Virology 255, 86–93 (1999)
Article ID viro.1998.9557, available online at http://www.idealibrary.com on IDEAL®

A New Picornavirus Isolated from Bank Voles (*Clethrionomys glareolus*)[1]

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Received August 3, 1998; returned to author for revision September 22, 1998; accepted November 30, 1998

A previously unknown picornavirus was isolated from bank voles (*Clethrionomys glareolus*). Electron microscopy images and sequence data of the prototype isolate, named Ljungan virus, showed that it is a picornavirus. The amino acid sequences of predicted Ljungan virus capsid proteins VP2 and VP3 were closely related to the human pathogen echovirus 22 (approximately 70% similarity). A partial 5′ noncoding region sequence of Ljungan virus showed the highest degree of relatedness to cardioviruses. Two additional isolates were serologically and molecularly related to the prototype.

INTRODUCTION

Different members of the picornavirus family can induce myocarditis in several species including humans and rodents. Two epidemiologic observations led us to hypothesize that rodents or arthropods in northern Sweden might be the vectors of an infectious agent linked to myocarditis in human. First, an episode of six lethal cases of myocarditis in a cohort of approximately 200 elite orienteers occurred between 1989 and 1992 (Wesslen *et al.,* 1992). It was speculated that exposure to the agent could occur during pathfinding competitions in forested areas. Second, statistical evidence has recently been published (Niklasson *et al.,* 1998) indicating that the incidence of myocarditis tracks the 3- to 4-year population cycles of the bank vole (*Clethrionomys glareolus*) in Sweden. Similar data on fluctuations in animal population density led to the identification of rodents as vectors for nephropathia epidemica (NE) in Sweden in the 1930s (Myhrman, 1934) and for Korean hemorrhagic fever in the 1950s (Gajdusek, 1962). We have shown that for Puumala virus, the causative agent of NE, the infection rate in bank voles in the spring correlated with the vole density the previous autumn (Niklasson *et al.,* 1995).

Based on these observations we initiated a study with the aim to isolate new picornaviruses from small rodents with the long-term goal to find new etiologic agents causing myocarditis in humans. We report here the isolation and primary characterization of a novel picornavirus isolated from wild bank voles (*C. glareolus*).

RESULTS

Cytopathogenicity and infectivity in animals of the Ljungan virus isolates

Three virus isolates were selected based on reaction with the human serum panels and showing size and morphology compatible with picornaviruses in electron microscopy. The first isolate was named Ljungan 87-012 after the Ljungan River in Medelpad County, Sweden where the animals were trapped. The second and third isolates originated from animals trapped outside Umeå in Västerbotten County and were designated 174F and 145SL, respectively. Virus could be isolated from both saliva/lung homogenate and feces from the same animal. All three isolates originated from *C. glareolus.* The Ljungan virus isolates produced a mild cytopathogenic effect without cell lysis. Indeed, it was possible to propagate infected cell cultures repeatedly after infection and detect viral antigen and RNA after several passages. Suckling mice inoculated with the isolates died in 3–5 days.

Morphology of the virus particles

Virus particles, 27 nm in diameter, were spherical with an almost featureless surface and appeared singly or in small aggregates on the grids. A representative picture of Ljungan virus 87-012 is seen in Fig. 1. In rare cases, the stain penetrated the particles, causing them to appear as empty shells.

[1] Sequence data from this article have been deposited with the GenBank database under Accession No. AF020541.

