Molecular characterization of human rhinovirus field strains isolated during surveillance of enteroviruses

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Human rhinoviruses (HRVs), which are the most frequent causative agents of acute upper respiratory tract infections, are abundant worldwide. We have identified HRV strains in environmental specimens collected in Finland, Latvia and Slovakia during the surveillance of polio- and other enteroviruses. These acid-sensitive HRV strains were isolated under conditions optimized for growth of most of the enteroviruses, i.e. in stationary human rhabdomyosarcoma cells incubated at 36 °C. Phylogenetic analysis of the sequences derived from the partial 5' non-coding region and the capsid region coding for proteins VP4/VP2 and VP1 showed that the HRV field strains clustered together with prototype strains of the HRV minor receptor group. Partial sequences of the 3D polymerase coding region generally followed this pattern, with the exception of a set of three HRV field strains that formed a subcluster not close to any of the established HRV-A types, suggesting that recombination may have occurred during evolution of these HRV strains. Phylogenetic analysis of the VP4/VP2 capsid protein coding region showed that the 'environmental' HRV field strains were practically identical to HRV strains recently sequenced by others in Australia, the United States and Japan. Analysis of amino acids corresponding to the intercellular adhesion molecule-1 receptor footprint in major receptor group HRVs and also in the low-density lipoprotein receptor footprint of minor receptor group HRVs showed conservation of the 'minor receptor group-like' amino acids, indicating that the field strains may have maintained their minor receptor group specificity.

INTRODUCTION

Human rhinoviruses (HRVs) belong to the family Picornaviridae and together with human enteroviruses (HEV) and a number of non-human enterovirus strains comprise the largest genus (Enterovirus) in the family (Knowles, 2008). Clinical illnesses caused by HRV are typically mild and self-limiting upper respiratory tract infections or common colds, but HRVs have also been increasingly associated with other clinical consequences of respiratory tract infection such as acute otitis media (Nokso-Koivisto et al., 2004; Pitkaranta et al., 1998; Vesa et al., 2001), acute community-acquired sinusitis (Pitkaranta et al., 1997, 2001), bronchiolitis (Hayden, 2004), pneumonia (Papadopoulos, 2004) and exacerbations of existing respiratory disorders such as asthma (Gern, 2002) and chronic obstructive pulmonary disease (Greenberg, 2002).

HRV encompasses 100 designated serotypes, including two antigenically distinct subtypes of serotype HRV1 (HRV1A and HRV1B) (Hamparian et al., 1987; Kapikian et al., 1967, 1971). The current taxonomy is based on genetic relationships of HRV. Genetic analysis of VP4/VP2 (Savolainen et al., 2002a) and VP1 (Laine et al., 2005; Ledford et al., 2004) capsid protein coding regions showed that the 100 serotypes cluster into two genetically distinct species, HRV-A and HRV-B. One HRV strain originally assigned as HRV87 belongs to the enterovirus species HEV-D (Blomqvist et al., 2002; Ishiko et al., 2002; Oberste et al., 2004). Recent studies, which have applied direct RT-PCR and sequencing to clinical specimens, have discovered a third genetic clade that is distinct from HRV-A and HRV-B and is preliminarily called HRV-C (Arden et al., 2006; Kistler et al., 2007a; Lamson et al., 2006; Lau et al., 2007; Lee et al., 2007; McErlean et al., 2007; Savolainen-Kopra et al., 2009).

HRVs are composed of an icosahedral protein capsid composed of 60 copies of protomers, each of which comprise a single molecule of four capsid proteins VP1–VP4, VP1 being the most exposed. An approximately 7200 bp single-stranded positive-sense RNA includes non-coding regions (NCR) at both the 5' and 3' ends and a single open reading frame residing between the NCRs.
HRV, like other RNA viruses, is predisposed to pronounced genetic variation, mostly due to the lack of proofreading capacity of the RNA-dependent RNA polymerase. Complete genome sequences of 46 prototype strains of HRV-A and HRV-B have been generated recently (Kistler et al., 2007b; Tapparel et al., 2007). According to this limited set of HRV sequences, it has been suggested that the main force driving the genetic diversification of HRV may be genetic drift rather than intra- or interserotypic recombination, which, in contrast, are common in closely related HEV genomes (Brown et al., 2003; Lindberg et al., 2003; Norder et al., 2002; Santti et al., 1999). In HEV, the recombination sites are usually located in genomic regions encoding non-structural proteins, but have also occasionally been found in the VP1 capsid protein coding region (Blomqvist et al., 2003; Martin et al., 2002).

