THE PATHOGENESIS OF CACHE VALLEY VIRUS IN THE OVINE FETUS

A Dissertation

by

ALINE RODRIGUES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Veterinary Pathology

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Approved by:

Co-Chairs of Committee,	John F. Edwards
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ABSTRACT

The Pathogenesis of Cache Valley Virus in the Ovine Fetus. (December 2011)

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Cache Valley virus (CVV) induced malformations have been previously reproduced in ovine fetuses; however, no studies have established the CVV infection sequence of the cells targeted by the virus or the development of the antiviral response of the early, infected fetus that results in viral clearance before development of immunocompetency. To address these questions, ovine fetuses at 35 dg were inoculated in utero with CVV and euthanized at 7, 10, 14, 21 and 28 dpi. On postmortem examination arthrogryposis and oligohydramnios were observed in some infected fetuses. Morphologic studies showed necrosis in the central nervous system (CNS) and skeletal muscle of earlier infected fetuses and hydrocephalus, micromyelia and muscular loss in later infected fetuses. Using immunohistochemistry and in situ hybridization, intense CVV viral antigenic signal was detected in the brain, spinal cord, skeletal muscles and fetal membranes of infected fetuses. Viral signal decreased in targeted and infected tissues with the progression of the infection.

To determine specific cell types targeted by CVV in the CNS, indirect immunofluorescence was applied to sections of the CNS using a double labeling technique with antibodies against CVV together with antibodies against neurons,

astrocytes and microglia. CVV viral antigen was shown within the cytoplasm of neurons in the brain and spinal cord. No viral signal was observed in microglial cells; however, infected animals had marked microgliosis.

The antiviral immune response in immature fetuses infected with CVV was evaluated. Gene expression associated with an innate, immune response was quantified by real-time, quantitative PCR. Upregulated genes in infected fetuses included ISG15, Mx1, Mx2, IL-1, IL-6, TNF-α, TLR-7 and TLR-8. The amount of Mx protein, an interferon stimulated GTPase capable of restricting growth of bunyaviruses, was elevated in the allantoic and amniotic fluid in infected fetuses. ISG15 protein expression was significantly increased in target tissues of infected animals. B lymphocytes and immunoglobulin-positive cells were detected in lymphoid tissues and in the meninges of infected animals. This demonstrated that the infected ovine fetus is able to stimulate an innate and adaptive immune response before immunocompetency that presumably contributes to viral clearance in infected animals.

DEDICATION

This work is dedicated to my loving husband Leo and to my family, for their love, encouragement and support throughout my career.

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NOMENCLATURE

BRA	Brain
BVDV	Bovine viral diarrhea virus
CNS	Central nervous system
CPE	Cytopathic effect
CVV	Cache Valley virus
DG	days of gestation
DPI	days post infection
H&E	Hematoxylin and eosin
IHC	Immunohistochemistry
ISG	Interferon stimulated gene
ISH	In situ hybridization
MEM	Minimal essential medium
MSS	Musculoskeletal system
Mx	Myxovirus resistance factor
PAMP	Pathogen associated molecular pattern
qPCR	quantitative polymerase chain reaction
SKM	Skeletal muscle
SPC	Spinal cord
$TCID_{50}$	50% Tissue culture infection doses
TLR	Toll like receptor

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CHAPTER I

INTRODUCTION, SIGNIFICANCE AND LITERATURE REVIEW

Significance

Fetal loss and abortions are common problems in the livestock industry. Frequently caused by genetic defects, toxins, hormonal imbalances and infectious agents, they are responsible for significant economic loss.[34] CVV is an infectious agent that causes abortion and fetal malformations in sheep and infrequently in other ruminants. Occasionally, CVV causes outbreaks of fetal losses.[29-31,39,44] Serologic surveys have shown that CVV is endemic in United States and infects many, large mammalian species.[16,20,22,31,39,57,95,96,103,122] Both single cases and outbreaks of fetal loss and malformations have been described in sheep,[29-31,39] goats[40] and occasionally cattle in Texas[33] and many states (most recently in Kansas,[45] with anecdotal reports from North Dakota[119] and South Dakota).[67]

As this disease can affect reproductive performance in ruminants in the United States, it is important to understand the pathogenesis of this virus, the progression of the infection and the development of an immune response in infected ruminant fetuses. This

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This dissertation follows the style of Veterinary Pathology.

data could further contribute to understanding of disease pathogenesis and could spur development of new diagnostic techniques to detect infected animals. Because this infectious agent is associated with rare cases of human encephalitis,[25,128] understanding the pathogenesis of CVV infection may be relevant to the study of fetal malformations caused by viruses in humans.[23]

