Polymerase Chain Reaction and Sequencing for Typing Rhinovirus RNA

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Primers were designed and tested for their ability to distinguish rhinoviruses from enteroviruses. A primer set derived from the 5'-UTR/VP coding region junction was able to amplify all the rhinovirus serotypes tested. Enteroviruses were either not amplified by these primer pairs or produced a band of larger size that could easily be discriminated from the rhinovirus-specific product. In contrast, primers embedded in the 5'-UTR region alone were able to amplify both rhinovirus and enterovirus RNA. It is shown that rhinoviruses could be specifically typed by sequencing the amplicon derived from this 5'-UTR set. The sequences of the 5'-UTR region of ten previously unsequenced rhinoviruses were derived. The sequences obtained cluster into two groups: 1B, 41, 15, 30, 63, 31, 56, and 44; and 17, 69, and 70. Amplicons from serotypes 17, 69, and 70 also group by sequence with the equivalent region of HRV14 from the genetic database, while the others group with 2 and 89. © 1994 Wiley-Liss, Inc.

KEY WORDS: enteroviruses, picornaviruses, 5'-UTR-VP coding region

INTRODUCTION

There are more than 100 rhinovirus serotypes within the family Picornaviridae [Gwaltney et al., 1989]. Rhinoviruses infect the upper respiratory tract, cause typical common cold illness, and are implicated in other respiratory conditions [Balfour-Lynn et al., 1992]. Rhinoviruses are traditionally distinguished from other members of the Picornaviridae by their acid lability, and can be typed by neutralisation with specific antisera after growth in tissue culture. Some are untypeable and probably represent new serotypes [Couch, 1992].

Improved means of characterising the rhinoviruses should help specific diagnosis and contribute to research into the common cold. Such methods should also clarify the number of viruses in the genus. Polymerase chain reaction (PCR) genetic amplification provides an obvious way of investigating respiratory virus infections and has already been described for adenoviruses, coronaviruses, influenza viruses, parainfluenza virus,

and respiratory synctial virus [Becker and Darai, 1992; Persing et al., 1993]. Several groups have described assays for rhinovirus RNA using primers from conserved sequences within the 5'-untranslated region (UTR) of the genome [Arruda and Hayden, 1993; Bruce et al., 1990; Gama et al., 1988, 1989; Hyypiä et al., 1989; Olive et al., 1990].

The 5'-UTR of the picornaviruses contains the internal ribosomal entry site (IRES) and the conserved sequences are probably essential for this function [Jang et al., 1990]. The length of the UTR differs between the enteroviruses and the rhinoviruses [Agol, 1991] and thus provides a means of differentiating the 5' PCR amplicons of these viruses [Olive et al., 1990]. While a set of PCR primers that amplifies the cDNA of all enteroviruses and rhinoviruses is useful, it would also be useful to be able to detect the two groups of viruses separately. We have attempted to do this by designing and testing sets of rhinovirus-specific primers from other (coding) regions of the genome. In addition we have investigated sequencing of the PCR products as a means of typing the virus from which the amplified RNA originated. This can only be successful for those viruses for which sequence information is available. At the time of writing, the sequences of the 5' end of 11 enteroviruses (PV1, 2, 3; CAV9, 21, 24; CBV1, 3, 4, 5; EV70) and 4 rhinoviruses (HRV1B, 2, 14, 89) are available in the genetic databases (GenBank and EMBL). We have sequenced the 5' amplicons of 10 previously unsequenced rhinoviruses (HRV15, 17, 30, 31, 41, 44, 56, 63, 69, 70) and 1, HRV1B, that was previously sequenced to demonstrate the usefulness of this technique as a means of virus identification. This work extends knowledge of the genomic sequence diversity of this group of viruses.
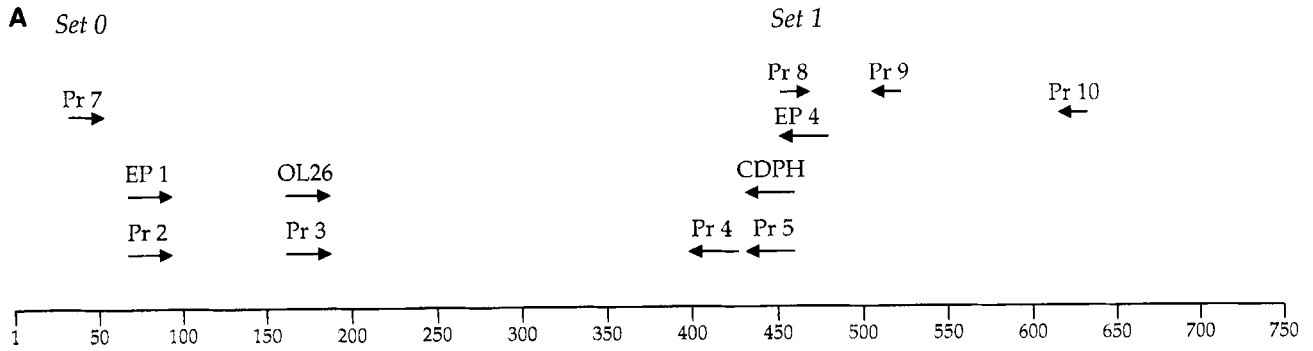
MATERIALS AND METHODS
Viruses

