Genetic predisposition for developing equine arteritis virus carrier state: adopting proper biosecurity measures when breeding mares to a persistently infected stallion

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Abstract

Outbreaks of equine viral arteritis (EVA) have occurred around the world and there is evidence of an increase in global incidence of the disease in past years. These outbreaks have had important economic consequences in terms of direct financial losses to the equine industry. Following natural infection, 10 - 70% of stallions can become persistently infected carriers of equine arteritis virus (EAV) and continuously shed virus in their semen. Carrier stallions have a central role in maintenance and perpetuation of the virus and are responsible for initiating major outbreaks of EVA. Furthermore, occurrence and spread of EAV has been facilitated by increasing horse movement around the world for competition and breeding and by trade of fresh or frozen semen and embryos. Control and preventive measures include prophylactic vaccination and control of movement of stallions used for natural or artificial breeding by serological testing and subsequent virologic assessment of their semen before initiation of each breeding season, as well as during pre-export and import quarantines. Here, we review the latest findings regarding genetic predisposition of stallions to become long-term EAV carriers, current laboratory tests for diagnosis of EAV in semen, as well as biosecurity measures that should be implemented when breeding mares to persistently infected stallions.

Keywords: Equine arteritis virus, EAV, equine viral arteritis, EVA, persistent infection, biosecurity

Introduction and background

Equine viral arteritis (EVA) is a reproductive and respiratory disease that affects members of the family *Equidae,* which includes horses, mules, donkeys and zebras.[1-10] EVA is caused by equine arteritis virus (EAV), a single-stranded, positive sense RNA virus that belongs to the family *Arteriviridae*, genus *Alphaartevirus* in the order *Nidovirales.*[1,11,12] The molecular biology of EAV, and clinical features and pathogenesis of EVA have been recently reviewed in several publications.[6,13-18] The virus is distributed throughout the world; most EAV infections are subclinical in nature and may go undiagnosed. However, some virulent strains periodically cause marked outbreaks of disease and they could be associated with abortion, neonatal mortality and establishment of persistent infection in stallions.[2,8,19-27] The incubation period varies from 2 -14 days (usually 6 - 8 days after venereal exposure) and the disease is clinically characterized by fever (> 41°C), depression, leukopenia, excessive lacrimation, anorexia, conjunctivitis, rhinitis and nasal discharge, urticaria of the head, neck, trunk, and hind limbs, and edema, which is most pronounced over the eyes (supraorbital), the abdomen, prepuce, scrotum, mammary glands, and hind limbs.[3,4,9,27-40] Abortion is a frequent outcome in naïve pregnant mares and generally occurs 10 - 30 days after exposure to EAV and at any time between 3 and 10 months of gestation.[2,23,27,35,37,41,42] Congenital infection in neonatal foals is characterized by severe fulminating bronchointerstitial pneumonia. Foals that are 1 to 3 months old can develop a progressive "pneumoenteric" syndrome, leading to death frequently associated with secondary bacterial infections.[24,25,43,44]

Most importantly, a variable proportion of acutely infected stallions (10 - 70 %) may become persistently infected.[22,45] The virus only persists in the stallion's reproductive tract and is continuously and exclusively shed in semen after clinical recovery from the acute phase of the infection (carrier state).[29,34, 45] The carrier state is testosterone-dependent and there is no evidence of persistent infection in mares, geldings or foals.[27,46-48] Persistently infected stallions shed EAV in their semen for either a short interval (ranging from several weeks to < 1 year post infection; defined as short term shedders or short term carriers) or a long interval (> 1 year post infection to lifelong; defined as long term persistently

infected shedders or carriers) without adverse effects on semen quality or reproductive capacity.[22,34,49-51] In these stallions, EAV is only detectable in the reproductive tract and viral persistence occurs, despite the presence of high serum neutralizing and mucosal antibodies.[22,34,50,52] Viral shedding in semen frequently begins at 5 days post infection; viral titers in seminal plasma are variable but frequently high, ranging from 10^1 to > 10^7 PFU/ml.[22,29,34] Analysis of multiple tissues from the reproductive tract of EAV carrier stallions unequivocally confirmed the ampullae as the primary site of EAV persistence. Dual immunohistochemistry staining confirmed EAV localizes in vimentin positive fibrocytes and mononuclear cells (T and B lymphocytes and macrophages) but not in the glandular epithelium.[34]

