

HIGHLY DIVERSE TYPE OF EQUINE ARTERITIS VIRUS (EAV) FROM THE SEMEN OF A SOUTH AFRICAN DONKEY: SHORT COMMUNICATION

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Equine arteritis virus (EAV) was detected by RT-nested PCR in semen samples from a naturally infected South African donkey. Sequence analysis of the amplified ORF5 fragment revealed only 60 to 70% nucleotide identity to a panel of EAV reference sequences. The unique donkey EAV sequence was also found to be stable during passage in horses. The sequence data reported in this study indicate that the South African donkey variant might represent a new genotype of EAV. The distinct genetic properties of the South African asinine strain of EAV suggest a divergent evolution of this arterivirus in various host species or, alternatively, a possible role for African donkeys in the emergence of EAV in horses.

Key words: EAV, arterivirus, genetic diversity, donkey

Equine arteritis virus (EAV) is a member of the Arteriviridae family, together with the porcine reproductive and respiratory syndrome virus (PRRSV), simian haemorrhagic fever virus (SHFV) and lactate dehydrogenase-elevating virus (LDV) (Snijder and Meulenberg, 1998). The families Arteriviridae and Coronaviridae constitute the order Nidovirales which, besides EAV that emerged in 19th century (Pottie, 1888), contains several new pathogens such as PRRSV (emerged in the 1980s) (Plagemann, 2003), porcine respiratory coronavirus (emerged in the early 1980s) (Laude et al., 1993) and severe acute respiratory

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syndrome coronavirus (SARS, emerged in 2002) (Rota et al., 2003). Exposure to EAV usually results in subclinical infection, but it may also give rise to a disease termed equine viral arteritis (EVA) which is associated with abortions in pregnant mares.

The EAV genome consists of a single positive-stranded RNA, containing nine open reading frames (ORF) (Snijder et al., 1999). The GP₅ envelope glycoprotein encoded by ORF5 contains the major neutralisation determinants, and is variable between EAV isolates (Balasuriya et al., 1997; Stadejek et al., 1999; Hornyák et al., 2005; Mittelholzer, Stadejek, Johansson, Baule, Ciabatti, Hannant, Paton, Autorino, Nowotny and Belák (unpublished results). The majority of the currently available ORF5 sequences have been obtained from European and North American horses and, when genetically compared to each other, they form a number of clusters of related sequences (Stadejek et al., 1999; Hornyák et al., 2005; Mittelholzer, Stadejek, Johansson, Baule, Ciabatti, Hannant, Paton, Autorino, Nowotny and Belák, unpublished results). Very little is known about EAV strain diversity on other continents and in equids other than horses. This information would be relevant for a better understanding of EAV evolution, more proper virus classification and for improved vaccine development (Castillo-Olivares et al., 2001 and 2003).

To address the issues outlined above, we analysed a 518 nt segment of the EAV ORF5 from seven semen samples collected three times between November 1993 and March 1994 (Paweska, personal communication) from a donkey in the Republic of South Africa (Paweska et al., 1995). Also, analysis was done on the ORF5 segment of EAV obtained from the plasma of a horse stallion intranasally inoculated with naturally infected donkey semen (Paweska et al., 1996). Total RNA extracted with TRIzol reagent (Invitrogen) directly from semen or plasma samples was reverse transcribed as previously described (Stadejek et al., 1999). Previously described nested PCR primers (Stadejek et al., 1999) failed to provide amplification from this cDNA. Therefore, we designed a new external primer set for the first round of the nested PCR: GL105F (nucleotide positions 11250-11271 of the full-length sequence of the Bucyrus strain, GenBank accession number NC_002532: 5' GCT GAC GGA TCG CGG CGT TAT T 3') and GL673R (11840-11818: 5' ATA GTG GGC CTA CCT GGG ACT AA 3'). Nested PCR was performed in a DNA Thermal Cycler (Perkin-Elmer). The reaction mixture comprised 5 µl of 10 × PCR buffer II (Perkin-Elmer); 1.5 mM MgCl₂; 200 µM dNTP (each); 20 pmol of sense and antisense primers; 1 U AmpliTaq DNA polymerase (Perkin-Elmer) and 5 µl of the template, making a total of 50 µl. In the first round of nested PCR with 5 µl of cDNA as template the aforementioned primers GL105F and GL673R were used and in the second round of nested PCR with 5 µl of the first round PCR product as template, the set of previously published nested primers was used: CR2 (sense primer) 11272 5' GCC A(A or G)T TTG CTG CGA TAT GAT GA 3' and EAV32 (antisense

