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Epizotiology and phylogeny of equine arteritis virus in hucul horses

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Abstract

The aim of the study was to determine the situation of equine arteritis virus (EAV) infections in hucul horses. A total of 176 horses (154 mares and 22 stallions) from the biggest hucul horse stud in Poland were tested. Antibodies against EAV were detected in 97 (55.1%) horses. The EAV seroprevalence among mares was 53.2% while in stallions - 68.2%. The percentage of positive mares increased with their age, thus amongst the mares of less than 2 years of age the percentage was 32.5%, while in the group of 3-5 years old increased to 59.4% and in the mares in the age of 6-10 years and older than 10 years 89.5% and 95% were seropositive, respectively. Among 11 seropositive stallions five were supposed to be shedders of EAV with their semen. It is likely that those persistently infected stallions were the reservoirs of the virus in the stud. Genetic studies using of ORF5 gene showed high homology between the viruses detected in the semen of those stallions what suggested lateral transmission between the stallions sharing the same stable. Persistent infection in an immature stallion, which has not yet been used for breeding, was established as a result of infection via respiratory route. Phylogenetic analysis confirmed that all hucul viruses shared the same ancestor and as most of EAV strains dominating in Polish horse population belonged to the European origin EAV subgroup (EU-1).

Keywords: hucul horse, equine arteritis virus, prevalence, phylogeny

1. Introduction

Equine arteritis virus (EAV) is one of the major viral pathogens of horses. It is the causative agent of equine viral arteritis (EVA), a contagious disease of horses and other equid species. The virus belongs to the genus *Arterivirus* of the family *Arteriviridae* in the *Nidovirales* order (Cavanagh, 1999; Snijder & Meulenberg, 1998). The viral genome consists of a single stranded, positive-sense RNA which includes 9 known open reading frames (ORFs). ORF5 encodes viral membrane glycoprotein GP5, which contains epitopes responsible for virus neutralisation (Balasuriya et al., 1997). Due to the ORF5 variability, this gene is used as target for phylogenetic analyses of EAV, whereas the majority of diagnostic RT-PCRs are based on the primers derived from the most conservative ORF7 gene encoding nucleocapsid protein N. EAV was first isolated during an outbreak of respiratory disease and abortion on a Standardbred farm in Bucyrus, Ohio in 1953 (Doll et al., 1957). There is only one recognized serotype of EAV but field strains of the virus vary significantly in their virulence. Exposure to EAV most frequently results in asymptomatic infection but certain strains can cause disease of varying clinical severity characterized by anorexia, depression, fever, dependant limb oedema, ocular and nasal discharge and conjunctivitis. The virus can also cause abortion in pregnant mares. If exposure to virus occurs in late phase of pregnancy the mare may not abort but newborn foal is infected and may die within a few weeks with symptoms of interstitial pneumonia or pneumoenteritis. Most infected horses recover without complications but up to 10-70% of stallions infected with EAV can subsequently become carriers and constantly shed the virus in their semen through several weeks, months or years (Timoney et al., 1986 and 1987). Such stallions are main reservoirs

of EAV in the equine population, spreading the virus to the naïve mares as well as transmitting it laterally to other horses sharing same stables (Guthrie et al., 2003).

The virus is distributed worldwide, but seroprevalence of EAV infection of horses varies between countries and different breeds. Since the first EVA cases described in Ohio in 1953, the disease spread over the world. The only countries that are considered to be free of EAV infections are Japan and Island. First EVA cases in Poland were confirmed in 1976-1977 in the thoroughbred horse stud on the south of the country and the first EAV strain was named Wroclaw-2 (Golnik & Michalak, 1978). In the recent years, the intensified horse trade, poor execution of biosecurity rules and lack of unified control regulations caused increase in dissemination of EAV. Eichhorn et at. (1995) observed significant elevation of the percentage of EAV seropositive horses in the horse population in Germany from 1.8% in late 80s' to almost 20% in 1995. Increase of EAV infection in horses was also described in Sweden and Italy (Glaser et al., 1997). In many of the EVA serologic studies carried out in Europe more than 20% of animals were infected with the virus (Glaser et al., 1997), except Great Britain where the percentage wasn't exceeding 2%, however the abortion storms and the mortality in the young foals caused by EAV are still a problem there (Newton et al., 1999). The significance of EAV infections is often underestimated by the horse breeders in Poland, however the incidence of abortions in mares caused by EAV infection could be estimated as one third of all the abortion cases in our country (Golnik & Sordyl, 2004). In Hungary, Szeredi et al. (2005) found that 10% of the abortions were due to EVA. Generally the percentage of EAV seropositive animals is considerably higher in Standardbreds then in other breeds. Very little is known about epidemiology of EAV infection in hucul horses. Huculs are a primitive breed of small mountain horses. It is one of the oldest primitive

