

**Reverse transcriptase-polymerase chain reaction  
for the detection of equine rhinitis B viruses and cell  
culture isolation of the virus**

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**Summary.** Equine rhinitis B virus (ERBV), genus *Erbovirus*, family *Picornaviridae* occurs as two serotypes, ERBV1 and ERBV2. An ERBV-specific nested reverse transcriptase-polymerase chain reaction (RT-PCR) that amplified a product within the 3D<sup>pol</sup> and 3' non-translated region of the viral genome was developed. The RT-PCR detected all 24 available ERBV1 isolates and one available ERBV2 isolate. The limit of detection for the prototype strain ERBV1.1436/71 was 0.1 50% tissue culture infectious doses. The RT-PCR was used to detect viral RNA in six of 17 nasopharyngeal swab samples from horses that had clinical signs of acute febrile respiratory disease but from which ERBV was not initially isolated in cell culture. The sequences of these six ERBV RT-PCR positive samples had 93–96% nucleotide identity with six other partially sequenced ERBV1 isolates and one ERBV2. ERBV was isolated from one of the six samples at fourth cell culture passage when it was shown that the addition of 20 mg/mL MgCl<sub>2</sub> to the cell culture medium enhanced the growth of the virus. This isolated virus was antigenically similar to ERBV2.313/75. Determination of the nucleotide sequence of the P1 region of the genome also indicated that the isolate was ERBV2, and it was therefore designated ERBV2.1576/99. This is the first reported isolation of ERBV in Australia. The study highlights the utility of PCR for the identification of viruses in clinical samples that may initially be considered negative by conventional cell culture isolation.

**Introduction**

Equine rhinitis B virus (ERBV), formerly equine rhinovirus 2, was first isolated in Switzerland [26], and the genomic sequence of the prototype isolate, P1436/71, was determined [31], resulting in its classification in a new genus, *Erbovirus* [15].

Equine rhinovirus 3, prototype P313/75, was also isolated in Switzerland [26] and following sequencing of the genome was shown to be a second serotype in the genus *Erbovirus*, designated ERBV2 [12, 15]. The ERBV1 serotype comprises two distinct phylogenetic groups, one of which is phenotypically acid stable and the other, acid labile [1].

ERBV1 and ERBV2 have been isolated from horses in Switzerland, UK, USA, Canada and Japan over an approximately 30-year period [5, 9, 19, 20, 27]. ERBV1- and ERBV2-neutralising antibodies are present in approximately 50–80% of horses in Switzerland, Austria, UK, Canada, New Zealand, The Netherlands and the United Arab Emirates [4, 5, 7, 11, 16, 19, 24, 29], and similar rates occur in Australian horses (unpublished). ERBV1 and ERBV2 have been isolated from horses with acute febrile respiratory disease with clinical signs that include fever to 41 °C for one to three days, serous nasal discharge, anorexia, oedema of the legs, lethargy, and pain and swelling of the lymph nodes of the head and neck. ERBV1 and ERBV2 subclinical infection and subsequent seroconversion were also recognized [2, 26].

We describe the development of an ERBV-specific RT-PCR that detected all of 24 available previously cultivated ERBV1 isolates and one ERBV2 isolate. The RT-PCR was used to detect ERBV in horses with acute febrile respiratory disease. We show that the inclusion of 20 mg/mL MgCl<sub>2</sub> in the cell culture medium significantly enhances the growth of ERBV in cell cultures.

## Materials and methods

### *Viruses and cells*

Viruses used to develop and validate the RT-PCR are listed in Table 1. A stock of each virus was prepared by inoculation of monolayer cell cultures of rabbit kidney (RK13) cells as previously described [1]. Other cells used for virus culture, including virus isolation attempts, were Vero cells (passage 130–200), RK13 cells (passage 195–250) and equine foetal kidney (EFK) cells (passage 4). Cells were cultured in either Eagle's Minimal Essential Medium (MEM, Sigma-Aldrich) with 10 mM NaHCO<sub>3</sub> and 50 µg/mL ampicillin (Sigma-Aldrich) or, when it was shown that MgCl<sub>2</sub> enhanced ERBV growth in cell culture, in Leibowitz' L-15 medium (L-15, Sigma-Aldrich) with 50 µg/mL ampicillin and 20 mg/mL MgCl<sub>2</sub>. Each of the media included 0.5% foetal bovine serum (FBS, JRH Biosciences).

