucleotides for binding of primer RV-F1 were observed. This suggests that the negative result for HRV72 may have been due to suboptimal primer binding. Similar to this drawback in the present study, the difficulty in designing primers that detect all HRV types and the necessity for more than one pair of primers for detection of all HRVs were described in a recent study on HRV-specific primers (Faux et al. 2011).

The assay was designed to distinguish between HEVs and HRVs by designing separate probes for differential detection of the viruses. However, one quality assessment sample containing HRV42 gave a positive signal only with the EV probe. Furthermore, samples containing HEV71, CVB3, CVA9, CVA16, CVA21, PV3 and echoviruses 11, 16 and 30, were positive with both the EV probe and RV probe. Together, these results imply the incapability of the duplex RT-PCR assay in distinguishing between HEVs and HRVs. Cross-amplification of the viruses, resulting from the sequence similarity between the 5’UTRs of HEVs and HRVs, is commonly reported (Capaul & Gorgievski-Hrisoho 2005; Gharabaghi et al. 2011; Harvala et al. 2012; Lai et al. 2003), and primers and probes that best distinguish between the viruses may not be the most sensitive ones (Johnston et al. 1993). Moreover, differential detection of HEVs and HRVs in respiratory samples in daily virus diagnosis may not be necessary, but supplemental assays can be used to differentiate
the viruses when clinically indicated. Apart from the cross-amplification described above, no other nonspecific signal was detected in the analysis of quality assurance samples, but a positive result was indicative of either HEV or HRV.

2 REAL-TIME MULTIPLEX RT-PCR ASSAY FOR DETECTION OF RSV AND hMPV AND EVALUATION OF ANTIGEN DETECTION FOR hMPV (II)

A real-time duplex RT-PCR assay for simultaneous detection of RSV and hMPV in respiratory samples was developed. The analytical specificity of the assay was evaluated in analysis of quality assurance samples (Table 5), results of which are presented in section 2.1.2. Moreover, 647 respiratory samples (Table 4) were analysed with the real-time duplex RT-PCR assay and the detection of hMPV and RSV in comparison to DFA and the RVP Fast assay was assessed. Analysis of the clinical samples yielded information on the incidence of hMPV as a respiratory pathogen in the Finnish population. The results of the clinical samples are described further below in section 4.

2.1 Performance of the real-time duplex RSV/hMPV RT-PCR assay (II)

Two primer pairs for real-time detection of RSV and hMPV in respiratory samples were designed, and amplification was performed in a one-tube RT-PCR with Tth polymerase at 60 °C. For optimization of the reaction conditions described in section 5.3, the viral RNA transcripts described in section 4.2 were utilized. Reagents and cycling conditions similar to those described for the real-time duplex HEV/HRV RT-PCR were applied to enable simultaneous detection of four respiratory viruses.
2.1.1 Analytical sensitivity and precision of the real-time duplex RSV/hMPV assay (II)

To define the detection limit of the real-time duplex RSV/hMPV RT-PCR, RNA transcripts of the viral sequences were produced, as described in section 4.2. In the duplex RT-PCR, a detection limit of 100 RNA transcripts per reaction, corresponding to $10^3$ copies per ml of sample for RSV and hMPV subtype A virus, was achieved. For hMPV subtype B virus, 1000 RNA transcripts equalling $10^4$ copies per ml of sample was detected in repeated analysis. The analytical sensitivity of the duplex RT-PCR assay was decreased in contrast to the corresponding single RT-PCR assays that achieved detection of 50 RNA transcripts of RSV and subtype A hMPV and 150 RNA transcripts of subtype B hMPV per reaction. However, the analytical sensitivity achieved for the duplex RT-PCR was sufficient for analysis of respiratory samples and in the order of that obtained by others (Kuypers et al. 2005).

In the precision study of the assay, parallel detection of RSV and hMPV subtype A and B RNAs exhibited intra-assay CV of the C\textsubscript{T} values, ranging from 3.63% to 3.99%. In interassay analysis, values from 3.78% to 5.80% were found, with the highest variance detected for the lowest concentration analysed. These values demonstrate both satisfactory repeatability and reproducibility of the assay and altogether indicate reliable performance of the assay in also detecting viruses present at low concentrations.

2.1.2 Analytical specificity of the real-time duplex RSV/hMPV assay (II)

To further assess the performance of the real-time duplex RSV/hMPV RT-PCR, 32 quality assurance samples were analysed. The results obtained for the samples of the 2006 and 2007 RSV and hMPV panels were consistent with the data provided by the organizer. The assay also detected samples weakly positive for RSV and hMPV subtypes A and B. Moreover, no false-positive results were detected among the samples containing AdV or HEV or the
negative controls analysed. Taken together, the results of the quality assurance samples imply high analytical sensitivity and specificity of the assay designed.