breeds described in Poland. The breed originates from the Hucul region in the East Carpathian Mountains. It is a region situated in the mountainous basin of the Prut and Cheremosh rivers, in the East Carpathians. At present it is located in the borderland between Ukraine and Romania. The breed was formed under the influence of harsh mountain climate in areas poor in feed and offering very primitive habitat conditions. Therefore hucul horses are known for their physical endurance, low feed requirement, fertility, longevity and resistance to illnesses. In the past they were used as saddle and pack horses but today mostly for mountain recreation and hippotherapy. Hucul are included in the protected gene fund of original and primitive animal breeds of FAO. To protect breed purity, coordination of the breeding goals and plans as well as the origin control, the Hucul International Federation (HIF) was founded in 1994 at Balice near Cracow in Poland as a result of cooperation between breeders from Austria, Hungary, Czech, Slovakia, Romania, Ukraine and Poland.

The purpose of the study was to investigate prevalence of EAV infection, the frequency of carrier state in stallions and the genetic variability among strains of EAV isolated from hucul horses in Poland.

2. Materials and methods

2.1. Sample collection

Most of the samples for the study were collected in the period of 2006-2008 from the biggest hucul horse stud in Poland. Mares with foals and stallions used for breeding were kept in the main stables whereas yearlings and immature stallions in other farm buildings located in the walking distance from the previous ones. No history of respiratory disorders, abortions or decrease in the pregnancy rate was notified

previously and any clinical signs of disease in the stud horses were unapparent in the time of sampling. None of the horses was vaccinated against EAV.

For serological tests 176 blood samples were collected including 154 samples from mares and 22 from stallions. Blood was collected in vacutainers, allowed to clot and centrifuged at 2000 x g for 10 min and serum was decanted to the fresh tubes. Subsequently all samples were tested with virus neutralization test. After that 11 serologically positive stallions were checked for the presence of EAV in their semen. Altogether 11 semen samples from 10 adult stallions sharing the same stable and one young male standing in the separate building of the same holding were collected.

2.2. Virus neutralisation test (VNT)

VNT was performed according to the procedure described in the OIE Manual (Timoney, 2004). All sera were inactivated for 30 min at 56°C. Subsequently, sera samples were added to the 96-well microtitre plate and serial twofold dilutions in serum-free cell culture medium (Eagle MEM) were made starting from a 1:2 dilution. To prepare working dilution of the stock virus containing from 100 to 300 $TCID_{50}$ per 25 µl, the reference Bucyrus strain (ATCC VR-796) was diluted in Eagle MEM with guinea-pig complement at a final concentration of 10%. Such dose of the virus was added to every well containing relevant serum dilutions, except the test serum control wells. The plates were shaken gently and incubated for 1 h at 37°C. After that time 50 µl of cells suspension RK13 (ATCC CCL-37) with concentration approximately 10^5 cells/ml were added to each well. The plates were sealed with parafilm and incubated for 3-5 d at 37°C in a humid atmosphere of 5% CO_2 and read under microscope for the presence of cytopathic effect (CPE). A titre of 1:4 or greater was considered as positive.