### *RT-PCR*

Viral RNA was extracted using a QIAamp Viral RNA Extraction kit (Qiagen) according to the manufacturer's directions. Reverse transcription was performed using 11 µL of RNA template and 1 µL of 10 µM oligo-(dT)<sub>15</sub> reverse primer heated to 70 °C for 5 min, centrifuged briefly and cooled quickly on ice. Samples were then incubated at 50 °C before the addition of 0.5 µL of Superscript II-RNase H reverse transcriptase (Invitrogen), 0.5 µL RNAGuard (Amersham Biosciences), 1 µL 10 mM dNTP, 2 µL 100 mM DTT, and 4 µL 5× RT buffer. After incubation at 50 °C for 45 min, reactions were inactivated at 70 °C for 5 min and either used undiluted, or diluted 1:10 with water. If stored for later use, samples were heated to 70 °C and vortexed immediately prior to use. PCR reactions were prepared in 200-µL PCR tubes (CLP Biological), with 25 µL final reaction volume with 400 nM of each primer, 10 units Taq polymerase (Promega), Taq buffer, 3.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP

**Table 1.** Equine rhinitis viruses used in the validation of an RT-PCR for detection of all erboviruses

No.	Serotype	Designation	Passage no.	Isolation year	Origin	Reference
1	ERBV1	1436/71	pp11 <sup>a</sup>	1971	Berne, Switzerland	[26]
2		201/71	13	1971	Berne, Switzerland	[26]
3		208/71	9	1971	Berne, Switzerland	[26]
4		917/71	10	1971	Berne, Switzerland	[26]
5		955/71	6	1971	Berne, Switzerland	[26]
6		1014/71	11	1971	Berne, Switzerland	[26]
7		11/74	5	1974	Berne, Switzerland	[26]
8		293/74	8	1974	Berne, Switzerland	[26]
9		322/74	7	1974	Berne, Switzerland	[26]
10		328/74	4	1974	Berne, Switzerland	[26]
11		513/74	4	1974	Berne, Switzerland	[26]
12		263/75	4	1975	Berne, Switzerland	[26]
13		271/75	3	1975	Berne, Switzerland	[26]
14		622/75	3	1975	Berne, Switzerland	[26]
15		367/75	5	1975	Berne, Switzerland	[26]
16		379/75	5	1975	Berne, Switzerland	[26]
17		426/75	4	1975	Berne, Switzerland	[26]
18		KP/92	NA <sup>b</sup>	1992	New York, USA	D. F. Holmes, unpublished
19		NAV II	9	1988	Kentucky, USA	W. H. McCollum and P. J. Timoney, unpublished
20		CW/88	7	1988	New Hampshire, USA	W. H. McCollum and P. J. Timoney, unpublished
21		57-14	9	1989	Kentucky, USA	W. H. McCollum and P. J. Timoney, unpublished
22		58-13	9	1989	Kentucky, USA	W. H. McCollum and P. J. Timoney, unpublished
23		83-11	9	1989	Kentucky, USA	W. H. McCollum and P. J. Timoney, unpublished
24		9051-7	9	1989	Kentucky, USA	W. H. McCollum and P. J. Timoney, unpublished
25	ERBV2	313/75	8	1975	Berne, Switzerland	[26]
c1	ERAV	393/76	pp22	1976	South Australia	[28]
c2		967/90	pp11	1990	South Australia	M. J. Studdert and N. P. Ficorilli, unpublished