primer) 5' TGG GCC TAC CTG GGA CTA ACA AC 3' 11836. Amplification of each PCR round involved 30 cycles of denaturation at 94 °C for 45 sec, primer annealing at 55 °C (first round) or 58 °C (second round) for 1 min and primer extension at 72 °C for 1.5 min.

PCR fragments of the predicted size were amplified from all the semen and plasma samples (not shown), purified using the QIAquick PCR Purification Kit (QIAGEN) in accordance with the manufacturers' recommendations, and subjected to cycle sequencing with the primers CR2 and EAV32.

The obtained nucleotide sequences were compared to 26 diverse ORF5 sequences from GenBank. Selection of the reference sequence set involved construction of a phylogenetic tree of all EAV ORF5 sequences available in GenBank (not shown). For comparative analysis presented in this paper only most phylogenetically distant sequences were used, representing all the genetic subgroups of EAV recently defined by Mittelholzer et al. (unpublished results). Also, ORF5 sequences of EAV from South African horses and Italian donkeys were included (Table 1).

The CLUSTAL W 1.83 programme was used for sequence alignment (Thompson et al., 1994), and the DNADIST programme of the PHYLIP 3.6b package (Felsenstein, 1989) was applied to establish the genetic distance between pairs of the sequences based on Kimura's 2-parameter method. The phylogenetic tree was constructed using NEIGHBOR programme from the PHYLIP package. Bootstrap sampling was carried out on 100 replicate data sets to assess the confidence limits of the branching pattern. The identity between the sequences was estimated with MegAlign 5.01 programme from the Lasergene package (DNASTAR Inc., USA) by CLUSTAL W method (Higgins and Sharp, 1989).

Intriguingly, the sequence from the semen of the naturally infected South African donkey was very different from the reference sequences obtained from viruses in horses and donkeys in North America and Europe, and from virus in a South African Lipizzaner (Fig. 1). The new South African asinine sequence of EAV had only 61.2% nucleotide identity to the American S1368 sequence (Fig. 1). In contrast, the two most diverse horse sequences, Hungarian H21 and American S1368, shared 74.5% nucleotide identity. Passaging the asinine virus once in horse was associated with minimal nucleotide differences (Fig. 1, compare sequences J1-931125 and J25-941109-3).

Several ORF5 sequence analyses allowed separation of the EAV strains into a number of genetic groups, with type I viruses found mostly in North America, while type II viruses were mostly found in Europe (Stadejek et al., 1999; Hornyák et al., 2005). The ORF5 sequences reported from Italian donkeys were clustered together with European sequences obtained from horses, indicating that these donkeys had become infected from horses (Stadejek et al., 1999; Ramina et al., 1999). In contrast, the South African donkey EAV variant seems

to represent a separate type of this virus, and expands the diversity of EAV ORF5 sequences from 74.5% to 61.2% identity. This resembles the diversity seen between the two recognised genotypes of another Arterivirus, PRRSV, where European (Lelystad Virus) and North American (VR-2332) prototype strains have approximately 56.7% nucleotide identity in ORF5. This report is the first and so far only evidence for a genetically unique South African donkey variant of EAV. If more strains similar to the one described here could be discovered, this would justify the separation of EAV into at least two genotypes, as in the accepted PRRSV taxonomy. Furthermore, for a definite classification the whole genome of this unique donkey EAV variant has to be determined.

