Sequence variation divides *Equine rhinitis B virus* into three distinct phylogenetic groups that correlate with serotype and acid stability

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Equine rhinitis B virus (ERBV), genus *Erbovirus*, family *Picornaviridae*, occurs as two serotypes, ERBV1 and ERBV2, and the few isolates previously tested were acid labile. Of 24 ERBV1 isolates tested in the studies reported here, 19 were acid labile and five were acid stable. The two available ERBV2 isolates, as expected, were acid labile. Nucleotide sequences of the P1 region encoding the capsid proteins VP1, VP2, VP3 and VP4 were determined for five acid-labile and three acid-stable ERBV1 isolates and one acid-labile ERBV2 isolate. The sequences were aligned with the published sequences of the prototype acid-labile ERBV1.1436/71 and the prototype ERBV2.313/75. The three acid-stable ERBV1 were closely related in a phylogenetic group that was distinct from the group of six acid-labile ERBV1, which were also closely related to each other. The two acid-labile ERBV2 formed a third distinct group. One acid-labile ERBV1 had a chimeric acid-labile/acid-stable ERBV1 P1 sequence, presumably because of a recombination event within VP2 and this was supported by SimPlot analysis. ERBV1 rabbit antiserum neutralized acid-stable and acid-labile ERBV1 isolates similarly. Accordingly, three distinct phylogenetic groups of erboviruses exist that are consistent with serotype and acid stability phenotypes.

INTRODUCTION

Three acid-labile equine picornavirus serotypes isolated from horses with acute upper respiratory disease were originally classified as members of the genus *Rhinovirus*, family *Picornaviridae*. The first recognized serotype, now called *Equine rhinitis A virus* (ERAV), formerly Equine rhinovirus 1, was reclassified in the genus *Aphthovirus*, family *Picornaviridae* as the only non-*Foot-and-mouth disease virus* (FMDV) member of that genus (Pringle, 1999). The other two, ERBV1 and ERBV2, which are serotypes of *Equine rhinitis B virus* (ERBV), have been isolated in Switzerland, the UK, the USA, Canada and Japan over an approximately 30 year period (Carman *et al.*, 1997; Fukunaga *et al.*, 1983; McCollum & Timoney, 1992; Mumford & Thomson, 1978; Steck *et al.*, 1978). ERBV1- and ERBV2-neutralizing antibodies are present in approximately 50–80 % of horses in Switzerland, the UK, Canada, New Zealand, the Netherlands and the United Arab Emirates (Burrows, 1979; Carman *et al.*, 1997; de Boer *et al.*, 1979; Holmes *et al.*, 1978; McCollum & Timoney, 1992; Rose *et al.*, 1974; Wernery *et al.*, 1998) and similar rates occur in Australian horses (W. D. Black, unpublished data). Both viruses have been isolated from horses with acute febrile respiratory disease with clinical signs that include fever to 41 °C for 1–3 days, serous nasal discharge, anorexia, oedema of the legs, lethargy, and pain and swelling of the lymph nodes of the head and neck. Untreated, there is often secondary bacterial infection predisposing to abscessation of regional lymph nodes. ERBV1 and ERBV2 subclinical infection and subsequent seroconversion have also been recognized (Burrell *et al.*, 1996; Steck *et al.*, 1978).

Based on genomic sequence (Wutz *et al.*, 1996), ERBV1.1436/71, formerly Equine rhinovirus 2, was recognized as the prototype ERBV of a new genus *Erbovirus* (Pringle, 1999). The genetically related but serologically distinct ERBV2.313/75 (Huang *et al.*, 2001), formerly Equine rhinovirus 3, was recognized as the only other member of this genus (King *et al.*, 2003b). The family *Picornaviridae* comprises nine recognized genera. The definitions of these nine genera are based on characteristics including host range, disease, phylogenetic relatedness and biophysical properties such as virion density, acid stability and antigenicity (King *et al.*, 2000, 2003a, b; Mayo, 2002; Pringle, 1999). Historically, the property used to distinguish rhinoviruses from enteroviruses was acid stability, in that typically enteroviruses survive the acid conditions of the stomach and infect the gut, whereas rhinoviruses are rapidly inactivated below pH 6 and are associated only with infections of the upper respiratory tract (Couch, 1990; Hamparian, 1979).

The GenBank/EMBL/DDBJ accession numbers of the sequences reported in this paper are AY606988–AY606998.

Picornaviruses such as human rhinoviruses (HRV) and FMDV vary greatly in nucleotide sequence and can be classified into genetic lineages according to time and geographical boundaries (Knowles & Samuel, 2003; Savolainen *et al.*, 2002a, b). The Swiss prototype (P) isolates ERBV1.1436/71 and ERBV2.313/75 (Steck *et al.*, 1978) may not be representative of the spectrum of erboviruses that exist in nature in diverse locations and with time of isolation.

The virus capsid structural proteins, VP1, VP2, VP3 and VP4, are encoded by the P1 region of the virus genome and are associated with the various biophysical and serological characteristics of picornavirus capsids. There is a precedent for using P1 region nucleotide or amino acid sequence analysis in studies of picornaviruses including the molecular epidemiology and phylogeny of enteroviruses, *Hepatitis A virus* (HAV), FMDV, HRV, ERAV, ERBV1 and ERBV2 (Huang *et al.*, 2001; Knowles & Samuel, 2003; Oberste *et al.*, 1999a; Robertson *et al.*, 1992; Savolainen *et al.*, 2002a; Varrasso *et al.*, 2001; Wutz *et al.*, 1996). Rhinoviruses (Savolainen *et al.*, 2002a, b) and teschoviruses (Zell *et al.*, 2001) each form complex cross-neutralizing groups that are phylogenetically related to various degrees and these may be an example of how picornaviruses such as erboviruses might exist. In the studies presented here, the P1 nucleotide sequence was determined and the antigenic and biophysical properties of a number of erboviruses isolated over a period of years and from different geographical locations were characterized. It was found that sequence variation among erbovirus isolates places them in three distinct groups that correlate with acid stability and serotype.