HRV are classified into two groups according to receptors used in cellular attachment and entry. Twelve HRV-A serotypes (HRV1A, 1B, 2, 23, 25, 29, 30, 31, 44, 47, 49 and 62), the minor receptor group, use members of the low-density lipoprotein receptor (LDLR) family, which are proposed to bind their ligands by electrostatic interactions with negatively charged ligand-binding domains (Gruenberger et al., 1995; Hofer et al., 1994; Marlovits et al., 1998; Vlasak et al., 2005b). The cellular receptor for the remaining HRV-A serotypes and all the HRV-B viruses (the major receptor group) is a cell surface member of the immunoglobulin supergene family called intercellular adhesion molecule 1 (ICAM-1) (Greve et al., 1989; Staunton et al., 1989; Tomassini et al., 1989). Recently, a third receptor, heparan sulfate, was shown to operate as an alternative receptor for some of the major group strains of HRV-A species (Vlasak et al., 2005a; Khan et al., 2007). The HRV strains genetically classified as the proposed HRV-C species have not been successfully propagated in cell cultures and thus their receptor specificity is currently unknown.

So far, both the biological and phylogenetic studies on individual HRV strains have focused almost entirely on HRV prototype strains isolated decades ago and the information on the HRV strains circulating today is scarce. In this study, we describe HRV field strains that were isolated from either environmental or clinical specimens during surveillance of polioviruses and other enteroviruses, initially regarded as non-poliovirus enteroviruses (NPEVs) and identified as HRV by molecular methods (Blomqvist et al., 2008). The HRV field strains were sequenced in four distinct genomic regions in order to investigate their genetic relationship with HRV prototype strains. In addition, the deduced amino acids of known HRV receptor footprints were used to evaluate the receptor specificity of the HRV field strains.

**METHODS**

**Cell lines and prototype virus strains.** Human rhabdomyosarcoma (RD) cells and recombinant mouse L cells expressing human poliovirus receptor (L20B) were provided by the WHO Polio Labnet. HRV prototype strain HRV1B was obtained from the National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. Poliovirus type 1 (PV1)/Sabin strain was kindly provided by J. Martin (National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, UK). Human embryonic skin cells were prepared locally by standard techniques from legally aborted embryos and used between passages 10 and 25.

**Isolation of HRV field strains.** The clinical HRV strains were isolated by standard enterovirus isolation techniques according to the guidelines of the Polio Laboratory Manual (WHO, 2004). The environmental HRV strains were isolated from sewage samples regularly collected in Finland, Latvia and Slovakia. Before virus isolation, 500 ml raw sewage sample was concentrated by using a two-phase separation method according to the procedure recommended by the WHO (2003). The concentrates were analysed for the presence of polioviruses and NPEVs by standard techniques (WHO, 2004). Briefly, for NPEV isolation, stationary cultures of RD cells in minimal essential medium (MEM) supplemented with 2 or 5 % fetal calf serum (FCS) were inoculated with 0.5 ml sewage concentrates and incubated at 36 °C. The inoculated cell cultures were inspected daily for cytopathic effect (CPE). The RD cultures showing 100 % CPE were freeze–thawed and subjected to one further passage in RD and L20B cells. The specimens that induced CPE in RD but not L20B cells were regarded as NPEV and the RD cultures with 100 % CPE were selected for molecular identification.

