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Short communication

Sequence analysis of ORFs 5, 6 and 7 of equine arteritis virus during persistent infection of the stallion—A 7-year study

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ABSTRACT

Nucleotide and amino acid sequences of ORFs 5, 6 and 7 of EAV during persistent infection in the stallion of the Malopolska breed were analysed in the study. A total of 11 blood and semen samples were collected between 2004 and 2011. The titre of specific EAV antibodies in this carrier stallion was maintained at a high level throughout the study and was equal approximately 1:128. The sequence analysis of ORF5 showed 16 variable sites including 12 with synonymous substitutions and 4 with non-synonymous substitutions. The degree of nucleotide sequence identity among the strains ranged from 98.92% to 100%, whereas amino acid homology ranged from 98.06% to 100%. Ten substitutions were identified including 7 with synonymous mutations and 3 with non-synonymous mutations in ORF6. The degree of similarities among the strains ranged from 94.55 to 100% and from 98.41% to 100% at the level of nucleotide and amino acid sequence, respectively. Only a single point mutation at position 255 of ORF7 (99.6% identity) was found in nucleotide sequences of these strains. Phylogenetic analysis showed that all strains present in the semen of this carrier stallion created a separate cluster of "quasi-species" within the second European subgroup of EAV.

1. Introduction

Equine arteritis virus (EAV) is the causative agent of equine viral arteritis (EVA), a respiratory and reproductive disease of horses and other equid species. The virus belongs to the genus *Arterivirus* of the family *Arteriviridae* in the *Nidovirales* order (Cavanagh, 1999). The viral genome consists of a single stranded, positive-sense RNA of approximately 12.7 kb in length which includes 10 known open reading frames (ORFs). The two most 5′-proximal ORFs (1a and 1b) occupy approximately three-quarters of the genome and encode two replicase polyproteins. The remaining 3′ one-quarter contains eight ORFs which encode eight structural proteins: E, GP2, GP3, GP4, 5a, GP5, M and N, respectively (de Vries et al., 1992; Firth et al., 2011). Based on phylogenetic analysis of the ORF5 sequences all strains of EAV were classified into two major groups: the North American group and the European group. The last one could be further divided into two subgroups, European subgroup 1 (EU-1) and European subgroup 2 (EU-2). (Balasuriya et al., 1995b; Mittelholzer et al., 2006; Stadejek et al., 1999; Zhang et al., 2007).

In Poland EVA is on the list of registrable diseases and all clinical outbreaks of the disease should be reported to the General Veterinary Inspectorate. The first outbreak of EVA in Poland was described at a thoroughbred stud in 1976–1977. The main clinical manifestations of the infection were abortions in pregnant mares and sudden death of foals (Golnik and Michalak, 1979). In the following years the presence of EAV specific antibodies was detected in horses of different breeds (Golnik and Sordyl, 2004; Rola et al., 2011). Despite the fact that a high percentage of horses was infected with EAV no any official reports on the occurrence of new clinical outbreaks of EVA in horses were

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published. It may mean that EAV strains circulating in horses in Poland are less virulent than in other countries.

The purpose of the study was to characterize the genetic changes in ORFs 5, 6 and 7 of EAV during persistent infection of the stallion of the Malopolska breed and compare them with genetic sequences of the highly pathogenic strains isolated in other countries.

2. Materials and methods

2.1. Samples

A total of 11 blood and semen samples were collected from a persistently infected, 18-years-old stallion of the Malopolska breed. It was one of the EAV carriers identified in our previous study (Larska and Rola, 2008). Samples were collected between 2004 and 2011 as indicated below (11/2004 [month/year], 08/2005, 11/2005, 03/2006, 10/2006, 12/2006, 01/2007, 11/2009, 02/2010, 04/2010 and 06/2011).

2.2. Serology

The virus neutralization test (VNT) was performed according to the OIE recommendations (Timoney, 2008).

