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Detection of related positive-strand RNA virus genomes by reverse transcription/ polymerase chain reaction using degenerate primers for common replicase sequences

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Abstract

A set of degenerate sense and antisense primers were designed on the basis of short segments with identical amino acids in the predicted ORF 1b replicase proteins of lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus, strain Lelystad virus (PRRSV-LV), which are members of a new group of positive-strand RNA viruses. Reverse transcription/polymerase chain reaction amplification using this set of degenerate primers yielded products of the expected size from the genomes of all three viruses. It also yielded a product of appropriate size from the genome of another strain of PRRSV (VR2332), the ORF 1b sequence of which is unknown, but the 3' end of the genome of which differs from that of the PRRSV-LV genome by about 50%. No products were generated from the genome of simian hemorrhagic fever virus (SHFV), another member of this virus group. However, an appropriate product was generated with a second set of degenerate primers which was designed from the same ORF 1b segments of LDV, EAV and PRRSV-LV as the first set but on the basis of human codon preferences. Sequence analysis showed that the amplified SHFV ORF 1b segment exhibited about 50% nucleotide identity with the corresponding segments of ORF 1b of LDV, EAV and PRRSV. The results show that these and other degenerate primer sets might be useful for the search of related viruses in other mammalian species.

Keywords: Degenerate primers for RT/PCR; Genus *Arterivirus*; Lactate dehydrogenase-elevating virus

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Lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV), porcine reproductive and respiratory syndrome virus (PRRSV) and simian hemorrhagic fever virus (SHFV) belong to a new group of positive-stranded RNA viruses, presently classified as the genus *Arterivirus* (reviewed in Plagemann and Moennig, 1992; Plagemann, 1996). These viruses are enveloped and have a diameter of 40–60 nm. Their genomic RNA is 13–15 kb and gene expression is via formation of 6 or 7 subgenomic mRNAs. They also possess common biological properties: they replicate primarily in macrophages and can establish long-term asymptomatic persistent infections in their hosts. These viruses were discovered only accidentally or because of the appearance of variants with increased pathogenic potential. The discovery of LDV was based on a 5- to 10-fold increase in plasma lactate dehydrogenase (LDH) activity caused by LDV infection in mice. SHFV establishes long-term asymptomatic infections in several genera of African monkeys and it was discovered because accidental transmission in colonies causes a generally fatal hemorrhagic fever in Asian macaque monkeys. EAV and PRRSV infections are most often subclinical but infection can result in severe respiratory symptoms and reproductive failure in their respective hosts (Meredith, 1993; Timoney and McCollum, 1993). The reasons for the variability in the outcome of EAV and PRRSV infections have not been elucidated but probably reflect the existence of variants with different pathogenic potential.

The existence of related viruses in mice, swine, horses and monkeys suggests that additional viruses of this group infect other species. Such viruses may have escaped detection because they generally cause only subclinical infections. However, if this is the case, variants with increased pathogenicity may emerge under certain environmental conditions, as apparently has been the case with PRRSV, since PRRS as a disease appeared only recently, independently in 1987 in the U.S. and in 1990 in Western Europe (Meredith, 1993). On the other hand, additional viruses of this group may already be associated in other species with diseases of unknown etiology.

It seems important, therefore, to develop procedures for the detection of such related viruses in other species. Serological tests seem of no use for this purpose because antibodies to LDV, EAV, SHFV and PRRSV do not cross-react (Plagemann and Moennig, 1992). The problem is further complicated by the very restricted host ranges of these viruses which make their isolation by propagation in cell culture difficult. LDV replicates only in a subpopulation of mouse tissue macrophages; SHFV and PRRSV replicate only in cultures of alveolar macrophages of their respective hosts and clones of a single African green monkey kidney cell line (MA104) but not all viral isolates infect MA104-derived cells; and EAV infects horse macrophages and a number of lines of kidney cells from different species (Plagemann, 1996). These difficulties have led us to develop a molecular method for the detection of viruses of this group. It is based on our finding of segments with identical amino acids in the ORF 1b replicase proteins of the two strains of LDV, LDV-C (Godeny et al., 1993) and LDV-P (Chen et al., 1993; Palmer et al., 1995), one strain of EAV (Bucyrus strain; den Boon et al., 1991) and one strain of PRRSV (Lelystad virus, LV; Meulenbergh et al., 1993) that have been sequenced.

