Genetic clustering of all 102 human rhinovirus prototype strains: serotype 87 is close to human enterovirus 70

Carita Savolainen, Soile Blomqvist, Mick N. Mulders† and Tapani Hovi

Enterovirus Laboratory, Department of Microbiology, National Public Health Institute (KTL), Mannerheimintie 166, FIN-00300 Helsinki, Finland

Human rhinoviruses (HRV), common agents of respiratory infections, comprise 102 designated serotypes. The genetic relationships of HRV prototype strains and the possibility of using genetic identification of a given HRV field strain were studied. Genomic sequences in the VP4/VP2 region were obtained from all 102 prototype strains. Phylogenetic analysis included 61 recently isolated Finnish field strains. Seventy-six out of the 102 prototype strains clustered in the HRV genetic group A and 25 in group B. Serotype 87 clustered separately and together with human enterovirus 70. The ‘percentage’ interserotypic differences were generally similar to those between different enterovirus serotypes, but for six pairs of HRV serotypes they were less than 10%. The maximum variation in genetic group A was 41% at the nucleotide level and 28% at the amino acid level, and in genetic group B 34% and 20%, respectively. Judging from the observed interserotypic differences, the 61 Finnish field isolates might represent as many as 19 different serotypes. One cluster of the field strains did not directly associate with any of the prototype strains and might represent a new serotype. However, larger numbers of field isolates of known serotype need to be characterized, possibly also in the VP1 region, to evaluate the feasibility of genetic typing of HRV strains.

Introduction

Human rhinoviruses (HRV) belong, together with enteroviruses and several other virus genera, to the family Picornaviridae, and thus share many common features, including a non-enveloped icosahedral capsid, a messenger-sense RNA genome and partial nucleotide and amino acid sequence identity (Rueckert, 1996). HRV have been shown to be the major group of causative agents of mild respiratory infections, e.g. the common cold (Rueckert, 1996; Mäkelä et al., 1998). The common cold is the most frequently occurring acute disease in humans and one of the main reasons for short time absence from work or school, with a major economical impact. Besides the common cold, HRV have been associated with more serious diseases, such as acute otitis media (Arola et al., 1988) and pneumonia in children (Abzug et al., 1990) as well as exacerbation of asthma (Johnston et al., 1995). Despite the overall economical and medical importance of HRV, little is known about the circulation and relative impact of individual serotypes. So far, 102 different serotypes of HRV have been determined. The large number of serotypes has hampered the use of conventional serotyping in the routine diagnosis of HRV infections.

HRV serotypes can be grouped in different ways, on the basis of shared structural and biological properties, e.g. receptor specificity (Abraham & Colonno, 1984; Uncapher et al., 1991), sensitivity to antiviral agents (Andries et al., 1990), immune cross-reactivity (Cooney et al., 1982) or genetic similarity (Duechler et al., 1987; Horsnell et al., 1995). Several groups have published studies on improved methodology for the detection of HRV infection, including molecular amplification methods like PCR and NASBA (Gama et al., 1989; Hyypää et al., 1989; Arruda & Hayden, 1993; Ireland et al., 1993; Johnston et al., 1993; Halonen et al., 1995; Samuelson et al., 1998; Andeweg et al., 1999, Blomqvist et al., 1999). These studies,
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Fig. 1. A dendrogram showing genetic relationships of different human rhinovirus and enterovirus groups in the 420 nt region encoding VP4/VP2. GenBank accession numbers for previously published enterovirus sequences: CV-A2, AJ296215; CV-A16, NC 001612; CV-A21, NC 001428; CV-B1, NC 001472; CV-B5, NC 001342; Echo1, X89531; EV70, NC 001430; EV71, NC 001769; PV1, NC 002058.

however, were designed to recognize HRV as a group, and not the individual serotypes. Molecular methods, e.g. partial genome sequencing, have been used in rapid genetic typing of the closely related group of enteroviruses (Oberste et al., 1999a, b). In addition, molecular analysis of wild-type poliovirus strains is routinely used in the Global Poliovirus Eradication Initiative in the follow-up of the programme (Kew et al., 1995). A rapid molecular identification method might provide a possibility for investigation of potential differences in pathogenicity between the different HRV serotypes and strains.