2.3. Viral RNA extraction and RT-PCR

Total RNA was extracted directly from seminal plasma using TRI Reagent (Sigma Aldrich). The semen samples were first tested in diagnostic RT-PCR using set of primers flanking 395 bp fragment of ORF7, previously described by Belák et al. (1994). For this purpose single tube kit Access RT-PCR System (Promega) was used. RT-PCR reaction mix consisted of 10 µl of AMV/Tfl 5x reaction buffer, 1 µl of 10 mM dNTP mix, 50 pM of sense and antisense primer, 2 µl of 25 mM $MgSO_4$, 5 U of AMV reverse transcriptase, 5 U Tfl DNA polymerase and 2 µl of extracted RNA. The mixture was supplemented with sterile water to a final volume of 50 µl. The reverse transcription was performed at 48^{o}C for 45 min and followed by short inactivation at 94^{o}C. The amplification was run in 35 cycles of denaturation at 94^{o}C for 45 s, primer hybridisation at 52^{o}C for 1 min and elongation at 72^{o}C for 1.5 min. The final elongation step was run at 72^{o}C for 10 min. The amplified products were visualized by gel electrophoresis in 2% agarose gel in TAE buffer at 100 V. For the sequencing and the studies of virus phylogeny the primers 11080P (5'TTGTGGCTATAGTTTATGTTC3') and 12664N (5' GGTTCCTGGGTGGCTAATAACTAC3') were used enabling amplification of 1608 nt fragment consisting whole ORF5 of EAV (Balasuriya et al., 2004). RT-PCR was performed with similar conditions described above. RT-PCR products were purified with QIAquick Gel extraction kit (Qiagen) and sequencing with ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

2.4. Phylogenetic analysis

The nucleotide sequences were aligned using ClustalW module of PHYLIP-based BioEdit platform (Felsenstein, 1989; Hall, 1999). The similarity matrix was generated using BLOSUM62 in BioEdit program. Neighbour Joining (NJ) tree was

constructed and bootstrapped on the set of 1000 replicates in MEGA software v. 4.1 (Felsenstein, 1985; Tamura et al., 2007). The choice of the reference EAV strains from NIH GenBank was guided by the closest relationship and geographic criteria. Simplified group division (EU-1, EU-2 and NA) was used according to Zhang et al. (2007).

The comparative analysis of deduced GP5 structure was done to predict possible differences in the neutralization potential of different EAV strains. The hydrophobic profiles of GP5 were constructed using Kyte-Doolittle scale in ProtScale and N-glycosylation sites (inferred on the basis of presence of N-X-S/T sites, where X is not praline) were predicted in NetNGlyc both tools available at ExPASy server (Gasteiger et al., 2003).

3. Results and discussion

A total of 176 horses were tested and the antibodies against EAV were detected in 97 (55.1%) animals. Out of 154 mares tested, 82 (53.2%) reacted positively in VNT. The ratio of positive mares increased progressively with their age. In mares of less than 2 years of age 32.5% of 83 tested had specific EAV antibodies. In remaining groups the percentage of infected individuals was higher and ranged from 59.4% in mares of 3-5 years old to 89.5% and 95% in mares in the age of 6-10 years and older than 10 years, respectively (Tab. 1). Among stallions 15 (68.2%) out of 22 tested possessed neutralizing antibodies towards EAV. In the group of 3-5 years old stallions only one was serologically positive. Eight out of 10 tested were positive in stallions of 6-10 years old. In the last group containing stallions older than 10 years specific antibodies were detected in 6 out of 7 tested. The titres of antibodies in seropositive stallions ranged from 1:32 to 1:512 but in EAV carriers