METHODS

Viruses and cells. ERAV and ERBV isolates used in this study are listed in Table 1. A stock of each virus was prepared by inoculation of monolayer cell cultures of RK13 cells (passage 195–250) and at 75–100 % cytopathic effect, flasks were frozen. Samples were then thawed and the cell culture lysates were clarified by centrifugation, ampouled and stored at −70 °C. Cells were grown in Eagle's minimal essential medium (MEM; Sigma-Aldrich) with 10 mM $NaHCO_3$ and 50 μg ampicillin (Sigma) ml^{-1} with 2 % fetal bovine serum (FBS; CSL). For the large-scale culture and purification of ERBV1.1436/71, Vero cells were used.

Virus stability at acid pH. Samples (30 μl) of each virus were added separately to 30 μl 100 mM HEPES at pH 3·0 then mixed. As a control, 30 μl of each virus was similarly added to 100 mM HEPES at pH 7·0. The assay was similar to that used in previous studies of acid stability among equine rhinitis viruses (Mumford &

Table 1. Origins of equine rhinitis viruses

Serotype	Isolate/year	Origin	Reference
ERBV1	1436/71	Berne, Switzerland	Steck *et al.* (1978)
	201/71	Berne, Switzerland	Steck *et al.* (1978)
	208/71	Berne, Switzerland	Steck *et al.* (1978)
	917/71	Berne, Switzerland	Steck *et al.* (1978)
	955/71	Berne, Switzerland	Steck *et al.* (1978)
	1014/71	Berne, Switzerland	Steck *et al.* (1978)
	11/74	Berne, Switzerland	Steck *et al.* (1978)
	293/74	Berne, Switzerland	Steck *et al.* (1978)
	322/74	Berne, Switzerland	Steck *et al.* (1978)
	328/74	Berne, Switzerland	Steck *et al.* (1978)
	513/74	Berne, Switzerland	Steck *et al.* (1978)
	263/75	Berne, Switzerland	Steck *et al.* (1978)
	271/75	Berne, Switzerland	Steck *et al.* (1978)
	622/75	Berne, Switzerland	Steck *et al.* (1978)
	367/75	Berne, Switzerland	Steck *et al.* (1978)
	379/75	Berne, Switzerland	Steck *et al.* (1978)
	426/75	Berne, Switzerland	Steck *et al.* (1978)
	NAV II/88	Kentucky, USA	W. H. McCollum and others, unpublished
	NS-CW/88	New Hampshire, USA	W. H. McCollum and others, unpublished
	57-14/89	Kentucky, USA	W. H. McCollum and others, unpublished
	58-13/89	Kentucky, USA	W. H. McCollum and others, unpublished
	83-11/89	Kentucky, USA	W. H. McCollum and others, unpublished
	9051-7/89	Kentucky, USA	W. H. McCollum and others, unpublished
	KP/92	Massachusetts, USA	D. F. Holmes, unpublished
ERBV2	313/75	Berne, Switzerland	Steck *et al.* (1978)
	1576/99	Melbourne, Australia	W. D. Black and others, unpublished
ERAV	393/76	Morphetville, Australia	Studdert & Gleeson (1978)

Thomson, 1978). After the addition of virus in MEM, the pH increased to pH 3·6. This is below pH 6·0, the pH at which ERBV1.1436/71 is inactivated (Newman *et al.*, 1977). The mixtures were incubated at room temperature for 1 h, then 60 µl 200 mM HEPES at pH 7·0 was added to each sample. The samples were serially diluted in 10-fold steps in Leibowitz' L-15 medium (ICN Sigma-Aldrich) with 20 mM $MgCl_2$, 50 µg ampicillin ml^{-1} and 2 % FBS. Virus titres were determined using 96-well microtitre trays and RK13 cells in L-15 medium with four replicates for each virus dilution (Studdert *et al.*, 1970). Virus titres were calculated as $TCID_{50}$ ml^{-1} (Karber, 1931). Acid stability was defined as the loss of $<10^3$ $TCID_{50}$ ml^{-1} at pH 3·6 for $\geqslant$1 h relative to the control at pH 7·0 (Mumford & Thomson, 1978).

RT-PCR. Viral RNA was prepared from virus-infected cell culture supernatants using a QIAamp Viral RNA extraction kit according to the manufacturer's instructions (Qiagen). Reverse transcription (RT) was performed using Superscript II RNaseH− reverse transcriptase according to the manufacturer's instructions (Invitrogen). PCRs were prepared with a 25 µl final reaction volume with 400 nM of forward and reverse primers as listed in Table 2, 1 U HiFi Platinum *Taq* polymerase (Promega), reaction buffer as supplied, 200 µM of each of dATP, dCTP, dGTP and dTTP, and 1 µl of a 1/10 dilution of cDNA template. PCR thermocycler conditions were: 60 s at 95 °C, then 35 cycles of 60 °C for 30 s and 68 °C for 60 s per kbp of expected product.

