

# Real-time RT-PCR for the detection and quantitative analysis of equine rhinitis viruses

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## Summary

**Reasons for performing study:** Equine rhinitis viruses (ERV) cause respiratory disease and loss of performance in horses. It has been suggested that the economic significance of these viruses may have been underestimated due to insensitive methods of detection.

**Objectives:** To develop a sensitive, rapid, real-time RT-PCR (rRT-PCR) assay suitable for the routine diagnosis and epidemiological surveillance of the A and B variants of ERV.

**Methods:** TaqMan primer probe sets for ERAV and ERBV were designed from conserved regions of the 5' UTR of the ERV genome. Over 400 samples from both clinically affected and asymptomatic horses were employed for validation of the assays. ERAV samples positive by rRT-PCR were verified by virus isolation and ERBV positive samples were verified by rRT-PCR using a different set of primers.

**Results:** The detection limit of the rRT-PCR for both viruses was 10–100 genome copies. Of 250 archival nasal swabs submitted for diagnostic testing over a 7 year period, 29 were ERAV positive and 3 were ERBV positive with an average incidence rate per year of 10 and 1.5%, respectively. There was evidence of co-circulation of ERAV and ERBV with equine influenza virus (EIV). Of 100 post race urine samples tested, 29 were ERAV positive by rRT-PCR. Partial sequencing of 2 ERBV positive samples demonstrated that one was 100% identical to ERBV1 from a 270 bp sequence and the other was more closely related to ERBV2 than ERBV1 (95% compared to 90% nucleotide identity in 178 bp).

**Conclusions:** The rRT-PCR assays described here are specific and more sensitive than virus isolation. They have good reproducibility and are suitable for the routine diagnosis of ERAV and ERBV.

**Potential relevance:** These assays should be useful for investigating the temporal association between clinical signs and rhinitis virus shedding.

## Introduction

Equine rhinitis viruses (ERV) are endemic in horse populations worldwide (McCollum and Timoney 1992; Burrell *et al.* 1996;

Carman *et al.* 1997; Wernery *et al.* 1998; Black *et al.* 2007a). These positive sense RNA respiratory viruses are members of the *Picornaviridae* family. The variants ERAV and ERBV1 were originally assigned to the genus *Rhinovirus* of the family *Picornaviridae* and were known as equine rhinovirus 1 and 2. Subsequent to nucleotide sequence determination in the 1990s, ERAV was re-classified in the genus *Aphthovirus* on the basis of its similarity to the sole member of this genus, foot-and-mouth disease virus (FMDV). ERBV1 was designated the prototype of a new genus *Erbovirus* (Li *et al.* 1996; Wutz *et al.* 1996; Stanway *et al.* 2005) along with another serologically distinct prototype ERBV2 (Huang *et al.* 2001). ERAV, ERBV1 and ERBV2 are acid labile viruses. A group of ERBV1-related acid stable viruses were recently re-classified as ERBV3 viruses based on sequence analysis of their viral capsid proteins (Black *et al.* 2005; Black and Studdert 2006).

Equine rhinitis virus infection is of significance in horses most notably in the racing sector. It has been reported that approximately 87% of susceptible horses in the Newmarket area of the UK become infected with ERAV each racing season (Powell *et al.* 1978) and a seroprevalence of 57 and 71% for ERAV and ERBV1, respectively, was recorded among Thoroughbred yearlings in Kentucky (McCollum and Timoney 1992). In Australia, the UK and Ireland, serological evidence suggests that ERAV is most common in 2-year-old horses shortly after they enter training yards (Burrows 1968; Powell *et al.* 1978; Klaey *et al.* 1998; Black *et al.* 2007a). ERV infection has been recorded as subclinical and the cause of mild to severe upper respiratory tract disease similar to the common cold in man (Plummer and Kerry 1962; Hoffer *et al.* 1972, 1978; Holmes *et al.* 1978; Steck *et al.* 1978). Generally, horses make a spontaneous recovery within 5–7 days in uncomplicated infection but secondary bacterial infection may prolong recovery and have a significant impact on performance (Burrows 1969; Steck *et al.* 1978).

Early, specific diagnosis is essential to the appropriate management of equine respiratory disease. Traditionally, ERV has been detected by virus isolation in susceptible cell lines such as Vero (African green monkey kidney) or Rabbit Kidney-13 (RK-13) cells. Virus has been isolated from nasal swabs, blood, faeces and urine (Plummer and Kerry 1962; Hoffer *et al.* 1972; McCollum and Timoney 1992). ERV can also be detected serologically by measurement of a rise in antibody titre in paired sera by serum

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neutralisation (Burrows 1968; Steck *et al.* 1978) or complement fixation (Burrell *et al.* 1996; Klaey *et al.* 1998). These established techniques are time consuming and laborious and do not facilitate prompt intervention and modification of training regimes. Furthermore, in the investigation of respiratory disease, the detection by RT-PCR of ERAV strains that were not detected in cell culture, led to the suggestion that the prevalence of this virus and hence its economic significance, may have been underestimated (Li *et al.* 1997). At the time of writing, only conventional RT-PCR assays were available for ERV (Black *et al.* 2007b; Dynon *et al.* 2007). However, shortly before submission a real-time duplex RT-PCR assay was published by Mori *et al.* (2009).

The aim of this study was to develop a sensitive, rapid, real-time RT-PCR (rRT-PCR) assay, suitable for the routine diagnosis and epidemiological surveillance of ERAV and ERBV to evaluate the pathogenic potential of these viruses.

## Materials and methods

### Cells and viruses

Samples of ERAV and ERBV1 were both kindly provided by the Animal Health Trust (AHT), Newmarket in 1988 and were used as reference viruses. These viruses were not genetically characterised by the AHT. Sequence data were generated for a total of 1370 nucleotides of the ERAV genome (Fig 1a,b: see [www.evj.co.uk/supinfo](http://www.evj.co.uk/supinfo)) and demonstrated 99.9% nucleotide identity with ERAV strain NM11/67 (Accession no. FJ607143 submitted to GenBank by Tuthill *et al.* 2009) i.e., there is a single nucleotide difference at position 1920 of FJ607143. The VP1 gene of this virus was also sequenced by Wernery *et al.* (2008) (Accession no. EF204771) and comparison of amplicon AII to this reference sequence shows 100% nucleotide identity (Fig 1b: [www.evj.co.uk/supinfo](http://www.evj.co.uk/supinfo)). Sequence data were obtained for a total of 934 nucleotides of the ERBV genome (Fig 1c,d: [www.evj.co.uk/supinfo](http://www.evj.co.uk/supinfo)) and demonstrated 100% nucleotide identity with the prototype ERBV1 strain P1436/71 (Accession no. NC\_003983, Wutz *et al.* 1996).

