Formerly unclassified, acid-stable equine picornaviruses are a third equine rhinitis B virus serotype in the genus *Erbovirus*

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Acid-stable equine picornaviruses (ASPs) were identified as a distinct serotype of equine picornaviruses that were isolated from nasal swabs taken from horses with acute febrile respiratory disease in the UK and Japan, and were placed in the group of unclassified picornaviruses. The nucleotide sequence of the P1 region, encoding the capsid proteins, was determined for three ASP isolates from the UK and the sequences were aligned with published sequences of *Equine rhinitis B virus* (ERBV), genus *Erbovirus*, including acid-labile ERBV1 and ERBV2 and the recently identified acid-stable ERBV1. The ASPs belong to the same phylogenetic group, composed of five acid-stable ERBV1 isolates. ERBV1 rabbit antiserum neutralized the ASP isolates at approximately 1/10 titre relative to acid-stable and acid-labile ERBV1 isolates, supporting prior findings that ASPs are a distinct serotype, albeit cross-neutralizing weakly with ERBV1. The genus *Erbovirus* therefore presently comprises three serotypes: ERBV1, ERBV2 and the proposed ERBV3.

The family *Picornaviridae* is a large family of viruses with over 230 serotypes (Stanway et al., 2005). The picornavirus capsid is dodecahedral, composed of 60 copies of each of three main structural proteins, VP1, VP2 and VP3, and usually one small structural protein, VP4, that are encoded within the P1 region of the genome (Racaniello, 2001).

Presently, the family *Picornaviridae* comprises nine genera, although others are proposed or listed as ‘unassigned’ (Stanway et al., 2005). The definitions of these genera are based on differences, including host range, disease and biophysical properties such as virion density and pH sensitivity, and structural, antigenic and phylogenic relatedness (Stanway et al., 2005). Several picornavirus-like viruses remain unclassified in that they share some properties with picornaviruses, but not enough for final classification. Included among these viruses are the so-called acid-stable equine picornaviruses (ASPs), which were isolated in the UK (Mumford & Thomson, 1978) and Japan (Fukunaga et al., 1983) from horses with or without acute febrile respiratory disease.

The first recognized equine rhinitis virus was *Equine rhinitis A virus* (ERAV) (Plummer, 1962), which, based on genomic sequence analysis (Li et al., 1996; Wutz et al., 1996), was reclassified in the genus *Aphthovirus* in 1999 (Stanway et al., 2005). The second recognized equine rhinitis virus, *Equine rhinitis B virus*, was first isolated in Switzerland (Steck et al., 1978) and the sequence of the genome of the prototype isolate, P1436/71, was subsequently determined (Wutz et al., 1996), which resulted in its classification in a new genus, *Erbovirus*, from ‘Equine rhinitis B virus’ (Stanway et al., 2005). A third equine picornavirus serotype, prototype P313/75, was first isolated in Switzerland (Steck et al., 1978) and, following sequencing of the genome, was shown to be a second serotype in the genus *Erbovirus*, designated ERBV2 (Huang et al., 2001; Stanway et al., 2005). The ERBV1 serotype comprises two distinct phylogenetic groups, one of which is phenotypically acid-stable and the other acid-labile (Black et al., 2005).

ASP, ERAV, ERBV1 and ERBV2 have each been isolated from horses with similar clinical signs, which included fever to 41°C for 1–3 days, serous nasal discharge changing to mucopurulent, anorexia, oedema of the legs, lethargy, swelling and abscessation of lymph nodes of the neck with pain on palpation (Fukunaga et al., 1983; Hofer et al., 1973; Mumford & Thomson, 1978).

We show that the P1 nucleotide sequences encoding the structural proteins of three so-called ASPs, isolated in the UK and representing a previously described distinct picornavirus serotype, are related closely to a group of acid-stable ERBV1 isolates within the genus *Erbovirus* and belong to the same phylogenetic cluster. We propose that ASPs be designated ERBV3 within the genus *Erbovirus*. 