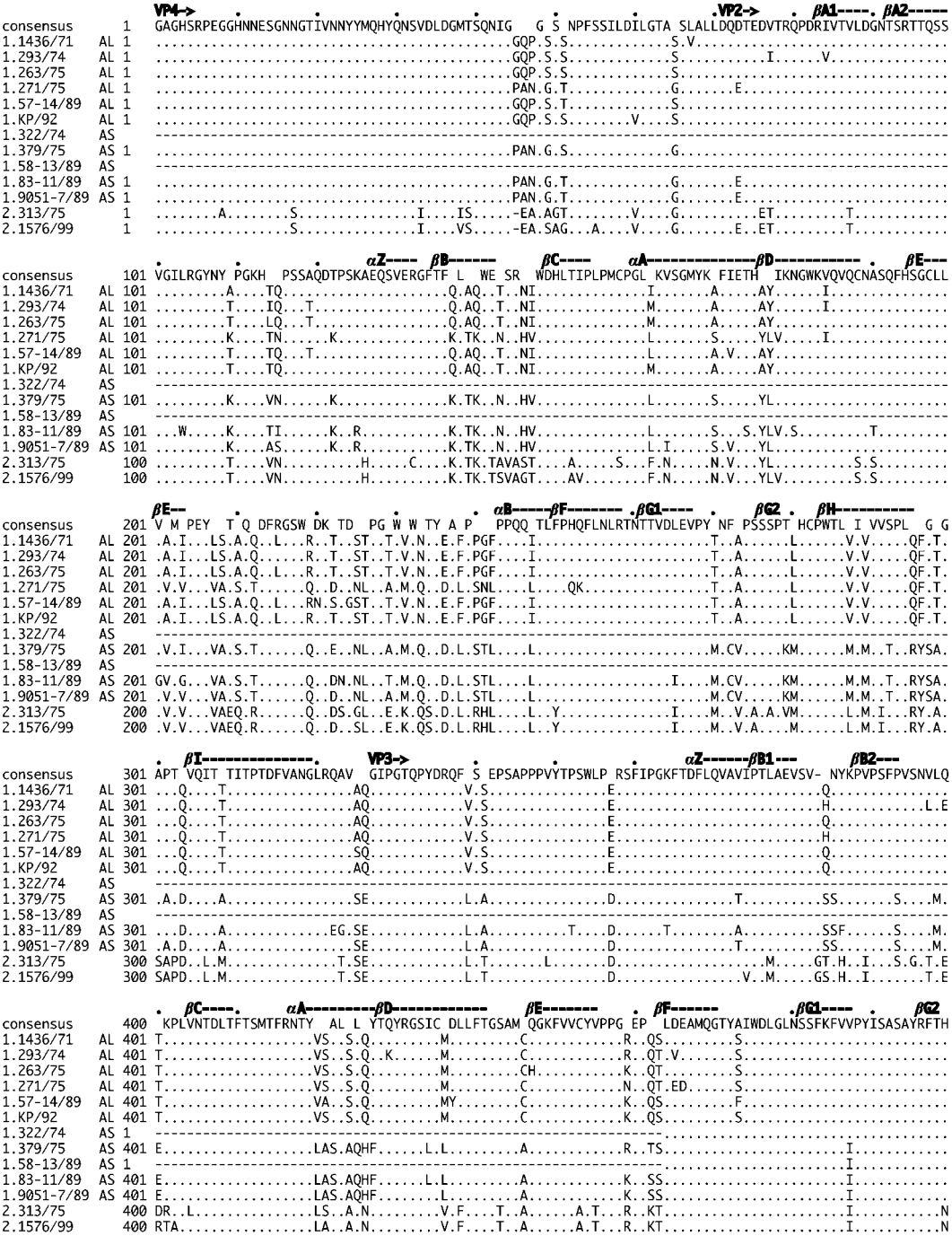
Cloning and sequencing of PCR products. PCR products were separated by agarose gel electrophoresis and extracted using a QIAquick kit according to the manufacturer's instructions (Qiagen). Purified DNA samples amplified from ERBV1 isolates 271/75 and 293/74 were then A-tailed using *Taq* polymerase (Sambrook *et al.*, 1989) and ligated into pGEM-T according to the manufacturer's instructions (Promega). Ligated products were transformed into *Escherichia coli* DH5α cells using a Gene Pulser (Bio-Rad). Plasmid DNA was purified from three separate insert-positive clones using Flexi-Prep kits according to the manufacturer's instructions (Amersham). All other virus isolates were sequenced in triplicate directly from PCR products. Big-Dye Terminator versions 2 and 3.1 sequencing reaction kits were used according to the manufacturer's instructions (Applied Biosystems) and processed using Perkin Elmer Sequencer model 377 machines (Australian Genome Research Facility). Plasmid DNA was initially sequenced in triplicate using vector-specific primers (Promega). PCR products were initially sequenced with the primers used to amplify the DNA (Table 2). Sequences were analysed by the SeqEdit software package (version 1.01; Applied Biosystems) and the GeneWorks software package (version 2.5; Oxford Molecular Group). Specific primers were designed based on previous sequencing results and sequencing was repeated in triplicate using the original DNA template material with at least 50 bp overlap between each sequencing dataset.

