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DETECTION, IDENTIFICATION AND MOLECULAR VARIATION OF HUMAN ENTEROVIRUSES

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*We are the heroes of our time,
but we're dancing with
the demons in our minds*

(Måns Zelmerlöw, Heroes)

ABSTRACT

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Detection, Identification and Molecular Variation of Human Enteroviruses

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Picornaviruses are the most common human viruses and the identification of the picornaviruses is nowadays based on molecular techniques, for example, reverse transcriptase polymerase chain reaction (RT-PCR). One aim of this thesis was to improve the identification of picornaviruses, especially rhino- and enteroviruses, with a real-time assay format and, also, to improve the differentiation of the viruses with genus-specific locked nucleic acid (LNA) probes. Another aim was to identify and study the causative agent of the enterovirus epidemics that appeared in Finland during seasons 2008-2010.

In this thesis, the first version of picornavirus qRT-PCR with a melting curve analysis was used in a study of rhinovirus transmission within families with a rhinovirus positive index child where rhinovirus infection was monitored in all family members. In conclusion, rhinoviruses spread effectively within families causing mostly symptomatic infections in children and asymptomatic infections in adults. To improve the differentiation between rhino- and enterovirus the picornavirus qRT-PCR was modified with LNA-incorporated probes. The LNA probes were validated with picornavirus prototypes and different clinical specimen types. The LNA probe-based picornavirus qRT-PCR was able to differentiate all rhino- and enteroviruses correctly, which makes it suitable for diagnostic use.

Moreover, in this thesis enterovirus outbreaks were studied with a well-observed method to create a strain-specific qRT-PCR from the typing region VP1 protein. In a hand-foot-and-mouth-disease (HFMD) outbreak in 2008, the causative agent was identified as CV-A6 and when the molecular evolution of the new HFMD CV-A6 strain was studied it was found that CV-A6 was the emerging agent for HFMD and onychomadesis. Furthermore, unusual E-30 meningitis epidemics that appeared during seasons 2009 and 2010 were studied with strain-specific qRT-PCR. The E-30 affected mostly adolescents and was probably spread in sports teams.

Keywords: enterovirus; rhinovirus; RT-PCR; LNA probe; coxsackievirus A6; echovirus 3

TIIVISTELMÄ

Riikka Österback

Ihmisten enterovirusten toteaminen, tunnistus ja molekylaarinen variaatio

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Virusoppi,
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2015

Pikornavirukset ovat laajin ihmisessä infektoita aiheuttava virusryhmä, johon kuuluvien virusten tunnistaminen perustuu nykyään käänteiskopiointi-polymeraasiketjureaktioon (RT-PCR). Yhtenä tämän väitöskirjan tavoitteista oli parantaa pikornavirusten, etenkin rino- ja enterovirusten tunnistusta. Ensin muutettiin rino- ja enterovirusten tunnistus reaaliaikaiseen polymeraasiketjureaktiomuotoon (qPCR), jossa erottelu tapahtuu PCR-tuotteen sulamispisteanalyysillä, ja virusten välistä erottelua paranneltiin vielä lukkiutuvilla nukleinihap-pokoettimilla (LNA). Toiseksi väitöskirjassa tutkittiin vuosina 2008 – 2010 Suomessa olleita enterovirusepidemioita.

Pikornavirusten qRT-PCR:a ja sulamispisteanalyysia käytettiin tutkimuksessa, jossa seurattiin rinoviruksen leviämistä perheessä. Rinovirus leviää tehokkaasti lapsiperheissä aiheuttaen oireisen infektion useimmiten lapsissa, vaikka perheen aikuisissa infektio voi olla oireeton. Pikornavirusten qRT-PCR:a paranneltiin vielä erottelevilla LNA-koettimilla. Koettimet validoitiin pikornavirusten tyyppikannoilla ja erilaisilla kliinisillä näytteillä. LNA-koettimet osoittautuivat tehokkaiksi rino- ja enterovirusten erottelijoiksi ja ne mahdollistavat testin käytön rino- ja enterovirusten diagnostiseen toteamiseen.

Lisäksi tutkittiin enterovirusepidemioita, epidemiakannalle kehitettiin enterovirusten VP1-proteiinin tyyppitysalueelta viruskantaspesifinen qRT-PCR. Vuonna 2008 olleen enterorokkoepidemian aiheuttajaksi tunnistettiin CV-A6, joka osoittautui molekyyliepidemiologisesti uudeksi enterorokkokannaksi. CV-A6:n aiheuttamaan enterorokkoon liittyi uutena enterorokon oireena myös kynsien lähtö. Myös sekä 2009 että 2010 esiintyi epätavallinen E-30 aiheuttama aivokalvontulehdusepidemia, ja tähän epidemiaan kuuluneita näytteitä tutkittiin viruskantaspesifisellä qRT-PCR:lla. E-30 aiheuttamaa aivokalvontulehdusta esiintyi eniten teini-ikäisissä, ja luultavasti virus levisi urheilujoukkueissa.

Avainsanat: enterovirus; rinovirus; RT-PCR; LNA koetin; coxsackievirus A6; echovirus 3

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ABBREVIATIONS

AOM	acute otitis media
BAL	bronchoalveolar lavage
BLAST	basic local alignment search tool
CODEHOP	consensus-degenerate hybrid oligonucleotide primer
CPE	cytopathic effect
CRE	<i>cis</i> -active RNA elements
CSF	cerebrospinal fluid
C_t	threshold cycle
CV-A	coxsackievirus A
CV-B	coxsackievirus B
dNTP	deoxynucleotidetriphosphate
E	echovirus
EIA	enzyme-linked immunosorbent assay
EV	enterovirus (strain)
HFMD	Hand-foot-and-mouth-disease
HPeV	parechovirus
IRES	internal ribosome entry site
LBM	Lim Benyesh-Melnick
NASBA	nucleic acid sequence-based amplification
NPA/NPS	nasopharyngeal aspirate/swab
ORF	open reading frame
PV	poliovirus
RNA	ribonucleic acid
RV	rhinovirus (strain)
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
T_m	melting temperature
VPg	viral protein
VP1	viral protein 1
5'NCR	5'-non-coding region
3'NCR	3'-non-coding region

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV).

- I. Österback R, Tevaluoto T, Peltola V, Susi P, Hyypiä T, Waris M. Simultaneous detection and differentiation of human rhino- and enteroviruses in clinical specimens by real-time PCR with locked nucleic acid probes. *Journal of Clinical Microbiology*, 2013 Dec;51(12):3960-7.
- II. Peltola V, Waris M, Österback R, Susi P, Ruuskanen O, Hyypiä T. Rhinovirus transmission within families with children: Incidence of symptomatic and asymptomatic infections. *The Journal of Infectious Diseases* 2008, Vol. 197, No. 3: 382-389.
- III. Österback R, Vuorinen T, Linna M, Susi P, Hyypiä T, Waris M. Coxsackievirus A6 and hand, foot, and mouth disease, Finland. *Emerging Infectious Diseases* 2009 Sep; 15(9):1485-8.
- IV. Österback R, Kalliokoski T, Lähdesmäki T, Peltola V, Ruuskanen O, Waris M. Echovirus 30 meningitis epidemic followed by an outbreak-specific RT-qPCR. *Journal of Clinical Virology*, 2015 Aug;69:7-11.

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1. INTRODUCTION

Picornaviruses belong to the large *Picornaviridae*- family, which is currently divided into 26 genera. Picornavirus family members can infect humans and animals and are spread throughout the world. Picornaviruses are (*pico*-) small (*rna*) RNA viruses with an icosahedral capsid surrounding the naked single-stranded positive-sense RNA genome. Several picornaviruses cause infections in humans and a majority of them belongs to the *Enterovirus* genus. The genus *Enterovirus* currently consists of 12 species: *Enterovirus A* (EV-A), EV-B, EV-C, EV-D, EV-E, EV-F, EV-G, EV-H, and EV-J; as well as *Rhinovirus A* (RV-A), RV-B, and RV-C. The traditional enteroviruses belong to the enterovirus species A to J; coxsackieviruses A and B, (CV-A and CV-B) echoviruses (E) and enteroviruses (EV). Rhinovirus species A and B consist of the old rhinovirus types. A newly discovered species RV-C is the most ascending one of the different enterovirus species.

Enterovirus infections are common diseases worldwide and they particularly affect children. Rhinoviruses and some enterovirus types cause upper and lower respiratory tract infections with mild symptoms from a running nose to a sore throat. Occasionally, the rhinoviruses can cause severe infections from bronchiolitis to sepsis-like illness. Furthermore, asthma exacerbation has been associated with rhinovirus infections. Rhinoviruses are transmitted in direct human-to-human contact via aerosol droplets or from contaminated surfaces. The symptoms of enterovirus infections can range from respiratory tract infections, herpangina, rash, and hand-foot-and-mouth-disease (HFMD), to gastroenteritis, as well as the more severe diseases meningitis and encephalitis. Enteroviruses are mostly transmitted via the fecal-oral route and they replicate in the gastrointestinal track. Enteroviruses also cause severe worldwide epidemics and they are the most common viruses causing absences from schools and work, thus encumbering the healthcare system and causing extensive economic effects.

In this thesis, the transmission of rhinoviruses between family members was studied and the detection method was also upgraded with more virus species specific new LNA-incorporated probes. Moreover, the enterovirus outbreaks during 2008-2010 were studied.

The identification of enteroviruses has traditionally been based on virus culture and neutralization antibodies. Nowadays, molecular identification methods have taken over as the standard method. The diagnostic identification of enteroviruses is primary based on RT-PCRs targeting the highly conserved 5'NCR region of the enterovirus genome. The evolution of the enterovirus genus generates constant maintenance and improvement requirements on the diagnostic identification methods. The typing of enteroviruses is important during epidemics and in epidemiology studies as well as in molecular evolutionary research. Enterovirus serotypes are defined according to the antigenic properties of VP1 protein. The enterovirus VP1 genetic region is heterogeneous, which creates challenges to designing new typing methods. Hence, new tools to study enterovirus outbreaks as well as improvements on the identification methods are constantly needed, and outbreak studies are necessary to gain a better understanding of the enterovirus transmission and epidemiology.

2. REVIEW OF LITERATURE

2.1. Picornaviruses

The order *Picornavirales* consists of 29 *Picornaviridae* genera, of which seven genera include viruses that cause infections in humans (Adams *et al.*, 2013; Knowles, 2012). Picornaviruses are small non-enveloped RNA-viruses and the RNA genome is single-stranded with positive sense orientation. According to the new classification, a majority of the human picornaviruses, including rhinoviruses, belong to the genus *Enterovirus*. Other human picornaviruses are parechoviruses, belonging to the genus *Parechovirus*; hepatitis A, belonging to the genus *Hepatovirus*; Aichi virus belonging to the genus *Kubuvirus*; and kobuvirus-related klasse- and saliviruses, belonging to the genus *Salivirus*. Saffold viruses belong to the genus *Cardiovirus* and cosaviruses to the genus *Cosavirus* (Adams *et al.*, 2013) (Tables 1 and 2).

This thesis covers the human-infecting picornaviruses, i.e., entero- and rhinoviruses that were formerly known as human entero- and rhinoviruses and in this thesis enterovirus stands for enterovirus species and rhinovirus for rhinovirus species. During this thesis project occurred coxsackievirus A6 (CV-A6) and echovirus 30 (E-30) epidemics in Finland.

2.2. Enteroviruses

The human enteroviruses were originally classified as polioviruses (PV), coxsackie A viruses (CV-A), coxsackie B viruses (CV-B), echoviruses (E), and enteroviruses (EV). Current enterovirus taxonomy is based on genomic relatedness, dividing human enteroviruses into four species, namely *Enterovirus A-D* (Knowles, 2012). Today, 104 human enteroviruses belong to those four *Enterovirus* species (Table 2), while the members of the other *Enterovirus* species (*E-J*) infect nonhuman primates. The sequence-based classification has increased our knowledge of human enteroviruses and their relatedness with each other as well as with the nonhuman primates-associated enteroviruses (Harvala *et al.*, 2014).

Enteroviruses are common worldwide circulating pathogens and, almost all over the world, enterovirus infections occur with a seasonal pattern with peaks in early summer to late autumn (Pallansch, 2013). Enteroviruses cause a wide range of diseases, e.g., infections in the central nervous system, hand-foot-and-mouth disease (HFMD), myocarditis, herpangina, rashes, and respiratory diseases infecting both upper and lower respiratory tracts.

The species in the genus *Enterovirus A* consist of 21 types, 11 CV-As and 10 EVs (Table 2). The endemic *Enterovirus A* strains are coxsackievirus A16 (CV-A16) and enterovirus 71 (EV-A71), and they are associated with herpangina and hand-foot-and-mouth disease. Furthermore, EV-A71 has been associated with severe neurological cases of aseptic meningitis, encephalitis and poliomyelitis-like paralysis and it has been linked to mortality - in Asia, Australia, Europe and America (Honkanen *et al.*, 2013a; Palacios and Oberste, 2005).

All known 28 Es, six CV-Bs, 24 EVs and coxsackievirus A9 (CV-A9) belong to the species *Enterovirus B* (Table 2). The members of the *Enterovirus B* group are often associated with meningitis, myocarditis, and encephalitis, and they cause infections of the central nervous system. E-30 is a common pathogen in viral meningitis outbreaks worldwide (Lee and Davies, 2007; Pallansch, 2013).

