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Abstract

7 Phylogenetic analysis was performed on the sequences of 44 Polish isolates of equine
8 arteritis virus that were isolated from the semen of stallions from national and private studs,
9 collected during 2001-2005. These sequences were also compared with 41 reference strains
10 previously described and commonly used in phylogenesis. On the basis of the nucleotide
11 sequence analysis of the ORF5 gene, encoding the glycoprotein GP5, it was demonstrated that
12 the Polish EAV isolates belonged to two subgroups and showed the closest relationship to the
13 European strains. Similar results were obtained using the nucleotide sequences of the ORF7
14 gene. The nucleotide identity between the ORF5 and ORF7 sequences of all Polish isolates
15 was in the range of 80.1 – 99.0% and 93.6 – 100% respectively. The analysis of genetic
16 diversity within the ORF5 sequences enabled a retrospective epizootic investigation. This
17 study suggested that some of the EAV shedding stallions were probably infected before they
18 were moved to Poland.

19 KEYWORDS: equine arteritis virus, phylogenetic analysis, stallion

1. Introduction

20 Equine arteritis virus (EAV) is present in many horse populations throughout the world.
21 The course of an EAV infection is usually subclinical. 30-60% of stallions become persistent
22 carriers and can shed EAV within their semen for several weeks, months or even years.
23 Mating of susceptible mares with EAV shedding stallions can result in embryo resorptions,
24 abortions, infertility and sometimes death of the newborn foals, which leads to large
25 economic losses in horse breeding. Persistently infected stallions are considered as the main
26 reservoir of the virus, hence the control of the disease should be based on the identification of
27 these infected animals and the limitation of their use to breed previously infected or
28 vaccinated mares only. Risk of EAV transmission from persistently infected stallions to other
29 horses in the same holding by other than venereal routes should be also considered (Balasuriya
30 et al., 1999; Guthrie et al., 2003). Although most EAV strains are of low virulence, outbreaks
31 of equine viral arteritis with severe clinical symptoms still occur (Larsen et al., 2001; Szeredi
32 et al., 2005). The studies showed that over 30% of abortions in pregnant mares in Poland
33 could have been caused by EAV infections and the virus was a more frequent cause of
34 abortions than equine herpes virus 1 (Golnik and Sordyl, 2004). The first outbreak of equine
35 viral arteritis (EVA) in Poland was described at a thoroughbred stud in 1976/77. The strain
36 isolated at the outbreak was named Wroclaw-2 (Golnik and Michalak, 1978). In the following
37 years the presence of EAV specific antibodies was detected in 24% of stallions tested at the
38 national reproduction centres in Poland (Golnik, 2000). Recent serological investigations
39 among Polish stallions showed a decrease in the percentage of EAV infected to 17.4%. The
40 probable cause of this drop was the efficient implementation of the regulations enforcing the
41 requirement to test the stallions for EAV at the national reproduction centers in Poland at the
42 beginning of 2001.

43 EAV belongs to the family *Arteriviridae* of the order *Nidovirales* (Cavanagh, 1997). The
44 viral genome is composed of a single-stranded positive sense RNA enclosed in an icosahedral
45 nucleocapsid and surrounded by a proteolipid envelope. To date nine open reading frames
46 (ORFs) in the EAV genome have been described which encode the viral replicase (ORFs 1a
47 and 1b), two non-structural proteins GP3 and GP4 (ORFs 3 and 4) and five structural proteins
48 E, GP2b (formerly G_S), GP5 (formerly G_L), M and N encoded by ORFs 2a, 2b, 5, 6 and 7
49 respectively (Snijder et al., 1999; Snijder and Meulenberg, 1998). Although only one
50 serotype of EAV has been identified, field isolates can differ in their virulence, pathogenicity
51 and antigenic properties. ORF5, encoding N-glycosylated protein GP5, is one of the most
52 variable genes in the EAV genome. The GP5 ectodomain is the main antigen recognized by
53 neutralizing antibodies (Chirnside et al., 1995). Specific reactions of neutralizing antibodies to
54 viral antigen are up regulated by binding into four main sites (A-D) of GP5 glycoprotein
55 (Balasuriya et al., 1997). Comparative analysis of the deduced GP5 amino acid sequence of
56 EAV strains revealed three distinct variable regions V1-V3 (Balasuriya et al., 1995b). Genetic
57 diversity of the ORF5 sequence is commonly used in phylogenetic analyses of EAV as well
58 as some other *Arteriviridae* like porcine respiratory and reproductive syndrome virus
59 (Balasuriya et al., 1995a; Stadejek et al., 1999 and 2002; Hornyak et al., 2005; Larsen et al.,
60 2001).

