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

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Keywords: equine arteritis virus, genetic variation, open reading frame, RT-PCR

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Abstract

Samples originating from a group of 14 horses experimentally infected with a variant of equine arteritis virus (EAV) Bucyrus strain, termed large plaque variant (LP3A1+) were analysed. These included 182 nasal swabs collected from day 1 to day 14 post-infection (p.i), and 21 virus isolates obtained from white blood cells of horses that showed a secondary viraemia between day 30 and day 72 p.i. In order to understand the reason for this virus behaviour, genetic stability of the virus was studied by comparison of partial open reading frame 5 (ORF5), specifying glycoprotein 5 (GP5). Viruses with amino acid mutations in GP5 were used for further amplification and sequencing of a fragment encompassing ORFs 2b, 3, and 4. The sequences of the virus obtained from nasal swabs shared complete homology with the inoculated virus showing that the ORF5 gene of LP3A1+ was genetically stable during the first two weeks p.i. However, a number of mutations were found in the ORF5 of virus isolates obtained from day 30 p.i. when compared to the original inoculated virus. These mutations mainly clustered in antigenic neutralization site C within variable region 1 of the GP5 ectodomain. ORFs 2b and 3 had mostly silent substitutions and were more stable, whereas ORF4 showed non-conservative substitutions. Our results show that ORFs 4 and 5 of the inoculated LP3A1+ virus were subject to positive selection, most likely driven by immunological pressure, during the course of infection. However, genetic variation did not appear to be the underlying reason for the onset of a second wave of viraemia. It is plausible that the virus had been "trapped" at immune privilege sites, following initial infection, and was triggered to replicate by an unknown stimulus, therefore causing reappearance of viraemia.

Introduction

Equine arteritis virus (EAV) is an enveloped RNA virus belonging to the family *Arteriviridae* of the order *Nidovirales*. It was first isolated in 1953 from lung tissue of aborted foetus during an outbreak in Bucyrus, USA. It has become worldwide in distribution, due to the growing movement of international horse trade and sport. The virus is transmitted primarily through respiratory and venereal routes. Stallions are infected with EAV mostly by respiratory route and around 60% of them become persistently infected for several years or a lifetime. In persistently infected stallions, the virus is mainly present in the reproductive tract, especially in the ampulla of the vas deferens. Mares become infected with EAV through breeding to carrier stallions (Timoney & McCollum, 1993) or following artificial insemination with infected semen. Although most naturally infected horses show little or no clinical signs, manifestations of disease may vary greatly depending on the virus strain and the animals' condition. Clinical signs of EAV infection include anorexia, fever, depression, excessive lacrimation, conjunctivitis, nasal discharge, urticaria and edema of the limbs and scrotum. Furthermore, pregnant mares may abort.

The EAV genome is a positive-stranded, polyadenylated RNA molecule of 12.7 Kb (den Boon *et al*., 1991). The proximal two thirds of the genome contains two large open reading frames (ORF) ORFs 1a and 1b, which encode the "replicase" polyproteins. These polyproteins are post-translationally processed by three virus-encoded proteases into twelve mature products, the non-structural proteins (nsp), nsp1 to nsp12 (Snijder *et al*., 1994; van Dinten *et al*., 1996). The remaining proximal one third of the genome contains seven overlapping ORFs (2a, 2b, 3, 4, 5, 6, and 7), which are transcribed into a nested set of subgenomic RNAs, and translated into the structural proteins of the virus (de Vries *et al*., 1992). These include the nucleocapsid protein (N) and a group of membrane proteins comprising minor (E, GP2b, GP3, and GP4) and major (GP5 and M) components of the viral envelope (Wieringa *et al*., 2003). The major viral envelope proteins, GP5 and M associate into disulfide-linked heterodimers, a process that is essential for virus infectivity (Snijder *et al*., 2003).

The importance of ORF5 in the host's immune response has been highlighted recently. The ORF5 encoded GP5 has been shown to express the neutralization sites of EAV, by using polyclonal antibodies from infected horses and monoclonal antibodies with specificity for the GP5 ectodomain (Balasuriya *et al*., 1997; Chirnside *et al*., 1995; Deregt *et al*., 1994; Nugent *et al*., 2000). Balasuriya *et al*. (1997) identified four distinct neutralization sites in the GP5 ectodomain, which include sites A (aa 49), B (aa 61), C (aa 67-90), and D (aa 99-106). Except for site A, sites B, C, and D are all located in the variable region 1 (V1) encompassing amino acid 61-121 (Balasuriya *et al*., 1995). Amino acid mutations in these four sites can change the neutralization phenotype of the virus (Balasuriya *et al*., 1997).