<sup>a</sup>pp passage post plaque purification<sup>b</sup>NA not available

(Promega), and 1  $\mu$ L of cDNA template. The primers were designed based on the published sequences of the prototype viruses ERBV1.1436/71 [31] and ERBV2.313/75 [12]. Primers used in the first round were: 400 nM of primers ERBV.OUTER.f (TTTTGATGCTTCA CATTCTCC) and ERBV.OUTER.r (CGCTGTACCCTCGGTCCTACTC), to which 5  $\mu$ L of the undiluted RT mixture was added as template. One drop of mineral oil was added to the reactions. Aerosol-resistant micropipette tips (CLP Biological) were used throughout. PCR cycle conditions were: 1 min at 95 °C, then 35 cycles of 30 sec at 60 °C, 45 sec at 72 °C, and 30 sec at 95 °C. 2  $\mu$ L of the resulting first-round PCR mixture was diluted in 1 mL of Tris-HCl buffer (pH 7.5), and 5  $\mu$ L was then added to the second-round PCR reactions as template. Second-round PCR reactions were performed as for the first-round PCR, but using primers ERBV.INNER.f (CTTACTAYGAATGTGARGGGGC) and ERBV.INNER.r (GCCTCGGCGAGTGAAGAG). Cultured ERBV isolates were diluted 1:1000 in transport medium prior to viral RNA extraction to avoid overloading the nested RT-PCR. Amplicons were analysed in 1% agarose gels as previously described [1]. Purification of amplified viral cDNA for sequencing were also as previously described [1]. Typically, for sequencing 1 pmol of primer, 4  $\mu$ L of BDT3.1 (Applied Biosystems) and 60–90 ng of template DNA was used per sequencing reaction. Both strands of at least three separate clones were sequenced.

#### *Sensitivity of the ERBV specific nested RT-PCR*

Dilutions of ERBV1.1436/71 were used to determine the sensitivity of the specific RT-PCR. The virus titre of the ERBV1.1436/71 sample was determined, and the sample was then diluted to obtain 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 TCID<sub>50</sub> per 140  $\mu$ L, i.e., 2, 1, 0, -1, -2, -3 and -4 log<sub>10</sub>TCID<sub>50</sub> per 140  $\mu$ L. These samples were then each subjected to RNA extraction and nested RT-PCR. The nested RT-PCR minimally detected 0.1 TCID<sub>50</sub> of ERBV1.1436/71 in both rounds of the PCR. While there was no absolute difference in the detection limit of the first- and second-round PCR, the amount of amplified DNA appeared much greater in the second round of the RT-PCR than the first round under the conditions described (data not shown).

#### *Nasopharyngeal swab and serum samples*

Nasopharyngeal swabs samples from 17 horses that had acute febrile respiratory disease that were obtained as part of another study (unpublished) were examined using the RT-PCR. Nasopharyngeal swabs were obtained using a modification of a method previously described [27]. Briefly, Dacron 'Nu Gauze' compress swabs (Johnson & Johnson) were used for mucous collection. Following collection, swabs were placed in viral transport medium (MEM supplemented with non-essential amino acids, 3% FBS, 11 mM NaHCO<sub>3</sub>, 50  $\mu$ g/mL ampicillin, 100  $\mu$ g/mL gentamicin, and 5  $\mu$ g/mL Fungizone (Sigma-Aldrich)). The samples were transported on ice, then filtered through a sterile 0.45  $\mu$ m membrane filter (Millipore) and stored at -70 °C. Serum samples were also obtained at the time of the nasopharyngeal swab, and a second sample was obtained approximately 2 weeks later. The samples were tested for the presence of viruses by inoculation onto RK13, Vero and EFK cells. When the ERBV-specific nested RT-PCR was satisfactorily validated, the nasopharyngeal swab samples were re-tested specifically for the presence of ERBV.

#### *Virus isolation*

For virus isolation, nearly confluent Vero, RK13 and EFK cell monolayers cultured in 25-cm<sup>2</sup> flasks were used. After the cell culture growth medium was removed, 1 mL of filtered nasopharyngeal swab sample was inoculated onto the cells and incubated at 37 °C for 1 h. 5 mL of maintenance medium (MEM at pH 7.5 with 0.5% FBS, 50  $\mu$ g/mL ampicillin and

11 mM NaHCO<sub>3</sub>) was then added to each flask, and the flasks were incubated at 37 °C for 3 days with daily examination for CPE using an inverted microscope. The samples were passaged three times, using 1 mL of the cell culture supernatant from the previous passage as inoculum, then stored at -70 °C.

#### *SN antibody*

Assays for SN antibody were performed in sterile, 96-well, flat-bottom microtiter trays as previously described [1]. An ERBV1.1436/71 rabbit antiserum used in SN and immunofluorescence assays was prepared as previously described [1]. Immunofluorescence assay staining of RK13 cells infected with ERBV1.1436/71 and ERBV2.1576 were performed using rabbit ERBV1.1436/71 antiserum [1], biotinylated swine anti-rabbit immunoglobulin (Dako), and streptavidin- fluorescein isothiocyanate (Dako) as previously described [18].