61 The data presented in this study is the first attempt to characterise the genetic diversity of
62 EAV isolates present in horses in Poland using the analysis of ORF5 and ORF7 sequences
63 from 44 EAV strains isolated from persistently infected stallions present at 18 different horse
64 studs. Additionally the analysis of phylogenetic relationship as a tool for tracing back the
65 source of EAV infection was demonstrated.

2. Materials and methods

66 44 semen samples, which proved positive for EAV isolation in RK 13 cell culture
67 (ATCC CCL-37) and in diagnostic RT-PCR assays for EAV, collected between 2000 and
68 2005, were used in this survey. The material was obtained from stallions originating from
69 small, private stables, national horse studs (over 200 horses in holding) and stallion depots
70 from 11 (out of 16) regions of Poland (Table 1).

71 Total RNA was isolated directly from the semen samples of stallions using TRI Reagent
72 (Sigma) according to the manufacturer's instruction. The samples were archived at -70°C
73 following RT-PCR. Two pairs of oligonucleotides were used to obtain sequences of ORF7
74 and ORF5. The primers OEVA14a and OEVA 15, flanking a 395 bp fragment from within
75 the nucleocapsid gene (ORF7) of EAV have been described previously (Belak et al; 1994).
76 The primers - MLEAV1 (5'TCTTTTACGACTGGTACGTTGG3') and MLEAV2
77 (5'AAAATCCCGTCACCACAAA3') were designed using the reference sequence of EAV
78 reference strain Bucyrus (Accession number DQ846750, starting at nt 11116 for the forward
79 primer and nt 11920 for the reverse primer) with the program OLIGO v. 6.71 (Molecular
80 Biology Insights, Inc., USA) and then used to amplify fragment of 822 bp corresponding to a
81 region of ORF5. For further sequence comparison a shorter region of 519 bp (nt 11296-11814
82 of the EAV strain Bucyrus genome) as used for of most of reference ORF5 sequences was
83 compared.

84 The RT-PCR assays were carried out using the one-tube Access RT-PCR System kit
85 (Promega) using the conditions described previously (Larska and Rola, 2004). Sequencing
86 was conducted using a modified Sanger method (dye terminator sequencing) (Sanger et al.,
87 1977) using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)
88 according to the instructions of the manufacturer. Furthermore, the DNA fragments were
89 purified using a QIAquick PCR Purification kit (Qiagen) following analysis in a 16-capillary
90 sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and the results were

91 analysed using GeneScan [®]Analysis Software. Phylogenetic analysis was done using the
92 BioEdit platform based on PHYLIP (Felsenstein, 1973; Hall, 1997). To reconstruct
93 phylogenetic trees a maximum likelihood algorithm was applied. The tree bootstrapping was
94 carried out on 1000 replicate data sheets in SEQBOOT program from PHILIP package and
95 drawn using TreeView v. 1.6.6 software. Data from 46 previously published reference
96 sequences of EAV from GenBank NCBI, NIH including 32 sequences of the ORF5 gene and
97 16 sequences of the ORF7 gene originating from 41 European and North American EAV
98 strains were used in the phylogenetic studies. The nucleotide sequences of the Polish EAV
99 isolates were deposited in GenBank under the accession numbers: EF102348-EF102432.