The most studied human enteroviruses, the polioviruses (PVs) 1-3 are included in *Enterovirus C* species. Furthermore, *Enterovirus C* species comprise nine CV-As, and eleven EVs (Table 2). Novel species of *Enterovirus C* (EV-C104, EV-C117, and EV-C118) have been isolated from patients with pneumonia and otitis media and EV-C109 has been isolated from a patient with influenza-like symptoms (Yozwiak *et al.*, 2010). Furthermore, fatal acute flaccid paralysis has been found to be caused by an *Enterovirus C* genotype (EV-C105) (Richter *et al.*, 2013). The effort to eradicate the poliovirus through vaccinations has been going on since 1962, and the vaccinations have reduced the poliomyelitis cases to 400 confirmed infections worldwide in 2013. In 2014, there were still eight countries (Pakistan, Nigeria, Afghanistan, Equatorial Guinea, Iraq, Cameroon, Syrian Arab Republic, and Ethiopia) where the wild-type polioviruses (WPV) have been detected (WHO, 2014).

Table 1. Genera of the picornavirus family, approved by ICTV in March 2014.

Genus <i>Picornavirus</i>	Host
<i>Aphthovirus</i>	Animals species
<i>Aquamavirus</i>	Animals species
<i>Avihepatovirus</i>	Animals species
<i>Avisivirus</i>	Animals species
<i>Dicripivirus</i>	Animals species
<i>Erbovirus</i>	Animals species
<i>Gallivirus</i>	Animals species
<i>Hunnivirus</i>	Animals species
<i>Megrivirus</i>	Animals species
<i>Mischivirus</i>	Animals species
<i>Mosavirus</i>	Animals species
<i>Oscivirus</i>	Animals species
<i>Pasivirus</i>	Animals species
<i>Passerivirus</i>	Animals species
<i>Rosavirus</i>	Animals species
<i>Sakobuvirus</i>	Animals species
<i>Kunsagivirus</i>	Animals species
<i>Sapelovirus</i>	Animals species
<i>Senecavirus</i>	Animals species
<i>Teschovirus</i>	Animals species
<i>Tremovirus</i>	Animals species
<i>Sicinivirus</i>	Animals species
<i>Enterovirus</i>	Human and animals species
<i>Parechovirus</i>	Human and animals species
<i>Hepatovirus</i>	Human and animals species
<i>Kobuvirus</i>	Human and animals species
<i>Cosavirus</i>	Human and animals species
<i>Cardiovirus</i>	Human
<i>Salivirus</i>	Human

Enterovirus D species include enterovirus 68 (EV-D68; formerly known as RV-87), enteroviruses 70 and 94 (EV-D70, EV-D94), and enterovirus 111 (EV-D111) capable of infecting both humans and chimpanzees (Table 2). RV-87 was reclassified as EV-D68 according to the genetic and antigenic affinity to the prototype strain of EV-D68 (EV-D68 Fermon) and the other *Enterovirus D* prototypes (Blomqvist *et al.*, 2002b). However, EV-D68 does not have the acid resistance feature, which is typical for enteroviruses. Therefore, EV-D68 has similar epidemiological and biological patterns as rhinoviruses and it has been reported as a causative agent in respiratory infection outbreaks (Lu *et al.*, 2014; Oberste *et al.*, 2004a).

2.2.1. Coxsackievirus A6

A type of *Enterovirus A*, CV-A6, has been identified from feces in the late 1940s. The CV-A6 specimens were collected from patients with poliomyelitis-like infections in a town called Coxsackie in Greene County in the U.S. state of New York. The coxsackieviruses were identified studying the pathogenicity of poliovirus-like viruses in suckling mice (Dalldorf, 1953).

CV-A6 and other coxsackie A viruses proliferate at least in rhabdomyosarcoma (RD) cell line (Schmidt *et al.*, 1975). CV-A6 has been associated with respiratory tract infections, aseptic meningitis, and herpangina, as well as HFMD often occurring in late summer and autumn (Richter *et al.*, 2006; Yamashita *et al.*, 2005). In a large Finnish surveillance study only six cases of CV-A6 were identified between 2000 and 2005 (Blomqvist *et al.*, 2008).

2.2.2. Echovirus 30

Echo (*Enteric Cytopathic Human Orphan*) viruses belongs to the *Enterovirus B* species, and of the echoviruses, E-30 is the main virus to cause aseptic meningitis worldwide in a pattern with 3-5 years intervals (Khetsuriani *et al.*, 2006). In the last decade, E-30 has caused an aseptic meningitis epidemic in several countries in Europe: Italy (Milia *et al.*, 2013), Spain (Trallero *et al.*, 2010), Greece (Mantadakis *et al.*, 2013), Serbia (Cosić *et al.*, 2010), France (Lévêque *et al.*, 2010), Latvia (Perevoscikovs *et al.*, 2010), and Finland (Savolainen-Kopra *et al.*, 2011) in Asia, in South-Korea (Hyeon *et al.*, 2013), China (Xiao *et al.*, 2013; Yang *et al.*, 2013), Taiwan (Ke *et al.*, 2011), and in South, Central and North America (dos Santos *et al.*, 2011; Khetsuriani *et al.*, 2006; Martinez *et al.*, 2012). Molecular evolution studies of E-30 have divided the E-30 viruses into eight lineages. Research has shown that instead of the geographical appearance of E-30 being the factor dividing the virus into different clusters, the time of evolution appears to be significant (McWilliam Leitch *et al.*, 2009). Evidently, between outbreaks, E-30 infects only sporadically and bursts to an outbreak when a new population without any herd immunity appears (Oberste *et al.*, 1999a).

2.3. Rhinoviruses

Rhinoviruses, formerly known as human rhinoviruses, have been reclassified as members of the genus *Enterovirus* and renamed as *Rhinovirus A*, *B*, and *C* (RV-A, RV-B, and RV-C, respectively) (Knowles, 2012). Rhinoviruses were identified in the 1950s, when studies to cure the common cold started. They are the most common and widely spread year-round cold viruses. The species *Rhinovirus A-C* consist of over 160 genotypes. At the moment, the RV-A species include 80, RV-B species 32 and RV-C species 54 virus types (www.picornaviridae.com). Rhinoviruses cause respiratory tract infections (mostly common cold-associated symptoms) and otitis media (Monto *et al.*, 2001; Mäkelä *et al.*, 1998; Peltola *et al.*, 2008). Lower respiratory tract infections, such as, pneumonia, bronchiolitis, wheezing, and asthma (Khetsuriani *et al.*, 2008) have also been linked to rhinoviruses. They are the most common infection agents to cause absence from schools and work with significant economic effects. Currently, there are no antivirals or vaccines for the treatment or prevention of rhinovirus infections.

A novel *Rhinovirus C* species was discovered when the reverse transcription polymerase chain reaction (RT-PCR) method became the main method to identify picornaviruses. Because RV-Cs do not grow in a traditional cell culture, the identification has to be based on molecular methods. Nowadays, there are over 50 classified RV-C types and RV-Cs have been found even in specimens collected in the 1980s (Linder *et al.*, 2013). The novel RV-C types are not new viruses, they were only not discovered until the identification methods were improved. The rhinovirus classification is regularly based on the sequencing of the VP1 coding region, or VP4 and part of VP2 coding regions. Furthermore, sequencing of the 5'NCR is also used for classification. However, the 5'NCR sequences give useful information on the virus species, but do not alone determinate the genetic type (Kiang *et al.*, 2008; Lee *et al.*, 2007; Savolainen-Kopra *et al.*, 2009c; Simmonds *et al.*, 2010a). The new clade of rhinovirus was proposed after a MassTag PCR study of specimens from patients with influenza-like symptoms, where the VP4 genome sequences showed diversity among rhinovirus isolates (Lamson *et al.*, 2006), and after a study of adult asthma patients with rhinoviruses clustering into a new clade (Kistler *et al.*, 2007). The first complete genome sequences (RV-C1, RV-C2, RV-C3, RV-C4, RV-C5, and RV-C6) of RV-Cs were published between 2006 and 2008 and a distinct clade of RV named RV-C was presented (Lau *et al.*, 2007; McErlean *et al.*, 2008; McErlean *et al.*, 2007).

RV-Cs cannot replicate in standard virus culture cell lines, and only experiments in sinus mucosal tissue have succeeded in growing RV-C. Thus, the enigma of RV-C cell entry and binding to receptor(s) remains unsolved. A comparison of the amino acid profile of RV-C with known rhinovirus receptor binding sites (ICAM-1 and LDLR receptor binding sites) and the replication of *in vitro* transcript RV-C RNA in HeLa cell line implicate the existence of RV-C-specific receptor(s) (Bochkov *et al.*, 2011; Hao *et al.*, 2012). Bochkov *et al.*, 2015, recently reported that cells expressing human cadherin-related family member 3 support the binding and replication of RV-C.

RV-Cs have been reported to associate with severe illnesses, for example, pneumonia, pericarditis, wheezing, and asthma (Broberg *et al.*, 2011; Cox *et al.*, 2013; Drysdale *et al.*, 2014; Gern, 2010; Tapparel *et al.*, 2009c) and also found in common infections, such as otitis media (Savolainen-Kopra *et al.*, 2009b). A study of hospitalized children with severe respiratory symptoms suggested that RV-C types may cause viremia more often than RV-A and RV-B types. Therefore, it is suggested that RV-C types can cause more severe symptoms than the other rhinovirus types (Fuji *et al.*, 2011). Additionally, RV-C has been found in feces, mostly from infants and children under 2 years of age with respiratory tract infections and from elderly people with gastrointestinal infections (Broberg *et al.*, 2011; Harvala *et al.*, 2012; Savolainen-Kopra *et al.*, 2013).

2.4. Other human picornaviruses

Other human picornaviruses belong to the genera *Parechovirus*, *Hepatovirus*, *Cosavirus*, *Cardiovirus*, *Salivirus*, and *Kobuvirus*. Before genetic reclassification in the 1990s parechoviruses 1 and 2 (HPeV-1 and HPeV-2) were known as human echovirus 22 and 23 (Hyypiä *et al.*, 1992). After the reclassification they formed the new genus *Parechovirus*. Currently, 16 parechoviruses (HPeV 1-16) have been identified and eight of them (HPeV 1-8) have been completely sequenced, whereas the others (HPeV 9-16) have been identified according to the partial sequencing of the VP1 region of the genome (www.picornaviridae.com). HPeVs have been associated with gastroenteritis and sepsis-like syndrome in children (Esposito *et al.*, 2014b; Kolehmainen *et al.*, 2014).

Hepatitis A virus (HAV) is the only member in the genus *Hepatovirus* and it causes hepatitis. HAV is transmitted via the fecal-oral route from contaminated food or water supply (Cristina and Costa-Mattioli, 2007). Human cosavirus (common-stool-associated-picornavirus) has been isolated in Pakistan from stool specimens of children with symptoms of acute flaccid paralysis, and in Australia from children with diarrhea. At least four species of cosaviruses have been identified, *Cosavirus A-D* (Holtz *et al.*, 2008; Kapoor *et al.*, 2008). The genus *Cardiovirus* includes Vilyuisk human encephalomyelitis virus (Lipton, 2008) and Saffold viruses. Currently, 11 Saffold virus genotypes have been identified, mostly from patients with gastrointestinal symptoms and respiratory infections (Blinkova *et al.*, 2009; Itagaki *et al.*, 2011). Aichivirus, a member of the genus *Kobuvirus* causes diarrhea in humans and is transmitted by seafood (Nielsen *et al.*, 2013c). Salivirus and Klassevirus, members of the genus *Salivirus*, are novel picornaviruses identified from stool specimens of children with diarrhea (Greninger *et al.*, 2009; Li *et al.*, 2009).

Table 2. Picornavirus genera, species and human genotypes in June 2015.

Genus	Species	Previous name	Human genotypes
Enterovirus	Enterovirus A	Human enterovirus A	CV-A2 to CV-A8, CV-A10, CV-A12, CV-A14, CV-A16, EV-A71, EV-A79, EV-A89 to EV-A92, EV-A114, EV-A119 to EV-A121
	Enterovirus B	Human enterovirus B	CV-B1 to CV-B6, CV-A9, E-1 to E7, E-9, E-11 to E-21, E-24 to E-27, E-29 to E-33, EV-B69, EV-B73 to EV-B75, EV-B77 to EV-B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107, EV-B111
	Enterovirus C	Human enterovirus C	PV1 to PV-3, CV-A1, CV-A11, CV-A13, CV-A17, CV-A19 to CV-A22, CV-A24, EV-C95, EV-C96, EV-C99, EV-C102, EV-C104, EV-C105, EV-C109, EV-C113, EV-C116, EV-C117, EV-C118
	Enterovirus D	Human enterovirus D	EV-D68, EV-D70, EV-D94, EV-D111 (also in chimpanzees)
	Enterovirus E	Bovine enterovirus A	
	Enterovirus F	Bovine enterovirus B	
	Enterovirus G	Porcine enterovirus B	
	Enterovirus H	Simian enterovirus A	
	Enterovirus J	Unassigned simian enteroviruses	
	Rhinovirus A	Rhinovirus A	RV-A1, RV-A2, RV-A7 to RV-A13, RV-A15, RV-A16, RV-A18 to RV-A25, RV-A28 to RV-A34, RV-A36, RV-A38 to RV-A41, RV-A43 to RV-A47, RV-A 49 to RV-A51, RV-A53 to RV-A68, RV-A71, RV-A73 to RV-A78, RV-A80 to RV-A82, RV-A85, RV-A88 to RV-A90, RV-A94, RV-A96, RV-A101 to RV-A109
	Rhinovirus B	Rhinovirus B	RV-B3 to RV-B6, RV-B14, RV-B17, RV-B26, RV-B27, RV-B35, RV-B37, RV-B42, RV-B48, RV-B52, RV-B57, RV-B69, RV-B70, RV-B 72, RV-B79, RV-B83, RV-B84, RV-B86, RV-B91 to RV-B93, RV-B97, RV-B99 to RV-B106
	Rhinovirus C	Rhinovirus C	RV-C1 to RV-C54
Hepatovirus	Hepatitis A virus	Enterovirus 72	Hepatitis A
Kobuvirus	Aichivirus A	Aichi virus	Aichivirus 1
	Aichivirus B	Bovine kobuvirus	
	Aichivirus C	Porcine kobuvirus	
Parechovirus	Human parechovirus		HPeV 1-16
	Ljungan virus		
	Sebokele virus		
Cardiovirus	Theilovirus		VHEV, Saffold virus 1-11
	Encephalomyocarditis virus		
Cosavirus	Cosavirus A-E)	Decaviruses	HCoSV-A1 - 24
Salivirus	Salivirus A		Salivirus, Klassevirus 1