#### *Nucleotide sequence accession numbers*

Nucleotide sequences determined in this study for the 3D<sup>pol</sup>-3'UTR RT-PCR products from the six nasal swabs are GenBank accession nos. AY607003–AY607008. The nucleotide sequences for the same region of four of the ERBV1 isolates listed in Table 1 are GenBank accession nos. AY606999–AY607002. The P1 region sequence for the isolated virus, ERBV2.1576/99 is GenBank accession no. AY606998.

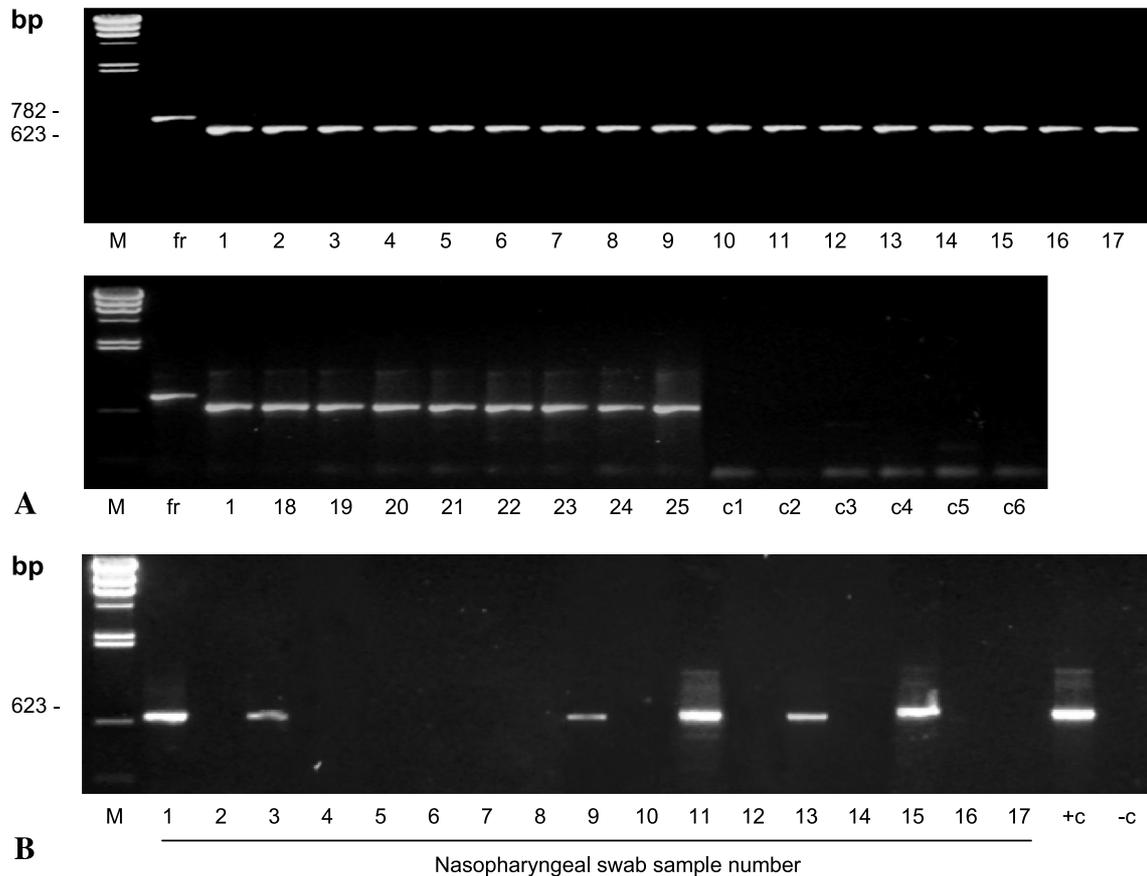
## **Results**

### *Development of an ERBV specific nested RT-PCR*

The RT-PCR amplified a product from all of 24 ERBV1 isolates and one ERBV2 isolate (ERBV2.313/75) as listed in Table 1 (Fig. 1). The products of both the first and second rounds of the PCR were of the expected size. The RT-PCR did not amplify any product from either of two tested ERAV isolates, nor was any product amplified from uninfected RK13, Vero or EFK cells, or media control.