3. Results and discussion

100 The ORF5 sequence based phylogenetic tree (Fig. 1A) was constructed and the
101 subgroups were assigned according to the division proposed by Mittelholzer et al. (2006). The
102 44 Polish isolates belonged to two European clusters, namely EAV-1 and EAV-3, as observed
103 with 32 other reference ORF5 sequences. None of the isolates studied belonged to North
104 American subgroup of EAV strains, namely EAV-2. Polish isolates M0/1, N2/1, N2/3, E1/1,
105 L1/5 and L1/7 together with the strains isolated from horses in Denmark (DK97-1 - GenBank
106 No. AF247539), Germany (A755 – AF099818; B076 – AF099819), Norway (NEAV-3 –
107 AF099837), UK (96/7982 – AF099846) and the USA which were imported from Europe prior
108 to isolation (CA97 – AF118783, CW01 – AY349168) belong to the EAV-3 subgroup with a
109 bootstrap value of 81.7%. The M0/1 strain was isolated from a 17-years old Angloarab
110 stallion, which was imported into Poland from Germany in 2000. The high similarity of this
111 strain to two German strains and lack of connection to other Polish strains suggests that this
112 stallion could have been infected with the virus before coming to Poland. The other 5 Polish
113 isolates from EAV-3 subgroup which probably had the same ancestor but where isolated from

114 the stallions from three geographically distant studs. The other 38 Polish isolates all belonged
115 to the European EAV-1 subgroup in which three separate branches were observed in the tree
116 (Fig. 1A). One branch included isolate H5/1 and some strains from Hungary (N15/03 –
117 AY359212; S269/03 – AY359207) and Austria (Vienna – AF099811) (Holyoak et al., 2005;
118 St-Laurent et al., 1997). The H5/1 strain was isolated from the semen of a Selle francais
119 stallion, which was imported to Poland from France some time before the testing. Its lack of
120 similarity to other Polish strains but its close relationship with Hungarian and Austrian strains
121 suggests that the virus infection may have happened before arrival in Poland. Two other
122 Polish isolates P0/1 and P2/4 and the Polish strain S-436 (AF099839), which was isolated
123 from the semen of Angloarabian stallion from Poland in 1988, showed the greatest similarity
124 to the AZ878 strain (AF065824) which originated in Arizona in 1987. Therefore it could be
125 concluded that the outbreak in Arizona could have been caused by the same parental EAV
126 strain that produced the three Polish strains. The stallion infected with the P0/1 was the
127 possible source of infection for the P2/4 animal as these two stallions were bred and kept in
128 the same stud. It suggests that horizontal transmission between stallions inside the depots can
129 occur (Guthrie et al., 2003; Balasuriya et al., 1999). The other 35 Polish isolates represented
130 the third branch of the EAV-1 group together with 10 reference strains. Whilst three isolates
131 (L1/8, L1/9 and D1/1) were closely related to the previously described strain 6547/96
132 (AF099841) isolated from an aborted foetus in Poland in 1996, some other isolates (A0/1,
133 B1/1, W1/1 and X3/1) were descendents of the same strain from which evolved the Polish
134 strain 5499/94 (AF099840) and the Italian strains ITA92 (U38598) and I13 (AY453310).

135 An almost identical division of isolates as observed with the ORF5 based tree was
136 obtained in the phylogenetic tree constructed with the ORF7 sequences (Fig. 1B).
137 Comparison of the ORF5 and ORF7 trees showed that the overall substitution rate within
138 nucleocapsid gene was around ten times slower than the rate of evolutionary changes in

139 glycoprotein GP5 gene (genetic distance bars at Fig. 1A and B). In the ORF5 sequences an
140 average of 34.2% of substitutions resulted in amino acid changes, whereas only 12.5%
141 substitutions in the ORF7 sequence were found to be non-synonymous.

142 No ORF5 sequences of Polish isolates had any deletions or insertions relating the nt
143 sequence of EAV reference strain Bucyrus. The extent of nucleotide identity between the
144 amplified ORF5 gene sequences of Polish isolates compared to the reference Bucyrus strain
145 sequence within the 519 nt gene fragment of Polish isolates to Bucyrus strain ranged from
146 79.7 to 85.7% which corresponded to 74 – 105 point mutations. The largest number of
147 nucleotide substitutions in comparison to Bucyrus ORF5 sequence was seen in the L1/7
148 sequence, whereas the H5/1 sequence showed the highest similarity to this reference strain.
149 The deduced GP5 sequences of Polish isolates and Bucyrus strain differed in 49 to 94 amino
150 acid positions out of 173 residues in total (81,9-90,6%), whereas the identity of the GP5
151 amino acid sequences between Polish isolates ranged from 82.5 to 98,8%. The differences
152 were localised mostly in the V1 region, in particular within the neutralisation sites B, C and D
153 (Balasuriya et al., 1997). Moreover characteristic amino acid substitution sites in comparison
154 with Bucyrus strain sequence in the variable fragments V1 and V3 of the GP5 protein were
155 found for strains in the European groups EAV-1 and EAV-3 (data not shown). They coded for
156 substitutions in the positions of amino acid residues 155 (I→V), 158 (I→L) and 208 (V→I or
157 V→T) in the strain that belonged to EAV-1, whereas all the strains from the EAV-3 group had
158 the same, unique mutations in amino acid residues 151 (I→V) and 174 (A→I). Similar
159 mutation sites were observed by Balasuriya et al. (1995b). The group-specific substitutions
160 could be used for the future in EAV strain typing as has being introduced in PRRSV strain
161 differentiation by RFLP or PCR using group-specific oligonucleotides (Oleksiewicz et al.,
162 1998).