**Molecular identification of HRV.** Total RNA was extracted from 100 μl of infected cell cultures with the RNeasy Mini kit (Qiagen) or the E.Z.N.A. Total RNA kit (Omega Bio-Tek). RNA (1 μl) was used in one-step RT-PCR in order to amplify an approximately 350 nt segment from the VP1 capsid protein coding region, exactly as described by Oberste et al. (2003). The PCR amplicons were purified by using the QIAquick kit (Qiagen) before sequencing (see below).

**Purification of the HRV field strains.** HRV field strains were purified by plaque assay. Serial 10-fold dilutions were prepared from virus strains and 100 μl of each dilution was used to inoculate monolayer cultures of RD cells on six-well plates. After adsorption for 30 min at 36 °C, the unadsorbed virus inocula were removed, the cells were overlaid with 2 ml 0.5 % carboxymethyl cellulose in MEM supplemented with 2 % FCS and the plates were incubated at 36 °C in the presence of 5 % CO2. Individual plaques were collected on day 2 or 3 and passaged once in RD cells at 36 °C in MEM supplemented with 2 % FCS. RD cells with 100 % CPE were freeze–thawed three times and clarified by low-speed centrifugation. The supernatants were collected and stored at −20 °C before use in the following experiments.

**Acid sensitivity.** Acid sensitivity of selected HRV strains was assessed by a standard method (Couch, 1991). Briefly, equal aliquots of plaque-purified virus strains were incubated in 0.1 M citric acid buffer, pH 4, and in 0.1 M phosphate buffer, pH 7, for 1 h at 36 °C, and then neutralized with 0.5 M phosphate buffer, pH 7.2. The infectivity of the acid-treated and untreated viruses was determined by titration in RD cells at 36 °C. Prototype virus strains HRV1B and PV1/Sabin were used as acid-sensitive and acid-resistant controls, respectively.

**RT-PCR and sequencing.** Total RNA was isolated from plaque-purified HRV field strains with the RNeasy Mini kit (Qiagen) or the E.Z.N.A. Total RNA kit (Omega Bio-Tek). RNA (1 μl) was reverse-transcribed and amplified using the same procedure that was used for the partial VP1 (Oberste et al., 2003), but the primers were changed for each genomic region to be amplified and sequenced. The primers for a 570 nt segment from the 5′ NCR were HRVA 5′ NCR 5′-GCTTGCCCTATGACGTTGTC-3′ and HRVA 3′ NCR 5′-TGTAGAGGTTTGGTTCATGC-3′ and for a segment of 515 nt from the 3′ NCR, the primers were HRVA 5′ NCR 5′-GTGAAATCAGGCTCCACAGC-3′ and HRVA 3′ NCR 5′-CATACTGGTGGCGTGGTTAA-3′.
sequencing primers (Lu et al., 2008), those for the VP4/VP2 capsid protein region were 9895 and 9565 (Savolainen et al., 2002b) and those for the VP1 coding region were as described previously (Vlasko et al., 2003). The primers for an approximately 600 nt region spanning the 3’ part of the 3D polymerase gene were as described previously (Savolainen et al., 2004). The PCR amplicons were purified by using the QIAquick kit (Qiagen) before use in sequencing with the corresponding RT-PCR primers (ABI Prism BigDye Terminator Cycle Sequencing; Applied Biosystems).

Phylogenetic analysis. Raw sequence data were analysed using Vector NTI Suite 10 (Invitrogen), assembled using ContigExpress and aligned using AlignX software. Multiple sequence alignments were made with CLUSTAL_X 1.83 (Thompson et al., 1997) and MEGA 4.0 (Tamura et al., 2007) using default parameters. The phylogenetic trees were estimated with MEGA 4.0 using the neighbour-joining (NJ) method (Saitou & Nei, 1987) with the maximum composite likelihood model (Tamura et al., 2007). Bootstrapping was performed with 1000 replicates (Hillis & Bull, 1993). Amino acid sequence alignment from the VP3/VP1 capsid protein region was manually edited to include only the set of ten non-linear amino acids corresponding to the ICAM-1 footprint before the NJ tree was estimated with MEGA 4.0 (Fig. 3a).