We have recently shown that HRV strains isolated from a cohort of small children during two successive years show a striking genetic divergence but segregate in the two known genetic clades of HRV (Savolainen et al., 2001). Our aim of this study was to explore the possibility of genetic typing of HRV strains. For this purpose we have partially sequenced all of the HRV prototype strains.
Methods

HRV prototype and field strains. We obtained the HRV prototype strains from the ATCC; Haartman Institute, Helsinki University; Janssen Pharmaceuticals; and National Institute for Public Health and the Environment, The Netherlands. In addition to the prototype strains of the 101 recognized serotypes, strain Hanks, considered to represent an additional serotype (Andries et al., 1990), was available for the current study through the courtesy of F. Hayden, Charlottesville, VA, USA. The clinical specimens were collected between 1994 and 1996 in the Tampere region, Finland, as a part of the Finnish Otitis Media studies as described before (Blomqvist et al., 1999; Vesa et al., 2001; Savolainen et al., 2001). The prototype strains and the field isolates were passaged once on HeLa-Ohio cells at 33 °C. Cultures with full cytopathic effect were freeze-thawed three times and clarified by centrifugation at 235 g for 20 minutes. The supernatant was collected and stored in 1 ml aliquots at −70 °C.

RNA isolation and RT–PCR. The RNA was isolated from 100 μl cell culture homogenate with the RNeasy Total RNA kit (Qiagen). Upon purification, RNA was eluted from the columns with 30 μl diethyl pyrocarbonate-treated H2O and subsequently stored at −70 °C. RT–PCR was performed using primer pair 9565-reverse and 9895-forward (9565 position 1083–1058 according to HRV1b by Hughes et al., 1988), 5′ GCA TCI GGY ARY TTC CAC CAC CAN CC 3′; 9895 position 534–560 in HRV1b, 5′ GGG ACC AAC TAC TTT GGG TGT CCG TGT 3′; I = inosine; Y = T, C; R = G, A; N = A, G, C, T producing a fragment of 549 nt spanning the hypervariable part of the 5′ NCR, the entire VP4 gene and the 5′ terminus of the VP2 gene. cDNA synthesis and PCR were carried out as described previously (Mulders et al., 2000). The PCR products were visualized after electrophoresis on an ethidium bromide-stained 2% agarose gel.

Sequence analysis. PCR products were purified with a PCR purification kit (QIAquick, Qiagen) in the case of a single band in the electrophoresis, or in the case of multiple bands using a gel extraction kit (QIAquick, Qiagen). The purified products were stored at −20 °C or directly used in the cycle sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems) with the same forward and reverse primers as used in the RT–PCR. An automated DNA sequencer was used for sequencing (ABI PRISM, model 377). Sequence data were analysed with Sequencing Analysis (version 3.1). Bootstrap analysis was performed using GONI (version 1.0) for pairwise comparisons. Multiple sequence alignments were made using CLUSTAL W, part of the PHYLIP program suite (version 10, Genetics Computer Group, Madison, WI, USA), and CLUSTALX. The analysed fragment was 420 nt in the VP4/VP2 region (207 nt in VP4 and 213 nt in VP2). Distance matrices were estimated using the Dnalist and Protist programs, part of the PHYLIP (Phylogeny Inference) package (version 3.572c; Felsenstein, 1993), using the maximum likelihood model of nucleotide substitution. Dendrograms were drawn using the neighbour-joining option in NEIGHBOR (PHYLIP), and were visualized using NJPLLOT (TREE VIEW (version 1.5.3)). Bootstrap analysis was performed using SEQBOOT (PHYLIP) with 100 or 1000 replicates. For prototype strains HRV31, HRV32, HRV40, HRV60, HRVHanks2102 and HRV79 the analysed sequence was shorter than the standard 420 nt. However, practically identical trees were seen after replacing the terminal ‘deletion’ with a series of N, or after editing the complete sequences to the length of the shortest one before analysis.