The organism

CVV belongs to the family *Bunyaviridae*, *Orthobunyavirus* genus, Bunyamwera group. This family contains more than 250 viruses distributed in five genera, four of which, *Orthobunyavirus*, *Nairovirus*, *Phlebovirus* and *Hantavirus*, are recognized to infect mammals, and one, *Tospovirus,* that infects plants.[127] With the exception of the hantaviruses that are transmitted by rodent secretions, most of the *Bunyaviridae* are transmitted by arthropods. The viruses within the *Orthobunyavirus* genus are transmitted by mosquitoes. The genus includes several viruses that cause disease and encephalitis in humans, including the viruses of the California group, such as California encephalitis virus, La Crosse virus, Jamestown Canyon virus and Snowshoe hare virus. In addition, two members of the Simbu group are considered significant pathogens of domestic animals, Akabane virus, which causes abortions and fetal malformations in ruminants,[11,126,127] similar to the disease caused by CVV, and Aino virus, which has been serologically implicated as causing congenital defects in calves and lambs.[109]

The virions in this family are 80 to 120 nm in diameter and primarily spherical with glycoprotein projections embedded in the envelope lipid bilayer. The viral genome is composed of three, single-stranded RNA segments, large (L), medium (M) and small (S). These segments are complexed with the nucleocapsid protein to form individual L, M, and S ribonucleocapsids. A virion-associated polymerase of unknown specific location is necessary to copy the negative-sense, viral genome into positive-sense, messenger RNA found in virions. In most cases, bunyaviruses use only a negative–sense coding strategy; however, some bunyaviruses use a combination of a negative-sense and ambi-sense coding strategy when coding the S and M segments. The S segment of the *Orthobunyavirus* genus members encodes the nucleocapsid protein and the nonstructural protein in overlapping reading frames[98] within the same cRNA. The M segment encodes two envelope glycoproteins in a single open reading frame (ORF). A single, large ORF is used to encode the L segment of the L protein that functions as the viral polymerase.[11,14,126,127]

Briefly, the replication process of bunyaviruses is as follows:[126,127]

1. Attachment (interaction of viral proteins and host receptors);
2. Entry, mediated by endocytosis;
3. Uncoating in acidic endocytic vesicles, and viral membrane fusion with endosomal membranes;
4. Transcription of viral mRNA through a virion-associated polymerase (using host cell-derived primers);
5. Translation of mRNA (L, M, S);

6. RNA replication, membrane-associated (synthesis of cRNA, that serves as template for cRNA);
7. Transport to golgi, assembly and glycosylation;
8. Migration of golgi vesicles and fusion with the plasma membrane and subsequent release of mature virions.

Bunyaviruses cause cytopathic effect in cultured cells and are generally cytolytic for targeted cells in vertebrate hosts. However, in invertebrate hosts, little or no cytolysis is observed in cells and tissues. The vertebrate host cell responds to infection by triggering interferon pathways, such as those of type 1 interferon, that result in activation of regulatory signals and an antiviral state in the infected cells.[60,97,116] Another mechanism used by the host cells to control the infection and degrade specific viral mRNA sequences is the synthesis of small, interfering RNAs from the cleavage of double stranded RNA.[111,127,136] The viruses, however, try to prevent activation of the interferon pathway at the level of cellular transcription.[78,149] Bunyaviruses also have been demonstrated to inhibit the host cell machinery by inhibiting protein synthesis and activation of caspases leading to cellular apoptosis.[126,127,1,12,32,111]

Bunyavirus pathogenesis

According to the tissue tropism of orthobunyaviruses, the clinical disease in infected hosts can be simplistically divided into two forms: infantile and adult infection with encephalitis, and placental and fetal infection with malformations and fetal death.

In both forms, the virus is introduced into the bloodstream by an insect vector during a blood meal. The first round of replication occurs within the cells in the superficial dermis and is followed by spread of the virus to target organs. Infection results in invasion of the central nervous system (CNS) or, in ruminants, in transplacental infection of the fetus.[109]

Members of the Bunyamwera group may cause a relatively mild infection in adult hosts, despite rare cases of encephalitis described in some hosts during spontaneous and experimental human infections.[109,137] Members of the California group cause severe encephalitis in children. Experimental studies with viruses in the California group have demonstrated that after subcutaneous inoculation, the virus replicates in smooth muscle cells of blood vessels and later enter the reticulo-endothelial system with a short 1-3 day viremia. After primary viremia, the viruses cross the blood-brain barrier with active viral replication occurring in endothelial cells.[72,109] Ability of the virus to invade the CNS to cause severe disease (meningitis, meningoencephalitis or encephalitis) presumably depends on the age of the host and the virus strain or serotype.