The proteins encoded by the genomes of these viruses are colinear and exhibit considerable amino acid identities (Plagemann, 1996). For example, the amino acid identities for the predicted LDV-P proteins with those of LDV-C, PRRSV-LV and EAV range from 75 to 99%, 33 to 57% and 27 to 40%, respectively (Plagemann, 1996). However, the greatest degree of amino acid identity is observed for the predicted ORF 1b proteins which contain common replicase, helicase and zinc finger motifs. Especially striking is the amino acid identity in a segment of the ORF 1b proteins containing the universal replicase motif **D...D...SG...T...N...SDD...** (in bold face in Fig. 1); 109/284–287 amino acids or 38% are identical in all four viruses and there are short segments of five and more amino acids (Fig. 1, 1–5, overlined) with complete amino acid conservation (see + signs in Fig. 1). This amino acid identity suggests that these segments may play important roles in the function of the replicase protein. We have focused on two of the longest stretches with amino acid identity (segments 3 and 5, Fig. 1) and have generated sense and antisense degenerate oligonucleotide primers representing these segments of LDV-P, PRRSV-LV and EAV (Fig. 2A) with which reverse transcription (RT)/polymerase chain reaction (PCR) should generate specific products from the genomes of these viruses and perhaps the genomes of related other viruses. The anticipated PCR product using these primers should be 170 nucleotides long and products of this size were indeed obtained from the genomes of all three viruses (see below).

LDV-P was harvested from plasma of 1-day LDV-infected mice (Chen et al., 1994). EAV, the European isolate of PRRSV (LV) and the U.S. isolate of PRRSV (VR2332; Murtaugh et al., 1995) and SHFV were propagated in cultures of CI-2621 cells. A vaccine strain of EAV (Timoney and McCollum, 1993) was kindly provided by Dr. W.H. McCollum, and PRRSV-LV and PRRSV-VR2332 and CI-2662 cells by Dr. T. Molitor, SHFV and MA-104 cells were kindly provided by Dr. P. Jahrling. Culture fluid was harvested from cell cultures infected with EAV, PRRSV or SHFV at 1–2 days post-infection (p.i.) and clarified by centrifugation at about 10,000 *g* for 10 min. The virions were collected by centrifugation through a layer of 0.5 M sucrose in TNE (50 mM NaCl, 10 mM Tris HCl, 10 mM EDTA, pH 7.4) at 35,000 rpm in an SW41 rotor (Beckman ultracentrifuge) for 4–5 h.

Genomic RNA was extracted from the virions by using RNAzol™ (Tel-Test, Inc., Friendswood, TX) according to the instructions provided by the supplier of the reagent. Samples of RNA were denatured by heating at 100°C for 20 s and reverse transcribed using random hexanucleotides and RNase H⁻ superscript reverse transcriptase (GIBCO BRL, Gaithersburg, MD). Segments of the first-strand products were amplified by PCR as described previously (Chen et al., 1993) using the degenerate primers shown in Fig. 2. Briefly, an initial denaturation step of 2 min at 94°C was followed by 35 to 40 cycles of annealing at 42°C for 1 min, extension at 72°C for 3 min and denaturation at 94°C for 30 s. A final step of annealing at 42°C for 1 min and extension at 72°C for 10 min was performed to complete the reaction. The PCR products were cloned into the SmaI site of Bluescript IIKS(+) (Stratagene, La Jolla, CA) which had been modified for TA cloning (Chen et al., 1993) or into a TA cloning vector from Invitrogen (San Diego,

ORF1b

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      1                             2
      ++ ++++++ + ++      + + + + + + + + + + + + + + + + + + + +
EAV 249 YnLSTQGFVLPsVfsmVRaylkeeIGdAPPLyLPSTvPsKNSqAGINGaeFPtKs1QSYclIDdMvsqsmKs
LV 245 YDLSTQGFVLPGLr1VRRfifghIGkAPPLFLPSTYPAKNSMAGINGGRFPtKdvs1PeIDeMCaRAVKE
LDV-P 243 FDLSRQGFVLPGLYLVRRYLsRLIGvRRRLfMPSYPAKNSMAGINGGRFP1twlQSHPDIDALCRACeE
LDV-C 244 FDLSRQGFVLPGLYLVRRYLcRLvGiRRRLf1PSTYPAKNSMAGINGGRFPthvvQSHPDIDALCeRACKE

