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Evaluation of neutralization patterns of the five unique Argentine equine arteritis virus field strains reported

M. G. ECHEVERRÍA1,2,3*, S. DÍAZ2,3, G. E. METZ1,3, M. S. SERENA1,3, C. J. PANE1,4, E. NOSETTO1,3

1Departamento de Virología; 2IGEVET; 3CONICET; 4CICPBA; Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Argentina.
*Correspondence. E-mail: gecheverria@fcv.unlp.edu.ar

ABSTRACT
Equine viral arteritis (EVA) is a contagious viral disease that frequently causes mild or subclinical infections in adult horses. Only one EAV serotype has been described. However, there are differences in antigenicity, pathogenicity and neutralization characteristics of virus field strains. The interaction of two viral proteins, GP5 and M, is critical for infectivity and amino acid changes in the GP5 sequences have an effect on the neutralizing phenotype, regardless of other viral proteins. The objective of the present study was to evaluate the neutralization phenotypes of the 5 unique Argentine EAV strains reported and to compare them with the neutralization phenotypes of the EAV-UCD reference strain, with special emphasis on the analysis of M and GP5 proteins. The strains had a similar neutralization phenotype pattern when anti-EAV serum, derived from EAV seropositive horses, was used in the analysis. Meanwhile, low titers were observed when equine polyclonal anti-EAV reference sera were used in the assay. Argentine strains have almost the same amino acid substitutions, with the exception of LP01 strain, that mainly involves the first variable region V1 especially in neutralization sites B and C. However, they are fairly different from the EAV-UCD strain. Nevertheless, the nucleotide and amino acid differences observed among the Argentine strains LP02/R, LP02/C, LP02/P and LP-LT-ARG did not show any variations in the neutralization phenotype.

Key words: Equine arteritis virus, Cross, virus neutralization, GP5 and M proteins

INTRODUCTION
Equine viral arteritis is a worldwide disease which may cause sporadic respiratory disease and sudden death in foals, abortion in mares, and mild or subclinical infections in adult horses (10). Adult stallions may become a major source of the infection that eliminates virus in semen for variable periods (21). Ongoing genetic and phenotypic assessment of viruses contained in the semen of carrier stallions is critical for persistent infection; not only are these stallions the essential natural reservoir of the equine arteritis virus (EAV), but it would appear that genetic diversity among strains of EAV is generated during the course of persistent infection in the carrier stallion. The EAV genome is a positive-stranded RNA of approximately 12.7 kb that includes 9 open reading frames (ORF), which codify 7 structural proteins. The former GL (GP5) enve-lope glycoprotein contains 3 variable and 4 constant regions, expresses the major neutralization determinants and is encoded by ORF5, whereas ORF 6 encodes the unglycosylated envelope M protein. Both proteins GP5 and M, form a disulfide-linked heterodimer structure (Cys-
8 of M and Cys-34 of GP5) that is critical for the infectivity, maturation and the expression of the antigenic epitopes of the viral particle, as well as for induction of anti-EAV neutralizing antibodies (4, 16). Although there is apparently only one EAV serotype, antigenic variation among field strains based on their virulence and neutralization phenotype has been described (2, 6, 21). It has been reported that amino acid changes in the GP5 sequences have an effect on the neutralizing phenotype (3, 11), regardless of the effects of other viral proteins. Four neutralization sites in the amino terminal ectodomain of the GP5 protein have been described, which include amino acid residues 49 (A), 61 (B), 67-90 (C) and 99-106 (D) (1, 6). Previous studies demonstrated that although sites B, C and D are the major neutralization sites, other minor neutralization epitope sites occur. Site C is considered the major neutralizing epitope site, although there is considerable conformational interaction of the four sites (A-D) (1). Even though the M protein does not have variable and constant regions, it is well known that the C-terminal part of this protein (between amino acids 88 and 162) is the strongest antigenic region (12). The objective of the present study was to evaluate the neutralization phenotypes of the 5 unique Argentine strains of EAV (LP01, LP02/R, LP02/C, LP02/P and LT-LP-ARG) reported and to compare them to the neutralization phenotype of EAV-UCD reference strain originally derived from the prototype American Bucyrus strain, with special emphasis on the analysis of M and GP5 proteins. Ongoing documentation of genetic diversity is critical for the improvement of molecular diagnostics and for the rational design of new generation vaccines.