2.2 Antigen detection of hMPV (II)

For DFA detection of hMPV, four commercial antibodies were tested, all of which showed clear positive signals for hMPV subtypes A1, A2, B1 and B2 on the control slides provided in the D³ DFA Metapneumovirus Identification Kit. The antibodies showed different staining patterns, with the D³ DFA reagent producing the faintest fluorescence. Although no nonspecific staining was detected on the negative control slides, analysis of the clinical samples revealed that anti-hMPV (Argene) and Imagen™ hMPV (Oxoid) antibodies resulted in nonspecific signals that were difficult to interpret. This background staining was observed in 27 (48.2%) and 7 (12.5%) of the 56 NPA samples analysed with the antibodies, respectively, and made finding of true positive cells difficult. For this reason, the remaining 294 samples were screened only with the D³ DFA Metapneumovirus Identification Kit and hMPV DFA Reagent (Light Diagnostics™). The D³ DFA reagent resulted in diffuse staining in contrast to the more granular fluorescence observed for the Light Diagnostics hMPV DFA reagent, but staining with both antibodies was easy to interpret. A full assessment of the two antibodies would, however, have required analysis of a larger number of hMPV-positive samples. The results of the DFA detection of hMPV from the clinical samples are described in further detail in section 4.2.

3 PERFORMANCE OF THE RVP FAST ASSAY (III)

In the present study, the RVP Fast assay was implemented as part of the routine testing repertory of the Virology Laboratory of HUSLAB, Helsinki University Central Hospital, and the performance of the assay was evaluated in analysis of quality assurance samples and respiratory samples. All respiratory samples were analysed with the RVP Fast assay in
parallel with DFA for AdV, IAV, IBV, RSV and PIV1–3, the real-time duplex RSV/hMPV RT-PCR assay and the real-time duplex HEV/HRV RT-PCR assay. Analytical performance of the RVP Fast assay was evaluated by comparing the results obtained for the respiratory samples with those achieved with DFA and RT-PCR.

3.1 Analytical specificity of the RVP Fast assay (III)

For assessment of the analytical specificity of the RVP Fast assay, 11 quality assurance samples of a NATRVP-2 Global Panel (Table 5) were analysed. All viruses present in the panel and covered by the RVP Fast assay were detected and no cross-reactivity occurred. The NATRVP-2 Global panel contained only one representative strain of each virus. However, analysis of a greater number of samples with more representative viruses of HEVs and HRVs would have been of particular interest in assessing the ability of the RVP Fast assay to detect viruses of the large genus *Enterovirus*. This would also have enabled a more comprehensive comparison of the RVP Fast assay and the real-time duplex HEV/HRV RT-PCR assay. Since assessing the performance of the RVP Fast assay with the 2010 QCMD samples was interfered with by the viral transport medium containing foetal calf serum (Luminex Technical Support Group in the final report of the QCMD 2010 HRV and hCoV Programme), the 2010 QCMD panels available to the study were not analysed by the RVP Fast assay.

3.2 Experiences with the RVP Fast assay in analysis of clinical samples (III)

The performance of the RVP Fast assay was also evaluated in analysis of a total of 373 respiratory samples collected in 2009–2011. Twelve of these samples exhibited invalid results for the MS2 internal control in initial analysis, but reanalysis of these samples resulted in a total of 370 samples (99.2%) with a valid result. The three samples with invalid results for MS2 were excluded from further analysis. Failure of the lambda external run control was not seen during analysis of the sample set. Together the results of the MS2
RNA and lambda phage DNA showed that the failures of the assay were due to faulty nucleic acid extraction or RT reactions (Merante et al. 2007). Indeed, valid results for the failed samples were obtained after extraction and amplification anew were performed, but not when only the postamplification steps were repeated. Excess evaporation of the reaction mixture observed after RT-PCR of the failed samples makes faulty cDNA synthesis the most plausible cause of the invalid results.

Within the 2009–2010 sample set analysed, the number of samples positive for at least one virus of the DFA panel increased from 70 (23.8%) with DFA to 81 (27.6%) positive samples with the RVP Fast assay. With all targets of the broad detection spectrum of the RVP Fast assay included, 179 positive samples, corresponding to a detection rate of 60.9%, were obtained. Similar increases in the detection rates over those of conventional methods have been described in previous studies with the RVP Fast assay (Gadsby et al. 2010; Gharabaghi et al. 2011) and the original version of the assay, the RVP Classic assay (Gharabaghi et al. 2011; Mahony et al. 2007). Considering all results of the RVP Fast assay and DFA, a 60.4% agreement between the results with a kappa value of 0.297 (P < 0.001) was achieved. This indicates only fair agreement between the results of the two assays, which is further supported by the P-value of 0.049 obtained in McNemar’s test. Together, the data show a significant increase in the detection rate of the RVP Fast assay in contrast to that of DFA.