2.3. RNA extraction and RT-PCR

Total RNA was extracted directly from seminal plasma using TRI Reagent (Sigma Aldrich) according to the manufacturer's instruction. PCR reactions were performed in two stages and Titan One-Tube RT-PCR kit (Roche) was used for each of them. The first RT-PCR with primers 11080P and 12644 N (Balasuriya et al., 2004) was performed to amplify a 1608 bp fragment of EAV genome, encompassing ORF5 to ORF7 genes.

Nested PCR with three pairs of primers was used to obtain sequences of ORFs 5, 6 and 7 genes. The primers MLEAV1 and MLEAV2 flanking a 822 bp fragment of ORF5, M1 and M10 flanking a 449 bp fragment of ORF6 and OEVA14a and OEVA15 flanking a 395 bp fragment of ORF7 have been previously described by Larska and Rola (2008), Sekiguchi et al. (1995) and Belák et al. (1994), respectively (Fig. 1).

2.4. Sequence and phylogenetic analysis

Products of nested PCR were purified with QIAquick Gel extraction kit (Qiagen) and suspended in a total volume of 20 μl of DEPC water. Sequencing reactions were performed using Applied Biosystems BigDye® Terminator v3.1 kit (Life Technologies) by Genomed company (Poland). The sequencing was done on both DNA strands separately using the same forward and reverse primers as for RT-PCR testing. For sequence analysis, a construct of consensus sequences and phylogenetic analysis, BioEdit Sequence Alignment Editor v7.08 was used. Analysed EAV nucleotide and predicted amino acid sequences were compared with sequences of previously isolated EAV strains acquired from the NCBI GenBank database. Phylogenetic trees were constructed using a neighbour – joining method with NJplot v4.32. The nucleotide sequences reported in this study were submitted to GenBank under the accession numbers JX984449–JX984459.

3. Results and discussion

The titre of specific EAV antibodies in this carrier stallion was maintained at a high level throughout the study. Except for the period from March 2006 to January 2007 (titre 1:64) the VNT titre was 1:128. These results confirmed that the carrier stallions have high titres of neutralizing antibodies that do not prevent establishment of persistent EAV infection.

Sequencing of ORF5 showed 16 variable sites in analysed region of GP5 gene, including 12 with synonymous substitutions (nt 177, 231, 234, 237, 394, 471, 507, 519, 585, 603, 612, 672) and 4 with non-synonymous substitutions (nt 31, 270, 302, 422) (Table 1). Eleven of the substitutions were located in variable regions of the gene (V1–V3), 4 in conserved regions (C1–C4) and 1 in a signal sequence. The degree of nucleotide sequence identity among EAV strains ranged from 98.92% to 100%, whereas amino acid homology ranged from 98.06% to 100%.

In ORF6 10 substitutions were identified including 7 with synonymous mutations (49, 136, 175, 202, 218, 289, 295) and 3 with non-synonymous mutations (111–112, 244, 359) (Table 1). The degree of similarities among the strains ranged from 94.55 to 100% and from 98.41% to 100%

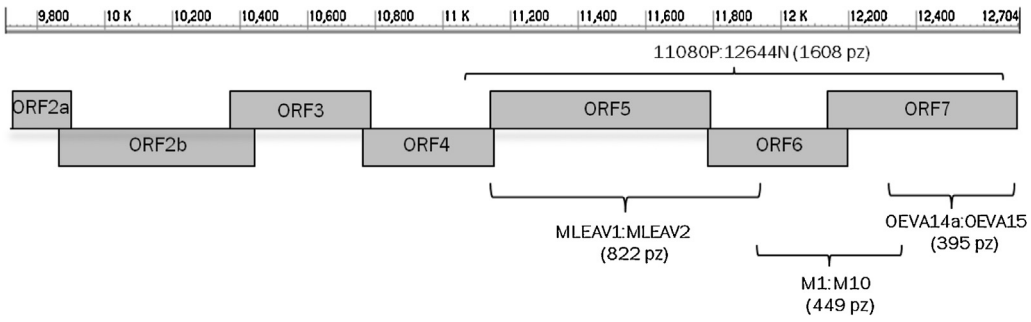

Fig. 1. A diagram showing the location of the primers used for amplification and sequencing of ORFs 5, 6 and 7 of EAV strains detected in the semen of the carrier stallion.