they were 1:128 or greater. Previous studies of the seroprevalence of EAV antibodies tested in Polish stallions ranged between 17.4 and 24% in the last 20 years (Golnik & Sordyl, 2004). Our testing of around 8000 horses between 1998 and 2006 showed that the percentage of EAV infected horses in Poland is at the level of around 18% (data not published). Interestingly in the same study the highest seroprevalence was detected among hucul horses (over 35%). Any breed predilections to EAV infection were never proven, however some studies suggest the higher incidence of EAV infections in standardbred horses in comparison to thoroughbreds (Newton et al., 1999). The high incidence of EAV infections established in this study is quite surprising as hucul horses are primitive breed, originally reared in harsh mountain climates and still kept free at Polish highlands all year around. The horses are known for their excellent endurance, fertility and resistant to diseases. Nevertheless absence of EVA clinical signs in this stud before and during the study could be explained probably by low pathogenicity of the viruses circulating or by high natural immunity of the horses linked to high seroprevalence or breed resistance. Interestingly, higher EAV seroprevalence at the 68.2% level was observed in the hucul stallions, while 53.2% of mares were seropositive. Additionally, the percentage of seropositive mares increased significantly with their age, which shows that the presence of EAV in this population is continuous (Burki et al., 1992).

Stallions are usually the reservoirs of the virus and can shed it continuously for months or even all along their life. Presence of the viral RNA was detected in semen of 5 (45.5%) among 11 seropositive stallions tested. A prominent RT-PCR product of expected size of 395 bp was observed in semen samples of 4 breeding stallions sharing the same stable (Fig. 1, lanes 7-10) and in the immature one which was not used for breeding yet and was kept separately (Fig. 1, lane 11). Same size

RT-PCR product was also obtained for positive control (Bucyrus strain added to negative semen). No amplification was observed in the remaining samples of semen and in the negative control. The sensitivity and specificity of the RT-PCR assay used in the study were determined previously (Larska & Rola, 2003). Detection limit of RT-PCR in our experiment corresponded to 10 $TCID_{50}$ of the reference strain Bucyrus. For genetic characterization of hucul's EAV strains RT-PCR with primers flanking a 518 bp fragment of ORF5 gene including most variable segments V1-V3 was used. Phylogenetic tree was constructed on the similarities between RNAs from semen of the hucul stallions and 31 reference strains selected from NCBI GeneBank database. In the constructed NJ tree, the hucul viruses created separate branch within European subgroup-1 (EU-1) cluster (Fig.2). The comparative sequence analysis of the genomes of the viruses detected in the hucul horses showed high similarities on the level of nucleotide (95.3 – 99.6%) and amino acid sequence (95.3-99.4%) amongst them which suggests that all five viruses shared common ancestor. Four of them (PLhc09/1-PLhc09/4) were detected in the semen of the stallions sharing the same stable. Very high similarity of these viruses suggests in-contact lateral transmission of EAV between the stallions inside the stable or carried by the personnel from EAV infected mares located in other buildings of the same stud. Similar situation was described in Lipizzaner stallions in South Africa where EAV transmission trough the fomites such as clothing, box bedding or equipment contaminated with the semen from shedding stallion was speculated (Guthrie et al., 2003). Same findings were supported by the study of EVA outbreak in Pennsylvania in 1996 (Balasuriya et al., 1999) and high homology of EAV strains isolated from the semen of persistently infected stallions from the same stallion reproduction centres in Poland (Larska & Rola, 2007). Furthermore, the fifth virus (PLhc09/5) originated

from young hucul stallion which has not yet been used for breeding and which was kept separately in another stable. The presence of EAV in its semen indicates respiratory route of infection. When compared to other reference strains, the hucul viruses were closely related at the similarity level of 94.2 to 95.9% to the previously described EAV strains detected in the semen collected from persistently infected stallions from different Polish studs in years 2000-2001 (PLB01/1, PLA00/1, PLN02/2, PLN02/2, PLK02/7) as well to the 5499/94 and 6547/96 EAV strains isolated from aborted foetus in the Standardbred stables in 1994 and 1996 (Stadejek et al., 1999, personal communication with W. Golnik). Hucul viruses demonstrated the highest similarity with foreign European strains from Italy (I13 – 94%, ITA92 and 874V/97– 95%) and viruses (A, R, G – 92%) isolated from the EVA outbreak in Warmblood horse stables in Pennsylvania, USA in 1998, after importing European stallion persistently infected with EAV (Balasuriya et al., 1995 and 1999; Mittelholzer et al., 2006; Stadejek et al., 1999). The differences in ORF5 sequence between these hucul isolates and Bucyrus strain as well as the first Polish strain Wroclaw-2 from 1976 were at the level of 15%. Sequence analysis revealed single amino acid changes in variable fragments V1 and V2 of deduced GP5 sequences between particular strains (Fig. 3) (Balasuriya et al., 1997). No changes in neutralization site A of V1 (49 aa) were observed (not shown), whereas in the 61 aa (B site) K present in 3 hucul viruses was substituted with N and E in PLhc09/1 and PLhc09/4, respectively. Again minor or no changes in the C (67-98 aa) and D neutralization sites between hucul viruses were present. Significant changes, however were observed in GP5 of PLhc09/5 virus isolated from the young stallion which showed variations in C site in V1, other between 172 and 175aa of V2 fragment and additional single change in 115 aa (E→X) of conservative sequence of

