Theriogenology 82 (2014) 1068–1079

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Semen quality of stallions challenged with the Kentucky 84 strain of equine arteritis virus

Juliana R. Campos [a], Patrick Breheny [b,1], Reno R. Araujo [c], Mats H.T. Troedsson [a], Edward L. Squires [a], Peter J. Timoney [a], Udeni B.R. Balasuriya [a,*]

[a] Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, Lexington, Kentucky, USA
[b] Department of Biostatistics, University of Kentucky, Lexington, Kentucky, USA
[c] University of Sao Paulo, Laboratory of Molecular Morphophysiology and Development, FZEA-USP, Pirassununga, Sao Paulo, Brazil

ARTICLE INFO

Article history:
Received 8 January 2014
Received in revised form 11 June 2014
Accepted 5 July 2014

Keywords:
Equine arteritis virus
EAV
Equine viral arteritis
EVA
Semen quality
Semen evaluation

ABSTRACT

Equine arteritis virus (EAV) is the causal agent of equine viral arteritis (EVA), a respiratory and reproductive disease of equids. Some strains of EAV can cause fever, leukopenia, and dependent edema of the limbs, scrotum, and preputium in the acutely infected stallion. We hypothesized that fever and scrotal edema observed during the acute phase of the infection, but not the presence of EAV, have an adverse effect on semen quality. A group of seven stallions were intranasally inoculated with the Kentucky 84 (KY84) strain of EAV. Stallions were monitored for clinical signs of EVA until 42 days postinoculation (dpi). Semen was collected every other day for the first 15 days and 2 times a week up to 79 dpi. Additional samples were collected at 147, 149, and 151 dpi. Semen from each stallion was evaluated on the basis of motion characteristics, total number of spermatozoa, membrane integrity, and morphology. Virus infectivity titers were determined in RK-13 cells. Significant decreases in sperm quality were observed between 9 and 76 dpi. LOWESS (locally weighted scatterplot smoothing) curves for each horse were fit and integrated to quantify spermatozoa exposure to fever, virus, and edema over a period of 67 days before each ejaculation. Linear mixed models were then fit to isolate the effects of each factor on semen quality. Scrotal edema and fever were found to exert independent effects on all the semen quality parameters ($P \leq 0.002$), whereas virus seems to exert little to no direct effect, as virus titers remained high long after semen quality returned to baseline.

1. Introduction

Equine arteritis virus (EAV) is the causal agent of equine viral arteritis (EVA), a respiratory and reproductive disease of equids. First isolated from a lung of an aborted fetus in 1953 [1,2], EAV is transmitted mainly by respiratory [2,3] and venereal routes [4]. Although most EAV strains cause subclinical infection after infection, some can cause moderate to severe clinical signs [5–7]. The clinical signs of EVA are characterized by fever, nasal and ocular discharge, conjunctivitis, depended edema, leukopenia, and abortion in pregnant mares [2,3]. The 1984 EVA outbreak in Kentucky's Thoroughbred breeding population generated widespread interest and major concern to the equine industry [3]. Epidemiologic data collected during that outbreak strongly confirmed establishment of long-term persistence of EAV in stallions and the

* Corresponding author. Tel.: +1 859 218 1124; fax: +1 859 257 8542.
E-mail address: ubalasuriya@uky.edu (U.B.R. Balasuriya).
[1] Present address: Department of Biostatistics, College of Public Health, 145 North Riverside Drive, University of Iowa, Iowa City, IA 52242, USA.

0093-691X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2014.07.004

importance of the carrier stallion in the dissemination and perpetuation of the virus. After EAV infection, a variable proportion of stallions (30%–70%) can become carriers and continuously shed virus in their semen for varying periods [4,8]. The virus persists in the male reproductive tract, specifically in the accessory glands with highest infectivity titers found in the ampulla of the vas deferens [9], despite the presence of high titers of neutralizing antibodies to EAV in serum [4,8,9]. Isolation of virus from the pre-ejaculatory fluid of semen from persistently infected stallions was not successful, whereas it could be recovered from the sperm-rich fraction of the ejaculate [8]. The precise mechanism of EAV persistence in the reproductive tract of stallions is unclear; however, it has been shown that establishment and maintenance of infection are testosterone dependent [10,11]. Persistently infected stallions play a major role in the transmission of EAV to mares either by natural breeding or artificial insemination with fresh-cooled or frozen semen [4,8,12]. Recently, it has been reported under experimental conditions that EAV can be transmitted to naive recipient mares *via* embryo transfer from a donor mare inseminated with EAV-infective semen [13].