Rhinoviruses were grown in fibroblast cells (WI-38, MRC5, or HEL). When complete cytopathic effect was

A *Set 0*   *Set 1*
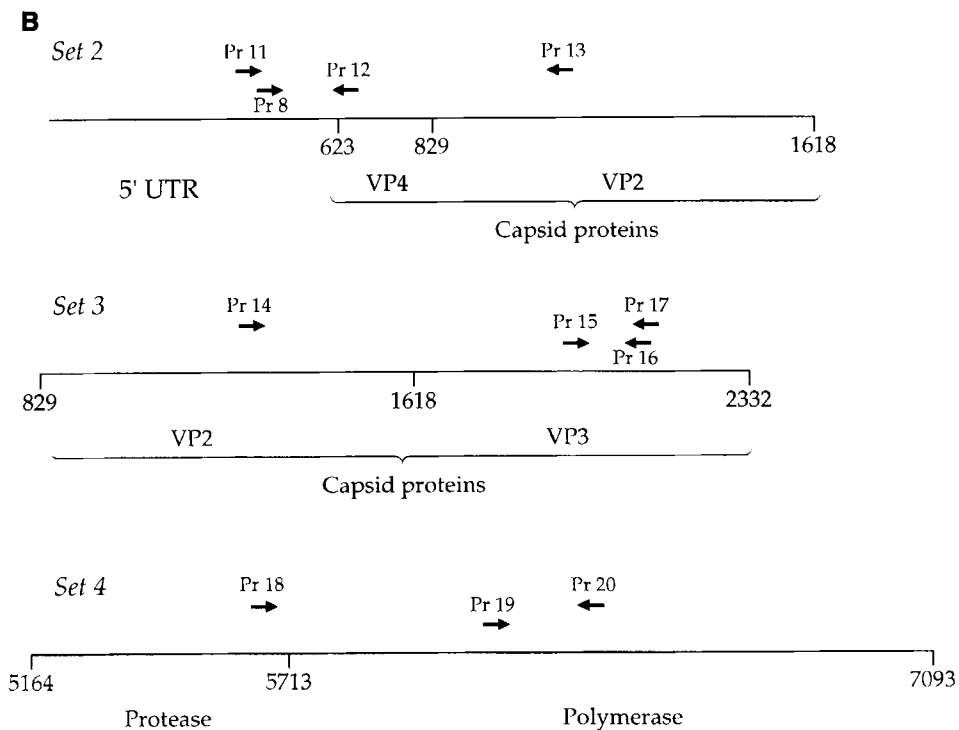

Fig. 1. Illustration of the approximate locations of the primers used in this study. **A:** PCR primers in the 5' UTR; **B:** primer sets selected for rhinovirus-specific potential.

evident the total virus was harvested by freezing and thawing the cell cultures. Enteroviruses were grown in a Vero continuous cell line. Cell debris was removed by

centrifugation and the clarified supernatant centrifuged at high speed to give a virus pellet, which was stored at −70°C.