### *Detection of ERBV in nasopharyngeal swab samples*

Products of the expected length were amplified from six of 17 nasopharyngeal swab samples (1506/99, 1525/99, 1576/99, 1605/99, 1630/99 and 1654/00) using the ERBV-specific nested RT-PCR (Fig. 1), and there was no evidence that the primers amplified products from any other viruses that may have been present in the swab samples. There was a high background in the first-round RT-PCR (data not shown) such that detection and nucleotide sequencing was based on the second-round product. These samples were previously considered ERBV negative based on primary isolation attempts in cell cultures. The DNA amplified from each of these six putative ERBV-positive swabs was sequenced by direct cycle sequencing. The nucleotide sequences were only partially complete for three of the six samples (1506/99, 1605/99 and 1525/99) due to the small amount of pure template material obtained from the swab samples. For comparison, the nucleotide sequences of the region amplified by the nested RT-PCR were determined for four known ERBV isolates, i.e., ERBV1.293/74, ERBV1.263/75, ERBV1.57-14/89 and ERBV1.9051-7/89, as listed in Table 1.



**Fig. 1. A.** ERBV-specific nested RT-PCR that detects all available previously isolated ERBV as listed (1–25) in Table 1. The second-round RT-PCR product (623-bp) is shown. A single 782-bp first-round product (fr) for ERBV1.1436/71 was included for comparison. The negative controls (c1–c6) were subjected to RNA extraction and fully nested RT-PCR as for the virus samples and were: c1 was ERAV.393/76, c2 was ERAV.967/90, c3 was RK13 cell lysate, c4 was Vero cell lysate, c5 was EFK cell lysate, c6 was virus transport medium. M was 750 ng of bacteriophage  $\lambda$  DNA digested with *Hind*III included as a base pair marker. **B.** Detection of ERBV in equine nasopharyngeal swab samples by fully nested RT-PCR. Nasopharyngeal swab samples from horses 1–17 are shown. The six positive samples (lanes 1, 3, 9, 11, 13, and 15) were from horses 1506/99, 1525/99, 1576/99, 1605/99, 1630/99 and 1654/00 respectively. The 623-bp positive control (+c) was ERBV1.1436/71. The negative control (–c) was virus transport media that was subjected to RNA extraction and RT-PCR. M was 750 ng of bacteriophage  $\lambda$  digested with *Hind*III included as a base pair marker

The nucleotide sequences were then aligned with the published sequences of ERBV1.1436/71 and ERBV2.313/75 (data not shown), and the percentage nucleotide identity between these sequences was determined. There was 93–96% nucleotide sequence identity between the nucleotide sequences of all six ERBV isolates and the six DNA products amplified from nasopharyngeal swabs. The 3D<sup>pol</sup> and 3'NTR regions were highly conserved amongst the ERBV sequences

consensus	1	ĈSATSI LNCLMNNIVLRAAA YDVFNTNYEEGLAFLTYGDDVLLCANQPLPLERFRKSLAKLGYTITPADKSSIFPEVSTLĀDVVFLKRRFKPDEEFPFLF
1436/71	1	.....V.....
293/74	1	R.....
263/75	1	R.....V.....
57/14	1	.....
9051/7	1	.....
313/75	1	.....F.....Q.....
1506/99	1	.....
1525/99	1	.....V.....
1576/99	1	.....
1605/99	1	.....
1630/99	1	.....L.....
1654/00	1	.....
ERAV	1	..C...TVL..VYIL..MMKA.E.F.PD.IQVIC...C.IASDLEIDFQKLVPFSSF.QV..T...TDF.KLT-..SE.T...A.V..G---AFY
EMCV	1	..A...M..TI...II..GL.LTYK.F.FD.VKV.S...L.VAT.YQ.DFDKV.A...T..K...N.T.T..LN...E.....K...--K.G.-.Y
consensus	101	RPVMDVRNLQDHL CYAKPGTLREKLLSTTDĻAVHLGPLEYĀRLFRPFVEVGYCVRPFGDĀRRĀWL RNFDL
1436/71	101	K.....R.....A.....
293/74	101	.....
263/75	101	.....
57/14	101	K.....
9051/7	101	.....
313/75	101	K.....
1506/99	89	K.....H.....
1525/99	67	K.....A.....
1576/99	101	.....
1605/99	91	.....
1630/99	101	.....Y.....
1654/00	101	.....Y.....
ERAV	97	K...KT.EAI.SFVR...QA...VAQ..G.CE.EQ.E...E..AGM-.Y.PTWRL.L.LQ..MKLGĈ
EMCV	98	...NREA.EAM.S.YR...S...T.I.M...S.KQ..D...A..R...VV.PS.ESVEYR.RSL.W-