GP5 (Fig. 3). Hydrophobicity profile and N-glycosilation sites were analysed to establish whether the differences in GP5 sequence had impact on its neutralizing phenotype between hucul viruses. No significant changes in the hydrophobic index calculated by Kite-Doolittle's algorithm were observed (figures not shown). Two potential N-glycosilation sites starting with the asparagines in position 56 aa (NCS) and 81 aa (NNT) of GP5 were present. Despite the difference in position 82 aa (N→D) in GP5 of PLhc09/5, the protein sustained the second N-glycosilation site. The amino acid differences between the hucul viruses in neutralization sites B, C and D of variable V1 region of GP5 may suggest emergence of EAV variants with distinct neutralization phenotypes. The evolution of EAV quasispecies as a response to the selective pressure was described for EAV during persistent infection in the stallions (Balsuriya et al., 1999 and 2004; Hedges et al., 1999), however it is possible that novel phenotypic variants can arise as a response to the summary of selective pressures of immunological status of whole stud in which single EAV strain is circulating. Nevertheless genetic analysis showed to be useful tool, suggesting single EAV source of the infection in the stud. This also arguments strengthening of the biosecurity measures to be crucial in the control of EAV infection inside the stud. NJ dendrogram showed that all the EAV detected in hucul stallions were closely related to the viruses of European origin EU-1. Viruses of this cluster seem to be endogenous in the population of horses in Poland (Larska & Rola, 2008), whereas EAV described previously which belong to EU-2 (Larska and Rola, 2008) were probably introduced to our country by persistently infected stallions imported from West European countries. In summary, the hucul isolates belonged to EU-1 cluster which seems to be endogenous in Poland.

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Table 1

Prevalence of EAV infection among mares and stallions according to their age

Age/years	Results				
	Mares		Stallions		
	No of tested	VNT+ (%)	No of tested	VNT+ (%)	PCR+ (%)
0-2	83	27 (32.5)	0	0	0
3-5	32	19 (59.4)	5	1 (20.0)	1 (100.0)
6-10	19	17 (89.5)	10	8 (80.0)	4 (50.0)
>10	20	19 (95.0)	7	6 (85.7)	0