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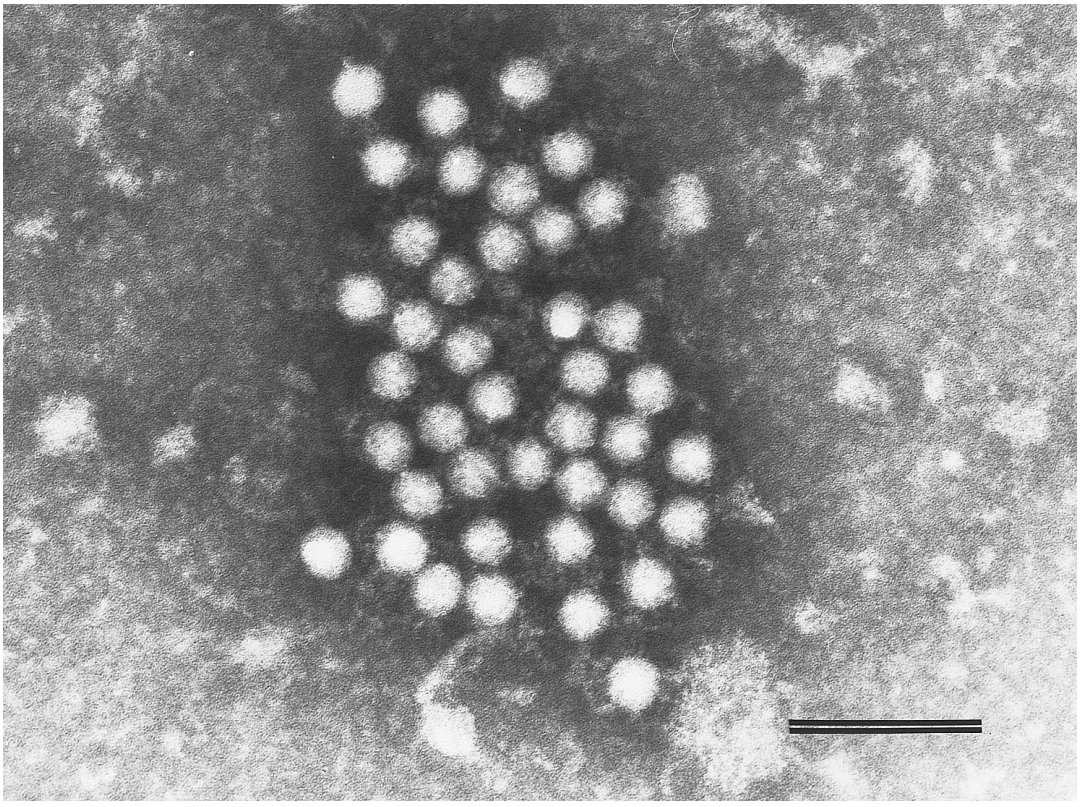

FIG. 1. Brain homogenate from suckling mice infected with Ljungan virus 87-012 examined by electron microscopy. Virus particles, 27 nm in diameter, are spherical with an almost featureless surface. Scale bar, 100 nm (magnification 280,000×).

Antigenic cross-reactions of the Ljungan virus with echovirus 22 and members of the cardiovirus group

The antigenic relationships between the Ljungan viruses 87-012, 145SL, and 174F, echovirus 22, Theiler's murine encephalomyelitis virus (TMEV), and encephalomyocarditis virus (EMCV) were determined using cross-immunofluorescence test (Table 1). Although all three Ljungan virus isolates were found to be related, 145SL was distinct from 87-012 and 174F. The cross-reactions between TMEV and EMCV were extensive, as expected, but the antigenic cross-reactions of Ljungan viruses with TMEV/EMCV or echovirus 22 were very weak or absent. Four of the five sera from the myocarditis patients and one of the five human control sera showed immunofluorescence test (IFT) reactivity at a dilution of 1:32 with one of the Ljungan virus isolates (Table 2).

TABLE 1

Cross-IFT Using Virus Infected GMK or Vero Cells

	Virus					
Antiserum	87-012	174F	145SL	EMCV	TMEV	Echovirus 22
87-012	2048	2048	1024	<16	<16	<16
174F	256	256	16	<16	<16	<16
145SL	256	256	2048	<16	<16	<16
EMCV	<16	<16	<16	128	64	<16
TMEV	<16	<16	<16	256	256	<16
Echovirus 22	<16	<16	<16	<16	<16	1024

Note. Immune animal sera were titrated using two-fold dilutions starting from 1:16. Antisera to Ljungan virus isolates 87-012, 145SL, and 174F were raised in mice, for TMEV and EMCV in guinea pigs, and for echovirus 22 in a monkey. The titer is given as the reciprocal value of the highest serum dilution being IFT positive.

Molecular analysis of the Ljungan virus isolates

A 2118-nucleotide-long region covering part of the 5′ noncoding region (NCR) and genes for capsid proteins VP2 and VP3 of the Ljungan virus 145SL isolate was sequenced and used for prediction of structural elements and the polyprotein sequence (Fig. 2).

The closest relative in the 5′NCR was Mengo virus, which showed 52% identity in the region covering nucleotides 219–679 (Fig. 3). In this genome region, known to comprise the internal ribosome entry site (IRES) in cardioviruses, similar predicted secondary structure elements were also found in the Ljungan virus. When partial 5′NCR sequences (303 nt) of the three Ljungan virus isolates were compared, they clustered relatively tightly together, suggesting that they represent different strains (Figs. 3 and 4).

