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

4
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25 **Abstract**

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

41

42 Keywords: equine arteritis virus, genetic variation, ORF

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49 **1. Introduction**

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

62

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

73

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

84

85 **2. Materials and Methods**

86 *2.1 Animal infection experiment*

87 Fourteen Welsh Mountain ponies (castrated male and mares) used in this study were part
88 of a larger vaccination and challenge study which will be reported more fully elsewhere.
89 Six ponies were vaccinated with a GL subunit vaccine (Castillo-Olivares et al., 2001) and
90 eight remained as naïve controls (see Table 2 for allocation of each group). The vaccinated
91 animals received 2 doses 4 weeks apart and the challenge occurred 5 months later when
92 the antibody levels had dropped to undetectable levels by the VN test. All ponies were
93 kept in an isolated barn, and were challenged with 10^6 TCID₅₀ of LP3A+ by
94 intranasopharyngeal nebulisation of virus suspension. The ponies were initially swabbed
95 every day for 14 days, and bled every other day until day 21. In view of the observation of
96 persistent viraemia in some ponies, blood sampling was continued from day 30 until three

97 consecutive samples were negative by virus isolation, whereupon the specific animal was
98 released from the study. All samples were stored at -80°C until analysis.

99

100 *2.2 RNA extraction and reverse transcription (RT)*

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

109

110 *2.3 PCR amplification of partial ORF5 gene*

111 Two sets of primers (Table 1) were used to amplify ORF5 gene by a nested PCR using
112 *Taq* DNA polymerase (Ampli Taq, Perkin-Elmer). The first PCR mix contained 32 μl of
113 sterile distilled water, 5 μl of 10x PCR buffer, 1 μl of 10 mM dNTP mix, 1 μl of each
114 external primer, 3 μl of 25 mM MgCl_2 , 1U of *Taq* polymerase and 5 μl of cDNA. The
115 thermal profile included initial denaturation at 94°C for 2 min, followed by 30 cycles of
116 denaturation at 94°C for 45 sec, annealing at 60°C for 55 sec, and extension at 72°C for 90
117 sec, and a final extension at 72°C for 7 min. Two microliter of the first PCR product was
118 used for the second PCR in a volume of 50 μl . The same thermal profile was used for the
119 second PCR except that the primers were annealed to the templates at 58°C for 60 sec.

120

121 *2.4 PCR amplification of ORFs 2b, 3, and ORF4*

122 The Elongase amplification system (Invitrogen, USA) was used to amplify a fragment of
123 1712 bp encompassing ORFs 2b, 3, and 4. The reaction mix contained 1 µl of 10 mM
124 dNTP mix, 2 µl of each primer, 5 µl of cDNA, 5 µl of 5x buffer A and B to adjust a final
125 MgCl₂ concentration to 1.5 mM, 29 µl of sterile distilled water, and 1 U Elongase enzyme
126 mix (a mixture of *Taq* and *Pyrococcus* species GB-D thermostable DNA polymerases).
127 The cycling profile included initial denaturation at 95°C for 2 min, followed by 35 cycles
128 of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec, and extension at 68°C for
129 3 min, and a final extension at 68°C for 10 min.

130

131 *2.5 Sequencing and sequence analysis*

132 Sequencing reactions were performed with an ABI PRISM BigDye Terminator v3.1 Cycle
133 Sequencing Kit (Applied Biosystems). Following precipitation of extension products,
134 capillary electrophoresis of the sequencing products was carried out in a 3100 genetic
135 analyzer, as described previously (Liu et al., 2006). The nucleotide sequences were
136 analysed with multiple programs of the DNASTAR package (DNASTAR, Inc., v5.0.3,
137 Madison, WI, USA). The nucleotide sequences have been deposited in the GenBank under
138 the accession numbers: EU289380-EU289388.