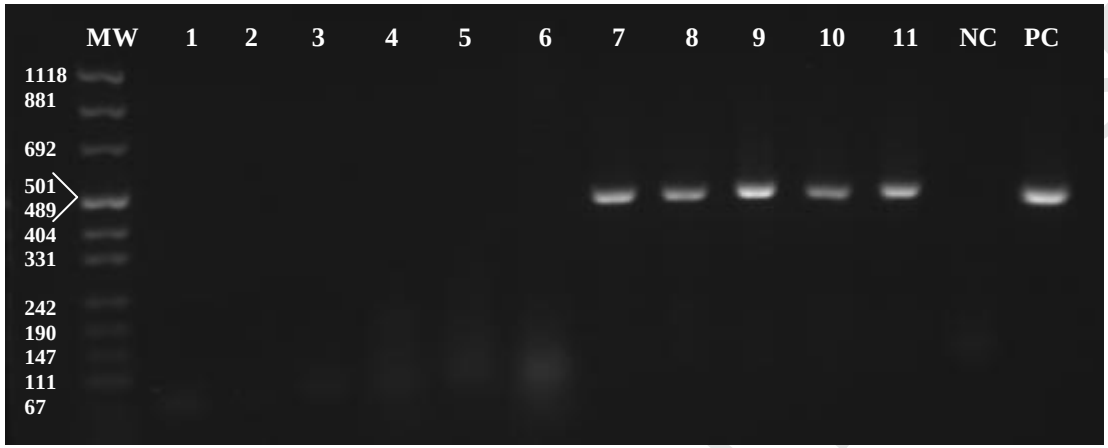

Fig. 1. Detection of EAV in semen samples collected from hucul stallions. MW – molecular weight marker, lines 1- 11 semen samples, NC - negative control (semen from seronegative stallion, previously tested for the presence of EAV), PC - positive control (semen spiked with Bucyrus EAV reference strain in the final titre of 10^3 $TCID_{50}$).