Persistently infected stallions have a central role in maintenance, perpetuation and evolution of the virus in the horse population. The occurrence and spread of EAV has been facilitated by increasing horse movement around the world for competition and breeding purposes and by trade of fresh or frozen semen and embryos.[26,53-58] Development of the carrier state in stallions directly reduces commercial value of these animals, with higher costs for breeding and barriers in national and international movement of persistently infected stallions and commercialization of their semen and embryos.

Genetic Predisposition of Stallions for Developing EAV Carrier State

Recently, it has been demonstrated that establishment of EAV long-term persistent infection (LTPI) correlates with in vitro susceptibility of a subpopulation of $CD3^+$ T lymphocytes to EAV infection;[33,59] consequently, stallions with the $CD3^+$ T lymphocyte susceptibility phenotype are at higher risk of becoming long-term, persistently infected carriers compared to those that lack this phenotype. A genome wide association study (GWAS) demonstrated that these phenotypes are associated with the CXCL16 gene located in equine chromosome 11 (ECA11).[60] Subsequently, studies identified 2 allelic variants of CXCL16 (namely CXCL16S and CXCL16R) that differ by 4 nonsynonymous nucleotide substitutions in exon 1 and have a very strong association with 2 $CD3^+$ T lymphocyte phenotypes and with either establishment of long-term persistence (CXCL16S) or the early viral clearance in stallions (CXCL16R).[61] Of the 2 encoded proteins, the CXCL16S isoform has EAV receptor activity and is associated with the $CD3^+$ T lymphocyte susceptible phenotype and establishment of LTPI in stallions, whereas the CXCL16R isoform lacks receptor activity and results in a $CD3^+$ T lymphocyte resistant phenotype and early viral clearance in stallions (short term carriers).[61,62] Moreover, the secretory from of the CXCL16 protein is the ligand for the chemokine receptor CXCR6 that is predicted to be expressed on $CD4^+$ and $CD8^+$ T cells, NKT cells and NK cells. Thus, it has been suggested that equine CXCL16 and CXCR6 may be 2 major cellular proteins associated with the EAV carrier state in stallions. Specific mechanisms mediating modulation of the CXCL16/CXCR6 axis and viral immune evasion in the male reproductive tract are currently under investigation in our laboratory.

Since genetic studies clearly established a very strong correlation between stallion genotype and likelihood of establishment of long-term persistence following infection, we recently developed an allelic discrimination quantitative PCR (qPCR) for CXCL16 genotyping (Balasuriya and Graves, 2018, unpublished). This assay detects presence of the susceptibility allele (CXCL16S*)*, allowing identification of stallions more prone to become long-term carriers if exposed to EAV. Therefore, it is recommended that sexually mature colts and stallions with the susceptibility allele should be vaccinated yearly against EAV to prevent establishment of the carrier state following natural infection.

Local inflammatory and mucosal antibody responses to EAV during long-term persistent infection

Interestingly, the primary site of EAV LTPI is in the ampulla of the reproductive tract rather than immunologically privileged tissues (i.e. testis). Recent studies characterized viral tropism as well as local inflammatory and mucosal antibody responses to EAV infection during the LTPI.[34,50,63] Using immunohistochemistry (IHC) and dual immunofluorescence, it has been demonstrated that EAV has a specific tropism for vimentin-positive stromal cells (e.g. fibrocytes and tissue macrophages) and $CD8^+$ T and $CD21^+$ B lymphocytes, but not for cytokeratin-positive glandular epithelial cells in the male reproductive tract, with the highest number of virus infected cells within the ampullae of the ductus deferens. Viral persistence is also associated with a moderate to severe, multifocal lymphoplasmacytic