In cardioviruses, the codon initiating the translation of

TABLE 2

Indirect Immunofluorescence Titers in Patients with Myocarditis (M) Tested for the Presence of Antibodies to the Three Ljungan Virus Isolates

	87-012	145SL	174F
M1	32	8	<8
M2	32	<8	<8
M3	32	32	<8
M4	<8	32	<8
M5	<8	<8	<8
C1	32	<8	<8
C2	<8	<8	<8
C3	<8	<8	<8
C4	<8	<8	<8
C5	<8	<8	<8

Note. Control patients (C) were matched for age, sex, and area of residence. The titer is given as the reciprocal value of the highest serum dilution that was IFT-positive.

the polyprotein is preceded by a stem-loop structure that could also be predicted in the Ljungan virus sequence. The stem-loop is followed by a pyrimidine stretch also found in cardioviruses and other picornaviruses (Fig. 2). The first AUG codon in good sequence context for translation initiation (Kozak, 1989) was followed by another potential initiation site 66 nucleotides downstream in the same reading frame. Ljungan virus may thus utilize the two AUG codons in a fashion analogous to aphthoviruses, known to initiate translation at two separate codons 81 nucleotides apart (Sangar *et al.,* 1987).

Although the VP0 precursor is usually proteolytically processed to capsid polypeptides VP4 and VP2 in mature virions in most picornaviruses, this cleavages does not appear to take place in echovirus 22 (Hyypiä *et al.,* 1992; Stanway *et al.,* 1994). According to the amino acid sequence homology, the Ljungan virus is closely related to echovirus 22 and may also contain VP0 as a major capsid protein. No consensus myristylation in the N terminus of VP0 corresponding to VP4 in other picornaviruses is found in contrast to most members of the family. This observation is also analogous with results obtained with echovirus 22 (Stanway *et al.,* 1994). The predicted amino acid sequences of Ljungan virus (145SL) VP2 and VP3 capsid proteins were compared with representatives of other picornaviruses whose three-dimensional structures are known and with echovirus 22 (Fig. 5). Sufficient similarity was found, making alignment of the polypeptide sequences possible and suggesting that these Ljungan virus proteins share the eight-stranded antiparallel beta-barrel structure found in the major capsid polypeptides of members in the picornavirus family. The closest similarity in primary structure terms in the VP2 and VP3 region was seen between Ljungan virus and echovirus 22 (approximately 70%; Fig. 6). These two viruses also have a similar predicted extension to the amino terminus of VP3 and, together with aphthoviruses, lack the long loop structure between beta strands E and F in VP2 when compared to other picornaviruses.

DISCUSSION

Our attempts to isolate novel viral pathogens from rodents led to the discovery of three viruses that were shown to belong to picornaviruses by using electron microscopy and sequence analysis. Picornaviruses have a protein capsid containing three major polypeptides (VP1–3) and one minor polypeptide (VP4) (Stanway, 1990). These surround a single-stranded RNA (7–8 kb) genome that is directly translated to a polyprotein and subsequently processed to the mature capsid polypeptides and nonstructural proteins needed for virus replication in the infected cells. There is a relatively long (approximately 750 nt) 5′NCR preceding the translation initiation site in the genome that exhibits extensive secondary structure and contains conserved sequence motifs. The members of the picornavirus family are classified in different genera, mainly on the basis of their physicochemical and pathogenetic properties.

Recent sequence analysis has further clarified the position of individual viruses in the five previously established picornavirus genera (aphtho-, cardio-, entero-, hepato-, and rhinoviruses) and revealed a new genus represented by echoviruses 22 and 23 (Hyypiä *et al.,* 1992; Stanway *et al.,* 1994), tentatively named parechoviruses. Aphthoviruses cause foot-and-mouth disease in cattle, cardioviruses (e.g., EMCV, Mengo virus, and TMEV) infect rodents, while enteroviruses (e.g., polioviruses), hepatoviruses (hepatitis A virus), and rhinoviruses (causative agents of the common cold) as well as echoviruses 22 and 23 are important human pathogens.

Molecular data clearly confirmed that Ljungan virus is a picornavirus and that it shares properties both with other members of the family found in rodents and with the human pathogen echovirus 22. In the 5′NCR, the closest relatives are the members of the cardiovirus genus, which may reflect the ability of Ljungan viruses to replicate in rodents. Major structural elements participating in stem-loop structure formation and playing a role in the initiation of translation, employing the internal ribosomal entry site (IRES) in cardioviruses and other picornaviruses, can also be found in the Ljungan viruses. On the other hand, the capsid proteins share considerable similarity with the capsid proteins of echovirus 22, which may correlate with their potential ability to infect humans. Clinical infections caused by echovirus 22 are common and in most cases involvement of respiratory and gastrointestinal tracts is seen (Grist *et al.,* 1978). Currently there is no evidence of the occurrence of echovirus 22 infections in other hosts than human. However, the results reported here suggest that a larger group of related

