EVALUATION OF THE SAFETY OF VACCINATING MARES AGAINST EQUINE VIRAL ARTERITIS DURING MID OR LATE GESTATION OR DURING THE IMMEDIATE POSTPARTUM PERIOD AND INFECTION OF EMBRYOS FOLLOWING INSEMINATION OF DONOR MARES WITH EQUINE ARTERITIS INFECTIVE SEMEN

By

CHARLES C. BROADDUS

Bachelor of Arts in Economics
University of Virginia
Charlottesville, Virginia
1996

Doctor of Veterinary Medicine
Auburn University
Auburn, Alabama
2003

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2011

EVALUATION OF THE SAFETY OF VACCINATING MARES AGAINST EQUINE VIRAL ARTERITIS DURING MID OR LATE GESTATION OR DURING THE IMMEDIATE POSTPARTUM PERIOD AND INFECTION OF EMBRYOS FOLLOWING INSEMINATION OF DONOR MARES WITH EQUINE ARTERITIS INFECTIVE SEMEN

Dissertation Approved:

G. Reed Holyoak

Dissertation Adviser

Anthony Confer

Sanjay Kapil

Steven Cooper

Outside Committee Member

Mark E. Payton

Dean of the Graduate College

ACKNOWLEDGMENTS

The vaccination study was supported by a grant from Fort Dodge Animal Health. Co-authors and contributors to the published paper include Udeni B. R. Balasuriya, BVSc, PhD; Jena L. R. White, Peter J. Timoney, MVB, PhD; Rebecca A. Funk, DVM, MS; and G. Reed Holyoak, DVM, PhD, DACT. Mark Payton, PhD provided assistance with the statistical analysis.

The embryo transfer study was supported by a grant from the American Quarter Horse Foundation. This work was also supported by the Frederick Van Lennep Chair endowment fund at the Maxwell H. Gluck Equine Research Center, and intramural research funding from the Gluck Equine Research Center and the Kentucky Agricultural Experiment Station, College of Agriculture, University of Kentucky. Co-authors and contributors to the published paper include Udeni B. R. Balasuriya, BVSc, PhD; Peter J. Timoney, MVB, PhD; Jena L. R. White; Chelsea Makloski, DVM, MS, DACT; Kelly Torrisi, DVM; Mark Payton, PhD; and G. Reed Holyoak, DVM, PhD, DACT.

Oklahoma State University veterinary students too numerous to mention helped in animal management, sample collection, and sample processing, and these studies could not have been completed without their help.

TABLE OF CONTENTS

Chapter Page

PART 1-VACCINATION STUDY: Evaluation of the safety of vaccinating mares against equine viral arteritis during mid or late gestation or during the immediate postpartum period

I. VACCINATION STUDY ABSTRACT 1

II. VACCINATION STUDY INTRODUCTION.....3

III. VACCINATION STUDY MATERIALS AND METHODS.....6

IV. VACCINATION STUDY RESULTS.....13

V. VACCINATION STUDY DISCUSSION.....21

VI. VACCINATION STUDY REFERENCES 30

VII.VACCINATION STUDY APPENDICES.....37

PART 2-EMBRYO TRANSFER STUDY: Infection of embryos following insemination of donor mares with equine arteritis virus infective semen

VIII. EMBRYO TRANSFER STUDY ABSTRACT.....48

IX. EMBRYO TRANSFER STUDY INTRODUCTION 50

X. EMBRYO TRANSFER STUDY MATERIALS AND METHODS.....54

XI. EMBRYO TRANSFER STUDY RESULTS 64

XII. EMBRYO TRANSFER STUDY DISCUSSION.....69

XIII.VACCINATION STUDY REFERENCES 76

XIV.VACCINATION STUDY APPENDICES 84

LIST OF TABLES

Table Page

1 (Vaccination study) 39
2 (Vaccination study) 41
3 (Vaccination study) 43
4 (Vaccination study) 45

1 (Embryo transfer study) 85
2 (Embryo transfer study) 87
3 (Embryo transfer study) 89

LIST OF FIGURES

Figure Page

1 (Embryo transfer study) 84

CHAPTER I

VACCINATION STUDY ABSTRACT

Objective—To determine whether it is safe to vaccinate pregnant or postpartum mares with a commercial modified-live virus vaccine against equine arteritis virus (EAV).

Design—Randomized controlled study.

Animals—73 mares and their foals.

Procedures—All mares were vaccinated with a commercial modified-live virus vaccine. Mares were vaccinated during mid gestation, during late gestation, 2 or 3 days after parturition, or were not vaccinated. Foaling outcomes were recorded and serum, blood, milk, and nasopharyngeal samples were collected.

Results—All mares vaccinated in mid gestation foaled without any problems; 21 of 22 mares had antibody titers against EAV at time of foaling. Of the 19 mares vaccinated in late gestation, 3 aborted; antibody titers against EAV were detected in 13 of 15 mares from which serum was obtained at the time of foaling. All postparturient vaccinates were seronegative at foaling; all of them seroconverted after vaccination. No adverse effects were detected in any of their foals.

Conclusions and Clinical Relevance—When faced with a substantial risk of natural exposure to EAV, it would appear to be safe to vaccinate healthy pregnant mares up to 3 months before foaling and during the immediate postpartum period. Vaccinating mares during the last 2 months

of gestation was associated with a risk of abortion; this risk must be weighed against the much greater risk of widespread abortions in unprotected populations of pregnant mares naturally infected with EAV.

CHAPTER II

VACCINATION STUDY INTRODUCTION

Equine viral arteritis is an infectious disease of equids caused by EAV. Although most primary cases of EVA are subclinical infections and therefore unnoticed by horse owners, some outbreaks may be associated with the appearance of influenza-like clinical signs, abortions, and interstitial pneumonia in neonatal foals.[1,2] The risk of abortion in pregnant mares and the potential for persistent infection in stallions have important economic ramifications for the horse breeding industry. Equine arteritis virus is an enveloped RNA virus belonging to the family Arteriviridae, which also includes another viral disease of major veterinary concern, PRRS.[3,4] The EAV and PRRS viruses are readily inactivated by common disinfectants and detergents. Equine arteritis virus survives for only 2 or 3 days at 37^{o}C, although it may survive for at least 75 days at 4^{o}C.[5]

Equine viral arteritis is a disease of worldwide concern, with serologic evidence of infection being recorded in North and South America, Europe, Australia, Africa, and Asia.[1,6] However, the seroprevalence of EAV infection varies greatly among countries and among equine populations within a country. The 1998 National Animal Health Monitoring System equine survey[7,8] reported that only 2.0% of unvaccinated horses in the United States were seropositive to EAV, in contrast to horses imported into California (mostly European Warmbloods) that had a seropositive rate of 18.6%. The seroprevalence of EAV infection varies widely among horse breeds in the United States, with approximately 80% of Standardbreds and 5.4% of

Thoroughbreds being seropositive. Most other breeds are believed to have a seroprevalence of < 2%.[1,9–13] Differences in prevalence among breeds may be associated with differences in management practices. The low overall rate of seropositivity in some horse breeds has enhanced the risk of widespread transmission of the EAV in those breeds during outbreaks of EVA. This was exemplified during the extensive outbreak of EVA in Thoroughbreds in Kentucky in 1984 and in the more recent multistate outbreak in Quarter Horses in the United States in 2006 and 2007.

Transmission of EAV among horses is principally via the respiratory or venereal routes.[1,14–18] Outbreaks have frequently resulted from breeding a naïve mare with EAV-infective semen; the virus is subsequently disseminated to other naïve horses on the premises via the respiratory route. Naturally infected stallions may become persistent carriers of EAV. Establishment and maintenance of the carrier state is a testosterone-dependent event, with the virus localized to the accessory sex glands.[18–20] Persistently infected stallions may be short-term (< 3 months), intermediate (3 to 7 months), or long-term (7 months to several years) carriers.[15,19] Carrier stallions can play a major role in widespread dissemination of the infection and are the primary natural reservoir of the virus.[6]

Subsequent to aerosol infection in a horse, EAV spreads to the lungs and bronchial lymph nodes, and then enters the circulation to be disseminated throughout the body within 2 days.[21] Following infection, virus can be isolated from the nasopharynx for 2 to 14 days, from the buffy coat for 2 to 21 days, and from serum or plasma for 7 to 9 days. An inability to isolate virus from serum or plasma after 7 to 9 days is associated with the appearance of antibodies at that time.[17] Equine arteritis virus has not been isolated from an infected horse longer at > 28 days after infection, except in the semen of carrier stallions.[18,22,23]

The hallmark histologic finding associated with EAV infection is arteritis, and the vascular injury likely results from direct virus-mediated injury to the endothelium and muscularis media of affected vessels.[24–26] The resulting vasculitis is characterized by marked fibrinoid necrosis of small muscular arteries, and the increased vascular permeability leads to hemorrhage and edema around these vessels.[27,28]

A cornerstone of current EVA prevention and control programs in the United States involves the targeted use of an MLV vaccine, which has been commercially available since 1985. This vaccine was derived from an experimental vaccine against EVA that was developed many years earlier by serial passage of the experimentally derived highly pathogenic Bucyrus strain of EAV 131 times in primary horse kidney cells followed by 32 times in primary RK cells (ie, HK131-RK32).[29] However, the original MLV vaccine was not fully attenuated in that it induced abortion in 2 late-gestation mares when injected directly into the fetus or the amniotic sac. Subsequently, a vaccine with a higher passage history (131 times in primary horse kidney cells followed by 111 times in primary RK cells [ie, HK131-RK111]) was tested and found to be safe when used in pregnant pony mares.[30] The current commercially available vaccine is derived from the latter of these experimental vaccines; it has a passage history of HK131-RK111-Eq Dermis 24.[31] On the basis of findings in the original experimental vaccine study, the manufacturer recommends that pregnant mares should not be vaccinated until after foaling.[29]

Despite the manufacturer's recommendation to refrain from use of the vaccine in pregnant mares, there have been reports of its use in pregnant mares without ensuing complications. There are also anecdotal reports of a few late-term abortions in mares that had been previously vaccinated with the MLV vaccine, but without any evidence directly linking vaccination with the abortions.[32] The study reported here was conducted in an attempt to establish whether vaccinating mares during mid or late gestation with an MLV vaccine against EVA would result in abortion and to determine the safety of vaccinating mares within a few days after foaling.

CHAPTER III

VACCINATION STUDY MATERIALS AND METHODS

Animals—A total of 73 mares were used in the study. Mares were predominantly Thoroughbreds or American Quarter Horses with a small number of Arabians and 1 Gypsy Cobb mare. Mares ranged in age from 5 to 25 years of age, and they were pregnant or had foaled recently. None of the mares had a history of gestational or peripartum complications. All of them were confirmed negative for serum neutralizing antibodies against EAV at the beginning of the study. Owner consent was obtained for all client-owned animals used in the study. The study was approved by and conducted in accordance with the guidelines of the Oklahoma State University Institutional Animal Care and Use Committee.

Procedures—All mares were maintained at 1 of 2 breeding premises in central Oklahoma for the duration of the study. Mares in group 1 (n = 22 mid-gestation mares) were vaccinated, housed, and foaled at a commercial breeding premises in Guthrie, Oklahoma. Mares in group 2 (n = 19 late-gestation mares) were vaccinated, housed, and foaled at the commercial breeding premises (2) or at the Center for Veterinary Health Sciences Ranch in Stillwater, Oklahoma (17). All of the mares in group 3 (n = 28 postparturient mares) were vaccinated, housed, and foaled at the Center for Veterinary Health Sciences Ranch. The control group consisted of 4 mares (2 were housed at the commercial breeding premises and 2 were housed at the Center for Veterinary Health

Sciences Ranch) that were not vaccinated. Neither premises had a prior history of EAV infection as determined on the basis of routine serosurveillance testing.

Mares in group 1 were vaccinated between 142 and 83 days before foaling, and mares in group 2 were vaccinated between 68 and 2 days before foaling. Mares in group 3 were vaccinated within 3 days after foaling. The mares in group 3 were subdivided into 2 subgroups: 16 mares and their foals were turned out to pasture following vaccination (pasture subgroup), and 12 mares were housed indoors in individual stalls (4.6 X 7.6 m [15 X 25 feet]) with their foals for 10 days following vaccination (which enforced close physical contact [stall subgroup]), after which these mares and foals were also turned out to pasture. Details of individual mares including the day of vaccination relative to the day of foaling are provided in Table 1.

Vaccination of mares—Mares were vaccinated against EVA by IM administration of a single dose of a commercially available MLV vaccine.[a] Mares were vaccinated in accordance with the manufacturer's instructions.

Management and collection of samples from mares and foals—Management and sample collection were based on pregnancy status at the time of vaccination.

GROUPS 1 AND 2 (PREPARTUM VACCINATES)

Following vaccination, each mare was monitored daily to evaluate general health as well as pregnancy status. As the anticipated date of parturition approached, mares were moved into a foaling stall. Each mare was frequently observed in the foaling stall through parturition.

Blood samples (5 mL) were collected via jugular venipuncture from each mare at the time of parturition (0 hours), 12 hours later, and 30 days after parturition. Blood samples (5 mL) were similarly collected from each foal immediately after birth before they nursed the dam (if possible), 12 hours later, and 30 days after parturition. The precolostral blood samples were obtained from the foals via venipuncture of the umbilical vein or jugular vein; blood samples were obtained from the foals at 12 hours and 30 days after parturition via jugular venipuncture. Samples were allowed to clot, and the serum was harvested and stored at –20°C. Milk samples (10 mL) were collected from each mare at time 0, 12 hours later, and 30 days after parturition.

GROUP 3 (POSTPARTUM VACCINATES)

Rectal temperature, pulse rate, and respiratory rate were recorded for each mare and foal at the time of parturition, 12 hours after parturition, 48 hours after parturition, and on days 1, 3, 5, 7, 14, and 28 after vaccination. The type of samples collected and the sample collection schedule was the same for mare-foal pairs of both the stall and pasture subgroups. Blood samples were collected from mares at the time of vaccination and on days 14 and 28 after vaccination for serum harvest and use in a VN test to detect development of antibodies against EAV. Blood samples were also collected from mares into EDTA-containing tubes for separation of peripheral blood mononuclear cells for use in virus isolation; these blood samples were collected on the day of parturition and days 1, 3, 5, 7, 14, and 28 after vaccination. Blood samples for serum harvest were collected by jugular venipuncture from the foals at the time of vaccination and on days 14 and 28 after vaccination for use determining possible exposure to vaccine virus. Nasopharyngeal swab specimens were collected from mares on the day of vaccination and days 1, 3, 5, 7, 9, 11, 14, and

21 after vaccination by use of sterile gauze swabs and placed in viral transport medium, as described elsewhere.[33]

CONTROL MARES

The control mares were housed and handled similarly to the mares in groups 1 and 2, except that they were not vaccinated. Blood samples were collected from the mares and their foals at the same times as for the horses of groups 1 and 2.

VN titers—Serum and milk samples were subjected to VN tests to detect antibodies against EAV. Serum VN antibody titers were determined in accordance with the method described in another study.[34]

Virus isolation—Virus isolation from buffy coat and nasopharyngeal swab specimens was performed in RK-13 cells in according with a recommended protocol.[35] Briefly, blood samples collected into EDTA anticoagulant were centrifuged at 500 X *g* for 10 minutes. Plasma and buffy coat cells were aspirated and placed in 15-mL conical centrifuge tubes. Buffy coat cells were pelleted by centrifugation at 1,500 X *g* for 10 minutes at 4°C. The plasma was aspirated, and the WBC pellet was resuspended in 5 mL of EMEM. The cell suspensions were frozen at –80°C until virus isolation was performed. Nasopharyngeal swab specimens in viral transport medium were vortexed and then filtered through a 0.45-µm syringe filter. Filtrates were frozen at –80°C until virus isolation could be performed. Virus isolation from peripheral blood mononuclear cells, filtrates of nasopharyngeal swab specimens, and clarified 10% fetal tissue suspensions was attempted in both high- and low-passage RK-13 cell lines in accordance with a recommended

protocol.[35] Briefly, serial 10-fold dilutions (10^{-1} to 10^{-3}) of each sample were made in supplemented EMEM, and 1 mL of each dilution was inoculated into 2 X 25-cm^2 flasks containing confluent monolayers of RK-13 cells. Flasks were incubated at 37°C for 1 hour and then were overlaid with EMEM supplemented with 0.75% carboxymethyl cellulose. Flasks were incubated at 37°C and evaluated for the appearance of viral cytopathic effect on days 3 and 4 after inoculation. If there were no detectable cytopathic effects, a second passage was performed on day 4. The RK-13 cell monolayers were fixed and stained with a 1% crystal violet solution containing 1% formaldehyde on day 5 after inoculation in the case of the first passage in cell culture and on day 4 after inoculation in the case of the second passage in cell culture. Tissue culture fluid was harvested and stored at –80°C until used for viral RNA extraction.