2.5. Structure and genomic organization of enteroviruses

Enteroviruses are non-enveloped RNA viruses approximately 30 nm in diameter with an icosahedral capsid surrounding the positive-sense single-stranded genome. The enterovirus genome consists of approximately 7 500 bp with a noncoding region (NCR) at both the 5' and 3' end. The length of 5'NCR is between 600 and 1200 nucleotides, depending on the picornavirus genus. The 5'NCR contains motifs that are important in viral RNA synthesis, such as VPg (genome linked viral protein) a small, approximately 23 amino acid long protein covalently linked to the 5' end of the 5'NCR and with an important role in triggering the replication. Moreover, the 5'NCR contains a cloverleaf secondary structure that binds to the 40s ribosomal unit. This internal ribosomal entry site (IRES) directs the messenger RNA translation in picornaviruses. The enterovirus polypeptide is encoded with a single open reading frame (ORF). The ORF is first cleaved into protein precursors P1, P2, and P3 and, thereafter, by viral proteases 2A^{pro} and 3C^{pro} into viral capsid proteins VP1, VP2, VP3, VP4 (P1), nonstructural proteins 2A, 2B, 2C, 3A, 3B, and 3C, as well as 3D polymerase (P2 and P3). The short, approximately 45 to 130 bp long 3'NCR consists of a polyadenylated (poly(A)) tail. The 3'NCR contains an RNA synthesis directing secondary structure. However, the whole 3'NCR is not necessary for infections (Figure 1A). The viral capsid proteins VP1, VP2, and VP3 are located on the outer surface of the virus particle and VP4 is located on the inner surface of the capsid. These four viral proteins form a protomer, and the whole virus capsid consists of 60 protomer units forming an icosahedral structure (Figure 1B). The nonstructural proteins are involved in the translation and replication of the virus (Ehrenfeld, 2010; Racaniello, 2007).

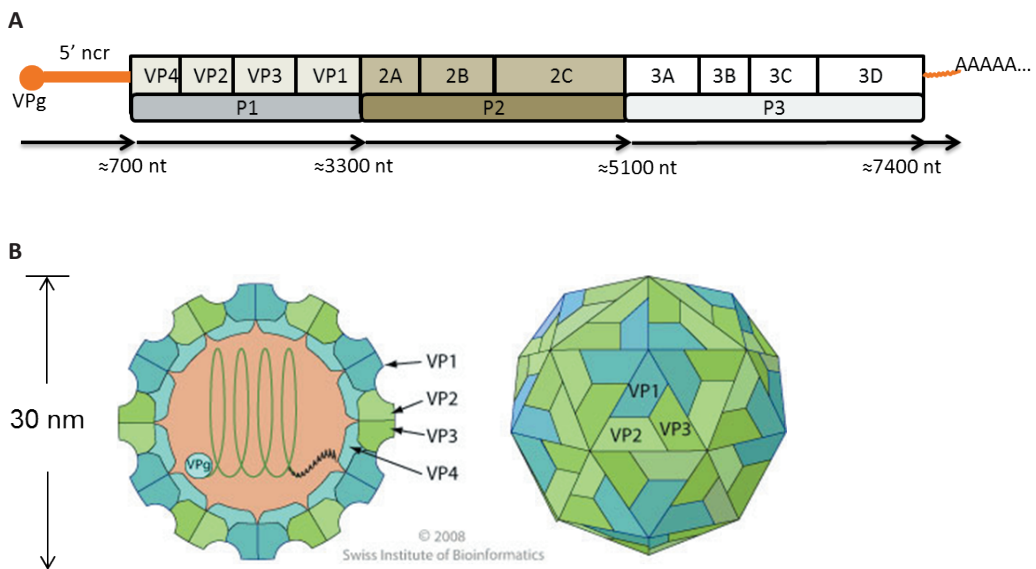


Figure 1. Genome (A) and structure (B) organization of enteroviruses. Figures modified from ViralZone: www.expasy.org/viralzone, Swiss Institute of Bioinformatics.

2.6. Enterovirus replication

The viral infection process starts with an attachment of the virus to the cell surface receptors. Host cell membrane contains several different types of cell surface proteins that act as contact proteins for the viruses and assist the infection by serving as receptors. Enterovirus receptors are well studied and several virus-specific receptors are known. Enterovirus receptors can be immunoglobulin family members, integrins, lipoproteins, and/or complement regulatory proteins. For example, intercellular adhesion molecule-1 (ICAM-1), an immunoglobulin family member, serves as the receptor for most of the RV-A and RV-B types (the major receptor group), and a low-density lipoprotein receptor (LDLR) for a handful of the RV-A and RV-B types (the minor receptor group) (Vlasak *et al.*, 2005). Polioviruses bind to the immunoglobulin-like adhesion receptor CD155, also known as the poliovirus receptor (PVR). Coxsackievirus and adenovirus receptor (CAR), ICAM-1, and complement decay-accelerating factor CD55 (DAF) are receptors for coxsackieviruses, and echoviruses are known to use the DAF receptor as the contact protein on cell surface (Ehrenfeld, 2010; Tuthill *et al.*, 2010). A virus binding to its receptor induces a conformational change and the RNA penetrates the cell membrane entering the host cell cytoplasm. In the cytoplasm, the VPg is cleaved, the ORF is translated, and viral proteases 2A^{Pro} and 3C^{Pro} break down the viral polypeptide via protein precursors (P1, P2, and P3) to viral proteins (VP1-VP4, 2A-2C, and 3A-3D). In the synthesis of picornavirus RNA, the positive-sense RNA genome is produced with 3D^{pol}, an RNA dependent RNA polymerase, via a complementary negative strand RNA in the replication vesicle. The replication of picornavirus viral RNA is assisted by *cis*-active RNA elements (CRE): for example, the poliovirus has four CREs located in the 5'NCR, ORF, 3'NCR, and 3' poly(A) tail (Steil and Barton, 2009). The capsid proteins are assembled to the icosahedral capsid around the viral RNA before new viruses are released (Racaniello, 2007).

2.7. Transmission and pathogenesis of enteroviruses

Enteroviruses are transmitted via the fecal-oral route or from human to human via droplets directly or indirectly in contaminated hand contact. Infective rhinovirus aerosols are spread by coughing, sneezing, singing or even breathing (Gralton *et al.*, 2013). Rhinoviruses are inoculated by intranasal or conjunctival routes and replication occurs in the nasal epithelium or pharyngeal mucosa. Rhinoviruses can live on surfaces for several hours and on healthy skin for a couple of hours (Jacobs *et al.*, 2013). The symptoms of rhinovirus infections are located at the replication sites, mostly in the upper or lower respiratory tract. The symptoms can appear within one to two days after inoculation and can last for one to two weeks in immunocompetent individuals. The ambient temperature for rhinovirus reproduction is 33°C, the temperature in the nasal cavity and pharynx. However, the higher temperature in the lower respiratory tract (37°C) is not a barrier for rhinovirus replication. Rhinovirus replications in the lower respiratory tract have been demonstrated in an experimental infection of adults with RV-A16 (DeMore *et al.*, 2009; Papadopoulos and Johnston, 2000).

Rhinovirus replication in the lungs can initiate asthma exacerbations, chronic obstructive disease (COPD), pneumonia, and bronchiolitis (Papadopoulos and Johnston, 2000). Although rhinoviruses can replicate in the airway epithelial cells *in vitro* without any cytopathology effects, an infection can cause edema in the airway epithelial cells and result in airway obstruction and mucosa secretion *in vivo* (Gern, 2010). In upper respiratory tract infections, rhinitis and nasal blockage are the common symptoms caused by an inflammatory response of neutrophils stimulating mucus secretion and vascular permeability (Kennedy *et al.*, 2012). It is not uncommon to detect rhinovirus RNA in the nose of asymptomatic patients (Nokso-Koivisto *et al.*, 2002). Commonly, the rhinovirus RNA-positive asymptomatic patient has a family member with common cold symptoms (Jartti *et al.*, 2008). It has been suggested that common cold symptoms caused by rhinoviruses are driven by the host immune system response against the virus (Kennedy *et al.*, 2012). When rhinoviruses replicate in the airway epithelial cells, the cells induce the expression of proinflammatory chemokines, which induce inflammatory cells, neutrophils, lymphocytes, and eosinophils and, thereby, cause an inflammation in the airways (Hershenson, 2013).

Rhinovirus viremia has been detected in children with severe symptoms and a high virus count in the nasopharyngeal swab (NPS) specimens (Esposito *et al.*, 2014a). Already several decades ago, rhinovirus viremia was connected with sudden death of infants (Williams *et al.*, 1984), but as reports of the findings are uncommon, rhinovirus viremia is a controversial issue. However, there are suggestions that the RV-C types are more viremic than RV-A and RV-B types.

Enteroviruses spread mostly by fecal-oral transmission. However, transmission via water is a probable route for spreading in areas with poor water and sewage infrastructure, whereas oral-oral transmission is a probable general route for enterovirus transmission in developed countries. Hemorrhagic conjunctivitis caused by enterovirus 70 (EV-B70) and coxsackievirus A 24 (CV-A24) are exceptions to the transmission route rule, as they are probably not transmitted via the fecal-oral route, but are more likely to be transmitted via direct eye secretion contact or via swimming pools (Aubry *et al.*, 2012). HFMD commonly caused by CV-A16 and EV-A71 can also be transmitted by the vesicular fluids. Enteroviruses spread effectively in public events, schools, and sports team activities causing even endemic outbreaks (Pallansch, 2013). Almost all enteroviruses can replicate in oral-pharyngeal mucosa or tonsils before they enter the gastrointestinal tract or bloodstream in the viremic state. The enteroviruses are acid stable and pass through the intestinal tract to the feces being able to cause secondary-site infections at other locations (Pallansch, 2013). Enterovirus can enter the central nervous system via axons or by passing through the blood-brain-barrier (BBB). The central nervous system entry and the neural pathway have been studied with poliovirus in mouse models (Ren and Racaniello, 1992; Yang *et al.*, 1997). Furthermore, some enteroviral symptoms, e.g., exanthema and cardiac disease, can be caused by the host immune response (Palacios and Oberste, 2005).

2.8. Clinical manifestations and epidemiology of enteroviruses

Common cold is the most commonly occurring disease and is most likely to be caused by rhinoviruses. Common cold is usually a self-limited disease with symptoms of rhinorrhea, sore throat, cough, headache, and even fever. Rhinovirus infections are mostly detected after the school starts in the fall, as well as in the springtime and several rhinovirus types are detected during the seasonal peaks. According to a cohort study, RV-Cs were detected more frequently in cases of severe illness than other rhinoviruses during the winter (Linder *et al.*, 2013). In another cohort study, severe rhinovirus infections were also observed during the winter months, but both RV-A and RV-C types were detected (Lee *et al.*, 2012).

Acute otitis media (AOM) is a common childhood disease often caused by bacteria, for example, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*, but it can also be caused by viruses. In several studies picornaviruses, mostly rhinoviruses, have been associated with AOM. According to a Finnish cohort study, almost one-half of the AOM episodes were caused by rhinoviruses (Blomqvist *et al.*, 2002a). Another Finnish study showed that AOM is not only a sole bacterial or virus disease, but that co-infections are also quite common. Co-infections of virus and bacteria were found in almost 70% of middle ear fluid specimens collected from children with AOM and tympanostomy tubes. Picornaviruses was the main group of viruses detected in the co-infection cases (Ruohola *et al.*, 2006).

Rhinovirus replication in the lower respiratory tract is associated with wheezing disease, which is suggested as a cause in contributing to the development of asthma (Gern, 2010; Jartti and Gern, 2011; Kotaniemi-Syrjänen *et al.*, 2003). In recurrent wheezing episodes a rhinovirus finding is very common. A large cohort study demonstrated a 10-fold risk to develop asthma during childhood if the child has suffered from rhinovirus infections and wheezing during the first three years of life (Jackson *et al.*, 2008). Bronchiolitis is the most common rhinovirus-associated lower respiratory track illness in children. Studies of hospitalized children with acute bronchiolitis have demonstrated that 16-26% of them suffered from rhinovirus infection (Cox and Le Souëf, 2014). In addition to bronchiolitis, rhinoviruses are known to cause pneumonia, an inflammatory condition of the lung alveoli. According to the study of Daleno *et al.*, 2013, the members of the species RV-A were the most common rhinovirus types to cause rhinovirus pneumonia.