      ++ +                               + + +                               ++ ++ ++
EAV 321 NIQTaTmaTcKrQYCSKYKiRsILGTNNyIgLgIRacLSGVTAfQkagkdgSPiYLGKsKF dpipapdky
LV 317 NWQVTVPCTLKKQYCSKpKTRTRILGTNNFIALahRSALSGVTQAFMK KawkSPIaLGKNKfKehLhctvaGR
LDV-P 315 HWQVTVPCTLKKQYCSKSKTRTRILGTNNFVALgLRsALSGVTQGFm RKGIGtPicLGKNKFTPLPvrigGR
LDV-C 316 HWQVTVPisLcdsaligSrphatLvlvpaigLvLaSALyfcTQGSM RsGIGsPIYLGnNKFTPLPtkvisR

      3                             4                             5
      + + + + + + + + + + + + + + + + + + + + + + + + + + + + + + + +
EAV 392 CLEtDLeSCDRSTPA1VRWFatNLifELAGqpElvhSYVLNCCHDLVvagsvAFTKRGLSSGDPiTSiSNT
LV 388 CLEADLASCDRSTPA1VRWFvaNLLyELAGeEeYlPSYVLNCCHDLVaTqdGAFTKRGLSSGDPVTSvSNT
LDV-P 386 CLEADLASCDRSTPA1IRWFTTNLLFELAGaEEWIPSYVLNCCHDvVSTMSGCFDKRGLSSGDPVTSiSNT
LDV-C 387 CLEADLASCDRSTPA1IRWFTTNLLFELAGpEEWIPSYVLNCCHDaVSTMSGCFDKRGLSSGDPVTSvSNT

      + + + + + + + + + + + + + + + + + + + + + + + + + + + + + +
EAV 464 iYSLV1YtQHMLCgLegyfpE1aEkyLdgsLelrDmfkyvrvyiYSDdvVLtptnqhyaasfdrwVPHLQa
LV 460 VYSLVIYAQHMVLSAL kmGHEIG1KFLLeeqLkfEDL1EiQPmLVYSDd1VL YaErptfPNYHwVVeHLDL
LDV-P 458 VYSLiIYAQHMVLSA FRCGHKIGGLFLqDSLEMEQLFELQPLLvYSDdVV fYnESdELPNYHFFVDHLDL
LDV-C 459 VYSLvIYAQHMVLSA FRCGHkvGGLFLrDSLEMEQLFELQPLLvYSDdVV 1yDESsELPNYHFFVDHLDL

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Fig. 1. Comparison of the amino acid sequences of a homologous segment of the ORF 1b proteins of LDV-P, LDV-C, PRRSV-LV and EAV. The sequences for the genomes of LDV-P, LDV-C, PRRSV-LV and EAV (Bucyrus strain) and for their predicted protein products are from the GenBank data base (accession numbers U151146, L13298, M96262, and X53459, respectively). Sequence alignment was conducted by the algorithm of Needleman and Wunsch (1970). + = amino acids that are identical in the segments of all four viruses. Segments with five or more identical amino acids are overlined (1–5) and the replicase motif D...D...SG...T...N...SDD is in boldface letters.

CA). The cloned cDNAs were sequenced in both directions using the Sequenase Version 2.0 (USBC, Cleveland, OH).

PCR products of the predicted size (170 nucleotides) were generated from the genomes of LDV-P, PRRSV-LV, PRRSV-VR2332 and EAV (Fig. 3) using the set of degenerate sense and antisense oligonucleotides A1479/A1480 (Fig. 2A). All products comigrated with the PCR product generated with the same primers from a cDNA clone of LDV-P (4–11) which encompasses the segment of the replicase gene to be amplified (Kuo et al., 1991; Fig. 3).

A.