Viral RNA extraction and real-time RT-PCR—Viral RNA was directly isolated from samples of tissue culture fluid by use of a commercial kit.[b] Briefly, tissue culture fluid samples were placed in microcentrifuge tubes and centrifuged at 13,800 X *g* for 2 minutes; then 140 µL of supernatant was removed and used for nucleic acid extraction in accordance with the manufacturer's instructions. Viral nucleic acid was eluted in 60 µL of nuclease-free water and stored at –80°C.

A 1-tube real-time RT-PCR assay[c] was performed with RT-PCR master mix[d] in a real-time PCR system.[e] The primers and probes used were identical to those previously described.[36,37] Every sample was tested in duplicate. Briefly, 25 µL of RT-PCR mixture for each reaction contained 12.5 µL of 2X master mix without uracil-N-glycosylase, 40X reverse transriptase[f] and RNase inhibitor mix,[g] 900nM forward and reverse primers (0.45 µL), 250nM probe (0.625 µL), 5.35 µL of nuclease-free water, and 5 µL of test sample RNA. Thermocycling conditions were used for

the standard mode as per manufacturer's recommendation (30 minutes at 48°C, 10 minutes at 95°C, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute). Each RT-PCR assay included a negative control sample without RNA (contained the reaction mix with 5 µL of water [no template]) and positive control samples.

Postmortem examination—Gross and histologic examination of any aborted fetuses was performed at an animal disease diagnostic laboratory.[h] Tissues were fixed in neutral-buffered 10% formalin and embedded in paraffin, and sections were stained with H&E. Specimens of fetal membranes and fetal lung, heart, and liver were collected for attempted virus isolation and viral nucleic acid extraction and stored at –80°C. Tissues were tested for evidence of infection with EHV-1, EHV-4, and *Leptospira* spp. Fluorescent antibody staining and examination of fetal tissues for EHV-1 and EHV-4 was performed on approximately 10-µm-thick tissue sections which were stained with caprine origin anti–EHV-1 and anti–EHV-4 polyclonal antiserum conjugated to fluorescein isothiocyanate[i] as described in standard protocols and procedures. Sections were counter-stained with Evans blue dye, which caused cells with positive results to stain fluorescent green and cells with negative results to stain brick red. Aerobic bacteriologic culture of fetal stomach contents, fetal lungs, or fetal membranes was performed on blood agar, MacConkey agar, and phenol-ethyl-alcohol agar.[38] Heart blood samples were also obtained from each fetus for serologic examination.

Statistical analysis—All statistical analyses were conducted with a commercially available statistical program.[j] Repeated-measures ANOVA[k] was used to assess the effects of parturition group and time. Simple effects of parturition group (control vs group 1 vs group 2) for a given time were calculated, and comparisons of the groups were performed with pair-wise *t* tests.[l,m]

Titer values were transformed with a logarithmic (base 2) function prior to calculation of the ANOVA. Means and SEs of the raw titer values were reported. Significance was set at values of $P < 0.05$ for all comparisons.

CHAPTER IV

VACCINATION STUDY RESULTS

Foaling outcomes—All 22 mares in group 1 foaled without difficulty or assistance, and each mare gave birth to a live foal. Serum IgG testing was performed on samples obtained from 20 foals at 12 hours after birth; all foals had IgG concentrations > 800 mg/dL. Although IgG concentrations were not determined for 2 foals, these foals were healthy at birth and remained healthy throughout the 30-day study period. Two foals were healthy at birth and had IgG concentrations > 800 mg/dL; however, both foals died before 30 days after birth, and regrettably neither was submitted for necropsy.

Three mares in group 2 aborted. One mare aborted 9 days after vaccination, a second mare aborted 11 days after vaccination, and a third mare aborted 38 days after vaccination. The remaining 16 mares in group 2 foaled without difficulty or assistance, and all foals, except for 1, had a serum IgG concentration > 800 mg/dL in samples obtained at 12 hours after birth. The 1 foal had a serum IgG concentration < 400 mg/dL at 12 hours after birth and was administered 1 L of plasma; this foal thrived throughout the 30-day study period. Two foals were euthanized at approximately 2 weeks after birth (one of these foals had evidence of severe abdominal pain, and an area of devitalized intestine was found during necropsy; the other foal was weak at birth,

dehydrated, and unable to stand, but no significant findings were detected in this foal during necropsy).

All 28 mares in group 3 gave birth to healthy foals without difficulty or assistance. Twenty-five of these foals had an IgG concentration > 800 mg/dL in samples obtained 12 hours after birth. Each of the 3 other foals required a transfusion of 1 L of plasma to increase the serum IgG concentration to > 800 mg/dL. All foals in this group remained healthy throughout the 30-day study period.

All mares in the control group gave birth to healthy foals without difficulty or assistance. All foals had an IgG concentration > 800 mg/dL in samples obtained at 12 hours after birth.

Clinical findings—The mares and foals in all groups were monitored after parturition. Mares and foals were generally bright, alert, and active throughout the 28-day period after vaccination, except for the 2 aforementioned foals in group 2 that were euthanized. Mares and foals of group 3 were closely monitored following vaccination, with frequent assessments of rectal temperature, pulse, respiration, and overall demeanor. No significant difference from the reference range was detected for mean rectal temperature, pulse rate, or respiratory rate recorded at birth, 12 hours after parturition, 48 hours after parturition, and 1, 3, 5, 7, 14, and 28 days after vaccination.

Results of VN tests—All mares were seronegative for EAV at the beginning of the study. Serologic data were available on almost all mares and foals over the study period. All of the

mares in group 1, except for 1 mare, were seropositive (titer ≥ 1:4) to EAV at foaling; titers for the seropositive mares ranged from 1:16 to ≥ 1:512 (Table 2). That 1 mare was seronegative at the time of foaling but seropositive (titer ≥ 1:512) 12 hours later. Samples were obtained at 30-days after parturition from 18 mares, all of which were still seropositive. The mean VN titer for the mares differed significantly between the control group and group 1 as well as between the control group and group 2 at 0 hours, 12 hours, and 30 days.

All 22 foals in group 1 were seronegative (titer < 1:4) at birth, but all were seropositive 12 hours later. All of the foals available for sample collection at 30 days after parturition were still seropositive. The mean VN titer for foals differed significantly between the control group and group 1 as well as between the control group and group 2 at 12 hours, whereas there was no significant difference between the control groups or group 1 and 2 at 0 hours. At 30 days after parturition, the mean VN titer for foals of group 1 was significantly different from that of the control group, whereas the mean VN titer did not differ significantly between the control group and group 2.

Similarly, data for milk samples were available for most mares. All mares in group 1 had antibodies against EAV in the colostrum at the time of foaling, with 18 of 22 mares having a titer ≥ 1:512. Milk samples collected 12 hours after foaling had antibodies against EAV for all of the mares, although there was a fourfold or greater decrease in antibody titer in many samples. Samples collected at 30 days after parturition did not have antibodies against EAV in 7 of 22 mares and had titers between 1:4 and 1:32 in 8 of 22 mares; the remaining 7 mares were not available for collection of milk samples. Mean VN titer in milk was not significantly different among all groups at 0 hours and was significantly different among all groups at 12 hours; at 30

days, the mean VN titer in milk for group 1 was significantly different from that of the control group, whereas the mean VN titer did not differ significantly between the control group and group 2.

Fifteen of 19 mares in group 2 were available for collection of milk samples. In those 15 mares, 13 were seropositive and 2 were seronegative at the time of foaling (Table 3). Fifteen foals from which samples were collected at the time of birth were seronegative. However, serum obtained before colostral suckling from 3 foals were positive for antibodies against EAV. The 4 control mares had negative results for antibodies against EAV in serum and milk samples obtained throughout the study.

Milk was obtained from 15 mares in group 2 at the time of foaling, with 11 milk samples having positive results and 4 samples having negative results for antibodies against EAV. The milk samples with negative results were collected from 4 mares that were vaccinated 2, 4, 4, and 19 days before foaling, respectively. The milk samples obtained 12 hours after parturition still had negative results for 3 of these 4 mares (mares vaccinated on 2, 4, and 4 days before foaling, respectively; samples were not obtained from the other mare). All group 2 milk samples collected at 30 days after parturition had negative results or had low titers ($\leq$ 1:16), except for 1.

The 28 mares and 27 foals comprising group 3 from which samples were available were negative for antibodies against EAV at the time of foaling. All of the mares responded following vaccination with detectable VN titers at days 14 and 28 after vaccination. All of the foals, except for 2, were seronegative at days 14 and 28 after parturition. Both seropositive foals were in the

pasture subgroup. The remainder of the foals in the pasture subgroup and the stall subgroup of foals were negative for antibodies at birth and 14 and 28 days after foaling (Table 4).

Virus isolation and PCR results—Results of virus isolation for nasopharyngeal swab specimens and buffy coat specimens obtained from the mares in group 3 were determined. Because there were a number of samples that were not collected, the data set was not complete for all mares. All samples with positive results for virus isolation were confirmed by use of real-time RT-PCR assay.

Twelve of 28 mares in group 3 had positive results for virus isolation of buffy coat specimens or nasopharyngeal swab specimens. Equine arteritis virus was isolated from the buffy coat of 9 mares in the period immediately following vaccination, 8 of which were in the pasture subgroup (the remaining mare with the positive results was in the stall subgroup). Virus was isolated from the nasopharynx of 5 mares, including 2 from the pasture subgroup and 3 from the stall subgroup. Two of the 12 mares with positive results for EAV had virus isolated from both the buffy coat specimens and nasopharyngeal swab specimens. Most of the virus isolations were from specimens collected within the first few days after vaccination.

Two foals seroconverted to EAV after vaccination of their respective dams; both mares had positive results for virus in the buffy coat specimens. However, EAV was not detected in nasopharyngeal swab specimens collected from either of these mares. Both of the mares were in the pasture subgroup and had positive results for virus isolation only 1 time (day 1 after vaccination).

Postmortem examination—Fetuses aborted by 3 mares in group 2 were submitted for a complete postmortem evaluation. The fetus from 1 mare was close to full term (approx 300-330 days of gestation). Gross examination of that fetus revealed multiple nonspecific lesions, including mild, multifocal, hemorrhage in the thymus and heart; mild mesenteric lymphadenopathy; and mild, acute, multifocal hemorrhage of the allantoic sac and chorioallantois in conjunction with chorioallantoic edema. The fetal membranes weighed 5.1 kg (11.2 lb). Histologically, lesions consistent with arteritis were observed as well as stromal neutrophilic infiltration and fibrinous inflammation of the chorioallantois and amniotic sac; arteritis with neutrophilic and fibrinous perifunisitis; mild hemorrhages of the kidneys, thymus, and adrenal glands; and hyperplasia of the lymphoid follicles of the spleen and mesenteric lymph nodes. The lungs were atelectic and not aerated, and meconium was found in the lumen of the alveoli and airways. Lung tissue from the foal had positive results for EAV by use of the PCR assay. The fetus was seronegative for antibodies against EAV, EHV-1, and *Leptospira* spp. Fluorescent antibody staining of sections of the liver yielded negative results for EHV-1 and EHV-4 antigens. Aerobic bacteriologic culture of fetal stomach contents also yielded negative results.

Necropsy of the aborted fetus and fetal membranes from a second mare that aborted revealed heavy (8.5 kg [18.7 lb]), edematous fetal membranes, a nearly full-term fetus, adrenal gland hemorrhage, and diffusely atelectic lungs. Histologic examination revealed chronic inflammatory lesions around blood vessels in the heart and in the stroma of the thickened areas of the fetal membranes. The fetus and fetal membranes had positive results for EAV by use of PCR assay. The fetus was seronegative for EAV, EHV-1, and several serovars of *Leptospira interrogans* (Canicola, Grippotyphosa, Icterohemorrhagica, Pomona, Bratislava, and Hardjo). Fluorescent

antibody staining of sections of the lungs and liver yielded negative results for EHV-1 and EHV-4 antigens. Aerobic bacteriologic culture of fetal stomach contents also yielded negative results.

Fetal membranes were the only tissue available for evaluation following the abortion of the third mare. The fetal membranes were heavy (8.6 kg [18.9 lb]), with marked thickening and edema of the entire allantochorion in the region of the pregnant horn and adjacent uterine body. Histologic examination confirmed stromal edema and congestion in the thickened areas of the fetal membranes. The fetal membranes had positive results for EAV by use of PCR assay. Aerobic bacteriologic culture of the fetal membranes yielded a moderate growth of *Klebsiella pneumonia*, α-*Streptococcus* spp, *Escherichia hermanii*, *Raltonia picketii*, *Acinetobacter wolfii*, and *Enterobacter gergoviae*; all were considered bacterial contaminants. Fungal culture of the fetal membranes yielded a growth of a small number of colonies of *Penicillium* spp. The fetal membranes had negative results for EHV-1 and EHV-4 by use of PCR assay. Leptospiral evaluation revealed titers against several *L interrogans* serovars (Grippotyphosa, 1:1,600; Icterohemorrhagica, 1:400; and Bratislava, 1:400); results were negative for several other *L interrogans* serovars (Canicola, Hardjo, and Pomona). The mare had a titer of 1:64 to EAV.

CHAPTER V

VACCINATION STUDY DISCUSSION

The economic impact of an outbreak of EVA can be substantial, as was the case following outbreaks on Thoroughbred breeding farms in Kentucky in 1984 and the multistate outbreak in Quarter Horses in 2006 and 2007. Breeding farms can be especially hit hard economically. The question frequently arises as to the best time to vaccinate broodmares against EVA to minimize or prevent the risk of widespread abortions. The primary objective of the study reported here was to provide an answer to this question.

To address this objective, mares were vaccinated during pregnancy (mid or late gestation) or immediately after parturition, which are often the time points when breeders want to vaccinate mares to protect them against the risk of natural infection with EAV. The only commercially available vaccine against EVA[a] in the United States was used; this vaccine is not recommended for use in pregnant mares. The vaccine insert indicates that pregnant mares should not be vaccinated until after foaling. Furthermore, pregnant mares should not be vaccinated during the last 2 months of gestation because a few instances of fetal invasion by vaccine virus have been detected after vaccination during this period. It is preferable to vaccinate mares when they are not pregnant; however, when pregnant mares are threatened by a high risk of natural exposure, vaccination may be undertaken with considerably less risk of abortion attributable to vaccination than is inherent for natural infection. Owners should be advised of the possibility of fetal

infection before vaccinating pregnant mares. The results of the present study support this recommendation.

The findings of the study reported here are consistent with those in another study[39] in which investigators found that the vaccine virus was associated with an isolated abortion in a mare recently vaccinated with the commercially available vaccine.[a] In that study,[39] there was homology between the nucleotide sequence of open reading frame 5 of the isolated virus and that of the vaccine virus. The mare aborted during the midst of an extensive outbreak of EVA on a Thoroughbred breeding farm in Illinois in 1994. In contrast to this finding, results of a subsequent study[31] were reported for the outcome after vaccinating 5 pregnant mares with the commercially available vaccine[a] between 51 and 85 days prior to foaling. Vaccination against EVA with the MLV vaccine did not result in abortion in any of the vaccinated mares. As emphasized by the authors of that study,[31] this finding must be interpreted with caution in light of the small number of horses involved. It is worth mentioning that following the 2006 and 2007 multistate outbreak of EVA, there was widespread use of the same MLV vaccine in pregnant mares with no published reports of confirmed abortions associated with vaccination.