Enterovirus season occurs during the summer and autumn, commonly beginning during the summer months and accelerating when the schools start in the fall. Enteroviruses affect mostly children and adolescent. Apparently, in severe enteroviral diseases the infection rate is higher in males than in females. The most common circulating types of enteroviruses are echoviruses, E-7, E-9, E-11, and E-30, as well as coxsackieviruses CV-B1, CV-B2, CV-B5, and CV-A9 ((CDC), 2006; Khetsuriani *et al.*, 2006; Pallansch, 2013). The above mentioned enteroviruses cause mainly aseptic meningitis and other neurologic symptoms, e.g., encephalitis and acute flaccid paralysis. Aseptic meningitis is a viral infection of the meninges with symptoms of headache, fever, vomiting, and photophobia. Other symptoms of enterovirus infections are myocarditis, HFMD, neonatal sepsis, hemorrhagic

conjunctivitis. Myocarditis, the inflammation of the heart tissue, has been associated with coxsackie B viruses, in general with CV-B3 (Cooper, 2009). CV-A24 and EV-D70 are the main enteroviruses causing the contagious hemorrhagic conjunctivitis (Palacios and Oberste, 2005). However, CV-A24 induced outbreaks have occurred mostly in tropical and subtropical locations (Aubry *et al.*, 2012). Enteroviral infections are common in infants: for example, coxsackie B viruses, E-9, and E-11 are reported to cause sepsis-like illness in newborn babies (Harvala *et al.*, 2011; Piralla *et al.*, 2014). Recently, the rarely detected rhinovirus-like EV-D68 has caused severe respiratory illness outbreaks in children and the virus has even been associated even with fatal cases (Lu *et al.*, 2014; Stephenson, 2014). It has been suggested, that enteroviruses may be associated with the progress of type 1 diabetes and it has also been proposed that, especially for coxsackievirus B and echoviruses, links between diabetogenic enterovirus types and epidemic patterns exist (Craig *et al.*, 2013; Oikarinen *et al.*, 2011).

2.9. Laboratory diagnostics of enteroviruses

2.9.1. Specimens

To improve the diagnostics of viral illnesses, the specimens must be collected at the site of the symptoms. To get good quality specimens, they should be collected within a few days of the onset of the symptoms. In most cases, the amount of the virus is highest in the acute phase of the illness. Hence, timing and the specimen type are important in identifying specific viral diseases. Specimens for molecular detection should be immediately transported in dry and clean vials to the laboratory. Specimens for virus culture should be transported in a virus culture transport medium in stable temperature to keep the virus viable and the cultivation should be started immediately in the laboratory.

In upper respiratory tract infections, primary specimens are nasopharyngeal swab, aspirate, and nasal or throat swab. There are recommendations to use flocked swabs in collection of viral specimens (Pallansch, 2013). The flocked swabs are made from synthetic fibers, for example, Nylon or Dacron or the semi-synthetic fiber Rayon. The fibers are applied by flocking around the swabs, the brush-like formation enlarging the contact surface of the swab and improving the collection of viral specimens. The flocked swab is patient-friendly and superior compared to the standard cotton swab, nasal wash or aspirate (Munywoki *et al.*, 2011; Waris *et al.*, 2013). In lower respiratory tract infections, sputum, bronchoalveolar lavage fluid (BAL), or tracheal/bronchial fluid are possible specimens for viral diagnosis (Jacobs *et al.*, 2013; Waris *et al.*, 2013).

In enteroviral meningitis or in other infections affecting the central nervous system, the CSF is the main specimen to be collected (Lee and Davies, 2007). Serum samples are mainly used for antibody testing, but in the viremic state enteroviruses can be found in the sera/ blood with molecular detection methods. HFMD can be diagnosed from the vesicular fluid swabs and enteroviruses have also been identified from tissue specimens. Enteroviral

hemorrhagic conjunctivitis can be analyzed from a conjunctival swab, and enteroviruses have also been identified from tears (Aubry *et al.*, 2012; Yin-Murphy *et al.*, 1985). Because enteroviruses replicate in the gastrointestinal tract, feces are suitable specimens for enterovirus diagnostics, as long as it is taken into account that the excretion of enteroviruses in the feces can persist for several weeks after the acute infection (Pallansch, 2013). Also some rhinovirus types, especially members of the species RV-C, can apparently survive in the gastrointestinal tract, and they have been identified from the feces of children and elderly people with symptoms of enteric infections or severe pneumonia (Broberg *et al.*, 2011; Harvala *et al.*, 2012; Honkanen *et al.*, 2013b; Lau *et al.*, 2012; Tapparel *et al.*, 2009c).

2.9.2. Virus culture and serology of enteroviruses

Entero- and rhinoviruses have been traditionally detected and cultivated grown in specific cell culture lines. This golden standard method is still very useful, at least as a reference method as well as in research use. Picornaviruses can be cultured from several specimen types, e.g. feces, CSF, tissue, BAL, or nasal or throat swab. Generally, entero- and rhinoviruses can infect several specific cell lines, for example, human foreskin fibroblasts (HFF), human lung carcinoma cells (A549), cervical cancer cells (HeLa), human lung fibroblasts (MRC-5), rhabdomyosarcoma cells (RD), and human colon carcinoma cells (Caco-2). The inoculated cell cultures are traditionally grown in roller tube cultures at 35-37°C, those for rhinoviruses at 33°C, and examined under microscope for conformational changes in the cell layer. The cytopathic effect (CPE) can manifest reveal as rounded, swollen, or shrunken cells, or the cells may be clustered together or destroyed. Picornavirus proliferation takes from a few days up to several weeks and, therefore, virus culturing is a time- and labor-consuming method of detecting entero- and rhinoviruses (Leland and Ginocchio, 2007).

Enterovirus replication in cell culture can be confirmed and typed with neutralization antisera; by immunofluorescence with type-specific monoclonal antibodies; or with molecular methods, e.g., RT-PCR and sequencing. Formely the World Health Organization (WHO) recommended the use of Lim Benyesh-Melnick (LBM) neutralization panel for the neutralization test. The LBM panel consists of pooled antibodies for the 59 most common enterovirus types and for two parechoviruses, HPeV-1 and HPeV-2. (Bendig and Earl, 2005; Egbertson and Mayo, 1986). However, the panel only covers a half of the currently known enterovirus types and it is very time-consuming. Comparison studies of traditional virus cultures with modern molecular detection methods show the overpowering sensitivity and specificity of the latter (Terletskaia-Ladwig *et al.*, 2008). Nowadays the enterovirus typing recommendation is based on molecular methods (WHO, 2015). Moreover, there are entero- and rhinoviruses (e.g., RV-Cs), which do not grow in traditional cell cultures or which do not produce a visible CPE. Nevertheless, the isolation of picornaviruses in cell cultures will still be needed in the future, because it helps us to understand the pathogenicity and other behavior of the viruses and their different types.

The multitude of entero- and rhinovirus types are challenging for the serological diagnostics. Serological tests are not highly relevant in the detection of primary entero- or rhinovirus infection. The challenge in picornavirus serology is the lack of knowledge of the specificity of antibody-antigen compatibility (Barclay and Al-Nakib, 1987; Blomqvist *et al.*, 2002a; Samuelson *et al.*, 1993). There is no universal entero- or rhinovirus antigen to cover antibody responses of all known genotypes. However, some entero- and rhinovirus antigens have cross-reactivity with each other or with other picornaviruses. To completely understand the coverage and cross-reactivity more antigen and antibody studies are needed. Nevertheless, several methods have been developed to detect entero- or rhinovirus antibody responses from sera or nasal mucus. The detection of the entero- or rhinovirus IgM antibodies proves the acute infection and the detection of IgG antibodies from paired sera specimens with a significant increase in the titres confirms a past or recent infection. (Barclay and Al-Nakib, 1987; Boman *et al.*, 1992; Samuelson *et al.*, 1993; Terletskaia-Ladwig *et al.*, 2000). Complement fixation and hemagglutination inhibition methods were formerly used to measure entero- or rhinovirus antibodies, whereas enzyme-linked immunosorbent assay (EIA) is a more modern method. In an enterovirus meningitis study, EIA proved to be a more sensitive method for the detection of enterovirus IgM antibodies than the complement fixation method. In the EIA test, CV-B5 and E-9 antigens were used, and the complement fixation test utilized a commercial picornavirus antigen (Terletskaia-Ladwig *et al.*, 2000). Even a type-specific IgM antibody test has been developed, e.g., for the detection of CV-A16 IgM in HFMD patients but, unfortunately, the cross-reactivity of CV-A16 antigen with other HFMD-causing enterovirus antibodies was not studied (Xu *et al.*, 2011).

2.9.3. Molecular detection methods

Advances in molecular-based detection methods have improved the diagnostics of entero- and rhinovirus infections. The direct detection of the virus genome without the need of a pathogen culture has enhanced pathogen detection and even enabled the identification of new types and species, for example, the discovery of RV-C. In addition to this, molecular-based detection can be performed with smaller sample volumes than viral culturing. Several molecular techniques for entero- and rhinovirus detection have been described, for example, real-time and conventional RT-PCRs, nested and semi-nested RT-PCRs, and nucleic acid sequence-based amplification (NASBA).

In NASBA, the detection of RNA is based on targeting the primers and probes in isothermal conditions with reverse transcriptase enzyme. The final product in NASBA is DNA and the advantage of NASBA technique is that the primers and probes do not bind to genomic DNA (Fox *et al.*, 2002; Heim and Schumann, 2002; van Doornum *et al.*, 2007). NASBA has been used in several entero- and rhinoviruses identification studies mostly in comparison with commercial or in-house RT-PCRs (Costa *et al.*, 2008; Landry *et al.*, 2003; Loens *et al.*, 2003; van Doornum *et al.*, 2007). According to the reports, there is no significant difference in the sensitivity or specificity between the two amplification methods. The disadvantage in the comparison between NASBA and RT-PCR techniques was the utilization of different target sites for the primers and probes.

Polymerase chain reaction (PCR) has become the most popular method for the detection of picornaviruses. The advantage of PCR is its ability to detect very small amounts of viral genome within a few hours. The most important part of the PCR assay is the design of the primers and their targeting properties. Similarly to other RNA viruses, the target genome of entero- and rhinoviruses must be transcribed to complementary DNA (cDNA) before a PCR assay. Usually the reverse transcription is performed in a separate reaction but, nowadays, there are solutions to perform the RT-reaction in the same procedure as PCR (Dierssen *et al.*, 2008; Nielsen *et al.*, 2013b). The first reports of diagnostic picornavirus RT-PCR are from the late 1980s (Gama *et al.*, 1989; Gama *et al.*, 1988; Hyypiä *et al.*, 1989) and since then, various PCR applications for entero- and rhinoviruses have been described: these have been both conventional and, more recently, real-time instrument assays (Dominguez *et al.*, 2008; Iturriza-Gómara *et al.*, 2006; Kares *et al.*, 2004; Lönnrot *et al.*, 1999; Steininger *et al.*, 2001b; Volle *et al.*, 2012). Several approaches to differentiate between the two related picornaviruses, entero- and rhinoviruses, have been developed. A highly popular approach of differentiation is the designing of virus-specific probes or even primers (Kares *et al.*, 2004; Lu *et al.*, 2008; Tapparel *et al.*, 2009b). In real-time assays the probes are usually labeled with a fluorophore attached to the 5'-end and a quencher attached to the 3'-end of the oligonucleotide probe. In the conventional assays the specific detection with probe hybridization can be performed in an EIA assay (Lauwers *et al.*, 2002), in line blot (Zhou *et al.*, 2009), or in liquid hybridization assay with lanthanide-labeled probes (Lönnrot *et al.*, 1999). Other methods for identification and typing include sequencing, (Deffernez *et al.*, 2004; Iturriza-Gómara *et al.*, 2006), melting curve analysis, or mass spectrometry analysis of the PCR product (Chen *et al.*, 2014; Nguyen *et al.*, 2013).

2.9.4. Picornavirus 5'NCR qRT-PCR

Fortunately, entero- and rhinoviruses are highly conserved at the 5'NCR. The conserved area enables the design of robust RT-PCR assays for virus detection. The most conserved site in picornavirus 5'NCR is the IRES that directs the RNA translation. Several combinations of primers are designed to target the 5'NCR and the assays have been commonly used with good results in picornavirus diagnostics (Loens *et al.*, 2006; Lu *et al.*, 2008; Lönnrot *et al.*, 1999; Miller *et al.*, 2007; Vuorinen *et al.*, 2003). However, the highly identical 5'NCRs of entero- and rhinoviruses generate challenges for specific identification between the two picornavirus genera. There are many different strategies to overcome the complexity of homogenous 5'NCR. One option is to design the assay to be as genus-specific as possible, e.g., by adapting the assay for a combination of different specimen type and clinical symptoms: this has been done in an enterovirus RT-PCR assay for CSF specimens from patients with meningitis or encephalitis (Archimbaud *et al.*, 2009; Dierssen *et al.*, 2008; Volle *et al.*, 2012). Another strategy is to design primers and probes from the most conserved region of the 5'NCR to cover all known entero- and rhinovirus types. These so-called panentero or universal picornavirus assays are good for screening specimens, but a second assay or sequencing of the PCR product is required to achieve differentiation if the probes are designed to cover all entero- and rhinovirus types (Lönnrot *et al.*, 1999; Tapparel *et al.*, 2009a).

In some reports of enterovirus-specific RT-PCR assays, at least one primer or probe is from the conserved region of 5'NCR. In one of these reports, rhinovirus-positive clinical specimens were negative in the assay, and only some cultivated rhinovirus prototypes were weakly positive in the enterovirus-specific RT-PCR (Nijhuis *et al.*, 2002). In other reports, the primers were from the conserved area but the separation was achieved with an enterovirus-specific probe (Kares *et al.*, 2004) or a rhinovirus-specific probe (Lu *et al.*, 2008) depending on the aim of the assay. According to the positions of the primers and probes in these reports, the detection of rhinoviruses should not be challenging. In a report by Nijhuis *et al.*, 2002, the positions of the forward primer and probe are the same as those of the forward primer and reverse primer in a picornavirus RT-PCR assay, which successfully recognized rhinoviruses (Lönnrot *et al.*, 1999; Vuorinen *et al.*, 2003). The only difference is the position of the reverse primer, but according to basic local alignment search tool (BLAST) the primer aligns with clinical rhinovirus isolates. Kares *et al.*, 2004, used an enterovirus-specific probe to differentiate enteroviruses from rhinoviruses. This probe has a slightly overlapping sequence with several rhinovirus prototypes and clinical strains, which may cause challenges in differentiation. Furthermore, the specificity of the probe with all enterovirus prototypes is not known. A rhinovirus-specific probe designed by Lu *et al.*, 2008 also has matches or overlaps with enterovirus strains in the GenBank. The disadvantage with picornavirus 5'NCR primer pairs is the possibility of an amplification of the human genomic sequences, which may cause false-positive specimen detections (Bochkov and Gern, 2012).