**Fig. 2.** Deduced amino acid sequence alignment of the 3D region amplified by a nested RT-PCR. The alignments are of six selected viruses as listed in Table 1 and of sequences from 1506/99, 1525/99, 1576/99, 1605/99, 1630/99, 1654/00 that were obtained directly from the relevant nasopharyngeal swab samples from horses with acute respiratory disease. Sequences for ERAV.PERV/62 and EMCV are included for comparison. Indicated are gaps (—) due to alignment incompatibility (ERAV and EMCV only) or due to lack of sequence data (ERBV N- and C-termini only). Conserved amino acids are also indicated (.)

in general in that no correlation could be discerned between the individual nucleotide sequences and serotype (ERBV1 or ERBV2), known phenotypes such as acid stability, or geographic location or year of isolation. There were several nucleotide differences between each of the sequences of the ERBV isolates and the nasopharyngeal swab samples, however, suggesting that they were each from different samples rather than due to the cross-contamination of laboratory samples.

To facilitate comparison with other more distantly related picornaviruses and to allow for the redundancy of codons, the 12 ERBV and swab nucleotide sequences were translated into amino acids and were aligned with ERAV and EMCV amino acid sequences of the 3D<sup>pol</sup> gene (Fig. 2). The amino acid alignment was then used to determine percentage amino acid identities and similarities between each sequence. The deduced amino acid sequences obtained from the nasopharyngeal swab samples were very similar to each other (99.4% amino acid identity; 98.8–100% amino acid similarity), and were very similar to ERBV sequences (96.5–100% amino acid identity; 97.6–100% amino acid similarity). In comparison to other known picornavirus sequences, the sequences from the swab samples were most similar to the sequence of EMCV (51.8–52.4% amino acid identity; 61.8–62.4% amino acid similarity), and were relatively dissimilar to the sequence of ERAV (38.8–41.2% amino acid identity; 50.6–51.8% amino acid similarity). The nasopharyngeal swab samples were therefore concluded to be positive for

ERBV of unknown serotype and acid stability phenotype. Subsequently it was shown that the isolated virus (1576/99) was ERBV2 (see below) and acid labile.

#### *Correlation between ERBV seropositivity and RT-PCR results*

Each of the six ERBV-positive samples came from horses that were seropositive for either ERBV1 or ERBV2, or both viruses. There was, however, no clear association between ERBV RT-PCR-positive horses and seroconversion to ERBV1 or ERBV2, although some rises in SN antibody titres were observed between acute and convalescent serum samples.

#### *Virus isolation from nasopharyngeal swab samples*

Virus isolation in cell culture had been attempted for all 17 nasopharyngeal swab samples, but no CPE was visible during three passages on RK13, Vero and EFK cells. The cell culture supernatants from each passage and each cell type were then stored at  $-70^{\circ}\text{C}$ . The ERBV-specific RT-PCR was used retrospectively on the cell culture supernatant samples from four of the six original nasopharyngeal swab samples that were ERBV positive by RT-PCR, to determine if ERBV was present in cell culture without visible CPE. Only one nasopharyngeal swab sample, 1576/99 Da/PC, was putatively ERBV positive by RT-PCR in the second and third passage in Vero and RK13 cells, but not EFK cells (data not shown). This indicated that an ERBV had been isolated from the nasopharyngeal swab sample and had been cultured in Vero and RK13 cells despite the absence of visible CPE. Furthermore, this indicated that not all cell types e.g., EFK, supported the growth of this virus under the conditions used. Despite many attempts to isolate virus from the other RT-PCR-positive samples in all three cell types and using the modified culture conditions, only the one isolate was obtained.