Amino acids	C	D	R	S	T	P	A	A1479/
Nucleotides	5'-TGT	GAC	CGC	TCC	ACC	CCG	GC-3'	5' (+) primer
		T	G	AGA		C		for LDV, LV, EAV
Amino acids	G	L	S	S	G	D	P	/A1480
Nucleotides	3'-CCA	AAC	AGT	AGT	CCT	CTG	GG-5'	3' (-) primer
	G	G	C	G	C	A		for LDV, LV, EAV
			A					

B.

Amino acids	C	D	R	S	T	P	A	A1481/
Nucleotides	5'-TGC	GAC	AGG	TCC	ACC	CCC	GC-3'	5' (+) human primer
		C	C	AG		T		
			A			A		
Amino acids	K	R	G	G	L	S	S	/A1482
Nucleotides	3'-TTC	TCC	CCG	CCG	GAC	TCG	TC-5'	3' (-) human primer
	T	G	A			AG		
			G					

Fig. 2. Degenerate primers designed from the segments 3 and 5 in Fig. 1. The primers in set A contain all nucleotides present in the sequences of LDV-P, EAV (Bucyrus strain) and PRRSV-LV. The degenerate primers in set B were designed from the segments but according to human codon preferences (Wada et al., 1992).

In the case of SHFV, the virus we had initially received replicated poorly in cultures of MA-104 cells; virus titers did not exceed 10^5 infectious units/ml of culture fluid as determined in an endpoint dilution assay in MA-104 cells and only a portion of the cells seemed to be killed even at 3 days p.i. Analysis of RNA extracted from the cell culture fluid indicated the presence of only small amounts of 15 kb genomic RNA in addition to two prominent species of RNAs of about 2.7 and 1.6 kb and two minor RNAs with intermediate molecular weights (Fig. 4A, lane 1). The origin(s) of the smaller RNAs is not known. The 1.6-kb RNA could be 18S rRNA, but it and some of the other RNAs could be defective interfering (DI) RNAs (M.A. Brinton, personal communication).

In view of the possible presence of DI RNAs that could restrict the replication of SHFV in our MA-104 cell cultures, we attempted to eliminate the smaller RNAs. We approached this task using endpoint dilution passages since, in our hands, SHFV failed to form plaques on MA-104 cell monolayers. In each passage the culture fluid was titrated and the medium from the culture that was inoculated with the highest dilution inducing cytopathogenic effects by 3 days p.i. was used to inoculate a fresh culture. After 7 passages, the virus titers of harvested culture fluids were still only about 10^5 infectious units/ml. No specific RT/PCR products were generated with RNA extracted from virus harvested from the culture fluid of such cultures with the primer set A1479/A1480 (Fig. 2A) in spite of several

