Equine rhinitis B virus: a new serotype

Jin-an Huang,¹ Nino Ficorilli,¹ Carol A. Hartley,¹ Rebbecca S. Wilcox,¹ Marianne Weiss² and Michael J. Studdert¹

¹ Centre for Equine Virology, School of Veterinary Science, The University of Melbourne, Victoria 3010, Australia
² Institute of Veterinary Virology, University of Berne, Berne, Switzerland

Equine rhinovirus serotype 3 isolate P313/75 was assigned, with an unclassified genus status, to the family Picornaviridae. The sequence from the 5-prime poly(C) tract to the 3-prime poly(A) tract of P313/75 was determined. The sequence is 8821 bases in length and contains a potential open reading frame for a polyprotein of 2583 amino acids. Sequence comparison and phylogenetic analysis suggest that P313/75 is most closely related to the prototype equine rhinitis B virus (ERBV) strain P1436/71, formerly named equine rhinovirus type 2. A high degree of sequence similarity was found in the P2 and P3 regions of the two genomes. However, the deduced amino acid sequences of the P1 region of P313/75 and ERBV strain P1436/71 contained significant differences, which presumably account for the serological segregation of the two viruses. It is suggested that P313/75 can be classified as a new serotype of the genus Erbovirus, tentatively named ERBV2. Seroepidemiological data indicate that ERBV2 infection of horses may be common (24%) in Australia.

Equine rhinitis viruses (formerly named equine rhinoviruses) are known to cause clinical and subclinical upper respiratory infections in horses worldwide (Carman et al., 1997). Among those viruses that were formerly called equine rhinoviruses (classified as members of the family Picornaviridae, genus Rhinovirus), two serotypes were identified (Flammini & Allegri, 1970; Newman et al., 1973; Steck et al., 1978). Based on sequence analysis in particular (Li et al., 1996; Wutz et al., 1996), serotype 1 has been renamed equine rhinitis A virus (ERAV) and reclassified as a member of the genus Aphthovirus, whereas serotype 2 has been renamed equine rhinitis B virus (ERBV) and reclassified as the sole member of the new genus Erbovirus. In early studies, a third serotype designated P313/75 was tentatively identified (Steck et al., 1978). Serum neutralization assays indicated that P313/75 was not neutralized by either ERAV or ERBV antisera, although P313/75 is reported to possess similar physico-chemical properties (Steck et al., 1978). P313/75 is presently assigned to the family Picornaviridae, but has an unclassified genus status due to the lack of available sequence information.

In this study, we determined and analysed the sequence from the poly(C) to the poly(A) tract of P313/75. Based on the sequence similarity and serological data, we propose that P313/75, formerly known as equine rhinovirus serotype 3, should be classified as a distinct serotype of the genus Erbovirus and tentatively named ERBV2; the ERBV1 prototype is represented by strain P1436/71. We also provide preliminary seroepidemiological data indicating that ERBV2 commonly infects horses in Australia.

P313/75 was obtained at passage 7. The virus stock used in this study was treated with chloroform and further plaque-purified in rabbit kidney (RK13) cells. Viral RNA was extracted from a P313/75-infected cell lysate using the Viral RNA Extraction kit from Qiagen. The RNA was reverse-transcribed into cDNA using SuperScript II (Gibco), according to the supplier’s instructions. The cDNA was then used for PCR. Initially, two sets of primers based on the 3A and 3D sequences of the ERBV prototype strain P1436/71 were used. Other primers, including oligo(dT)13 and oligo(dC)16, were subsequently designed based on the sequence of P1436/71 or the newly acquired sequences of P313/75.

Primary RT–PCR experiments using primer sets based on the 3A and 3D regions of the ERAV.P393/76 and ERBV.P1436/71 genomes showed that only the ERBV primers produced PCR products from P313/75 (data not shown). Sequences of the amplified P313/75 3A and 3D regions were found to share about 90% nucleotide similarity with those of ERBV.P1436/71.