Vero, RK-13 and primary equine embryonic lung (EEL) cells were propagated at 37°C in an atmosphere of 5% CO<sub>2</sub> in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS), 100 u/ml penicillin and 100 µg/ml streptomycin (all GIBCO)<sup>1</sup>. For virus isolation and propagation, the cells were maintained in the same medium supplemented with 2% FCS (maintenance medium).

### Clinical samples

Details of samples employed to validate the ERV rRT-PCR assays and to examine primer probe specificity can be found in Table 1. Two-hundred-and-fifty archival nasal swabs dating from

### See [www.evj.co.uk/supinfo](http://www.evj.co.uk/supinfo) for Figure 1

Fig 1: Animal Health Trust reference ERV strains used in this study aligned with published sequence data. (a) ERAV strain sequence data (AI) aligned with isolate NM11/67 (Accession number FJ607143), amplicon AI corresponds to nucleotides 505 to 1178 of FJ607143; (b) ERAV strain sequence data (AII) aligned with isolate NM11/67 (Accession numbers FJ607143 and EF204771), amplicon AII corresponds to nucleotides 1705 to 2352 of FJ607143. (c & d) ERBV strain sequence data (BI and BII) aligned with prototype ERBV1 strain P1436/71 (Accession number NC\_003983). Amplicon BI corresponds to nucleotides 1003 to 1406 and amplicon BII corresponds to nucleotides 1870 to 2399 of NC\_003983. \* Represents identical nucleotides.

**TABLE 1: Samples employed to validate the sensitivity and specificity of the ERV rRT-PCR assays**

| Sample                    | Number tested | Pathogen                |
|---------------------------|---------------|-------------------------|
| Nasal swab                | 250           | Unknown*                |
| Urine                     | 100           | Unknown                 |
| Nasal swab                | 10            | EHV1                    |
| Nasal swab                | 10            | EHV4                    |
| <i>Post mortem</i> tissue | 12            | EHV1 or EHV4            |
| Nasal swab                | 12            | EIV                     |
| Nasal/buccal/genital swab | 8             | EIA                     |
| Nasal swab                | 25            | 5 EIV positive premises |
| Nasal swab                | 25            | 7 EHV positive premises |

\*Query respiratory disease.

2001–2007 were selected to examine the prevalence of ERV in Ireland. These swabs were collected from horses suffering from respiratory disease and submitted to the diagnostic laboratory of the Virology Unit of the Irish Equine Centre. No virological diagnosis was made. Virus was not isolated in RK-13 and EEL cells. PCR/RT-PCR for equine herpes virus 1 (EHV1), equine herpes virus 4 (EHV4) and equine influenza virus (EIV) were negative. One-hundred urine samples were collected from competing horses at Irish racetracks to examine the prevalence of virus shedding in clinically normal horses. Fifty-two samples comprising swab material and *post mortem* tissue from horses that were previously identified as EIV, equine infectious anaemia virus (EIAV) or EHV1/4 positive were used to examine assay specificity. Finally, 25 nasal swabs from premises affected by EIV and 25 nasal swabs from premises affected by EHV1 or EHV4 in Ireland were also tested to detect co-circulation of viruses.

### Isolation of virus from clinical samples

Nasal swabs were submitted to the laboratory in 5 ml of chilled virus transport medium, which consisted of sterile phosphate-buffered saline (PBS) supplemented with 100 u/ml penicillin, 100 µg/ml streptomycin, 5 mg/ml amphotericin B and 2% FCS. Urine samples and *post mortem* tissues were submitted to the laboratory in leak proof containers without additives. Nasal swabs were squeezed out with sterile forceps and tissues were homogenised. Approximately 0.1 g of *post mortem* tissue was homogenised in 5 ml of maintenance medium. Nasal fluids, urine and tissue homogenates were clarified by low-speed centrifugation prior to cell culture inoculation. For routine virus isolation, 25 cm<sup>2</sup> flasks of near-confluent RK-13 or EEL cells were inoculated with 0.5 ml nasal fluid/tissue homogenate. For the isolation of ERAV from rRT-PCR positive samples, 25 cm<sup>2</sup> flasks of near-confluent Vero cells were inoculated with 0.5 ml of urine or nasal fluid. The inoculum was allowed to adsorb for 1 h at 37°C. At the end of the adsorption period the inoculum was removed, the monolayer was washed with 3 ml sterile PBS and 5 ml maintenance medium was added. The monolayer was examined for the presence of cytopathic effect (CPE) and the medium was changed daily. When no evidence of infection was seen after 7 days the culture was frozen, thawed, sonicated and clarified prior to passage of 0.5 ml on fresh cell monolayers for a further 7 days.

### Extraction of RNA from clinical samples

Viral RNA was extracted from 140 µl sample material using QIAamp Viral RNA Mini kit<sup>2</sup> according to the manufacturer's

**TABLE 2: rRT-PCR ERV assay primer and probe sequences**

| Name <sup>a</sup>     | Sequence (5' to 3')        |
|-----------------------|----------------------------|
| ERAV 468F             | CCA GGT AAC CGG ACA GCG    |
| ERAV 569R             | GGC AGC GCT ACC ACA GG     |
| ERAV 508 <sup>b</sup> | CAT TGC TCT GGA TGG TGT    |
| ERBV 77F              | TGA TGC TTG GCT CTC AGA AA |
| ERBV 189R             | GCA AAC GAC CAA CAC ATC AA |
| ERBV 171 <sup>b</sup> | CTT CCA ACT AAA CCC        |
| ERBV 530F             | GTG GTA GCG CTG TGT GAG TT |
| ERBV 709R             | GAC ATG TGT GCA TTC AG     |

<sup>a</sup>Numbered from the 5' end of reference genomes for ERAV and ERBV (GenBank accession numbers NC\_003982 and NC\_003983 respectively);

<sup>b</sup>5'FAM labelled MGB probes.

instructions. The extraction procedure was also carried out on nuclease free water as a negative control and, on an appropriate positive control.