MATERIALS AND METHODS

Virus and cross-virus neutralization assay

The EAV-UCD reference American strain was kindly provided by Dr H. McCollum, University of Kentucky, USA (5), and has been used for diagnosis in our laboratory since 1980. Although the strain was originally derived from the prototype Bucyrus virus, the passage history of the strain is not fully documented. The 5 Argentine strains had been isolated in our laboratory in 2001 (LP01 in Farm A), 2002 (LP02/R, LP02/C and LP02/P –named LP02 group– in Farm B) and in 2007 (LT-LP-ARG in Farm C) and partially characterized in several studies (7, 8, 13). These strains were cultured and titrated in rabbit kidney cells (RK13). Briefly, a stock of each strain (second passage) was cultured, clarified, stored in aliquots at −70 °C and then titrated using the same cell line. Thirteen serum samples from stallions detected EAV positive by virus neutralization (VN), from our sera bank, and the reference EAV antisera (Anti-Bucyrus) were used to phenotypically characterize the 5 Argentine strains of EAV. Five of the thirteen serum samples belonged to horses where the virus isolation had been previously performed and named: “R”, “C”, “P” and “A” for LP01, LP02/R, LP02/C, LP02/P and LT-LP-ARG strains, respectively. Cross neutralization tests were performed as described by International Standards (20). Briefly, 14 polyclonal heat-inactivated sera were diluted in serum-free medium 1:2 serial dilutions. The samples were mixed with an equal volume of each EAV strain (100 TCID50) and after 1 hr of incubation in 5% CO2, 100 µl of RK13 cells (3 x 105 cells/ml) were added and incubated under same conditions for 72 hs more. Cross-virus neutralization tests were performed as a single batch to avoid variation in the proceeding of the technique. Neutralization titers are expressed as the inverse of the antibody highest dilution providing at least 50% protection of RK13 cell monolayers against 100 TCID50 of virus.

RT-PCR

Viral RNA was extracted from virus infected RK13 cell cultures infected with each strain with Trizol (Invitrogen) and precipitated with isopropanol. Five microliters of RNA resuspended in distilled water were used for cDNA synthesis. For the RT step, cDNA was obtained using reverse transcriptase and random hexamers (Promega and Biodynamics, respectively). For PCR amplification of the M protein gene, a pair of primers was designed based on RefSeq NC-002532: MFor 5’ GGATGGAGCCATAGATTGATGAGTTTCAT’3’ (position 11898-12009) and MRev 5’ TTGATACCTGTAGGGTGATGGC’ (position 12366-12386). For the GP5 protein gene, primers GL105F and GL673R were used according to Mittelhozer et al. (14). PCR reactions were carried out in a final volume of 50 µl containing 5 µl of the cDNA, 3 µl of MgCl2 (25 mM), 5 µl 10X PCR buffer, 1.25 U Taq polymerase, 1 µl dNTP mix (0.2 mM each) –Promega– and 2 µl of each primer (20 pm each). Denaturation, annealing and extension consisted of 35 cycles at 94 °C 45 seconds, 53 °C or 60 °C (M and GP5 PCR cycles respectively) 1 min and 72 °C 90 seconds respectively. Each PCR product (10 µl samples) was examined on 2% agarose gels on TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). The gels were examined under UV light after ethidium bromide staining.

Sequence analysis

Previously to the sequencing analysis of the M and GP5 genes, the PCR products were purified by a PCR purification kit (Promega). Products of the M gene (ORF 6) were cloned in TOPO TA 2.1 cloning vector (Invitrogen) and then used to transform competent Top10 cells. Two positive clones were selected for nucleotide sequencing using vector primers (M13 primers). For the GP5 gene (ORF 5), direct sequencing was done using internal primers CR2 and EAV32 as previously described (18). Sequencing analysis was performed by using the dye-terminator method (MegaBACEtm 1000, Instituto de Genética Veterinaria, IGEVET, CONICET CCT, La Plata). The sequences alignments of M and GP5 genes were carried out by using the Clustal X version 1.92 software (19). To predict N-glycosylation in GP5 sequences, the NetNGlyc Server analysis by Technical University of Denmark (http://www.cbs.dtu.dk/services/NetNGlyc/) was performed. This program predicts asparagines to be N-glycosylated according to the sequons Asn-Xaa-Ser/Thr (where Xaa in n pro), with a threshold 0.5.