ampullitis with extensive involvement of T lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$ and $CD25^+$), clusters of $CD21^+$ B lymphocytes, diverse Ig secreting plasma cells, macrophages ($Iba1^+$, $CD163^+$ and $CD204^+$) and dendritic cells ($CD83^+$).[34,50] Most importantly, infiltrating lymphocytes have enhanced expression of CXCL16 and CXCR6.[49,63] Moreover, transcriptome analysis of the ampulla of EAV LTPI stallions demonstrated that the local $CD8^+$ T lymphocyte response is predominantly orchestrated by a specific subset of transcription factors (mainly EOMES, PRDM1 [BLIMP-1], NFATC2, TBX21 [T bet]), which are associated with presence of the CXCL16S allele and likely related to a local Th1 response.[63] Also, there was upregulation of T cell exhaustion related markers and homing chemokines/chemokine receptors (including CXCL16/CXCR6).

Extensive characterization of the antiEAV mucosal antibody response in long-term carrier stallions and comparative analysis with the serological response identified major differences in immunoglobulin isotypes in seminal plasma.[50] The serological response is primarily mediated by virus-specific IgM and IgG1, although virus-specific serum IgA, IgG3/5, IgG4/7, and IgG6 isotype responses are not detected. In contrast, EAV specific immunoglobulin isotypes in seminal plasma included IgA, IgG1, IgG3/5, and IgG4/7. Interestingly, seminal plasma IgG1 and IgG4/7 possess virus neutralizing activity similar to that of serum IgG1, whereas seminal plasma IgA and IgG3/5 do not. However, virus neutralizing IgG1 and IgG4/7 in seminal plasma are not effective in preventing viral infectivity and mechanism(s) enabling EAV to evade neutralizing mucosal antibody response in semen is unknown.

Role of microRNA in EAV long term persistent infection in stallions

MicroRNAs are noncoding RNA molecules ~ 22 - 24 nucleotides in length that participate in posttranscriptional regulation of gene expression through specific degradation of mRNA targets or blockade of their translation.[64] Recently, we investigated the role of equine seminal exosome-associated miRNAs and their potential role during EAV LTPI in the reproductive tract.[49] Seminal exosomes participate in cell to cell communication by specific cargo delivery, which may include proteins (e.g. cytokines, growth factors), specific lipids, and coding and small noncoding RNAs with potential regulatory functions including microRNAs (miRNAs).[65,66] Our studies demonstrated that EAV LTPI is associated with specific downregulation of seminal exosome associated miRNA eca-mir-128, along with enhanced expression of CXCL16 in the male reproductive tract. Interestingly, target evaluation and in silico analysis identified CXCL16 mRNA as a putative target of eca-mir-128; furthermore, we determined that expression of these (CXCL16 and seminal exosome-associated eca-mir-128) in the male reproductive tract are inversely correlated. Based on these findings, we inferred that seminal exosome-associated eca-mir-128 is implicated in regulation of the CXCL16/CXCR6 axis in the reproductive tract of persistently infected stallion. However, further investigation is warranted to identify its specific mechanism in modulating the CXCL16/CXCR6 axis in the reproductive tract of the EAV long-term carrier stallion. How other eca-mir-128 regulated pathways contribute to modulating the CXCL16/CXCR6 axis favoring long-term EAV persistent infection in the stallion reproductive tract is not well known. In summary, recent studies indicate that pathogenesis of EAV persistence in the male reproductive tract is complex and involves multiple host factors.