Immunofluorescence was performed in attempts to visualize cells infected with ERBV.1576/99. RK13 monolayer cell cultures inoculated with either ERBV1.1436/71 or ERBV.1576/99 showed similar clusters of rounded cells with strong cytoplasmic fluorescence when probed with rabbit antiserum to ERBV1.1436/71, but not with preimmune rabbit serum (data not shown).

After the fourth passage in cell culture the new isolate ERBV.1576/99 produced complete CPE in established monolayers of RK13 cells in MEM after 16–24 h. When cultures were inoculated with less than 100 TCID<sub>50</sub>, ERBV.1576/99 in DMEM formed small plaques in RK13 cells (approximately 50 to 250  $\mu\text{m}$  diameter, as estimated by comparison with an hemocytometer grid), after which the plaques stopped increasing in size. The plaques were not visible 3 days after incubation, and this was possibly related to the growth of surrounding cells into the previously clear plaque areas. This confounded the analysis of the ERBV isolate 1576/99 by virus titration and SN assays. This was also observed in the culture of ERBV2.313/75 in RK13 and Vero cells under similar conditions.

To improve the visibility of the ERBV plaques, virus titration assays were performed using various culture conditions including varying temperature,

media, and supplementation with different solutes, conducted in 10-well replicates and repeated three times. The titre of the ERBV isolate 1576/99 was higher at 37 °C in RK13 cells in L-15 medium ( $5.0 \log_{10} \text{TCID}_{50}/\text{mL}$ ) compared to DMEM medium ( $4.5 \log_{10} \text{TCID}_{50}/\text{mL}$ ). The titre was higher in L-15 medium at 33 °C ( $5.5 \log_{10} \text{TCID}_{50}/\text{mL}$ ), relative to 37 °C ( $4.5 \log_{10} \text{TCID}_{50}/\text{mL}$ ), but the virus plaques remained small in each. All subsequent assays were performed at 37 °C. The titre was higher in L-15 at 37 °C with 20 mM  $\text{MgCl}_2$  ( $6.8 \log_{10} \text{TCID}_{50}/\text{mL}$ ), or 0.5 mM  $\text{ZnCl}_2$  ( $6.8 \log_{10} \text{TCID}_{50}/\text{mL}$ ) relative to the same conditions without  $\text{MgCl}_2$  supplementation ( $4.8 \log_{10} \text{TCID}_{50}/\text{mL}$ ). In the presence of  $\text{MgCl}_2$  and  $\text{ZnCl}_2$ , the plaques were very large and visible (approximately 250–1000  $\mu\text{m}$ ), but the uninfected cells also had some slight cytopathology presumably due to the toxicity of the solutes.  $\text{ZnCl}_2$  was cytotoxic at 2 mM, causing complete destruction of the RK13 cell monolayer. Other solutes such as  $\text{CuSO}_4$ ,  $\text{NH}_4\text{SO}_4$ ,  $\text{MnCl}_2$ , glutathione and DEAE, were assayed, but were either cytotoxic or had little effect on the virus titre at the concentrations used. Therefore, RK13 cells at 37 °C in L-15 medium with 20 mM  $\text{MgCl}_2$  supplement were used as the standard conditions for analyzing the ERBV isolate 1576/99 in titration and SN assays.

#### *Antigenic relationships between ERBV1, ERBV2 and isolate 1576/99*

ERBV.1576/99 was neutralized similarly to ERBV2.313/75 in an SN assay using monospecific ERBV1 and ERBV2 antisera from naturally infected horses. An ERBV1.1476/71 rabbit antiserum did not neutralize 1576/99, and rat antiserum prepared against ERBV2.313/75 neutralised the isolate with a pattern similar to ERBV2.313/75 (data not shown). The isolate was therefore designated ERBV2.1576/99.

### **Discussion**

During the design of ERBV-specific primers for an RT-PCR, several different primer sets were tested for suitability. These included primers based within the 3A and 3D<sup>pol</sup> regions, which amplified various ERBV isolates including ERBV2.313/75, allowing the sequencing of this virus and its subsequent identification as an erbovirus [12]. The 3' non-translated region (3' NTR) and the 3D<sup>pol</sup> sequences were highly conserved between all ERBV sequences. The 3' NTR contains a 43-base-long stem-and-loop structure, s2m, [13], that is highly conserved between several viruses including human, sheep, cat, avian, turkey, and pig astroviruses, as well as avian infectious bronchitis and the human severe acute respiratory syndrome (SARS) coronaviruses (GenBank AY278741), but not in picornaviruses, except erboviruses. The RT-PCR also amplified the expected product from three isolates of a distinct serotype of acid-stable equine picornavirus that has been proposed as an erbovirus (unpublished).