Phylogenetic analysis. Nucleotide sequences were translated into amino acid sequences using the standard table of mammalian codons, and amino acid sequence alignments were generated by the CLUSTAL_X 1.83 software package (Thompson *et al.*, 1997) using 'gap' and 'gap extension' penalties of 3·0 and 0·1, respectively (Fig. 1). Data from these alignments were then used to generate matrices of amino acid sequence identities and similarities (%) by the MacBoxshade software package (version 2.15E; Institute for Animal Health, Pirbright, UK). Phylogenetic analysis was performed on the aligned amino acid sequences using the CLUSTAL_X 1.83 software package with 1000 bootstrapping replicates and data were converted into phylogenetic trees with the TREEVIEW 1.6.6 software package (Page, 1996). Predictions of the cleavage sites of the erbovirus polyprotein were based on predictions of the likelihood of aphthovirus $3C^{pro}$ and $3CD^{pro}$ cleavage of amino acid sequences (cleavability score) and the likelihood of amino acid sequences being

Table 2. Oligodeoxynucleotide primers

Nucleotide position*	Genomic region†	Sequence (5′–3′)	F/R‡
908	5′ UTR	TTGTTCTATGGTGACRATGGC	F
1379	L	GCACGGGTAGACAAGTGGCT	F
2255	VP2	TTCCTGGTGAGTGGAAGTGG	F
2301	VP2	CCCCAGCAACTAACACTC	F
2315	VP2	CTTACACTTTACCCGCATCAGTT	F
2395	VP2	ACIARAGTCCAIGGRCARTG	R
2557	VP3	TCGGCTGAAAGAAACTGCCTGTC	R
2651	VP3	CTTCATACCAGGAAAGTTCAC	F
2700	VP3	GTTCCCACTATGGCTGAAG	F
2768	VP3	CATAGAGGTAAAGGTTAAGTCTG	R
2861	VP3	TATAGRGGITCIATTTGYATGGA	F
2974	VP3	TAGAATTTAAACCCAGATCCCA	R
3080	VP3	CGCATCAGCCTACCGTTTCAC	F
3369	VP1	CTTTTCAGGCTTTGCCAACTC	F
3566	VP1	ACGGGGCGCCANCCNACCC	R
3740	VP1	CAAGTGCAGATTTCCATTCC	F
3863	VP1	CAGGCAACAARTYAACATTGC	R
4219	2A	GCCGGGGTTAAGTTCAACATC	R
4244	2B	CCGGGCATCACTGACCAAATAG	R

*Nucleotide position of the 3′ end of the primer relative to the ERBV1.1436/71 sequence (Wutz *et al.*, 1996).
†Genomic region where primer binds.
‡F, Forward, sense primer; R, reverse, antisense primer.



on the protein surface (surface score) using the NetPicoRNA neural net algorithm (Blom *et al.*, 1996). Analysis of the putative recombinant amino acid sequence (see Fig. 3) was with the SimPlot software package (Lole *et al.*, 1999).

Antiserum. Rabbit antiserum to purified ERBV1.1436/71 was prepared in a New Zealand White rabbit. Purified ERBV1.1436/71 (50 μg) in 500 μl was injected intradermally at several sites into a 6-month-old rabbit. The rabbit was inoculated with UV-inactivated virus in Freund's complete adjuvant (Sigma) on day 0, and on days 21 and 42 the rabbit was similarly inoculated with inactivated virus in Freund's incomplete adjuvant. On day 119, the rabbit was boosted with purified live virus without adjuvant. Blood was taken on day 161 and the serum was stored in 500 μl aliquots at −20 °C.

Serum neutralization (SN) assay. SN assays were performed as previously described (Studdert *et al.*, 1970). Briefly, sera were diluted 1 : 10 in medium and inactivated for 30 min at 56 °C. Sera were diluted in twofold steps then added to 96-well microtitre trays before the addition of 50 μl containing approximately 100 $TCID_{50}$ of each virus. The mixtures were incubated at 37 °C for 30 min before the addition of RK13 cells.

Accession numbers. As part of this study the following erbovirus P1 sequences were determined and submitted to GenBank/EMBL/DDBJ and are used within the Figures and Tables presented: ERBV1.293/74 (AY606988), ERBV1.322/74 (partial P1 sequence only; AY606989), ERBV1.263/75 (AY606990), ERBV1.271/75 (AY606991), ERBV1.379/75 (AY606992), ERBV1.KP/92 (AY606993),