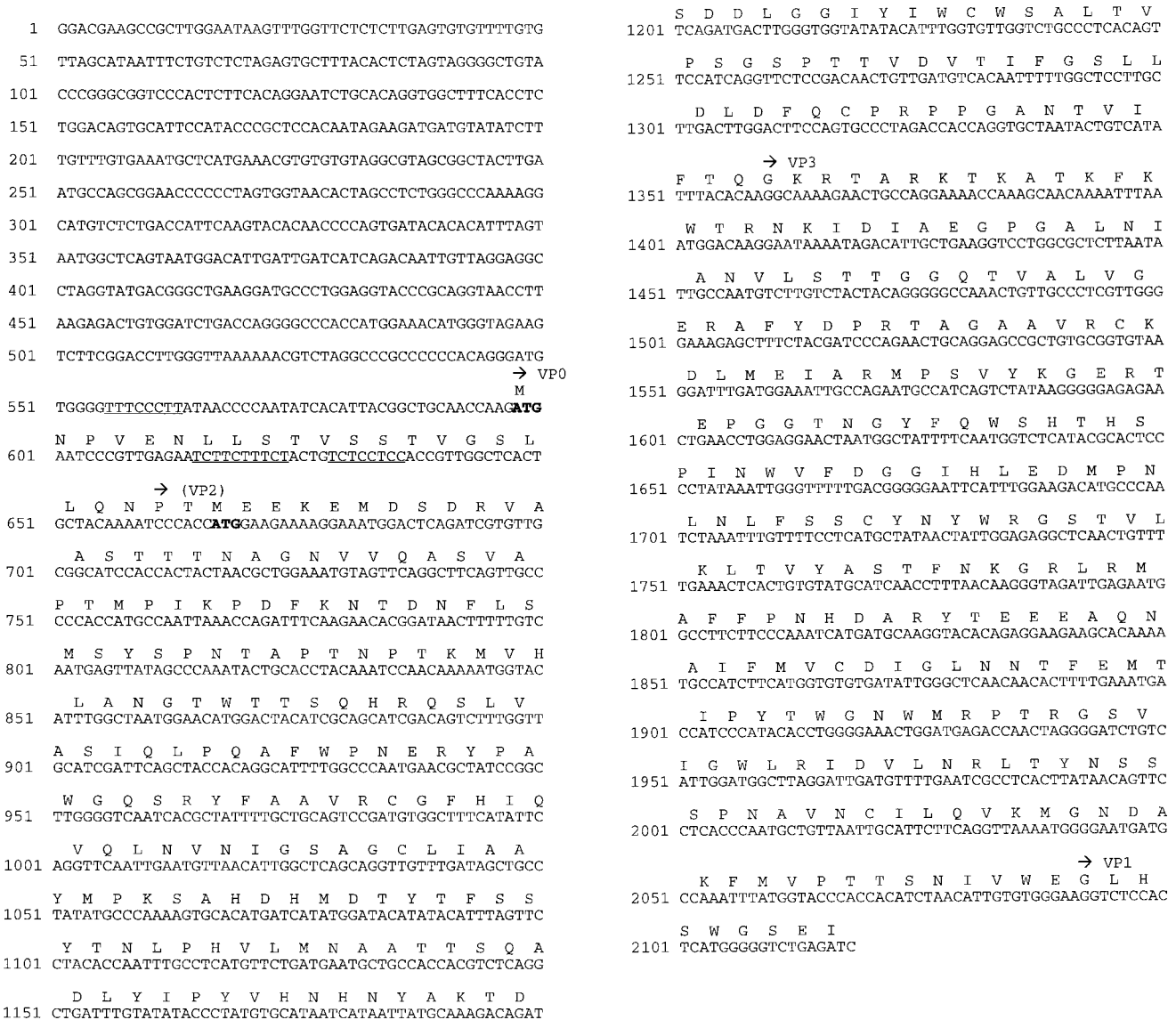

FIG. 2. Partial nucleotide sequence of the genome of the Ljungan virus isolate 145SL. The sequence covers most of the 5′ noncoding region and genes coding for capsid polypeptides VP0 and VP3 on the basis of comparison with other picornavirus genomes. Pyrimidine-containing regions are underlined, the two putative initiation codons are shown in boldface type, and the predicted proteolytic processing sites of the polyprotein are indicated.

picornaviruses may exist in different animal species and that they could also be causative agents of human infections with currently unknown etiology. Colonized bank voles experimentally infected with Ljungan virus showed no signs of disease, supporting the idea that they could be the reservoir of the virus although other species may carry the virus too. The virus could be detected by RT-PCR 4 weeks after the experimental infection but the antibody responses measured by IFT in these PCR-positive animals were either very weak or undetectable (data not shown). On the other hand, high antibody levels could be detected in mice using the same infection protocol. Thus, it is evident that the immunological response differs in various species and it is possible that human infection may occur without a high titer response.