The new South African asinine variant of EAV represented by seven independent but nearly identical sequences (Fig. 1) was previously extensively characterised by experimental infection studies in donkeys and horses. It was shown to be poorly transmissible to horses and of low pathogenicity in both species including pregnant mares (Paweska et al., 1995, 1996, 1997*b*; Paweska, 1997*a, b*). Importantly, our data show that the asinine strain sequence was stable during passage in horse, supporting the existence of a unique South African donkey EAV variant that is distinct from EAVs from horses and donkeys in Europe and North America. This interpretation is perhaps further supported by the finding that there is a widespread distribution and long-standing presence of EAV among South African donkeys (Paweska and Barnard, 1993; Paweska et al., 1997*a*), but a very restricted prevalence of seropositive horses in this country (Paweska, personal communication).

In contrast to our findings, it was previously reported that the sequence of 1569 nucleotides of the 3' end of the new South African donkey EAV strain genome, including ORF5, was nearly identical to the American prototype Bucyrus isolate (Paweska et al., 1995). The cause of this discrepancy remains unknown, but considering the close similarity of the previously determined 'asinine' sequence to the ubiquitous laboratory strain, contamination in the previous study might be suspected.

In summary, modification of the first-round PCR primers improved the sensitivity of the previously described RT-nested PCR method (Stadejek et al., 1999), and enabled us to amplify very diverse EAV ORF5 sequences such as the one from the South African donkey reported here. Finally, with the new asinine sequence, the similarity among South African EAV ORF5 sequences is now as low as 66.4%, thus exceeding that observed in any other country or even continent (Fig. 1 and not shown), and indicating that EAV might have existed longer on the African continent than in Europe or North America.

Table 1
Summary of sequences used for phylogenetic analysis

Name	Geographical origin	Year	Animal origin	GenBank accession number
Vienna	Austria	1964	Horse	AF099811
A4	Austria	1998	Horse	AY453278
DK97-7	Denmark	1997	Horse	AF247545
EAV 86 P	France	1986	Horse	AF099815
H9	Hungary	unknown	Horse	AY453294
H11	Hungary	unknown	Horse	AY453296
H13	Hungary	unknown	Horse	AY453298
H15	Hungary	unknown	Horse	AY453300
H16	Hungary	unknown	Horse	AY453301
H21	Hungary	unknown	Horse	AY453306
135VE2/95	Italy	1995	Horse	AF099824
1489V/96	Italy	1996	Donkey	AF099829
3308V/96	Italy	1996	Donkey	AF099830
I16	Italy	1995	Horse	AY453313
NEAV-1	Norway	1988	Horse	AF099835
J1-931125	RSA	1993	Donkey	AY956596
J2-931125	RSA	1993	Donkey	AY956597
J3-931209	RSA	1993	Donkey	AY956598
J4-931209	RSA	1993	Donkey	AY956599
J5-940309	RSA	1994	Donkey	AY956600
J6-940309	RSA	1994	Donkey	AY956601
J7-931125	RSA	1993	Donkey	AY956602
J25-941109-3	RSA	1994	Horse	AY956603
RSA4	RSA	1996	Horse	AY453335
S2	Sweden	1999	Horse	AY453340
S3	Sweden	1999	Horse	AY453341
S4	Sweden	1999	Horse	AY453342
S6	Sweden	2000	Horse	AY453344
Bibuna	Switzerland	1964	Horse	AF099843
Bucyrus	USA	1953	Horse	X53459
KY63	USA	1963	Horse	U38599
S1368	USA	1994	Horse	AF099848
S1512	USA	1995	Horse	AF099850
S1711	USA	1996	Horse	AF099853

Sequences J2-931125, J2-931125, J3-931209, J4-931209, J5-940309, J6-940309, J7-931125 and J25-941109-3 were obtained as part of this study. The remaining sequences were selected for comparative purposes from GenBank to represent the most diverse accessions