163 No deletions or insertions were found in the 333 nt ORF7 sequences of 44 Polish
164 isolates. The similarity of ORF7 sequences of Polish isolates to Bucyrus strain ranged from
165 93.3 to 96.6% (11-22 nt changes). Up to 8 nucleotide differences were found between ORF7
166 sequences of the Polish isolates.

167 The present report was designed to analyse, for the first time, the genetic relationship
168 between EAV strains isolated from the stallions of different breed and age that were collected
169 at different dates and from geographically distinct horse studs in Poland. Continued
170 phylogenetic investigation could reveal further genetic variability of EAV strains circulating
171 in the Polish horse population due to international and intercontinental transport of horses,
172 especially after Poland joined the European Union Community. This study revealed that most
173 of the strains isolated from persistently infected stallions originating from the major Polish
174 horse breeding farms are highly similar to the EAVs isolated in the 80's and 90'. To date
175 only one Polish isolate (PLD76) was linked to North American EAV strains which showed
176 the closest relationship to the Bucyrus and vaccine Arvac strain. (Balasuriya et al, 1995a).
177 The PLD76 strain was isolated from a thoroughbred horse in Wroclaw in 1976/77 but
178 although it shares the same year and place of isolation as the Wroclaw-2 strain, the genetic
179 analysis showed that these strains are not related. In our study we sequenced the Wroclaw-2,
180 which we obtained thanks to the courtesy of W. Golnik, and we confirmed its relationship to
181 European subgroup according to division by Stadejek et al. (1999). In the previous study
182 Wroclaw-2 strain have not clustered within none of the EAV-1 and EAV-3 groups although it
183 showed higher relationship to the European strains. Moreover none of the Polish isolates
184 studies showed similarity to this strain.

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Figure legend

Fig. 1. Maximum likelihood phylogenetic trees based on the analysis of the ORF5 (A) and ORF7 (B) nucleotide sequence of the Polish EAV isolates and related reference sequences. The scale corresponds to the number of expected substitutions per site. The bootstrapping of ORF5 tree was done in 1000 duplicates and shown as percentage.