139

140 **3. Results**

141 *3.1 PCR amplification and sequence analysis of EAV ORF5*

142 The ORF5 gene was amplified by nested RT-PCR from 182 nasal samples. The viral
143 ORF5 gene was detected from nasal discharges for a period of 8-13 days (Table 2). At day
144 14 p.i, viral RNA could not be detected in nasal swabs from any of the infected horses.

145 Amplification was obtained from 20 out of 21 virus isolates from blood samples taken
146 from day 30 to day 72 p.i.

147

148 A total of 84 PCR products were used for sequencing, comprising 66 from nasal swab
149 samples (from day 2, 4, 6, 8, 10, and 12 p.i.) and 18 from virus isolates recovered from
150 blood samples (from day 30 to day 72 p.i.). Sequence alignments showed little nucleotide
151 variation of sequences from nasal swab samples: 65 out of the 66 sequences were identical
152 to the input virus (LP3A+). Only one sequence (0071-10) had a nucleotide change (A to
153 G), which led to a predicted amino acid change from Asparagine (N) to Aspartic acid (D)
154 at position 81 of GP5. This change would abolish the second glycosylation site, which is
155 present in the majority of EAV field isolates (Stadejek et al., 1999). The alignment of the
156 remaining 18 sequences (obtained from virus isolates of blood samples collected from 30
157 days p.i.) revealed 8 variants, with a total of 22 nucleotide substitutions.

158

159 The deduced amino acid sequences of those virus isolates with non-synonymous changes
160 were aligned with the sequence of LP3A+. As shown in Fig. 1, 12 out of 14 amino acid
161 mutations occurred in the first two variable regions: V1 (aa 61-121), and V2 (aa 141-178).
162 One mutation occurred between V1 and V2, another (V to I at aa 217 of sequence 5407-
163 50) occurred in V3 (aa 202-222; not shown in the Figure 1). These substitutions mainly
164 grouped in the V1 region, especially at site C, previously identified as a crucial antigenic
165 neutralization site.

166

167 *3.2 PCR amplification and sequence analysis of EAV ORFs 2b, 3, and 4*

168 A fragment of around 1.8 kb was amplified with the Elongase enzyme mix from nine
169 selected viruses whose GP5 had amino acid changes in regard to the original virus LP3A+.
170 The final sequence was assembled from 4 overlapping sequence readings. The alignment
171 of the nucleotide sequences with the original LP3A+ revealed sequence variation of each
172 ORF less than 0.3%, 0.6%, and 0.7% for ORFs 2b, 3 and 4, respectively. As shown in
173 Table 3 (the virus is indicated as “horse number-day p.i”), of the three ORFs, ORF4 had
174 the highest proportion of coding changes (4/6), followed by ORF3 (3/7) and ORF2b (3/9).

175

176 In order to investigate the heterogeneity of sequences within the input virus and selected
177 clinical samples, a PCR-XL-TOPO vector (Invitrogen, USA) was used for cloning ORF5
178 PCR products (generated using Elongase) from the LP3A+ virus stock and from samples
179 obtained at different time points from ponies 665f and 0071. Some sequence variation was
180 detected between individual clones (5-16 clones per sample) for a given sample, i.e. 12 out
181 of 16 clones of 665f-10, and 5 out 5 clones of 665f-44 had single nucleotide changes from
182 the consensus. For pony 0071, all 0071-10 clones had the same nucleotide change
183 (corresponding to amino acid substitution N81D) identified from the uncloned PCR
184 product, confirming this was the predominant sequence for this sample. Moreover, the
185 identical nucleotide change was identified in 5 out of 15 clones of the LP3A+ input virus.
186 This suggests that a proportion of the original input virus population included the N81D
187 variant.

188

189 To exam whether virus isolation could have been responsible for introduction of changes
190 due to replication in tissue culture, the LP3A virus stock was propagated for three
191 passages. Viral RNA was extracted and subjected for same RT-PCR amplification.

