Phylogenetic analysis of human rhinovirus capsid protein VP1 and 2A protease coding sequences confirms shared genus-like relationships with human enteroviruses

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Phylogenetic analysis of the capsid protein VP1 coding sequences of all 101 human rhinovirus (HRV) prototype strains revealed two major genetic clusters, similar to that of the previously reported VP4/VP2 coding sequences, representing the established two species, *Human rhinovirus A* (HRV-A) and *Human rhinovirus B* (HRV-B). Pairwise nucleotide identities varied from 61 to 98 % within and from 46 to 55 % between the two HRV species. Interserotypic sequence identities in both HRV species were more variable than those within any *Human enterovirus* (HEV) species in the same family. This means that unequivocal serotype identification by VP1 sequence analysis used for HEV strains may not always be possible for HRV isolates.

On the other hand, a comprehensive insight into the relationships between VP1 and partial 2A sequences of HRV and HEV revealed a genus-like situation. Distribution of pairwise nucleotide identity values between these genera varied from 41 to 54 % in the VP1 coding region, similar to those between heterologous members of the two HRV species. Alignment of the deduced amino acid sequences revealed more fully conserved amino acid residues between HRV-B and polioviruses than between the two HRV species. In phylogenetic trees, where all HRVs and representatives from all HEV species were included, the two HRV species did not cluster together but behaved like members of the same genus as the HEVs. In conclusion, from a phylogenetic point of view, there are no good reasons to keep these two human picornavirus genera taxonomically separated.

### INTRODUCTION

Human rhinoviruses (HRV; genus *Rhinovirus*, family *Picornaviridae*) are small non-enveloped icosahedral viruses containing one copy of an approximately 7200 nt long single-stranded messenger-sense RNA genome. The RNA molecule contains a long single reading frame encoding an approximately 2180 aa long polypeptide, flanked by 5' and 3' non-translated regions and a polyadenylate tail in the 3' terminus (Stanway *et al*., 1984). The capsid is 24–30 nm in diameter and is composed of 60 identical copies of each of the four coat proteins, VP1, VP2, VP3 and VP4. In HRV serotype 14 (HRV14) they comprise 290, 262, 236 and 68 aa, respectively (Stanway *et al*., 1984). The exterior of the viral capsid is built up of the three larger proteins (VP1, VP2 and VP3) while the smallest VP4 protein lies inside the capsid (Rossmann *et al*., 1985). VP1 is the most variable of all picornavirus capsid proteins, contains major neutralizing antigenic sites, and most residues of the known receptor attachment sites. The non-structural region of the genome encodes proteins for polyprotein cleavage and RNA synthesis (Rueckert, 1996).

A total of 100 distinct HRV serotypes were systematically characterized and designated with numbers from HRV1 to HRV100, where serotype HRV1 is further divided into two antigenic subtypes 1A and 1B, and the latest suggested new serotype Hanks has no number (Andries *et al*., 1990; Couch, 1996; Rueckert, 1996). Human rhinovirus serotypes were originally defined by neutralization reactions (Rueckert, 1996). In principle, members of a distinct serotype are neutralized by homologous antisera but not by heterologous antisera. However, a number of cross-reactions between certain sets of HRV serotypes have been reported (Cooney *et al*., 1982).

In total, seven complete HRV genomes [HRV1B (Hughes *et al*., 1988); HRV2 (Skern *et al*., 1985); HRV9 (Leckie, 1987); HRV14 (Stanway *et al*., 1984); HRV16 (Lee *et al*., 1995); HRV85 (Stanway, 1989) and HRV89 (Duechler *et al*., 1987)] as well as all human enterovirus (HEV) genomes have been sequenced (Ryan *et al*., 1990; Brown *et al*., 2003;
Oberste et al., 2004a, b, c). Human rhinoviruses are closely related to HEV, share the same virion morphology, an identical expression strategy, a similar genome organization and have more than 50% nucleotide sequence identity taking the entire genome into account (Rueckert, 1996; King et al., 2000). The main reported differences between these genera are the relative acid lability and thermostability of HRVs, and the primary infection sites (Rueckert, 1996). While different HEVs may initiate their infection at both gastrointestinal and respiratory mucosa, HRVs are considered to be restricted to the respiratory mucosa. Based on >70% amino acid identity in the structural protein coding region (VP1–VP4), human rhinoviruses are divided into two species Human rhinovirus A (HRV-A) and Human rhinovirus B (HRV-B), and the officially reported 64 human enterovirus serotypes into five species Human enterovirus A to D and Poliovirus (HEV-A to D and PV) (King et al., 2000). Genetically, the HRV14 serotype belonging to the HRV-B species was reported to be more closely related to human enteroviruses and especially to polioviruses than to the HRV-A species (Stanway et al., 1984).