Detection of RSV, hMPV, HEV and HRV in clinical samples was performed by the RVP Fast assay in comparison to the duplex real-time RT-PCR assays optimized in the study. An increase in the overall detection rate of these viruses by the RVP Fast assay over RT-PCR was observed, since the number of positive samples increased from 133 (45.2%) by the RT-PCR assays to 141 (48.0%) by the RVP Fast assay. This increase was, however, due to the detection rates of HEVs and HRVs. Further detailed data on individual viral targets are described below in section 4.
To assess the impact of the real-time duplex RT-PCR assays developed in the study and the RVP Fast assay on diagnosis of respiratory tract infections, a total of 647 respiratory samples sent for routine virus diagnostics to HUSLAB, Helsinki University Central Hospital laboratory from November 2007 to June 2008 (350 samples) and from December 2009 to April 2010 (297 samples) were analysed (Table 9). Three samples of the latter sample set were excluded from further analysis due to invalid internal control results in the RVP Fast assay, and therefore the results below are described for the remaining 294 samples. The 350 samples dating back to 2007–2008 were analysed with DFA for RSV and hMPV and the real-time duplex RSV/hMPV RT-PCR assay. The 294 samples of the 2009–2010 sample set were analysed with DFA for AdV, IAV, IBV, RSV and PIV1-3, the RVP Fast assay, the real-time duplex RSV/hMPV RT-PCR assay and the real-time duplex HEV&HRV assay. Findings from analysis of the 2009–2010 sample set are shown in Figure 4. Due to the low incidence of IAV, IBV and PIVs in the aforementioned sample sets, 42 respiratory samples that were positive for IAV by real-time RT-PCR (Rönkkö et al. 2011; Ward et al. 2004), and 34 samples positive for either IBV or PIV3 with DFA, were analysed in evaluation of the RVP Fast assay (Table 9).

Table 9. Summary of the methods used for analysis of the respiratory samples.

<table>
<thead>
<tr>
<th>Methods used</th>
<th>Time period and number of respiratory samples collected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2007-2008 2009-2010 2010-2011 2010-2011</td>
</tr>
<tr>
<td></td>
<td>350 samples 294 samples 34 samples 42 samples</td>
</tr>
<tr>
<td>DFA</td>
<td>IIa         III       III       NAb</td>
</tr>
<tr>
<td>hMPV DFA</td>
<td>II          NA         NA         NA</td>
</tr>
<tr>
<td>HEV/HRV RT-PCR</td>
<td>NA          III        NA         NA</td>
</tr>
<tr>
<td>RSV/hMPV RT-PCR</td>
<td>II          III        NA         NA</td>
</tr>
<tr>
<td>RVP Fast</td>
<td>NA          III        III        NA</td>
</tr>
<tr>
<td>IAV RT-PCR</td>
<td>NA          NA         NA         III</td>
</tr>
</tbody>
</table>

a) II and III, samples and results originally described in the corresponding original publication
b) NA, method not applied in analysis of the sample set
4.1 Detection of HEVs and HRVs (III)

The efficiency of the RVP Fast assay in detection of HEVs and HRVs was examined in comparison with the real-time duplex HEV/HRV RT-PCR assay developed (Figure 4). Of the 294 samples collected in 2009–2010, both RT-PCR and the RVP Fast assay found HEV/HRV in 55 samples (Table 10). Of these, six samples were RT-PCR-negative in the initial analysis but became HEV-positive in reanalysis when primer EV-F2 was used. Primers EV-F2 and RV-F2 were used only in reanalysis of samples, whereas EV-F1 and RV-F1 were used for the initial analysis because these primers were thought to achieve differential detection of HEVs and HRVs. Discordant results were obtained for 25 samples. Seven RVP-negative samples were positive for HEV/HRV in RT-PCR with a mean C\textsubscript{T} of 34.6, and 18 RVP-positive samples remained negative in RT-PCR. Similar to these discrepant RVP-negative results, reduced detection of the viral targets was described for both the RVP Classic assay (Merante et al. 2007) and the RVP Fast assay (Gadsby et al. 2010) in samples with low viral load corresponding to C\textsubscript{T} values of >35.

<table>
<thead>
<tr>
<th></th>
<th>PCR-positive</th>
<th>PCR-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>RVP-positive</td>
<td>55</td>
<td>18</td>
</tr>
<tr>
<td>RVP-negative</td>
<td>7</td>
<td>214</td>
</tr>
</tbody>
</table>

Based on the findings described above, the RVP Fast assay displayed a sensitivity of 88.7% and specificity of 92.2% compared with the real-time multiplex RT-PCR assay (Table 1 in III). Detection rates of 21.1% and 24.8% were obtained by the real-time duplex HEV/HRV RT-PCR assay and the RVP Fast assay, respectively. The results of the two assays were 91.5% concordant, with a kappa value of 0.760 (P < 0.001), indicating a substantial agreement of the results. In statistical analysis, however, a significant difference was detected in McNemar’s test (P = 0.043). Together with a kappa value barely below the 0.8 limit value for almost perfect agreement, this implies a weak but true decrease in the
detection rate of the real-time RT-PCR assay in comparison to the RVP Fast assay. Taken together with the results from analysis of the quality assurance samples (section 1.2.2), doubts were raised on the capability of the real-time RT-PCR to detect all representative viruses of HEVs and HRVs, although the effect of additional freezing and thawing of the nucleic acid extracts before the RT-PCR may have resulted in false-negative results of some samples. Similar to our results, superior detection rates for HEV/HRV by the RVP Classic assay (Pabbaraju et al. 2008) and the RVP Fast assay (Gadsby et al. 2010) in comparison to in-house assays have been described. Moreover, a broad detection range of HRV serotypes for the RVP Classic assay was reported recently (Chandrasekaran et al. 2012).