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Table 1

Description of the origin of Polish EAV strains used in present study

| Strain designation ^a | Abbreviation ^b | Collection date | Stallion data | | | |
|---------------------------------|---------------------------|-----------------|------------------|--------------------|-------------------|---------------------|
| | | | Age ^c | Breed ^d | Stud ^e | Region ^f |
| PLA00/1 | A0/1 | 2000 | 3 | pony | A | WM |
| PLB01/1 | B1/1 | 2001 | 14 | xo | B | MA |
| PLC01/2 | C1/1 | 2001 | 7 | PCB | C | KP |
| PLD01/3 | D1/1 | 2001 | 13 | wlkp | D | WM |
| PLE01/4 | E1/1 | 2001 | 4 | PCB | E | MP |
| PLF01/5 | F1/1 | 2001 | 5 | oo | F | PD |
| PLG02/1 | G2/1 | 2002 | 6 | PCB | G | MA |
| PLH05/1 | H5/1 | 2005 | 15 | sf | H | SL |
| PLK02/1 | K2/1 | 2002 | 7 | xo | K | LU |
| PLK02/2 | K2/2 | 2002 | 3 | xo | K | LU |
| PLK02/3 | K2/3 | 2002 | 3 | xo | K | LU |
| PLK02/4 | K2/4 | 2002 | 4 | xo | K | LU |
| PLK02/5 | K2/5 | 2002 | 6 | PCB | K | LU |
| PLK02/6 | K2/6 | 2002 | 13 | xo | K | LU |
| PLK02/7 | K2/7 | 2002 | 13 | x | K | LU |
| PLK02/8 | K2/8 | 2002 | 5 | xo | K | LU |
| PLK02/9 | K2/9 | 2002 | 17 | PCB | K | LU |
| PLL01/1 | L1/1 | 2001 | 7 | wlkp | L | KP |
| PLL01/2 | L1/2 | 2001 | 3 | sl | L | KP |
| PLL01/3 | L1/3 | 2001 | - | wlkp | L | KP |
| PLL01/4 | L1/4 | 2001 | 15 | wlkp | L | KP |
| PLL01/5 | L1/5 | 2001 | 10 | PCB | L | KP |
| PLL01/6 | L1/6 | 2001 | 6 | xo | L | KP |
| PLL01/7 | L1/7 | 2001 | 14 | wlkp | L | KP |
| PLL01/8 | L1/8 | 2001 | 13 | wlkp | L | KP |
| PLL01/9 | L1/9 | 2001 | 8 | wlkp | L | KP |
| PLM00/1 | M0/1 | 2000 | 17 | xo | M | LU |
| PLM00/2 | M0/2 | 2000 | 10 | oo | M | LU |
| PLN02/1 | N2/1 | 2002 | 10 | PCB | N | WM |

| Strain designation ^a | Abbreviation ^b | Collection date | Stallion data | | | |
|---------------------------------|---------------------------|-----------------|------------------|--------------------|-------------------|---------------------|
| | | | Age ^c | Breed ^d | Stud ^e | Region ^f |
| PLN02/2 | N2/2 | 2002 | 13 | wlkp | N | WM |
| PLN02/3 | N2/3 | 2002 | 16 | wlkp | N | WM |
| PLP00/1 | P0/1 | 2000 | 9 | oo | P | MP |
| PLP01/1 | P1/2 | 2001 | 10 | sl | P | MP |
| PLP01/2 | P1/3 | 2001 | 8 | xo | P | MP |
| PLP02/1 | P2/4 | 2002 | 5 | xx | P | MP |
| PLR04/1 | R4/1 | 2004 | 11 | KWPN | P | MP |
| PLR05/1 | R5/2 | 2005 | 11 | KWPN | P | MP |
| PLS01/1 | S1/1 | 2001 | 6 | wlkp | S | ZP |
| PLS01/2 | S1/2 | 2001 | 15 | sp | S | ZP |
| PLS01/3 | S1/3 | 2001 | 8 | PCB | S | ZP |
| PLT03/3 | T3/1 | 2003 | 7 | oo | T | SW |
| PLW01/1 | W1/1 | 2001 | 21 | xx | W | WM |
| PLX03/1 | X3/1 | 2003 | 7 | wlkp | X | WP |
| PLZ03/1 | Z3/1 | 2003 | 5 | PCB | Z | PM |

1 ^a strain names contain of three letters for Poland (PL) and stud of origin (A-Z) and then the
2 two last digits of isolation year and the number of the isolate from the stud; ^b Abbreviation of
3 Polish strain names used in the text and phylogenetic trees; ^c years; ^d xo-Anglo-Arab; PCB-
4 Polish cold blood; wlkp-Wielkopolski horse; oo-Purebred Arabian; sf-Selle francais; x-half
5 Thoroughbred ; sl-Silesian horse ; xx-English Thoroughbred ; KWPN-Dutch Warmblood; ^e A
6 – H stand for small private studs; M, T and W were national horse stud farms; the remaining
7 eight – national stallion depots; ^f Voivodship MW – Warmian-Masurian; MA – Masovian;
8 KP – Kuyavian-Pomeranian; MP – Lesser Poland; PD – Podlachian; SL – Silesian; LU –
9 Lublin; ZP – West Pomeranian; SW – Swietokrzyskie; WP – Greater Poland
10 (Wielkopolskie); POM – Pomeranian. The abbreviations were used according to HASC and
11 the regions described in detail at http://en.wikipedia.org/wiki/Voivodeships_of_Poland.