RESULTS

Isolation and identification of HRV strains from environmental and clinical samples

All HRV field strains analysed in this study (Table 1) were identified, by using molecular methods, from the NPEV isolates gathered during surveillance of polio- and other enteroviruses, as described previously (Blomqvist et al., 2008). The first ‘environmental’ HRV strains were isolated from sewage samples in 2001 in the National Polio Laboratories in Riga, Latvia, and in Bratislava, Slovakia. The virus isolates were untypable by neutralization with intersecting pools of enterovirus antisera and were subsequently sent to the National Institute for Health and Welfare (THL), Helsinki, Finland, for molecular identification. Since 2001, 11 additional HRV strains were isolated from sewage specimens in Slovakia. The environmental surveillance in Finland yielded ten HRV strains during 2001–2007 as well. The clinical HRV strains were isolated in Reykjavik, Iceland, and sent to THL, Helsinki, for molecular identification. The HRV field strain FIN90-1592 was isolated in THL, Helsinki, in 1990; it was recorded as an untypable enterovirus and identified as HRV by molecular methods in 2007.

For molecular identification of the virus strains, sequences of approximately 350 nt were produced from the VP1 capsid protein-coding region and compared to the nucleotide database (GenBank) by using BLAST analysis. The best scores for query sequences were 87–94 % nucleotide identity to prototype strains of HRV minor receptor group in the species HRV-A.

Acid sensitivity of HRV field strains

Traditionally, HRV are distinguished from HEV by their sensitivity to acidic conditions, in contrast with most HEV strains which maintain their infectivity at a low pH. Fourteen initially isolated HRV field strains were tested for acid sensitivity by using a standard protocol (Table 2). All HRV field strains, as well as the prototype strain HRV1B, lost their infectivity after a 1 h acid treatment (pH 4.0 at 36 °C), while PV1/Sabin remained unaffected under the same conditions.

Table 1. Description of HRV field strains characterized in this study

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Specimen:</th>
<th>Collection date</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVA01-E10596</td>
<td>Latvia</td>
<td>2001</td>
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<td>SVK01-E93</td>
<td>Slovakia</td>
<td>2001</td>
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<td>09/12/2002</td>
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<td>SVK03-E95</td>
<td>Slovakia (Skalica)</td>
<td>08/10/2003</td>
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<td>SVK03-E132</td>
<td>Slovakia (Vrakuna)</td>
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</tr>
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<td>19/05/2003</td>
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<tr>
<td>ICE07-1337</td>
<td>Iceland</td>
<td>2007</td>
</tr>
</tbody>
</table>

Phylogenetic analysis of genetic relationships of HRV strains

To study the phylogenetic relationships of the HRV field and prototype strains of HRV-A, partial 5’ NCR, VP4/VP2 capsid protein-coding regions, complete VP1 with the flanking regions of VP3 and 2A and partial 3D polymerase coding regions were sequenced from all HRV field strains. The complete set of 75 HRV-A prototype sequences was
HRV prototype strains and seven field strains of cluster 3 HRV strains divided into two different branches in the phylogenetic trees constructed from the other three genomic regions. In contrast, cluster 3 strains, with the phylogenetic trees constructed from either the VP4/VP2 or complete VP1 coding regions, respectively. Corresponding amino acid identities were 97.7–100 % in VP4/VP2 and 88.5–97.5 % in VP1, respectively. When using these three genomic regions for comparisons, the closest prototype strain for each HRV field strain was invariably a minor receptor group HRV strain. In the partial 3D polymerase coding region, the closest prototype strain for 25 of the 28 HRV field strains studied was a minor receptor group strain with 90.3–94.8 % nt and 96.8–98.7 % aa identities. In contrast, three HRV field strains, FIN02-E1, SVK05-E344 and LVA01-E10596, formed a subcluster, which was not close to any of the minor receptor group HRV prototype or any other HRV-A prototype strains for which 3D polymerase sequences were available.