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are: ERBV3.4442/75 (DQ108383); ERBV3.4/75 (DQ108384); ERBV3.2484c/75 (DQ108385).
Virus isolates used in this study were ERBV1.1436/71 and ERBV2.313/75 (Steck et al., 1978), acid-stable ERBV1.9051-7/89 (W. H. McCollum and others, personal communication; Black et al., 2005), ASP.4442/75, ASP.R4/75 and ASP.2484c/75 (Mumford & Thomson, 1978). The viruses were cultured in RK13 cells and cell-culture lysates were clarified by centrifugation and stored at −70 °C. Virus titrations and stability assays were performed as described previously (Black et al., 2005; Mumford & Thomson, 1978). RT-PCR amplification and nucleotide sequence determination were as described previously, as were the preparation of a purified virus antiserum and Western blot analysis (Black et al., 2005).

A Western blot of purified ASP.4442/75 and ERBV1.1436/71 probed with ERBV1.1436/71 rabbit antiserum demonstrated cross-reactivity of ASP proteins with ERBV1.1436/71 rabbit antibody, further suggesting a close antigenic relationship between these viruses (Fig. 1). VP1 of ASP.4442 appeared to be slightly less reactive than that of ERBV1.1436/71, whereas VP2 and VP3 of both viruses reacted strongly and were similar. No VP4 (predicted to be ~7.5 kDa) was detected by Western blot, which was also found when using the ERBV1.1436/71 rabbit antiserum to probe various other ERBV isolates (data not shown). An unexpected protein was observed with an approximate molecular mass of 27 kDa. Other studies of six ERBV1 and ERBV2 isolates have similarly found an approximately 27 kDa protein that copurified with virions through sucrose gradients and, in ERBV1.1436/71, this protein was also observed after trypsin treatment (unpublished data). Studies are in progress to identify this protein more conclusively. Cellular and ERAV capsid proteins were not detected by the ERBV1 rabbit antiserum in Western blot.

A serum neutralization assay was performed on representative isolates of each group of viruses by using ERBV1.1436/71 rabbit antiserum and the results were expressed as the reciprocal of the highest dilution of serum that neutralized 100–178 50 % tissue culture infective doses (TCID50) of the respective virus. The acid-labile ERBV1.1436/71 and the acid-stable ERBV1.9051-7/89 isolates were neutralized similarly at a serum neutralizing (SN) antibody titre of 5619. The ERBV2.313/75, ASP.4442/75 and ASP.R4/75 isolates were neutralized similarly at an SN antibody titre of 178, whilst the ASP.2484c/75 isolate was neutralized at an SN antibody titre of 100.

The neutralization-assay results demonstrated that the three ASP isolates were partially neutralized by ERBV1.1436/71 rabbit antiserum to an extent similar to that for ERBV2.313/75, indicating that these two serotypes cross-neutralize weakly with ERBV1 while remaining distinct from each other, as reported previously (Fukunaga et al., 1983; Mumford & Thomson, 1978). The three ASP isolates were confirmed to be acid-stable, with no loss of virus titre at pH 3–6 after 1 h at room temperature relative to control virus suspensions held at pH 7 (results not shown), and these results are similar to those reported by Mumford & Thomson (1978). The results are also comparable to those for recently identified acid-stable ERBV1 isolates, which contrasted with acid-labile ERBV1 isolates (Black et al., 2005).

The nucleotide and deduced amino acid sequences of the P1 regions of the three ASP isolates were determined and aligned. An alignment of the VP1 amino acids of some representative isolates is presented (Fig. 2). Phylogenetic trees were determined based on the entire P1 region nucleotide and amino acid sequences (Fig. 3). The phylogenetic trees indicated that the three ASPs cluster within the same phylogenetic group composed of acid-stable ERBV1 isolates, despite belonging to distinct serotypes. Phylogenetic trees based on VP1 sequences alone (data not shown) were similar in branching to the trees produced from the entire P1 sequences, as shown in Fig. 3, and rates of variation among the ASP sequences and various ERBV1 and ERBV2 sequences were similar to those observed previously for erbovirus sequences (Black et al., 2005).