Experimental studies in pregnant ewes infected with Akabane virus in the Simbu group, have shown that after intravenous inoculation, the virus invades placental tissues within 1 day and the fetal tissues within 5-7 days after inoculation.[109,110] Experimentally infected ewes develop a short viremia with development of serum neutralizing antibodies independent of the route of inoculation (subcutaneous, intravenous or intrauterine).[105,108-110] The development of lesions in the fetus will depend on the stage of fetal development when infected. If infection occurs before immunocompetency,

Akabane virus can cause severe necrosis in developing fetal tissues, including fetal brain, skeletal muscle, trophoblasts and placental membrane epithelium. If the fetus survives the infection musculoskeletal system (MSS) and CNS malformations occur.[26,106]

Cache Valley Virus

CVV was first described and isolated from a *Culiseta inornata* mosquito pool collected in 1956 in Cache Valley, Utah, an like other *Bunyavirus*, CVV caused encephalitis when inoculated intracerebrally into newborn mice.[69] Since then, CVV has been isolated from several mosquito genera, including *Aedes*, *Anopheles*, *Psorophora*, *Coquilettidia* and *Culex*, from numerous states in the USA,[15,21,22,39,65,79,90,95,96,123] Canada[57,71] and in other countries in the Americas.[3,13,20,38,49,152,154] Although unproven, *Culiseta inornata* is considered the principal vector of CVV.[65] As with most arboviruses, it is believed that effector molecules in mosquito saliva enhance CVV transmission and infection.[42]

CVV naturally infects a wide variety of animal species, and serologic and/or virologic evidence of infection has been described in sheep,[29-31,39,43,44] cattle,[122,153] horses,[24,79,96] deer,[16,103] goat,[40] pig, raccoons, woodchuck,[20] caribou, turtles,[21] rabbits[2,17] and foxes.[98] Serologic evidence of human infection with rare cases of meningoencephalitis are also described.[25,128,23,41,93,142,148]

In sheep, CVV is responsible for embryonic and fetal death, stillbirths and congenital malformations including hydrocephalus, hydranencephaly, microencephaly, porencephaly, torticollis, scoliosis, arthrogryposis, oligoamnion and flexural deformities.[29,30,34,39,44] In adult sheep, the infection is usually subclinical.[34,39]

The fetal disease can be experimentally reproduced in sheep by intrauterine inoculation of CVV. Reproducing fetal infection has proved itself to be difficult when CVV is inoculated intravenously into pregnant sheep.[30,34,39] Experimental infection in pregnant ewes can result in fever, depression and innapetence.[30,39] Similar to Akabane virus infection,[102] the ovine fetus is susceptible to development of fetal malformations if CVV is inoculated between 28 and 48 days of gestation (dg). Embryonic death and mummification occur if the virus is inoculated before 28 dg. If the virus is inoculated after 48 dg, no malformations are observed.[30,39] In Akabane virus-infected fetuses, virus can be identified in placentomes 24 hours after infection, indicating that this may be the portal of infection of the fetus.[110] The infection of the fetal membranes with CVV can cause oligohydramnios and oligohydroallantois, possibly resulting in fetal akinesia that could contribute to development of arthrogryposis.[39] In experimental infections, the virus is cleared from the tissues of infected fetuses within 4 to 6 weeks after infection.[94]

Serum neutralization is the most commonly used test to identify viral exposure. Because no transfer of maternal antibodies to fetuses occurs *in utero*, the identification of viral neutralizing antibodies in precolostral heart blood implies *in utero* exposure and infection. Virus isolation from blood can be used to identify infected animals; however, the viremia in adult sheep is short. Virus can be isolated from tissues of early infected

fetuses, but viral recovery from term fetuses is unlikely, because the virus is cleared from tissues within a few weeks after infection.[34,39] Polymerase chain reaction (PCR) has been used to identify RNA from CVV in infected cell cultures and tissues from infected animals and humans.[18,19,25,34,128]

Experimental infection of CVV in mice does not result in infection or antibody production if the virus or the virus with the vector saliva is inoculated subcutaneously. Infection and antibody production occurred only into sites that had been previously fed by mosquitoes. In the same study, CVV infection in pregnant mice did not result in fetal death or malformations in pups.[42]