TABLE I. Primers Used for Rhinovirus RNA Amplification

Name	Sequence[a]	Location[b]	Polarity[c]
5'-UTR (set 0)			
Pr2[d]	CGGTACCTTTGTACGCCTGTT	63-79	S
Pr3[e]	CAAGCACTTCTGTTTCCC	167-184	S
Pr4	AGGCTCTTCACACCATGT	425-409	AS
Pr5	CCGCATTCAGGGGCCGGAGGACT	464-442	AS
EP4[f]	TTAGGATTAGCCGCATCCAG	474-455	AS
5'-UTR/VP4 (set 1)			
Pr7	GGTGTTGTACTCTGTTA	43-59	S
Pr8	GAATGCGGCTAACCTTAACCCT	457-478	S
Pr9	TCCCGCAATTGCTCATTACGAC	532-511	AS
Pr10	ATACCTGTGCACCCATG	638-622	AS
5'-UTR/VP4/VP2 (set 2)			
Pr11	TGTGAAGAGCCGCGTGTGCT	415-434	S
Pr8	GAATGCGGCTAACCTTAACCCT	457-478	S
Pr12	TAGATACCTGTGCACCCATG	641-622	AS
Pr13	AGTGCATCTGGTAATTTCCA	1086-1067	AS
VP2/VP3 (set 3)			
Pr14	CTCACACACCCAGGTGA	1265-1281	S
Pr15	TATACCCACTGGACTGG	1934-1950	S
Pr16	CCCACATCCCACACAACATG	2091-2070	AS
Pr17	GATTGCAAACCCACATCCCA	2100-2081	AS
Polymerase (set 4)			
Pr18	AGAGATGGATTTTCAGC	5663-5672	S
Pr19	AATATGGTGTTGACTTGCC	6153-6172	S
Pr20	CCTACTGCTGAACCAGT	6329-6345	AS

[a]All 5' → 3'.
[b]Human rhinovirus 1B, GenBank accession number D00239 [Hughes et al., 1988].
[c]S = sense; AS = antisense.
[d]Similar to EP1 [Gow et al., 1991].
[e]Similar to OL26 [Gama et al., 1989].
[f]EP4 [Gow et al., 1991].

RNA Extraction, Reverse Transcription, and Nested Amplification

Viral RNA was extracted from pelleted virus using a silica-guanidium thiocyanate method [Boom et al., 1990; Gibson et al., 1993]. The synthesis of cDNA from the RNA recovered from the silica-guanidium thiocyanate and subsequent PCR was as described previously [Gibson et al., 1993] in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 1 mM each dNTP, 3.3 μM random hexamer, 16 U RNasin, and 200 U M-MLV reverse transcriptase in RNase-free H_2O (USB) for 10 min at room temperature, then 45 min at 37°C. After denaturation by boiling and quick cooling, 20 μl of cDNA was added to 80 μl first round PCR mix (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 5 pmol of each outer primer, and 2.5 U of Taq polymerase) and amplified for 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 3 min. Secondary amplification was performed on 2 μl of the primary mix in 50 μl final volume with 2.5 mM $MgCl_2$ and 25 pmol of each inner primer for 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Fifteen microliters of the final product was electrophoresed on a 3% 3:1 NuSieve agarose gel (FMC) and stained by ethidium bromide.

Primers

Primers previously described in the literature [Gama et al., 1989; Gow et al., 1991; Hyypiä et al., 1989] and designed inhouse were used (Fig. 1, Table I). Programs used for primer design were MultiAlin (Cherwell Scientific; [Corpet, 1988]), the B&L Utilities (Busch and Lucas, Wissenschaftliche Software), GeneJockey, and Lasergene. Five sets of primers were designed (Table I): a set in the 5'-UTR for amplification of both the enteroviruses and the rhinoviruses (set 0); a set in the 5'-UTR/VP4 gene for the amplification of the rhinoviruses (set 1); and three sets in the coding genes for the amplification of the rhinoviruses (sets 2, 3, and 4).

Primers were tested against cloned DNA sequences, pBR322/HRV1B and pBS/HRV14 [Hughes et al., 1988; Stanway et al., 1984], which were the kind gifts of Dr. G. Stanway, University of Essex.