Our previous studies of HRV prototype strains and a set of field isolates in the genomic regions of VP4/VP2 (Savolainen et al., 2002a) showed that all but one of the 102 prototype strains clustered into the established species of HRV-A and HRV-B. Serotype 87 (HRV87) was observed as an outlier compared with other HRV serotypes and a closer analysis revealed that entero virus 68 (HEV68), belonging to the HEV D species, was the closest relative both genetically and antigenically (Blomqvist et al., 2002; Savolainen et al., 2002a, b).

We have sequenced the complete VP1 capsid protein region and partial regions of the flanking proteins VP3 and 2A of 96 human rhinovirus prototype strains enabling analysis of the relationships of all established HRV serotypes. We have recently reported capsid protein VP1 sequences of the 10 serotypes that used the very low density lipoprotein receptor (VLDL-R) for cell binding and entry (Vlasak et al., 2003). While this manuscript was in preparation, Ledford and co-workers published a report based on an independent complete dataset on HRV VP1 sequences with a special view on serotype-specific sensitivities to the VP1 binding antiviral drug pleconaril (Ledford et al., 2004). In this report we analyse general features of the nucleotide and deduced amino acid sequences of VP1, phylogenetic relationships between all human rhinovirus prototype strains in the VP1 region and in the sequenced part of the 2A region, and especially, the relationships between human rhinoviruses and human enteroviruses.

METHODS

Viruses. Ninety-six HRV prototype strains were obtained from the National Institute for Public Health and the Environment, The Netherlands; Janssen Pharmaceuticals, Beerse, Belgium; American Type Culture Collection, Rockville, USA, and the Haartman Institute, University of Helsinki, Finland. The virus strains were passaged once in HeLa-Ohio cells at 33 °C, and cultures with cytopathic effect were freeze-thawed three times and clarified by centrifugation at 230 g for 10 min.

RT-PCR and sequencing. Viral RNA was extracted from infected HeLa-Ohio cells by using RNeasy Total RNA kit (Qiagen). The VP1 gene flanked by the partial VP3 and 2A genes were amplified with overlapping RT-PCR reactions with primers for VP3, VP1 and 2A (see Supplementary Table 1 in JGV Online). RT-PCR was carried out as described previously (Oberste et al., 1999a, b, 2000). PCR products were purified for sequencing either directly with QIAquick PCR Purification kit (Qiagen) when a single band was observed or, in the case of multiple bands, after extraction from the 1% agarose gel with a QIAquick Gel Extraction kit (Qiagen). Cycle sequencing was achieved with same or different primers with their locations ranging from VP3 to 2A region and the Big Dye Terminator Cycle Sequencing kit version 1.1 (Applied Biosystems). An automated DNA sequencer ABI 310 (Applied Biosystems) was used for sequencing.

Sequence/phylogenetic analysis. All obtained nucleotide and deduced amino acid sequences were aligned by using the multiple sequence alignment program CLUSTALX, version 1.83 (Thompson et al., 1997). Alignments were generated with the default parameters and adjusted manually due to differences in the lengths of the VP1 gene and a high divergence between the HRV serotypes. CLUSTALX was also used for calculating nucleotide and amino acid identity matrices. Consensus sequences were inferred with GENEDOC program, version 2.6.002 (Nicholas & Nicholas, 1997) from the multiple amino acid sequence alignments produced with CLUSTALX. Phylogenetic relationships were inferred from the aligned nucleotide sequences with the neighbour-joining algorithm of Saitou and Nei (Saitou & Nei, 1987) implemented in the CLUSTALX program. Phylogenetic trees were constructed under Kimura two-parameter nucleotide substitution model excluding the positions with gaps. A bootstrapping option was selected with 1000 number of bootstrap trials. Inferred phylogenetic trees were drawn by using NIPILOT program (Perriere & Gouy, 1996) and radial trees with TREEVIEW, version 1.6.6 (Page, 1996).