Figure 4. Findings of respiratory viruses from the 2009–2010 sample set with DFA, RT-PCR and RVP used for analysis.

4.2 Detection of RSV and hMPV (II, III)

The performances of the RVP Fast assay and the real-time duplex RT-PCR assay in detection of RSV and hMPV were assessed in analysis of respiratory samples. Moreover, four commercial reagents for detection of hMPV by DFA were assessed. In all, 644 samples were analysed with the real-time duplex RT-PCR assay and DFA for the presence of RSV.
Of these, 112 samples (17.4%) were found positive for RSV in the duplex RT-PCR assay (Table 11). In DFA, a positive result was obtained for 95 (80.5%) of the RT-PCR-positive samples and for two RT-PCR-negative samples. With PCR as a reference assay, these findings resulted in 84.8% sensitivity and 99.6% specificity for DFA. As expected, the detection rate of the real-time duplex RT-PCR was significantly higher than that of the DFA routinely used for respiratory virus diagnosis. The circulation pattern of RSV is discussed further below and monthly distribution of the positive findings is described further in Figure 5.

### Table 11. Findings of RSV from analysis of the clinical samples.

<table>
<thead>
<tr>
<th>RT-PCR and DFA</th>
<th>DFA and RVP</th>
<th>RT-PCR and RVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR+ DFA+</td>
<td>DFA+ RVP+</td>
<td>PCR+ RVP+</td>
</tr>
<tr>
<td>PCR+ DFA-</td>
<td>DFA+ RVP-</td>
<td>PCR+ RVP-</td>
</tr>
<tr>
<td>PCR- DFA+</td>
<td>DFA- RVP+</td>
<td>PCR- RVP+</td>
</tr>
<tr>
<td>PCR- DFA-</td>
<td>DFA- RVP-</td>
<td>PCR- RVP-</td>
</tr>
</tbody>
</table>

| Sample sets 2007–2008 and 2009–2010 analysed |
| Sample set 2009–2010 and 34 additional samples analysed |
| One sample not tested in DFA |

Of the aforementioned 644 samples, the 294 samples collected in 2009–2010 were analysed for RSV, utilizing the RVP Fast assay, duplex RT-PCR and DFA (Figure 4). An additional 34 samples collected in 2011 were analysed with the RVP Fast assay and DFA. In the 2009–2010 sample set, a total of 61 samples (20.7%) were positive for RSV (Table 11). Discordant results were obtained for eight samples, four of which were RVP-negatives, two with positive results in both DFA and RT-PCR (mean $C_T$ 22.0) and the other two were positive only by RT-PCR (mean $C_T$ 36.0). Since the possibility of false-negative results by RVP for samples with $C_T$ values over 35 in real-time PCR (Merante et al. 2007) occurs in RSV detection (Gadsby et al. 2010; Pabbaraju et al. 2008), the actual intriguingly divergent results are the two samples with a mean $C_T$ of 22.0 and positive also by DFA. Unfortunately for the study, these samples were not available for reanalysis by the RVP Fast assay.
Similar to the results published by others, a great proportion (21.1%, 12 samples) of the RSV-positive samples contained one or more coinfecting virus (Pabbaraju et al. 2011b). In the 294 samples analysed, RSV detection rates of 18.4%, 19.4% and 20.1% were obtained by DFA, RVP and RT-PCR, respectively. An agreement of 97.6% between the results of the RVP Fast assay and DFA, and 98.0% agreement of the results by RVP and RT-PCR were achieved with kappa values of 0.910 (P < 0.001) and 0.936 (P < 0.001), respectively. The kappa values obtained imply an almost perfect agreement between the results. This was further supported by McNemar’s test, which found no significant difference in detection of RSV between the RVP Fast assay and DFA (P = 0.289) or RVP and RT-PCR (P = 0.687). For the RVP Fast assay, sensitivities of 96.3% and 93.2%, using DFA and RT-PCR as the reference methods, respectively, were achieved (Table 1 in III). The specificities of the RVP Fast assay in comparison to the DFA and RT-PCR, were 98.2% and 99.1%, respectively.