**Global distribution of HRV strains**

The global relationships of the HRV field strains were assessed by analysing the VP4/VP2 capsid protein coding region as, at present, most HRV sequence data are available on this region (Kistler et al., 2007a; McErlean et al., 2007; Savolainen et al., 2002b). The HRV field strain VP4/VP2 sequences produced in this study were first aligned with all HRV sequences available in GenBank and the sequences that clustered together with minor receptor group HRV prototype strains were selected for more detailed analysis (Fig. 2). As a result of the GenBank search, one, four and five additional HRV field strains were tagged onto the minor receptor group clusters 1, 2 and 3, respectively. Due to the limited length of some of the retrieved sequences, the subsequent phylogenetic comparison was based only on 324 nt beginning at the 5’ end of the VP4 capsid protein-coding region.

Phylogenetic analysis revealed seven distinct HRV field strain branches, which had inter-strain nt identities higher than 97.5 %. In the minor receptor group, cluster 1 HRV strains isolated in Iceland, Slovakia and Australia in 2003 and again in Slovakia in 2004 and 2005 were 97.9–99.7 % and 100 % identical at the nt and aa levels, respectively. Six more branches of closely related HRV field strains were seen in the minor receptor clusters 2 and 3, two of which contained HRV strains sequenced from three different continents. Seven HRV field strains, which originated from either Finland (2003, 2005 and 2006), Australia (2003) or the United States (2004), shared ≥99.4 % nt identity. The closest prototype strain to available and was used in the analysis of 5’ NCR, VP4/VP2 and VP1 regions, whereas only 56 HRV-A prototype strain sequences were available for analysis of the 3D polymerase region. The clustering of HRV strains based on these four genomic regions is shown in Fig. 1a–d.

In the VP1 region, HRV field strains clustered into three distinct minor receptor group HRV clusters 1 to 3, previously described by Vlasak et al. (2003), supported by high bootstrap values (Fig. 1c). The clustering was exactly identical in phylogenetic trees constructed from either nucleotide or deduced VP1 amino acid sequences (data not shown). Clusters 1 (represented by prototype strains HRV1A and HRV1B and including six HRV field strains) and 2 (prototype strains HRV2, HRV23, HRV30 and HRV49 and 12 HRV field strains) also grouped similarly in the phylogenetic trees constructed from the other three studied genomic regions. In contrast, cluster 3 strains, with six HRV prototype strains (HRV25, HRV29, HRV31, HRV44, HRV47 and HRV63) and ten HRV field strains, were similar in only two capsid regions. The 16 cluster 3 HRV strains divided into two different branches in the phylogenetic tree constructed from partial 5’ NCR sequences (Fig. 1a). In the 3D polymerase region, the six HRV prototype strains and seven field strains of cluster 3 formed two branches equal to the 5’ NCR, whereas three HRV field strains, FIN02-E1, SVK05-E344 and LVA01-E10596 clustered together but separate from the HRV prototype strains of cluster 3 or other minor group HRV prototype strains (Fig. 1d).

Individual HRV field strains had 96–98.3 %, 86.9–93.8 % and 88.6–91.8 % nt identity to their closest HRV prototype strain at the partial 5’ NCR, VP4/VP2 and complete VP1 coding regions, respectively. Corresponding amino acid identities were 97.7–100 % in VP4/VP2 and 88.5–97.5 % in VP1, respectively. When using these three genomic regions for comparisons, the closest prototype strain for each HRV field strain was invariably a minor receptor group HRV strain. In the partial 3D polymerase coding region, the closest prototype strain for 25 of the 28 HRV field strains studied was a minor receptor group strain with 90.3–94.8 % nt and 96.8–98.7 % aa identities. In contrast, three HRV field strains, FIN02-E1, SVK05-E344 and LVA01-E10596, formed a subcluster, which was not close to any of the minor receptor group HRV prototype or any other HRV-A prototype strains for which 3D polymerase sequences were available.