RESULTS AND DISCUSSION

VN tests using the EAV-UCD strain were performed with sera belonging to stallions “RE”, “R”, “C”, “P” and “A” and all of them resulted positive to EAV antibodies (with titers of 8 and 16). The neutralization phenotype of each Argentine strain was characterized by cross microneutralization assay using a panel of polyclonal anti-EAV equine sera. The results of cross VN tests are shown in Table 1. Each polyclonal anti-EAV equine serum was able to neutralize its homologous virus, but not always did it neutralize the other viruses at the same titer as that neutralized by the own virus (between 16 to 512). A four-fold or greater difference in VN titer between EAV viruses was considered as a significant change in the neutralization pheno-
Table 1. Neutralization titers of polyclonal EAV antibodies and anti-Bucyrus sera to Argentine strains and EAV-UCD reference strain

<table>
<thead>
<tr>
<th></th>
<th>EAV-UCD</th>
<th>LP01</th>
<th>LP02/R</th>
<th>LP02/C</th>
<th>LP02/P</th>
<th>LT-LP-ARG</th>
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<td>4</td>
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<td>8</td>
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<td>128</td>
<td>32</td>
<td>128</td>
<td>128</td>
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<td>256</td>
<td>512</td>
<td>128</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>“C”</td>
<td>8</td>
<td>128</td>
<td>512</td>
<td>128</td>
<td>128</td>
<td>512</td>
</tr>
<tr>
<td>“P”</td>
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<td>128</td>
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Figure 1: Alignment of the 162 amino acid sequences of the M protein of laboratory and field strains of EAV. The Cys in position 8 are in bold letter.
type. Equine anti-EAV serum derived from a horse inoculated with the original Bucyrus strain neutralized all the viruses but at a low extent with mean titers ranging from 4 to 16. Virus neutralization of the EAV-UCD reference strain was the weakest of all strains, and was poorly neutralized by heterologous antisera. Regardless of the antigenic differences among the viruses, determined by amino acid sequences analysis of ORF5, a relatively high level of virus specific antibodies cross-reacted with the homologous or related viruses. However, a low level of cross-virus neutralization was observed when using the reference viral strain.

For both GP5 and M proteins, the cDNA was independently and specifically amplified by RT-PCR using the RNA purified from cell cultures infected with each strain as template. This single-round RT-PCR generated a unique product observed as a sharp, visible band on the agarose gels stained with ethidium bromide. In each case, bands of identical size were obtained from both the positive control (EAV-UCD reference strain) and the Argentine strains.

The nucleotide sequence data reported in this paper for both ORFs were previously submitted to the GenBank database and assigned the following accession numbers: complete ORF 6 DQ333586 (LP01), DQ333587 (LP02/R), DQ333588, (LP02/C) DQ333589 (LP02/P) and for partial ORF 5 DQ435439, DQ435440, DQ435441, DQ435442 and EU622889 for LP01, LP02/R, LP02/C, LP02/P and LT-LP-ARG respectively.