The possibility that P313/75 PCR products and sequences were obtained as a result of contamination of PCR reagents by...
Fig. 1. Unrooted phylogenetic trees inferred using the maximum-likelihood method for nucleotide sequences of (A) the P1 region and (B) the 3D region of viruses representing the nine genera of the Picornaviridae family. The computer software programs ECustalW, ESeqboot, EDnaML and EConsensus from the University of Wisconsin Genetics Computer Group were used to construct the trees. The sequences were aligned and bootstrapped. Data sets (100) from the bootstrap were used by EDnaML to infer phylogenetic trees. The final trees were derived from EConsensus and from comparison with published trees (Marvil et al., 1999). The genus to which each virus belongs is indicated in bold typeface. The viruses and the corresponding GenBank accession numbers for the sequences are: Erbovirus ERBV2.P313/75 (AF361253) and ERBV1.P1436/71 (X96871); Cardiovirus EMCV.pv21 (X74312) and TMEV.BeAn8386 (M16020); Aphthovirus FMDV.A12 (M10975), FMDV.C-S8 (AF274010), FMDV.01K (AF347668), FMDV.SAT2 (AI251473) and ERAV.393/76 (L43052); Hepatovirus HAV.HM-175 (M14707) and AEV.calnek (AI225173); Enterovirus EV-71 (U22521) and PV-2 (X00595); Rhinovirus HRV-14 (X01087); Parechovirus HPeV-2 (AI005695); Kobuvirus AiV (ABO10145); and Teschovirus PTV-1 (AJ011380).

Table 1. Relationship between ERBV2.P313/75 and some picornaviruses at the nucleotide and amino acid level

<table>
<thead>
<tr>
<th>Virus</th>
<th>5′ UTR</th>
<th>L</th>
<th>VP4</th>
<th>VP2</th>
<th>VP3</th>
<th>VP1</th>
<th>P2</th>
<th>P3</th>
<th>3′ UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERBV1.P1436/71</td>
<td>90</td>
<td>73</td>
<td>75</td>
<td>67</td>
<td>71</td>
<td>62</td>
<td>81</td>
<td>89</td>
<td>98</td>
</tr>
<tr>
<td>ERAV.393/76</td>
<td>48</td>
<td>40</td>
<td>50</td>
<td>51</td>
<td>53</td>
<td>53</td>
<td>43</td>
<td>50</td>
<td>48</td>
</tr>
<tr>
<td>EMCV</td>
<td>50</td>
<td>50</td>
<td>45</td>
<td>47</td>
<td>50</td>
<td>50</td>
<td>40</td>
<td>42</td>
<td>48</td>
</tr>
<tr>
<td>FMDV.01K</td>
<td>52</td>
<td>44</td>
<td>56</td>
<td>49</td>
<td>52</td>
<td>49</td>
<td>51</td>
<td>45</td>
<td>43.5</td>
</tr>
</tbody>
</table>

The percentage nucleotide identity and amino acid similarity (in parentheses) was calculated using the computer program Gap. The S fragment, poly(C) tract and poly(A) tail were not included in the calculations. The gaps between the sequences were not considered to be differences. The duplicated 3B sequences of FMDV were not removed in the percentage calculation. The GenBank accession numbers for the sequences are X96871 (ERBV1.P1436/71), AF347668 (ERAV.393/76), AJ74312 (EMCV) and X00871 (FMDV.01K).

an ERBV template was ruled out by further testing of the reagents used. The likelihood that ERBV was still present in the P313/75 pool was eliminated, as RT–PCR using ERBV-specific primers for regions within P1 failed to amplify the expected products. Cross-neutralization assays also showed that antisera to either ERAV or ERBV did not neutralize P313/75 (data not shown).

Based on the P313/75 3A and 3D sequence information and several ERBV primers, we were able to amplify segments of the P313/75 genome. PCR products were cloned into a