A total of 16 samples (2.5%) of the 644 samples analysed in the study were positive for hMPV by the real-time duplex RSV/hMPV RT-PCR assay (Table 12). Four samples (1.1%) of the 2007–2008 sample set analysed with the duplex RT-PCR assay and DFA were PCR-positive for hMPV, and two of the samples were clearly also positive by the DFA. One RT-PCR-positive sample was considered DFA-negative, because the fluorescence detected only with the Argene anti-hMPV reagent was interpreted as nonspecific staining similar to that seen in several other samples with this antibody. The fourth RT-PCR-positive sample was also considered as DFA-negative, although when re-examined, the sample was clearly negative only with D³ DFA reagent and the weak fluorescence interpreted as a nonspecific signal was also observed with the other hMPV reagents. This sample had a high C_T of 37.3 in the duplex RT-PCR assay. The figures described above resulted in a low sensitivity of 50% for all reagents evaluated, but interpretation of this result must be made with caution due to the low number of hMPV-positive samples analysed. Indeed, in contrast to our results, higher sensitivities of 85.4% (Landry et al. 2008) and 63.2% (Aslanzadeh et al. 2008) for the Light Diagnostics hMPV reagent and the Imagen™ hMPV, respectively, were described in clinical samples. The D³ hMPV DFA reagent that was originally reported to have a sensitivity of 73.9% (Gerna et al. 2006b; Percivalle et al. 2005), also attained
sensitivity of 59.0–95.2% in previous studies on clinical samples (Aslanzadeh et al. 2008; Gerna et al. 2007; Jun et al. 2008; Vinh et al. 2008). Since subjective interpretation of results is a well-known weakness of DFA, in the present study, a second examination by another microscopist for all samples with positive or suspected results with at least one of the reagents evaluated, was performed to minimize the influence of subjective interpretation.

Table 12. Findings of hMPV from analysis of the clinical samples.

<table>
<thead>
<tr>
<th>RT-PCR and DFA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RT-PCR and RVP&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR+ DFA+</td>
<td>PCR+ RVP+</td>
</tr>
<tr>
<td>PCR+ DFA-</td>
<td>PCR+ RVP-</td>
</tr>
<tr>
<td>PCR- DFA+</td>
<td>PCR- RVP+</td>
</tr>
<tr>
<td>PCR- DFA-</td>
<td>PCR- RVP-</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>346</td>
<td>282</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sample set 2007–2008 analysed
<sup>b</sup> Sample set 2009–2010 analysed

In the analysis of clinical samples, the Argene anti-hMPV reagent and Imagen™ hMPV reagent showed background staining that made finding of true positive cells difficult and resulted in specificities of 48.0% and 86.5% for the antibodies, respectively, when 56 samples were analysed. This is in contrast to a previously reported specificity of 100.0% for the Imagen™ hMPV reagent (Aslanzadeh et al. 2008). Similar to the 99.4–99.8% and 100% sensitivities described for the D<sup>3</sup> DFA (Aslanzadeh et al. 2008; Jun et al. 2008) and the Light Diagnostics reagent (Landry et al. 2008), a sensitivity of 100% was achieved for both of the antibodies in the present study. Although interpretation of the results on the sensitivity is limited, the results on the specificity of the DFA reagents are reliable, since the sample set consisted of a large number of samples positive for other respiratory viruses, as well as samples that were DFA-negative for common respiratory viruses. Equal specificity of the D<sup>3</sup> hMPV DFA reagent and the Imagen™ hMPV reagent was reported in a study by Azlanzadeh and coworkers (2008), although a smaller number of clinical samples was analysed with the the Imagen™ hMPV reagent. No other studies that report simultaneous evaluation of more than one DFA reagent for hMPV have been published. In the present
study, the low prevalence of hMPV-positive samples was an evident limitation, and a greater number of positive samples would have required a more comprehensive assessment of the performance of the hMPV antibodies applied.

The 2009–2010 sample set of 294 samples was tested for hMPV by the duplex RT-PCR assay and the RVP Fast assay (Figure 4). Twelve of the samples (4.1%) were positive for hMPV in RT-PCR. Of these, one very weakly positive sample (C<sub>T</sub> 44.0), with an elevated MFI of 132 (threshold 200), was missed by the RVP Fast assay. With the figures described above, no difference of statistical significance (P = 0.500) in the detection of hMPV by the RVP Fast assay and the real-time multiplex RT-PCR assay was observed.

Of particular interest in the present study was the prevalence of hMPV among respiratory samples sent for daily virus diagnostics to HUSLAB. In analysis of two random selections of the samples, the 2007–2008 and 2009–2010 sample sets, relatively low prevalences of 1.1% and 4.1% of hMPV were observed. This finding is in agreement with two Finnish studies reporting detection rates of 1.3% (Heikkinen et al. 2008) and 4.0% (Jartti et al.