Results

Genetic clustering of HRV prototype strains

All previously non-sequenced 97 HRV prototype strains were sequenced across the VP4/VP2 interval. In the phyl-

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Fig. 2. For legend see facing page.
genetic analysis, the sequences of all but one of the prototype strains clustered in the two previously known phylogenetic clades (Horsnell et al., 1995), with 76 serotypes in the HRV1b-related group (group A) and 25 in the HRV14-related group (group B). Serotype 87 appeared to belong to the enterovirus species D represented by enterovirus 70 (Fig. 1 and Table 1). The identity of HRV87 was confirmed in a neutralization assay by monospecific antiserum obtained from the ATCC (data not shown). Because of the wide range of variation between the strains, the phylogenetic trees were constructed separately for the two groups (Figs 2 and 3). The trees also include the previously studied, recent Finnish field isolates (Savolainen et al., 2001).

The maximum variation in genetic group A was 41% at the nucleotide level and 28% at the amino acid level (distance matrices not shown). The variation maxima for genetic group B, the HRV14 group, were 34% and 20%, respectively. Distinction between different prototype strains was highly variable. For six heterologous pairs of strains in group A (Fig. 2; HRV25/62, HRV29/44, HRV8/95, HRV1a/1b, HRV31/32 and HRV21/Hanks2102), the difference was less than 10%. For only four strains, the closest heterologous prototype strain was more than 25% different. For a vast majority of the prototype strains, there is a representative of at least one heterologous prototype within the range of 20%. In group B, there were no heterologous pairs of prototype strains closer than 13%, but also here the smallest interserotypic difference was rarely more than 25%.

Clustering of field strains with the prototype strains

Using aligned sequences in the VP4/VP2 region, most of the clinical isolates could be relatively easily linked to a single prototype strain. In all but one out of the 24 field strains in group A, a prototype strain was closer than 10% (Fig. 2), while four out of the 37 group B field strains remained outside this threshold difference. These strains isolated in September 1996 formed a distinct cluster of their own and did not definitely associate with any of the prototype strains (Fig. 3). Bootstrap values for demarcation between the recent field isolates on one hand and the closest prototype strain on the other were usually very high, suggesting evolutionary difference. Judging from the observed interserotypic difference between the prototype strains, the 61 field strains could represent as many as 19 different serotypes.

Discussion

We have shown in this paper that all but one of the prototype strains of HRV cluster in the two previously known major genetic clades (Horsnell et al., 1995), which is also the basis of the present taxonomic definition of HRV species. Serotype 87, already previously known to differ from all other HRV serotypes by receptor specificity, clustered close to enterovirus 70. HRV-A, constituting the HRV1b-related genetic group, included about three-quarters of the prototype strains while HRV-B, the HRV14-related group, comprised the remaining quarter of the prototype strains. Interserotypic genetic distances in the capsid protein coding region were similar to those of enteroviruses, and clustering of 61 recent field isolates suggested that a method for genetic identification of HRV strains might be a feasible goal, as has been reported for enteroviruses (Oberste et al., 1999b).

Our current results are in full agreement with previously published clustering of 21 strains in VP2 (Horsnell et al., 1995). The ratio of the prototype strains segregating into the two HRV species was 3:1, while the distribution of group A and B strains among the 61 recent Finnish field isolates was different, with a clear majority in group B. As noted before (Savolainen et al., 2001), the intra-epidemic diversity of HRV strains was remarkable, with several different serotype-like clusters circulating concomitantly in a cohort of about 300 children younger than 2 years of age living in a given suburb.

The clustering of HRV87 with HEV-D was a striking observation but in line with the report of Andeweg et al. (1999) who showed that HRV87 sequences in the 5′NCR cluster separately from those of the remaining HRVs. The identity of HRV87 was confirmed by specific neutralization with a valid monotypic antiserum obtained from the ATCC, and the typical rhinovirus character of acid-lability was also demonstrated by a standard test (Couch, 1992). HRV87 was definitely acid-labile, typical of rhinoviruses, while EV70 was acid-stable, typical of enteroviruses (data not shown). It has been known for a long time that HRV87 differs from the other HRVs in receptor specificity (Uncapher et al., 1991). The receptor is sialidase-sensitive, which has also been reported for EV70 (Utagawa et al., 1982). These observations have prompted further investigations on the relatedness of HRV87 to human enteroviruses (to be reported separately), and may pose taxonomic problems concerning criteria for differentiation between HRV and enteroviruses.