ORF5 is also associated with EAV evolution. During a 10-year persistent infection of stallions, ORF5 along with ORF3 were shown to evolve more rapidly than ORFs 2 and 4, whereas ORFs 6 and 7 were highly conserved throughout the period (Hedges *et al*., 1999). Due to this feature, ORF5 is the gene of choice for studies on EAV diversity and evolution (Stadejek *et al*., 1999; Balasuriya *et al*., 1997).

The large plaque variant (LP3A) of EAV Bucyrus strain was derived by plaque purification of the original stock of the pleural fluid isolate virus (Wescott *et al*., 2001). *In vivo* characterization of the LP3A virus has shown that it induces a viraemia lasting for 6-7 days, accompanied by pyrexia for 6-11 days with a maximum temperature of 40-40.5°C, and virus nasal shedding for 8-11 days (Castillo-Olivares *et al*., 2001). Curiously, a virus stock (LP3A1+) that was obtained by one additional cell culture passage of the LP3A virus in equine embryonic cells was shown to be more virulent than the LP3A virus. In order to characterise the *in vivo* biology of this LP3A1+ virus, an experimental infection was done with a group of 14 horses. A particular finding was that despite development of neutralization antibodies within two weeks p.i, the horses underwent a second period of viraemia from day 30 p.i. The present studies focused on the genetic comparisons of viruses obtained from the horses throughout the experimental period.

Materials and Methods

Virus and horses

A panel of 14 horses was experimentally infected with a variant of the EAV Bucyrus pleural fluid isolate, termed LP3A1+. Nasal swabs were taken daily from day 1 to day 14 p.i. from each horse. The virus isolates were obtained by inoculating rabbit kidney (RK-13) cells with white blood cell fractions collected from 9 horses, between day 30 and day 72 p.i. The procedure for experimental infection and virus isolation has been described (Castillo-Olivares *et al*., 2001).

RNA extraction

Total RNA was isolated from 182 nasal swab extracts and 21 virus isolates using the TRIzol LS Reagent (Invitrogen, USA), according to the manufacturer's instructions. Briefly, 250 µl of samples were mixed with 750 µl of TRIzol reagent and incubated for 5 min at room temperature. 200 µl of chloroform were added, and the tubes were shaken vigorously for 15 sec and then centrifuged at 13 500 rpm (model 1-15K with 12132 rotor from Sigma, Germany) for 15 min. The RNA was collected from the aqueous phase and precipitated with 500 µl of isopropanol at -20°C overnight. RNA was pelleted by centrifugation at 13 500 rpm for 20 min at 4°C and washed with 1 ml of cold 75% ethanol, air dried, and subsequently resuspended in 30 µl of DMPC water.

Reverse transcription (RT)

A mix of 5 µl RNA, 2 µl of random hexamers (Pharmacia) and 3 µl of sterile distilled water was denatured at 65°C for 10 min and then chilled on ice. An RT mix comprising 4 µl of 5x first strand buffer (Invitrogen), 2 µl of 0.1 M DTT, 2 µl of 10 mM dNTP mix (Amersham Biosciences), 1 U of RNAguard (Amersham Biosciences) and 200 U of Moloney murine leukaemia virus reverse transcriptase (Invitrogen) were added. The reverse transcription was performed at 37°C for 90 min followed by the inactivation of the enzyme at 95°C for 5 min.

PCR amplification of partial ORF5 gene

Two sets of primers were used to amplify ORF5 gene by nested PCR using *Taq* DNA polymerase (Ampli Taq, Perkin-Elmer), as shown in Table 1. The first PCR reaction mix contained 32 µl of sterile distilled water, 5 µl of 10x PCR buffer, 1 µl of 10 mM dNTP mix, 1 µl of each external primer, 3 µl of 25 mM $MgCl_2$, 1U of *Taq* polymerase and 5 µl of cDNA. The thermal profile included initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 55 sec, and extension at 72°C for 90 sec and a final extension at 72°C for 7 min. The second PCR reaction mix contained the same amount of buffer, *Taq* polymerase, $MgCl_2$, inner primers, and dNTP mix plus 2 µl of the first PCR product, and sterile distilled water to make a volume of 50 µl. In the second PCR program the primers were annealed to the templates at 58°C for 60 sec. The PCR products (8 µl) were run on 2% agarose gel stained with ethidium bromide, and the DNA bands were visualized under UV light.