**Table 2.** Infectivity of selected HRV strains in RD cells after acid treatment (pH 4, 1 h, at 36 °C) and without acid treatment (pH 7)

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Log10 virus titre TCID50 (0.025 ml)⁻¹</th>
<th>pH 4</th>
<th>pH 7</th>
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<tr>
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</tr>
<tr>
<td>PV1/Sabin†</td>
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</table>

*HRV1B was used as an acid-sensitive control.
†PV1/Sabin was used as an acid-resistant control.

**Fig. 1.** Phylogenetic analysis of nucleotide sequences in partial 5’ non-coding (a), VP4/VP2 (b), VP1 capsid protein (c) and partial 3D polymerase coding regions (d). The trees were constructed with MEGA 4.0 using the NJ method, Maximum composite likelihood model and 1000 bootstrap replicates. The HRV field strains from this study are marked with filled circles (●). Clusters 1, 2 and 3 are indicated by bars and numbers.
these strains was HRV49. Eight field strains sequenced from either Europe (from Iceland in 2003, Slovakia in 2004 and 2005 and Finland in 2005), Japan (2003) or the United States (2003 and 2004) shared \( \geq 98.5 \% \) nt identity.

**Amino acids specific for minor and major receptor group HRV**

To estimate the conservation of the receptor specificity of HRV field strains, amino acids residing in either ICAM-1 or LDLR footprints were studied. We have previously shown that by aligning a set of ten non-linear VP3 and VP1 amino acids predicted to participate to ICAM-1 binding (Kolatkar et al., 1999), the HRV-A prototypes can be clearly separated into two major clusters that correspond to the major and minor receptor groups (Laine et al., 2006). VP3 sequences long enough for the ICAM-1 footprint analysis were obtained from 26 HRV field strains. As shown in the dendrogram constructed from this non-linear set of amino acids (Fig. 3a), the HRV field strains strictly clustered into three main branches together with the minor receptor group HRV prototype strains.

The BC and HI loop amino acids were aligned in order to study the conservation of amino acids shown to interact with the very-low-density lipoprotein (VLDL)-receptor (Verdaguer et al., 2004). Overall, the cluster-specific lengths of the amino acid loops were strictly conserved in HRV field and prototype strains (Fig. 3b). Three HRV2 amino acids proved to interact with VLDL-receptor reside in the BC loop and the other three in the HI loop. When the corresponding amino acids in the HRV field strains were compared with all the minor receptor group HRV prototypes, individual substitutions were found in 15 HRV field strains, in three of the six amino acid positions.

**DISCUSSION**

In this study, we have shown for the first time that HRV are present in environmental samples. HRV were isolated from sewage in conditions optimized for isolation of polio- and other enteroviruses and were recorded as untypable NPEVs prior to identification as HRV by molecular methods. The isolated HRV strains were acid sensitive, as is typical for HRVs. Genetically these HRV field strains clustered to the minor receptor group of HRV, the classification was also supported by conservation of amino acids involved in the predicted minor and major receptor footprints. GenBank searches showed that practically identical HRV strains were also sequenced during the same study years in other parts of the world. Highly similar HRV field strains were also isolated from clinical specimens in the present study.

Environmental surveillance for polio- and other enteroviruses is based on the fact that both symptomatic and asymptomatic patients may excrete these viruses in stool for several weeks. The viruses remain infective in sewage and can be detected by, for example, virus isolation. In contrast, HRV replication is mainly restricted to upper airways and the infectivity of acid-sensitive HRV is destroyed in the low pH of the stomach. However, despite the sensitivity to acid, HRVs are otherwise known to be as stable as enteroviruses. HRV load in nasal secretions is high and the virus is easily transferred to fingertips and environmental surfaces where HRV may remain infective and transmissible for several days (Gwaltney & Hendley, 1982; Hendley & Gwaltney, 1988; Hendley et al., 1973; Winther et al., 2007). We cannot be certain how HRV ends up in sewage. It is conceivable that HRVs derived from respiratory secretions are carried into sewage by discarded used tissues or in waste water. HRV may also be passed to