Several viruses have been detected in the semen of a number of animal species, some of which can have direct or indirect detrimental consequences on the male reproductive tract and on semen [14]. Semen quality of stallions experimentally infected with the Kentucky 84 (KY84) strain of EAV was decreased for a period of time after inoculation with the virus, but it was not confirmed that the negative effect on semen parameters was because of the direct effect of the presence of virus in semen [15]. Thus, the objectives of this study were to determine (1) the effect on various semen parameters of stallions experimentally infected with the KY84 strain of EAV using computer-assisted sperm analysis (CASA) and differential interference contrast (DIC) microscopy and (2) if possible changes observed in semen quality result from direct effects of the presence of virus in the semen or from the indirect effects of fever and scrotal edema during the acute phase of infection in the stallion.

2. Materials and methods

2.1. Cells and viruses

The high passage rabbit kidney cell line (RK-13 KY; passage level 399–409) was maintained in Eagle's minimum essential medium (EMEM; Mediatech, Inc., Manassas, VA, USA) supplemented with 10% ferritin-supplemented bovine calf serum (Hyclone Laboratories, Logan, UT, USA), 100 U/mL penicillin and streptomycin (Mediatech, Inc.), and 1 μg/mL amphotericin B (Sigma–Aldrich, St. Louis, MO, USA). The virulent KY84 strain of EAV was used as the challenge virus [6,16]. The KY84 strain of EAV has been shown to establish persistent infection in the reproductive tract of stallions and to cause moderate to severe clinical signs of EVA infection in horses [9]. The modified live virus vaccine strain of EAV (ARVAC; Zoetis Animal Health Inc., Kalamazoo, MI, USA) was used as challenge virus in the microneutralization assay.

2.2. Stallions

Seven sexually mature (4–16-year old) stallions of mixed breed were included in the study (stallions IDs: L136–L142). Horses were obtained from a local commercial vendor and acclimated to their new environment for approximately 2 months before the study commenced. During this period, animals were accommodated in individual paddocks and trained to mount a mare or a phantom for collection of semen into an artificial vagina. All stallions exhibited good libido and normal sexual behavior. All were confirmed seronegative (titer $<$1:4) for EAV neutralizing antibodies several times before intranasal inoculation with the KY84 strain of EAV using a previously described protocol [17]. The animals were housed in individual stalls in an isolation facility for the duration of the study at the University of Kentucky Maine Chance Farm, Lexington, KY, USA. The study was carried out in accordance with an Institutional Animal Care and Use Committee–approved protocol at the University of Kentucky, Lexington, KY, USA (protocol number 2011-0888).

2.3. Experimental inoculation of stallions and clinical examination

Stallions were inoculated intranasally with 3.75×10^5 plaque-forming units (PFU) of the KY84 strain of EAV in 5.0 mL of EMEM using a fenestrated catheter passed *via* the posterior nares into the nasopharynx as previously described [5]. All stallions were examined and clinical parameters were recorded by the same veterinarian. Scrotal edema was classified on a scale from 0 to 5 and recorded as absent (0), mild (1–2), moderate (3), and severe (4–5). Preinoculation (7, 5, and 2 days before experimental challenge) clinical examinations were performed once daily to determine baseline values for body temperature and also to certify that the parameters were within normal limits before experimental challenge of the stallions. Specifically, fever and scrotal edema were monitored twice daily (every 12 hours) for the first 15 days after infection, and the highest value of the day was recorded. Clinical signs continued to be monitored once per week during the following 4 weeks of the experiment (at 21, 28, 35, and 42 days postinoculation [dpi]). Blood samples were collected at 0, 2, 4, 6, 8, 10, 12, 14, 21, 28, 35, and 42 dpi to determine individual serum neutralizing antibody responses to EAV. The neutralizing antibody titers were determined as described by Senne et al. [17].

2.4. Semen collection

Two ovariectomized mares previously vaccinated with the commercial modified live virus vaccine against EVA (ARVAC; Zoetis Animal Health, Inc.) (respective antibody titers 1:256 and 1:512) were used to "tease" the stallions. Each stallion was allowed to mount either one of the mares or a breeding phantom to enable semen collection in an artificial vagina (Botucatu model; Botupharma, Botucatu, SP, Brazil). The artificial vagina was disinfected with a disinfectant (Roccal-D Plus; Pfizer Inc., New York, NY, USA) between collections. A disposable liner lubricated with