Nucleotide sequence accession numbers. The accession numbers of human rhinovirus sequences (HRV1b, HRV2, HRV14, HRV16 and HRV89), and those of non-HRV picornaviruses obtained from GenBank and used in sequence analysis are listed in the Supplementary Table 2 in JGV Online. There was an error in our published VP4/VP2 sequence of HRV31 (Savolainen et al., 2002b). A correct version was deposited in the GenBank in June 2003 with the accession no. AF343583.

RESULTS

General aspects of HRV VP1 sequences

Amplified PCR products of approximately 1350 bp comprising the VP1 gene and flanking parts of the VP3 and 2A genes were obtained from 96 HRV prototype strains. All HRV prototypes were successfully amplified with the primer pair 92378–92379 (see Supplementary Table 1 in JGV Online) with the resulting amplicon covering the VP3/VP1 junction. The VP1-2A region was more complicated and amplification of all strains required the use of multiple forward and two reverse primers. The sequences flanking the VP1 gene covered more than 180 nt (60 aa) of the 3’ end of the VP3 gene and approximately 240 nt (80 aa) of the 5’ part of the 2A gene. The corresponding sequences of HRV1B, HRV2, HRV14, HRV16 and HRV89 were
obtained from GenBank and included in this analysis. In total, 101 HRV prototype VP1 sequences were analysed. When compared with the HRV VP1 sequences recently published by Ledford and co-workers (Ledford et al., 2004) our dataset in most cases showed an identity ranging from 100% down to a single amino acid difference. In some cases, however, the difference reached 1% level with the most striking one being a relative amino acid insertion in the DE loop of HRV6 in our sequences as compared to those published by Ledford and co-workers.

The borders of the VP1 protein sequences were predicted from alignments of the deduced amino acid sequences with the previously reported VP1 cleavage sites of HRV1B, HRV14 and HRV16 (Stanway et al., 1984; Hughes et al., 1988; Gradi et al., 2003). Within both HRV species, the VP3/VP1 cleavage site was conserved with amino acid residues Q/N-P-[IV]-E in HRV-A (see Supplementary Fig. S1a in JGV Online) and A-L-[EMPT]-E/G-[FL] in HRV-B (see Supplementary Fig. S1b in JGV Online), respectively. The C terminus of the VP1 protein was more variable in HRV-A without any conserved amino acids close to the cleavage site, even though the last residue was always hydrophobic. In contrast, the predicted N terminus of 2A was more conserved and started with G-P-[ST], with only HRV43 and HRV75 having threonine instead of serine at position +3. Within the HRV-B species the VP1/2A cleavage site appeared to be conserved presenting as Y/G.

**Genetic clusters of HRV serotypes**

Multiple sequence alignment was used to evaluate genetic relationships between HRV prototype strains. Both nucleotide and amino acid identities in the VP1 sequences clustered in two main groups according to the established two HRV species as previously reported for other genomic regions (Horsnell et al., 1995; Savolainen et al., 2002b, 2004) and recently also for VP1 by others (Ledford et al., 2004). Within the HRV-A, the between-serotype pairwise nucleotide identities ranged from 61 to 98%, and the corresponding amino acid identities from 58 to 98%, with HRV8 and HRV95 being the most, and HRV45 and HRV94 the least identical two strains. The corresponding values in the species HRV-B were from 67 to 87% and from 67 to 93%, respectively. Between the human rhinovirus species the nucleotide and amino acid identities varied between 46 and 55%, and 37 and 45%, respectively.

Phylogenetic analysis of VP1 sequences of all HRV prototype strains revealed a clear distinction between the two HRV species (Fig. 1). As regards within-species clustering of the HRV sequences, two lesser groups of HRV-A strains segregated from the bulk of HRV-A with high bootstrapping values: HRV8, HRV95 and HRV45 formed one group (designated here as HRV-A’) and 16 other strains the second group (Fig. 1, HRV-A”). All HRV-A’ strains had a relatively short VP1 sequence (282–283 aa) whereas the HRV-A” strains presented with relatively long sequences (287–296 aa). HRV-B strains also segregated into two main groups and both these groups further into two subgroups with high bootstrapping values (Fig. 1).