Fig. 2. Alignment of the predicted amino acid sequences of ERBV2.P313/75 and ERBV1.P1436/71 (Wutz et al., 1996) polyprotein. Predicted proteolytic cleavage sites, indicated by arrows (>) or <), are identical between the two viruses, except for the VP2/3 cleavage site where EG occurs in ERBV2.P313/75 and QG occurs in ERBV1.P1436/71. Identical (|), highly conserved (;), less conserved (:) and deleted (-) residues are indicated.
Fig. 2. For legend see facing page.
pGEM-T vector using a cloning kit from Promega. The ABI BigDye Terminator sequencing protocol was followed to determine sequences. Gaps in the sequence between segments were filled by RT–PCR using primers based on the newly acquired sequences of P313/75. The 3′ end was amplified with an oligo(dT)13 and a 3D-based primer, while an oligo(dC)16 and an L region-specific primer were used to obtain the 5′ sequence. Successful PCR amplification of the expected products from P313/75 using the primers oligo(dT)13 and oligo(dC)16 confirmed the presence of both poly(A) and poly(C) tracts in the P313/75 genome. The 3′ end poly(A) tract is a common feature of all picornaviruses and the poly(C) tract near the 5′ end is found in some other members of the *Picornaviridae* family.

The initial cloned version of the P313/75 sequence was found to be out of frame due to two missing nucleotides in a stretch of CA repeats within the VP1-coding region. The problem was resolved by sequencing the PCR product directly. In fact, the sequence of the whole P1 region of P313/75 was reconfirmed by directly sequencing the PCR products. Overall, each base was sequenced at least three times.

The total region that was sequenced was 8821 bases in length and was located between the 5′ poly(C) and the 3′ poly(A) tracts of P313/75; this region has the potential to code for 2583 amino acids. The P313/75 sequence can be divided from the 5′ end to the 3′ end into 5′ UTR–L–P1–P2–P3–3′ UTR–poly(A) tail. Attempts to obtain the sequence of the S region located at the extreme 5′ end upstream of the poly(C) tract were unsuccessful.

For DNA and protein analyses, the computer software programs Blast, Clustree, EClustalW and EDnalML were accessed via the Australian National Genomic Information Service. Phylogenetic trees based on the P1 and 3D nucleotide sequences of picornaviruses representing the nine genera were inferred using the maximum-likelihood method. These trees (Fig. 1) clearly showed that P313/75 and ERBV.P1436/41 formed a tight cluster. The 3D nucleotide sequences of these viruses appeared to be significantly closer than P1 in distance between P313/75 and ERBV.P1436/41. Similar observations can be made with different serotypes of foot-and-mouth disease viruses (FMDV).

Other analyses based on sequence alignments, in particular of the 5′ and 3′ UTR, P2 and P3 regions, also showed that P313/75 had the highest similarity to ERBV.P1436/71 (Table 1). Indeed, the deduced amino acid sequences in the P2 and P3 regions were almost identical between P313/75 and P1436/71 (Fig. 2). Also, P313/75 and ERBV.P1436/71 were found to share some common features. P313/75 maintained the three ATG codons present in ERBV.P1436/71 at the start of the putative open reading frame, with the first ATG being predicted to be the most likely initiation codon. The length of the 3′ UTR and the coding regions for P2 and P3 were identical. The common RNA motif identified in the 3′ UTR was retained (Jonassen et al., 1998) and the predicted RNA folding patterns in the 5′ UTR were very similar (data not shown). The predicted polyprotein cleavage sites were preserved between the two viruses (Fig. 2).

However, P313/75 contained some obvious variations from ERBV.P1436/71. P313/75 was shorter than P1436/71 by seven nucleotides and six amino acids, respectively, due to sporadic insertions and deletions spanning the sequence between the poly(C) tract and 2A. P313/75 was actually larger than P1436/41 in the 5′ UTR by 11 nucleotides, including eight additional contiguous nucleotides downstream of the poly(C) tract, whereas P313/75 was shorter in the L and P1 regions by a net loss of 18 nucleotides. The six amino acids that would have been encoded by these 18 nucleotides were distributed in L (−1), VP4 (−1), VP3 (+1) and VP1 (−5).

The sequence of the P1 region of P313/75 also was substantially different from that of ERBV.P1436/71. The nucleotide identity and amino acid similarity over the whole P1 region between the two viruses were 67% and 81%, respectively. The scores between P313/75 and other members of the *Picornaviridae* family over the same region were much lower, with most below 45%. Further analysis showed that the amino acid changes between P313/75 and ERBV.P1436/71 were located mostly within VP1 and VP2, with 76% and 80% similarity, respectively.