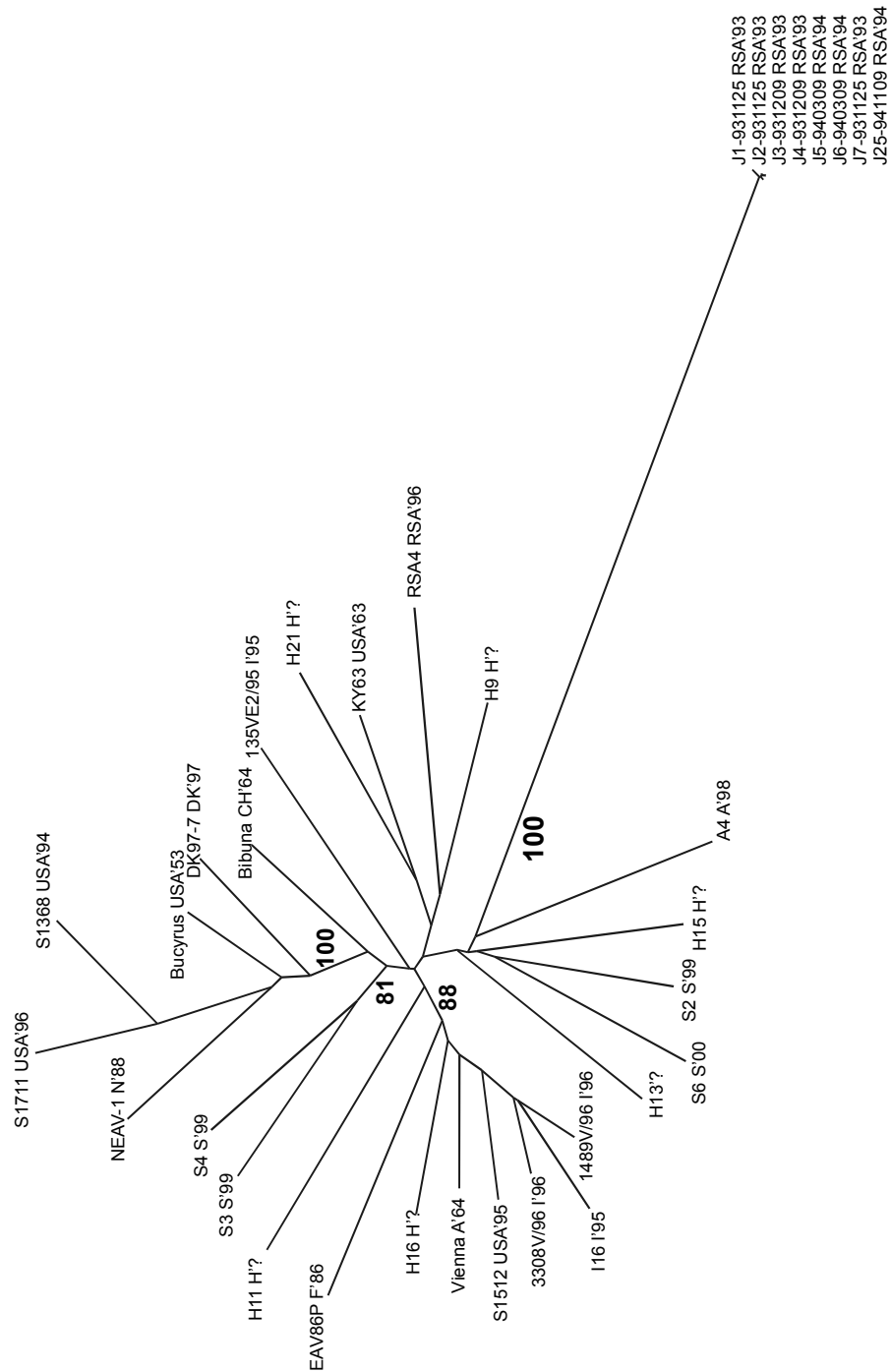


Fig. 1. A naturally infected South African donkey harboured a unique EAV type. Phylogenetic tree of ORF5 sequences made with NEIGHBOR programme of PHYLIP package. The bootstrap values adjacent to the nodes represent the percentage of 100 trees that supported the clustering

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