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**Fig. 2.** Phylogenetic analysis of nucleotide sequences in a 324 bp region spanning from the beginning of the VP4 capsid protein coding region to nt 117 of VP2. Sequences were analysed as in Fig. 1. The HRV strains isolated in this study are marked with filled circles (●) and those sequenced by others (Kistler et al., 2007a; McErlean et al., 2007; GenBank accession no. AB162765) with filled squares (■).
sewage by excretion in stool, but in this case, the viruses have to either be protected against the low pH in the stomach by an as-yet unknown mechanism or retain their infectivity after the return to a favourable pH. The physical conditions in sewage do not seem to destroy HRV nor do they destroy HEV. According to the WHO recommended procedure for the treatment and concentration of sewage specimens, the pH of the raw sewage is measured upon arrival in the laboratory; in our laboratory at least, the pH of sewage specimens has, without exception, been neutral. HRV field strains characterized in this study were isolated in conditions typical for optimal growth of most enteroviruses. The HRV strains induced pronounced CPE in stationary RD cell cultures following incubation at 36°C, which substantially differs from the traditional isolation practice for HRV (incubation in rolling HeLa Ohio cells at 33°C). The effective replication of some HRV prototype strains at 36°C, which is the temperature in lower airways, has also been described previously (Mosser et al., 2002; Papadopoulos et al., 1999; Puro et al., 2005). The isolation of only HRV strains that genetically belonged to the minor receptor group may be explained by the selectivity of the RD cell line. We inoculated RD cells with a collection of clinical HRV strains originally isolated in HeLa Ohio cells at 33°C (Blomqvist et al., 1999) and determined that these were genetically close to either HRV-A or HRV-B major receptor group HRVs (Savolainen et al., 2002b), but no CPE was produced upon incubation at 36°C (data not shown). In contrast, when a selection of

Fig. 3. Analysis of selected amino acids involved in the ICAM-1 receptor footprint in major receptor group rhinoviruses (a) and the footprint of LDLR family in minor receptor group HRVs (b). (a) A set of ten non-linear VP3 and VP1 amino acids in the ICAM-1 footprint [amino acids according to HRV16 are: Thr3179, Pro3180, Asp3181, Thr3182, Ser3185, Gly1148, Ile1151, Arg1205, Val1209 and Asp1213 (Kolatkar et al., 1999)] were aligned and phylogeny was inferred by using MEGA 4.0. Minor receptor group HRV strains are shown in bold. HRV field strains are indicated by filled circles (●). (b) Amino acids in BC and HI loops of VP1 were aligned with MEGA 4.0. HRV prototype strains are shown in bold. Amino acids contacting the VLDL-receptor in HRV2 are indicated by arrows (Verdaguer et al., 2004). HRV field strain residues located in the corresponding sites but differing from the prototype strain residues are shown in white type on a black background.
plaque-purified HRV field strains isolated in this study were inoculated into stationary HeLa Ohio cells at 36 °C, CPE was induced, at least when a high multiplicity of infection was used (data not shown). We did not attempt to isolate HRV from environmental specimens in HeLa Ohio cells at 33 °C. It is very likely that the major receptor group HRV strains exist in the sewage as well, but the isolation procedure optimized for HEV does not support their growth.

Although we have clearly demonstrated in this study that HRV are present in environmental samples, their isolation has been infrequent. Approximately 60 sewage specimens were collected and analysed annually in Finland during the study years (2001–2007). Depending on the number and type of cell lines used in virus isolation, about 30–90 different HEV strains were gathered annually. In contrast, rhinovirus was isolated only once in 2002, 2003, 2004, 2006 and 2007. In 2005, in addition to one HRV strain that was isolated from the sewage sample collected in the city of Tampere, four different HRV strains were isolated from two successive sewage samples collected in the southwestern part of Finland. All of these HRV strains from 2005 were isolated between August and September, which has previously been shown to be the high season for HRV in Finland (Vesa et al., 2001). The second cluster of HRV isolations was in Skalica, Slovakia, in October–November 2005. This was probably due to the intensified sampling because of the simultaneous presence of vaccine-derived poliovirus type 2 in the sewage from Skalica (Cernakova et al., 2005). The isolation of genetically distinct HRV strains from concomitant or successive specimens was not surprising, since multiple serotypes (Fox et al., 1975; Gwaltney et al., 1968; Monto & Cavallaro, 1972) or multiple genetically identified types (Peltola et al., 2008; Savolainen et al., 2002a) are known to co-circulate in a given population during each HRV season. In addition to the co-circulation of different HRV strains in restricted regions, the simultaneous existence of practically identical HRV strains in distinct locations was seen. This is in accordance with the observations made in epidemiological studies performed several decades ago (Gwaltney et al., 1968; Stott, 1969). The current analysis was performed on the most conserved part of the capsid, VP4, which may partly explain the genetic closeness of the geographically distant strains. However, as earlier demonstrated (Laine et al., 2005) and also confirmed in this study, partial VP4/VP2 and VP1 regions yield comparable results when determining the closest genetic type of HRV.