192 Sequencing of these PCR products showed no nucleotide difference among these
193 sequences.

194

195 **4. Discussion**

196 The normal course of EAV infection is generally characterized by a viraemia which does
197 not last longer than 14 days p.i. However, in certain cases viraemia can be more prolonged
198 (McCollum et al., 1994; Neu et al., 1988). In this study, a proportion of ponies
199 experimentally infected were found to have viraemia lasting up to 72 days post-infection.
200 This could correspond to a low level, persistent viraemia at a level around the thresholds
201 for detection by virus isolation and PCR amplification, or alternatively periodic waves of
202 viraemia. Since the levels of viraemia were not quantitated, it is therefore not known
203 whether there was significant fluctuation in the level of viraemia between days 3 and 72
204 p.i. For several other viruses, periodic waves of viraemia have been found to be associated
205 with sequence variation that often corresponds to changed antigenic determinants. Hedges
206 et al. (1999) reported genetic divergence with emergence of novel phenotypic variants of
207 EAV during persistent infection of stallions. In this study, we investigated genetic
208 variation during the prolonged viraemia observed for EAV, concentrating on
209 determination of the sequence of a known neutralising antibody target (GP5).

210

211 Based on the sequence analysis of ORF5, it is clear that the virus is genetically stable
212 during the first two weeks p.i. Nucleotide variation of ORF5 was only detected in a single
213 sample out of 66 PCR products from nasal swabs of 14 ponies within the first two weeks.
214 Sequencing multiple ORF5 clones PCR amplified from the input virus stock identified an
215 equivalent sequence to 0071-10 (corresponding to amino acid substitution N81D) as a

216 minor component of the original input virus population. This suggests that the sequence of
217 0071-10 (and of 072c-30, a blood isolate recovered 30 days p.i.) may have been selected
218 from the original input virus pool, rather than arising during *in vivo* diversification. Our
219 result is consistent with the findings of Balasuriya et al. (1999), who showed a 99.9% to
220 100% sequences identity of ORF5 of EAV from 11 foetal tissues of 9 foals collected
221 during an EAV outbreak. In contrast, during the prolonged period of viraemia, several
222 mutations were observed in ORF5. Since these mutations were clustered in two variable
223 regions of GP5 with most amino acid substitutions in the neutralization site C, it is
224 plausible that there is a link between the mutations and immune selection of variants
225 during the prolonged viraemic phase. Genetic variation has been demonstrated among
226 field isolates and laboratory strains of EAV and by selection of neutralization resistant
227 variant *in vitro* (Balasuriya et al., 1995a, 1995b; Chirnside et al., 1995; Hedges et al.,
228 1996).

229

230 It should be noted that the sequences determined during the prolonged viraemic phase (day
231 30 onwards) were from virus isolates rather than primary clinical samples (whereas the
232 sequences determined during the first two weeks of infection were direct from nasal swab
233 material). It is possible, therefore, that the process of virus isolation resulted in a
234 'bottleneck', such that a limited subset of virus variants present in the original population
235 grew out of the virus isolation cultures. However it is unlikely that the process of virus
236 isolation *per se* imposed a selective pressure resulting in the selection of variants, since a
237 round of three passages of EAV in RK-13 cells was not found to result in selection of
238 sequence variants. Furthermore, the positions of the mutations seen in GP5 are consistent

239 with selection in response to host antibody pressure. Indeed, the pattern of mutations seen
240 in our study is similar to those observed by Balasuriya et al. (1999).

241

242 However, immune selection, if present, does not explain the fact that none of the
243 mutations became fixed i.e. present in sequential samples. In particular, 0071 displayed 4
244 different sequences on day 31, 50, 57, and 72 p.i. It seems that sequences changed from
245 one sequence type to another in a very short time period. This may be a consequence of
246 sampling a subset of the entire virus population at any given time. Alternatively, over the
247 time-span of the experiment there may have been relatively rapid fluctuations in the
248 predominance of particular sequence variants in the virus population and a longer time
249 period may be required for particular mutations to become fixed in the population.

