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# Mapping B-cell epitopes in equine rhinitis B viruses and identification of a neutralising site in the VP1 C-terminus

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ABSTRACT

*Erbovirus* is a genus of the family *Picornaviridae* and equine rhinitis B virus (ERBV) is the sole species. Erboviruses infect horses causing acute respiratory disease and sub-clinical and persistent infections. Despite the high seroprevalence and worldwide distribution of these viruses, the pathogenesis and antigenic structure of the three ERBV serotypes (ERBV1, 2 and 3) is poorly understood. To characterise linear epitopes on ERBV structural proteins, a set of fusion proteins were expressed in *Escherichia coli*. These proteins were tested in Western blot and ELISA and reactive proteins were also used to identify neutralisation epitopes. VP1 contained serotype specific epitopes whereas VP2 was highly cross-reactive across the serotypes. The C-terminus of VP1 accounted for most of the reactivity of full-length VP1 and was also the location of a neutralising site in each serotype.



## 1. Introduction

Respiratory disease in horses is of major economic importance, particularly in the performance horse industry. One of the lesser-studied equine respiratory pathogens is equine rhinitis B virus (ERBV). Equine rhinitis B virus has been isolated from horses with clinical signs including fever to 41 °C for 1–3 days, nasal discharge, anorexia, oedema of the legs, lethargy, and swelling and abscessation of lymph nodes of the neck with pain on palpation (Fukunaga et al., 1983; Hofer et al., 1972; Mumford and Thomson, 1978). The ability of ERBV to establish persistent infection over an 18–24-month period (Burrows and Goodridge, 1978; Mumford and Thomson, 1978) and associated reports of subclinical infection with ERBV have suggested a role for these viruses in the exacerbation of infection with other respiratory viruses and with secondary bacterial infections (reviewed by Carman et al., 1997).

Initially, ERBV was classified as a *Picornavirus* with the equine rhinoviruses (Hofer et al., 1972; Steck et al., 1978). Analysis of genome sequence identity led to the reclassification of ERBV as the sole species in the genus *Erbovirus*, family *Picornaviridae* (Pringle, 1999). There are three known serotypes, ERBV1, ERBV2 and ERBV3 that vary in their acid stability (Horsington et al., 2011). All three serotypes have been isolated from horses worldwide and neutralising antibodies have been detected in 50–80% of horses tested and in all age groups (Black et al., 2007; Carman et al., 1997; Dunowska et al., 2002; Dynon et al., 2007; Fukunaga et al., 1981; Holmes et al., 1978; McCollum and Timoney, 1992; Mumford and Thomson, 1978; Rose et al., 1974; Steck et al., 1978; Wernery et al., 1998). Simultaneous infection with multiple serotypes has been reported and neutralising antibodies to two or all three serotypes in the one horse is common (Black et al., 2007; Dynon et al., 2007; Horsington et al., 2011).

Antibodies are the major effectors of protection against picornavirus infection, although cell-mediated immunity plays an essential role in the immune response to at least some of these viruses (Glezen et al., 1969; McCullough

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et al., 1992; Pay and Hingley, 1987; Rossi et al., 1991). While many neutralising epitopes in picornaviruses are conformational, linear epitopes are found in a number of viruses including Theiler's murine encephalomyelitis virus (TMEV) (Inoue et al., 1994; Kim et al., 1992), poliovirus (PV) (Blondel et al., 1983; Chow et al., 1985; Hoatlin et al., 1987), Enterovirus 71 (Foo et al., 2007), human rhinovirus (Skern et al., 1987) and foot and mouth disease virus (FMDV) (Giavedoni et al., 1991; Mateu et al., 1989; Strohmaier et al., 1982). The external capsid proteins, VP1, VP2 and VP3, share a wedge-shaped, eight-stranded, β-barrel structure. Differences exist in the size and conformation of the connecting loops between the strands and the extensions of the N- and C-termini, and antigenic sites appear limited to the surface exposed loops (reviewed by Mateu, 1995; Rueckert, 1985). In many picornaviruses the VP1 protein is the major site of antigenic epitopes and is the target for neutralising antibodies, although antigenic sites have also been detected in VP2 and VP3 (reviewed by Inoue et al., 1994; Mateu, 1995; Usherwood and Nash, 1995).