![Figure 5. Monthly incidence of RSV and hMPV findings in sample sets collected in 2007–2008 and 2009–2010.](image)
2004b) in paediatric study populations. The prevalence of hMPV differed between the two sample sets analysed and variation in prevalence of hMPV from season to season has also been reported by others (Aberle et al. 2008; Rafiefard et al. 2008; Walsh et al. 2008; Williams et al. 2006). Data on the occurrence of RSV and hMPV between November 2007 and June 2008 show a pattern, in which RSV appears to circulate earlier and for a longer period than hMPV (Figure 5). Similar results were also obtained for the sample set from December 2009 to April 2010. Circulation of hMPV occurs in springtime (García-García et al. 2007; Choi et al. 2006) and later epidemiological peaking of hMPV compared with the RSV (Aberle et al. 2010; Heininger et al. 2009; Madhi et al. 2007; Rafiefard et al. 2008; Robinson et al. 2005) has also been reported.

4.3 Detection of influenza viruses (III)

The 294 samples collected in 2009–2010 were analysed for influenza virus utilizing the RVP Fast assay and DFA. The RVP Fast assay found 4 samples of the sample set that were positive for IAV, all unsubtypeable by the assay (Table 13 and Figure 4). Three of these samples were also positive in DFA. An unsubtypeable IAV result of the RVP Fast assay was highly indicative of the 2009 pandemic IAV(H1N1) (Ginocchio & St George 2009; Vinikoor et al. 2009). Indeed, three of the samples available for further testing were positive in a real-time RT-PCR assay specific for the nonstructural and haemagglutinin genes of the novel IAV(H1N1) 2009 of swine origin (Rönkkö et al. 2011). The fact that all four samples date from early December 2009 suggests that all four samples were truly positive for the novel IAV(H1N1) 2009. IBV was not detected in the sample set.

To further assess the detection of IAV by the RVP Fast assay, 15 samples positive for seasonal IAV by real-time RT-PCR targeting the matrix gene (Ward et al. 2004), 19 samples positive for IAV(H1N1) 2009 by real-time RT-PCR (Rönkkö et al. 2011) and eight samples negative for both viruses, were analysed with the RVP Fast assay (Table 13). Concordant results for the negative samples were obtained by RVP, whereas two of the
samples PCR-positive for seasonal IAV (mean $C_T$ 37.2) remained negative by RVP. The RVP Fast assay was unable to type one of the positive samples. Moreover, only 12 (63.2%) of the samples positive for IAV(H1N1) 2009 by RT-PCR were positive in the RVP Fast assay. The samples missed by the assay had a mean $C_T$ of 32.8 in RT-PCR. The figures described above result in 76.2% concordance, with a kappa value of 0.514 ($p = 0.125$), indicating only moderate agreement of the results of real-time RT-PCR and RVP in detection of IAV. Indeed, a significant difference between the results of the assays was confirmed in McNemar’s test ($P = 0.004$). Taken together, the results show a significant decrease in the detection rate of the RVP Fast assay in comparison to the real-time RT-PCR assays utilized, and call for adjusting the IAV component of the assay to also cover the novel IAV(H1N1) 2009 virus.

Table 13. Findings of IAV from analysis of the clinical samples.

<table>
<thead>
<tr>
<th>DFA and RVP$^a$</th>
<th>RT-PCR$^b$ and RVP</th>
<th>RT-PCR$^c$ and RVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFA+</td>
<td>DFA+</td>
<td>DFA-</td>
</tr>
<tr>
<td>RVP+</td>
<td>RVP-</td>
<td>RVP+</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$) Sample set 2009–2010 analysed  
$^b$) The assay described by Ward et al. 2004 applied for analysis of 42 samples  
$^c$) The assay described by Rönkkö et al. 2011 applied for analysis of 42 samples

To assess the performance of the RVP Fast assay in detection of IBV, 34 samples collected in 2011 that were positive for IBV or PIV3 in DFA, were analysed with the assay. Two of the 22 samples positive for IBV in DFA remained negative by RVP, resulting in 91% concordance of the results by the two assays. Although low sensitivity of the RVP Fast assay in detection of IBV has been described previously (Gharabaghi et al. 2011), the two discordant findings of our study cannot be generalized for the performance of the assay. The figures from analysis of a total of 328 samples described above result in sensitivity of 90.0% and specificity of 100% for the RVP Fast assay in comparison with DFA.
4.4 Detection of AdV (III)

Of the 294 samples collected in 2009–2010 and the 34 samples collected in 2011, the RVP Fast assay found 9 samples positive for AdV, 4 of which were also positive by DFA (Table 14 and Figure 4). Additionally, one RVP-negative sample appeared positive by DFA. Since we wanted to further assess the performance of the RVP Fast assay in detection of AdV, two strains of AdV3, one AdV5 strain and one AdV7 strain, isolated from patient samples, were analysed. These strains are representatives of AdV species B1 and C commonly detected in respiratory samples. AdV 3 and AdV5 were detected by the RVP Fast assay, but the AdV 7 strain remained negative.

Table 14. Findings of AdV from analysis of the clinical samples.