In contrast with HEVs (whose molecular typing is widely used because the VP1 capsid protein coding sequences and neutralization-determined serotypes exactly correspond), the correlation of serologic and genetic HRV types has not been thoroughly studied. We have previously proposed the term ‘the closest prototype strain’ for assigning the molecular type for HRV field strains (Savolainen et al., 2002b) until more sequence data becomes available and the molecular classification scheme can be agreed. In this study, the clustering of HRV field strain sequences closest to only one of the HRV prototypes was obvious in VP1, but was almost identical in other studied genomic regions. However, three HRV strains (SVK03-E132, FIN05-E12942 and ICE07-1337) clustered strictly together with both HRV29 and HRV44 in all four sequenced regions, forming a clade of five virus strains. Previously, the prototype strains HRV29 and HRV44 have been shown to be antigenically related with reciprocal neutralizing ability (Cooney et al., 1973, 1982). If the HRV serotype classification scheme is renewed according to the accumulating sequence data, the virus pair HRV29/HRV44 might be considered as a single HRV serotype/type (Ledford et al., 2004).

Recombination has been considered to play a significant role in genetic diversification of HEV, but its importance in HRV strain diversification is currently unclear because of the limited amount of HRV sequences available (Kistler et al., 2007b; Simmonds & Welch, 2006). By analysing four distinct genomic regions from the set of 28 HRV field strains in this study, we noticed the inconsistent clustering of some HRV field and prototype strains in the 5’ NCR and 3D polymerase coding region as compared to the capsid region. This might be a consequence of recombination events in the HRV strains in the past. A more recent recombination may have occurred during the evolution of the HRV field strains that clustered together with HRV31 from other studied regions, but these formed a subcluster separate from the HRV-A prototype strain sequences available for analysis. However, the complete genome sequences of these strains are required to confirm the recombination. On the other hand, 25 of the 28 characterized HRV field strains clustered together with the same HRV prototype strain in all studied genomic regions, indicating that recombination has been rare in this set of HRV field strains.

Since the genetic data indicated that all HRV field strains identified in this study probably belong to the HRV minor receptor group, the deduced amino acids of the known receptor footprints were analysed in more detail. The conservation of the ‘minor receptor group-like’ residues in the ICAM-1 footprint of the major receptor group HRV predicts that these HRV field strains have not evolved to use the major receptor ICAM-1. It has been shown previously that the lysine (Lys) 1224 in the HI loop of VP1 is strictly conserved in all minor receptor group HRV prototypes (Laine et al., 2005; Ledford et al., 2004; Vlasak et al., 2003) and is believed to be essential but not sufficient for HRV attachment to members of the LDLR family (Vlasak et al., 2005b). This Lys was also conserved in 24 of 28 HRV field strains characterized in this study, but was substituted with arginine (Arg) in the remaining four HRV strains. The HRV field strains with this Lys to Arg substitution clustered close to the prototype strain HRV1B in all genomic regions and, despite this one substitution, the other amino acids involved in the LDLR footprint were fully conserved. Whether this substitution
affects the receptor specificities of these HRV field strains remains to be investigated.

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REFERENCES