#### *Real-time RT-PCR primers and probes*

A multiple sequence alignment of ERV sequences deposited in GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) was analysed and conserved regions amongst different strains were highlighted. A combination of Applied Biosystems software Primer Express<sup>3</sup> and the online application Primer<sup>3</sup> (Rozen and Skaletsky 2000) were employed for primer and probe analysis and design. Primer probe sets for ERAV and ERBV detection were chosen, details of which can be found in Table 2. The ERAV and ERBV primers and probes are located in the 5' UTR region of the ERV genome and have 100% sequence identity with 8 published ERAV strains and 4 published ERBV strains (2 of ERBV1 and 2 of ERBV2). The ERAV primers, 468F and 569R, in conjunction with 5' FAM labelled MGB probe ERAV 508, produce a 118 bp RT-PCR product. The ERBV primers 77F and 189R together with 5' FAM labelled MGB probe ERBV 171, produce a 132 bp RT-PCR product.

#### *Real-time RT-PCR assays for detection of ERV*

Real-time RT-PCR was carried out on the ABI TaqMan 7500<sup>3</sup> platform using the one-step EZ RT-PCR kit<sup>3</sup> as recommended by the manufacturer. A 25  $\mu$ l reaction consisted of 1x buffer, 3 mmol/l manganese acetate, 300  $\mu$ mol/l of each dNTP except for 600  $\mu$ mol/l dUTP, 0.6  $\mu$ mol/l of primers, 0.2  $\mu$ mol/l probe, 0.01 u/ $\mu$ l AmpErase UNG, 0.1 u/ $\mu$ l of rTth DNA polymerase and 5  $\mu$ l RNA. Conditions were UNG treatment at 50°C for 2 min, reverse transcription at 60°C for 30 min, deactivation of UNG at 95°C for 5 min and amplification with 50 cycles of 95°C for 15 s and 60°C for 1 min.

#### *Determination of rRT-PCR assay sensitivity using in vitro transcribed RNA*

The ERAV and ERBV1 reference viruses obtained from the AHT were propagated in Vero and RK-13 cells respectively and employed for production of *in vitro* transcribed (IVT) RNA. RNA was extracted from virus preparations and 16  $\mu$ l reverse transcribed in a 40  $\mu$ l reaction consisting of 1x AMV buffer and 0.625 u/ $\mu$ l of AMV RT<sup>4</sup>, 1  $\mu$ mol/l reverse primer (Table 2, ERAV 569R and ERBV 189R), 200  $\mu$ mol/l of each dNTP and 0.5 u/ $\mu$ l

RNase inhibitor<sup>3</sup>. Reverse transcription was carried out at 42°C for 45 min, followed by denaturation at 95°C for 5 min (G-Storm)<sup>5</sup>. A 50  $\mu$ l PCR reaction contained 0.125 u/ $\mu$ l AmpliTaq DNA polymerase, 1x buffer and 200  $\mu$ mol/l of each dNTP<sup>3</sup>, 0.5  $\mu$ mol/l of each primer (Table 2, ERAV 569R and 468F, ERBV 189R and 77F) and 12.5  $\mu$ l cDNA. An initial denaturation was carried out at 95°C for 5 min, followed by amplification with 30 cycles of 95°C held for 15 s, 55°C for 30 s and 72°C for 20 s and a final elongation at 72°C for 5 min.

RT-PCR products for ERAV and ERBV (118 and 132 bp, respectively) were purified (QIAquick PCR Purification Kit)<sup>2</sup> and cloned into vector pCR-4-TOPO<sup>1</sup> according to the manufacturer's recommendations. Purified plasmid DNA (QIAprep Spin Miniprep kit)<sup>2</sup> was linearised with *Sbf*I, gel purified (QIAquick Gel Extraction kit)<sup>2</sup> and employed as template for T7 RNA polymerase *in vitro* transcription (RiboMAX)<sup>6</sup>. The reaction was carried out according to the manufacturer's instructions except that additional DNase (3 u/ $\mu$ g RNA) was added to the RNA prior to purification in order to completely remove input plasmid DNA.

The *in vitro* transcribed RNA was checked for removal of DNA in a minus RT, PCR only reaction where there was no amplification signal. The concentration of the RNA transcript was determined by measuring the absorbance at 260 nm and the copy number subsequently calculated using the formula: Number of copies =  $(X \text{ g}/\mu\text{l} * 6.022 * 10^{23}) / (\text{no. of bases} * 340 \text{ Daltons/base})$ . Intra-assay variation was calculated using 3 replicates of the ERAV or ERBV transcript at copy numbers 10<sup>6</sup>, 10<sup>4</sup> and 10<sup>2</sup> within the same rRT-PCR run. The analysis was repeated on 3 different occasions. Inter-assay variation was calculated based on the rRT-PCR assays from 3 different days.

#### *Real-time RT-PCR quantification of ERV RNA in clinical samples*

In order to quantify the amount of virus in positive samples, serial log dilutions of the ERV transcripts were used to generate standard curves ranging from 10<sup>6</sup> to 10<sup>2</sup> copies of ERAV/ERBV. The amount of ERV RNA in 40 positive samples was then determined from the curve in a rRT-PCR using the EZ RT-PCR kit as described previously. The samples, 20 swabs and 20 urines, were chosen at random. The 20 urine samples were ERAV positive. Of the 20 nasal swabs 17 were ERAV positive and 3 were ERBV positive.

#### *Verification of rRT-PCR positive samples*

Samples positive for ERAV by rRT-PCR were verified by virus isolation on Vero cells followed by immunofluorescence (IF) staining. For convenience, flasks of Vero cells for IF were frozen at -70°C. Cells were pelleted by centrifugation at 130 x g for 5 min and suspended in 2 ml of MEM medium. The cell suspension (200  $\mu$ l) was then fixed to a glass slide (Cytospin 4)<sup>7</sup> and fixed with acetone for 10 min. Negative control slides consisted of Vero cells inoculated with PBS. Specific ERAV equine antiserum (20  $\mu$ l) diluted 1:20 was added as the primary antibody (kindly provided by the AHT, Newmarket). The slides were incubated in a moist chamber for 1 h at 37°C then washed 3 times in PBS. Fluorescein labelled goat anti-horse IgG( $\gamma$ )<sup>8</sup> (20  $\mu$ l) diluted 1:50 was used as the secondary antibody and again slides were incubated in a moist chamber for 1 h at 37°C then washed 3 times in PBS. Stained slides were examined using a UV microscope (Nikon Optiphot)<sup>9</sup> and the presence or absence of fluorescing cells was determined.