2.9.5. Typing of enteroviruses

The traditional method to define enterovirus type is virus isolation followed by LBM neutralization test (Bendig and Earl, 2005). The neutralization test pool covers less than half of the currently known enterovirus types. Nowadays, enterovirus types are identified by partially or completely sequencing the approximately 900-nucleotide-long VP1 protein. As the VP1 sequences are heterogeneous between enteroviruses, the antigen properties of the VP1 define the enterovirus type. The VP1 sequence is compared with the VP1 sequences of known types, e.g. in BLAST search of the Genbank. In sequence comparison the VP1 nucleotide sequence identity with a prototype virus must exceed 75% to confirm a type, whereas in the case of a nucleotide sequence identity below 70% a new enterovirus type may be indicated (Oberste *et al.*, 2004b; Pallansch M.A, 2013). The large numbers of enterovirus types and the heterogeneity among their VP1 sequences generate challenges in creating assays for typing. Several alternatives for typing enteroviruses from virus cultures or directly from clinical specimens have been developed and designed. The assays differ in the length of amplified sequences and location of primers on the VP1 protein (Blomqvist *et al.*, 2008; Caro *et al.*, 2001; Casas *et al.*, 2001; Norder *et al.*, 2001; Oberste *et al.*, 2000; Oberste *et al.*, 1999b; Palacios *et al.*, 2002; Thoelen *et al.*, 2003). However, the most frequently used method to type enteroviruses is a semi-nested PCR assay with a consensus-degenerate hybrid oligonucleotide primer (CODEHOP) (Chiang *et al.*, 2012; Nix *et al.*, 2006; Ortner *et al.*, 2009). The enterovirus CODEHOP primers have a long sequence-specific adhesion part in the 5'end to add stability and increase the annealing temperature and an approximately

10 base pair long degenerated consensus region in the 3' end to add variability to the primer. The CODEHOP primers are composed on the basis of the amino acid sequences of enterovirus VP1 protein (Nix *et al.*, 2006). Another method to solve the variability problem among the VP1 sequences is to design species-specific primers for typing, similar to those created by Leitch *et al.*, 2009, for *enterovirus A* and *enterovirus B* specific assays.

Another two capsid protein regions, VP4 and VP2, have been used with good results for enterovirus typing (Ishiko *et al.*, 2002; Nasri *et al.*, 2007). In a comparison study, enterovirus sequencing of VP1, VP2, and VP4 regions was performed and the results were compared using the VP1 sequence result as a reference. According to the comparison both VP1 and VP2 sequences are suitable for enterovirus typing and the VP4 sequence gave correct results with viruses belonging to the EV-A genus (Perera *et al.*, 2010).

To avoid the sequencing procedure and to enhance the direct typing of enteroviruses, microarray-based typing assays have been developed (Chen *et al.*, 2006; Susi *et al.*, 2009). However, the microarray approaches are limited to previously defined types and they do not cover all enterovirus types. The sequencing will be essential in discovering new enterovirus types in the future.

In the classification of rhinoviruses there are recommendations as to how much the types can vary genetically in the VP1 and VP4/VP2 regions of the genome. To define the virus type in a rhinovirus-positive clinical specimen the sequence should cover 90% of the VP1 or VP4/VP2 genome region and a minimum of 87% nucleotide sequence identity for VP1 and 90% for VP4/VP2 is required to identify the type. For the proposal of new rhinovirus prototypes at least the complete VP1 genome sequence or, alternately, the complete genome sequence should be available (McIntyre *et al.*, 2013; Simmonds *et al.*, 2010a). The sequencing of the rhinovirus VP4/VP2 genome region (Savolainen *et al.*, 2002) is widely used in typing rhinoviruses in clinical specimens (Jin *et al.*, 2009; Linsuwanon *et al.*, 2009; Savolainen-Kopra *et al.*, 2009a; Wisdom *et al.*, 2009), whereas the sequencing of rhinovirus VP1 genome is mostly used in identifying and analyzing new RV-C types (Simmonds *et al.*, 2010a, b).

The sequencing of the rhinovirus 5'NCR is a highly popular method, because it is sensitive and easy to perform. However the 5'NCR sequence alone does not determinate new enterovirus strains, a capsid protein sequence is always needed (Savolainen-Kopra *et al.*, 2009c). The 5'NCR sequence can be used, for example, for screening specimens and typing rhinoviruses to RV-A, RV-B, or RV-C species when the nucleotide homology exceeds 96% (Miller and Mackay, 2013). Several assays to genotype rhinovirus from the 5'NCR have been developed and validated with rhinovirus prototypes and clinical isolates (Bochkov *et al.*, 2014; Kiang *et al.*, 2008; Lee *et al.*, 2007). Kiang *et al.*, 2008, compared the 5'NCR assay results with genotyping results from VP4/VP2 region and 99% of tested clinical specimens indicated same genotype. Lee *et al.* 2007 compared 5'NCR and VP1 sequencing results of clinical specimens also with good results, indicating the usefulness of 5'NCR in typing rhinoviruses.

3. AIMS OF THE STUDY

The aims of this thesis were to study and improve molecular methods to identify entero- and rhinoviruses, the picornavirus family members. For this purpose, the well-observed picornavirus 5'NCR RT-PCR with universal picornavirus primers was improved with locked nucleic acid (LNA) probes to properly differentiate rhino- and enteroviruses. Picornavirus detection was also improved with the development of virus-specific qRT-PCRs from the typing region VP1. The virus-specific qRT-PCRs were used in the studies of enterovirus epidemics in Finland in 2008-2010.

The specific aims of the thesis were:

1. To study rhinovirus transmission within families with picornavirus 5'NCR qRT-PCR and melting curve analysis.
2. To improve the 5'NCR qRT-PCR and the differentiation of entero- and rhinoviruses with LNA probes.
3. To identify and study the causative agent of a HFMD epidemic in Finland in 2008.
4. To study the large Echovirus 30 epidemic in Finland in 2009 and 2010.

4. MATERIALS AND METHODS

4.1. Prototypes and other positive controls

Prototypes used in this thesis were from the archives of the University of Turku, Department of Virology and, they were originally acquired from the American Type Culture Collection (ATCC, USA). The viruses have been cultured in LLC, RD, HeLa or CaCo cells. The archive consists of 163 enterovirus prototypes, seven prototype cDNAs cloned to recombinant plasmids, and eight transcribed RNAs received from elsewhere (Chang *et al.*, 1989; Dahllund *et al.*, 1995; Hughes *et al.*, 1988; Jenkins *et al.*, 1987; McLeish *et al.*, 2012; Stanway *et al.*, 1984). Parechoviruses 1-6 (HPeV-1 - HPeV-6) were clinical isolates and were provided by Dr. Katja Wolthers, Academic Medical Center, Amsterdam, The Netherlands (Benschop *et al.*, 2008) (Table 3).

Table 3. Prototypes and positive controls

Control type	Species	Types*	Study
Prototypes	RV-A (75)	RV-A1 RV-A1b, RV-A2, RV-A7 - RV-A13, RV-A15, RV-A16, RV-A18 - RV-A25, RV-A28 - RV-A34, RV-A36, RV-A38 - RV-A41, RV-A43 - RV-A47, RV-A49 to RV-A51, RV-A54 - RV-A68, RV-A71, RV-A73 - RV-A78,-A80 - RV-A82, RV-A85, RV-A88 - RV-A90, RV-A 94 - RV-A96, RV-A98, RV-A100	
	RV-B (25)	RV-B3 - RV-B6, RV-B17, RV-B26, RV-B27, RV-B35, RV-B37, RV-B42, RV-B48, RV-B52, RV-B57, RV-B69, RV-B70, RV-B72, RV-B79, RV-B83, RV-B84, RV-B86, RV-B91 - RV-B93, RV-B97, RV-B99	I
	EV-A (12)	CV-A2 - CV-A8, CV-A10, CV-A12, CV-A14, CV-A16 EV-A71	
	EV-B (35)	CV-B1 - CV-B6, CV-A9, E1 - E-7, E-9, E-11 - E-21, E-24 - E-27, E-29 - E-33	
	EV-C (15)	PV-1 to PV-3, CV-A1, CV-A11, CV-A13, CV-A15, CV-A17 - CV-A19 CV-A20a and b, CV-A21, CV-A22, CV-A24	
	EV-D (1)	EV-D68	
	HPeV	HPeV-1 - HPeV-6	
Plasmids	RV-A	RV-A1b and RV-A85	
	RV-B	RV-B14	I
	EV-A	CV-A16	
	EV-B	CV-B4, CV-A9, E-11	
Transcripts	RV-A	RV-A16	
	RV-B	RV-B14	
	RV-C	RV-C40, RV-Cpat19	I
	EV-A	CV-A16	
	EV-B	E-7, E-30	
	EV-C	CV-A21	
	EV-D	EV-70	

*Types RV-A1b and E-11 also in study II, III, and IV; RV-A16 purified prototype in study II; CV-A6, CV-A16, and EV-A71 in study III; and E-30 in study IV.

4.2. Clinical specimens

Nasal swabs for the rhinovirus transmission study were received from patients hospitalized on the pediatric infectious diseases ward of Turku University Hospital (Turku, Finland) and from their family members (II). CV-A6 were detected in enterovirus-positive vesicular fluid, feces, throat swabs, tracheal aspirate, and nails sent to the diagnostic laboratory at the Department of Virology at the University of Turku from Pirkanmaa Hospital District (Tampere), Pori Central Hospital, Central Ostrobothnia Central Hospital (Kokkola), and Turku University Hospital (III). Clinical specimens in the validation study of rhinovirus and enterovirus qRT-PCR with differentiating LNA probes were received from the diagnostic service unit of the Department of Virology at the University of Turku material collection (I). E-30 was detected in CSF specimens from patients with meningitis received at the diagnostic service unit of Department of Virology at the University of Turku (IV).

4.3. Primer, probe and assay design

4.3.1. Virus-specific primers

To study the enterovirus epidemics caused by CV-A6 in 2008 and 2009, and E-30 in 2009 and 2010, virus-specific primers from the VP1 region were designed. The primer design was performed using sequences obtained from specimens of the epidemic seasons by sequencing the partial enterovirus VP1 with primers described by others (Nix *et al.*, 2006). The specific primers were designed using Primer3, a primer designing tool (<http://primer3.ut.ee/>). Primers lengths were optimized to approximately 20 bp and the optimal melting temperature (T_m) of the primers was set to 60°C and optimum percentage of Gs and Cs (GC%) in primers was set to 50%.

4.3.2. LNA probes and BOXTO

Locked nucleic acid probes were designed from the 5'NCR sequences of rhino- and enterovirus prototypes obtained from the GenBank database. To reduce the number of variable bases and still keep the differentiation ability between the probes, the probes were designed to the length of 13 bases. To increase the probe melting temperature and the differentiation ability, four to five DNA bases were replaced with locked nucleic acid (LNA) bases (Table 4). LNA is a 2'-O,4'-C-methylene-linked bicyclic ribofuranosyl nucleotide locked in a C3'-endo conformation and able to hybridize to both DNA and RNA, as well as increase the denaturation temperature of the duplex (Petersen *et al.*, 2000). LNA probes were designed with the T_m calculator provided by Exiqon (<http://lna-tm.com>) with the conditions set to 115 mM salt, 0.5 μ M target probe, and melting temperature fixed at 70°C. Rhinovirus LNA probes (Rlp) were labeled at the 5' end with 6-carboxyfluorescein (FAM), enterovirus LNA probes (ENp) were labeled with indodicarbocyanine (Cy5), while they both were labeled at the 3' end with an appropriate Dark Quencher (DQ) (Table 4). A nonspecific asymmetrical cyanine dye BOXTO (4-[6-(benzoxazole-2-yl-(3-methyl)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-quinolinium chloride) (TATAA Biocenter) was also included in the

LNA probe assay to visualize primer amplification and melting curve analysis. BOXTO binds to the double stranded DNA and it has a maximum absorbance at 515 nm and emission at 552 nm. The absorbance is measured with the JOE/yellow channel, and BOXTO is a suitable dye to be combined with LNA or hydrolysis probes, for example, TagMan (Ahmad, 2007; Lind *et al.*, 2006).

4.4. Nucleic acid extraction and qRT-PCRs

4.4.1. Nucleic acid extraction and RT-reactions

In the rhinovirus transmission study (II), nasal swabs were collected, placed into dry and sterile vials, and transported at room temperature to the laboratory. Prior to the extraction of nucleic acids, 1 ml PBS was added to the tube and mixed. The nucleic acids were extracted from 150 µl and eluted to 50 µl volume with E.Z.N.A Viral RNA isolation Kit (Omega Bio-Tek) according to the manufacturer's protocol. In the other studies, NucliSens EasyMag (BioMérieux, Boxtel, the Netherlands) automated total nucleic acid extractor was used with an elution volume of 55 µl (I, III, IV).