PCR amplification of ORFs 2b, 3, and ORF4

The Elongase amplification system (Invitrogen, USA) was used to amplify a fragment of 1712 bp encompassing ORFs 2b, 3, and 4. The reaction mix contained 1 µl of 10 mM dNTP mix, 2 µl of each primer, 5 µl of cDNA, 5 µl of 5x buffer A and B to adjust the final $MgCl_2$ concentration to 1.5 mM, 29 µl of sterile distilled water, and 1 U Elongase enzyme mix (a mixture of *Taq* and Pyrococcus species GB-D thermostable DNA polymerases). The cycling profile included initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec, and extension at 68°C for 3 min, and a final extension at 68°C for 10 min. The PCR products (8 µl) were run on 0.8% agarose gel stained with ethidium bromide, and the DNA bands were visualized under UV light.

Sequencing and sequence analysis

The PCR products were purified by using Qiaquick PCR purification kit (Qiagen) according to manufacturer's instructions. The BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for the sequencing reactions, which were performed in a 20 µl volume containing 2 µl BigDye, 1.8 µl of each primer, 6 µl of half-Buffer, 0.8-4 µl of DNA template, and filtered Super-Q water. The thermo profile was 25 cycles of denaturation at 96°C for 15 sec, annealing at 50°C for 10 sec, and extension at 60°C for 4 min. The extension products were precipitated with 2 µl of 3 M sodium acetate (pH 4.6), and 50 µl of cold 95% ethanol. After incubation at room temperature for 15 min, the tubes were centrifuged at 13 000 rpm for 20 min, and the pellets were washed once with 250 µl of 70% ethanol. The pellets were dried, resuspended in 12 µl of deionised formamide. Capillary electrophoresis was carried out in an ABI 3100 genetic analyzer (Applied Biosystems). Sequence data were collected automatically with the software provided by the manufacturer. The nucleotide sequences were analysed with multiple programs of the DNASTAR package (DNASTAR v5.0.3, Madison, WI, USA).

Results

PCR amplification and sequence analysis of EAV ORF5

The ORF5 gene was amplified by nested PCR from 154 out of 203 total samples. During this experimental infection, the viral ORF5 gene could be detected by RT-PCR from horses' nasal discharges for 8-13 days p.i (Table 2; the virus identification is indicated as "horse number-day p.i"). At day 14 p.i, no EAV could be detected in nasal swabs from all of the infected horses. However, between day 30 and 72 p.i, viral RNA was detected again in supernatants of RK-13 cells that had been inoculated with horse white blood cells, and undergone standard three passages for virus isolation. Two horses (0071 and 665f) had EAV in the blood for longer time than the others.

A total of 84 PCR products were used for sequencing, including 66 from day 2, -4, -6, -8, -10, and -12 p.i, and 18 from day 30 to day 72 p.i. The sequence alignment showed that the ORF5 kept quite stable during the first two weeks p.i: 65 sequences had no nucleotide change and thus were identical to the original sequence of LP3A1+. Only one sequence (0071-10) had one nucleotide change (A to G) at day 10, the last day when ORF5 could be detected from nasal swabs of this horse. The amino acid also changed from Asparagine (N) to Aspartic acid (D) at position 81 of GP5, which corresponds to a normal glycosylation site in this protein.

The alignment of the remaining 18 sequences (obtained from day 30 p.i) revealed 22 nucleotide substitutions in these sequences. Most of these substitutions clustered between nucleotide positions 200-290, and 510-540, which correspond to two variable regions in the encoded GP5.

The deduced amino acid sequences of viruses from day 30 p.i were also aligned with GP5 sequence of LP3A1+. As shown in Fig. 1, 12 out of 15 amino acid mutations occurred in the first two variable regions: V1 (aa 61-121), and V2 (aa 141-178). One mutation occurred between V1 and V2, another (V to I at aa 217 of sequence 5407-50) occurred in V3 (aa 202-222; not shown in Figure). These substitutions mainly grouped in the V1 region, especially in site C, which was identified as a crucial antigenic neutralization site.

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

A fragment of around 1.8 kb was amplified with the Elongase enzyme mix from 9 selected viruses whose GP5 had amino acid changes in regard to the original LP3A1+ strain. The final sequence was assembled from 4 sets of the sample.