All ERBV positive samples were verified by one-step rRT-PCR performed on the Roche Lightcycler<sup>4</sup> with a second set of primers, ERBV 530F and 709R (Table 2). The light cycler RNA amplification kit, SYBR Green I<sup>4</sup> was employed, giving a mix of 5.0 mmol/l MgCl<sub>2</sub>, 5X reaction mix, 0.4 µl enzyme mix, 0.5 µmol/l of each primer, 4.0 µl RNA and H<sub>2</sub>O to 20 µl. Real-time RT-PCR conditions were 55°C for 10 min, 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 55°C for 10 s and 72°C for 10 s. Fluorescence data were acquired at the end of each cycle and a dissociation stage was performed (95°C for 0 s, 65°C for 10 s and a 0.1°C/s rise to 95°C) to identify positive melting peaks. Where sufficient amplicon was generated, ERBV positive samples were sequenced (Qiagen)<sup>10</sup> with the same primers used in the RT-PCR.

## Results

### Detection of ERV in Ireland

Of the 250 archival nasal swabs tested for ERV, 29 were ERAV positive and 3 were ERBV positive (Table 3). Over the 7 years examined there was an average incidence rate per year of 10% for ERAV and 1.5% for ERBV. Of the 100 urine samples tested by rRT-PCR, 29 were ERAV positive but all were ERBV negative. Of the 52 swabs and *post mortem* tissues which were positive for other pathogens (Table 1), there was one case of co-infection where an EIV positive swab tested positive for ERBV. Concurrent infections

**TABLE 3: Prevalence of ERV in nasal swabs taken in Ireland 2001–2007**

| Year  | No. tested | ERAV <sup>a</sup> | ERBV <sup>+</sup> | ERAV % + | ERBV % + |
|-------|------------|-------------------|-------------------|----------|----------|
| 2007  | 52         | 5                 | 1                 | 9.6%     | 1.9%     |
| 2006  | 33         | 1                 | 0                 | 3.0%     | 0%       |
| 2005  | 18         | 1                 | 1                 | 5.5%     | 5.5%     |
| 2004  | 31         | 1                 | 0                 | 3.2%     | 0%       |
| 2003  | 30         | 3                 | 0                 | 10.0%    | 0%       |
| 2002  | 56         | 13                | 0                 | 23.2%    | 0%       |
| 2001  | 30         | 5                 | 1                 | 16.6%    | 3.3%     |
| Total | 250        | 29                | 3                 |          |          |

<sup>a</sup>+ is positive for ERV.

**TABLE 4: Reproducibility of rRT-PCR assays with ERV transcripts**

| RNA copy number         | Occasion | Coefficient of variation (%) |            |
|-------------------------|----------|------------------------------|------------|
|                         |          | Intra-assay                  | Interassay |
| ERAV<br>10 <sup>6</sup> | 1        | 0.84                         | 1.50       |
|                         | 2        | 2.30                         |            |
|                         | 3        | 0.93                         |            |
| 10 <sup>4</sup>         | 1        | 4.26                         | 1.46       |
|                         | 2        | 0.71                         |            |
|                         | 3        | 4.43                         |            |
| 10 <sup>2</sup>         | 1        | 0.73                         | 2.69       |
|                         | 2        | 0.36                         |            |
|                         | 3        | 1.38                         |            |
| ERBV<br>10 <sup>6</sup> | 1        | 0.04                         | 1.06       |
|                         | 2        | 1.32                         |            |
|                         | 3        | 1.22                         |            |
| 10 <sup>4</sup>         | 1        | 0.15                         | 3.00       |
|                         | 2        | 1.12                         |            |
|                         | 3        | 2.08                         |            |
| 10 <sup>2</sup>         | 1        | 0.15                         | 1.76       |
|                         | 2        | 1.70                         |            |
|                         | 3        | 3.12                         |            |

Percent coefficient of variation = (s.d./mean)\*100.

(1 ERAV and 1 ERBV) were detected on 2 premises affected by EIV. Virus was not isolated from any of the nasal swabs that tested positive for ERV by rRT-PCR. Virus was isolated from 17 of the 29 urine samples that were positive by rRT-PCR and from an additional 3 urine samples that were negative by rRT-PCR.

### Quantitative real-time RT-PCR sensitivity and variability

The limit of detection for both ERV transcripts was 10–100 copies/µl. Attempts made at ERAV/ERBV multiplexing using FAM and VIC labelled probes resulted in a loss of sensitivity for ERAV. Intra-assay variation ranged from 0.36–4.43% for ERAV and 0.04–3.12% for ERBV (Table 4). The coefficient of variation for interassay variation was ≤3.0% for both rRT-PCR assays. The RNA standard curves for both assays were consistently linear ( $r^2 = 1$ ) in the range of  $1 \times 10^6$  to  $1 \times 10^2$  copies/µl. The viral load (copies/ml) of 20 ERAV positive urine samples and 20 ERV positive swab samples (17 ERAV and 3 ERBV) was calculated (Fig 2). The amount of ERAV in the 20 urines examined ranged from  $2 \times 10^4$  to  $7 \times 10^9$  copies/ml. Of the 20 ERV positive nasal swabs, the amount of ERAV ranged from  $2 \times 10^4$  to  $1.5 \times 10^7$  copies/ml. Two of the ERBV positive swabs contained  $2 \times 10^6$  copies/ml and the third contained  $4.5 \times 10^5$  copies/ml.

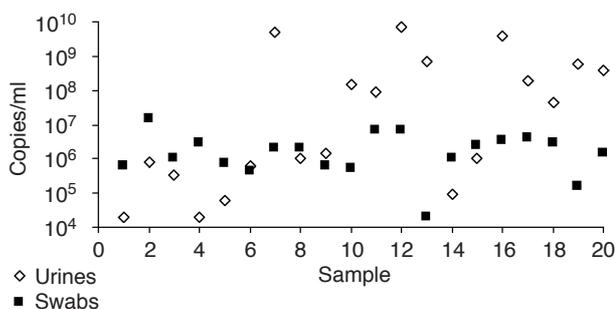
### Sequence data

RT-PCR products for 3 of the 5 ERBV positive samples were sequenced (Fig 3). Sequence data were obtained for the TaqMan RT-PCR products for samples 656, 351 and 517. For this RT-PCR product (Fig 3a), there are 9 nucleotides that differ between reference ERBV1 and ERBV2 strains and ERBV2 has one additional nucleotide. These differences are all located in a 19 bp sequence in this amplicon. For this RT-PCR product, sample 656 is 100% identical to ERBV1 and samples 517 and 351 have 98% and 96% nucleotide identity respectively with ERBV1.