Reverse transcription reactions were performed with the reverse primers (Table 4): 5'NCR RT-reaction and 5'NCR sequencing RT-reactions with ENRI reverse primer 4- (I-IV) (Lönnrot *et al.*, 1999); CV-A6 RT-PCR with CV-A6vp1 reverse primer (III); enterovirus VP1 RT-reaction with AN-88 reverse primer (I, III, IV) (Nix *et al.*, 2006); and E-30-specific RT-reaction with E-30 reverse primer (IV). The U RevardAid H Minus M-MuL V reverse transcriptase (Fermentas, St. Leon-Rot, Germany) and RicoLock inhibitor (Fermentas, St. Leon-Rot, Germany) were used as the RT enzyme and RNase inhibitor in the RT-reactions. The RT-reactions were performed at 42°C for 1h and the cDNAs were used immediately or frozen at -20°C prior to use.

Table 4. Primers and probes.

Primers	Sequence	Study
5'NCR reverse primer 4-	5'-GAAACACGGACACCCAAAGTA-3'	I-IV
5'NCR forward primer 3+	5'-CGGCCCTGAATGCGGCTA A-3'	I-IV
5'NCR sequencing forward primer U2+	5'-CAAGCACTTCTGTTTCCCC-3'	I-IV
AN-88 enterovirus VP1 reverse primer	5'-TACTGGACCACCTGGNGGNAYRWACAT-3'	I, III, IV
AN-89 enterovirus VP1 forward primer	5'-CCAGCACTGACAGCAGYNGARAYNGG-3'	I, III, IV
CV-A6vp1 reverse primer	5'-ACTCGCTGTGTGATGAATCG-3'	III
CV-A6vp1 forward primer	5'-CGTCAAAGCGCATGTATGTT-3'	III
E-30 VP1 reverse primer	5'-ACCCTGTAGTTGCCACGTA-3'	IV
E-30 VP1forward primer	5'-ATAGTGTGCCCCGCTCTA-3'	IV
Probes		
RV LNA probe Rlp1	5'-(FAM)-TYGGIYCCATCC-DQ1-3'	I
RV LNA probe Rlp2	5'-(FAM)-TCGGIYCCGTC-DQ1-3'	I
HEV LNA probe ENp1	5'-(Cy5)- TCGGITCCGCTG-DQ3-3'	I
HEV LNA probe ENp2	5'-(Cy5)- TCGGITCCGCCAC-DQ3-3'	I

LNA bases underlined; Y = T or C; R=A or G, W= A or T; N=A, T, C, or G; X= deoxyinosine; FAM, 6-carboxyfluorescein; Cy5, indodicarbocyanine; DQ, dark quencher. The references for 5'NCR primers and AN88 and AN89 primers are mentioned in the text.

4.4.2. Quantitative polymerase chain reactions and melting curve analysis

Quantitative PCRs (Table 5) were performed with Rotor-Gene 3000 or 6000 instrument (Qiagen) from 5 µl of cDNA in a total volume of 25 µl. The amplification conditions were designed according to the primer pair annealing temperature and PCR master mix requirements. The 5'NCR PCRs, VP1 PCR, and virus-specific PCRs were performed with QuantiTect Sybr Green PCR master mix (Qiagen) or with Maxima Sybr Green PCR master mix (Fermentas), which are measured with the FAM channel of the instrument. In the quantitative 5'NCR LNA probe assay QuantiTect Probe master mix (Qiagen) was used. In PCRs with Sybr Green detection a melting curve analysis was performed immediately after the

Table 5. Real-time RT-PCR conditions and reagents.

PCR	Master mix	Conditions	Study
5'NCR qRT-PCR (ENRI)	QuantiTect Sybr Green PCR master mix (Qiagen)	95°C 15 min, 45 cycles: 95°C 15s 65-55°C 30s (touchdown 1°C/ 1 st 10 cycles) 72°C 40s and T _m analysis from 72°C to 95°C	II
5'NCR qRT-PCR (ENRI)	Maxima Sybr Green PCR master mix (Promega)	95°C 10 min, 45 cycles: 95°C 15s 65-55°C 30s (touchdown 1°C/ 1 st 10 cycles) 72°C 40s and T _m analysis from 72°C to 95°C	I,III, IV
5'NCR qRT-PCR (with probes) (BOXER)	QuantiTect Probe master mix (Qiagen)	95°C 15 min, 50 cycles: 95°C 15s 65-55°C 30s (touchdown 1°C/ 1 st 10 cycles) 72°C 40s and T _m analysis from 72°C to 95°C	I
5'NCR sequencing qRT-PCR	QuantiTect Sybr Green PCR master mix (Qiagen)	95°C 15 min, 45 cycles: 95°C 10s 65-55°C 30s (touchdown 1°C/ 1 st 10 cycles) 72°C 40s 72°C 4min and T _m analysis from 72°C to 95°C	II
5'NCR sequencing qRT-PCR	Maxima Sybr Green PCR master mix (Promega)	95°C 10 min, 45 cycles: 95°C 10s 65-55°C 30s (touchdown 1°C/ 1 st 10 cycles) 72°C 40s 72°C 4min and T _m analysis from 72°C to 95°C	I,III, IV
Enterovirus VP1 qRT-PCR	Maxima Sybr Green PCR master mix (Promega)	95°C 10 min, 45 cycles: 95°C 15s 50°C 30s 72°C 40s and T _m analysis from 72°C to 95°C	I,III, IV
CV-A6 VP1 qRT-PCR	Maxima Sybr Green PCR master mix (Promega)	95°C 10 min, 45 cycles: 95°C 15s 60°C 30s 72°C 45s and T _m analysis from 72°C to 95°C	III
E-30 VP1 qRT-PCR	Maxima Sybr Green PCR master mix (Promega)	95°C 10 min, 45 cycles: 95°C 15s 60°C 30s 72°C 45s and T _m analysis from 72°C to 95°C	IV

PCR amplification in the Rotor-Gene instrument. In the melting curve analysis the double-stranded PCR product is monitored during stepwise heating; in the melting temperature of the PCR product, the DNA dissociates and the highly fluorescent bound dye is released and turned to a low fluorescent state leading to a large reduction in fluorescence.

4.4.3. Sequencing

The 5'NCR sequencing RT-PCR was performed from the same cDNA as the 5'NCR ENRI RT-PCR. The 5'NCR sequencing RT-PCR was performed with the ENRI sequencing forward primer U2+ amplifying a 397-bp-long amplicon. The partial VP1 products were produced with enterovirus VP1 qRT-PCR, CV-A6, or E-30-specific qRT-PCR. The amplicons were purified either with agarose gel QIAquick PCR purification kit (Qiagen) (I-III) or NucleoSpin® 96 PCR Clean-up kit (Macherey-Nagel) (IV). The sequencing was performed in the DNA Sequencing Service of the Turku Centre for Biotechnology (Turku, Finland) (I-IV). Sequences were analyzed with NCBI Basic Local Alignment Tool (BLAST) (I-IV), sequence alignments were constructed with the ClustalW program (<http://www.ebi.ac.uk/Tools/msa/clustalw>), and phylogenetic analysis was performed with MEGA version 4 (III) or 6 (IV) software (www.megasoftware.net) by using Jukes-Cantor algorithm and the neighbor-joining method.

5. RESULTS

5.1. Picornavirus 5′NCR qRT-PCR (I)

The well-served conventional picornavirus 5′NCR RT-PCR with liquid hybridization probes to differentiate between entero- and rhinoviruses (Lönnrot *et al.*, 1999) was upgraded to real-time format and performed with SYBR Green as the double-stranded DNA dye. In the 5′NCR qRT-PCR, the separation of entero- and rhinoviruses is performed with a melting curve (T_m) analysis. Each run contained RV-A1b and E-11 prototypes as positive controls. The separation point between entero- and rhinoviruses was defined from the T_m value of RV-A1b prototype + 1.5°C. Specimens below the separation point were considered as rhinoviruses and above as enteroviruses. This separation point resulted from the testing of 42 picornavirus prototypes, 17 rhinovirus strains (RV-A1b, RV-A2, RV-A9, RV-A11, RV-A12, RV-A13, RV-A16, RV-A22, RV-A29, RV-A36, RV-A38, RV-A39, RV-A56, RV-A59, RV-A66, RV-B3, RV-B48), and 25 enterovirus strains (CV-A1, CV-A2, CV-A3, CV-A7, CV -A10, CV-A13, CV-A15, CV-A16, CV-A21, CV-B3, CV-B4, CV-B5, CV-A9, E-9, E-6, E-7, E-9, E-11, E-18, E-24, E-30, EV-D68, PV-1, PV-2, PV-3). From the tested prototypes, only three enteroviruses (E-1, E-7, and CV-A13) and four rhinoviruses (RV-A13, RV-A36, RV-A16, and RV-B48) gave an indistinct T_m (Figure 2).

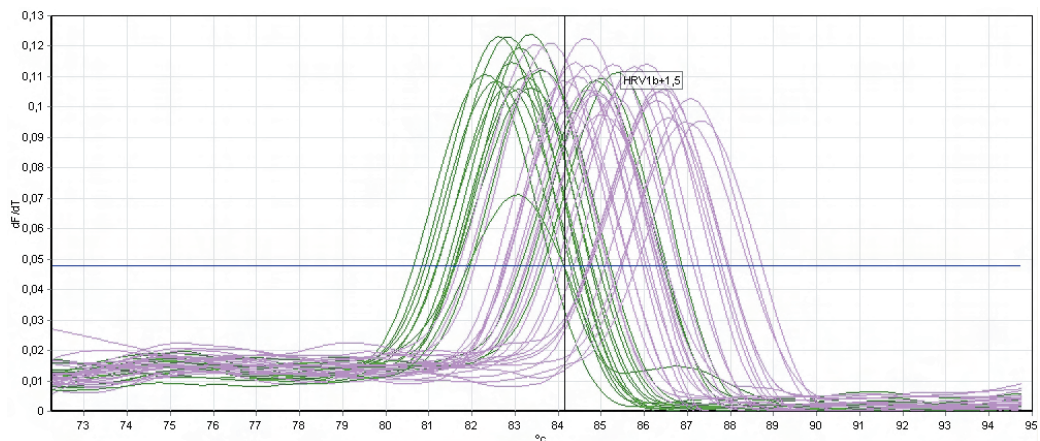


Figure 2. Melting curve analysis of 42 picornavirus prototype products produced with 5′NCR qRT-PCR. Rhinoviruses are shown in green peaks and enteroviruses are shown in pink peaks.

In the liquid hybridization with lanthanide chelate-labelled probes the prototypes gave correct results; the exceptions being an RV-A12 prototype, which was negative and an RV-A2 prototype, which was positive with both the EU-labelled rhinovirus probe and Sm-labelled enterovirus probe. However, 44 (28%) of the 61 clinical specimens positive for picornavirus 5′NCR primers were negative in the liquid hybridization assay. A sequence analysis of the liquid hybridization-negative specimens indicated non-hybridization of the probes with RV-C types. In real-time assay with SYBR Green, 51 (84%) of the 61 clinical specimens were correctly typed as rhino- or enteroviruses with the T_m analysis.

5.2. Improvement of picornavirus 5'NCR qRT-PCR (I)

The picornavirus 5'NCR qRT-PCR with melting curve analysis was improved with designing probes that were better able to differentiate between rhino- and enteroviruses. The probes were designed from alignments of sequenced prototypes and sequences obtained from GeneBank. Probes were designed to target the 5' end of the reverse primer, a 13-base-long region with two variable bases to differentiate between rhino- and enteroviruses (I/ Figure 1). LNA bases were included to increase the melting temperature of the short probes to 70°C. The rhinovirus probes were labeled with FAM, and enterovirus probes with Cy5 at the 5' end, and all were labeled at the 3' end with dark quencher (DQ) (Table 4). The dsDNA dye BOXTO was added to the assay to monitor probe-negative PCR products. Hence, the assays consist of measuring the rhinovirus LNA probe-positive specimens with the green channel (FAM), enterovirus LNA probe-positive specimens with the red channel (Cy5), and primer amplification and melting curve analysis with the yellow channel (BOXTO) of the instrument (I/ Figure 2).

The validation and assessment of analytical specificity of the assay and the LNA probes were performed with 163 enterovirus prototypes including both entero- and rhinoviruses, seven enterovirus cDNA cloned plasmids, nine *in vitro*-transcribed RNAs covering the enterovirus genera A-D and rhinovirus genera A-C (Table 3), and 118 clinical specimens covering nine different specimen types (I/ Table 2). All prototypes and other controls were correctly identified with the LNA probes, were amplified with BOXTO, and had a melting point typical to virus-specific amplicons as compared to primer-dimers or other non-specific amplicons. A scatter plot analysis demonstrated the correlation between prototypes' C_t values with the LNA probes and BOXTO (I/ Figure 3 A and B). Of the clinical specimens used in the validation, 23 were cultured before identification with 5'NCR qRT-PCR. The correct virus type of the clinical specimens were ensured with 5'NCR or VP1 sequencing; for some specimens reference results were obtained from the Enterovirus Laboratory, NPHI, Helsinki, Finland. LNA probes unambiguously differentiated between clinical specimens. All the enterovirus specimens were positive with enterovirus probe and with BOXTO dye, with the exception of CV-A24-positive conjunctival fluid that was negative for BOXTO and had a high C_t value with HEV probe indicating a low concentration of CV-A24 virus in the specimen. CV-A6 and RV-C were found in one nasal swab with C_t -values on both probe channels and on the BOXTO channel. A nasal swab positive for HEV-D type EV-D68 amplicon was reactive with both probes and BOXTO. Two specimens were double positive for enteroviruses, one with CV-A9 and CV-A24, and one with oral polio vaccine strains PV-2 and PV-3 (Quality control specimens, treated as clinical specimens) (I/ Table 2).