Evolution of EAV during persistent infection

Despite the presence of neutralizing antibodies in serum and seminal plasma, EAV persists in the reproductive tract of carrier stallions and is transmitted via semen. EAV evolves during persistent infection in the reproductive tract, leading to emergence of genetic variants with distinct neutralization phenotypes due to amino acid substitutions in major viral neutralization sites located in the GP5 major envelope glycoprotein, conferring the ability to successfully escape humoral immunity.[20,21,26,58,67,68] However, these investigations did not provide a complete picture of EAV evolution during long-term persistent infection, because they were confined either to a few selected viral open reading frames (ORF5 or ORFs 2 - 7) or based on consensus genomic sequences. Availability of next generation sequencing technology has overcome these deficiencies and allowed identification of rapidly evolving regions of the viral genome likely to contribute to survival of EAV in the reproductive tract of

experimentally and naturally infected stallions. Recently, sequential viruses isolated from nasal secretions, buffy coat cells and semen of experimentally infected and naturally infected EAV carrier stallions (followed for 726 days and 7 and 10 years post infection, respectively) have been deep sequenced to elucidate the intra-host micro evolutionary process after a single transmission event.[69] Analysis of variants from nasal secretions and buffy coat cells during acute infection indicated a lack of extensive positive selection; however, characteristics of the mutant spectra were different in the two sample types. In contrast, the virus population in the semen during acute infection has undergone a selective bottleneck, as reflected by a reduction in population size and multiple sites in the virus genome that were under diversifying selection. During persistent infection, there is extensive genome-wide purifying selection which shaped the variant diversity in stallion reproductive tract. Furthermore, during persistent infection, extensive genome wide purifying selection shaped variant diversity in the stallion reproductive tract. Overall, the nonstochastic nature of EAV evolution during persistent infection was driven by active intra-host selection pressure. Among the open reading frames within the viral genome, ORF3, ORF5 and the nsp2 encoding region of ORF1a accumulated the majority of nucleotide substitutions during persistence, with ORF3 and ORF5 having the highest intra-host evolutionary rates. EAV evolved at a rate of 4×10^{-4} nucleotide substitutions/site/year during natural infection in the reproductive tract. These findings provide a novel insight into the evolutionary mechanisms of EAV, identifying critical regions of the viral genome likely associated with establishment and maintenance of persistent infection in the stallion reproductive tract.

Identification of EAV carrier stallions

Current EVA control and prevention strategies partly rely on identification of EAV carrier stallions. The current approach for initial identification of EAV carrier stallions is dependent on demonstration of anti-virus neutralizing antibodies in serum. If the stallion is determined to be seropositive (neutralizing antibody titer e 1:4) and has no certified history of vaccination against EVA or confirmation of seronegative status prior to initial vaccination, virologic assessment of their semen either by virus isolation in cell culture or demonstration of viral nucleic acids by molecular diagnostic testing is required to determine their infection status and potential carrier state.[52,70-72] An alternative method for identification of EAV carrier stallions or infective semen is to perform test breeding of the stallion (or semen derived from the stallions) to 2 naïve mares. Briefly, this method consists of test breeding 2 seronegative (naïve) mares twice, each on 2 consecutive days (total of 4 covers). The mares should be kept in quarantine and tested for the presence of neutralizing antibodies to EAV after 14 and 28 days post breeding. If the test is performed properly and strict quarantine is maintained, seroconversion in both mares bred to the stallion is evidence of EAV infection and thus, indicates that the stallion (or the semen) is persistently infected. There could be 2 outcomes: (i) If both mares seroconvert, the stallion is a carrier of EAV and should be reported to the state veterinarian (please refer below for guidelines to breed EAV carrier stallions to a seropositive mare); or (ii) If both mares remain seronegative, identify the stallion as a "seropositive nonshedding stallion" or "seropositive noncarrier". Stallion is qualified for breeding but needs to be vaccinated annually. However, this method of screening stallions for EAV carrier state is expensive, time consuming and not practical for routine identification of carrier stallions.