Formerly, the only possibilities of recognizing new viral agents were to isolate them in experimental animals or in tissue culture. More recently, it has also become possible to use molecular techniques for identification of new pathogens that cannot be cultivated, at least by using standard techniques. Despite this, the specific etiology of several human diseases with expected association with infections is still unclear. As seen in this work and other studies, there are numerous potential human pathogens circulating in the environment. Our isolation experiments from only a limited number of bank

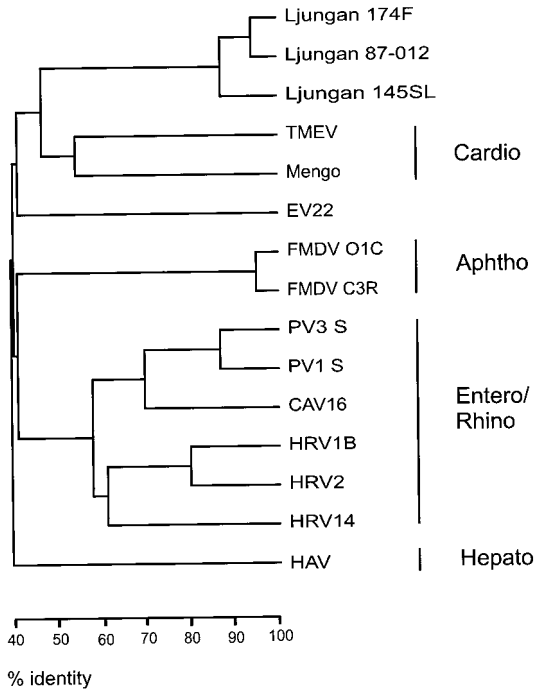

FIG. 3. Molecular relationships between the Ljungan virus isolates and representatives of other picornaviruses in a partial sequence of 303 nucleotides in the 5′ noncoding region. Theiler's murine encephalomyelocarditis virus (TMEV) and Mengo virus represent the most closely related cardioviruses.

voles resulted in the recognition of three related picornavirus strains. We believe that an important strategy to be used to identify novel human viral pathogens is to study their suspected reservoir using epidemiological and environmental data, to use a wide variety of cell lines for isolation, and to indicate the presence of viruses not only by CPE but also by immunodetection using sera from patients with the disease under investigation.

While thus far we have been unable to demonstrate a pathogenetic role of the Ljungan virus isolates as the causative agents of human disease, our present virologic analysis of the isolates demonstrates that the bank vole *C. glareolus* is a host for a new picornavirus closely related to the human pathogen echovirus 22. This finding highlights the need for a better understanding of the viral ecology of murine as well as other animal species in nature.

MATERIALS AND METHODS

Trapping of animals

Small rodents were trapped at several locations in northern Sweden and transported live to the Swedish Institute for Infectious Disease Control. Animals were bled using ether anesthesia and killed. Organs were immediately removed and stored at −70°C until tested for the presence of viruses. A total of 53 *C. glareolus* and 28 *Microtus agrestis* were tested.

Virus isolation

Saliva mixed with lung homogenate and feces were analyzed separately from each animal. The material was inoculated into T25 flasks of confluent BHK-21 cells. The cells were blind passaged twice a week during a 2-week period. At the end of this period, or earlier if signs of CPE occurred, the cells were removed from the flasks with a rubber policeman, placed onto 10-well spot slides, air-dried, and acetone-fixed. The cells were then stained with a panel of 10 human sera including that from five athletes deceased from myocarditis and bled at autopsy and 5 control sera. All the samples (saliva–lung and feces separately) were tested individually by an indirect IFT as described below, using the sera at a 1:10 dilution. Cells showing CPE or positive reaction by IFT were selected for further analysis. This included electron microscopy, intracerebral inoculation of 1-day-old suckling mice, antigenic characterization, and sequence analysis.

Viruses, antisera, and serological procedures

The ATCC TMEV (VR 995), EMCV (VR 129B), and echovirus 22 (strain Harris) prototypes were used as references. Antisera to Ljungan virus 87-012, 145SL, and 174F isolates were raised in mice (NMRI), to TMEV and EMCV in guinea pigs (Dunkin Hartley), and to echovirus 22 in a monkey. The animals were injected intraperitoneally with a cell culture supernatant (BHK-21 cells) and serum was collected 4–6 weeks later. Preimmunization sera were tested individually while postimmunization sera were pooled from all infected animals.