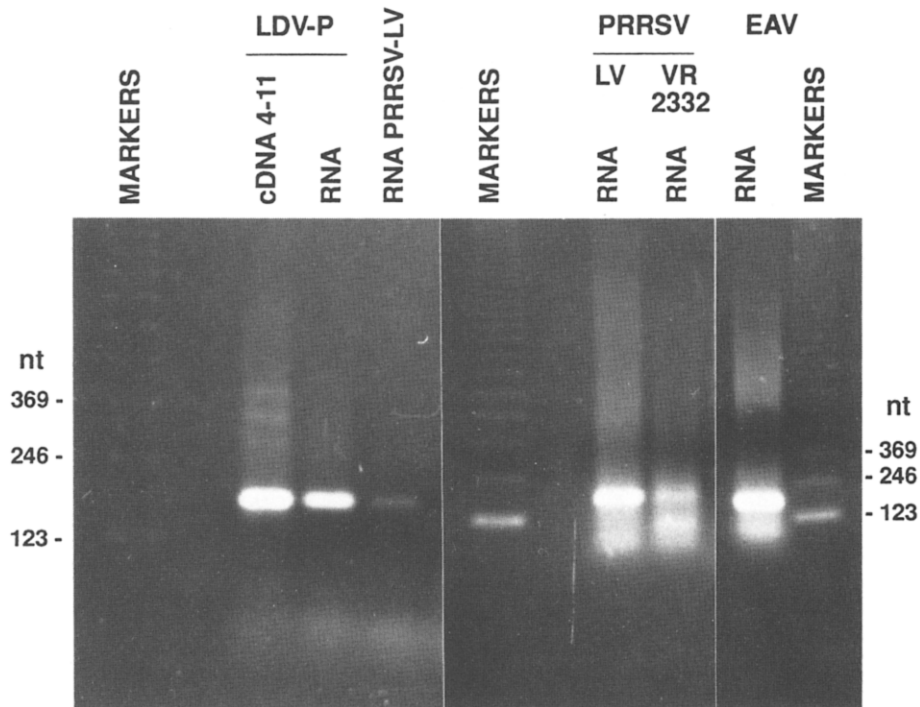


Fig. 3. PCR products generated from LDV cDNA 4–11 and first-strand cDNAs generated by RT from genomic RNAs of LDV-P, PRRSV-LV, PRRSV-VR2332 and EAV (vaccine strain). The PCR products were generated with degenerate primer set A (see Fig. 2).

attempts. However, products were generated from SHFV RNA with a second degenerate primer set designed for the same ORF 1b replicase segments on the basis of human codon preferences (A1481/A1482; Fig. 2B). With these primers a product of the predicted size (161 nucleotides) was generated from SHFV RNA (data not shown, see later) but not from LDV-P RNA. The product was TA cloned and two independent clones were sequenced (see later).

In the course of these studies we observed that SHFV replicated much more efficiently in CI-2621 cells than in MA-104 cells; CI-2621 represents a clone of MA-104 cells which has been isolated for the propagation of PRRSV but has not been previously used in SHFV studies. SHFV infection resulted in complete destruction of CI-2621 cell cultures by 1–2 days p.i. and the virus formed plaques in cultures of these cells in 1–2 days. This permitted plaque purification of the virus. SHFV was plaque purified three consecutive times. When then propagated in CI-2621 cells, 1- or 2-day culture fluid consistently contained about $2\text{--}3 \cdot 10^7$ PFU/ml. RNA extracted from SHFV harvested from such culture fluids was largely free of the smaller RNAs (Fig. 4A, lane 2). A RT/PCR product of the expected size (161 nt) was generated from this RNA with the A1481/A1482 primer