When analyzing the predicted amino acid sequences (Figure 1), the M ectodomain contains a Cys residue at position 8 in all strains analyzed, and another Cys residue is present in position 95 in Argentine strains while in the EAV-UCD strain is phenylalanine. No insertions or deletions were detected in this ORF. The amino acid changes were seen in the following positions: 30 (Leu - Ile), 42 (Leu - Met), 43 (Ala - Thr), 80 (Val - Leu), 81 (Met - Ile), 87 (Met - Val), 88 (Met - Leu) and 122 (Ile - Val). In position 114, one amino acid substitution (Pro - Ser) in EAV-UCD and LP02/P and in position 154, Ala - Thr in EAV-UCD, LP02/C and LT-LP-ARG strains were observed. Some critical amino acid substitutions on the M protein are responsible for the attenuation of the ARVAC vaccine strain of EAV. The substitutions of amino acids 38, 49, 71, 81, 122, 150 and 154 appear to be related to attenuation of the virus, being amino acids 81, 122 and 154 critical for this ORF (23). In our study, we found the following substitution on the M protein: Met in position 81 (EAV-UCD), Ile in 122 (EAV-UCD) and Ala in 154 (EAV-UCD, LP02/C and LT-LP-ARG). Although we found

Figure 2: Alignment of the amino acid sequences of the critical neutralization regions of GP5 proteins of Argentine and reference laboratory EAV strains. The conserved glycosylation (amino acid 56) site is in bold letter. Deleted amino acids are indicated by "..". Neutralization sites B, C and D (variable region Vj) are indicated in boxes.
changes in the same amino acid positions as those in Zhang et al. (23) they are not the same amino acid substitutions that could be responsible for the virulence of the virus.

Changes involving GP5 are detailed as follows. The deduced amino acid sequences (51-222) of the variable and conservative regions of the GP5 protein are illustrated in Figure 2. Regarding the first constant region (C\(_1\)), there is a deletion of 6 nt in the EAV-UCD strain, resulting in the loss of 2 amino acids and a substitution of Cys 57 only in LP02/R strain (Trp). Cys in positions 63, 66 and 80 as well as C\(_2\) and C\(_3\) regions remained invariable in all the virus strains. When analyzing the V\(_1\) region (amino acids 61-121), neutralizing site B (amino acid 61) is variable in the LP01 strain (Lys – Glu), neutralizing site C (amino acids 67-90) is hypervariable and neutralizing site D (amino acids 99-106) remains invariable among the strains. The substitutions of amino acids in neutralizing site C are as follows: position 67 (Glu - Val or Thr), 71 (Asp - Glu), 73 (Val - Ile), 76 (Phe - Ile), 79 (Gly - Asp), 82 (Asn - Asp), 83 (Thr - Ala), 84 (Tyr - His), 85 (Ser - Ala), 87 (Pro - Ser), 89 (Ala - Ser), 90 (Thr - Glu). As it was demonstrated by Balasuriya et al. (1) the amino acid substitutions at 61 and 67 position can alter other epitopes. The rest of changes involving the V\(_1\) region are located in amino acid 119 (Phe - Val) and 121 (Met - Leu). Argentine strains have almost the same substitutions with the exception of the LP01 strain, involving the first variable region V\(_1\) especially in neutralization sites B and C, but are quite different when compared to the EAV-UCD strain. When analyzing the putative N-linked glycosylation sites, the six strains possess asparagine sites at amino acids 56 (conserved and critical to virus infectivity) and 81 (variable), and as it was described, the asparagine in position 81 is present in the majority of EAV fields isolated (6, 18) and possibly related to persistent infection and reduced virulence. It has been previously reported that the loss of glycosylation sites of another arterivirus, the lactate dehydrogenase-elevating virus protein, alters the virulence and cellular tropism of the virus (17). It is unknown whether the glycosylation changes in the GP5 protein play an analogous role in the case of EAV (22). However, according to the prediction glycosylation program used here, the asparagine present in position 81 is not enough to glycosylate for the tripeptide obtained after translation in LP01, LP02/P (NNT) and EAV-UCD (NNA). If it is taken into account that glycosylation reduces virulence, LP01, LP02/P and EAV-UCD strains could be more virulent than LP02/R, LP02/C and LT-LP-ARG strains, which have probed to glycosylate in both positions. When analyzing V\(_2\) and V\(_3\) regions (amino acids 141-178 and 202-222 respectively), substitutions are in positions 141, 154, 155, 158, 171, 174, 175, 178, 208 and 217. It is important to remark that the sequence strain has been used in our laboratory since 1980 and only suffers RK13 cell culture passages once a year. This reference strain changed only 4 nt of the 519 analyzed, which led to predicted amino acid changes from Phe to Ile at position 76 and Asp to Asn at position 81, both in neutralization site C. This result suggests that ORF 5 readily mutates during passage in cell cultures (5). There was no information about the sequence of EAV-UCD ORF 6 at the time of the virus isolation.