For the larger Lightcycler amplicon (Fig 3b), there are 16 nucleotides that differ between reference ERBV1 and ERBV2 strains. Sequence data were obtained for the Lightcycler RT-PCR products for samples 656 and 351. There was insufficient product for sequencing sample 517. Sample 656 is 100% identical to ERBV1. However, sample 351 has 95% nucleotide identity with ERBV2 and 90% with ERBV1.

## Discussion

In this study, single rRT-PCR assays for the detection of ERAV and ERBV in diagnostic samples were developed and validated. The



**Fig 2:** Forty samples that were ERV positive by rRT-PCR, comprising 20 urine samples and 20 nasal swabs were chosen at random for viral load determination.

**Real-time RT-PCR ERBV positive samples sequence data**

**a) TaqMan amplicon**

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                                # #
ERBV1      GAGACCCGATTCTTTTGAGTCGAGACCATTTCCTCCCT-GAAGATTGCTTTTCTTCC
656        GAGACCCGATTCTTTTGAGTCGAGACCATTTCCTCCCT-GAAGATTGCTTTTCTTCC
517        GAGACCCGATTCTTTTGAGTCGAGACCATTTCCTCCCT-GAAGATTGCTTTTCTTCC
351        GAGACCCGATTCTTTTGAGTCGAGACCATTTCCTCCCTTAAAGATTGCTTTTCTTCC
ERBV2      GAGACCCGATTCTTTTGAGTCGAGAACCTTACCCCAACCCTTGAGATTGCTTTTCTTCC 60
          ***** * * ** ** *****

ERBV1      AACTAAACCCCTATTGATGTGTTGGTCGTTTGC
656        AACTAAACCCCTATTGATGTGTTGGTCGTTTGC
517        AACTAAACCCCTATTGATGTGTTGGTCGTTTGC
351        AACTAAACCCCTATTGATGTGTTGGTCGTTTGC
ERBV2      AACTAAACCCCTATTGATGTGTTGGTCGTTTGC 93
          *****
    
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**b) Lightcycler amplicon**

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                                # #
ERBV1      CTGTGTGAGTTGGCGGTGGATCCCCCTCGTGGTAACACGAGCCCCCGTGGCCAAAAGCCC
656        CTGTGTGAGTTGGCGGTGGATCCCCCTCGTGGTAACACGAGCCCCCGTGGCCAAAAGCCC
351        CTGTGTGAGTTGGCGGTGGACCCCCCTCGTGGCGACACGAGCCCCCGTGGCCAAAAGCCC
ERBV2      CTGTGTGAGTTGGCGGTGGAACCCCTCGTGGTGACACGAGCCCCCGTGGCCAAAAGCCC 60
          ***** * * * * *

                                # #
ERBV1      AGTGTTTACAGCACCTCTCACATCCAGGACGACCCCATCTGGCGCTCACTCTTAGTAGT
656        AGTGTTTACAGCACCTCTCACATCCAGGACGACCCCATCTGGCGCTCACTCTTAGTAGT
351        AGTGTTTGACAGCACCTCTCACATCCAGGGAGACCCACCTGGCGCTCACTCTAGTAAC
ERBV2      AGTGTTTACAGCACCTCTCACATCCAGGAAACCCACCTGGCGCTCACTCTTAGTAAC 120
          ***** * * * * *

                                # #
ERBV1      ATGGCTTAGTACGCATTAGGTGGTAAGCCGAGCTCTCCCTCGGCCTTGTCTGAATGC
656        ATGGCTTAGTACGCATTAGGTGGTAAGCCGAGCTCTCCCTCGGCCTTGTCTGAATGC
351        TCATCCTAGTACGTATTAGGTGGTAAGCCGAGCTCTCCCTCGGTCTTGTCTGAATGC
ERBV2      ACTTCCTAGTAGGTATTAGGTGGTAAGCCGAGCTCTCCCTCGGTCTTGTCTGAATGC 178
          ***** * * * * *
    
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*Fig 3: ERBV rRT-PCR positive samples were sequenced for diagnosis confirmation. Amplicons were generated by both the TaqMan (a) and Lightcycler (b) rRT-PCR assays. ERBV1 and ERBV2 reference sequences are from GenBank accession numbers NC\_003983 and NC\_003077 respectively. # Indicates nucleotides in the Irish isolates which are different to both ERBV1 and ERBV2 reference strains.*

assays were shown to be 100% specific with no cross reactivity in samples with recognised nontarget templates. They are more sensitive than virus isolation in cell culture. None of the ERAV or ERBV rRT-PCR positive nasal swabs were positive by virus isolation (0/35). This is in agreement with the findings of Mori *et al.* (2009) and Black *et al.* (2007b) where 0/14 and 1/6, respectively, ERBV rRT-PCR positive nasal swabs only, were positive by virus isolation. Our data provide further evidence to support the hypothesis that the significance of ERV has been underestimated due to the lack of sensitivity of traditional diagnostic techniques (Li *et al.* 1997). In this study, 59% of the ERAV rRT-PCR positive urines were positive by virus isolation. There was no apparent correlation between the viral RNA load in the urine sample and the likelihood of isolating virus. A minority of urine samples (3%) were negative by rRT-PCR, but positive by virus isolation. We excluded the possibility that these rRT-PCR negatives were due to the presence of PCR inhibitors in the sample material, as these urines spiked with EIV did not have reduced RT-PCR efficiency for the EIV. In addition, these false negative RT-PCR samples were not due to strain variation as the cell culture supernatants of these urines tested positive by this assay. This suggests that there was insufficient viral RNA in the sample for detection by rRT-PCR.

Partial sequence data (270 nucleotides) for one of the 5 ERBV positive samples (sample 656) demonstrated a 100% nucleotide

identity to an ERBV1 reference strain. However, sample 351 demonstrated greater identity with ERBV2 (95%) than ERBV1 (90%) in an amplicon of 178 nucleotides. This is the first time that a putative ERBV2 has been identified in Ireland, but further genetic characterisation is required to definitively type this virus. Two of the Irish ERBV positive samples had some unique nucleotides which were not common to either ERBV1 or ERBV2 reference sequences, highlighting the need for full genome sequencing of these viruses.