<table>
<thead>
<tr>
<th></th>
<th>DFA-positive</th>
<th>DFA-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>RVP-positive</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>RVP-negative</td>
<td>1</td>
<td>318</td>
</tr>
</tbody>
</table>

Whereas false-negative results in the RVP Fast assay have mainly been described for samples with low viral loads, for detection of AdV samples with high viral loads and low C_T values in parallel real-time PCR have also remained negative (Gadsby et al. 2010; Pabbaraju et al. 2011b). This has also been described for the RVP Classic assay and has been suggested to result from competitive inhibition by another viral target or mismatches with some of the AdV serotypes, of which serotypes 1 and 2 have been identified in RVP-negative samples (Pabbaraju et al. 2008; 2011a). Together with the data published by others, the results of the present study imply that detection of AdV species commonly occurring in respiratory infections may not be certain, and other measures for confirmation of negative results may be needed.
4.5 Detection of PIVs (III)

The respiratory samples collected in 2009–2010 were analysed for PIVs utilizing the RVP Fast assay and DFA. In all, 12 samples were positive for PIV1–3 (Table 15 and Figure 4), 11 of which were positive in the RVP Fast assay, including seven that were also positive by DFA. One sample was positive for PIV-1 only by DFA. PIV4 was not detected in the sample set.

Due to the low sensitivity described for the RVP Fast assay in PIV1–3 (Gharabaghi et al. 2011) and the low prevalence of PIVs in the 2009–2010 sample set, 34 additional samples collected in 2011 and positive for PIV3 or IBV in DFA were analysed. Of the 12 samples positive for PIV3 in DFA, 11 samples were RVP-positive (Table 15). Although the low prevalence of PIV1 and PIV2 limits interpretation of the data obtained in the present study, the results imply reliable detection of PIVs by the RVP Fast assay compared with the DFA.

Table 15. Findings of PIVs from analysis of the clinical samples.

<table>
<thead>
<tr>
<th>Target virus</th>
<th>Result with RVP</th>
<th>Result with DFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DFA-positive</td>
</tr>
<tr>
<td>PIV1</td>
<td>RVP-positive</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>RVP-negative</td>
<td>1</td>
</tr>
<tr>
<td>PIV2</td>
<td>RVP-positive</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>RVP-negative</td>
<td>0</td>
</tr>
<tr>
<td>PIV3</td>
<td>RVP-positive</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>RVP-negative</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^{a}\) One sample positive for PIV2 in DFA

4.6 Detection of hCoVs and hBoV (III)

Of the 294 samples collected in 2009–2010, the RVP Fast assay detected hCoVs in 26 samples, which corresponds to an overall detection rate of 8.8%. Of the individual targets,
HKU1 was detected in 15 samples (5.1%), 229E in 8 samples (2.7%) and OC43 in 3 samples (1.0%) (Figure 4). hCoV NL63 was not detected in the sample set and SARS-CoV is not tested by the RVP Fast assay. In all, the non-SARS-hCoVs constituted a significant proportion of the respiratory viruses detected and the prevalences in the range of 4.2–16.5% reported by the RVP Fast assay were observed (Gadsby et al. 2010; Pabbaraju et al. 2011b). Since no previous measures for detection of hCoVs in the routine testing algorithm of the Virology Laboratory of HUSLAB, Helsinki University Central Hospital existed, no comparison for detection of these viruses was available.

In the sample set of 2009–2010, 14 samples (4.8%) were positive for hBoV by the RVP Fast assay (Figure 4). Similar to the hCoVs, no comparison for these results was available, because hBoV was not covered in the respiratory virus diagnostics of HUSLAB. However, the incidence of the virus in our study population is in agreement with the detection rates of 2.1–4.1% obtained by the RVP Fast assay in previous studies (Gadsby et al. 2010; Pabbaraju et al. 2011b).

4.7 Mixed infections (III)

In analysis of the 2009–2010 sample set, a total of 26 (8.5%) samples with two or more viruses present were detected. The RVP Fast assay detected 19 dual infections and two triple infections. Additionally, a coinfecting virus was detected in five samples by RT-PCR. The viruses present in coinfections were HEV/HRV, RSV, hBoV, AdV, hCoV HKU1, hCoV OC43, hCoV 229E, hMPV, PIV1, PIV3 and PIV4, in descending order. All coinfections were missed by DFA. The 7.1% prevalence of mixed infections in the sample set is in agreement with a viral coinfection rate of 3.3–15.6% reported for symptomatic patients in other studies by the RVP Fast assay (Gadsby et al. 2010; Mahony et al. 2007; Pabbaraju et al. 2008, 2011b).
Molecular detection of viruses is increasingly being performed to achieve timely and sensitive detection of viral pathogens. The DFA panel currently utilized for viral respiratory infection diagnosis at HUSLAB, Helsinki University Central Hospital, offers detection of AdV, IAV, IBV, RSV and PIV1–3, which comprise only a portion of the viruses known as respiratory pathogens. The multiplex RT-PCR and liquid hybridization assay developed in the study enables detection of HEVs, HRVs and HPeVs in respiratory samples and, alternatively, detection of enteric picornaviruses HEVs, HPeVs, and AV in stool samples. The single RT-PCR and liquid hybridization assay developed in the study enables sensitive detection of HAV. Moreover, development of the two real-time duplex RT-PCR assays hasten detection of HEVs and HRVs, provide more sensitive detection of RSV than does DFA and further broaden the detection range of respiratory pathogens to also cover hMPV. Although the real-time multiplex RT-PCR assay for detection of HEVs and HRVs did not reliably distinguish between these picornaviruses, a positive result was nevertheless indicative of either virus present in a sample.