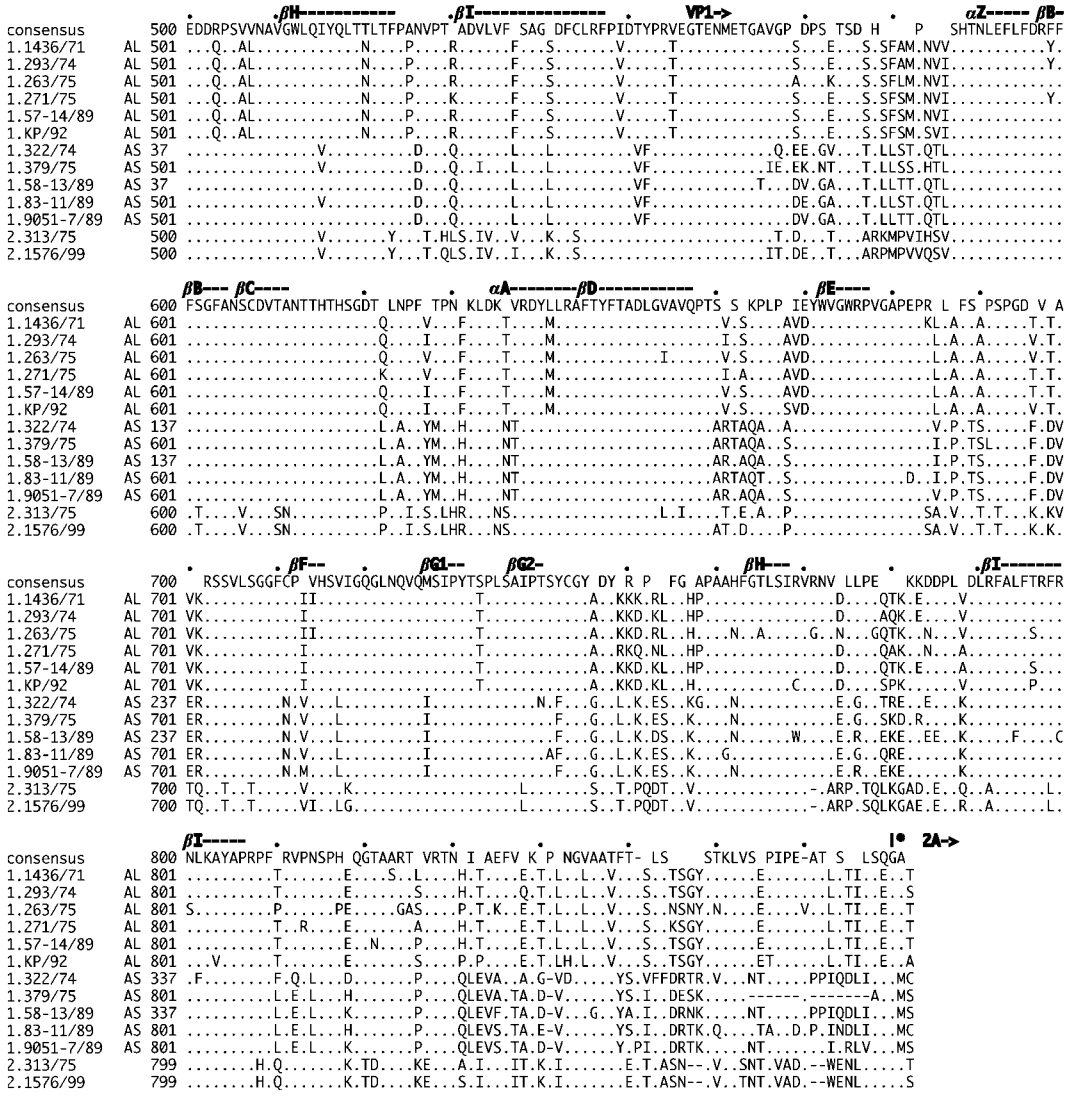


Fig. 1. Alignment of P1 amino acids of 11 ERBV1 isolates (prefixed by 1) and two ERBV2 isolates (prefixed by 2). The viruses used included six acid-labile (AL) ERBV1 isolates and five acid-stable (AS) ERBV1 isolates. The putative N termini of structural proteins (VP→), the putative α-helices and β-sheet regions of ERBV1.1436/71 (Wutz *et al*., 1996) (T. Skern, personal communication) and the newly proposed VP1/2A cleavage site (|*) are indicated. Also indicated are gaps (-) due to alignment incompatibility or due to lack of sequence data (the N terminus of isolates 322/74 and 58-13/89 only). Conserved amino acids are also indicated by a dot (.).

ERBV1.57-14/89 (AY606994), ERBV1.58-13/89 (partial P1 sequence only; AY606995), ERBV1.83-11/89 (AY606996), ERBV1.9051-7/89 (AY606997) and ERBV2.1576/99 (AY606998). The GenBank/EMBL/DDBJ accession numbers of other viruses used are: ERBV1.1436/71 (NC_003983) (Wutz *et al*., 1996) and ERBV2.313/75 (NC_003077) (Huang *et al*., 2001).

RESULTS

Acid-labile and acid-stable ERBV1 phenotypes

Virus isolates from Australia, Switzerland and the USA that were previously classified as ERBV1 (P1436/71-like) and ERBV2 (P313/75-like) by SN assays (Table 1) were tested for acid stability. Of 24 ERBV1 isolates and two ERBV2 isolates, five ERBV1 isolates were stable after being held at pH 3·6 for 1 h at room temperature. The five acid-stable ERBV1 isolates were: ERBV1.322/74, ERBV1.379/75, ERBV1.58-13/89, ERBV1.83-11/89 and ERBV1.9051-7/89 (Table 3).

Erboviruses cluster into three distinct phylogenetic groups

Virus isolates selected for sequencing were chosen to best represent diverse geographical locations (Australia,

Table 3. Stability of ERBV isolates at pH 3·6 for 1 h

Serotype	Isolate/year	Virus titre*		Acid stability
		pH 7·0	pH 3·6	
ERBV1	1436/71	7·4	<2·7	−
	201/71	6·9	<2·7	−
	208/71	5·8	<2·7	−
	917/71	7·2	<2·7	−
	955/71	7·4	<2·7	−
	1014/71	7·7	<2·7	−
	11/74	5·7	<2·7	−
	293/74	7·4	<2·7	−
	322/74	6·2	3·9	+
	328/74	6·9	<2·7	−
	513/74	6·2	<2·7	−
	263/75	6·2	<2·7	−
	271/75	7·2	<2·7	−
	622/75	6·6	<2·7	−
	367/75	6·0	<2·7	−
	379/75	6·9	5·4	+
	426/75	6·2	<2·7	−
	NAV II/88	6·4	<2·7	−
	NS-CW/88	5·7	<2·7	−
	57-14/89	7·2	<2·7	−
	58-13/89	7·4	7·2	+
	83-11/89	6·8	5·8	+
	9051-7/89	5·7	5·4	+
	KP/92	6·9	<2·7	−
ERBV2	313/75	6·9	<2·7	−
	1576/99	6·7	<2·7	−

*Measured as $\log_{10}$ $TCID_{50}$ ml^{-1}.