A previously described IFT protocol (Niklasson and LeDuc, 1987; Riggs, 1979) was used to test antibody titers. Briefly, spot slides were prepared by incubating

```
          1                                                  50
L174F     AGTCTAGTTT CATTCTGTGT GTGTTTGGCA CTGAAATTAT TTCTGTCTCT
L87-012   AGTCTAGTCT TATCTTGTAT GTGTCCTGCA CTGAACTTGT TTCTGTCTCT
L145SL    AGTTTGGTTC TCTCTTGAGT GTGTTTTGTG TTAGCATAAT TTCTGTCTCT
          *** * **    *  **  * ****  *   *   *  * **********

          51                                                 100
L174F     GGGGTGCTTT ACACTTCAGT AGGGGCTGTA CCCGGGCGGT GGGACTCTTC
L87-012   GGAGTGCTCT ACACTTCAGT AGGGGCTGTA CCCGGGCGGT CCCACTCTTC
L145SL    AGAGTGCTTT ACACTCTAGT AGGGGCTGTA CCCGGGCGGT CCCACTCTTC
           * ***** * *****  *** ********** **********    *******

          101                                                150
L174F     ACAGGAATNT GCACAGGTGG CTTTCACCTC TGGACAGTGC ATTCCACACC
L87-012   ACAGGAATCT GCACAGGTGG CTTTCACCTC TGGACAGTGC ATTCCACACC
L145SL    ACAGGAATCT GCACAGGTGG CTTTCACCTC TGGACAGTGC ATTCCATACC
          ******** * ********** ********** ********** ****** ***

          151                                                200
L174F     CGCTCCACAG TAGAAGATGA TGTGTGTCTT TGCTTGTGAA AAGCTTGTGA
L87-012   CGCTCCACGG TAGAAGATGA TGTGTGTCTT TGCTTGTGAA AAGCTTGTGA
L145SL    CGCTCCACAA TAGAAGATGA TGTATATCTT TGTTTGTGAA ATGCTCATGA
          ********   ********** *** * **** ** ******* * ***  ***

          201                                                250
L174F     AAATCGTGTG TAGGCGTAGC GGNTACTTGA GTGCCAGCGG ACNACCCCTA
L87-012   AAATCGTGTG TAGGCGTAGC GGCTACTTGA GTGCCAGCGG ATTACCCCTA
L145SL    AACGTGTGTG TAGGCGTAGC GGCTACTTGA ATGCCAGCGG AACCCCCCTA
          **   ***** ********** ** *******  ********* *   ******

          251        264
L174F     GTGGTAACAC TAGC
L87-012   GTGGTAACAC TAGC
L145SL    GTGGTAACAC TAGC
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FIG. 4. Alignment of partial sequence of the 5′NCR of three Ljunganvirus isolates. Asterisks show completely conserved positions.

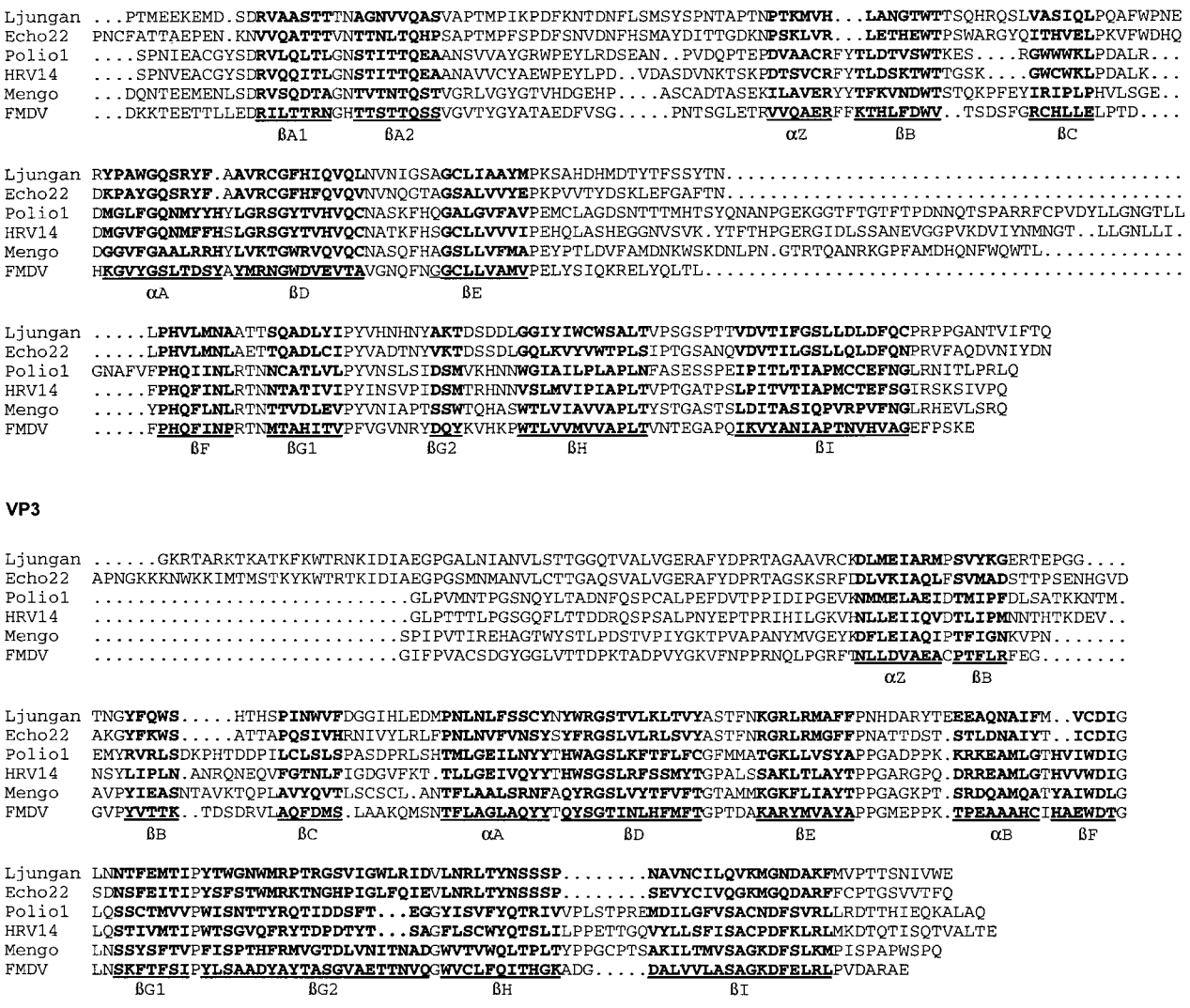