The rRT-PCR assays described here detected ERAV in 30 and ERBV in 5 of 300 nasal swabs collected over a 7 year period. The results suggest an association between these viruses and potential loss of performance in racehorses. Twenty-five (23 ERAV and 2 ERBV) of the 35 positive samples identified were submitted from racehorses suffering from loss of performance. Analysis of one flat training yard in Co. Kildare showed cases of ERAV respiratory disease primarily in 2-year-olds, in 3 consecutive years (2001–2003). One horse in this yard had 2 positive nasal swabs collected 18 days apart in February and March of 2002. The persistence of ERAV in the pharynx of some horses for at least one month after infection has been reported previously (Plummer and Kerry 1962). In the same Co. Kildare yard, there was also evidence of ERBV co-circulating with ERAV in 2001. The detection of the co-circulation of ERV and EIV in other yards (competition yards in Co. Down in 2003 and in Co. Limerick in 2007, and a racing

yard in Co. Meath in 2007) suggests that the multifactorial aetiology of some outbreaks of respiratory disease may currently be underestimated. A horse in the competition yard in Co. Down was infected simultaneously with ERBV and EI. Co-infection with equine rhinitis virus and equine herpes virus has been reported previously (Hoffer *et al.* 1978; Powell *et al.* 1978; Dynon *et al.* 2007) but, to the authors' knowledge, this is the first incidence of ERBV and EIV co-infection in horses.

The examination of urine samples collected from winning racehorses during a 4 month period (August–November 2006) indicated the high prevalence of ERAV infection in Ireland. In contrast, no ERBV was detected in urine. Thirty-two percent of urine samples were positive for ERAV, 29% by rRT-PCR and 20% by virus isolation. The latter results are similar to those of McCollum and Timoney (1992) who isolated ERAV from 17% of post race urine samples screened in Kentucky but did not isolate ERBV. They reported that the frequency of ERAV in the urine was distributed evenly among the sexes. Of the 100 racehorses screened in this study, 26% of males (19/72) and 36% of females (10/28) were shedding ERAV in their urine. McCollum and Timoney (1992) suggested that the persistent presence of the virus in the urine of some horses suggested a 'urinary carrier state'. The capacity to establish a persistent infection is a characteristic of FMDV the other member of the *Aphthovirus* genus (Alexandersen *et al.* 2003).

The rRT-PCR assays described here are more sensitive with a faster turnaround time compared to virus isolation for the detection of ERV. The higher incidence of ERV positive urine samples (32%) relative to nasal swabs (13%) coupled with the detection of concurrent infections with other viruses, indicates the need for further investigation of the epidemiology and the true clinical significance of these ubiquitous viruses. Real-time RT-PCR will be a useful tool for investigating the temporal association between clinical signs and rhinitis virus shedding.

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## Manufacturers' addresses

<sup>1</sup>Invitrogen, Paisley, Renfrewshire, UK.

<sup>2</sup>Qiagen, Valencia, California, USA.

<sup>3</sup>Applied Biosystems, Foster City, California, USA.

<sup>4</sup>Roche Diagnostics, Burgess Hill, West Sussex, UK.

<sup>5</sup>Gene Technologies, Braintree, Essex, UK.

<sup>6</sup>Promega Corporation, Madison, Wisconsin, USA.

<sup>7</sup>Thermo Scientific, Fremont, California, USA.

<sup>8</sup>KPL, Gaithersburg, Maryland, USA.

<sup>9</sup>Nikon, Kingston Upon Thames, Surrey, UK.

<sup>10</sup>Qiagen Sequencing Services, Hilden, Germany.

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# Real-time RT-PCR for the detection and quantitative analysis of equine rhinitis viruses

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**Keywords:** horse; equine rhinitis virus; diagnosis; quantitative RT-PCR; co-circulation