The alignment of the nucleotide sequences with the original LP3A1+ revealed less than 1.0% divergence. However, the sequence distances of each ORF in relation to LP3A1+ differed by less than 0.3%, 0.6%, and 0.7% for ORFs 2b, 3 and 4, respectively. As shown in Table 3, changes in both ORFs 2b and 3 were mostly silent, whereas 4 out of 6 substitutions in ORF4 resulted in amino acid shifts.

Discussion

Following normal course of infection with EAV, the virus is eliminated from the circulation within two weeks by the host immune response. Castillo-Olivares *et al*. (2001) showed that the virus had disappeared from horse blood within two weeks, and that pyrexia disappears from all of the horses by 12 days p.i. As shown in our results, no viral RNA could be detected at day 14 p.i from all of horses' nasal discharges. It was though surprising that virus could again be detected in the horses' blood from day 30 p.i, meaning development of a secondary, late viraemia.

The reason for the late viraemia is unclear. One plausible explanation is that the LP3A1+ virus could escape antibody neutralization by mutating the neutralization epitopes in the GP5, and thus can survive to reinitiate infection. However, the amino acid substitutions in GP5 occurred in 9 out of 18 virus sequences and 5 sequences had such substitutions at antigenic determinant site C, when compared to the inoculated virus. For instance, an amino acid mutation (Y to H) occurred at site C of both sequence 9509-43 and 0071-50. This mutation also occurred at the same site from various strains, as reported by Stadejek *et al*. (1999). Amino acid mutations in the V1 region have been correlated with the differences in the neutralization phenotype of EAV field strains (Balasuriya *et al*., 1995; 1998). Obviously, virus without mutations or with less efficiency at mutating their antigenic determinant sites would not survive clearance by host antibodies and would be eliminated from the host. In fact, during the course of this experiment, viruses both with and without mutations at antigenic determinant site C did exist in the infected horses from day 30 p.i. Based on this analysis, it is unlikely that neutralization escape mutants, would have reinitiated replication and, consequently be responsible for the late viraemia.

Another possibility is that the virus could have been "trapped" in tissues for example, at immunological privileged sites, presumably following systemic dissemination through macrophages. The virus could not be eliminated efficiently from such sites by neutralizing antibodies against EAV. The virus could have been triggered by some stimulus, for example stress, to start new cycles of replication therefore causing reappearance of viraemia. The nucleotide substitutions found in the analysed genes, could therefore have resulted from the virus repeated replication, under antibody pressure. What tissues could play a role in "sequestering" the virus and for how long it could remain in this state is unknown. Persistent infection with EAV is known to be established only in post pubertal stallions, as the infection has been shown to be testosterone dependent (Little *et al*, 1992). It would be of interest to determine whether other forms of virus persistence occur upon infection with EAV.

A parallel experiment has shown that ORFs 2b, 3, 4, and partial 5 sequences of EAV LP3A1+ are genetically stable during three serial passages in RK-13 cells, as the sequences from each passage were identical to the original sequence of LP3A1+ virus. This shows that the nucleotide changes in sequences of the isolates (from day 30 p.i.) were not induced by cell-passage but rather represent the viruses present in the blood of the horses.

The sequence alignment of ORF5 showed that the pattern of virus variation could not be maintained from day to day. For example, the nucleotide sequence of virus 665f-50 reversed to the sequence it had at day 30 p.i. after some nucleotide changes had occurred at day 44 p.i. RNA viruses exist as a population of quasispecies, with a dynamic balance between the original species and the mutants until the establishment of a master sequence is reached. It is possible that the time-span of the experiment was not enough to allow the fixation of the mutation.

The nucleotide substitutions in ORF2b, 3, and 4 were fewer than in ORF5. This may be related to the fact that their encoded glycoproteins are integral membrane proteins, which associate into disulfide-linked complexes in mature virions. Most parts of the complexes are probably buried in the lipid bilayer and thus are less exposed to evolutionary forces than the GP5, which is a surface glycoprotein. Another distinct characteristic was that substitutions in ORFs 2b and 3 were mostly silent, whereas the ones found in ORFs 4 and 5 resulted in amino acid shifts. These results show that ORFs 4 and 5 were under positive selection pressure, which led to emergence of virus variants in the course of infection. In contrast to the findings of Hedges *et al*. (1999) and Balasuriya *et al*. (2004), where ORFs 5 and 3 were subject to strong selection pressure in the host, our analysis indicated a negative pressure, and thus stabilizing of ORF3, as well as ORF2b. The studies of Hedges *et al*. (1999) and Balasuriya *et al*. (2004) were done on persistent infections in stallions, which may have different characteristics from the experimental infection of the present study. This could be a reason underlying the differences in ORF3 behaviour.