250

251 In conclusion, the ORF5 gene of LP3A+ was genetically stable during the first two weeks
252 p.i. The genetic variation of LP3A+ virus observed from day 30 p.i. mainly clustered in
253 the ectodomain part of GP5, particularly in the neutralization site C of the variable region
254 one. These mutations in the V1 region could be responsible for differences in the
255 neutralization phenotype of virus.

256

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259

260 **References**

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319 of equine arteritis virus. In: Sixth International Symposium on Positive Strand RNA
320 Viruses, Paris, France, May 28 – June 2, p. 1-79.

- 1 Table 1. Nucleotide sequence and position of primers used for PCR amplification and
 2 sequencing of EAV ORFs 2b, 3, 4, and partial ORF5.

Name	Position ^a	Nucleotide sequence (5'-3')
105	105-126	GCTGACGGATCGCGGCGTTATT
CR2	127-149	GCCARTTTGCTGCGATATGATGA
CR3	691-669	TGGGCCTACCTGGGACTAACAAC
673	695-673	ATAGTGGGCCTACCTGGGACTAA
BuF_GS	9743-9764	CCCGTGTGATGGGCTTAGTGTG
BuR_GS-2	10600-10585	GGGAACAATTAATACTAA
BuF_ORF3	10251-10273	TTGCACCGGCATTGEATCATAAT
BuR_ORF3	10829-10809	CGTGGCCCAATCCATGACTAA
BuF_ORF4	10629-10651	TTGCGTGCAAGTGGTTTGTAGTC
BuF_ORF4-1	11187-11167	CGCACCCCAAAGCAAGAATAG
BuF_GL2	10957-10975	GGAACACCCAAACGCTACT
BuR_GL-M	11675-11655	TAAGCGTAGCATAGGGTAGTA

^aBased on strain Bucyrus (GenBank accession no. NC_002532).

3

- 1 Table 2. PCR amplification of partial ORF5 gene of EAV from nasal swabs and virus
 2 isolates collected from 14 horses during the experimental period. “+” indicates positive
 3 result, and “-“ indicates negative result: samples that were not available is shown as “n”

	Vaccinated horses							Non-vaccinated horses						
d.p.i. ^a	1769	5407	0071	0d4d	222a	5764	7013	9509	072c	091b	197c	230c	445f	665f
1	+	+	+	+	+	+	+	+	+	+	+	+	+	-
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	-	+	+	+	-	n	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	-	n	+	+	n	+	+	+	+	-
8	+	+	+	+	+	n	+	+	n	+	+	+	n	+
9	-	+	+	+	+	+	+	-	+	+	+	+	n	+
10	+	-	+	-	+	+	+	-	+	+	+	-	+	+
11	+	-	-	-	-	+	-	-	n	+	n	-	+	-
12	-	-	-	-	-	n	+	-	n	+	+	+	n	+
13	-	-	-	-	-	n	+	-	-	-	+	-	-	+
14	-	-	-	-	-	-	-	-	-	n	-	-	-	-
30	+				+	+		+	+					
31			+	+	+									+
43								+						+
44		+												+
50		+	+	-	+									+
57			+											
60			+											
72			+											

^a days post infection.

1 Table 3. Summary of nucleotide and amino acid substitutions in ORFS 2b, 3, and 4

Viruses	ORF2b		ORF3		ORF4	
	nt change	aa change	nt change	aa change	nt change	aa change
	T→A (67)	L→M (23)	A→G (85)	N→D (29)		
0071-31	A→G (567)	I→M (189)				
	G→A (153)					
0071-57	T→C (456)					
			A→G (489)		A→G (95)	H→R (32)
					T→C (237)	
0071-72					A→G (67)	G→R (98)
	A→C (213)					
072c-30	C→T (380)					
1769-30	T→C (516)		T→C (34)			
			T→C (259)		C→T (355)	P→S (119)
			C→T (293)	S→F (98)		
5407-50			C→T (362)	T→I (121)		
665F-44			A→G (489)			
	A→T (313)	H→Y (105)			A→G (95)	H→R (32)
9509-43	C→T (396)				T→C (183)	

2