Sequencing PCR Products

PCR products were sequenced with one of the two primers used for amplification at a ratio of 3.2 pmol of primer to 0.8 pmol of amplicon using the Taq DyeDeoxy Terminator Cycle Sequencing Kit and 373A DNA Sequencer from Applied Biosystems. Prior to sequencing PCR mixtures were cleaned by chloroform extraction to remove the mineral oil overlay and by spin dialysis to remove excess primers and dNTPs using Amicon Centricon-100 microconcentrators [Sambrook et al., 1989]. Sequences were analysed and compared using the programs GeneJockey (Biosoft) and Lasergene (DNAStar). EMBL accession numbers for the sequences are Z29654–Z29664.

Fig. 2. Amplification of rhinovirus RNA with the different primer sets. Lanes 1–14 (top and bottom) and lanes 15–28 (top and bottom) are, respectively, HRV44; HRV70; HRV31; HRV41; HRV17; HRV30; HRV1B; EV70; HRV63; HRV62; HRV56; HRV15; HRV69; HRV72. M indicates molecular weight markers. Lanes 1–14 (top left): Amplification with primer set 1. Lanes 15–28 (top right): Amplification with primer set 2. Lanes 1–14 (bottom left): Amplification with primer set 3. Lanes 15–28 (bottom right): Amplification with primer set 4.

TABLE II. Amplification of Picornaviruses With Primer Sets*

	Enterovirus 5' set	Rhinovirus set 1	Rhinovirus set 2	Rhinovirus set 3	Rhinovirus set 4
Echo 1–9 11–34	+[a]	NT	−[b]	NT	NT
Polio 1–3 Sabin 1–3	+	NT	−	NT	NT
Coxsackie B1–6 A6, A9	+	NT	−	NT	NT
Entero 70	+	−	+[c]	+[c]	+/−
Entero 71	+	NT	−	NT	NT
Rhino 1B	+	+	+	+	+
15	+	+	+	+	+
17	+	+	+	+/−	+/−
30	+	+	+	−	+
31	+	+	+	−	+
41	+	+	+	−	−
44	+	+	+	+	+
56	+	+	+	+/−	−
62	+	+	+	−	+
63	+	+	+	+	−
69	+	+	+	−	+[d]
70	+	+	+	−	−
72	+	+	+	−	−

*NT = not tested.
[a]Except 13, 22.
[b]Except 7, 12, 16 (weak positive or nonspecific).
[c]Larger product.
[d]Smaller product.

RESULTS
Primer Testing

Four sets of primers were designed from the four available rhinovirus sequences in the GenBank/EMBL databases (HRV1B, 2, 14, and 89) to differentiate between enterovirus and rhinovirus infections. Multiple alignment programmes were used so that the primers would potentially amplify only rhinovirus cDNA. Set 1
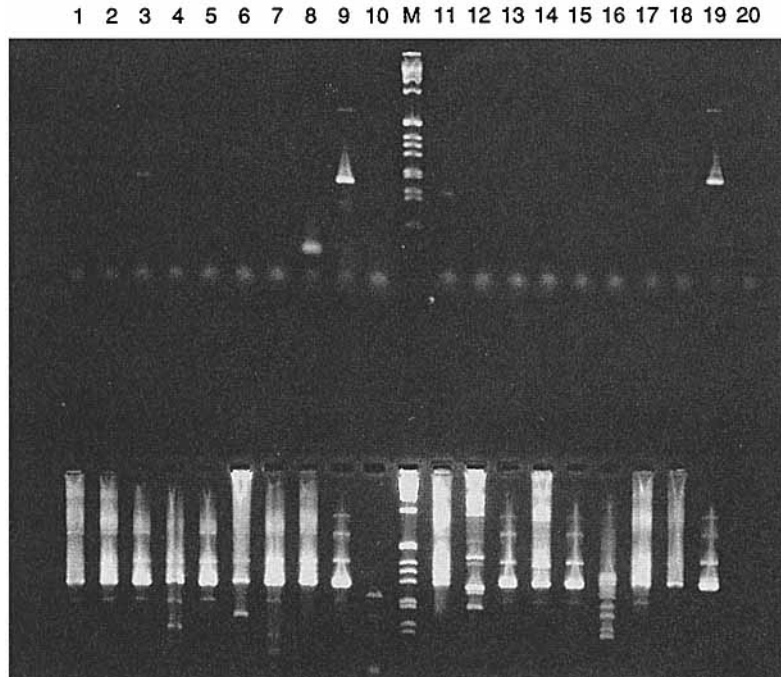
Fig. 3. Amplification of enteroviruses with either rhinovirus-specific primers or generic 5'-UTR primers. **Lanes 1–20 (top and bottom):** Respectively, EV14; EV15; EV16; EV17; EV18; EV31; EV20; EV21; HRV1B; negative control; EV32; EV23; EV24; EV25; EV26; EV27; EV33; EV29; HRV1B; negative control. M indicates molecular weight markers. **Top:** Amplification with set 1 rhinovirus-specific primers. **Bottom:** Amplification with generic 5'-UTR primers.