Studies in which the vaccine strain of EAV was administered to mares include those conducted by researchers instrumental in the development of the vaccine in the 1960s and 1970s. The original vaccine inoculated directly into a fetus or amniotic sac induced abortion in 2 mares.[29] A subsequent study[30] that involved the IM administration of a vaccine virus with a higher passage history did not result in any abortions in 18 mares vaccinated at 30 days of gestation to nearly the end of a full-term gestation. The present study is the largest study conducted to assess the effects of vaccination with the MLV vaccine against EVA in peripartum mares. Furthermore, the passage history of the vaccine virus was much higher than that of the modified-live experimental vaccines used in earlier studies.[29,30]

Of the 22 mares in group 1 (mares vaccinated in mid gestation), all gave birth to live healthy foals. Twenty foals remained healthy throughout the study. The 2 foals that died before 30 days after birth appeared to have died of apparently unrelated illness, notwithstanding the fact that a postmortem examination was not performed on either foal. Furthermore, there was no evidence of congenital EAV infection in that every foal was seronegative for antibodies against EAV at birth. All of the foals were seropositive at 12 hours after birth, which confirmed effective absorption of colostral-derived antibodies from their dams. One mare in this group was seronegative for EAV at the time of foaling, but both that mare and her foal had a titer ≥ 1:512 12 hours later, and the milk obtained from that mare at the time of foaling had positive results for antibodies (titer, ≥1:512). This discrepant result would strongly suggest that the sample identified as that of the mare at 0 hours was probably mislabeled and that it actually represented the sample obtained from the foal at 12 hours after birth. It is important that all foals were seronegative for EAV at birth and were subsequently seropositive at 12 hours and 30 days after parturition, which indicated that vaccination of mid-gestation mares does not result in exposure of the unborn fetus to vaccine virus and that colostrum does provide passive protection against EVA in the case of newborn foals.[40] The findings indicated that vaccination with the commercially available vaccine[a] between 83 and 142 days before the anticipated date of foaling does not compromise maintenance of pregnancy nor result in congenital infection of a the fetus.

It is important to consider the neutralizing antibody concentration that is sufficient to afford protection against wild-type EAV infection. In 1 study,[41] titers as low as 1:8 were fully protective, and titers of 1:4 were moderately protective, when horses were challenge exposed to a highly virulent Bucyrus strain of EAV. All mares in the study reported here had fully protective titers of 1:8 or higher at 30 days after parturition.

In contrast to the fact that none of the vaccinated mares in group 1 aborted, 3 of 19 mares in group 2 (all mares were vaccinated during late gestation) aborted. The mares in group 2

aborted 9, 11, and 38 days after vaccination, respectively. Tissues from all 3 aborted fetuses had positive results for EAV by use of virus isolation and PCR assay. In light of the fact that all foals in group 1 were seronegative at birth, it must be assumed that these foals did not have EAV infection in utero. In contrast, detection of EAV in tissues of the 3 aborted foals in group 2 was important. Considered in conjunction with other findings, it would appear that EAV was a factor in causing abortion in each of these 3 mares. Unfortunately, the aborted fetus from 1 mare was not recovered (the mare aborted unexpectedly in a pasture, and the fetus was not found). This mare also had a high titer to *L interrogans* serovar Grippotyphosa, with moderate titers also to *L interrogans* serovars Icterohemorrhagica and Bratislava. Because of the lack of any histologic confirmation of EAV infection and in light of the titers for leptospirosis, the abortion of that mare cannot unequivocally be attributed solely to EAV.

The mare that aborted while in a pasture and whose fetus was not recovered aborted 38 days after vaccination, as opposed to the other 2 mares that aborted 9 and 11 days after vaccination, respectively. The closer temporal association of the abortions with vaccination in those 2 mares in addition to the histologic findings in the aborted fetuses provided a stronger case for involvement of the vaccine strain of EAV in each of these abortions. The lesions of arteritis observed in the chorioallantois and umbilical cord in association with the positive PCR findings and nondetectable or marginal titers for leptospirosis would point to the vaccine strain of EAV as the likely cause of the abortion in the mare that aborted 11 days after vaccination. Similar findings, including chronic inflammation around the blood vessels in the heart and fetal membranes, for the mare that aborted 9 days after vaccination would also support this conclusion. The homology of the nucleotide sequence of open reading frames 2a to 7 of the isolated virus and the corresponding region of the vaccine virus implicated the vaccine strain of EAV as the cause of the abortions. It is worth mentioning that samples of fetal heart blood collected from the aborted fetuses of the mares that aborted 9 and 11 days after vaccination, respectively, were

negative for antibodies against EAV, which suggested that the immune system of these fetuses did not have time to respond to circulating vaccine virus before fetal death occurred.

Further supportive evidence of the possibility of in utero fetal infection with the vaccine virus was the finding of presuckle serum titers against EAV in a limited number of foals in group 2. In contrast to group 1, in which all of the foals were born seronegative to EAV, 3 of 18 foals in group 2 were born seropositive to EAV. The dams of these 3 foals were vaccinated 13, 36, and 68 days before foaling. The titers confirmed that in utero fetal infection with EAV had taken place. It is worth mentioning that all 3 foals were born healthy and remained healthy throughout the duration of the study. Because the fetal membranes from apparently normal births were not routinely collected and subjected to virus isolation, PCR assay, or histologic examination, it is unknown whether there may have been evidence of infection by EAV in the fetal membrane tissues of these 3 foals.

The virus isolated from each animal represented the vaccine virus rather than wild-type virus acquired during the course of the study. Although neither virus isolation nor PCR assay differentiates vaccine virus from wild-type virus, there was no other source of EAV during this study, except for the vaccination. Despite vigilant surveillance, EAV was not detected on any premises in Oklahoma during the time of the study. Neither the control mares nor other nonvaccinated horses on the breeding farms where the study was conducted seroconverted during the study.

The intent of the vaccination protocol used in mares in group 3 was to assess the responses of the mare and foal if the mare was vaccinated with the MLV vaccine within 2 to 3 days after foaling. All of the mares seroconverted as expected, and all of the foals (except for 2) remained seronegative throughout the study. The 2 foals that were seropositive were clinically normal on the basis of results of physical examination, and their heart rate, respiratory rate, and

rectal temperature were not significantly different from those of their cohorts. It was thought that close contact between the mare and foal during the immediate postpartum period might increase the chance of transmission of the vaccine virus from mare to foal. Surprisingly, the 2 foals with positive results for antibodies against EAV were both part of the pasture subset. It could be argued that mares and foals housed in pastures likely have similar patterns of physical contact to those confined in stalls. Twelve of 28 mares in group 3 that had positive results for EAV during virus isolation or PCR assay of either buffy coat specimens or nasopharyngeal swab specimens comprised mares from both the pasture subgroup (n = 9 mares) and the stall subgroup (3). Apparently, only 2 of the 12 virus-positive mares shed a sufficient amount of virus to infect their foals, as attested to by seroconversion of the foals. Neither of these 2 foals had any clinical signs of illness. The titers in the foals were the result of exposure to the vaccine virus by some undefined route, perhaps via the milk. On the basis of findings in the present study, it does not appear to be detrimental to vaccinate mares 2 to 3 days after foaling, although there is a low risk that the foal may become infected with the vaccine virus through contact with its vaccinated dam. It would be prudent to recommend against vaccinating a mare soon after foaling if there was any compromise of the foal's health. If there is a need to vaccinate a mare during the immediate postpartum period, blood should be collected from her foal prior to vaccination of the mare to document seronegative status, and the foal could then be considered potentially vaccinated on that date if detected seropositive to EAV 14 to 28 days after vaccination of the dam.

It was hypothesized that the 16 of 28 mares in group 3 that were not EAV-positive during virus isolation or PCR assay of buffy coat specimens or nasopharyngeal swab specimens had negative results because of enhanced clearance of vaccine virus associated with the peripartum period. The immune system of these mares may have been more aggressive in achieving pathogen clearance during the peripartum period, as suggested by vaccination studies in humans.[42,43] It is interesting that the CDC recommendation for vaccination of a pregnant woman

seronegative for antibodies against tetanus, diphtheria, and pertussis is to vaccinate during the postpartum period with tetanus toxoid, reduced diphtheria toxoid, and acellular pertussis vaccine.[42] Despite the lack of any documented complications associated with prepartum vaccination, the CDC believes it prudent to make this recommendation, presumably for the same reasons the manufacturer of the commercially available vaccine against EAV[a] makes the same recommendation. In studies[44–46] in dairy cows, it has been found that there are dramatic decreases in the percentage of peripheral blood CD4+ T cells as well as increased activity of CD8+ lymphocytes at parturition. Peripheral blood B-cell concentrations are highest immediately before parturition and lowest immediately after parturition.[44] Taken together, these findings indicate that parturition affects immunity. It is hypothesized that in the study reported here, it was the effect of foaling that led to the low rate of virus isolation in the group 3 mares after vaccination.

It is also important to consider the effects of the use of vaccines during pregnancy in other species. Pregnant sows vaccinated with an MLV vaccine for PRRS virus, another member [in addition to EAV] of the family Arteriviridae, had a decreased number of pigs born alive and weaned, compared with results for pregnant sows that were not vaccinated.[47] However, similar to the situation with EAV in naïve pregnant mares, this decrease in productivity is sometimes an acceptable alternative to the effects of disease caused by natural exposure to the PRRS virus. The reproductive performance of sows after vaccination against PRRS depends on the stage of gestation: the largest decreases in pigs born alive and weaned were detected in sows vaccinated during the last 4 weeks of gestation,[48] which again was similar to the most important reproductive dysfunction in the mares vaccinated during late gestation in the present study. In contrast to the results of these studies in pregnant sows vaccinated with a MLV vaccine against PRRS,[47,48] it has been found that pregnant cows vaccinated with certain MLV respiratory vaccines did not have any detrimental effects.[49] In that study,[49] pregnant cows vaccinated throughout gestation with a combination vaccine containing MLV components against bovine herpesvirus-1, bovine viral

diarrhea virus, parainfluenza virus-3, bovine respiratory syncytial virus, and inactivated cultures of *L interrogans* serovars Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, and Pomona had abortion rates similar to those of pregnant cows and heifers administered sterile water diluent during the same stages of gestation. Therefore, effects of vaccination on reproductive performance of a pregnant animal appear to depend on the vaccine.

Serologic results of the mares and foals in group 3 indicated that the mares became seropositive after vaccination, whereas the foals did not. Several reasons may have accounted for this, including the fact that when the mares developed vaccine-induced titers following vaccination 2 days after parturition, the foals were no longer able to absorb antibodies through the gastrointestinal mucosa. Transintestinal permeability for immunoglobulins of calves progresses to complete impermeability between 12 and 24 hours after birth, and foals have been found to have a similar time for gastrointestinal closure.[50,51] Although 12 of 28 mares in group 3 shed vaccine virus, the amount of vaccine virus shed may have been too low to exceed the threshold dose needed to infect and result in seroconversion in the foals, except for 2 of the foals.

Virus neutralization testing of milk samples revealed that the colostrum collected at foaling was antibody-positive for all mares in group 1. In group 2, all mares (except for 4) had positive results for antibodies against EAV in colostrums samples collected at foaling; the 4 mares that had negative results were vaccinated 2, 4, 4, and 19 days before foaling, respectively. The milk obtained 12 hours after foaling from 3 of those mares (vaccinated 2, 4, and 4 days before foaling respectively) still had negative results, whereas the milk of the other mare had a titer of 1:32 (low positive titer), perhaps because the mare was vaccinated only 19 days before foaling. Milk samples from the other 3 mares probably had negative results because a sufficient amount of time had not elapsed between vaccination and sample collection; available serum samples obtained from these 3 mares at 0 and 12 hours also had negative results, whereas the serum samples obtained from these mares at 30 days after parturition had titers of $\geq$ 1:512.

Analysis of the findings in the study reported here confirmed what has been the official recommendation for vaccination of peripartum mares with the MLV vaccine against EVA. Although vaccination of pregnant mares with MLV vaccines should be undertaken with caution, it appears that the risk of adverse consequences is minimal in mares vaccinated up to 3 months before foaling. Vaccination of these mares provides the potential benefit of colostral antibody protection for the foals, which does not appear to be evident in mares vaccinated soon after foaling. It is also apparent that mares may be vaccinated during the last 2 months of gestation and they will not necessarily abort; 16 of 19 mares in group 2 gave birth to healthy foals. If mare populations are under stress or have been exposed to other infections, vaccination against EAV during the last 2 months of gestation may result in abortion. However, for most circumstances, the risk of abortion is less likely. On the basis of these findings, and in the face of a high risk of natural exposure to EAV, the risk of vaccination-related abortion is far outweighed by the substantial risk of EVA-related abortion and the potential of widespread dissemination of the virus. This conclusion should be considered in the event of future EVA outbreaks.

CHAPTER VI

VACCINATION STUDY REFERENCES

1. Timoney PJ, McCollum WH. Equine viral arteritis. *Vet Clin North Am Equine Pract* 1993;9:295–309.

2. McCollum WH, Timoney PJ, Lee J Jr, et al. Features of an outbreak of equine viral arteritis on a breeding farm associated with abortion and fatal interstitial pneumonia in neonatal foals, in *Proceedings*. 8th Int Conf Equine Infect Dis 1998;559–560.

3. Cavanagh D. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. *Arch Virol* 1997;142:629–633.

4. Snijder EJ, Meulenberg JJ. The molecular biology of arteriviruses. *J Gen Virol* 1998;79:961–979.

5. Balasuriya UBR, MacLachlan NJ. Equine viral arteritis. In: Sellon DC, Long M, eds. *Equine infectious diseases*. Philadelphia: Saunders, 2006;153–164.

6. Holyoak GR, Balasuriya UBR, Broaddus CC, et al. Equine viral arteritis: current status and prevention. *Theriogenology* 2008;70:403–414.

7. National Animal Health Monitoring System. *Equine viral arteritis (EVA) and the US horse industry*. Fort Collins, Colo: USDA APHIS, 2000.

8. Hullinger PJ, Gardner IA, Hietala SK, et al. Seroprevalence of antibodies against equine arteritis virus in horses residing in the United States and imported horses. *J Am Vet Med Assoc* 2001;219:946–949.

9. Timoney PJ. Equine viral arteritis: epidemiology and control. *J Equine Vet Sci* 1988;8:54–59.

10. McCollum WH, Bryans JT. Serological identification of infection by equine arteritis virus in horses of several countries, in *Proceedings*. 3rd Int Conf Equine Infect Dis 1973;256–263.

11. McCue PM, Hietala SK, Spensely MS, et al. Prevalence of equine viral arteritis in California horses. *Calif Vet* 1991;45:24–26.

12. McKenzie J. Survey of stallions for equine viral arteritis. *Surveillance* 1996;16;17–18.

13. Moraillon A, Moraillon R. Results of an epidemiological investigation on viral arteritis in France and some other European and African countries. *Ann Rech Vet* 1978;9:43–54.

14. Doll ER, Knappenberger RE, Bryans JT. An outbreak of abortion caused by the equine arteritis virus. *Cornell Vet* 1957;47:69–75.

15. Timoney PJ, McCollum WH, Murphy TW, et al. The carrier state in equine arteritis virus infection in the stallion with specific emphasis on the venereal mode of virus transmission. *J Reprod Fertil Suppl* 1987;35:95–102.

16. Cole JR, Hall RF, Gosser HS, et al. Transmissibility and abortigenic effect of equine viral arteritis in mares. *J Am Vet Med Assoc* 1986;189:769–771.

17. McCollum WH, Prickett ME, Bryans JT: Temporal distribution of equine arteritis virus in respiratory mucosa, tissues and body fluids of horses infected by inhalation. *Res Vet Sci* 1971;12:459–464.

18. Timoney PJ, McCollum WH, Roberts AW, et al. Demonstration of the carrier state in naturally acquired equine arteritis virus infection in the stallion. *Res Vet Sci* 1986;41:279–280.

19. Little TV, Holyoak GR, McCollum WH, et al. Output of equine arteritis virus from persistently infected stallions is testosterone dependent, in *Proceedings*. 6th Int Conf Equine Infect Dis 1992;225–229.

20. Holyoak GR, Little TV, McCollum WH, et al. Relationship between onset of puberty and establishment of persistent infection with equine arteritis virus in the experimentally infected colt. *J Comp Pathol* 1993;109:29–46.

21. Henson JB, Crawford TB. The pathogenesis of virus-induced arterial disease-Aleutian disease and equine viral arteritis. *Adv Cardiol* 1974;13:183–91.

22. Neu SM, Timoney PJ, McCollum WH. Persistent infection of the reproductive tract in stallions experimentally infected with equine arteritis virus, in *Proceedings*. 5th Int Conf Equine Infect Dis 1987;149–154.