For serological testing, whole blood should be collected into Vacutainer® tubes without anti-coagulant (for separation of serum). Semen samples submitted for virologic assessment should contain the sperm-rich fraction of the ejaculate.[19,45] Preejaculatory fluids are inappropriate, since presence of virus in this fluid is inconsistent or variable. Frozen semen straws from EAV seropositive stallions should be tested for presence of virus by virus isolation or viral nucleic acids by molecular testing. Currently, virus isolation and virus neutralization test (VNT) are the world organization for animal health (OIE) prescribed tests for international trade (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals).[70] A competitive enzyme linked immunosorbent assay (cELISA) was recently developed and validated for detection of EAV-specific antibodies and could be considered as an alternative assay for serological diagnosis of EVA.[73-76] Even though virus isolation is the gold standard for detection of EAV in semen and the OIE prescribed test for international trade, performance of an RT-qPCR and a reverse

transcription insulated isothermal PCR (RT-iiPCR) described in literature have equal or higher sensitivity compared to virus isolation in semen samples.[77-89] Therefore, molecular assays could be used as an alternative method for EAV diagnosis.[77-79,88]

Biosecurity measures when breeding mares to a persistently infected stallion

EVA control and prevention measures are primarily based on identification of EAV carrier stallions and vaccination of susceptible horses, including stallions. Commercially available vaccines (modified live virus vaccine [MLV; ARVAC®Zoetis, Kalamazoo, MI] and inactivated vaccine [Artervac®], Zoetis) are protective and can prevent establishment of the carrier state, although ARVAC® (Zoetis) is better characterized than the inactivated product. It is extremely important to prevent sexually mature colts and stallions from being exposed to EAV during outbreaks of EVA. Spread of EAV could be prevented by implementing proper biosecurity control programs on breeding farms, racetracks, horse shows and veterinary clinics and hospitals. It is imperative to identify and isolate the index case and horses that come in contact, thereby minimizing or eliminating direct or indirect contact of susceptible horses with the secretions and excretions of EAV-infected horses. If an outbreak of EVA on a farm is suspected based on clinical signs and history, state veterinarian should be notified, affected and in-contact horses should be isolated, movement of horses on and off the farm should be discontinued (quarantine), at-risk horses should be vaccinated and breeding activity should be stopped to prevent further spread of virus. Stalls and equipment on the affected premises should be decontaminated with disinfectants (phenolic, chlorine, iodine or quaternary ammonium compounds). Quarantine is discontinued when no additional clinical cases of EVA or serologic evidence of infection are observed for 3 - 4 weeks. Stallions in exposed premises should be tested for shedding of the virus as described above and follow the guidelines described below during breeding of seropositive stallions to naïve mares.[15]

EAV carrier stallions have pivotal roles in transmission and maintenance of EAV infection in horse populations.[27,45] Therefore, outbreaks of EVA can be prevented by identification of persistently infected stallions and institution of management practices to prevent introduction of EAV-infected horses. The US Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS), Equine Viral Arteritis: Uniform Methods and Rules, describes minimum standards for detecting, controlling and preventing EVA, as well as minimum EVA requirements for interstate and intrastate movement of horses.[72]

Guidelines for prevention and control of EAV in breeding stallions

1. All stallions should be tested for neutralizing antibodies to EAV before they are vaccinated with MLV or inactivated EAV vaccines. Testing should be done at least 60 days prior to breeding.
2. A neutralization antibody titer of 1:4 or greater (VNT titer ≥ 1:4) is regarded as positive. Owners of stallions that are seropositive should provide a valid vaccination certificate (stallion should have been confirmed seronegative before first vaccination against EAV).
3. Nonvaccinated seropositive stallions (no vaccination history) for EAV (VNT titer ≥ 1:4) should be tested for carrier state by test breeding or testing semen samples for presence of EAV by virus isolation/molecular testing of 2 separate semen samples derived from 2 separate collections or by test breeding, as indicated above. If semen samples are negative for EAV by virus isolation/molecular testing, identify stallions as EAV noncarriers.
4. Management of EAV carrier stallions:
   Carrier stallions must be housed, handled and bred in a facility isolated from noncarrier stallions and mares.
   Carrier stallions should be approved by the State Veterinarian for breeding.
   Carrier stallions should be bred only to mares that are seropositive either by natural exposure or by vaccination with neutralizing antibody titers ≥ 1:64 determined at least 30 days prior to breeding.

5. If seronegative (titer < 1:4), stallions should be vaccinated with the MLV vaccine after collecting a serum sample as proof. The vaccination needs to be officially recorded.
6. If stallions were to be vaccinated for the first time, they should be isolated for 28 days post vaccination before the breeding season or semen collection for artificial breeding purposes can begin. Vaccinated stallions should receive annual boosters.