In the study, two commercial DFA reagents, D³ DFA Metapneumovirus Identification Kit (Diagnostic Hybrids, Inc.) and Human Metapneumovirus (hMPV) DFA Reagent (Light Diagnostics™), resulted in an appearance of hMPV-infected cells that was easy to interpret. Therefore, either one of these two antibodies was considered applicable for DFA detection of the virus in respiratory samples as part of routine diagnostics. However, a large-scale assessment of the two reagents involving a greater number of positive samples is needed.

To further broaden the diagnostic range of respiratory viruses, a bead-based suspension microarray test, the RVP Fast test, for detection of respiratory viruses was evaluated. The study found the overall performance of the test satisfactory, although results for all individual targets of the assay were not precise. Our results imply that detection of IAV(H1N1) 2009 and AdV by this assay is not definite, since some AdV strains present at high copy numbers may be missed and only 63.2 % of the samples positive for IAV(H1N1)
2009 were detected by the RVP Fast assay. In effect, this means that other detection measures for these targets need to be performed, and adjustments needed for these components of the RVP Fast assay put the superior flexibility of the suspension microarray approach to the test. The performance of the assay in detecting PIV1 and PIV2 was restricted by the low number of samples positive for these targets, and further assessment with a greater number of positive samples is required. Moreover, the sensitivity of the RVP Fast assay for PIV4, hCoVs and hBoV could not be assessed in this study design, because no reference method for comparison of the results was available.

Assessment of the analytical performance of the real-time duplex RT-PCR assays and the RVP Fast assay involved analysis of a total of 723 respiratory samples. Findings in analysis of two random selections of the samples sent for daily virus diagnostics, the 2007–2008 and 2009–2010 sample sets, revealed a prevalence of 2.4% for hMPV, implying a true need to detect the virus as part of routine diagnosis, whether this is done by RT-PCR or DFA. Furthermore, the 8.8% occurrence of the non-SARS-hCoVs in one sample set suggests that these viruses form a significant group of pathogens in samples sent for virus diagnosis and, therefore, should be diagnosed routinely. Moreover, since increasing evidence is accumulating on hBoV also being a real respiratory pathogen, detection of this virus, enabled by the RVP Fast assay, may also be of clinical relevance.

As expected, the superior sensitivity of the PCR assays resulted in greater detection rates of respiratory viruses than with DFA. When combined with the broad detection range of the RVP Fast assay, a nearly threefold overall detection rate of respiratory viruses was observed. Due to the sensitivity of PCR-based methods, a shift towards molecular detection of viruses changes the way of interpreting test results in contrast to the current situation utilizing the DFA. A negative result from the DFA cannot be interpreted as ruling out a suspected virus and a positive result is always a finding with clinical relevance. In molecular detection, interpretation of results is quite the opposite: a negative result of a reliably performed PCR-based analysis may be considered as absence of the pathogen in the sample, but a positive finding may be difficult to interpret, since viral genomes are
occasionally also detected in respiratory and stool samples of asymptomatic subjects, probably due to a distant or persistent infection. This phenomenon concerns, however, a minority of subjects and certain viruses, such as hBoV, and in most cases a positive PCR result, do correlate with clinical illness. This is also true for HRVs, for which recurrent or persistent infections are rare (Jartti et al. 2008; Kusel et al. 2006; Nokso-Koivisto et al. 2002). Together, this rationale suggests that a positive PCR result for HRV is generally of clinical relevance and reflects a current respiratory infection with or without symptoms. However, since differing virus quantities in samples of asymptomatic subjects and subjects with clinical illnesses have been suggested (Jansen et al. 2011), quantitative approaches and receiver-operating characteristic analysis may prove helpful in future interpretation of PCR results.

Evaluation of the RVP Fast assay demonstrated that adopting a PCR-based multiplex assay results in a remarkable increase in overall viral detection rate, in comparison to conventional methods. Together with automation, application of microarrays and other efficient means to discriminate individual PCR products facilitates emergence of high-throughput detection systems in virology laboratories. The sensitive detection of all the viruses of clinical relevance facilitates efficient infection control measures and appropriate patient management. With multiplex testing becoming more common, the clinical importance of mixed infections may be systematically studied and new associations of viral infection with chronic diseases may be unveiled. Moreover, collection of data on occurrence of all the viruses currently recognized of clinical relevance will enable a better understanding of seasonality, geographical distribution and risk groups of viral pathogens.
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