primers were in the 5'-UTR; set 2 in the 5'-UTR/VP4/VP2; set 3 in VP2/VP3; and set 4 in the polymerase gene (Fig. 1). These primers were tested against the RNA extracted from HRV1B and 12 unsequenced rhinoviruses (Table II). The activity of these four primer sets was compared with primers embedded in the conserved sequences at the 5' end of the picornavirus genome (see Fig. 1, Table I; Litton et al., personal communication). Only sets 1 and 2, and the "generic" 5'-UTR primers were able to amplify all the rhinovirus RNAs tested. Examples of the results obtained are shown in Figures 2 and 3.

To confirm the specificity of rhinovirus primer set 2, PCR was carried out on 51 different enteroviruses from tissue culture stocks (echoviruses 1–9, 11–34; EV70 and 71; CB1–6; CA6, CA9; wild type and Sabin vaccine PV1, 2, and 3; see Table 1). All the enteroviruses with the exception of echo 13 and echo 22 gave bands of the expected size using the 5'-UTR primers (set 0). None of the enteroviruses (apart from EV70) was amplified using the rhinovirus-specific primers (set 2). A band of larger size (approximately 300 base pairs, bp) on ethidium bromide-stained agarose gels was observed. This was clearly distinguishable from the enterovirus amplicon (185 bp).

The sensitivity of rhinovirus set 2 primers was investigated using two cloned genome targets (pBR322/HRV1B and pBS/HRV14). Approximately 100 genome copies of pBR322/HRV1B and 600 copies of pBS/HRV14

were detectable with the primary PCR outer primers, this yield was increased at least 10-fold in the nested reaction with the inner primers, matching the sensitivity of the 5'-UTR (set 0) primers (Litton et al., personal communication).