FIG. 5. Alignment of the predicted amino acid sequences of Ljungan virus capsid proteins VP2 and VP3 with the corresponding polypeptides of echovirus 22 and representatives of other picornaviruses with known three-dimensional structures (Stanway *et al.,* 1994). The known and predicted alpha helix and beta strand structures are indicated. EV 22, echovirus 22; PV1S, poliovirus 1 Sabin strain; CAV16, coxsackievirus A16; HRV1B, human rhinovirus 1B; Mengo, Mengo virus; FMDV, foot-and-mouth disease virus; HAV, hepatitis A virus.

virus in green monkey kidney (Ljungan isolates) or Vero cells (EMCV, TMEV, and echovirus 22) for 8 h to 10 days. At signs of discrete CPE, cells were removed from the flask with a rubber policeman and applied to microscope slides, air-dried, fixed in cold (4°C) acetone, and stored at −70°C until used. The titer was determined after the serum, diluted in PBS, was incubated on the slides at 37°C for 1 h in a moist chamber and bound antibodies were detected by FITC-conjugated goat anti-human IgG (Sigma, F-1641), rabbit anti-mouse (Dako, F0313), or goat anti-guinea pig immunoglobulin (Jackson ImmunoResearch Laboratories 106-095-003) for 1 h at 37°C.

Electron microscopy

Cell culture media or brain tissue homogenates were examined by negative contrast electron microscopy. A 10-μl droplet was incubated on a Formvar/carbon-coated grid for 1 min or, alternatively, 0.5-ml samples were centrifuged for 30 min at 20,000 *g* to remove cell debris, and finally the supernatants were pelleted directly onto grids in a Beckman Airfuge for 10 min at 160,000 *g.* The grids were stained with 2% phosphotungstic acid (pH 6.0) and examined in a Philips CM 100 electron microscope at a magnification of at least 46,000.

RT-PCR and sequencing

The Ljungan virus isolates 87-012, 145SL, and 174F were grown in the human lung carcinoma line A549 in roller bottles. Supernatants were filtered through 0.45-μm cellulose acetate filters (Costar) and the virus was pelleted at 20,000 *g* for 20 h at 4°C. RNA was isolated from the virus-containing pellets using the acid

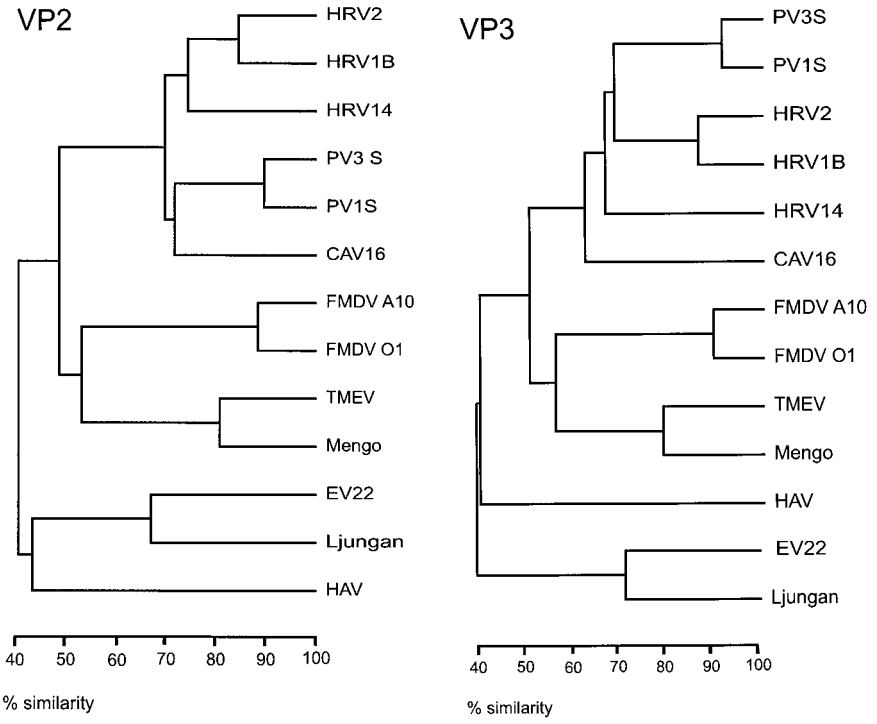

FIG. 6. Dendrograms based on predicted amino acid sequences of capsid proteins VP2 and VP3 exhibiting the genetic position of the Ljungan virus prototype strain among representatives of picornaviruses.