**Genetic relationships between HRV and HEV**

Phylogenetic analysis was carried out including VP1 coding sequences of all 101 HRVs, and 45 HEV strains selected to represent all four species supplemented with poliovirus 1 and 3, porcine enterovirus 9 and simian picornavirus 1. Both HRV-A and HRV-B clustered clearly among the HEV species without showing any closer relationship to each other than to the HEV-species (Fig. 1). This was especially evident in a star-formed tree (Fig. 1b).

As another approach to study the relationships, we calculated frequency distributions of pairwise nucleotide and amino acid identity values within and between different HRV and HEV species and representatives of three other human picornavirus genera (Van Regenmortel et al., 1997; Oberste et al., 1999b). This analysis included all designated HRV, HEV and poliovirus serotypes. The pairwise within-species identity values of HRV-A or HRV-B were very similar although relatively more variation was seen within HRV-A as also reported by Ledford et al. (2004) (Fig. 2a and b). While most of the within-species interserotypic identity values were less than 75%, a definite slope was seen in the distribution curve towards greater identities. In contrast, for all HEV species the values were less than 75% with a few exceptions [echovirus 1 and 8, coxsackievirus A11 and A15, coxsackievirus A13 and A18 (Fig. 2c), as reported before (Oberste et al., 1999b)]. On the other hand, a fair overlap was seen in the distributions of pairwise identities between heterologous members of the two HRV species (range at nucleotide level from 46 to 55% and at amino acid level from 37 to 45%) and pairs composed of a human rhinovirus and a human enterovirus serotype (range from 41 to 54%, and 32 to 48%, respectively). The pairwise identity values between a human rhinovirus and representatives of three other human picornavirus genera ranged from 31 to 42% at the nucleotide and from 12 to 26% at the amino acid identity (Fig. 2a and b).

In yet another approach in the analysis of HRV–HEV relationships deduced amino acid sequences were aligned with CLUSTALX followed by manual editing within HRV-A and HRV-B species, six poliovirus strains and 45 HEV serotypes, including representatives of all four species. Obtained alignments were analysed to reveal ‘fully conserved’ and ‘consensus’ residues, respectively, for the two HRV species separately, for the selected HEV strains and for the analysed polioviruses (Fig. 3). The term ‘fully conserved’ means here that in the alignment, all amino acid residues at a given position were identical. Likewise, a ‘consensus’ amino acid residue means that at least 65% of the analysed strains within the indicated group showed this residue at this position. The alignments revealed that within the HRV-A and HRV-B species there were 96 and 144 fully conserved amino acids in the VP1 sequence,
respectively (capital letters in Fig. 3). Altogether 64 aa residues were fully conserved between all rhinovirus prototype strains. Interestingly, HRV-B shared as many as 81 aa with the studied poliovirus strains, with 12 out of these also being fully conserved in the HRV-A species. HRVs and the studied HEV strains shared in total 37 aa (the HEV–HRV consensus sequence in Fig. 3), while multiple alignment of HRVs, HEVs and human parechovirus 2 revealed only seven fully conserved amino acids (data not shown).

Clustering of HRV prototype strains in the non-structural 2A protein gene

The non-structural 2A protein region, 435 nt in length, encodes a proteinase cleaving the precursor of the structural proteins (VP1–4) away from the nascent polypeptide (Rueckert, 1996). A phylogenetic analysis was carried out including all HRVs, one representative from each of the HEV species and porcine enterovirus B, covering approximately 240 nt from the 5' end of the 2A region. Virus strains for which the obtained reliable sequence was shorter were not included (HRV53, HRV12, HRV67, HRV86 and HRV99). The minimum and maximum nucleotide identities within the species HRV-A were 53 % between serotypes HRV45 and HRV80, and 99 % between serotypes HRV8 and HRV95, respectively. The corresponding amino acid identities were 52 and 99 %. There was 100 % amino acid identity between serotypes HRV98 and HRV54. In the HRV-B species the identities varied between 62 and 87 % at nucleotide level and 62 and 96 % at amino acid level. Between the species HRV-A and HRV-B the nucleotide identities varied from 37 to 52 % and the amino acid identities from 23 to 35 %, respectively.