The aim of this study was to better understand the antigenic relationship between ERBV serotypes and identify potential antigens for a serotype-specific ERBV antibody detection ELISA. Full-length VP1 and VP2 and a selection of proteins representative of the surface exposed loops were expressed as fusion proteins in *Escherichia coli* and were used to map linear B-cell epitopes. The antigenicity of the fusion proteins was tested in Western blot and ELISA, using sera obtained from ERBV-immunised rats and from naturally ERBV-infected horses. Linear neutralising epitopes were identified using these fusion proteins in competitive inhibition of neutralisation assays and virus neutralisation assays with polyclonal rat sera prepared against specific proteins.

## 2. Methods

### 2.1. Viruses and sera

Viruses used in this study included the prototype ERBV1 and ERBV2 strains, ERBV1.1436/71 and ERBV2.313/75 (Black et al., 2005), and the Australian ERBV3 isolate, ERBV3.2225AS (Horsington et al., 2011).

Rat sera to specific proteins or whole virus were prepared by subcutaneous inoculation with 10 μg of purified protein in Freund's complete adjuvant followed by a second inoculation of 10 μg purified protein in Freund's incomplete adjuvant 14 days later. Production of protein or virus specific antibodies was evaluated in ELISA and Western blot (data not shown). Blood was collected from the tail vein and the serum was stored at −20 °C. This work was approved by the Animal Ethics Committee of the University of Melbourne, Australia.

ERBV positive horse sera were obtained from samples submitted for serology to the Equine Infectious Disease Laboratory between 1994 and 2000. Two hundred sera were screened in virus neutralisation assays against ERBV1.1436/71, ERBV2.313/75 and ERBV3.2225AS. Two sera for each serotype that showed neutralisation titres for only one of the three serotypes were selected for use in this study.

### 2.2. Construction of ERBV plasmids

Areas of high amino acid sequence variation between serotypes were identified for expression in *E. coli* (Fig. 1A). A schematic representation of the fusion proteins used in this study is shown in Fig. 1B. Full-length VP1 and VP2 PCR products (for primers see Supplementary Table S10) of each serotype were cloned into pGEM-T (Promega) and subcloned into the pQE30 (Qiagen) vector using the BamHI and SalI sites, in frame with the His-tag. Smaller regions were expressed as glutathione-S-transferase (GST) fusion proteins. PCR products representing VP1 N-terminus (Nt), CD loop, EF loop, GH loop, HI loop and C-terminus (Ct), and the VP2 EF loop (for primers see Supplementary Table S10) of each serotype were cloned into pGEM-T and subcloned into the pGEX-4T2 (GE Lifesciences) vector using the BamHI and SalI sites, in frame with the GST tag. Constructs were sequenced to confirm the correct sequence and reading frame of inserts.

### 2.3. Expression of fusion proteins

Recombinant fusion proteins encoded by plasmids were expressed in Rosetta Gami (Novagen) cells after induction with 1 mM isopropyl β-D-1-thiogalactopyranoside at 37 °C. Cells were pelleted then lysed using BPER (PIERCE) to separate fractions containing soluble and insoluble proteins, as described by the manufacturer. Full-length proteins expressed from pQE30 were purified under native conditions for VP2 and under denaturing conditions with 8 M urea for VP1 (Qiagen, 2003). Purified, denatured proteins were refolded by 1 M step-wise dialysis. Proteins expressed by the pGEX system were purified in native form using glutathione–sepharose 4B beads (GE Lifesciences), as described by the manufacturer. The eluted fractions were examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), quantitated using protein assay dye reagent (Bio-Rad) and stored in aliquots at −70 °C. A His-tagged control protein unrelated to ERBV (hepatitis E virus capsid protein (Li et al., 2005)) and GST alone were also prepared as described above. Proteins were named based on the serotype of origin, the capsid protein of origin and the loop or terminus. For example, full-length ERBV1 VP1 is referred to as 1VP1 and the EF loop of the VP2 protein from ERBV3 is referred to as 3VP2-EF.

### 2.4. SDS-PAGE and Western blot

SDS-PAGE was performed as described by Laemmli (1970) using the BioRad Mini-Protean III system and 12.5% (w/v) acrylamide gels under reducing conditions and either stained with Coomassie brilliant blue or transferred to polyvinylidene fluoride (PVDF) membrane (Warner et al., 2001). Membranes were probed with rat or horse sera as primary antibodies, and bound antibodies detected with HRP-conjugated rabbit anti-rat (DAKO) or HRP-conjugated goat anti-horse IgG (KPL) secondary antibodies. All horse sera were pre-absorbed against GST/*E. coli* cell lysate prior to use to prevent non-specific