## Sequence data for Animal Health Trust ERAV and ERBV reference viruses

```
a)      FJ607143  TGTCCATTACCAGGTGATAAACTTAAGAAGATGGGCAGTTTCCATGAAGTTGTCAAAGCC 540
AI      -----AAGAAGATGGGCAGTTTCCATGAAGTTGTCAAAGCC 36
          *****

      FJ607143  CACCACCTGGTTAAGAATGGCTGGGATGTGGTTGTGCAGGTGAATGCCTCATTTGCTCAC 600
AI      CACCACCTGGTTAAGAATGGCTGGGATGTGGTTGTGCAGGTGAATGCCTCATTTGCTCAC 96
          *****

      FJ607143  TCAGGGCGCTGTGTGTAGCAGCAGTGCCGAATACGAACACACACATGAGAAAGCGCTT 660
AI      TCAGGGCGCTGTGTGTAGCAGCAGTGCCGAATACGAACACACACATGAGAAAGCGCTT 156
          *****

      FJ607143  AAGTGGTCTGAGCTTGAGGAACCAGCTTACACATACCAACAACCTTTCAGTCTTCCCCAC 720
AI      AAGTGGTCTGAGCTTGAGGAACCAGCTTACACATACCAACAACCTTTCAGTCTTCCCCAC 216
          *****

      FJ607143  CAGTTGCTAAATTTGAGGACAAATTCATCAGTACATTTGGTGATGCCCTACATTGGACCA 780
AI      CAGTTGCTAAATTTGAGGACAAATTCATCAGTACATTTGGTGATGCCCTACATTGGACCA 276
          *****

      FJ607143  GGCCCAACAACAATCTGACTTTGCACAACCCGTGGACCATTGTTATTTTAAATTTTGCT 840
AI      GGCCCAACAACAATCTGACTTTGCACAACCCGTGGACCATTGTTATTTTAAATTTTGCT 336
          *****

      FJ607143  GAATTGACAGGACCTGGCCAAACTGTGCTGTAACATATGTCGGTGGCTCCCATCGATGCA 900
AI      GAATTGACAGGACCTGGCCAAACTGTGCTGTAACATATGTCGGTGGCTCCCATCGATGCA 396
          *****

      FJ607143  ATGGTTAACGGGCTCTCCCAAATCCAGAGGCACCGATTAGAGTGGTGTCTGTGCCGTGAA 960
AI      ATGGTTAACGGGCTCTCCCAAATCCAGAGGCACCGATTAGAGTGGTGTCTGTGCCGTGAA 456
          *****

      FJ607143  TCAGACTCTTTCATGTCTTCAGTACCTGACAATTCGACTCCACTGTACCCCAAGGTTGTG 1020
AI      TCAGACTCTTTCATGTCTTCAGTACCTGACAATTCGACTCCACTGTACCCCAAGGTTGTG 516
          *****

      FJ607143  GTCCCGCCGCGCAAGTTCCTGGCCGGTTTACAAATTTTATTTGATGTGGCAAAACAGACA 1080
AI      GTCCCGCCGCGCAAGTTCCTGGCCGGTTTACAAATTTTATTTGATGTGGCAAAACAGACA 576
          *****

      FJ607143  TATTCATTTTGTTCATTTCTGGAAGGCCTTATTTTCGAGGTCCTAACACCTCTGGGGAC 1140
AI      TATTCATTTTGTTCATTTCTGGAAGGCCTTATTTTCGAGGTCCTAACACCTCTGGGGAC 636
          *****

      FJ607143  GAGCCACTGTTTCAGATGGATGTGTGCTCAGTGCAGGCTACATGGTACCTACGTA 1200
AI      GAGCCACTGTTTCAGATGGATGTGTGCTCAGTGCAGGCTACATGGTACCTACGTA----- 674
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|    |          |  |      |
|----|----------|--|------|
| b) | EF204771 | CACGTGCACACGGATGTCAGCTTCTTGCTTGACCGGTTCTTTGATGTTGAAACACTTGAG   | 132  |
|    | FJ607143 | CACGTGCACACGGATGTCAGCTTCTTGCTTGACCGGTTCTTTGATGTTGAAACACTTGAG   | 1740 |
|    | AII      | -----TTGCTTGACCGGTTCTTTGATGTTGAAACACTTGAG                      | 36   |
|    |          | *****  |      |
|    | EF204771 | CTTTCAAATTTGACAGGTTCTCCTGCCACACATGTTTGGATCCGTTTGGCTCGACTGCC    | 192  |
|    | FJ607143 | CTTTCAAATTTGACAGGTTCTCCTGCCACACATGTTTGGATCCGTTTGGCTCGACTGCC    | 1800 |
|    | AII      | CTTTCAAATTTGACAGGTTCTCCTGCCACACATGTTTGGATCCGTTTGGCTCGACTGCC    | 96   |
|    |          | *****  |      |
|    | EF204771 | CAACTGGCTTGGGCACGTCTGCTAAACACTTGCACCTACTTCTTTTCTGATTTGGAATTG   | 252  |
|    | FJ607143 | CAACTGGCTTGGGCACGTCTGCTAAACACTTGCACCTACTTCTTTTCTGATTTGGAATTG   | 1860 |
|    | AII      | CAACTGGCTTGGGCACGTCTGCTAAACACTTGCACCTACTTCTTTTCTGATTTGGAATTG   | 156  |
|    |          | *****  |      |
|    | EF204771 | TCAATTCAGTTTAAATTTACTACTACTCCATCCTCTGTTGGAGAAGGCTTCGTGTGGGTA   | 312  |
|    | FJ607143 | TCAATTCAGTTTAAATTTACTACTACTCCATCCTCTGTTGGAGAAGGCTTCGTGTGGGTA   | 1920 |
|    | AII      | TCAATTCAGTTTAAATTTACTACTACTCCATCCTCTGTTGGAGAAGGCTTCGTGTGGGTA   | 216  |
|    |          | *****  |      |
|    | EF204771 | AAGTGGTTCCTGTTGGAGCACCAACCAAGACTACAGATGCTTGGCAGTTAGAAGGAGGT    | 372  |
|    | FJ607143 | AAGTGGTTCCTGTTGGAGCACCAACCAAGACTACAGATGCTTGGCAGTTAGAAGGAGGT    | 1980 |
|    | AII      | AAGTGGTTCCTGTTGGAGCACCAACCAAGACTACAGATGCTTGGCAGTTAGAAGGAGGT    | 276  |
|    |          | *****  |      |
|    | EF204771 | GGAAATTCAGTTAGAATTCAACAATGGCCGTTGCAGGGATGTCCCCACTGTTGTGTTT     | 432  |
|    | FJ607143 | GGAAATTCAGTTAGAATTCAACAATGGCCGTTGCAGGGATGTCCCCACTGTTGTGTTT     | 2040 |
|    | AII      | GGAAATTCAGTTAGAATTCAACAATGGCCGTTGCAGGGATGTCCCCACTGTTGTGTTT     | 336  |
|    |          | *****  |      |
|    | EF204771 | AAGATTCAGGCTCTCGTTCACAAGCCTGTGGCTTCAGCGTGCCATATACATCTATGTGG    | 492  |
|    | FJ607143 | AAGATTCAGGCTCTCGTTCACAAGCCTGTGGCTTCAGCGTGCCATATACATCTATGTGG    | 2100 |
|    | AII      | AAGATTCAGGCTCTCGTTCACAAGCCTGTGGCTTCAGCGTGCCATATACATCTATGTGG    | 396  |
|    |          | *****  |      |
|    | EF204771 | CGTGTGTGCCAGTCTTTTACAATGGCTGGGGTGCACCTACCAAAGAAAAGGCAACCTAC    | 552  |
|    | FJ607143 | CGTGTGTGCCAGTCTTTTACAATGGCTGGGGTGCACCTACCAAAGAAAAGGCAACCTAC    | 2160 |
|    | AII      | CGTGTGTGCCAGTCTTTTACAATGGCTGGGGTGCACCTACCAAAGAAAAGGCAACCTAC    | 456  |
|    |          | *****  |      |
|    | EF204771 | AATTGGCTTCCTGGTGCACACTTTGGTTCCATTTTGTGCTGACTTCTGATGCGCATGACAAA | 612  |