In phylogenetic analysis the partial 2A sequences of the HRV-B strains clustered as a group among the different enterovirus species while the HRV-A clade formed a unique branch of its own (Fig. 4). Within HRV-A, the distinct subgroup HRV-A’ was at least as evident as in the VP1 region, while subgroup HRV-A” divided into two parts, still separated from the major HRV-A group. In addition, HRV1A and HRV1B appeared to have segregated out from the bulk of the HRV-A strains, and to join one part of the HRV-A” strains. In HRV-B only three subclusters were seen here as compared with four in the VP1 gene. Interestingly, one of the subgroups, including serotypes HRV69, HRV52, HRV17, HRV70, HRV91 and HRV48, showed amino acid patterns at the VP1/2A junction that were different from those of the rest of HRV-B strains (see Supplementary Fig. S1 in JGV Online). Likewise, the sequences of these strains at the VP3/VP1 cleavage site varied from those of the other HRV-B serotypes.

DISCUSSION

In this study, complete VP1 gene sequences of human rhinoviruses clustered into the two established species, HRV-A and HRV-B, as reported before (Ledford et al., 2004). Within both species the range of interserotypic VP1 sequence identities was relatively wide and different from that of human enteroviruses, which is more uniform. In phylogenetic analysis HRV-B was in both genomic regions close to polioviruses and the HEV species, and did not form a separate cluster with HRV-A. These observations have bearings on the evolution and taxonomy of HRVs as well as on molecular epidemiology of HRV infections.

The VP3/VP1 and the VP1/2A cleavage sites were deduced from alignment with previously sequenced HRV strains and were found to be on average conservative within each
Fig. 2. Histograms reflecting pairwise identity values of VP1 nucleotide (a) and amino acid (b) sequences between 101 human rhinovirus strains, 70 human enterovirus strains, and single representatives of three other picornavirus genera. The horizontal axis shows the identity percentage in pairwise comparisons adjusted to the nearest integer and the vertical axis the frequency of observations (column height). Codes for different pair categories: white, HRV versus human hepatitis A virus, human parechovirus 2 or simian picornavirus 1; black with white dots (middle peak), HRV-A versus HRV-B; light grey (middle peak), HRV versus HEV or poliovirus; black (right-hand peak), within HRV-A comparisons; white with horizontal stripes (right-hand peak), within HRV-B comparisons. (c) Similar analysis of within-species nucleotide identities of HEV-A (white), HEV-B (grey) and HEV-C+polioviruses (black).

Fig. 3. Consensus sequence alignment of HRV-A, HRV-B, and selected representatives of poliovirus and HEV-A to D species. Capital letter in sequence stands for a fully conserved amino acid and lower-case letter for a ‘consensus’ amino acid present in more than 65% of the analysed strains within the indicated group. Hyphen stands for greater variability of amino acids including relative deletions. Different shading grades indicate degree of amino acid similarity between the groups (black 100%, dark grey at least 80%, and light grey 60–80% of studied strains). The last line stands for a designated consensus sequence for the entire group of studied strains.
Fig. 4. A phylogenetic tree based on partial 2A protease coding sequences of all but five HRV prototype strains (HRV12, HRV53, HRV67, HRV86 and HRV99 not included), selected representatives from all human enterovirus species and porcine enterovirus B (PEV9). (HEV-A: CAV16, NC_001612; HEV-B: CBV1, NC_001472; HEV-C: CAV21, NC_001428; HEV-D: EV70, NC_001430; PV: PV1, NC_002058). For HRV-A subgroups, see the text.
HRV species. Because of the within-species variability of the 2A sequences one might have expected corresponding clustering of the amino acid sequences at the VP1/2A cleavage site. However, no general trend could be seen to support this view, except that within the HRV-B species one of the phylogenetic subgroups could be distinguished from the other HRV-B serotypes at the VP1/2A cleavage site.