Sequencing the HRV 5'-UTR PCR Amplicon

The sequence (approximately 270 bp) of part of the extreme 5' region of 11 HRVs was determined to investigate the sequence variation in these viruses and the feasibility of typing them by sequencing PCR amplicons. Both strands of the PCR amplicons were sequenced (Table III). The sequences clustered into two groups: serotypes 1B, 41, 15, 30, 63, 31, 56, and 44 showed approximately 90% similarity; and serotypes 17, 69, and 70 showed greater than 90% similarity with each other but shared only about 80% similarity with the other serotypes (Fig. 4, 5). Serotypes 17, 69, and 70 also grouped with the sequence of the equivalent region of HRV14 while the remainder of rhinoviruses sequenced in this study grouped with the previously sequenced HRVs (1B, 2, and 89). The sequences showed short conserved regions interspersed with variable domains. The sequence of HRV1B was compared with that already in the database (accession number D00239; Hughes et al., 1988) and was found to be identical apart from having an extra C at position 207 (between 380 and 381 of D00239).
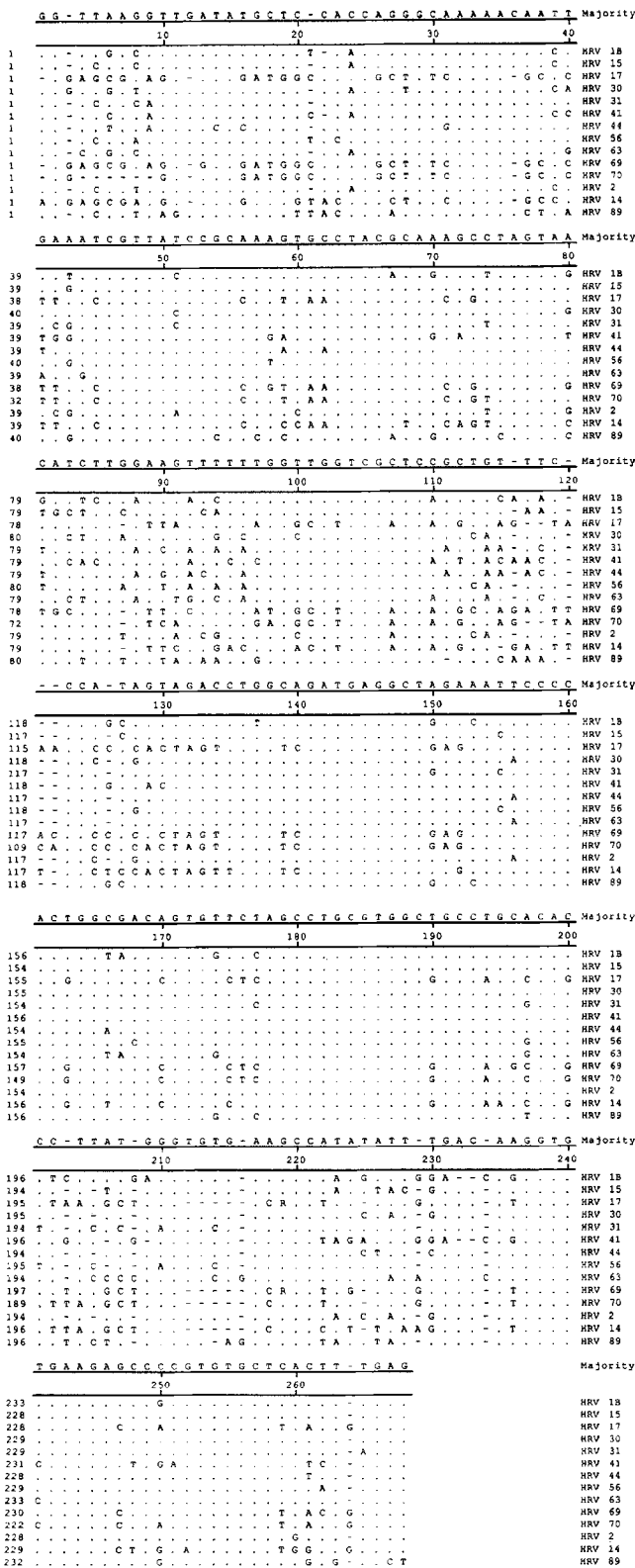
Fig. 4. Alignment of the sequences of the 5'-UTR amplicons of 11 HRVs determined in this study (1B, 15, 17, 30, 31, 41, 44, 56, 63, 69, 70) and the equivalent sequences from HRVs 2, 14, 19 from the GenBank/EMBL database. EMBL accession numbers are Z29654–Z29664. The sequences were aligned with the "MegAlign" module of Lasegene using the Clustal method with weighted residue table.

DISCUSSION

This study has focussed on the design and testing of primers from regions of the genome other than the 5'-UTR of the picornaviruses, which has been the usual PCR target [Arruda and Hayden, 1993; Bruce et al., 1990; Gama et al., 1989]. It has shown that it is possible to select primers that are capable of distinguishing between enterovirus and rhinovirus RNA. This will help to clarify the extent that rhinoviruses are involved in respiratory infections circulating in the community, for instance in studying those presenting to general practitioners.

As expected, and as others have shown [Gama et al., 1989; Hyypiä et al., 1989; Olive et al., 1990], primers embedded in the conserved sequences in the 5'-UTR were able to detect both enterovirus and rhinovirus RNA (set 0, Fig. 1, Table I). Of the primer sets chosen to be specific for the rhinoviruses, both sets 1 and 2 were able to amplify all the serotypes tested (Figs. 2, 3). Because of the slightly better efficiency of set 2 and the larger size of its amplicon (185 vs 76 bp), these primers were chosen to be tested against a range of enteroviruses. Specific amplification was not observed for any of them (Fig. 3). Thus, the set 2 primers may allow for differentiation between enterovirus and rhinovirus RNA merely by the presence or absence of a band of correct size on a gel. As we were not able to test all members of the Picornaviridae with these primers their full usefulness remains to be determined, and it may also be necessary to have a sequence confirmation for any ethidium bromide-stained band. Notwithstanding these reservations, the set 2 primers should provide a useful screening assay for rhinovirus RNA.