Switzerland and USA), year of isolation (1971–1999), serotype (ERBV1 and ERBV2) and the acid stability phenotype. The complete P1 nucleotide sequences of nine virus isolates and two partial P1 nucleotide sequences (from within VP3 to 2A) of ERBV1.322/74 and ERBV1.58-13/89 were determined, and the amino acid sequences were deduced and aligned with the published sequences of ERBV1.1436/71 and ERBV2.313/75 (Fig. 1). The predicted protein secondary structures indicated above the amino acid sequence alignments in Fig. 1 were as previously described for ERBV1.1436/71 (Wutz *et al.*, 1996) (T. Skern, personal communication).

Amino acids were less conserved between isolates in regions predicted to be within the surface loops than those in the predicted α-helices and β-sheets comprising the structural elements of the capsid proteins. The C terminus of VP1 contains a deletion of 14 aa in the acid-stable ERBV1.379/75 relative to ERBV1.1436/71, whereas other acid-stable ERBV1 isolates, ERBV1.83-11/89 and ERBV1.9051-7/89, had 1 or 0 aa deletions, respectively (Fig. 1). ERBV2.313/75 and ERBV2.1576/99 both had deletions of 4 aa, relative to ERBV1.1436/71, in the VP1 C terminus alone.

When the complete P1 amino acid sequences of isolates within each of the three groups were compared there was 93–94 % identity between the three acid-stable ERBV1 isolates, 90–97 % identity between the six acid-labile ERBV1 isolates and 94 % identity between the two ERBV2 isolates. When the P1 amino acid sequences were compared between each of the groups, there was 75–82 % identity between the acid-stable ERBV1 and the acid-labile ERBV1 isolates, 73–75 % identity between the acid-stable ERBV1 and the ERBV2 isolates, and 71–74 % identity between the acid-labile ERBV1 and the ERBV2 isolates. Amino acid variation between the three groups tended to cluster in the predicted VP1 C terminus and the VP2 and VP1 βE-βF surface loops.

To obtain additional sequence data for acid-stable ERBV1 isolates, the entire sequence of the VP1 region (approx. 972 nt) and part of the VP3 region (approx. 279 nt) of two other acid-stable ERBV1 isolates (ERBV1.322/74 and ERBV1.58-13/89) was determined. The deduced amino acid sequences of these two acid-stable isolates (Fig. 1) had 89–92 % identity with acid-stable ERBV1 isolate sequences and had less than 72 % identity with acid-labile ERBV1 and ERBV2 isolate sequences. It was concluded that all five acid-stable viruses were more similar to each other than they were to the acid-labile ERBV1 or ERBV2.

A phylogenetic tree based on the complete P1 region amino acid sequences of three acid-stable ERBV1, five acid-labile ERBV1 and two ERBV2 isolates was determined (Fig. 2). Three distinct groups of erboviruses were apparent. This tripartite arrangement of erboviruses contrasts with the single phylogenetic group of ERAV isolates previously recognized in studies of 10 ERAV sequences (Varrasso *et al.*, 2001). Consistent with the results of amino acid identity

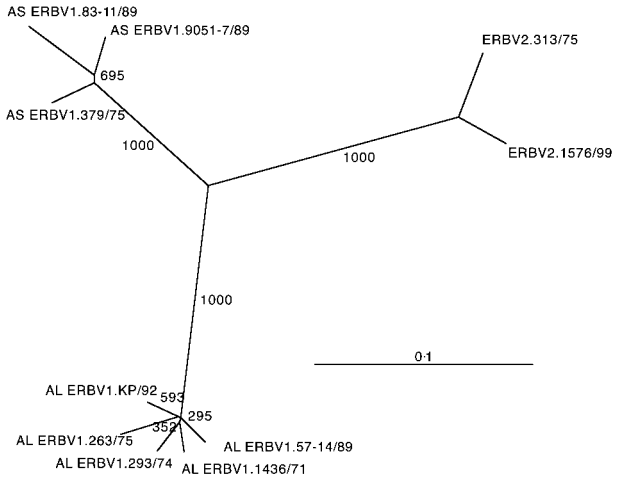


Fig. 2. Phylogenetic neighbour-joining tree using 1000 bootstrap replicates based on the deduced, complete P1 amino acid sequences of three acid-stable (AS) ERBV1, five acid-labile (AL) ERBV1 and two ERBV2 isolates.

and similarity, the three erbovirus phenotypes clustered into three distinct and separate phylogenetic branches, in which acid-stable ERBV1 and acid-labile ERBV1 P1 amino acid sequences were almost as phylogenetically distant from each other as they were from ERBV2 sequences. Furthermore, there was less conservation of P1 amino acid sequences within each individual phylogenetic erbovirus group than within the group of 10 ERAV sequences (Varrasso *et al.*, 2001). SimPlot analysis of each of the ERBV isolates for which complete P1 amino acid sequences are known was performed, but for clarity, only a representative number of sequences from each ERBV phylogenetic group and the putative recombinant ERBV1.271/75 are shown (Fig. 3). Regions of higher and lower amino acid similarity were observed over the length of these sequences, typically correlating to structural and non-structural loop regions, respectively, but it was apparent that the sequences were distributed in groups of acid-stable ERBV1, acid-labile ERBV1 and ERBV2. One exception was acid-labile ERBV1.271/75, which had high amino acid similarity to acid-stable ERBV1 sequences N-terminal of approximately midway into VP2 (approx. 95 %), but low amino acid similarity to acid-stable ERBV1 sequences C-terminal of VP2 (approx. 80 %).