23. Guthrie AJ, Howell PG, Hedges JF, et al. Lateral transmission of equine arteritis virus among Lipizzaner stallions in South Africa. *Equine Vet J* 2003;35:596–600.

24. Doll ER, Bryans JT, McCollom WH, et al. Isolation of a filterable agent causing arteritis of horses and abortion by mares; its differentiation from the equine abortion (influenza) virus. *Cornell Vet* 1957;47:3–41.

25. Jones TC, Doll ER, Bryans JT. The lesions of equine viral arteritis. *Cornell Vet* 1957;47:52–68.

26. Holyoak GR, Giles RC, McCollum WH, et al. Pathological changes associated with equine arteritis virus infection of the reproductive tract in prepubertal and peripubertal colts. *J Comp Pathol* 1993;109:281–93.

27. Coignoul FL, Cheville NF. Pathology of maternal genital tract, placenta, and fetus in equine viral arteritis. *Vet Pathol* 1984;21:333–340.

28. Estes PC, Cheville NF. The ultrastructure of vascular lesions in equine viral arteritis. *Am J Pathol* 1970;58:235–253.

29. Doll ER, Bryans JT, Wilson JC, et al. Immunization against equine viral arteritis using modified live virus propagated in cell cultures of rabbit kidney. *Cornell Vet* 1968;58:497–524.

30. McCollum WH. Vaccination for equine viral arteritis, in *Proceedings*. 2nd Int Conf Equine Infect Dis 1970;143–151.

31. Timoney PJ, Fallon L, Shuck K, et al. The outcome of vaccinating five pregnant mares with a commercial equine viral arteritis vaccine. *Equine Vet Educ* 2007;19:606–611.

32. Timoney PJ, Creekmore L, Meade B, et al. Multi-state occurrence of EVA—report of the committee on infectious diseases of horses: addressing equine viral arteritis in the United States, in *Proceedings*. 110th Annu Meet US Anim Health Assoc 2006;354–362.

33. Balasuriya UBR, Snijder E J, van Dinten LC, et al. Equine arteritis virus derived from an infectious cDNA clone is attenuated and genetically stable in infected stallions. *Virology* 1999;260:201–208.

34. Senne DA, Pearson JE, Carbrey EA. Equine viral arteritis: a standard procedure for the virus neutralization test and comparison of results of a proficiency test performed at five laboratories, in *Proceedings*. 89th Annu Meet US Anim Health Assoc 1985;29–34.

35. Timoney PJ. *World Organization for Animal Health (OIE): OIE manual of diagnostic tests and vaccines for terrestrial animals*. 6th ed. Paris, France: OIE, 2008.

36. Balasuriya UBR, Leutenegger CM, Topol JB, et al. Detection of equine arteritis virus by real-time TaqMan® reverse transcription-PCR assay. *J Virol Methods* 2002;101:21–28.

37. Lu Z, Branscum A, Shuck KM, et al. Comparison of two real-time reverse transcription polymerase chain reaction assays for the detection of equine arteritis virus nucleic acid in equine semen and tissue culture fluid. *J Vet Diag Invest* 2008;20:147–155.

38. Carter GR. Cultural Procedures Employed for Clinical Specimens. In: Carter GR, Cole JR, eds. *Diagnostic procedures in veterinary bacteriology and mycology*. 5th ed. San Diego: Academic Press Inc, 1990;15–17.

39. Moore BD, Balasuriya UBR, Nurton JP, et al. Differentiation of strains of equine arteritis virus of differing virulence to horses by growth in equine endothelial cells. *Am J Vet Res* 2003;64:779–784.

40. McCollum WH. Studies of passive immunity in foals to equine viral arteritis. *Vet Microbial* 1976;1:45–54.

41. McCollum WH. Responses of horses vaccinated with avirulent modified-live equine arteritis virus propagated in the E. Derm (NBL-6) cell line to nasal inoculation with virulent virus. *Am J Vet Res* 1986;47:1931–1934.

42. Murphy TD, Slade BA, Broder KR, et al. Prevention of pertussis, tetanus, and diphtheria among pregnant and postpartum women and their infants. *MMWR Recomm Rep* 2008;57:1–51.

43. Okuda M, Yamanaka M, Takahashi T, et al. Positive rates for rubella antibody in pregnant women and benefit of postpartum vaccination in a Japanese perinatal center. *J Obstet Gynaecol Res* 2008;34:168–173.

44. Van Kempen C, Mallard BA. Effects of peripartum stress and health on circulating bovine lymphocyte subsets. *Vet Immunol Immunopathol* 1997;59:79–91.

45. Kimura K, Goff JP, Kehrli ME, et al. Phenotype analysis of peripheral blood mononuclear cells in periparturient dairy cows. *J Dairy Sci* 1999;82:315–319.

46. Shafer-Weaver KA, Sordillo LM. Bovine CD8+ suppressor lymphocytes alter immune responsiveness during the postpartum period. *Vet Immunol Immunopathol* 1997;56:53–64.

47. Dewey CE, Wilson S, Buck P, et al. Effects of porcine reproductive and respiratory syndrome vaccination in breeding-age animals. *Prev Vet Med* 2004;62:299–307.

48. Dewey CE, Wilson S, Buck P, et al. The reproductive performance of sows after PRRS vaccination depends on stage of gestation. *Prev Vet Med* 1999;40:233–241.

49. Ellsworth MA, Brown MJ, Fergen BJ, et al. Safety of a modified-live combination vaccine against respiratory and reproductive diseases in pregnant cows. *Vet Ther* 2003;4:120–127.

50. Raidal SL, McTaggart C, Yovich JV, et al. Effect of withholding macromolecules on the duration of intestinal permeability to colostral IgG in foals, in *Proceedings*. 46th Annu Am Assoc Equine Pract Conv 2000;260–263.

51. Stott GH, Marx DB, Menefee BE, et al. Colostral immunoglobulin transfer in calves—period of absorption. *J Dairy Sci* 1979;62:1632–1638

CHAPTER VII

VACCINATION STUDY APPENDICES

Footnotes

a. Arvac, Fort Dodge Animal Health, Overland Park, Kan.

b. Qiagen, Valencia, Calif.

c. TaqMan RT-PCR assay, Applied Biosystems, Foster City, Calif.

d. TaqMan One-Step RT-PCR Master Mix, Applied Biosystems, Foster City, Calif.

e. 7500 Fast real-time PCR System, Applied Biosystems, Foster City, Calif.

f. MultiScribe Reverse Transcriptase, Applied Biosystems, Foster City, Calif.

g. RNase inhibitor, Applied Biosystems, Foster City, Calif.

h. Oklahoma Animal Disease Diagnostic Laboratory, Oklahoma State University, Stillwater, Okla.

i. VRMD, Pullman, Wash.

j. PC SAS, version 9.2, SAS Institute Inc, Cary, NC.

k. PROC MIXED, PC SAS, version 9.2, SAS Institute Inc, Cary, NC.

l. LSMEANS with SLICE, PC SAS, version 9.2, SAS Institute Inc, Cary, NC.

m. LSMEANS with DIFF, PC SAS, version 9.2, SAS Institute Inc, Cary, NC.

TABLE 1: Day of vaccination relative to day of foaling for the mares used in the EVA vaccination study.

GROUP 1[*]		GROUP 2[¥]		GROUP 3[§]	
Mare No.	Interval between vaccination and foaling (days)	Mare No.	Interval between vaccination and foaling (days)	Mare No.	Interval between foaling and vaccination(days)
1	-141	23	-25	43	2
2	-130	24	-41	44	2
3	-131	25	-54	45	2
4	-114	26	-68	46	2
5	-129	27	-13	47	2
6	-118	28	-11	48	2
7	-113	29	-36	49	2
8	-85	30	-9	50	3
9	-91	31	-2	51	2
10	-129	32	-36	52	2
11	-123	33	-53	53	2
12	-83	34	-38	54	3
13	-122	35	-52	55	2
14	-107	36	-18	56	3
15	-101	37	-4	57	2

16	-142	38	-4	58	2
17	-120	39	-19	59	2
18	-125	40	-11	60	2
19	-121	41	-40	61	2
20	-101			62	3
21	-96			63	2
22	-103			64	2
				65	2
				66	2
				67	2
				68	2
				69	2
				70	2

*Group 1(n=22) vaccinated between 142-83 days before foaling.

¥ Group 2 (n=19) vaccinated between 68-2 days before foaling.

§ Group 3 (n=28) vaccinated within 3 days of foaling.

Table 2—Results of VN testing of serum and milk samples from the mares and foals in Group 1 (mid-term prepartum vaccinates).

Mare No.	Milk VN Titer			Serum VN Titer (Mare)			Serum VN Titer (Foal)		
	0h	12h	1m	0h	12h	1m	0h	12h	1m
1	≥1:512	1:128	<1:4	1:256	1:256	1:128	<1:4	≥1:512	1:32
2	≥1:512	1:128	N/S	1:128	1:64	1:256	<1:4	≥1:512	1:32
3	≥1:512	1:128	N/S	1:512	1:256	N/S	<1:4	≥1:512	N/S
4	≥1:512	≥1:512	1:4	1:16	1:32	1:64	<1:4	1:16	1:32
5	≥1:512	1:32	<1:4	1:64	1:128	1:32	<1:4	1:256	1:64
6	≥1:512	1:256	1:4	1:64	1:128	1:128	<1:4	1:256	1:64
7	≥1:512	≥1:512	1:32	≥1:512	1:256	≥1:512	<1:4	1:256	1:512
8	≥1:512	1:256	<1:4	1:128	1:128	1:64	<1:4	≥1:512	1:32
9	≥1:512	1:64	<1:4	≥1:512	≥1:512	≥1:512	<1:4	≥1:512	≥1:512
10	N/S	1:256	1:4	≥1:512	≥1:512	≥1:512	<1:4	≥1:512	≥1:512
11	1:64	1:16	<1:4	1:128	1:128	1:256	<1:4	1:256	1:64
12	≥1:512	1:64	N/S	≥1:512	1:512	N/S	<1:4	≥1:512	N/S
13	1:256	1:32	N/S	≥1:512	≥1:512	N/S	<1:4	1:128	N/S

14	≥1:512	1:64	1:16	≥1:512	≥1:512	≥1:512	<1:4	≥1:512	1:128
15	≥1:512	1:128	1:8	≥1:512	1:256	1:256	<1:4	1:256	1:128
16	≥1:512	1:128	1:8	≥1:512	≥1:512	≥1:512	<1:4	≥1:512	≥1:512
17	1:256	1:16	N/S	1:128	1:16	1:16	<1:4	1:64	1:4
18	≥1:512	≥1:512	1:32	≥1:512	≥1:512	≥1:512	<1:4	≥1:512	≥1:512
19	≥1:512	1:16	<1:4	≥1:512	1:256	1:256	<1:4	1:64	1:32
20	≥1:512	1:64	<1:4	≥1:512	≥1:512	≥1:512	<1:4	1:256	1:16
21	≥1:512	1:16	N/S	1:128	1:128	1:128	<1:4	1:128	1:4
22	≥1:512	1:64	N/S	<1:4	≥1:512	N/S	<1:4	≥1:512	N/S

0h = Sampled at time of foaling. 12h = Sampled 12 hours after foaling. 1m = Sampled 1 month after foaling. N/S = Not sampled. VN titer = Reciprocal of serum neutralization titer to EAV.

Table 3—Results of VN testing of serum and milk from the mares and foals in Group 2 (late term prepartum vaccinates).

Mare No.	Milk VN Titer			Serum VN Titer (Mare)			Serum VN Titer (Foal)		
	0h	12h	1m	0h	12h	1m	0h	12h	1m
23	≥1:512	1:8	<1:4	≥1:512	1:256	1:64	<1:4	1:256	1:32
24	≥1:512	≥1:512	<1:4	≥1:512	1:256	≥1:512	<1:4	≥1:512	1:256
25	1:64	1:16	N/S	1:32	1:32	1:16	<1:4	1:32	1:16
26	≥1:512	1:32	N/S	≥1:512	1:256	N/S	1:16	1:256	N/S
27	≥1:512	N/S	1:16	1:128	N/S	1:256	1:4	N/S	<1:4
28	N/S	N/S	N/S	N/S	N/S	N/S	<1:4	N/S	N/S
29	1:256	1:256	1:4	≥1:512	1:256	1:32	<1:4	1:32	1:16
30	N/S	N/S	N/S	N/S	N/S	N/S	<1:4	N/S	N/S
31	<1:4	<1:4	1:256	<1:4	<1:4	≥1:512	<1:4	<1:4	N/S*
32	≥1:512	1:256	1:8	≥1:512	≥1:512	1:256	≥1:512	≥1:512	1:64
33	≥1:512	1:64	<1:4	1:32	1:128	1:8	<1:4	1:256	1:16
34	N/S	≥1:512	N/S	N/S	1:64	N/S	N/S	N/S	N/S
35	≥1:512	1:4	<1:4	1:64	1:128	1:32	<1:4	1:16	<1:4

36	≥1:512	1:128	<1:4	≥1:512	≥1:512	≥1:512	<1:4	1:128	1:32
37	<1:4	<1:4	1:4	N/S	<1:4	≥1:512	<1:4	<1:4	<1:4
38	<1:4	<1:4	1:16	<1:4	<1:4	≥1:512	<1:4	<1:4	<1:4
39	<1:4	1:32	1:8	1:64	1:32	≥1:512	<1:4	1:16	<1:4
40	1:64	1:16	1:4	1:64	1:128	1:256	<1:4	<1:4	<1:4
41	N/S	1:256	N/S	1:128	1:256	1:256	<1:4	1:256	N/S*
42A(control)	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4
42B(control)	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4

*Euthanized at approximately 1 month of age.

0h = Sampled at time of foaling. 12h = Sampled 12 hours after foaling. 1m = Sampled 1 month after foaling. N/S = Not sampled. VN titer = Reciprocal of serum neutralization titer to EAV.

Table 4—Virus isolation and serologic results on the mares and foals in Group 3 (postpartum vaccinates).

Mare no.	VN titer (mare)			VN titer (foal)			Virus isolation – buffy coat (mare)							Virus isolation – nasopharyngeal sample (mare)								
	0d‡	14d*	28d*	0d‡	14d*	28d*	-2d†	1d*	3d*	5d*	7d*	14d*	28d*	0d‡	1d*	3d*	5d*	7d*	9d*	11d*	14d*	21d*
43(P)	<1:4	≥1:512	1:256	<1:4	<1:4	<1:4	-	-	+	N/S	-	-	-	-	-	N/S	-	-	-	-	-	-
44(P)	<1:4	≥1:512	1:256	<1:4	<1:4	<1:4	-	-	-	-	-	N/S	N/S	-	+	-	-	-	N/S	N/S	N/S	N/S
45(P)	<1:4	1:256	≥1:512	<1:4	<1:4	<1:4	-	-	-	-	+	-	+	-	-	-	-	-	-	-	N/S	-
46(P)	<1:4	1:128	≥1:512	<1:4	N/S	<1:4	-	-	-	-	-	-	N/S	-	-	-	-	-	-	-	-	-
47(P)	<1:4	≥1:512	≥1:512	<1:4	<1:4	<1:4	N/S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48(P)	<1:4	≥1:512	≥1:512	<1:4	N/S	<1:4	-	-	N/S	-	-	-	N/S	-	-	-	-	-	-	-	-	-
49(P)	<1:4	≥1:512	1:256	<1:4	N/S	<1:4	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
50(P)	<1:4	1:256	1:128	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	N/S	N/S	-	-	-	-	-	-	N/S
51(P)	<1:4	1:128	1:64	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
52(P)	<1:4	N/S	N/S	<1:4	N/S	N/S	-	+	+	-	-	N/S	N/S	-	-	+	-	-	-	N/S	N/S	N/S

53(P)	<1:4	1:256	1:256	<1:4	N/S	≥1:512	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
54(P)	<1:4	1:256	1:64	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
55(P)	<1:4	1:64	N/S	<1:4	<1:4	N/S	-	-	-	-	-	-	N/S	-	-	-	-	-	-	-	-	N/S
56(P)	<1:4	1:256	N/S	<1:4	1:128	N/S	-	+	-	-	-	-	N/S	-	-	-	-	-	-	-	-	N/S
57(P)	<1:4	1:8	1:64	<1:4	<1:4	<1:4	-	-	N/S	Neg	-	-	-	-	-	-	N/S	-	-	-	-	-
58(P)	<1:4	1:128	1:256	<1:4	<1:4	<1:4	-	-	-	+	-	-	-	-	-	-	-	-	-	-	N/S	-
59(P)	<1:4	1:256	≥1:512	<1:4	<1:4	<1:4	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	N/S
60(S)	<1:4	1:256	≥1:512	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
61(S)	<1:4	1:256	1:128	N/S	<1:4	<1:4	N/S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
62(S)	<1:4	1:256	1:256	<1:4	<1:4	<1:4	N/S	N/S	-	-	-	-	-	-	-	-	-	-	-	-	-	-
63(S)	<1:4	≥1:512	1:256	<1:4	<1:4	<1:4	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
64(S)	<1:4	≥1:512	≥1:512	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N/S
65(S)	<1:4	≥1:512	≥1:512	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	N/S

66(S)	<1:4	1:256	1:256	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
67(S)	<1:4	1:32	1:64	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
68(S)	<1:4	≥1:512	1:256	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	
69(S)	<1:4	1:128	1:256	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
70(S)	<1:4	1:256	1:256	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N/S	
71(PC)	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
72(SC)	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

*Days post-vaccination of mares. †Days pre-vaccination of mares. ‡Day of vaccination of mare.