The ERBV-specific nested RT-PCR detected ERBV1.1436/71 at 0.1  $\text{TCID}_{50}$  per 140  $\mu\text{L}$  of sample. This corresponded to a limit of detection of 0.7  $\text{TCID}_{50}/\text{mL}$ . The detection of ERBV in six of 17 swabs tested, and the observation that ERBV1

and ERBV2 were isolated from the saliva of horses at titres from approximately 56–5600 TCID<sub>50</sub>/mL [9] suggests that the ERBV-specific nested RT-PCR is sensitive enough to detect ERBV in clinical samples, assuming that nasal swabs and saliva samples are similar. While the first and second rounds of the nested RT-PCR were similarly sensitive in detecting cell-cultured ERBV1.1436/75, the first round of the nested RT-PCR did not clearly detect ERBV in clinical samples later found to be positive in the second round. This was presumed to be due RT-PCR inhibitors in the nasal swab samples, often observed to have suspended contaminants prior to filtration, and also mis-priming equine and other nucleic acids, which can reduce specific amplification.

Only after successful virus isolation from one of four nasopharyngeal swab samples that were ERBV positive by nested RT-PCR was it determined that the virus growth in cell culture is significantly enhanced by modifications to the growth medium, particularly the inclusion of 20 mM MgCl<sub>2</sub>, which was not provided on initial culture of these swab samples. Efforts were then made to improve the culture conditions for ERBV in general and for ERBV2.1576/99 in particular. Plaques produced by human rhinoviruses were reported to be larger in the presence of 20 mM MgCl<sub>2</sub> and also when low bicarbonate medium was used [8], while zinc ions may inhibit the growth and or cytopathogenicity of rhinoviruses [23]. Cardiovirus growth in cell culture is enhanced by 40 mM magnesium ions [25]. Similarly, we found that the plaques formed by ERBV2.1576/99 and ERBV2.313/75 were larger and more visible in L-15 medium supplemented with 20 mM MgCl<sub>2</sub>. The apparent enhancement of ERBV2.1576/99 titre in medium supplemented with 20 mM MgCl<sub>2</sub> was possibly due to the effect on the cells, rather than on the virus *per se*, given the similarity of effects using ZnCl<sub>2</sub>. ERBV1.1436/71 was, however, stabilized at 50 °C by 1 M MgCl<sub>2</sub> [21]. Furthermore, the culture of ERBV was improved by the use of rapidly dividing cells [26].

The development of an ERBV-specific fully nested RT-PCR allowed the identification and first isolation of ERBV in Australia, demonstrating conclusively that ERBV is present in Australian horses. The ERBV-specific RT-PCR would facilitate detection of ERBV in nasopharyngeal swab samples by reducing the dependence on cell culture conditions and virus viability in samples. RT-PCR is advantageous because of high sensitivity and specificity, and the short time required to produce a result relative to established techniques such as virus isolation by cell culture, or the observation of seroconversion using paired serum samples and testing by ELISA or SN antibody assay [6, 10]. Seroconversion of convalescent horses to ERBV1 or ERBV2 may not be a reliable means of diagnosis given that approximately 80% of horses sampled had detectable SN antibody to either virus, albeit at low SN antibody titre, and that most seropositive horses had only mild (i.e., <4-fold) subsequent rises in SN antibody titre. ERBV has been isolated from horses without detectable SN antibody, and conversely, some horses were observed to seroconvert without isolation of ERBV [5], strengthening the need for a fast, reliable and sensitive method of detection, such as RT-PCR, that does not rely on a serum antibody response or virus isolation. The conclusive determination of ERBV as the cause of equine respiratory disease by detection of virus in nasopharyngeal samples

by nested RT-PCR is, however, also made difficult by the long-term persistent infection of horses with these viruses without causing apparent disease, as reported after the repeated isolation of ERBV1 over 2 years after two yearling colts were experimentally infected [3]. Conclusive demonstration of the association between ERBV and clinical disease in future studies would be greatly facilitated by the use of the nested RT-PCR described above.

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