The amino acids of the entire P1 regions of the three sequenced ASPs had 93–96 % identity with five sequenced acid-stable ERBV1 isolates, whereas the ASPs had only 77–78 % identity with three completely sequenced acid-labile ERBV1 and 75–77 % identity with two sequenced ERBV2 isolates. This is significant given that, when comparing P1 amino acids from within each group, there

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**Fig. 1.** Western blot of purified ASP.4442/75 (lane 1), ERBV1.1436/71 (lane 2) and RK13 cell lysate (lane 3) probed with ERBV1.1436/71 rabbit serum. Whole virus capsids were purified by ultracentrifugation in 20 mM MgCl2 through a 5 % sucrose cushion and the proteins were then denatured and separated by SDS-PAGE (15 % gel), transferred to a PVDF membrane, blocked and stained as described previously (Black et al., 2005). The molecular masses for each ASP.4442/75 structural protein predicted from the P1 sequence data are VP1 (35 kDa), VP2 (29 kDa), VP3 (26 kDa) and VP4 (7 kDa).
was 94–99% identity, indicating that ASP sequences were related as closely to acid-stable ERBV1 sequences as they were to other ASP sequences, but not related as closely to acid-labile ERBV1 or ERBV2 sequences.

An RT-PCR product was amplified from the three ASPs by using oligonucleotide primers 5'-TTTTGATGCTTCACTTCTCC-3' and 5'-CGGTGTAACCTCCTGCTTACTC-3', specific to the 3D and 3' non-translated regions of ERBV1 and ERBV2. The putative \( \alpha \)-helices and \( \beta \)-sheet regions for ERBV1.1436/71 are indicated above the consensus sequence (Wutz et al., 1996). Also indicated are gaps (-) due to alignment incompatibility and conserved amino acid residues (.). A dot is shown above the consensus sequence to indicate every tenth amino acid residue position where possible.

![Fig. 2. Deduced amino acid alignment of structural protein VP1 of ASP.4442/75, R4/75, 2484c/75, acid-stable (AS) ERBV1.83-11/89, 9051-7/89, acid-labile (AL) ERBV1.1436/71, 293/74 and ERBV2.1576/99. See Fig. 3 for GenBank nucleotide sequence accession numbers. RNA template was prepared by using a QiAamp Viral RNA Extraction kit, according to the manufacturer's instructions (Qiagen). Oligodeoxynucleotide primers, RT-PCR conditions and DNA sequencing were as described previously (Black et al., 2005). The putative \( \alpha \)-helices and \( \beta \)-sheet regions for ERBV1.1436/71 are indicated above the consensus sequence (Wutz et al., 1996). Also indicated are gaps (-) due to alignment incompatibility and conserved amino acid residues (.). A dot is shown above the consensus sequence to indicate every tenth amino acid residue position where possible.

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that these differences in antibody titres may correlate with the observation that ERAV, like other aphthoviruses, produces a viraemia, whereas ERBV, like human rhinoviruses, appears to remain localized to the upper respiratory tract.

An acid-labile ERBV1 strain, recently isolated from a horse in Australia with acute respiratory disease signs, had a P1 nucleotide sequence more similar to those of the phylogenetic group of acid-stable ERBV1 and ASPs than to those of the other two groups of acid-labile ERBV1 and ERBV2 (R. S. Wilcox, personal communication), suggesting that neither acid stability nor serotype is conserved within this phylogenetic group. Further analysis of more ERBV isolates from each phylogenetic group is required. These viruses, however, are rarely isolated, despite the high (>70%) prevalence of serum neutralizing antibody to each of ERBV1 and ERBV2 among horses in Australia (unpublished data) and elsewhere (Kriegshäuser et al., 2005).

The results presented report the complete P1 nucleotide sequences of several ASPs, isolated in the UK, that were previously included within a group of unclassified picornaviruses (Stanway et al., 2005). We show that these viruses belong to the same phylogenetic group as five acid-stable ERBV1 isolates, although they have weak serological cross-neutralization and definite serological cross-reactivity in Western blot with ERBV1.1436/71 rabbit antiserum, despite being of a distinct serotype. These so-called ASP isolates also have a degree of similarity to erbovirus isolates, including acid stability (Black et al., 2005) and the previously described disease association and sites of isolation, and also seroprevalence and the typical SN antibody titres described in earlier studies (Burrows, 1979; Fukunaga et al., 1983). We propose that the so-called ASPs be designated serotype ERBV3 in the genus Erbovirus. It seems notable, however, that the three serotypes fall into three non-corresponding phylogenetic groups: group 1, acid-labile ERBV1; group 2, ERBV2; and group 3, acid-stable ERBV1 and ERBV3.

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References