Negative = Negative for EAV after 2 blind passages in RK-13 cells. N/S = Not sampled. P = Pasture group. PC = Pasture control. S = Stall contact

CHAPTER VIII

EMBRYO TRANSFER STUDY ABSTRACT

The objective was to evaluate the potential risks associated with embryo transfer from mares bred with equine arteritis virus (EAV) infective semen. Twenty-six mares were embryo donors, whereas 18 unvaccinated and EAV antibody seronegative mares were embryo recipients. Of the 26 donor mares, 15 were unvaccinated and seronegative for antibodies to EAV and 11 were vaccinated for the first time with a commercially available modified live virus vaccine against EVA before breeding and subsequent embryo transfer. All donor mares were bred with EAV-infective semen from a stallion persistently infected with the virus. Twenty-four embryos were recovered 7 d post-ovulation; all were subjected in sequential order to five washes in embryo flush medium, two trypsin treatments, and five additional washes in embryo flush medium (prior to transfer). Twelve and seven embryos (Grades 1 or 2) were transferred from the non-vaccinated and vaccinated donors, respectively, and pregnancy was established in 3 of 12 and 2 of 7. Perhaps trypsin reduced embryo viability and pregnancy rate. The uterine flush fluid of 11 mares (9 of 15 and 2 of 11 from non-vaccinated and vaccinated donor groups, respectively) was positive for EAV by VI (confirmed by real-time RT-PCR); the wash fluid from the embryos of nine of these mares was negative following 10 washes

and two trypsin treatments. However, the embryo wash fluid from two mares was still positive for EAV after all 10 washes and the two trypsin treatments, and one embryo was positive for EAV. Two of 18 recipient mares had seroconverted to EAV 28 d after embryo transfer. Virus was not detected in any fetal tissues or fluids harvested after pregnancies were terminated (60 d). In conclusion, we inferred that the washing protocol of 10 washes and two trypsin treatments did not eliminate EAV from all embryos; due to limitations in experimental design, this requires confirmation. Furthermore, there may be a risk of EAV transmission associated with *in vivo* embryo transfer from a donor mare inseminated with EAV infective semen.

CHAPTER IX

EMBRYO TRANSFER STUDY INTRODUCTION

Equine viral arteritis (EVA) is a contagious disease of equids caused by equine arteritis virus (EAV). Although the majority of primary cases of EAV infection are subclinical and consequently undetected, some outbreaks may be associated with the appearance of influenza-like clinical signs, abortion in pregnant mares, and interstitial pneumonia in neonatal foals [1,2]. The risk of abortion in pregnant mares and the potential for persistent infection in stallions have substantial economic ramifications for the horse breeding industry. Equine arteritis virus is an enveloped RNA virus belonging to the family *Arteriviridae*, which also includes another important viral disease, porcine reproductive and respiratory syndrome (PRRS) [3,4]. These viruses are readily inactivated by common disinfectants and detergents. Equine arteritis virus survives for just 2 to 3 d at 37 °C, although it may survive up to 75 d at 4 °C [5].

Equine viral arteritis is a disease of worldwide concern, with serologic evidence of infection being reported in North and South America, Europe, Australia, Africa, and Asia [1,6]. However, the seroprevalence of EAV infection varies greatly between countries and among equine populations within countries. The 1998 National Animal Health Monitoring System (NAHMS) equine survey reported only 2.0% of unvaccinated horses in the United States were seropositive to EAV, in contrast to horses imported into California having a seropositive rate of 18.6% (mostly European Warmbloods) [7,8]. The seroprevalence of EAV infection varies widely

between breeds in the USA, with approximately 80% of Standardbreds and 5.4% of Thoroughbreds antibody positive. Most other breeds are believed to have a seroprevalence of <2% [1,9-13]. Differences in prevalence between breeds may in part be associated with differences in management practices. This was exemplified during the extensive outbreak of EVA in Thoroughbreds in Kentucky in 1984 [14] and in the more recent multistate occurrence in Quarter Horses in the USA in 2006-2007 [6,15,16].

Transmission of EAV between horses principally occurs via the respiratory and/or venereal routes [1,17-20]. Outbreaks have frequently resulted from breeding a naïve mare with EAV infective semen; the virus is subsequently disseminated to other naïve animals on the premises by the respiratory route. Naturally infected stallions may become persistent carriers of EAV. Establishment and maintenance of the carrier state is testosterone-dependent, with the virus localized to the accessory sex glands [21,22]. Persistently infected stallions may be short term (<3 mo), intermediate (3-7 mo), or long term carriers (7 mo to several years) [18,22]. Carrier stallions can play a major role in the widespread dissemination of the infection and are the primary natural reservoir of the virus [1,6,9].

In addition to the acknowledged risk of spread of EAV via semen, embryo transfer (ET) must also be considered as a potential route of spread. Equine arteritis virus is present in reproductive tract secretions of naturally infected mares [23], and these secretions are in contact with the developing embryo until it is collected from the donor mare approximately 7 d after breeding [24]. With the increasing number of ET being performed in some breeds, this procedure may pose a risk of transmission of EAV which has not been adequately determined.

Numerous studies have been conducted on the efficacy of various disinfection procedures for controlling microorganisms in the semen and embryos of humans and farm animals. Methods described include washing procedures, enzymatic treatments, antibiotics, immunological

methods, photosensitive dyes, antiviral agents, interferon, lactoferrin, and acidification [25]. One of the most common methods of disinfecting embryos involves trypsin; the method is detailed in the Manual of the International Embryo Transfer Society (IETS) [26]. Although trypsin effectively removed most bacteria and some viruses from embryos, it was ineffective in removing African swine fever virus and bovine mycoplasmas from *in vivo*-derived embryos [27-29]. It also failed to inactivate equine encephalomyelitis virus and swine influenza virus in infected cell cultures [30]. It is important to note that transmission of any virus through the ZP into the oocyte by the fertilizing sperm has not been reported in either cattle or horses [31]. With respect to the international shipment of bovine embryos, the risks from enzootic bovine leukosis virus, bovine herpesvirus-1, and bluetongue virus infective semen are negligible when embryos are processed in accordance with the IETS protocol. However, there is the potential that *in vivo*-derived embryos resulting from breeding with bovine viral diarrhea virus infective semen may still be contaminated with the virus [32]. A recent study indicated that bovine embryos, when washed according to the IETS guidelines, do not cause BVDV transmission to recipients or their offspring [33].

Equine embryos were treated with trypsin in several studies. In experiments involving embryo cryopreservation, trypsin increased the permeability of the capsule [34,35]. Trypsin also made the capsule more prone to loss during subsequent embryo handling; furthermore, absence of the capsule was detrimental to embryonic survival *in vivo* [36,37]. Trypsin has been used experimentally to successfully remove EHV-1 from exposed equine early blastocysts, whereas washing alone without trypsin did not successfully remove EHV-1 from embryos; however, neither study involved subsequent ET [38,39]. In contrast to its effectiveness on early blastocysts, trypsin did not decontaminate EHV-1-exposed expanded blastocysts or embryos surrounded only by the capsule [40].

It is not currently known whether EAV can be transmitted via ET. Therefore, the present study was conducted to assess the risk of transmission of infection associated with ET from non-EVA-vaccinated, as well as vaccinated donor mares, bred with EAV infective semen, and whether the internationally accepted protocol for washing embryos would successfully prevent spread of the virus through embryo transfer.

CHAPTER X

EMBRYO TRANSFER STUDY MATERIALS AND METHODS

Subjects and vaccination

A total of 44 mares were selected as embryo donors or recipients and used in the study. The large number of available mares facilitated the synchronization of embryo donors with embryo recipients. Synchronized mares were randomly allocated into two groups. Twenty six were selected as embryo donor mares, and 18 as embryo recipients. A single serum sample was obtained from each mare prior to vaccination or placebo treatment to confirm their seronegativity for antibodies to EAV. Donor mares were also tested for serum neutralizing antibodies to EAV on the day they were bred. Of the 26 donor mares, 15 were not vaccinated against EVA (Group D) and 11 were vaccinated for the first time before breeding and subsequent embryo transfer (Group VD). Mares in the VD group were given their first vaccination with a modified-live virus EVA vaccine (Arvac®, Pfizer Animal Health, New York, NY, USA) 28 d prior to being bred with virus infective semen (Day -28). Mares in the D group were administered a single placebo IM injection of sterile saline (0.9% NaCl) contemporaneously with vaccination of the VD mares.

Facilities

This study was approved and conducted in accordance with the guidelines of the Oklahoma State University Institutional Animal Care and Use Committee. All mares were maintained at the Center for Veterinary Health Sciences Ranch (CVHSR) in Stillwater, OK, USA for the duration of the study. These mares did not have contact with any EAV infected or exposed mares. When ovulation was imminent and the decision made to breed each donor mare, she was moved by trailer (observing standard biosecurity procedures) to a separate research facility isolation area, 1 km away, which was accessible by a separate entrance. The isolation area consisted of four individual paddocks (20 m x 40 m), separated from each other by a 5 m alley; each paddock had separate water and feed facilities. Recipient mares were moved to a separate paddock in this isolation area when they were considered ready to receive an embryo. Mares bred with EAV-infective semen were maintained separately from the recipient mares and there was no risk of indirect viral transmission via fomites. All recipient mares were placed in paddocks not previously occupied by EAV exposed mares.

Mare reproductive management

Each non-vaccinated mare was artificially inseminated with EAV infective semen and the recovered embryos were transferred to seronegative recipients. Once Group D was complete, the remaining mares selected as embryo donors were vaccinated against EVA and served as the VD group.

The estrous cycles of all mares within each group were synchronized as closely as possible with prostaglandin (10 mg dinoprost given im) administered to mares with a CL that was at least 6 d post-ovulation. Most mares began to exhibit estrus 4-7 d after prostaglandin treatment and individual mares were then examined for cycle synchrony. Ultrasonography was performed

daily on all mares once a follicle greater than 30 mm was detected. When the dominant follicle was ≥35 mm in the presence of diminishing endometrial edema and a softening cervix, either 2500 IU human chorionic gonadotropin (Intervet-Schering Plough, Millsboro, DE, USA) or 1.1 mg deslorelin (Essential Pharmacy Compounding, Omaha NE, USA) was administered to induce ovulation. Mares selected to serve as embryo donors were bred (24 h after hCG/deslorelin injection) with fresh chilled semen that was shipped by overnight delivery. Mares were moved to the isolation facility before they were bred. Both inseminated donor mares and potential recipient mares continued to be examined by ultrasonography to document the day of ovulation, with ultrasound examination for ovulation of bred mares being performed in the afternoon. The ultrasound machine and stocks used for mare restraint were thoroughly cleaned and disinfected with 1-Stroke Environ (Steris Corporation, St. Louis, MO, USA) between mares. The majority of mares ovulated 24-48 h after hCG or deslorelin injection, thus allowing multiple mares to ovulate within several days of each other.

In addition to the routine breeding described above, selected mares were treated with a superovulation protocol to increase the potential yield of embryos recovered. Mares were selected for this protocol depending on their individual cycle characteristics. Mares that had three or more follicles between 20 and 25 mm in diameter were selected to receive eFSH and undergo superovulation; mares that did not have a cohort of follicles did not receive superovulatory hormones, as there was minimal benefit in doing so. For mares that were selected for superovulation, ultrasonographic evaluations were performed daily, starting 5 d after ovulation, looking for the presence of multiple small follicles on both ovaries measuring approximately 20-25 mm that developed in response to endogenous FSH. When the largest follicle(s) in the cohort attained a diameter of 23 to 25 mm, eFSH therapy was started. A dose of 12.5 mg eFSH was given im every 12 h for approximately 3-4 d. On the second day of eFSH therapy, prostaglandin (10 mg dinoprost) was given. The eFSH therapy was discontinued when >50% of the cohort of

developing follicles were approximately 35 mm in diameter. Mares were then allowed to 'coast' without any hormone therapy for 24 h, and then given hCG (2,500 IU, IV) or deslorelin (1.1 mg), inseminated 24 h post hCG administration (Day 0), and monitored to determine day(s) of ovulation. A majority of mares with multiple ovulations had synchronous (i.e. same day) ovulations. Occasionally a mare ovulated one or more follicles one day and one or more follicles the next day.

The NV and VD groups of donor mares were exposed to EAV by insemination with extended semen from a known carrier stallion of proven fertility containing infectivity titers >1 x 10^5 plaque-forming units of EAV/mL of seminal plasma (Gluck Equine Research Center, Lexington, KY, USA). This virus has been sequenced and identified as EAV S3699 strain (GenBank Accession # GQ903796) [15]. The fresh chilled extended semen was stored at approximately 4 °C and shipped from the farm of origin to the CVHSR by overnight delivery as needed for each mare. Upon arrival, sperm motility and morphology were assessed; every sample of extended semen received had >50% progressive motility and >90% normal morphology at insemination. Each mare to be inseminated was inseminated once with the shipped semen on the morning it was received.

Embryo recovery, sampling, and transfer

Embryos were obtained by flushing the uterus 7-8 d after ovulation (Days 7 or 8 post insemination/virus exposure), using a commercial equine embryo flush solution (Emcare, Bio-Free, Auckland, New Zealand). Embryo flushing was performed in the afternoon 7 d after ovulation was documented (also in the afternoon). Six liters of pre-warmed (35-37 ^{o}C) flush solution were used to recover embryos. The uterus was infused with 1 to 2 L of the solution by gravity flow (via a 50 cm 37 french balloon tipped silicone catheter), then flushed back via

gravity flow out through a 75 µm in-line filter positioned above a 1 L collection cylinder (Partnar Animal Health, Port Huron, MI, USA). The process was repeated two or three additional times for a total of 6 L of flush solution per attempted embryo recovery. Recovery of fluid from the second and third uterine lavages was assisted by transrectal massage of the uterus. After the last of the medium was recovered, the fluid within the filter cup was poured into three or four sterile search dishes and some unused flush solution was used to rinse the filter into the search dishes. The medium contained in the search dishes was then examined under 7-15x magnification with a stereo dissection microscope. Embryos were measured using an eyepiece micrometer and designated by size < or > 200 µm and deemed as Grades 1 to 4 (1 = excellent, 2 = good, 3 = fair, and 4 = degenerated and nontransferable). Separate flush kits were used for each mare, including autoclaved catheters and disposable tubing and filters.