By contrast, neither the set 3 nor 4 primers were able to detect all the rhinovirus RNAs tested, although they were based on sequences conserved between the four full sequences available (1B, 2, 14, 89). This is perhaps not surprising, as sequence variation among the more than 100 rhinovirus genomes is likely to be great.

PCR will be particularly useful for rhinovirus identification if the amplicon can be rapidly sequenced and the information used to type the virus, as an alternative to culture and neutralisation with pools of antisera. The extent to which this can be done will depend on the sequences of appropriate PCR target regions being determined for cultured viruses of known serotype. (Assuming that the cloning and sequencing of the entire genome of all known picornavirus serotypes will not be performed in the near future.) The choice of an appropriate amplicon to sequence is determined by its size and the extent to which the primers can be expected to be conserved among the different serotypes of the virus genus and family. Obviously, the amplicons of primer sets 3 and 4 would not be appropriate as this study has shown their primers are insufficiently conserved. Although the sequence amplified by the set 2 primers would seem a good choice for rhinovirus genomic typing, the amplicon could be considered to be a little too short, at 185 bp, to generate sufficient information. We therefore chose to investigate the sequence divergence of the amplicon produced by the 5'-UTR
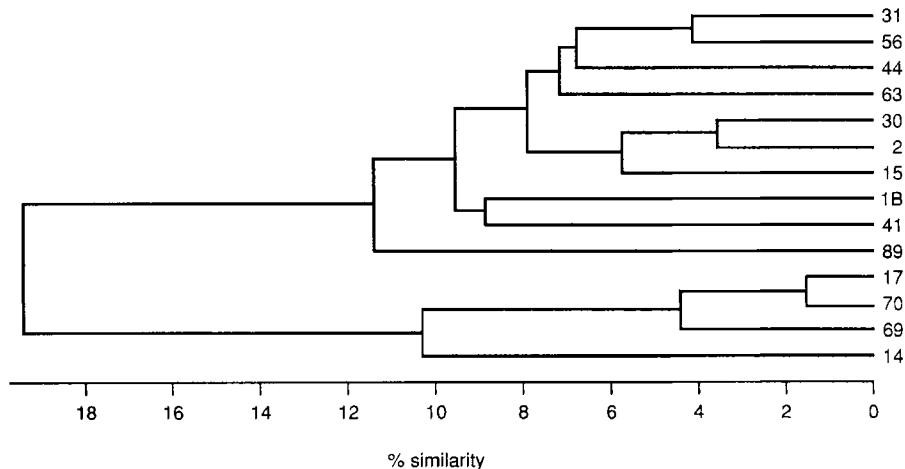
Fig. 5. Phylogenetic tree of the sequences from Figure 4, produced using the same program. The horizontal axis gives an estimate of the percentage dissimilarity of the sequences.

primers. This is just under 300 bp, depending on the specific virus target and the primers used, and is thus an ideal size for sequence determination on an average gel. Also, this sequence can be amplified with the same primers for both the enteroviruses and the rhinoviruses. When, ultimately, there is a database of the 5'-UTR sequences of all the members of the Picornaviridae it will allow typing by the PCR and sequencing method described in this work.

Sequence determination allows inferences to be made about the relatedness of viruses. This was not the main reason for this study and we do not consider that we have obtained suficient sequence for rigorous phylogenetic comparison; but it is nevertheless interesting to compare the sequences we have obtained. These, together with the other HRV sequences in the 5'-UTR already in the public domain, can be divided into two groups: 1B, 2, 15, 30, 31, 41, 44, 56, 63, and 89; and 4, 17, 69, and 70 (Fig. 5). The significance of this is not clear.

ACKNOWLEDGMENTS

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