Studies with FMDV have identified five overlapping neutralizing antigenic sites that involve surface oriented amino acids (Crowther et al., 1993; Kitson et al., 1990; Lea et al., 1994). Some of the important sequences implicated in FMDV neutralization were the βB–βC loop and the βE–α2B ‘puff’ in VP2, the βB ‘knob’ in VP3, and the βF–βC loop, the βG–βH loop and the carboxyl end of VP1. In comparison, the predicted secondary structures of the P1 sequences showed that the amino acid sequences in the regions implicated in FMDV neutralization were highly variable between P313/75 and P1436/71. Indeed, four of the six missing amino acids in P313/75 were located in the carboxyl end of VP1. In addition, the residues in the predicted βE–βF loop (amino acids 897–930) and the βH–βI loop (amino acids 988–1009) of VP1 were very different. These highly variable regions may explain the serological segregation between P313/75 and P1436/71.

The sequence and serological relationships between P313/75 and P1436/71 are reminiscent of those of distinct serotypes of FMDV. The amino acid identity in the P1 regions of different serotypes of FMDV ranged from 60 to 82%, while the identity in 3D was about 95%. A recent study demonstrated that the P1 regions of eleven ERAV isolates that belonged to a single serotype were at least 95% identical in their amino acid sequences (A. Varrasso, personal communication). P313/75 and ERBV.P1436/71 were over 95% identical at the amino acid level over the P2 and P3 regions and this observation, together with the phylogenetic analysis and other common features, argued strongly that the two viruses should be classified in the same *Erbovirus* genus. The low amino acid identities (72.5%) in P1, supported by the lack of cross
neutralization between the two viruses, indicate that they are distinct serotypes. Therefore, we propose that P313/75 should be classified as a member of the genus Erbovirus as a distinct serotype designated ERBV2, with P313/75 as the prototype strain; the prototype for ERBV1 is P1436/71.

ERBV2.P313/75 was isolated from a horse with a history of 6 days of intermittent fever that began 4 days after castration and an operation for an umbilical hernia in Switzerland (Steck et al., 1978). The clinical importance of ERBV2 for the horse industry requires further assessment. The close sequence similarity suggests that ERBV1 and ERBV2 probably evolved from the same ancestral strain not long ago, partly due to the infidelity of RNA polymerase and partly due to immune selection (Domingo et al., 1993; Fares et al., 2001; Haydon et al., 2001).

Although the study by Steck et al. (1978) resulted in the isolation of predominantly ERBV1 strains, it is likely that ERBV2 is as prevalent. We tested 258 horse sera for serum neutralizing antibodies and found that 24% of the samples were positive for antibody to ERBV2.P313/75. The neutralizing antibody titres of the positive horse sera were generally low, between 40 and 640, which also appeared to be true for horse sera positive for antibody to ERBV1 (W. Black, personal communication). In addition, it was found that a sample of horse serum with a neutralizing antibody titre of 640 to ERBV1.P1436/71 and < 20 to ERBV2.P313/75 showed some cross reactivity with ERBV2.P313/75 by immunofluorescence (data not shown), further confirming the close relationship between the two viruses. The observed cross reactivity could be due to non-neutralizing antibodies directed to the homologous P2 and P3 regions or to stretches of conserved sequences in P1 between the two viruses. The infrequent isolation of ERBV2 may be linked to the fact that its culture requirements are different from ERBV1 strains. It was noticed that ERBV2.P313/75 at passage 7 showed a different phenotype from ERBV1.P1436/71 at passage 13 or more in both Vero and RK13 cells. Under a methylcellulose overlay, ERBV2.P313/75 did not produce plaques on confluent monolayer cell cultures of either Vero or RK13 cells. In contrast, ERBV1.P1436/71 did produce plaques. However, ERBV2.P313/75 was found to form plaques of irregular shape on about 60% confluent RK13 monolayer cell cultures or when freshly seeded RK13 cells were used. It is not clear whether the phenotypes were the result of cell culture adaptation or properties of the wild-type viruses.

Funding for this project was provided by Racing Victoria and a Special Virology Fund. R.S.W. was a recipient of an Australian Postgraduate Award. We thank Wesley Black and Annalisa Varrasto for antisera and Cynthia Brown for technical help.

References


Received 30 May 2001; Accepted 31 July 2001