In conclusion, the mutations of LP3A1+ virus mainly clustered in the ectodomain part of GP5, which is under strong immunological pressure, and could have resulted in changing neutralization characteristics of the virus. The role of these variants in reinitiating infection, however is not clear as it appears that other factors could have contributed to onset of late viraemia. It would be of interest to investigate the occurrence of other forms of persistent infection following acute equine viral arteritis.

Acknowledgements

This study was funded by project FAIR CT98-4123. We thank Alia Yacoub for her kind assistance with sequencing.

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Table 1. Sequence and position of primers used for the PCR amplification and sequencing of EAV ORFs 2b, 3, 4, and partial ORF5 (positions based on the EAV Bucyrus strain; GenBank accession No. NC_002532)

Name	Position	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	GGGAACAATTAACTAA
BuF_ORF3	10251-10273	TTGCACCGGCATTGEATCATAAT
BuR_ORF3	10829-10809	CGTGGCCCAATCCATGACTAA
BuF_ORF4	10629-10651	TTGCGTGCAAGTGGTTTGTAGTC
BuR_ORF4-1	11187-11167	CGCACCCCAAAGCAAGAATAG
BuF_GL2	10957-10975	GGAACACCCAAACGCTACT
BuR_GL-M	11675-11655	TAAGCGTAGCATAGGGTAGTA

Table 2. PCR amplification of a part of EAV ORF5 gene from nasal swabs and virus isolates collected from 14 horses during the experimental period

Horse No.	1769	5407	7013	9509	0071	072c	091b	0d4d	197c	222a	230c	445f	665f	5764	Total No.
D 1	+	+	+	+	+	+	+	+	+	+	+	+	-	+	14
D 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14
D 3	+	+	+	+	+	-	N	+	+	-	+	+	+	+	13
D 4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14
D 5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14
D 6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14
D 7	+	+	+	+	+	N	+	+	+	-	+	+	-	N	12
D 8	+	+	+	+	+	N	+	+	+	+	+	N	+	N	11
D 9	-	+	+	-	+	+	+	+	+	+	+	N	+	+	13
D 10	+	-	+	-	+	+	+	-	+	+	-	+	+	+	14
D 11	+	-	-	-	-	N	+	-	N	-	-	+	-	+	12
D 12	-	-	+	-	-	N	+	-	+	-	+	N	+	N	11
D 13	-	-	+	-	-	-	-	-	+	-	-	-	+	N	13
D 14	-	-	-	-	-	-	N	-	-	-	-	-	-	-	13
D 30	+			+		+				+				+	5
D 31					+			+		+			+		4
D 43				+									+		2
D 44		+											+		2
D 50		+			+			-		+			+		5
D 57					+										1
D 60					+										1
D 72					+										1
Total	15	16	14	16	19	11	12	16	13	17	14	11	18	11	203

Table 3. Summary of nucleotide and amino acid substitutions found in ORFs 2b, 3, and 4.

Viruses	ORF 2b		ORF 3		ORF 4	
	nt change	aa change	nt change	aa change	nt change	aa change
0071-31	T→A (67) A→G (567) G→A (153)	L→M (23) I→M (189)	A→G (85)	N→D (29)		
0071-57	T→C (456)					
			A→G (489)		A→G (95) T→C (273)	H→R (32)
0071-72					A→G (67)	Q→R (98)
072c-30	A→C (213) C→T (380)					
1769-30	T→C (516)		T→C (34) T→C (259) C→T (293)	 S→F (98)	C→T (355)	P→S (119)
5407-50			C→T (362)	T→I (121)		
665f-44			A→G (489)		A→G (95) T→C (183)	H→R (32)
9509-43	A→T (313) C→T (396)	H→Y (105)				