Antigenic relationships among erboviruses

The antigenic relationships among 11 selected erbovirus isolates using ERBV1.1436/71 rabbit antiserum are shown in Table 4. The ERBV1 rabbit antiserum neutralized acid-stable ERBV1 isolates and acid-labile ERBV1 isolates similarly. The ERBV1 rabbit hyperimmune antiserum neutralized ERBV2 isolates to approximately 1/10 of the SN antibody titre at which ERBV1 isolates were neutralized.

Table 4. Neutralization of equine rhinitis viruses by ERBV1 rabbit antiserum

Virus type/phenotype	Isolate/year	ERBV1 rabbit antiserum*
Acid-labile ERBV1	1436/71	1 778
	293/74	562
	263/75	3 162
	271/75	1 778
	57-14/89	5 623
	KP/92	3 162
Acid-stable ERBV1	322/74	1 778
	379/75	1 778
	83-11/89	3 162
	9051-7/89	1 778
ERBV2	313/75	178
	1576/99	178
ERAV	393/76	<100

*Reciprocal of the highest dilution of serum that neutralized 63–200 $TCID_{50}$ of virus.

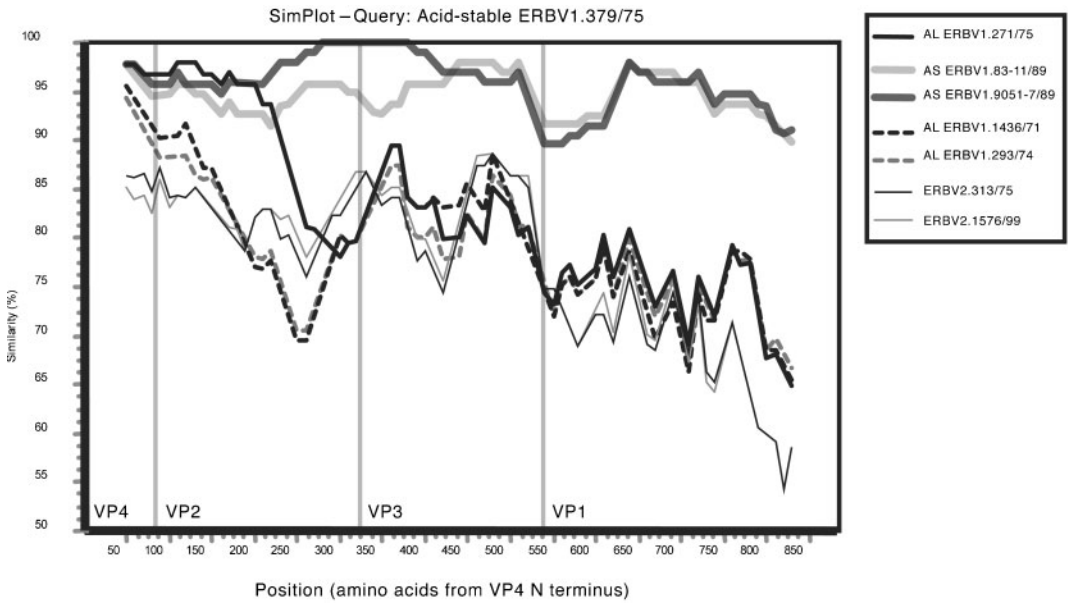


Fig. 3. SimPlot analysis showing amino acid similarity (%) of the complete P1 region deduced amino acid sequences of three of six acid-labile ERBV1 (including the putative recombinant acid-labile ERBV1.271/75), two acid-stable ERBV1 and two ERBV2 isolates. Acid-stable ERBV1.379/75 was used as the reference sequence against which all other sequences were compared using a moving mean window of 100 aa in 10 aa increments without Jukes–Cantor correction or gap-stripping. The approximate locations of the VP4, VP2, VP3 and VP1 N termini are indicated by vertical lines.

DISCUSSION

Two ERBV1 isolates from Berne, Switzerland, 1974–1975, and three ERBV1 isolates from Kentucky, USA, 1989, were unexpectedly found to be acid stable, remaining infectious after 1 h at pH 3·6. With further investigation, the complete and partial P1 nucleotide sequences of five acid-stable ERBV1, six acid-labile ERBV1 and one ERBV2 were determined and compared with the sequences of the two prototype viruses [ERBV1.1436/71 (Wutz *et al.*, 1996) and ERBV2.313/75 (Huang *et al.*, 2001)]. These 13 sequences segregate into three phylogenetic groups that correlate with acid stability phenotype and serotype. These groups are acid-labile ERBV1 and ERBV2, and acid-stable ERBV1. There was approximately 90–97 % amino acid identity between the ERBV P1 sequences within each ERBV phylogenetic group and approximately 71–82 % amino acid identity between all 13 ERBV P1 sequences, compared with >96·8 % amino acid identity between 10 ERAV P1 sequences (Varrasso *et al.*, 2001). Originally, only a few members of the ERBV and ERAV serotypes were tested for acid stability and since all were inactivated at pH 6·0 or below these viruses were classified as members of the genus *Rhinovirus* and named accordingly (Mumford & Thomson, 1978; Plummer, 1962; Steck *et al.*, 1978). In subsequent studies, serotype alone was used to classify new isolates (Fukunaga *et al.*, 1983; McCollum & Timoney, 1992). Other acid-stable picornaviruses have been isolated from horses in the UK, Germany and Japan (Bohm, 1964; Fukunaga *et al.*, 1983; Mumford & Thomson, 1978). These are included within the group of unclassified picornaviruses (King *et al.*, 2003a) and are members of a serotype distinct from ERBV1 and ERBV2 (Fukunaga *et al.*, 1983; Mumford & Thomson, 1978).