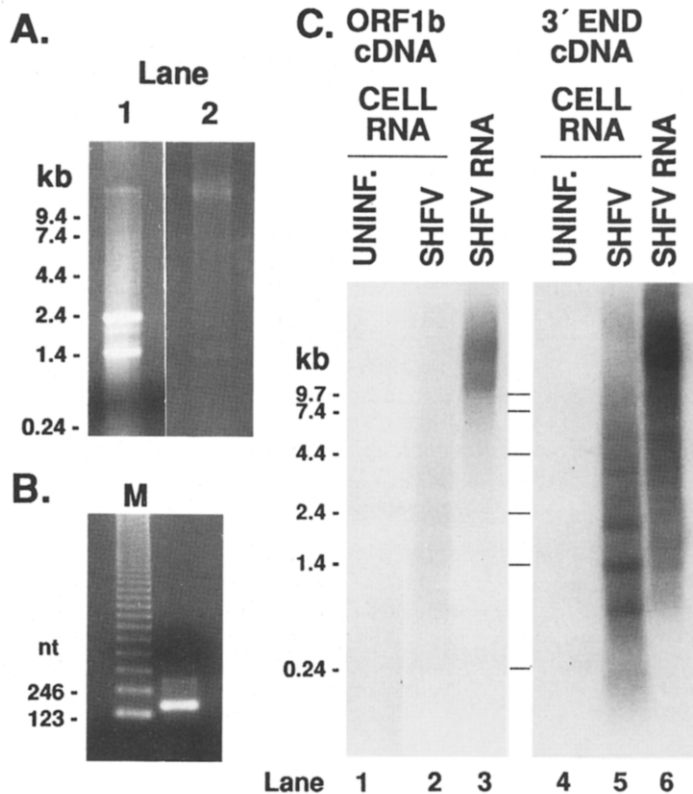


Fig. 4. Gel electrophoresis of RNA extracted from two preparations of SHFV (A), PCR product generated by RT/PCR from SHFV RNA using degenerate primer set B (B) and Northern hybridization analyses of genomic SHFV RNA extracted from SHFV virions (lanes 3 and 6) and RNA extracted from uninfected (lanes 1 and 4), and 1-day SHFV-infected (lanes 2 and 5) CI-2621 cells (C). (A) RNA was extracted from virions harvested from cultures of non-plaque-purified (lane 1) or plaque-purified SHFV virions (lane 2). (B) The PCR product was generated from RNA of the plaque-purified SHFV. (C) Northern hybridization analyses of RNA were conducted as described previously (Kuo et al., 1991) using as probes SHFV ORF 1b cDNA (see Fig. 5A; lanes 1–3) or a cDNA to the 3' end of the SHFV genome (see text; lanes 4–6). The cDNA probes were labeled by random priming using [α - 32 P]dATP as described previously (Chen et al., 1993).

set (Fig. 4B). The product was TA cloned and three additional clones sequenced. The sequences agreed well with each other and those of the two clones isolated earlier. The consensus sequence is shown in Fig. 5A. The only variability seen was in the segments representing the degenerate primer set (only the sequences of these segments of one clone are shown in Fig. 5A). This variability indicates the independent origin of the five clones that have been sequenced.

The consensus sequence of 121 nt between the two primers of the SHFV cDNA clones was colinear with those of LDV-P, LDV-C, EAV and PRRSV-LV and exhibited 52–56% nt identity with all of them (Table 1). The SHFV segment was

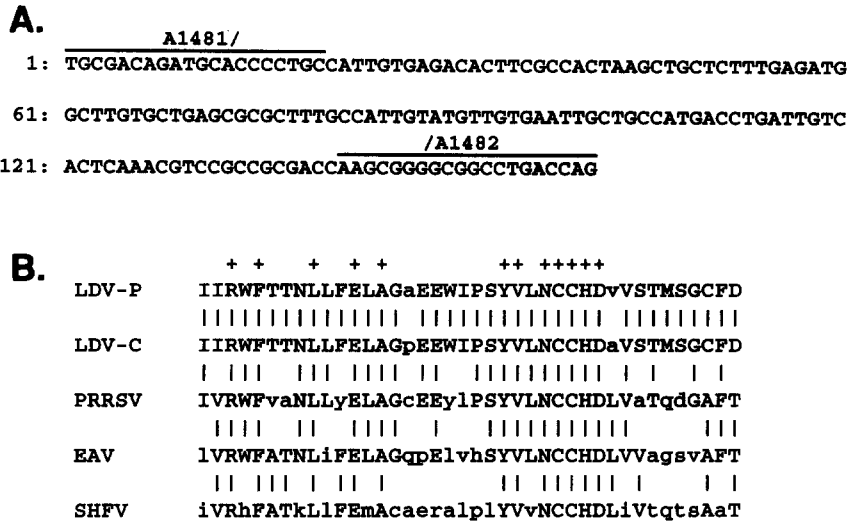


Fig. 5. Nucleotide consensus sequence of the RT/PCR-generated ORF 1b segment of SHFV (A) and comparison of the amino acid sequence of the predicted 40-amino-acid-long SHFV ORF 1b protein segment with the homologous segments of the ORF 1b proteins of LDV-P, LDV-C, PRRSV-LV and EAV (B). In A, the segments representing the A1481/A1482 degenerate primer set are overlined and in B, + = amino acids that are identical in the segments of all five viruses. The SHFV ORF 1b sequence has been submitted to the GenBank nucleotide sequence data bank and has been assigned accession number U28864.

slightly less related to those of LDV, PRRSV and EAV than the latter three to each other (56 - 60%). This was even more the case for the amino acid identities of the predicted ORF 1b protein segments of 40 amino acids (Fig. 5B) which varied between 45 and 53%, whereas those for LDV, EAV and PRRSV fell between 60

Table 1
Nucleotide identities between 121-nucleotide-long segments of the ORF 1b of LDV-P, LDV-C, PRRSV, EAV and SHFV and amino acid identities of the predicted protein sequences