Guidelines for breeding a mare to an EAV shedding stallion or insemination with EAV contaminated semen:

1. Stallions that are confirmed semen shedders and carriers of EAV can be used for breeding purposes, provided stringent requirements are met.[27,45,54,55,90]
2. Carrier stallions should be kept physically isolated and bred only to mares that are seropositive from either previous natural exposure or vaccination (not < 3 weeks previously). It is also critical that carrier stallions are kept isolated and collected separately to prevent contamination of collection equipment, teasers, and premises with ejaculate, because EAV can be transmitted to susceptible horses by indirect aerosol contact. When embryo transfer is used for breeding, it is highly recommended that both donor and recipient mares are vaccinated against EVA if the former are to be bred with EAV-infective semen.[56]
3. Mares to be bred to EAV carrier stallions should be tested for neutralizing antibodies at least 30 days prior to breeding.
4. Neutralizing antibody titer of ≥ 1:64 is regarded as protective against EAV; therefore, these mares can be bred to an EAV shedding carrier stallion (natural breeding) or insemination with EAV infective semen (artificial breeding) without being vaccinated.
5. Mares that are seronegative to EAV should be vaccinated with MLV vaccine and the vaccination recorded. Vaccinated mares should be isolated for 21 days and should not be bred during this period. After 21 days, breed the shedding stallion (natural breeding) or inseminate with EAV infective semen (artificial breeding).
6. Mares should be kept isolated from other nonvaccinated or seronegative horses for 3 weeks (21 days) after being bred to a shedding stallion or after insemination with infective semen.
7. Management of mares after annual booster vaccination: Annual booster vaccination 21 days prior to breeding is required and no isolation necessary following booster vaccination. There is no need for isolation of mares after breeding to a carrier stallion for the second time.

General guidelines for preventing stallions from becoming carriers of EAV

1. Standard biosecurity measures for the prevention of infectious diseases should be implemented on breeding farms e.g. horses that travel for competitions or comingle with horses coming from outside the farm should be vaccinated.
2. Stallions that are shuttled between Northern and Southern hemispheres should be tested for EAV (neutralizing antibodies and carrier state) by an accredited laboratory.
3. Prepubertal colts should be genetically tested for presence of the CXCL16 genotype (CXCL16SS or CXCL16SR) using an allelic discrimination real time PCR. Colts that carry the susceptible genotype (CXCL16S) should be vaccinated after 6 months of age. If no genetic testing is to be performed, all colts should be vaccinated between 6 and 12 months of age.
4. More widespread screening of stallion populations for EAV, as well as harmonization of various diagnostic tests (e.g. virus isolation, real time PCR, VNT and ELISA) as well as utilization of molecular approaches as prescribed tests for international trade by the OIE should be promoted and would be highly beneficial to satisfy current demands of the equine industry.

Conclusion

Equine arteritis virus continues to be an important infectious disease of horses; its occurrence can be associated with substantial economic loss to the equine industry due to abortions, neonatal mortality and establishment of the carrier state. There is sufficient evidence that increase in the incidence of EVA in past years is associated with movement of horses for breeding purposes and commercialization of semen. EVA prevention and control strategies are primarily focused on identification of carrier stallions; therefore, substantail efforts are being undertaken in this regard, as well as in trying to understand mechanisms of pathogenesis of EAV in the stallion reproductive tract. It is anticipated that these will identify host factors associated with persistent infection and may, in the future, enable strategies to induce viral clearance in carrier stallions other than surgical castration. Future research focused on improvement of diagnostic assays and vaccines is also of high priority. Implementation of strict EVA surveillance programs capable of identifying carrier stallions may lead to EAV eradication. However, considering large value of international trade of horses, semen, and embryos, enactment of EVA eradication programs will require joint international efforts towards implementation of a universal code of practice.

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Conflict of interest

The authors declare no conflict of interest.

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