Amino acid variation between erbovirus isolates occurred throughout the P1 sequence, but tended to occur more often in the predicted VP1 C terminus and the VP2 and VP1 βE-βF surface loops compared with the predicted α-helix and β-sheet structural elements. This is consistent with findings for other picornaviruses, including ERAV (Varrasso *et al.*, 2001). Whereas the precise amino acids responsible for conferring either acid stability or serotype specificity are unknown, the alignment of deduced erbovirus amino acid sequences (Fig. 1) shows clusters of amino acids that are conserved within phylogenetic subgroups, but vary between these subgroups, such as the N and C termini and the βC-αA loop of VP1, the βE-βF loops of VP1 and VP2, the βB-βC loop of VP2, the αA helices of VP1, VP2 and VP3, and the βH-βI loop of VP3.

There were many differences in the sequences of the C termini of VP1 between all sequenced isolates, including a 14 aa deletion in ERBV1.379/75 (Fig. 1). There were far fewer differences in the adjacent sequences, such as the βI sheet of VP1 (Fig. 1). Several regions had high levels of amino acid similarity (up to 100 %), as shown by SimPlot analysis of the aligned sequences (Fig. 3), but overall there was a general trend towards lower amino acid similarity (down to approximately 65 %) at the C terminus of VP1. The C terminus of VP1 extends beyond the surface of picornaviruses and therefore is not constrained by interaction with other structural proteins. Hence, these amino acid sequences are more free to drift randomly than those in more defined structural regions and may also interact with neutralizing antibodies, which may represent a selective pressure driving the divergence of the amino acid sequences within this region (Luo, 1997; Xie *et al.*, 1987).

Interestingly, the acid-labile ERBV1.271/75 has an amino acid sequence for VP4 and N-terminal of the βF sheet of VP2 that is more consistent with the acid-stable ERBV1 isolates within the alignment of amino acid sequences (Fig. 1) and by SimPlot analysis (Fig. 3). The amino acid sequence from the βF sheet of VP2 to the C terminus of VP1 is more consistent with the group of acid-labile ERBV1 isolates and is consistent with a recombination event within the βF sheet of VP2. Recombination events yielding chimeras have been observed in other picornaviruses such as HAV (Lemon *et al.*, 1991), poliovirus (Blomqvist *et al.*, 2003) and other enteroviruses (Martin *et al.*, 2002). That the ERBV1.271/75 recombination event occurred within the βF sheet of VP2 while the acid-labile phenotype was retained suggests that the acid-stable phenotype is associated with the amino acid sequences C-terminal of this site.

Erboviruses, like several other picornaviruses such as cardioviruses, aphthoviruses and teschoviruses, possess a 2A protein that mediates the 2A/2B cleavage by a unique mechanism and the VP1/2A junction is cleaved by $3C^{pro}$, which primarily cleaves between Gln/Gly or Glu/Gly residue pairs (Hughes & Stanway, 2000; Palmenberg, 1990; Ryan & Flint, 1997; Wutz *et al.*, 1996). It is noted that VP1/2A cleavage site of ERBV1.1436/71 predicted by Wutz *et al.* (1996) as Thr/Asn, which has a predicted cleavability score of 0·137 and a surface score of 0·616 (Blom *et al.*, 1996), is not conserved among the 13 erbovirus sequences (Fig. 1). However, three residues upstream of the originally predicted cleavage site, the Glu/Gly residues (cleavability score 0·443, surface score 0·746) or Gln/Gly residues (cleavability score 0·370, surface score of 0·655), are conserved among all 13 erbovirus sequences and are therefore more likely to be the cleavage site. This is supported by amino acid sequence comparisons with other picornaviruses (Doherty *et al.*, 1999) and implies that the 2A protein may be 19 aa, rather than 16 aa as predicted (Wutz *et al.*, 1996).

The cross-neutralization between acid-labile and acid-stable ERBV1 isolates indicates a close antigenic relationship between these erboviruses, despite obvious phenotypic and phylogenetic differences. These results are supported by studies of antigenic relationships using ERBV1 and ERBV2 antisera from naturally infected horses (data not shown).

In enteroviruses, VP1 sequence analysis has been used as a molecular surrogate for antigenic typing, since the VP1 sequence correlates to some extent with serotype (Oberste *et al.*, 1999b; Rico-Hesse *et al.*, 1987) and analysis of a 450 nt

region of over 100 prototype strains and clinical isolates revealed that, with one exception, homologous strains were >75 % identical to each other (Oberste *et al.*, 1999a). Among 152 strains of HAV, seven distinct phylogenetic groups (genotypes) were identified with >85 % nucleotide sequence identity within the VP1-2A junction region in each genotype (Robertson *et al.*, 1992). For FMDV, phylogenetic analysis of the C-terminal 128 aa of VP1 and adjacent 7 aa of the 2A of 51 SAT3-type viruses (Bastos *et al.*, 2003) or the nucleotides of VP1 of a large number of viruses as reviewed by Knowles & Samuel (2003) indicated that there were distinct virus lineages (topotypes) differing in nucleotide identity by >20 % that were evolving independently in different geographical localities within each serotype. Given that the 11 complete (and two partial) erbovirus P1 region nucleotide sequences available have >77 % identity with other members of the same phylogenetic group (i.e. <23 % difference in identity) and <71 % identity with members of the two other phylogenetic groups (i.e. >29 % difference in identity), it appears that these erboviruses segregate into several distinct phylogenetic groups in a manner similar to enteroviruses, HAV, FMDV and presumably other picornaviruses.

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