Viruses	Nucleotide identity (%) ^a				
	LDV-P	LDV-C	PRRSV-LV	EAV	SHFV
LDV-P		83	60	55	56
LDV-C	95		56	57	52
PRRSV-LV	67	67		59	54
EAV	65	60	63		52
SHFV	48	45	53	50	

Amino acid identity (%)^a

^a Nucleotide (top, right) and amino acid identities (bottom, left) were determined by the algorithm of Needleman and Wunsch (1970) to align sequences and dividing the number of identical residues by the total number of residues of the segment.

and 67% (Table 1). Thirteen amino acids were the same for all five viruses and the segment YVXNCCHD was conserved in all (Fig. 5B) indicating an important function of this segment in the replicase protein.

Since this ORF 1b segment is highly conserved, it seemed ideal for assessing potential evolutionary relationships between the four viruses, but none were apparent. For example, 9 amino acids were identical in the segments of LDV, PRRSV and EAV but differed from that of SHFV, 7 amino acids were identical in the segments of LDV, PRRSV and EAV but differed from that of EAV, 4 amino acids were identical in the segments of PRRSV, EAV and SHFV but differed from that of LDV, and 2 amino acids were identical in the segments of LDV, EAV and SHFV but differed from that of PRRSV.

The authenticity of the SHFV segment was demonstrated by Northern hybridization analyses. The ORF 1b cDNA hybridized to genomic RNA extracted from virions (Fig. 4C, lane 3) but not to the subgenomic mRNAs (Godeny et al., 1995) extracted from 1-day SHFV-infected CI-2621 cells (Fig. 4C, lane 2). For a control hybridization we prepared a 759-nt-long cDNA representing the 3' end of the SHFV genome on the basis of sequence information recently supplied by Godeny et al. (1995). The cDNA was generated by RT/PCR with genomic SHFV RNA and as primers sense oligonucleotide SH-1 (5'-GAGTGGTCATGGTAG-TATC-3'; nt 72–90 in the sequence provided by Godeny et al., 1995) and antisense oligonucleotide SH-2 (5'-TGGTGTGATGATAGCAGCA-3'; nt 830–812). This cDNA hybridized both to genomic SHFV RNA (Fig. 4C, lane 6) and the six subgenomic mRNAs present in infected cells (Fig. 4C, lane 5). We observed one additional RNA of about 4.2 kb (Fig. 4C, lane 5) which might represent 29S rRNA. No hybridization was observed with RNA from uninfected cells (Fig. 4C, lanes 1 and 4).

In summary, our results demonstrate that degenerate primer sets of sense and antisense primers to sequences representing common ORF 1b protein segments (Fig. 1) can be used to detect the genomes of all members of this group of positive-stranded RNA viruses. Of special interest is the finding that these degenerate primer sets generated products from the genomes of three viruses, namely PRRSV-VR2332, SHFV and the vaccine strain of EAV, for which no ORF 1b sequence information was available so that their sequences could not be incorporated into the design of the degenerate primer sets. This finding is not only significant in the case of SHFV, a quite distinct virus, but also in the case of PRRSV, since the known 3' end of the genome of PRRSV-VR2332 differs considerably from that of PRRSV-LV (by about 50%; Murtaugh et al., 1995). Similarly, the sequence of the EAV vaccine strain used in the present study may differ considerably from that of the wild-type Bucyrus strain that has been sequenced (den Boon et al., 1991) since the vaccine strain has been passaged about 300 times through cultures of various types of cells (Timoney and McCollum, 1993). It is also of interest that in the case of the SHFV genome, the degenerate primer set designed from genome sequences of LDV, PRRSV-LV and EAV yielded no RT/PCR product but that the primer set designed for the same genome segments on the basis of human codon preferences did yield products

which exhibited strong sequence homology with the equivalent segments of ORFs 1b of the other viruses of this group.

These results, therefore, indicate that these degenerate primer sets and others that can be readily designed from ORF 1b protein segments with identical amino acids (Fig. 1) might be useful in the search for related viruses in other species. This would be a novel approach for detecting existing unrecognized viruses with potential pathogenicity or new emerging viruses. In the case of the LDV/EAV/PRRSV/SHFV group, such a search may be favored by the fact that these viruses are known to cause long-term persistent infections.

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