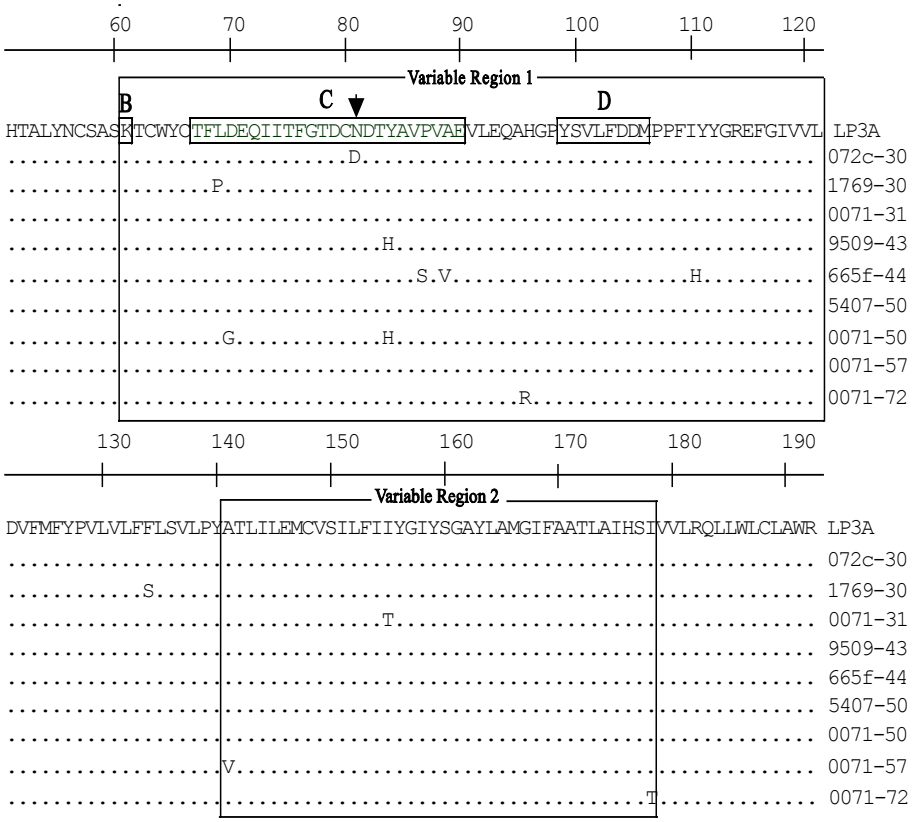

Fig. 1. The amino acid sequence alignment of partial EAV GP 5 ectodomain along with original LP3A sequence. Three neutralization sites (B, C, and D) located in the variable region 1 are shown. The glycosilation site is indicated with symbol ↓.

Acknowledgements

I am grateful to the Swedish Foundation for International Co-operation in Research and Higher Education (STINT), for giving me such valuable opportunity to take this International Master of Science programme in SLU, Sweden. I greatly appreciate Dr. Karin Östensson and Marie Sundberg for making my life and studies easy.

These studies were performed in the Department of Virology, the National Veterinary Institute, Uppsala, Sweden. I am grateful to the department headed by Prof. Berndt Klingeborn, for providing a friendly environment and good facilities for my work.

I also would like to express my gratitude to:

Prof. Sándor Belák, my supervisor, for giving me the opportunity to my start master study in virology, and inspiring discussions to guide the final part of the laboratory work.

Dr. Mikhayil Hakhverdyan, my co-supervisor, for teaching me laboratory skills, step by step, and taking care of my life here in Sweden.

Dr. Claudia Baule, for her extensive input for the EAV work and writing my thesis, followed by correction and discussion for more than ten times. I am happy to be the top one in your daily schedule.

Alia Yacoub, for her kind assistant with a lot of lab work, which made the work on EAV and BCoV go so smoothly.

Sara Hägglund, for her sincere help with my thesis, providing so many samples for the BCoV work. Could we “dig” more stories from the current work?

Dr. Frederik Widén, Mats Isaksson, Karin Ullman, Peter Thorén, and all in the Department of Virology, SVA and the Department of Biomedical Sciences and Veterinary Public Health, SLU for the help and for being so nice to me.

International students in this programme, Jate, Hos, Mon, Fernando, Suwicha, and Bhupender.

My beloved parents, for your many, many years’ hard work to support my study and life. No one could imagine how much you have done for me!!! My brother, Ruilu, my sisters, Yanling and Han, and your families for the love, support and encouragement.

My dear wife, Hongyan for her endless love and understanding. Life becomes brighter, funny, and more beautiful because of you!

Last but not the least, all of my friends, relatives, and teachers, seeing me growing up and providing all kinds of help.