5.2.1. Detection limits of 5'NCR qRT-PCR with LNA probes (I)

The limits of detection (LOD) of 5'NCR qRT-PCR with LNA probes were determined with a dilution series of *in vitro*-transcribed EV species A-D and RV species A-C (Table 3) (McLeish *et al.*, 2012) with $1,0 \times 10^4$ to 10 copies of RNA/ μ l in three separate assays. The LOD was at least 100 RNA copies/ μ l, for most of the species between 10 to 100 RNA copies/ μ l or less

than 10 RNA copies/ μ l. To assess the intra-assay repeatability of LNA probes, five replicates of 100 RNA copies/ μ l of each transcript were determined in the same assay (I/Table 3). The amplification efficiency was determined with a dilution series of plasmid-viruses from 5×10^7 to 5 copies of plasmid-virus/reaction. All plasmid-viruses could be detected at 5 copies/reaction and the amplifications were linear up to 5×10^7 copies/reaction. The efficiency was on average 0.99 (± 0.3 SD) for all plasmid-viruses with all three methods, i.e., 5'NCR qRT-PCR with SYBR Green and melting curve analysis, LNA probes, and BOXTO dye. The standard deviation of standard curve intercept Ct-values was smaller with LNA probe detection (0.63) than with either SYBR Green (1.42) or BOXTO (1.47). This was probably due to slight sequence-specific variation in the binding of the dsDNA dye to the amplicon.

5.3. Rhinoviruses in families (II)

Rhinoviruses causing the common cold affect everybody in human communities, and especially the infants and children. To study rhinovirus transmission within families, 24 families with more than one child were enrolled. At the beginning of the study, eight families had a rhinovirus-positive index child. During the 3-week follow-up time the families documented all respiratory symptoms in a diary, and nasal swabs, a total of six samples per person, were taken twice a week. The swabs were analyzed for rhinoviruses with the picornavirus 5'NCR qRT-PCR and melting curve analysis. In the families with a rhinovirus-positive index child all the siblings and one half of the parents had rhinoviruses in the nasal cavity. Asymptomatic rhinovirus infections were seen in siblings over 7 years of age and in parents within families with a symptomatic rhinovirus-positive member.

5.3.1. Load and characterization of rhinoviruses (II)

In the rhinovirus transmission study, the nasal swabs taken at the beginning of the infection were more often positive for rhinovirus than those taken at a later phase or after the infection. The copy numbers of rhinoviruses were higher in the swabs taken from symptomatic patients than in those taken from asymptomatic family members (median copy number $5.3 \log_{10}$ vs. $4.9 \log_{10}$ copies/swab). The rhinovirus-positive specimens were further analyzed with the 5'NCR sequencing qRT-PCR assay and the products were sequenced. The sequences from the same family were aligned and sequences with over 98% similarities in the nucleic acid composition were regarded as the same virus type. A phylogenetic tree was constructed and it showed infection of the same type of rhinovirus within each family indicating transmission within the family (II/Figure 2).

5.4. Coxsackievirus A6 a new endemic HFMD-virus (III)

A nationwide HFMD epidemic started in Finland in August 2008. The first specimens were received from two children and a parent with HFMD from the Central Hospital of Seinäjoki, Southern Ostrobothnia. Vesicular fluids from the patients were sent for analysis to the

Department of Virology, University of Turku. The specimens subjected to the picornavirus 5'NCR qRT-PCR produced positive results with melting points typical to enteroviruses. An enterovirus VP1 qRT-PCR was performed to achieve a sequence for type identification. The enterovirus sequences were identified with the BLAST search as CV-A6. A virus-specific qRT-PCR was developed for the detection of the circulating CV-A6 strain. The CV-A6 qRT-PCR was designed to amplify a 199-bp-long product from the VP1 genome. In the final analysis, 12 vesicle fluid samples, 23 throat swabs, 2 tracheal aspirate samples, 5 stool samples, 5 cerebrospinal fluid samples, and one nail sample from 44 patients with HFMD were CV-A6 positive during a period between August 2008 and February 2009. The relationship between the clinical CV-A6 strains was studied and the sequences were compared with prototypes of CV-A6 (Gdula), CV-A16 (G10), EV-A71 (BrCr), and newest clinical CV-A6 isolates from the GenBank. According to the phylogenetic analysis and sequence alignment all Finnish CV-A6 strains clustered in one cluster with a nucleotide identity between 97% - 100% (III/ Figure 2). In a comparison of the Finnish CV-A6 strains with the related enterovirus prototype strains, the nucleotide identity was 83% with CV-A6 (Gdula), 56% with CV-A16 (G-10), and 57% with EV-A71 (BrCr).

5.4.1. Onychomadesis (III)

In onychomadesis the nail plate is shed from the nail matrix. The painless onychomadesis can occur in finger and/or toenails and is associated with infections, medication, or autoimmune diseases (Bernier *et al.*, 2001). In the CV-A6 epidemic, onychomadesis was seen in HFMD patients approximately 4 to 8 weeks after the infection. The nail shedding was a new feature in HFMD infections and possibly typical for the CV-A6 HFMD strain (III/ Figure 1). Moreover, shed nails were analyzed from two siblings who contracted HFMD 8 weeks earlier. The nails were incubated in proteinase K at 56°C overnight and the nucleic acid extraction was performed from the supernatants. The nails were enterovirus positive in the picornavirus 5'NCR qRT-PCR and, furthermore, one nail sample was positive in the specific CV-A6 VP1 qRT-PCR. The sequence was identical to the circulating CV-A6 strains.

5.5. Echovirus 30 outbreak in Finland (IV)

E-30 is one of the most common viral pathogens to cause aseptic meningitis, and it has caused outbreaks worldwide for decades, probably also in Finland. There are no reports on E-30 epidemics in Finland before the 2009 outbreak (Savolainen-Kopra *et al.*, 2011). Between August 2009 and September 2010, the diagnostic service unit of the Department of Virology at the University of Turku received a total of 272 enterovirus-positive specimens of which 147 were CSF specimens. The patients were from Turku, Pori, Seinäjoki, Oulu, Lahti, and Kokkola areas. A virus-specific qRT-PCR assay targeting the VP1 genomic region was developed to investigate the E-30 outbreak. The outbreak occurred in two periods, the first wave between August and December 2009, and the second wave between July and September 2010. During the first wave, 75 enterovirus-positive CSF specimens were analyzed with the E-30-specific qRT-PCR and positive amplicons were sequenced. Sequences

were obtained from 54 specimens. The sequences were confirmed with the NCBI BLAST search tool. The alignments of the 54 sequences from 2009 showed 97-100% homology with each other. In July 2010, aseptic meningitis cases started to increase again in the Northern Finland (Oulu) and spread nationwide during the summer. During the second wave, 72 CSF specimens were enterovirus-positive in picornavirus 5'NCR qRT-PCR. The positive specimens were analyzed with E-30-specific qRT-PCR and positive PCR products were obtained from 52 specimens. The specimens were sequenced and the sequence homology between the 52 specimens was from 91% to 100%.

In 2009, E-30 was detected mostly in children and teenagers between 10 to 19 years of age (mean age 15y 8m), with male gender predominance (75 %). In 2010, the age distribution was greater, from 2 weeks up to 55 years of age, with the majority of cases in patients between 15 and 24 years of age (mean age 17y 6m) (Figure 5). No gender predominance was observed in the 2010 outbreak. Nucleotide homology between all (106) outbreak sequences was from 91 to 100%. In comparison of all outbreak sequences with E-30 reference strain Bastianni the nucleotide homology was between 73 to 79 %, and 89 to 97 % with E-30 strains (E30GB62 (FJ538769), E30GB63 (FJ538770), and E30FI31 (FJ538710)) from Genbank. Overall, E-30 was confirmed in 106 patient CSF specimens during the outbreaks.

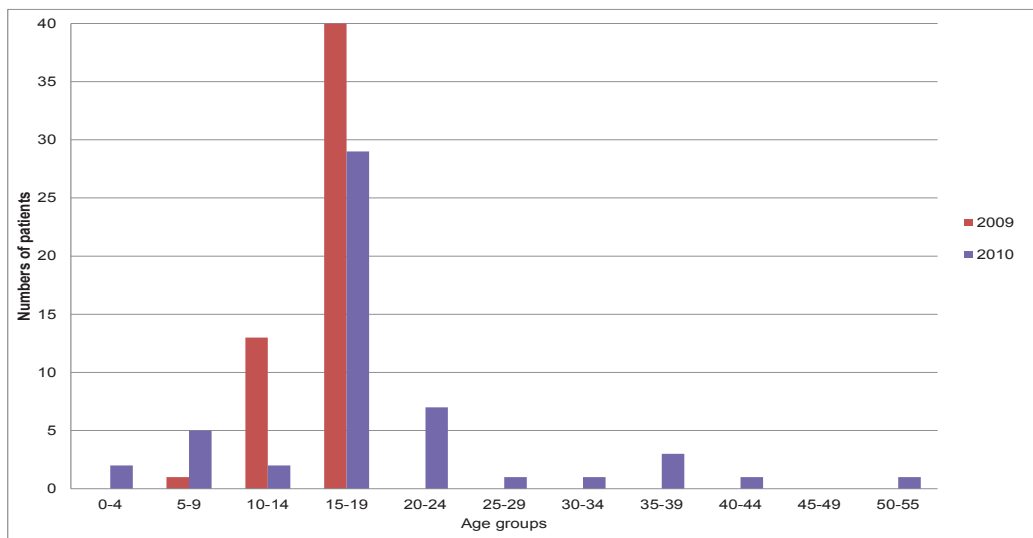


Figure 5. Age distribution of 106 patients with echovirus 30 meningitis in 2009 and 2010.

6. DISCUSSION

6.1. Picornavirus 5'NCR qRT-PCR

Rhino- and enteroviruses are highly conserved in the 5'NCR of the genome, thus the 5'NCR forms a suitable region for detection with universal picornavirus primers. The universal picornavirus primers recognize all currently known rhino- and enterovirus types. Several variants of primer pairs targeting the picornavirus 5'NCR have been published: some primer pairs are designed to produce small, under 150 bp-long PCR products (Lönnrot *et al.*, 1999; Nijhuis *et al.*, 2002; Steininger *et al.*, 2001a), whereas other primer pairs amplify almost the entire 5'NCR (Chiang *et al.*, 2012; Loens *et al.*, 2003). Additionally, there are picornavirus assays with either virus-specific or universal probes for rhino- and enteroviruses (Dierssen *et al.*, 2008; Kares *et al.*, 2004). While all the RT-PCR methods are suitable for the detection and identification of rhino- and enteroviruses, the best set of primers and/or probes as well as the method best covering all the over 260 enterovirus genus types known today still needs further study. The increasing numbers of new picornavirus types challenge the assays and, therefore, the constant regeneration of the methods is mandatory.

One of the starting points for this study was the observation of false-positive results with the enterovirus probe in the entero- and rhinovirus differentiating liquid hybridization assay after the 5'NCR RT-PCR with universal rhino- and enterovirus primers (Lönnrot *et al.*, 1999). There were also specimens which were negative with the probes but the primers amplified a PCR product according to agarose gel electrophoresis.

In study I, the 5'NCR qRT-PCR assay was first modernized to real-time qRT-PCR format with double-strand dye SYBR Green and a melting curve analysis. The melting curve analysis of the PCR product was performed to differentiate the rhino- and enteroviruses: the melting curve analysis directly differentiates rhinoviruses from enteroviruses with approximately 80% certainty. This method clearly outperformed liquid hybridization assay probes in rhinovirus identification. Unfortunately, the differentiation by T_m had its limitations with an overlap zone between rhino- and enteroviruses, and a part of the studied strains was misclassified. Therefore, new virus species-specific LNA probes were designed. The final real-time 5'NCR qRT-PCR assay with the LNA probes and dsDNA dye BOXTO unambiguously differentiated all the studied prototype and clinical strains. Several other picornavirus assays have been recently reported, (Dupouey *et al.*, 2014), compared a probe and BOXTO-assay with a Sybr Green assay for rhinovirus detection. The best sensitivity and specificity were obtained with the Sybr Green assay. Disadvantage of the combined probe and BOXTO-assay was the unspecific amplifications with BOXTO dye, and in addition to the binding site of the probe play a critical role in sensitivity and specificity of the assay. Very popular picornavirus multiplex assays have been simultaneous detection of parecho- and enteroviruses, some of the assays detect rhinoviruses but they are not differentiated from enteroviruses (Cabrerizo *et al.*, 2014; de Crom *et al.*, 2013; Nielsen *et*

al., 2013a; Pabbaraju et al., 2015). Even enterovirus strain specific multiplex assays have been reported, such as simultaneous detection and identification of EV-A71, CV-A16 and other enteroviruses (Thanh *et al.*, 2015; Zhang *et al.*, 2014). There are also commercial respiratory virus multiplex kits containing both entero- and rhinovirus detections. The large panels of respiratory viruses may affect the sensitivity and specificity of the multiplex assays (Cho *et al.*, 2013; Deng *et al.*, 2013). However, the developed probe and BOXTO assay is unique, because it is using the stable conserved primer sites amplifying both entero- and rhinoviruses with highest possible sensitivity and still capable of differentiating them with the specially designed LNA probes.

The 5'NCR qRT-PCR assay validation was performed according to the instructions in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines (Bustin *et al.*, 2009). The assay showed excellent sensitivity and repeatability, which were determined with *in vitro*-transcribed RNAs covering enterovirus species A-D and rhinovirus species A-C. Sensitivity was ≤ 10 , or 10-100 copies of RNA / μl , and the standard deviations (SD) of five replicates of 100 copies of RNA/ μl were from 0.2 to 1.8. The assay linearity and stability were studied with the cDNA-cloned plasmids and the amplification efficiencies were approximately 100%. Clinically significant specimen types such as CSF, NPA, nasal swab, feces, vesicular fluid, and tissue specimens were tested and all were found to be suitable assay matrices for the detection of rhino- and enterovirus. Moreover, the analytical specificity was validated with different viruses and no cross-reactions were seen with the other viruses. Therefore, the picornavirus real-time 5'NCR qRT-PCR with rhino- and enterovirus-specific LNA probes and BOXTO dye is suitable for clinical diagnostic use.