All collected embryos were washed according to the guidelines set out by the IETS [26]. Briefly, the washing protocol entailed 12 washes using 12 dishes (35 mm in diameter), each containing 2 mL of wash fluid. Washes 1-5 and 8-12 used fresh medium identical to that used to flush the embryos from the uterus. Proteolytic enzyme treatment between flush medium Washes 5 and 8 consisted of 2 washes (Washes 6 and 7) in Hank's balanced salt solution (pH 7.6 to 7.8) containing 0.25% porcine derived trypsin (Gibco, Carlsbad, CA, USA), with a total contact time of 60 to 90 s (~30 s per wash). Separate sterile micropipettes were used between wash wells, and the ambient temperature during treatments was approximately 27 ^{o}C. No more than 10 µL of wash medium containing the embryos (ensuring a 1:200 dilution) was transferred from one dish to another. If more than one embryo was collected from a mare, all embryos were washed together. Using a stereo microscope, all embryos were deposited at the 6 o'clock position in each dish, the embryos counted, each wash dish gently swirled counter-clockwise to fully disperse and disaggregate the embryos, and then very gently swirled in a clockwise direction depositing the embryos to the center of the dish. At the end of the wash procedure all embryos were examined

for defects within the zona pellucida (ZP) if present and graded for quality (1 = excellent or good, 2 = fair, 3 = poor, and 4 = dead or degenerating) according to the IETS standard [41]. Both ZP intact Grades 3 or 4 embryos and ZP defective embryos from each individual mare were grouped separately. In the case of large expanded blastocysts, the ZP became very thin, but the spherical nature of embryo and the lack of an expanded capsule were used as an indication that disappearance of the ZP had not occurred [42,43].

The embryo flush medium was filtered through a 0.45 µm filter (Partnar Animal Health) and the filtrate tested for infectious virus in cell culture. Similarly, Washes 1, 3, 5, 8, 10, and 12 were analyzed for the presence of virus in cell culture. Grades 1 or 2 embryos were transferred to seronegative recipient mares. Each recipient was donor matched according to estrous cycle synchrony and received one or two embryos from that donor (in some cases, there were not enough synchronized recipients available to accept more than one embryo transfer). When only one Grade 1 or 2 embryo was flushed from a donor mare, that embryo was transferred to a recipient. If two such embryos were recovered, one was transferred and one was harvested for examination for virus. If three or more Grades 1 or 2 embryos were recovered, no more than two embryos were transferred to one recipient; the remaining embryo(s) were either transferred to a second recipient or harvested for virological examination.

Grades 1 or 2 washed embryos were individually or doubly loaded into a 0.25 mL straw and transcervically transferred via a sheathed Cassou pipette and deposited into the lumen of the uterine body, or base of the uterine horn of an individual embryo recipient mare. The straw was checked before transfer via microscopy and after transfer by flushing the catheter tip and subsequent microscopic examination to ensure transfer of the embryo had occurred. Recipients were selected that had ovulated 1 d prior (+1) to 3 d after (0, -1, -2, and -3) the donor mare, with highest preference given to recipient mares that ovulated 2 d after the donor mares, followed by recipients that ovulated 1 d after the donor mare.

Pregnancy monitoring

Embryo recipient mares were examined via transrectal ultrasonography for pregnancy at 7 to 10 d post-transfer. The transducer was routinely cleaned with 70% isopropyl alcohol between examinations. Recipient mares were examined twice weekly via ultrasound to assess pregnancy status. If at any point the pregnancy was determined to be failing based on ultrasonographic appearance, the uterus was lavaged and the fluid tested for EAV. At 60 d gestation, all pregnancies were terminated via cervical dilation and uterine lavage with 2-6 L of saline. The fetus, fetal membranes, and allantoic and/or amniotic fluid were collected, frozen, and subsequently analyzed after initially making a tissue suspension when needed for viral infectivity by attempted isolation in RK-13 cells.

Monitoring of body temperature and clinical signs of embryo donor mares

Rectal temperatures were obtained on embryo donor mares on Days 0-14 and 28 (Day 0 = day of insemination with EAV-infective semen). Clinical signs in the embryo donor mares were also monitored and recorded on the same days, assessing the demeanor of the mares and noting the presence of any swelling or nasal discharge.

Statistical analysis

All statistical analyses were conducted using PC SAS Version 9.1 (SAS Institute, Cary, NC, USA). Analysis of variance procedures (PROC MIXED) were performed to assess the combined effects of day and vaccination on temperature. A repeated measures model was used,

with animal as the experimental unit for vaccination and day as the repeated factor. An autoregressive period 1 covariance structure was used to model the within animal variation. The simple effect of vaccination for each day was assessed (SLICE option in an LSMEANS statement) and P-values reported. Means and SEM for each day and vaccination combination were calculated and a significance level of 0.05 was used for all comparisons.

Virus isolation

The following samples were collected from each donor mare for attempted virus isolation (VI): nasopharyngeal swabs, vaginal swabs, and whole blood samples for buffy coat harvest on Days 0, 7, 14, and 28 (relative to AI). In addition, uterine flush samples, embryo wash samples, nontransferable embryos, and semen samples were subjected to VI testing. Virus isolation was attempted in RK-13 cells according to the standard OIE protocol [44]. Briefly, whole blood samples collected into tubes containing EDTA anticoagulant were centrifuged at 500 x g for 10 min. The plasma and buffy coat cells were aspirated and placed in 15 mL conical centrifuge tubes. The buffy coat cells were pelleted at 1500 x g for 10 min at 4 °C. Plasma was aspirated, and the white blood cell pellet was re-suspended in 5 mL of EMEM (Oklahoma Animal Diagnostic and Disease Laboratory). The cell suspensions were frozen at -80 °C until VI could be attempted. The nasopharyngeal swabs in viral transport medium were well vortexed before being filtered through a 0.45 µm syringe filter. Filtrates were frozen at -80 °C pending virus isolation. Nontransferable embryos were frozen in embryo flush medium at -80 °C until VI could be attempted. Virus isolation from PBMCs, nasopharyngeal swab filtrates, vaginal swab filtrates, nontransferable embryos, and clarified 10% fetal tissue suspensions was attempted in both high and low passage RK-13 cell lines, according to the OIE recommended protocol [43]. Briefly, serial decimal dilutions (10^{-1} to 10^{-3}) of each sample were made in supplemented EMEM and 1

mL of each dilution was inoculated into each of 2 × 25-cm^2 flasks containing confluent monolayers of RK-13 cells. Flasks were incubated at 37 °C for 1 h before being overlaid with EMEM supplemented with 0.75% carboxy methyl cellulose. Flasks were incubated at 37°C and checked for the appearance of viral cytopathic effect (CPE) on postinoculation Days 3 and 4. If there was no detectable CPE, a second blind passage was performed on Day 4. The RK-13 cell monolayers were fixed and stained with a 1% crystal violet solution containing 1% formaldehyde on post-inoculation Day 5 in the case of the first passage and on postinoculation Day 4 in the case of the second passage in cell culture. Tissue culture fluid (TCF) was harvested and stored at -80 °C for viral RNA extraction.

Viral RNA extraction and real-time Taq-Man® RT-PCR

Viral RNA was directly isolated from VI-positive TCF samples using a commercial kit. Briefly, TCF samples were microcentrifuged at 13,800 g for 2 min, and 140 μL of supernatant was removed and used for nucleic acid extraction according to the manufacturer's instructions (Quiagen viral RNA kit, Quiagen, Valencia, CA, USA). Viral nucleic acid was eluted in 60 μL of nuclease free water and stored at -80 °C.

A one-tube, real-time TaqMan RT-PCR assay was performed using the TaqMan One-Step RT-PCR Master Mix in a 7500 Fast Real-Time PCR System (Quiagen viral RNA kit, Quiagen). The primer and probe set used in the assay was identical to that previously described [45,46]. Every sample was tested in duplicate. Briefly, 25 μL of RT-PCR mixture for each reaction contained 12.5 μL of 2 × Master Mix without UNG (uracil-N-glycosylase), 40 × MultiScribe and RNase Inhibitor Mix, 900 nM of forward and reverse primers (0.45 μL), 250 nM probe (0.625 μL), nuclease free water (5.35 μL), and 5 μL of test sample RNA. The following thermocycling conditions were used under standard mode as per manufacturer's recommendation:

30 min at 48 °C, 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Each RT-PCR run included a negative control without RNA (containing the reaction mix with 5 μL of water [no template control]) and positive controls. The sensitivity of this assay was 10 RNA molecules [45,46].

Virus neutralizing titers

Upon arrival at the research facility, blood was collected from each mare to document that it was negative for neutralizing antibodies to EAV. In the course of the experiment, blood was collected from each donor mare by jugular venipuncture on 0, 7, 14, and 28 d postbreeding, allowed to clot, and the serum harvested for testing for antibodies to EAV. Blood was also collected from each recipient mare 14 and 28 d post embryo transfer, and serum tested to determine if seroconversion had occurred. Sera were screened for neutralizing antibodies to EAV according to the method of Senne et al [47].

CHAPTER XI

EMBRYO TRANSFER STUDY RESULTS

Embryo collection and transfer

Of the 26 embryo donor mares in this study, embryo recovery was not attempted on one mare in the non-vaccinated group, due to Grade 2 fluid accumulation in the uterus on 7 d after AI. A total of 24 embryos were obtained from the remaining 25 embryo donor mares. Sixteen embryos were recovered from 11 of the remaining 14 mares in the non-vaccinated group. Seven of the 11 vaccinated donor mares yielded a total of eight embryos upon uterine flushing. Embryo transfer results are summarized in Tables 2 and 3.

Of the 16 embryos obtained from the non-vaccinated group, 12 were transferred into 11 recipients (one recipient received two embryos). Four embryos were of unsatisfactory quality to transfer and were frozen for laboratory testing. Of the eight embryos obtained from the vaccinated group, seven were transferred into seven recipients, and one embryo was frozen for laboratory testing.

Resultant pregnancies of transferred embryos

Of the 11 mares receiving embryos obtained from the non-vaccinated donor mare group, three were pregnant at Day 14. One of these pregnancies, in mare R11, was lost between Days 14

and 24 (when she was subsequently examined). Fluid and tissues were recovered for virus isolation and rRT-PCR testing by uterine lavage when this pregnancy was noted to be failing. The other two pregnancies continued to be monitored and were allowed to progress to 60 d, at which time they were terminated and the fetuses and uterine fluid collected for diagnostic testing.

Of the seven mares receiving embryos from the EVA vaccinated donor mare group, two (mares R12 and R16) were pregnant at Day 14. Mare R16, though pregnant at 14 d, had lost the pregnancy by Day 21, at which time uterine lavage was performed to collect embryonic tissue and fluid for virus isolation and rRT-PCR. Mare R12's pregnancy progressed until 60 d, at which time the pregnancy was terminated and the fetus and uterine fluids were collected for diagnostic testing.

Body temperature results and assessment of clinical signs in embryo donor mares

Mean body temperatures of the non-vaccinated donor mares were significantly higher than the mean temperatures of the vaccinated donor mares on Days 2, 4-11, and 28. Mean temperatures of the two groups displayed the greatest difference on Days 6, 7, and 8, with non-vaccinated mares demonstrating mean temperatures of >1.1 °C higher than vaccinated mares on those days (Fig. 1).

Clinical signs suggestive of EVA were seen in conjunction with the elevated temperatures in many of the donor mares, both in those that were vaccinated as well as those not vaccinated against EVA. Clinical signs were more severe in nonvaccinated mares; nasal discharge was observed in 12 of 15 mares, with moderate to severe mucopurulent nasal discharge noted in 5/15 mares. Three of 15 mares in the non-vaccinated group displayed minimal swelling of their rear legs for 1-2 d. None of the non-vaccinated mares had swollen extremities on Day 28. In the vaccinated group, serous nasal discharge was noted in 6/11 mares on one or more days;

however, none of the mares developed a mucopurulent nasal discharge. None of the mares in the vaccinated group displayed any swelling of the extremities.

Virus detection and serologic results on semen and embryo donor mares

Six aliquots of semen were collected and frozen from the EAV carrier stallion over the period of the study to confirm his infectivity. All six samples from the stallion were EAV positive by VI and PCR.

All fetal tissues, allantoic, and amniotic or fetal wash fluids from the fetuses and fluids collected from the three 60-d pregnancies, as well as the flush fluid and tissues from the two mares that lost their pregnancies between Days 14 and 21 were negative for EAV by VI.

All mares in the non-vaccinated donor mare group were negative for antibodies to EAV on Day 0 (day of AI with EAV-infective semen). Thirteen of the 15 mares in the non-vaccinated group were seropositive by 28 d following insemination (Table 1). The majority of the mares that seroconverted did so by Day 14, although Mares D1 and D3 had not seroconverted by Day 28 after insemination. While these mares exhibited mucopurulent nasal discharge approximately 4-7 d post breeding, neither became febrile.

Based on attempted VI, 9 of 15 mare in the non-vaccinated group were positive for EAV from the buffy coat on Day 7 after AI, 8 of 15 were virus positive from nasal swab filtrates, and eight were EAV positive from vaginal swabs on post-insemination Day 7. Vaginal swabs from all the mares were virus negative on Day 0 (prior to breeding); a Day 0 sample was not available on Mare 13. Overall, 12 of 15 mares were positive by VI on either buffy coat, nasopharyngeal swab, or vaginal swab on either Days 7 or 14 post-insemination. All donor mares were VI negative on

buffy coat, nasopharyngeal swab, and vaginal swab by Day 28 following insemination. All virus positive results were confirmed by PCR assay (Table 1).

All 11 mares in the EVA vaccinated group of donor mares were positive for antibodies to EAV on the day of insemination. Titers ranged from 1:8 to ≥1:512. From Day 14 to the end of the test period (Day 28), all mares in this group had developed titers ≥1:512.

No infectious virus was detected in the buffy coat samples of mares in the VD group over the duration of the sampling period. Two of 11 nasopharyngeal swab samples were positive for EAV on attempted VI, one on Day 14 (mare VD20), and one on Day 7 (mare VD24). Four of 11 vaginal swab samples were EAV positive on Day 7. Buffy coat, nasopharyngeal swab, and vaginal swab specimens collected 28 d after insemination were VI negative. Positive VI results were confirmed by PCR assay (Table 1).

Embryo recipient serologic testing

All recipient mares that were blood sampled (samples were unavailable from two mares) were seronegative 14 d following embryo transfer. Of 18 mares sampled 28 d after embryo transfer, 16 of 18 were seronegative for antibodies to EAV. However, two mares, numbers R2 and R13, were seropositive 28 d following embryo transfer, with titers of 1:16 and 1:64 respectively (Table 2).

Virus detection results on embryo flush and wash samples

Virus isolation was performed on embryo collection (flush) fluids, the wash fluids, and the embryo itself, if it was not of satisfactory quality to be transferred. Nine of 15 flush fluid

samples from mares in the non-vaccinated group were positive for EAV by VI and PCR. Of the embryos recovered and washed from 11 mares, four of the wash fluid samples were virus positive after the first wash, and one was positive beginning with Wash five. Three samples of embryo wash fluid became negative starting with Wash 3 and remained negative through the rest of the washes. The wash fluid samples from two embryos from mares D3 and D4 were virus-positive in the case of Washes 5, 6, and 10. The embryo from mare D3 which was not transferred was negative for virus, whereas the embryo from mare D4 which was also not transferred was positive by VI and PCR.

Two of 11 flush fluid samples from the vaccinated donor mares were positive for EAV by VI and PCR. No embryo was recovered from Mare VD24. An embryo was recovered from Mare VD19, and all the washes were negative for EAV by VI. This embryo was transferred into mare R4 (Table 3).

CHAPTER XII

EMBRYO TRANSFER STUDY DISCUSSION

Embryo transfer (ET) in horses has become increasingly popular over the last decade, in part due to the easing of breed registration restrictions. The American Quarter Horse Association, for example, now permits registration of foals born as a result of embryo transfer. Another factor influencing the growing popularity of equine ET is the increased success of ET, in large measure due to improved technical proficiency of the veterinarians performing the procedure. In light of the popularity of embryo transfer, especially in the American Quarter Horse (AQH) breed, and the multistate occurrence of EVA involving the breed in 2006/07 [6,15,16], the risks of performing embryo transfer from a mare that has been bred with EAV-infective semen needed to be investigated. Efforts were made in this study to simulate the natural conditions that a mare would experience when bred with virus-positive semen.

The ability of the wash and trypsin treatment protocol to remove infectious virus from the embryos was assessed in two ways: (1) sampling and testing of the wash fluid; and (2) determining whether the embryo recipient mares seroconverted to EAV. That EAV was present 7 d after ovulation in the reproductive tracts of the majority of the NV group mares was a very important result, with 9 of 15 flush samples from this group virus positive by VI and PCR. Moreover, the rate of dilution of virus in those flushes would be high, making it remarkable that virus detection was positive. For flushes reported to be negative, perhaps the quantity of virus in

the flush was too low to be detected by methods used in this study. Regarding the VD group, 2 of 11 flush samples tested positive 7 d after ovulation; therefore, we inferred that vaccination reduced the frequency of EAV in the reproductive tract of vaccinated mares. Of the total of 18 washes/trypsin treatments (if multiple embryos were collected, they were washed/trypsin treated together), four samples from wash one were positive by VI and PCR, two of which were negative on subsequent washes. All four samples were from mares in the non-vaccinated group. Two samples continued to be virus positive up to and including Wash 10; neither embryo was of sufficient quality to transfer, therefore both were tested by VI and PCR, with one of the two testing positive. Therefore, we inferred that the wash protocol including trypsin treatment was not successful in all cases in removal of EAV from the embryo.