guanidinium–thiocyanate method as described earlier (Chomczynski and Sacchi, 1987). Synthesis of cDNA was performed under standard conditions using 1 μg of RNA, AMV reverse transcriptase (Boehringer Mannheim), and random 14-mer oligonucleotides as primers in a 20-μl reaction. Fragments of the viral 5′NCR cDNA were amplified using cardiovirus-specific consensus primers (sense) 5′-GGCCGAAGCCGCTTGGAATA-3′ and (antisense) 5′-GTGGCTTTTGGCCGCAGAG-3′, both primers modified after the EMCV2 and EMCV1 primers previously reported (Jongen *et al.,* 1993; Palmenberg, 1989). Amplification was carried out through 30 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 2 min.

A 1.8-kb fragment extending from the 5′NCR to the viral polyprotein sequences was obtained by PCR amplification of cDNA from the 145SL isolate. The primers were (sense) 5′-ACAGTGCATTCCACAC-3′ (SLJU1) or 5′-CCGCTCCACAATAGA-3′ (SLJU2) and (antisense) 5′-GATCTCAGAC-3′ (primer 118). The SLJU1 and SLJU2 primers are located immediately adjacent to each other and were chosen as consensus primers for the Ljungan isolates with as little homology as possible to the EMCV and TMEV group of viruses. The sequence of primer 118 has been published previously (Bauer *et al.,* 1993). The amplification conditions were 30 cycles at 94°C for 30 s, 42°C for 1 min, 72°C for 2 min. The amplified fragments were cloned into the pCRII T vector (Invitrogen).

The cDNA clones were sequenced using a *Taq* polymerase FS cycle sequencing kit (Perkin–Elmer) and the data were collected with an ABI Prism 310 genetic analyzer (Perkin–Elmer).

Sequence analysis

Nucleotide identities and amino acid similarities were calculated with the program GAP from the Genetic Computer Group Software package (GCG), (Devereux *et al.,* 1984). The nucleotide alignments and dendrograms were created with the program PILEUP (GCG).

ACKNOWLEDGMENTS

The study was supported by Stiftelsen Olle Engkvist, Byggmästare, the Swedish Environment Protection Agency (via the National Swedish Environmental Monitoring Programme), the National Board of Health and Welfare, the Academy of Finland, and the Sigrid Juselius Foundation.

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