The genetic variation of Argentine EAV strains obtained from semen mainly clustered in the ectodomain part of GP5, particularly in the neutralization site C. These mutations could be responsible for differences in the neutralization phenotype of Argentine vs prototype EAV-UCD. According to the results obtained by Zhang et al., 2008 (23) when comparing different strains of EAV (virulent and attenuated), the amino acids substitutions are located in positions, 72, 81, 100, 104, 106, 170 and 214, being 81, 100 and 104 critical positions. Even though the six strains analyzed in this work had the same amino acids at GP5 critical positions that indicate no attenuation, it is important to remark that the Argentine strains are phylogenetically related to European strains, whereas the reference strain EAV-UCD is a typical American strain, derived from the Bucyrus strain (5). The virulence phenotypes identified as amino acid substitutions that individually or collectively would appear to be directly related to attenuation, could be in other genes than GP5 and M.

As has been reported by Balasuriya et al. (5, 6), the neutralization of viruses isolated from the same geographic area sometimes varied due to a considerable antigenic variation among field strains of EAV. Our data reported here indicate that there are some antigenic variations among Argentine strains derived from the same farm in the same year (LP02 group). Several reports have also documented variations in the neutralization of field strains of EAV for monoclonal antibodies and polyclonal antisera (1, 9, 22). The EAV-UCD strain appeared to be different from Argentine strains as it was poorly neutralized by heterologous antisera of stallions from the infected farms. The sporadic occurrence of EVA around the world, indicates that asymptomatic and subclinical EAV infection of horses is very common, which is consistent with previous clinical reports where EAV infected stallions generally transmit low virulence viruses (15, 21).

In summary, amino acid substitutions at site B and C of the GP5 protein have an effect on the expression of conformational neutralizing epitopes, and similar changes are likely critical to generate antigenic variation among field strains of EAV. As it had been demonstrated by Balasuriya et al. (1), the amino acid substitutions at 61 and 67 positions can alter other epitopes. LP01 and LP02/C Argentine EAV strains had similar substitutions, as described by Balasuriya et al., 2004 -amino acids 61 and 67- (6). Assuming the importance of the GP5 protein in virus neutralization, we consider that this protein could be a candidate for a subunit or peptide vaccine, therefore characterization of virus neutralization sites could be an
advantage for a better insight into vaccine development. Prior to development of any such vaccine, however, it will be first desirable to comprehensively define the location of individual epitopes involved in neutralization among the strains circulating in Argentina and to identify and to establish whether these amino acids are consistently expressed in our field strains of EAV. This is a prerequisite to the logical development and design of improved vaccines and diagnostic methodologies.

Our results agree with those obtained by Balasuriya et al. (2), who observed heterogeneity in the group of EAV strains and antigenic variations of proteins involved in virus neutralization. Nevertheless, the differences found among the strains isolated in 2002 did not show any variations in the VN phenotype. We found differences between the Argentine EAV strains and the EAV-UCD reference strain involving other variable regions than V1. When analyzing the strain sequences, the substitutions of amino acids that individually or collectively appear, seem not to be directly related to attenuation, even when both proteins are involved in viral replication and immune response. Further studies are needed to determine the role of sequence variations and amino acid level in the virus tropism, antigenicity and virulence of the strains isolated in Argentina. Furthermore, it would be of interest to analyze the phylogenetic relationships among isolates worldwide, involving sequences of different ORFs. Because of the virus sensitivity, a neutralization test for detecting antibodies to EAV can be significantly influenced by several factors, especially the source and passage history of the virus strain used in this study. As was expressed by the OIE Terrestrial Manual, we consider that it would be pertinent to think about the possibility to include a European EAV reference strain as a second serotype. There are continuous efforts to bring greater uniformity in serological results among laboratories providing virus neutralization or other comparable serological assays for this disease (20).

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