Fig. 2. Neighbour-joining dendrogram of the nucleotide sequences of human rhinovirus group A clinical isolates and prototype strains in the 420 nt region encoding VP4/VP2. Numbers indicate bootstrap values for each branch as calculated using the Neighbour package (100 replicates). A distance matrix was calculated using DNACOR (maximum likelihood) and the dendrogram with JOINTREE. The isolation month and year of the field strains are shown. The sequences of the clinical isolates have accession numbers AY015114–AY015174. The sequences of the following clinical isolates appear in GenBank with a different code: 8643aug96 = 292aug96, 5700may95 = 7may95, 7931may95 = 21may95, 8169aug96 = 237aug96, 8452jul95 = 274jun95, 9257apr95 = 95apr95, 7746aug96 = 7746aug96, 7781nov95 = 206apr95, 7922mar96 = 21mar96. Members of the human rhinovirus minor receptor group are presented in bold. GenBank accession numbers for previously published sequences: HRV1b, NC001435; HRV2, NC00316; HRV6, NC001752; HRV89, A10937.
Fig. 3. For legend see facing page.
The patterns of antiviral drug sensitivity of HRV strains were previously suggested to follow the genetic grouping (Andries et al., 1990), a view partially supported by our current findings. Andries and coworkers divided HRV serotypes, after a very complex multivariate analysis, into antiviral sensitivity clusters A and B. All sequenced genetic group B strains belong to antiviral sensitivity cluster A, which also includes a few definite HRV-A serotypes, HRV8, HRV13, HRV18, HRV32, HRV43, HRV54 and HRV95, close to the intergroup border. According to receptor usage, HRV serotypes have been grouped in the major and minor receptor groups, respectively, and HRV87, which uses yet another molecule for cellular interactions (Uncapher et al., 1991). Both genetic groups were already previously known to include members of the major receptor group. All the ten minor receptor group members were now partially sequenced and found to belong to genetic group A. At the VP4/VP2 region they did not cluster more closely together (Fig. 2). However, since VP1 is known to be the counterpart for receptor binding in the case of HRV2 (Hewat et al., 2000), it would be intriguing to know if alignments made according to VP1 amino acid sequences would change the situation.

With the present data, further genetic subgrouping of HRV serotypes within either of the two established HRV species would appear difficult. Bootstrap values supporting the major branches were very small, especially in genetic group A (Fig. 2). However, differences between distinct serotypes were often, although not always, well supported. Most interserotypic differences in the VP4/VP2 region resemble those between enterovirus serotypes, but for a few pairs of HRV prototypes the difference was of the order of the intergenotypic differences within a given enterovirus serotype, or even less (Mulders et al., 2000). The strains labelled HRV8 and -95 appear almost identical, and the proposed new serotype represented by strain Hanks (Andries et al., 1990) clustered very close to the prototype strain of HRV21. Some similarity with previously reported antigenic cross-reactivity (Cooney et al., 1982) could be found in the clustering of HRV prototype strains. For instance, the reported cross-reactions between HRV2 and -49 and between HRV29, -44 and -62, respectively, are not surprising regarding their relatively close genetic similarity.

Clustering of the clinical isolates with each other on one hand, and with given prototype strains on the other, suggests that genetic typing of HRV strains might also be possible according to the lines previously described for human enteroviruses (Oberste et al., 1999a, b). For enteroviruses, sequence comparisons in the VP1 region give a more reliable serotype identification than those in the VP4/VP2 region, as the demarcation between interserotypic and intraserotypic variations is greater in VP1 (Oberste et al., 1999a; Mulders et al., 2000). The current results are promising, but much larger numbers of serotype-confirmed independent field isolates have to be analysed before it is possible to evaluate development of a genomic sequence-based identification system for HRV isolates.

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References


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Structural view.


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