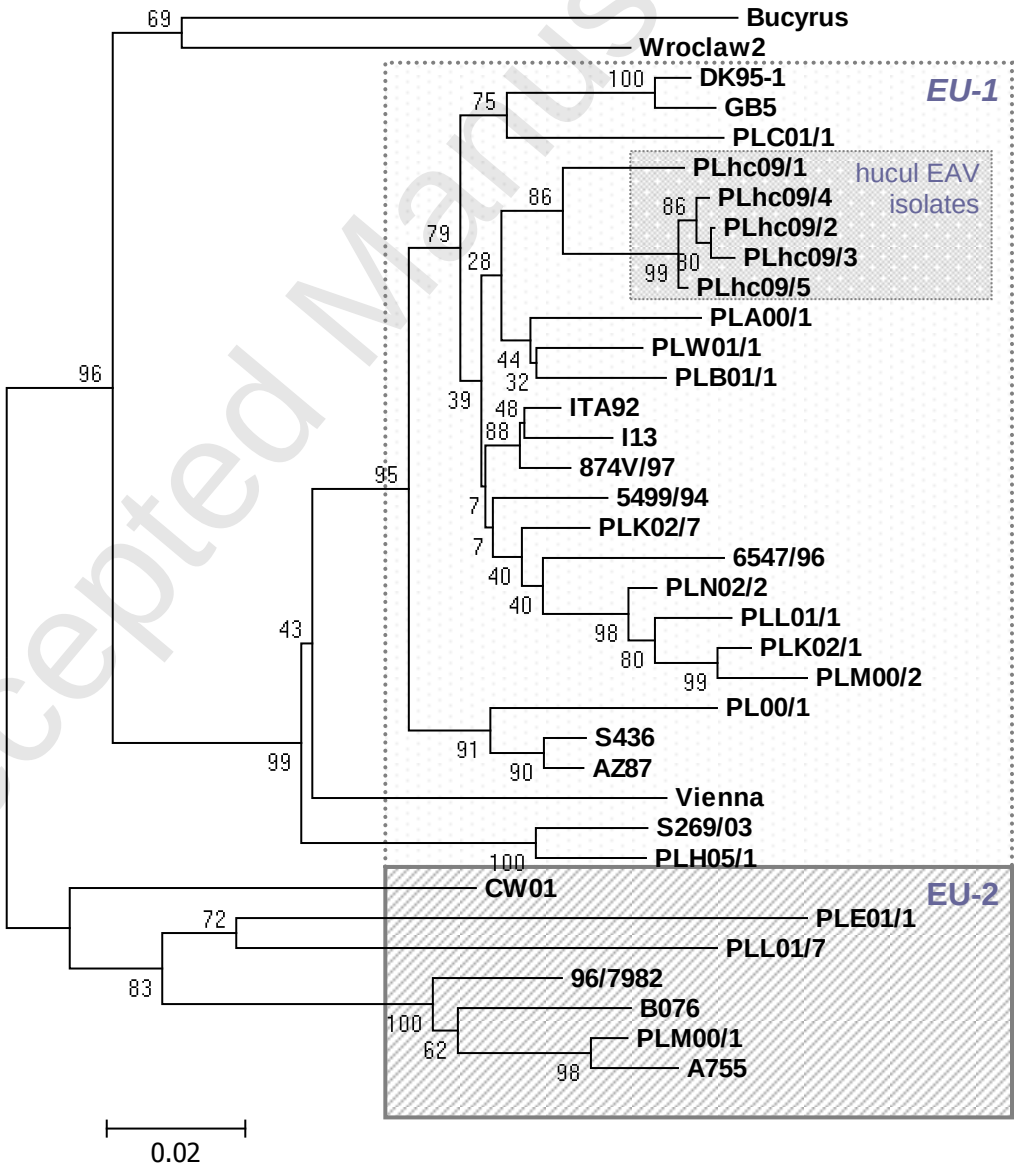

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Fig. 2. ORF5 phylogenetic tree constructed using neighbour joining method in MEGA v. 4.1 (Tamura, 2007). The numbers at the nodes annotates the percentage of confidence for identical sequence distribution when the tree was constructed 1000 times (Felsenstein, 1985). The names of EAV strains are in accordance with GenBank description. Differentially shaded fields indicate distinct two European subgroups (EU-1 and EU-2) and separate branch of EU-1 consisting of EAV detected in the semen collected from 5 different infected hucul stallions (from PL09hc/1 to PL09hc/5)

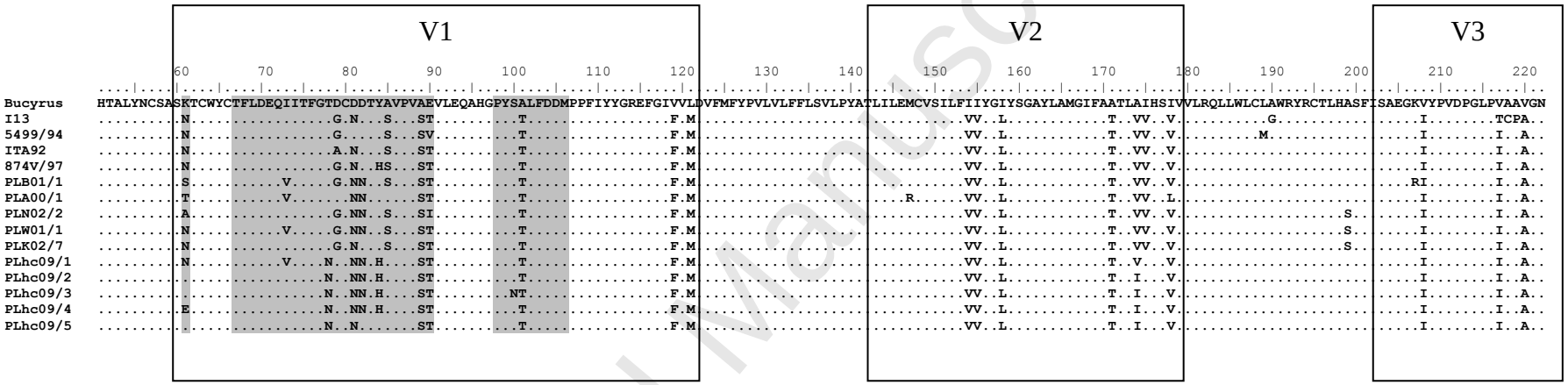

Fig. 3. The alignment of the deduced amino acid glycoprotein GP5 sequence of hucul EAVs (PLhc09/1-PLhc09/5) with selected, most similar European strains (Italian I13- AY453310.1, ITA92 - U38598.1; 874V/97 - AF099831) including Polish older (5499/94 - AF099840.1) and recent viruses (PLB01/1- EF102349.1, PLA00/1 - EF102348.1, PLN02/2 - EF102377.1, PLW01/1 - EF102389.1, PLK02/7 - EF102362.1) and EAV reference strain Bucyrus. Variable fragments of GP5 V1 (61-121aa), V2 (141-178aa) and V3 (202-222) were framed (Balasuriya et al., 1995). Neutralization sites of the protein (B-D) are grey shaded (Balasuriya et al., 1997; Balasuriya et al., 2007).