Phylogenetic analysis of the VP1 coding sequences of all the human rhinovirus prototypes revealed clustering of the strains in the two species Human rhinovirus A (76 strains) and Human rhinovirus B (25 strains), respectively, as reported previously on the basis of VP4/VP2 sequences (Savolainen et al., 2002b) and recently based on an independent dataset of VP1 sequences (Ledford et al., 2004). From the pairwise identity data of the VP1 coding nucleotide sequences, serotypes in the HRV-B species appeared more cohesive than those in the HRV-A species as previously suggested by Ledford and co-workers by analysing the deduced amino acid sequences (Ledford et al., 2004). Both HRV species appeared to segregate into distinct subgroups, as also noted for partial 3D sequences (Savolainen et al., 2004) but not seen for the VP4/VP2 sequences (Savolainen et al., 2002b). The designated subgroup HRV-A’ was also distinct in the partial 2A sequences while the other designated VP1 subgroup, HRV-A”, was joined with HRV1A and HRV1B. The translocation of these two strains from the bulk of HRV-A strains might be a result of recombination in the history of these strains.

The designated interserotypic borders for human enteroviruses correspond to identities of 75% at the nucleotide level and 88% at the amino acid level (Oberste et al., 1999a). Several within-species pairs of HRV serotypes showed much less mutual difference, as also indicated by Ledford and co-workers (Ledford et al., 2004). In some cases, there are also previous data on antigenic cross-reactivity between the indicated pairs. Accordingly, Ledford and co-workers suggested that serotype pairs HRV8–HRV95 and HRV21–HRVHanks, respectively, should each be classified in a single serotype. On the other hand, four pairs of HRV serotypes (HRV3–HRV14, HRV12–HRV78, HRV13–HRV41 and HRV36–HRV58) with previously reported reciprocal cross-reactions showed greater than 12% divergence at the amino acid level. Clearly, more studies are required to clarify the relationships between designated serotypes and pairwise VP1 sequence identities of HRV prototype strains. The observed differences between HRV and HEV in this respect might reflect different evolutionary history of the two virus genera and be somehow related to the different natural habitats and/or different natural courses of the infections.

VP1 coding sequences are known to represent the most suitable genomic region for identification of the serotype of enterovirus isolates (Oberste et al., 1999b), and in the classification of newly sequenced non-typeable picornaviruses (Oberste et al., 2001). A similar use of the human rhinovirus VP1 sequences for confirmed serotype identification seems so far impractical because of the apparently too close relationship of several designated serotypes. However, the capsid region sequences can be exploited in molecular epidemiology. Whereas several primer pairs were needed for amplification of the complete VP1 gene, the sequences covering more than 183 nt of the 3’ end of the VP3 gene and approximately 432 nt of the 5’ end of the VP1 gene were obtained with a single primer pair (92378–92379). The latter primers might also be suitable for amplification and sequencing of field isolates of human rhinovirus for molecular epidemiology. Sequence data from representative collections of recent HRV isolates are needed to complement the picture on the range of genetic variation of HRV strains.

The relationships between human rhinoviruses and enteroviruses were explored with pairwise identity analyses, consensus amino acid sequence comparisons, and phylogenetic tree constructions. Distribution of interserotypic pairwise nucleotide identity values of HRV-A–HRV-B pairs were not different from those of HRV–HEV pairs resembling a situation within a single genus. Strikingly, amino acid sequence comparisons revealed that the HRV-B species and polioviruses shared more conserved amino acid residues than HRV-A and HRV-B. This was also previously noted for HRV14 (Stanway et al., 1984). Likewise, phylogenetic analysis of both complete VP1 and partial 2A sequences placed HRV-A and HRV-B separately among the different HEV and poliovirus species. Similar results have been obtained previously using VP4/VP2 (Savolainen et al., 2002b) and 3D (Savolainen et al., 2004) regions. This close relationship between HEV and HRV has also been noted before on the basis of the few complete HRV sequences.

In conclusion, we have shown in this paper that complete VP1 and partial 2A sequences of HRV prototype strains cluster in the two established species like those of the other genomic regions of HRV analysed. The interserotypic identities in the VP1 show a similar range of variation as seen in the VP4/VP2 region, and are not likely to provide a means for unequivocal serotype identification, in contrast to the human enteroviruses, where the interserotypic differences are more distinct. Yet, VP1 sequences can be used for identification of field strains, for instance, using a ‘nearest prototype strain’ – definition, and in the molecular epidemiology of HRV infections. Analysis of interserotypic pairwise sequence identities proved that HRV-A and HRV-B strains are not closer to each other than they are to human enteroviruses. From a phylogenetic point of view, human rhinoviruses and human enteroviruses could be considered members of a single genus in the family Picornaviridae.

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