The second measure by which the ability of the wash and trypsin protocol to remove infectious virus from the equine embryo was assessed also confirmed ineffectiveness of the protocol in every case. Of the 18 mares in which embryos were transferred, all were seronegative on Day 14 post transfer; 16 were still seronegative on Day 28 post transfer, indicating that in most cases, the transfer of a washed and trypsin treated embryo did not result in EAV infection. However, two of the 18 recipient mares did seroconvert, indicating that EAV can be transmitted by embryo transfer, even when the embryo has been subjected to the accepted IETS wash and trypsin treatment protocol. Both of the seropositive mares received embryos derived from mares whose uterine flush fluid tested positive for EAV by PCR and VI, although virus was not detected in any of the washes of these two embryos. Perhaps the virus was tightly adherent to the embryo and could not be removed by the wash and trypsin treatment; it is also possible that the amount of available virus in the wash fluid was so small that it was undetectable. In support of this, that the two mares in question had only seroconverted by Day 28 post transfer, at which point their respective antibody titers were 1:16 and 1:64.

Not withstanding the two mares in which it was shown that EAV could be transmitted by ET, there were certain limitations to how this study was carried out. In light of the complex and expensive nature of the study, as well as state-imposed limitations on the use of a virus considered non-endemic in the state in which the study was conducted, individual isolation facilities were not available for recipient mares. The inability to achieve strict isolation of each mare may have increased the risk of cross-contamination between the paddocks accommodating donor and recipient mares respectively. The potential for this to occur was recognized by the researchers, and every effort was made to ensure that inadvertent transmission of the virus did not occur. The donor and recipient mares, while kept in the same isolation area, were maintained in completely separate paddocks, and all equipment was disinfected after use on each mare. Nevertheless, in spite of the biosecurity measures that were enforced, this possibility of transmission of EAV to the two mares that became infected other than by embryo transfer must be borne in mind when interpreting the results of this study.

It is difficult to determine from this study if EAV has an effect on fertility and early embryonic development, but the rate of embryos of unsatisfactory quality for transfer was high; 4 of 16 (25%) in unvaccinated mares and 1 of 8 (12.5%) in vaccinated mares. There was a similarly low rate of successful transfer from unvaccinated mares of 3 of 11 at Day 14 (with 1 of 3 subsequently dying) and vaccinated mares of 2 of 7 (with 1 of 2 subsequently dying); these low rates may have been due to trypsin treatment of the embryos or to virus contamination that may adversely affect embryonic development. Due to experimental limitations, a group of recipient mares receiving non-trypsin washed embryos derived from mares inseminated with EAV-negative semen was not included in the study. Perhaps the washing and trypsin treatment protocol which the embryos were subjected to after collection may have harmed the embryo and resulted in its failure to develop. The equine embryo is considered to lose its ZP approximately 24 h after entering the uterus, which often occurs at ~6.5 d after ovulation and capsule formation [43].

These embryos were collected at approximately 7 d after ovulation when many were in the expanded blastula stage, with a very thin ZP. Because of the thinness of the ZP, however, the process of losing the ZP was difficult to assess. The ZP may have been so thin as to fail to fully preclude the effect of trypsin on the trophectodermal layer. In other species such as cattle, exposure of embryos to trypsin during washing did not decrease embryonic survival [47-49]. In the cow, the embryo typically does not hatch until 9-11 d post ovulation, and so the continued presence of the ZP may protect the underlying blastocyst from the detrimental effects of trypsin. In some ways, the equine capsule is more resistant to degradation than the ZP. Whereas exposure of the ZP to acidic conditions (pH 2.0), basic conditions (pH 12.0), urea, high temperature (65 °C for 60 min or 80 °C for 30 min), mercaptoethanol, and dithiothreitol resulted in the solubilization of the ZP, it did not appear to affect the capsule at the concentrations used. However, the capsule was completely solubilized using a trypsin solution at pH between 7.5 and 9.0 [50,51]. Despite evidence of the adverse effect of trypsin on the capsule and the expectation that embryos at the time of recovery from donor mares would have only a thin ZP remaining, the trypsin wash method has been used consistently as the recommended method to remove infectious agents from embryos of other species. Although trypsin treatment of the ZP denuded embryos in this study may have detrimentally affected pregnancy rates, it was critical to determine if the trypsin and wash protocol as widely used by the IETS would effectively decontaminate the embryos of EAV, since this embryonic stage is commonly flushed in clinical practice [53]. In the same way that the presence or absence of the ZP may have influenced the effects of trypsin on the embryo, the interaction between the virus and the embryo could be different, depending on the presence or absence of the ZP. The stage of the embryo at time of collection also may be a critical factor with respect to both trypsin treatment and the ability of the wash protocol to remove EAV.

Of the five pregnancies diagnosed at 14 d, two were subsequently lost before the study was concluded on Day 60 post transfer. The two mares which lost their pregnancies did not

exhibit any detectable abnormalities on physical examination. Virus was not detected in the uterine flush fluid/fetal tissues, and neither mare became seropositive. Early embryonic death has not been shown to be a proven feature of EAV infection [54]. Based on the design of this study, the factor(s) responsible for the loss of pregnancies before Day 21 could not be determined. All three pregnancies that progressed until terminated at 60 d did so without problem, and EAV was not detected from any of the fetal tissues collected. This finding was consistent with the results of a study that assessed the effects of EAV-positive semen on mare fertility in which pregnancy rates were similar between mares bred with virus positive and virus negative semen [54]. In light of the low number of pregnancies following embryo transfer with embryos subjected to the IETS protocol, the effects of this protocol on the equine embryo, especially the inclusion of the trypsin treatment, needs to be further investigated.

Vaccination of the embryo donor mare (Group VD) appeared to reduce the likelihood of the virus being present in the uterus at the time the embryo was collected, 7 d after breeding, with only 2 of 11 uterine flush fluid samples virus positive from Group VD, compared with 9 of 15 virus positive uterine flush fluid samples from the non-vaccinated mare group. In addition, virus was not detected in any of the wash fluid samples from Group VD. These findings would support the recommendation to vaccinate mares to be bred with EAV-infective semen. Likewise, it would seem prudent to vaccinate embryo recipient mares in clinical practice. The embryo recipient mares were not vaccinated in this study so that they could be tested for the presence of EAV antibodies following embryo transfer.

Following breeding with EAV-infective semen, clinical signs displayed by the mares were recorded to assess the severity of the infection and to determine if vaccination provided protection from the development of clinical signs. Rectal temperatures of each mare were used as an objective measure of a clinical response from breeding with infective semen, and the elevated body temperatures seen in the NV mares were generally associated with the presence of nasal

discharge. With a significant difference of >1.1 °C between the NV and VD groups demonstrated in the mean body temperatures on Days 6, 7, and 8, it is apparent that breeding with this strain of EAV infective semen caused elevation in body temperature in unvaccinated mares and that vaccination clearly minimized the chance of developing an increased body temperature following breeding with EAV infective semen. The clinical signs observed were consistent with the elevated temperatures seen in the non-vaccinated donor group in comparison with the vaccinated donor group. The increased incidence of clinical signs consistent with EVA including the elevation in body temperature indicates that the challenge dose and strain of EAV used in this study were sufficient to elicit a clinical response. Based on the low numbers of pregnancies achieved following embryo collection from both the vaccinated and the unvaccinated mares, it does not appear that the elevated body temperatures seen in the non-vaccinated group had a detrimental effect on the viability of the embryos collected. However, the low number of pregnancies achieved in both groups limited the relevance of these findings.

The results of this study demonstrated that under the stated experimental conditions, EAV can be transmitted via embryo transfer even after the embryos have been washed and trypsin treated, in accordance with the IETS protocol. This was supported both by failure of the IETS protocol to decontaminate all washed and treated embryos of EAV, together with the fact that EAV was successfully transmitted by embryo transfer, albeit in a very limited number of cases; more detailed studies are needed to corroborate this finding. However, in view of the prohibitive expense of maintaining all the recipient mares in individual isolation facilities, the possibility of virus transmission from a source other than an infective embryo cannot be completely excluded. In light of the findings of this study, it is highly recommended that both donor and recipient mares be vaccinated against EVA if the former are to be bred with EAV-infective semen. Additionally, appropriate biosecurity measures need be implemented at time of breeding and embryo transfer to minimize the risk of inadvertent lateral spread of EAV infection

when using virus positive semen. Additional studies are warranted to further investigate the nature of EAV transmission via embryos to recipients and offspring.

CHAPTER XIII

EMBRYO TRANSFER STUDY REFERENCES

[1] Timoney PJ, McCollum WH. Equine viral arteritis. Vet Clin North Am Eq Pract 1993;9:295-309.

[2] McCollum WH, Timoney PJ, Lee J Jr, et al. Features of an outbreak of equine viral arteritis on a breeding farm associated with abortion and fatal interstitial pneumonia in neonatal foals. Eq Infect Dis VIII: Proc of the Eighth Int Conf 1998;559-60.

[3] Cavanagh D. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch Virol 1997;142:629-33.

[4] Snijder EJ, Meulenberg JJ. The molecular biology of arteriviruses. J Gen Virol 1998;79:961-79.

[5] Balasuriya UBR, MacLachlan NJ. Equine Viral Arteritis. In: Equine Infectious Diseases, Sellon, Long (Eds.), Saunders, 2006, pp. 153-64.

[6] Holyoak GR, Balasuriya UBR, Broaddus CC, Timoney PJ. Equine viral arteritis: current status and prevention. Theriogenology 2008;70:403-14.

[7] National Animal Health Monitoring System. Equine viral arteritis (EVA) and the US horse industry. USDA, APHIS, 2000.

[8] Hullinger PJ, Gardner IA, Hietala SK, et al. Seroprevalence of antibodies against equine arteritis virus in horses residing in the United States and imported horses. J Am Vet Med Assoc 2001;219:946-9.

[9] Timoney PJ. Equine viral arteritis: epidemiology and control, J Equine Vet Sci 1988;8:54-9.

[10] McCollum WH, Bryans JT. Serological identification of infection by equine arteritis virus in horses of several countries. Eq Inf Dis III: Proc of the Third Int Conf 1973;256-63.

[11] McCue PM, Hietala SK, Spensely MS, et al. Prevalence of equine viral arteritis in California horses. California Vet 1991;45:24-6.

[12] McKenzie J. Survey of stallions for equine arteritis virus. Surveillance 1996;17-8.

[13] Moraillon A, Moraillon R. Results of an epidemiological investigation on viral arteritis in France and some other European and African countries. Ann Rech Vet 1978;9:43-54.

[14] Timoney PJ. Clinical, virological and epidemiological features of the 1984 outbreak of equine viral arteritis in the Thoroughbred population in Kentucky, USA. Proc Grayson Found Int Conf of Thoroughbred Breeders Orgs on Equine Viral Arteritis: Dromoland Castle, Co. Clare, Ireland. 1984;24-33.

[15] Zhang J, Timoney PJ, Shuck KM, et al. Molecular epidemiology and genetic characterization of equine arteritis virus isolates associated with the 2006-2007 multi-state disease occurrence in the USA. J Gen Virol 2010;91:2286-301.

[16] Timoney PJ, Creekmore L, Meade B, et al. 2006 Multi-State Occurrence of EVA. Proc United States Anim Health Assoc 2007;354-62.

[17] Doll ER, Knappenberger RE, Bryans JT. An outbreak of abortion caused by the equine arteritis virus. Cornell Vet 1957;47:69-75.

[18] Timoney PJ, McCollum WH, Murphy TW, et al. The carrier state in equine arteritis virus infection in the stallion with specific emphasis on the venereal mode of virus transmission. J Reprod Fertil Suppl 1987;35:95-102.

[19] Cole JR, Hall RF, Gosser HS, et al. Transmissibility and abortigenic effect of equine viral arteritis in mares. J Am Vet Med Assoc 1986;189:769-71.

[20] McCollum WH, Prickett ME, Bryans JT: Temporal distribution of equine arteritis virus in respiratory mucosa, tissues and body fluids of horses infected by inhalation, Res Vet Sci 1971;12:459-64.

[21] Timoney PJ, McCollum WH, Roberts AW, et al. Demonstration of the carrier state in naturally acquired equine arteritis virus infection in the stallion. Res Vet Sci 1986;41:279-80.

[22] Little TV, Holyoak GR, McCollum WH, et al. Output of equine arteritis virus from persistently infected stallions is testosterone dependent. Eq Inf Dis VI: Proc of the Sixth Int Conf 1992;225-9.

[23] McCollum WH, Timoney PJ, Roberts AW, Willard JE and Carswell GD. Response of vaccinated and non-vaccinated mares to artificial insemination with semen from stallions persistently infected with equine arteritis virus. Eq Inf Dis V: Proc of the Fifth Int Conf 1988;13-8.

[24] Squires, EL McCue PM, Vanderwall D. The current status of equine embryo transfer. Theriogenology 1999;51:91-104.

[25] Bielanski A. Disinfection procedures for controlling microorganisms in the semen and embryos of humans and farm animals. Theriogenology 2007;68:1-22.

[26] Stringfellow DA. Recommendations for the sanitary handling of in-vivo-derived embryos. In: Manual of the International Embryo Transfer Society, Stringfellow DA, Seidel SM (Eds.), International Embryo Transfer Society, 1998, pp. 79-84.

[27] Singh EL. Disease control: procedures for handling embryos. Rev Sci Tech Off Int Epiz 1985;4:867-72.

[28] Riddell KP, Stringfellow DA, Panangula VS. Interaction of Mycoplasma bovis and Mycoplasma bovigenitalium with preimplantation bovine embryos. Theriogenology 1989;32:633-41.

[29] Bielanski A, Eaglesome MD, Ruhnke HL, Hare WC. Isolation of Mycoplasma bovis from intact and microinjected preimplantation bovine embryos washed or treated with trypsin or antibiotics. J In Vitro Fert Embryo Transf 1989;6:236-41.

[30] Malcolm H, Merrill MD. Effect of purified enzymes on viruses and Gram-negative bacteria. J Exp Med 1936;64:19-28.

[31] Wrathall AE, Simmons HA, Van Soom A. Evaluation of risks of viral transmission to recipients of bovine embryos arising from fertilization with virus-infected semen. Theriogenology 2006;65:247-74.

[32] Waldrop JG, Stringfellow DA, Galik PK, Riddell MG, Givens MD, Carson RL. Infectivity of bovine viral diarrhea virus associated with in vivo-derived bovine embryos. Theriogenology 2004;62:387-97.

[33] Bielanski A, Lalonde A, Algire J. Transmission of noncytopathic bovine diarrhea virus by infected semen to embryo donors and by embryos to embryo transfer recipients and offspring. Reprod Fertil Develop 2010;22:251.

[34] Legrand E, Krawiecki JM, Tainturier D, et al. Does the embryonic capsule impede the rreezing of equine embryos? Proc 5th Int Symp on Equine Embryo Transfer, Havemeyer Foundation Monograph 2000;3:95-6.

[35] Maclellan LJ, Carnevale EM, Coutinho da Silva MA, et al. Cyropreservation of small and large equine embryos pre-treated with cytochalasin-B and/or trypsin. Theriogenology 2002;58:717-20.

[36] Tharanasit T, Colenbrander B, Stout TAE. Effect of cryopreservation on the cellular integrity of equine embryos. Reproduction 2005;129:789-98.

[37] Stout TAE, Meadows SM, Allen WR. Stage-specific formation of the equine blastocyst capsule is instrumental in hatching and to embryonic survival *in vivo.* Anim Reprod Sci 2005;87:269-81.

[38] Hebia I, Fieni F, Duchamp G, et al. Potential risk of equine herpes virus 1 (EHV-1) transmission by equine embryo transfer. Theriogenology 2007;67:1485-91.