|    | FJ607143 | AATTGGCTTCCTGGTGCACACTTTGGTTCCATTTTGTGCTGACTTCTGATGCGCATGACAAA | 2220 |
|    | AII      | AATTGGCTTCCTGGTGCACACTTTGGTTCCATTTTGTGCTGACTTCTGATGCGCATGACAAA | 516  |
|    |          | *****  |      |
|    | EF204771 | GGAGGGTGCTACCTGCGGTATCGTTTTCCGCGCGCCAACATGTATTGCCCTCGCCCCATT   | 672  |
|    | FJ607143 | GGAGGGTGCTACCTGCGGTATCGTTTTCCGCGCGCCAACATGTATTGCCCTCGCCCCATT   | 2280 |
|    | AII      | GGAGGGTGCTACCTGCGGTATCGTTTTCCGCGCGCCAACATGTATTGCCCTCGCCCCATT   | 576  |
|    |          | *****  |      |
|    | EF204771 | CCGCCGGCTTTTACGCGTCCAGCGGACAAAACCAGACACAAATTTCCCACTAACATTAAC   | 732  |
|    | FJ607143 | CCGCCGGCTTTTACGCGTCCAGCGGACAAAACCAGACACAAATTTCCCACTAACATTAAC   | 2340 |
|    | AII      | CCGCCGGCTTTTACGCGTCCAGCGGACAAAACCAGACACAAATTTCCCACTAACATTAAC   | 636  |
|    |          | *****  |      |
|    | EF204771 | AAGCAG-----  | 738  |
|    | FJ607143 | AAGCAGTGTACC-----  | 2352 |
|    | AII      | AAGCAGTGTACCAATTACTCTCTCCTCAAATTTGGCTGGAGATGTTGAGAGCAACCTGGC   | 696  |
|    |          | *****  |      |

c) NC\_003983 CCTAAACAAGGACCTCTCCTTGACAACGAATGCCCCCTCCACTTGAATTGGCTGAATTT 1020  
BI -----CTTGAATTGGCTGAATTT 18  
\*\*\*\*\*

NC\_003983 CCAAACAAAGACAACAACACTGCTGGGTTGCGGCACTGTCTCACTATTACACCCGTGTGTGAC 1080  
BI CCAAACAAAGACAACAACACTGCTGGGTTGCGGCACTGTCTCACTATTACACCCGTGTGTGAC 78  
\*\*\*\*\*

NC\_003983 GTGACCAATCAGTGACTAAGGTCCTCAACCACCTTCTGGCATTCCGGTATTATCTCACG 1140  
BI GTGACCAATCAGTGACTAAGGTCCTCAACCACCTTCTGGCATTCCGGTATTATCTCACG 138  
\*\*\*\*\*

NC\_003983 GCTTGGCAGTCCATTCTCCAGACAGATTTGTTAACGGGTATTATCCTGCTGCTTTTGCC 1200  
BI GCTTGGCAGTCCATTCTCCAGACAGATTTGTTAACGGGTATTATCCTGCTGCTTTTGCC 198  
\*\*\*\*\*

NC\_003983 GTTGAAACAGGACTTTGTTCACGGGCCTTCCCCATGCAACAGCACGGGTATGTTAGGAAT 1260  
BI GTTGAAACAGGACTTTGTTCACGGGCCTTCCCCATGCAACAGCACGGGTATGTTAGGAAT 258  
\*\*\*\*\*

NC\_003983 GCCACCTCCCACCCCTACAACCTTTTGTCTTTGCTCTGAACCCGTCCCTGGTGAAGACTAT 1320  
BI GCCACCTCCCACCCCTACAACCTTTTGTCTTTGCTCTGAACCCGTCCCTGGTGAAGACTAT 318  
\*\*\*\*\*

NC\_003983 TGGCATGCAGTAGTTAAGGTGGACTTGTCTAGAACTGAGGCACGTGTAGACAAATGGCTT 1380  
BI TGGCATGCAGTAGTTAAGGTGGACTTGTCTAGAACTGAGGCACGTGTAGACAAATGGCTT 378  
\*\*\*\*\*

NC\_003983 TGCATTGATGATGACCGGATGTACCTTTTCCAGGCCCTCCAACCTCGTGTCAAGCTGGCTTCC 1440  
BI TGCATTGATGATGACCGGATGTACCT----- 404  
\*\*\*\*\*

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d)  NC_003983  TTGCGTGGCTACAAC TACGCACCAGGCAAACACACCCAGCCCTCCAGTGCTCAAGACACC 1920
    BII  -----TACAAC TACGCACCAGGCAAACACACCCAGCCCTCCAGTGCTCAAGACACC 51
           *****

    NC_003983  CCCAGCAAGGCTGAGCAGTCCGTTGAGCGAGGATTTACGTTTCAGCTTGCACAGTGGGAG 1980
    BII  CCCAGCAAGGCTGAGCAGTCCGTTGAGCGAGGATTTACGTTTCAGCTTGCACAGTGGGAG 111
           *****

    NC_003983  ACATCCAGAAACATTTGGGACCATTTGACAATCCCTCTTCCCATGTGCCCTGGTCTTATT 2040
    BII  ACATCCAGAAACATTTGGGACCATTTGACAATCCCTCTTCCCATGTGCCCTGGTCTTATT 171
           *****

    NC_003983  AAGGTCTCTGGAATGTACAAAGCTTTCATTGAAACCCACGCCACATTAAGAATGGGTGG 2100
    BII  AAGGTCTCTGGAATGTACAAAGCTTTCATTGAAACCCACGCCACATTAAGAATGGGTGG 231
           *****

    NC_003983  AAGATTCAGGTGCAGTGCAATGCCAGCCAGTTCACAGTGGCTGCCTTTTGGTTGCCATG 2160
    BII  AAGATTCAGGTGCAGTGCAATGCCAGCCAGTTCACAGTGGCTGCCTTTTGGTTGCCATG 291
           *****

    NC_003983  ATTCTCGAGTACCTTAGCACTGCACAACAGGATTTTCTTGGTTCGTGGCGAGACAAAACC 2220
    BII  ATTCTCGAGTACCTTAGCACTGCACAACAGGATTTTCTTGGTTCGTGGCGAGACAAAACC 351
           *****

    NC_003983  ACGGATTCTACACCCGGCAGTGGGTGTGGAACACATATGAGGCTTTTCCCCCGGTTTT 2280
    BII  ACGGATTCTACACCCGGCAGTGGGTGTGGAACACATATGAGGCTTTTCCCCCGGTTTT 411
           *****

    NC_003983  CCTCCTCAGCAGATTACCCTGTTTCCCCATCAATTTTAAATTTGAGAACTAACACAACG 2340
    BII  CCTCCTCAGCAGATTACCCTGTTTCCCCATCAATTTTAAATTTGAGAACTAACACAACG 471
           *****

    NC_003983  GTTGACCTTGAGGTCCCGTATACAAATTTTGCCCCCTCTTCAAGTCCTACTTTGCATGT 2400
    BII  GTTGACCTTGAGGTCCCGTATACAAATTTTGCCCCCTCTTCAAGTCCTACTTTGCATGT- 530
           *****
    
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Fig o1: Animal Health Trust reference ERV strains used in this study aligned with published sequence data. (a) ERAV strain sequence data (AI) aligned with isolate NM11/67 (Accession number FJ607143), amplicon AI corresponds to nucleotides 505 to 1178 of FJ607143; (b) ERAV strain sequence data (AII) aligned with isolate NM11/67 (Accession numbers FJ607143 and EF204771), amplicon AII corresponds to nucleotides 1705 to 2352 of FJ607143. (c & d) ERBV strain sequence data (BI and BII) aligned with prototype ERBV1 strain P1436/71 (Accession number NC\_003983). Amplicon BI corresponds to nucleotides 1003 to 1406 and amplicon BII corresponds to nucleotides 1870 to 2399 of NC\_003983. \* Represents identical nucleotides.