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# Analysis of ORFs 2b, 3, 4, and partial ORF5 of sequential isolates of equine arteritis virus shows genetic variation following experimental infection of horses

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**Abstract**

Samples from horses experimentally infected with the "large plaque variant (LP3A+)" of equine arteritis virus were analysed. These included 182 nasal swabs collected from day 1 to 14 post-infection (p.i.), and 21 virus isolates obtained from white blood cells of animals that showed a prolonged viraemia between days 30 to 72 p.i. In order to determine the genetic stability of the virus and particularly to characterise the genetic variants found during the prolonged viraemia, partial sequences of open reading frame 5 (ORF5) encoding glycoprotein 5 (GP5) were generated. Viruses with amino acid substitutions in GP5 were used for further amplification and sequencing of a fragment encompassing ORFs 2b, 3, and 4. The ORF5 nucleotide sequences of the virus present in 65 out of 66 nasal swabs were identical to that of the inoculated virus, suggesting that the ORF5 gene of LP3A+ was genetically stable during the first 2 weeks p.i. Contrary to this, a number of mutations were found in the ORF5 of virus isolates obtained from day 30 p.i. The mutations mainly clustered in antigenic neutralization site C within variable region 1 of the GP5 ectodomain. Sequence variability was also identified in ORFs 2b, 3 and 4, with ORF 4 having the highest proportion of non-synonymous changes (4/6).


*Keywords:* Equine arteritis virus; Genetic variation; ORF

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## 1. Introduction

Equine arteritis virus (EAV) is an enveloped RNA virus belonging to the family *Arteriviridae* of the order *Nidovirales*. The EAV genome is a positive-stranded, polyadenylated RNA molecule of 12.7 kb (den Boon et al., 1991). The proximal two thirds of the genome contains two large open reading frames (ORF) ORFs 1a and 1b, which encode the ''replicase'' polyproteins. These polyproteins are post-translationally processed by three virus-encoded proteases into twelve mature products, the non-structural proteins (nsp), nsp1 to nsp12 (Snijder et al., 1994; van Dinten et al., 1996). The remainder of the genome contains seven overlapping ORFs (2a, 2b, 3, 4, 5, 6, and 7), which are transcribed from a nested set of subgenomic mRNAs, and translated into the structural proteins of the virus (de Vries et al., 1992). These include the nucleocapsid protein (N) and a group of membrane proteins comprising minor (E, GP2b, GP3, and GP4) and major (GP5 and M) components of the viral envelope.

The major viral envelope protein GP5 has been shown to contain four distinct neutralization sites in its ectodomain, namely sites A (aa 49), B (aa 61), C (aa 67–90), and D (aa 99–106) (Balasuriya et al., 1997). Sites B, C, and D are all located in variable region 1 (V1), encompassing amino acids 61–121. Amino acid substitutions in these four sites can change the neutralization phenotype of the virus, and deletion of this region (aa 66–122) demonstrated an altered specificity of the neutralizing antibody responses, compared with those elicited by wild type virus (Castillo-Olivares et al., 2003). Furthermore, it has been demonstrated that a prototype subunit vaccine comprising bacterially expressed GP5 (ectodomain) is effective at stimulating virus neutralizing antibody and protecting against EAV challenge infection in horses (Castillo-Olivares et al., 2001).

The large plaque variant (LP3A) of EAV Bucyrus strain was derived by plaque purification of the original stock of the pleural fluid isolate virus (Wescott et al., 2001). A virus stock (LP3A+) was obtained by one additional cell culture passage of the LP3A virus in equine embryonic lung cells. During the course of vaccination and challenge experiments, it was observed that following intranasal infection with LP3A+, the virus could be re-isolated from the blood of a proportion of ponies even after 3 weeks post-infection (p.i.; up to day 72 p.i.). The present studies aimed to investigate the genetic stability of viruses obtained from the horses throughout the long experimental period (72 days), and particularly to characterise the genetic variants found during the prolonged viraemia.

## 2. Materials and methods

### 2.1. Animal infection experiment

Fourteen Welsh Mountain ponies (castrated male and mares) used in this study were part of a larger vaccination and challenge study which will be reported more fully elsewhere. Six ponies were vaccinated with a $G_L$ subunit vaccine (Castillo-Olivares et al., 2001) and eight remained as naïve controls (see Table 2 for allocation of each group). The vaccinated animals received 2 doses 4 weeks apart and the challenge occurred 5 months later when the antibody levels had dropped to undetectable levels by the VN test. All ponies were kept in an isolated barn, and were challenged with $10^6$ $TCID_{50}$ of LP3A+ by intranasopharyngeal nebulisation of virus suspension. The ponies were initially swabbed every day for 14 days, and bled every other day until day 21. In view of the observation of persistent viraemia in some ponies, blood sampling was continued from day 30 until three consecutive samples were negative by virus isolation, whereupon the specific animal was released from the study. All samples were stored at −80 °C until analysis.

### 2.2. RNA extraction and reverse transcription (RT)

The samples analysed in this study included 182 nasal swabs collected from day 1 to 14 p.i. and 21 virus isolates, derived by recovery of infectious virus by passage on RK13 cells, from white blood cells of animals that showed a prolonged viraemia between days 30 and 72 p.i. Total RNA was extracted, from either the primary clinical samples (nasal swabs) or tissue culture (virus isolates), using the TRIzol LS Reagent (Invitrogen, USA), following the manufacturer's instructions. Synthesis of cDNA was done by random priming and using M-MLV reverse transcriptase, as described previously (Liu et al., 2006).