6.2. Rhinovirus transmission (II)

Rhinovirus transmission from human to human occurs via aerosols or direct contact with nasal secretion. This is why the rhinoviruses are very common and spread effectively especially among children. It is presumed that children under school age suffer from approximately 10 rhinovirus infections per year (Gern, 2013). In a surveillance study of respiratory viruses among families rhinoviruses were the most detected virus in all age groups and children under five year of age were most likely to have a symptomatic infection and frequency of rhinovirus infections correlated with the family size. In addition, nearly 50% of all the rhinovirus episodes were asymptomatic (Byington *et al.*, 2015). There are also reports of rhinovirus outbreaks in health care facilities, where transmission occurred among health care workers and patients. More than one rhinovirus type were associated with the outbreaks (Cutino-Moguel *et al.*, 2014; Mubareka *et al.*, 2013).

Rhinovirus transmission was studied in families with a rhinovirus-positive child as an index case. The studied families had a minimum of two children and the families collected the nasal swab samples at home. The nasal swab specimens were taken twice a week during the 3-week study period and were sent by regular mail to the laboratory where the nasal swabs were analyzed with the picornavirus 5'NCR qRT-PCR.

In families with a rhinovirus-positive index child, all the siblings and half of the parents were rhinovirus-positive. When the clinical data was inspected, almost all the rhinovirus-positive children under 7 years of age had symptoms, whereas most of the rhinovirus-positive parents were asymptomatic. In conclusion, in families with a child with a symptomatic rhinovirus infection, the transmission to other family members is highly probable, but the appearance of clinical symptoms is more age dependent. Furthermore, other studies of respiratory viruses among families have also detected rhinoviruses as most common virus causing both symptomatic and asymptomatic infections, and the dynamic of rhinovirus infections correlates with age and the number of children in the family (Budge *et al.*, 2014; Byington *et al.*, 2015; Camargo *et al.*, 2012)

Another observation from the study (II) was that nasal swabs are a good sampling method for the detection of rhinoviruses. It has been demonstrated that rhinoviruses are stable in nasal swabs collected to dry and clean vials and are well detectable with qRT-PCR within four days after the collection; therefore, the nasal swab in a dry vial is a suitable method for the patients themselves to collect and mail the specimens to the laboratory (Waris *et al.*, 2013). Self-sampling is a good tool to use in transmission and surveillance studies of respiratory viruses. It can be used to supplement traditional clinical-based specimens collection and when the participants get good instructions for the self-sampling the quality of the specimens are same as in specimens collected in a healthcare unit (Byington *et al.*, 2015; Elliot *et al.*, 2015; Plymoth *et al.*, 2015).

6.3. Detecting and studying enterovirus outbreaks (III, IV)

Enterovirus outbreaks occur worldwide and they are mostly aseptic meningitis outbreaks caused by E-30 or other enteroviruses (Kim *et al.*, 2012; Kumar *et al.*, 2013; Mirand *et al.*, 2008); HFMD epidemics caused by EV-A71, CV-A16, or CV-A6 (Wu *et al.*, 2010); or hemorrhagic conjunctivitis epidemics caused by CV-A24 (Aubry *et al.*, 2012). There are several ways to identify and study the pathogens, e.g., culturing the specimens in immortalized cell lines and performing a neutralization assay or RT-PCR from a positive culture (Papadakis *et al.*, 2014). Several molecular methods are widely used to study enterovirus outbreaks, including RT-PCRs amplifying the conserved 5'NCR, nested RT-PCRs amplifying the VP1 region, or the sequencing of the PCR products (Chiang *et al.*, 2012; Iturriza-Gómara *et al.*, 2006; Nix *et al.*, 2006; van Doornum *et al.*, 2007).

In our outbreak study approach, the pathogen was first identified with a sensitive and reliable method. Enteroviruses were detected with the picornavirus qRT-PCR from the highly conserved region of the 5'NCR. Even the sequencing of the 5'NCR can help in determining the pathogen in an outbreak. For pathogen classification, a sequence from the VP1 protein is needed. There are several approaches to obtain the VP1 sequence, and here, CODEHOP primers designed by Nix *et al.*, 2006, were used. The CODEHOP primer assay has its limitations in the sensitivity when the PCR is performed directly from the clinical specimens, but cultured specimens are superior due to their large amount of virus. However,

good VP1 sequences from the clinical specimens were obtained with the CODEHOP primer assay. Virus-specific primers were designed according to the VP1 sequences and a new virus-specific assay was created to study the epidemic. Commonly, the virus-specific RT-PCRs are more sensitive than genus-specific RT-PCR assays. A strain-specific RT-PCR was designed both for targeting the VP1 region in the CV-A6 and studying the E-30 epidemic, and in both studies the PCR products were sequenced for confirmation. This method is a straightforward and rapid for the identification of viruses. In 2014, this approach was used to specifically identify EV-D68 to define its spread and disease associations in the USA and Europa (Khan, 2015; Poelman *et al.*, 2015).

6.3.1. Coxsackievirus A6 and HFMD (III)

Before 2009, HFMD outbreak reports have been associated with enterovirus strains CV-A16 or EV-A71. In recent years, particularly HFMD outbreaks caused by EV-A71 have been more frequent in Southeast Asia and Australia (Chen *et al.*, 2006; McMinn, 2002; Wang *et al.*, 2002) and, only sporadic cases of HFMD caused by other coxsackievirus A types have been reported (Cabral *et al.*, 1998; Miyazawa *et al.*, 2008), before our report on the CV-A6 outbreak in Finland in 2008 (III). In general, CV-A6 findings have been rare and mostly associated with herpangina (Lo *et al.*, 2011; Yamashita *et al.*, 2005).

In Finland, CV-A6 was identified on four occasions without known disease association in an enterovirus surveillance study in 2000-2007 (Blomqvist *et al.*, 2010). Several studies of CV-A6 outbreaks even with severe cases have been published after our report in 2009 (Sinclair *et al.*, 2014; Stewart *et al.*, 2013). Nowadays, HFMD outbreaks caused by CV-A6 have been reported in Spain (Cabrerizo *et al.*, 2014; Montes *et al.*, 2013), France (Mirand *et al.*, 2012), China (Lu *et al.*, 2012), Thailand (Puenpa *et al.*, 2013), Taiwan (Lo *et al.*, 2011), Japan (Kobayashi *et al.*, 2013; Wei *et al.*, 2011), India (Gopalkrishna *et al.*, 2012), and USA (Lott *et al.*, 2013). In these outbreaks, the symptoms were typical for HFMD with blisters in the hands, feet, and mouth. In addition to the symptoms mentioned above, CV-A6 caused widely spread exanthema, skin lesions in hands and feet, and blister eruptions in the buttocks.

In the CV-A6 HFMD outbreaks a common feature has been onychomadesis, nail shedding. In onychomadesis the nail plate separates from the nail matrix. Onychomadesis appears approximately 1 to 2 months after the onset of the infection, generally without any complications or pain (Davia *et al.*, 2011; Wei *et al.*, 2011). The association of HFMD and onychomadesis was presented in two earlier reports. (Bernier *et al.*, 2001; Clementz and Mancini, 2000). In study III, a causative agent was identified for the first time. Since then, several reports of HFMD associated with CV-A6 and later onychomadesis have been published (Davia *et al.*, 2011; Miyamoto *et al.*, 2013; Wei *et al.*, 2011), but study III is still the only one reporting extraction of the nucleic acid corresponding CV-A6 from the nails.

In some reports, HFMD caused by CV-A6 has been considered atypical and associated with more severe symptoms, including formation of large vesicles and vesicular rash in

the trunk, buttocks, and perioral face (Huang *et al.*, 2013; Lott *et al.*, 2013; Sinclair *et al.*, 2014). In addition, CV-A6 has been detected in a case of epididymitis after a HFMD infection (Vuorinen *et al.*, 2014).

Phylogenetic analysis of CV-A6 VP1 sequences suggests that the HFMD-associated strain may have diverged already in 1990's, at latest 2004, but the change in disease presentation was recognized first in study III, 2008 (Gaunt *et al.*, 2015). Today, the genomes of strains of HFMD-causing CV-A6 have been completely sequenced, strains originating from different parts of the world (Chung *et al.*, 2013; Gaunt *et al.*, 2015; Osterback *et al.*, 2014). The Finnish CV-A6 isolate showed features of the traditional HFMD viruses CV-A16 and EV-71. The common features were seen in the 2A protease genes *cis*-acting replication element and in the 3A and 3D sites of the polyprotein. Possibly the CV-A16 and EV-71 features in the Finnish CV-A6 isolate can explain the shift in symptoms from herpangina to more severe HFMD with onychomadesis (Osterback *et al.*, 2014) Isolated during a Hand-Foot-and-Mouth Disease Outbreak in Finland in 2008 (Osterback *et al.*, 2014). Analysis of whole genome sequences of CV-A6 strains circulating worldwide during the past 10 years have revealed eight recombinant forms of which two have strongest association with HFMD-like disease (Gaunt *et al.*, 2015). They represent strains resembling those identified in study III and those isolated in Scotland during an outbreak of eczema herpeticum in 2014 (Sinclair *et al.*, 2014).

6.3.2. Echovirus 30 outbreak (IV)

An aseptic meningitis outbreak caused by E-30 occurred in Finland in 2009; before this outbreak only sporadic cases of E-30 and a small outbreak in 1996 and 1997 were detected (Savolainen-Kopra *et al.*, 2011). E-30 has caused aseptic meningitis outbreaks also in other European countries during the last years. E-30 was the predominant type in several epidemics in Spain in the 21st century (Trallero *et al.*, 2010) and it also caused aseptic meningitis outbreaks in Latvia and Serbia in summer 2010 in Italy in 2012 and in France in 2013 (Cosić *et al.*, 2010; Milia *et al.*, 2013; Nougairède *et al.*, 2014; Perevoscikovs *et al.*, 2010).

In the E-30 outbreak study, the CSF specimens were first analyzed for enteroviruses with the highly conserved and competent 5'NCR qRT-PCR for picornaviruses. An E-30 outbreak-specific qRT-PCR was designed according to the sequence obtained from an E-30 positive CFS specimen (GenBank no: KJ179954). The sequence was performed with a qRT-PCR amplifying a 395-bp-long partial sequence of the enterovirus VP1 genome (Nix *et al.*, 2006). The E-30-specific qRT-PCR was designed to confirm the presence of the enterovirus during the outbreak season. The E-30 outbreak continued in two periods over a year, starting in Southwestern Finland in August 2009 and fading out during the following winter to peak again in the summer 2010. The 2009 wave affected mostly teenage boys between 15 and 19 years of age and the 2010 wave affected a wider population with no gender predominance. In France, E-30 affected mostly children under 10 years of age and all the sequences clustered in same genogroup (Nougairède *et al.*, 2014) and in Italy, the E-30 meningitis outbreak was among parents with children in the same class of a nursery school and according to

the phylogenetic analysis, E-30 belonged to same genogroup VII as the strain circulated in Finland 2009 (Milia *et al.*, 2013). There are several reports of E-30 outbreaks among group activities, in USA, E-30 caused meningitis among high school football players (Croker *et al.*, 2015) and in Japan, E-30 caused an outbreak in a high school baseball club members and their relatives (Hayashi *et al.*, 2009). Furthermore, E-30 outbreaks have been associated with swimming in pool or sea (Begier *et al.*, 2008; Faustini *et al.*, 2006) However, the E-30 outbreak in Finland correlated with previously reported outbreaks in temperate climates, with its summer-autumn seasonality, as well as age and gender distributions. Sport clubs and other outdoor activities were most likely places of transmission in the outbreak.

7. CONCLUSIONS

The world of picornaviruses is growing all the time, new pathogens are found and the old ones find new ways to proliferate and cause diseases. In this thesis, the identification of picornaviruses has been improved with several new molecular methods. First, the picornavirus RT-PCR assay was upgraded to a real-time format with the differentiation of rhino- and enteroviruses with a melting curve analysis. This method was used in the study of rhinovirus transmission in families with conclusions that the rhinovirus spread efficiently in families with children under seven years of age (II).

Secondly, the picornavirus 5'NCR qRT-PCR assay was improved to differentiate between rhino- and enteroviruses more specifically with virus-specific probes (I). The assay was designed to have incorporated LNA-probes, which increase the sensitivity and specificity of the assay. The LNA-probe assay recognized all the tested enterovirus prototypes and clinical specimens correctly. The assay was validated with all possible rhino- or enterovirus specimen types, which makes the assay suitable for diagnostic use.

When a picornavirus outbreak occurs it is desirable to have a reliable method to investigate the epidemic. In this thesis, the picornavirus outbreaks were studied with a virus-specific qRT-PCR method designed from the picornavirus typing area VP1, and the genetic relationship of the outbreak strains with each other and previous strains was also analyzed (III and IV). The method was proved to be very useful in rapidly identifying positive specimens and in studying the causative agent of the epidemic.

Moreover, the HFMD epidemic was caused by a new HFMD virus, CV-A6 (III). After our report of the HFMD outbreak caused by CV-A6 and the associated onychomadesis, there have been several reports of similar HFMD outbreaks and clinical cases caused by CV-A6; therefore, our findings and report on CV-A6 were groundbreaking in the field of infectious diseases and picornavirus research. The reported E-30 outbreaks also increased the understanding of aseptic meningitis outbreaks, their occurrence among particular age groups, and the spreading of the E-30 in out-of-school activities (IV). In conclusion, enteroviral outbreaks arise with intervals of a couple of years and straightforward plans and methods are needed to investigate the pathogen and its epidemiology.

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