[39] Hebia I, Duchamp G, Larrat M, et al. EHV-1 can be removed from exposed equine early-blastocysts (D6.5) by a bath of trypsin. Proc of the 23rd Ann Meeting of the European Assoc of Embryo Transfer, Alghero, 2007;174.

[40] Hebia I, Duchamp G, Larrat M, et al. Can equine early blastocysts (d6.5) and expanded blastocysts (d8) previously exposed to equine herpes virus 1 (EHV-1) be decontaminated by immersion in trypsin? Proceedings of the 7th International Symposium on Equine Embryo Transfer, Havemeyer Foundation Monograph 2008:40-1.

[41] Robertson I, Nelson RE. Certification and identification of the embryo. In: Manual of the International Embryo Transfer Society, Stringfellow DA, Seidel SM (Eds.), International Embryo Transfer Society, 1998, pp.103-16.

[42] Allen WR, Wilsher S. A review of implantation and early placentation in the mare. Placenta 2009;30:1005-15.

[43] Flood, P.F., Betteridge, K.J., Diocee, M.S. Transmission electron microscopy of horse embryos 3–16 days after ovulation. J Reprod Fertil 1982;32:319-27.

[44] Timoney PJ. Equine Viral Arteritis. In: World Organization for Animal Health (OIE): OIE manual of diagnostic tests and vaccines for terrestrial animals, OIE, 2008, pp. 904-18.

[45] Balasuriya UBR, Leutenegger CM, Topol JB et al. Detection of equine arteritis virus by real-time TaqMan® reverse transcription-PCR assay. J Virol Methods 2002;101:21-8.

[46] Lu Z, Branscum A, Shuck KM, et al. Comparison of two real-time reverse transcription polymerase chain reaction assays for the detection of equine arteritis virus nucleic acid in equine semen and tissue culture fluid. J of Vet Diag Invest 2008;20:147-55.

[47] Senne DA, Pearson JE, Carbrey EA. Equine viral arteritis: a standard procedure for the virus neutralization test and comparison of results of a proficiency test performed at five laboratories. Proc of the 89th Ann Mtg of the United States Anim Health Assoc 1985;29-34.

[48] Echternkamp SE, Kappes SM, Mauer RR. Exposure of bovine embryos to trypsin during washing does not decrease embryonic survival. Theriogenology 1989;32:131-7.

[49] Givens MD, Marley SD. Approaches to biosecurity in bovine embryo transfer programs. Theriogenology 2008;69:129-36.

[50] Stringfellow DA, Givens MD. Epidemiologic concerns relative to in vivo and in vitro production of livestock embryos. Animal Reprod Sci, 2000;60:629-42.

[51] Bousquet D, Guillomot M, Betteridge KJ. Equine zona pellucida and capsule: some physicochemical and antigenic properties. Gamete Res 1987;16:121-32.

[52] Oriol, J.G., Sharom, F.J., Betteridge, K.J. Developmentally regulated changes in the glycoproteins of the equine embryonic capsule. J Reprod Fertil 1993;653-64.

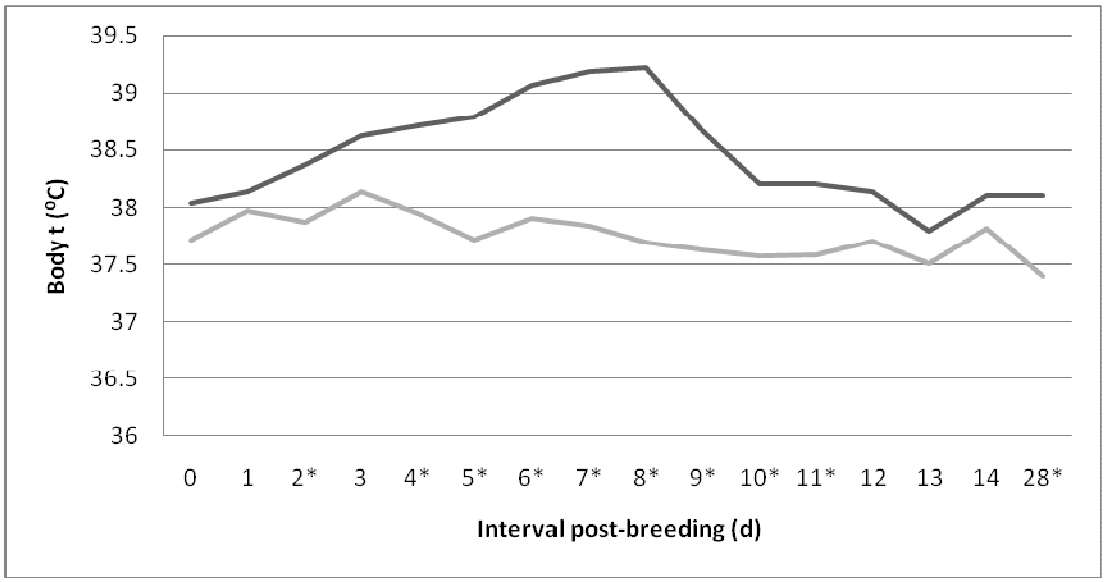
[53] Stout TAE, Meadows S, Allen WR. Stage-specific formation of the equine blastocyst capsule is instrumental to hatching and to embryonic survival in vivo. Animal Reprod Sci 2005;87:269-81.

[54] Raz T, Corrigan M, Loomis P, Card C. Effects of equine arteritis virus-positive semen on mare fertility. Animal Reprod Sci 2006;94:112-4.

CHAPTER XIV

EMBRYO TRANSFER STUDY APPENDICES

Fig. 1. Mean body temperatures (ºC) from the non-vaccinated embryo donor (D) group (black line) and vaccinated embryo donor (VD) group (grey line) in mares inseminated with equine arteritis virus infective semen.



Asterisks denote days on which mean temperatures differed between groups ($P<0.05$).

Table 1. Results of virus isolation (VI) and serologic testing (SNT) of donor mares inseminated with equine arteritis virus infective semen. Mares D1-D15 comprised the non-vaccinated group, whereas Mares VD16-VD26 comprised the initial vaccinated group.

Mare	SNT				Buffy Coat VI				Nasopharyngeal Swab VI				Vaginal Swab VI			
No.	0 d[a]	7 d	14 d	28 d	0 d	7 d	14 d	28 d	0 d	7 d	14 d	28 d	0 d	7 d	14 d	28 d
D1	<1:4	<1:4	<1:4	<1:4	-[b]	-	-	-	-	-	-	-	-	-	-	-
D2	<1:4	<1:4	1:16	1:8	-	+[c]	+	-	-	-	-	-	-	+	-	-
D3	<1:4	<1:4	<1:4	<1:4	-	-	-	-	-	-	-	-	-	-	-	-
D4	<1:4	<1:4	1:128	1:128	-	-	-	-	-	-	-	-	-	+	+	-
D5	<1:4	1:8	1:128	1:32	-	+	-	-	-	+	-	-	+	+	+	-
D6	<1:4	<1:4	1:32	1:8	-	+	-	-	-	+	+	-	-	+	-	-
D7	<1:4	<1:4	1:32	1:4	-	+	-	-	-	+	-	-	-	+	-	-
D8	<1:4	<1:4	1:128	1:32	-	+	-	-	-	-	-	-	-	-	+	-
D9	<1:4	<1:4	<1:4	1:32	-	-	-	-	-	-	-	-	-	+	-	-
D10	<1:4	<1:4	1:128	1:64	-	-	-	-	-	+	-	-	-	+	-	-
D11	<1:4	<1:4	1:64	1:64	-	+	-	-	-	+	-	-	-	-	-	-
D12	<1:4	1:8	1:64	1:64	-	-	-	-	-	-	-	-	-	-	-	-
D13	<1:4	<1:4	1:8	1:32	-	+	-	-	-	+	+	-	-	-	-	-

D14	<1:4	<1:4	1:32	1:256	-	+	-	-	-	+	-	-	-	-	-	-
D15	<1:4	<1:4	1:64	1:16	-	+	-	-	-	+	-	-	-	+	-	-
VD16	1:8	≥1:512	≥1:512	≥1:512	-	-	-	-	-	-	-	-	-	-	-	-
VD17	1:128	≥1:512	≥1:512	≥1:512	-	-	-	-	-	-	-	-	-	+	-	-
VD18	1:256	1:128	≥1:512	≥1:512	-	-	-	-	-	-	-	-	-	-	-	-
VD19	1:128	1:256	≥1:512	≥1:512	-	-	-	-	-	-	-	-	-	+	-	-
VD20	1:64	≥1:512	≥1:512	≥1:512	-	-	-	-	-	-	+	-	-	-	-	-
VD21	1:32	1:128	≥1:512	≥1:512	-	-	-	-	-	-	-	-	-	-	-	-
VD22	≥1:512	≥1:512	≥1:512	≥1:512	-	-	-	-	-	-	-	-	-	-	-	-
VD23	1:256	≥1:512	≥1:512	≥1:512	-	-	-	-	-	-	-	-	-	-	-	-
VD24	1:32	1:256	≥1:512	≥1:512	-	-	-	-	-	+	-	-	-	+	-	-
VD25	1:256	≥1:512	≥1:512	≥1:512	-	-	-	-	-	-	-	-	-	+	-	-
VD26	≥1:512	1:256	≥1:512	≥1:512	-	-	-	-	-	-	-	-	-	-	-	-

(a) d = days after insemination

(b) - = negative on VI

(c) + = positive on VI and confirmed positive by PCR

Table 2. Results of serologic testing of embryo recipient mares following embryo transfer.

Recipient mare No.	Day 14	Day 28	Embryo source (Donor No.)	Outcome	Presence of virus in donor mare flush fluid
R1	<1:4	<1:4	D7	Preg[a]	+[b]
R2	<1:4	1:16	D12	NP[c]	+
R3	<1:4	<1:4	D1	NP	-[d]
R4	<1:4	<1:4	VD19	NP	+
R5	NS[e]	<1:4	D5	NP	-
R6	<1:4	<1:4	D11	NP	+
R7	NS	<1:4	D5	NP	-
R8	<1:4	<1:4	D1	NP	-
R9	<1:4	<1:4	D9	NP	-
R10	<1:4	<1:4	D10	Preg	-
R11	<1:4	<1:4	D8	Preg	+
R12	<1:4	<1:4	VD23	Preg	-
R13	<1:4	1:64	D15	NP	+
R14	<1:4	<1:4	VD18	NP	-
R15	<1:4	<1:4	VD16	NP	-
R16	<1:4	<1:4	VD17	Preg	-
R17	<1:4	<1:4	VD25	NP	-
R18	<1:4	<1:4	VD20	NP	-

(a) Preg = pregnant

(b) + = positive on VI and confirmed positive by PCR

(c) NP = not pregnant

(d) - = negative on VI

(e) NS = not sampled

Table 3. Results of virus isolation and PCR testing of uterine flush fluid collected at time of embryo collection, subsequent washes of recovered embryos, and the embryo if not transferred; number of embryos recovered and disposition.

Donor No.	Flush	Wash 1	Wash 3	Wash 5	Wash 6	Wash 8	Wash 10	Number of embryos recovered and disposition
D1[a]	-[b]	-	-	-	-	-	-	3–Tr(R3)[c], Tr(R8),Tst(-)[d]
D2	+[e]	ENR[f]	ENR	ENR	ENR	ENR	ENR	0
D3	+	-	-	+	+	-	+	1 – Tst(-)
D4	+	+	+	+	+	-	+	1 – Tst(+)
D5	-	+	-	-	-	-	-	2 – Tr(R5), Tr(R7)
D6	-	ENR	ENR	ENR	ENR	ENR	ENR	0
D7	+	-	-	-	-	-	-	1 – Tr(R1)
D8	+	+	-	-	-	-	-	1 – Tr(R11)
D9	-	-	-	-	-	-	-	1 – Tr(R9)
D10	-	-	-	-	-	-	-	1 – Tr(R10)
D11	+	+	-	-	-	-	-	3-Tr(R6), Tr(R6), Tst(-)
D12	+	-	-	-	-	-	-	1 – Tr(R2)
D13	-	ENR	ENR	ENR	ENR	ENR	ENR	0
D14	+	ENR	ENR	ENR	ENR	ENR	ENR	0
D15	+	-	-	-	-	-	-	1 – Tr(R13)
VD16[g]	-	-	-	-	-	-	-	1-Tr(R15)
VD17	-	-	-	-	-	-	-	1-Tr(R16)
VD18	-	-	-	-	-	-	-	1-Tr(R14)
VD19	+	-	-	-	-	-	-	1-Tr(R4)
VD20	-	-	-	-	-	-	-	2-Tr(R18), Tst(-)

VD21	-	ENR	ENR	ENR	ENR	ENR	ENR	0
VD22	-	ENR	ENR	ENR	ENR	ENR	ENR	0
VD23	-	-	-	-	-	-	-	1-Tr(R12)
VD24	+	ENR	ENR	ENR	ENR	ENR	ENR	0
VD25	-	-	-	-	-	-	-	1-Tr(R17)
VD26	-	ENR	ENR	ENR	ENR	ENR	ENR	0

(a) Mares D1-D15 comprised the non-vaccinated donor group

(b) - = negative by VI

(c) Tr(x) = embryo transferred into mare x

(d) Tst(+ or -) = embryo not transferred and directly tested by VI and PCR

(e) + = positive by VI and confirmed by PCR

(f) ENR = embryo not recovered

(g) Mares VD16-VD26 comprised the initial vaccinated donor group

VITA

Charles Cuthbert Broaddus

Candidate for the Degree of

Doctor of Philosophy

Thesis: EVALUATION OF THE SAFETY OF VACCINATING MARES AGAINST EQUINE VIRAL ARTERITIS DURING MID OR LATE GESTATION OR DURING THE IMMEDIATE POSTPARTUM PERIOD AND INFECTION OF EMBRYOS FOLLOWING INSEMINATION OF DONOR MARES WITH EQUINE ARTERITIS INFECTIVE SEMEN

Major Field: Veterinary Clinical Sciences

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in veterinary clinical sciences at Oklahoma State University, Stillwater, Oklahoma in December, 2011.

Completed the requirements for the Doctor of Veterinary Medicine at Auburn University, Auburn, Alabama in 2003.

Completed the requirements for the Bachelor of Arts in Economics at the University of Virginia, Charlottesville, Virginia in 1996.

Experience:

Current employment is as the Program Manager for the Office of Veterinary Services, Virginia Department of Agriculture and Consumer Services, Richmond, Virginia.

Prior employment as a Theriogenology Resident and Lecturer at Oklahoma State University, Stillwater Oklahoma.

Prior employment in private veterinary practice in Mechanicsville, Virginia, and in Manchester, Georgia.

Professional Memberships: American Veterinary Medical Association, Virginia Veterinary Medical Association, American College of Theriogenologists, Virginia Academy of Food Animal Practitioners.

Name: Charles C. Broaddus Date of Degree: July, 2011

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EVALUATION OF THE SAFETY OF VACCINATING MARES AGAINST EQUINE VIRAL ARTERITIS DURING MID OR LATE GESTATION OR DURING THE IMMEDIATE POSTPARTUM PERIOD AND INFECTION OF EMBRYOS FOLLOWING INSEMINATION OF DONOR MARES WITH EQUINE ARTERITIS INFECTIVE SEMEN

Pages in Study: 90 Candidate for the Degree of Doctor of Philosophy

Major Field: Veterinary Clinical Sciences

Scope and Method of Study: Randomized controlled studies

Findings and Conclusions: All mares vaccinated in mid gestation foaled without any problems, but 3 mares vaccinated in late gestation aborted. Vaccinating mares during the last 2 months of gestation was associated with a risk of abortion; this risk must be weighed against the much greater risk of widespread abortions in unprotected populations of pregnant mares naturally infected with EAV. When embryo transfer is performed after breeding with EAV infective semen, it is apparent that the IETS washing protocol does not fully remove EAV virus from the embryo, as virus could be detected on a small number of embryos, and a minority of mares seroconverted following embryo transfer. In conclusion, we inferred that the washing protocol of 10 washes and two trypsin treatments did not eliminate EAV from all embryos; due to limitations in experimental design, this requires confirmation. Furthermore, there may be a risk of EAV transmission associated with *in vivo* embryo transfer from a donor mare inseminated with EAV infective semen.

ADVISER'S APPROVAL: G. Reed Holyoak