Ovine fetal immune system development

The gestation period of the ewe is approximately 145 days. In the ovine fetus, the development of erythropoiesis, myelopoiesis and megakaryopoiesis occurs in the yolk sac and liver at approximately 17 dg.[99] Cells expressing MHC class I and MHC class II can be identified by 19 and 25 dg, respectively.[145] At approximately 20 to 25 dg, lymphocyte production begins in the thymus. At 48-50 dg, lymphocytes are seen in the bloodstream. At 55-60 dg, lymphocytes are found in spleen and lymph nodes. At 75-80 dg, they are in the bone marrow and Peyer's patches. By 55-60 dg, primordial lymph nodes form in the prescapular region with the development of the lymphoid, erythroid, myeloid and megakaryocytic series. The lymph nodes become integrated into a lymphatic system at approximately 65 dg.[99]

Fetal cells have phagocytic activity by 17 dg. If bacteria or india ink are inoculated intravenously in fetuses at 20 dg, phagocytized material can be observed in cells in hepatic sinusoids, in blood monocytes, and in the endothelium of multiple organs. This phagocytosis presumably occurs in the absence of immunoglobulin opsonins. The development of complement is detected at 120 dg.[99]

The placenta in ruminants is syndesmochorial; thus, the uterine tissues and chorionic epithelium form a barrier that prevents transplacental passage of immunoglobulins from the ewe to the fetus.[145] Inducing placental vasculitis has been shown to allow antibodies to move from the dam to the fetus and placental fluids, but antibodies are unlikely to be identified in fetal serum.[112] Antibodies against bacteriophages can be identified as early as 35-40 dg. It is believed that there is a hierarchy of antibody response depending on the nature of the inoculated antigen.[131,132] However, some studies, have shown that antibody response is usually inconsistent and has only been measured as present (quantitative), but without evidence of specifity (qualitative). Even though ovine fetuses are able to respond to antigens between 60 and 75 dg, no production of effective IgG antibodies usually occurs after the fetus is older than 87 dg. Fetuses are incapable of producing antibodies against Salmonella organisms until birth, even though they are able to respond to the infection by producing what were interpreted as nonspecific immunoglobulins.[46,47] In experiments, when antigens are inoculated prior to the development of an adaptive immune response, immune tolerance usually is not observed in challenged fetuses.[47]

Specific viral neutralizing antibodies were found at 75 dg in fetuses directly inoculated after 50 days, and antibody appearance correlated with the elimination of viable virus from infected tissues.[94] In another study, IgG and IgM antibodies against Akabane virus only were identified after 76 dg.[106] A third study evaluating the immune response of ovine fetuses infected with Akabane virus demonstrated cells with IgG and IgM in the spleen of fetuses at 59-60 dg; however, no significant neutralizing antibody titer developed at that point.[102]

Cell-mediated immune response with allograft rejection has been described as early as 70 dg, and if ovine fetuses are given an allograft between 55-65 dg, the grafts were accepted, and no allograft immune response developed.[99] Development of a cellular infiltrate/proliferation in the brain of ovine fetuses infected with Akabane virus starting at 50 dg, was interpreted as a non protective immunity, although identity of the cells was not determined. However, increased cellular infiltration/proliferation failed to occur in fetuses exposed to Akabane virus before 40 dg.[94]

Innate immune response and bunyaviruses

Innate immunity creates epithelial barriers associated with innate immune response that block entry of microorganisms by cells, such as phagocytic cells (neutrophils and macrophages), dendritic cells and natural killer cells, and proteins of the complement system. The most important cellular reactions in innate immune response include inflammation, characterized by phagocytosis of pathogens by macrophages and

neutrophils, and an antiviral defense mediated by natural killer cells and dendritic cells. These cells recognize pathogen associated molecular patterns (PAMPS) on foreign pathogens which results in activation of transcription factors and produce type I interferon that stimulates production of antimicrobial cytokines and proteins by the leukocytes. Examples of PAMPS include toll like receptors (TLR) 7 and TLR8, which recognize single stranded RNA.[82]

The genes within the interferon pathway promote cell survival and inhibition of virus replication during a viral infection. Some interferon stimulated genes (ISG), such as ISG15, myxovirus resistance factor (Mx) and 2'5'-oligoadenylate synthetase (OAS) are shown to be produced in response to viral infection.[27,130] Using real-time quantitative PCR, upregulation of ISG and continuous stimulation of the innate antiviral response has been demonstrated in blood and tissues of fetuses and steers infected with bovine viral diarrhea virus (BVDV).[130]

The Mx protein is an interferon-stimulated GTPase. This GTPase is located in intracellular membranes and is involved in intracellular trafficking, membrane remodeling and fusion processes. Mx proteins can be upregulated by type I and type II interferons and are capable of restricting growth of several viruses, including influenza virus, measles virus and bunyaviruses within the bunyavirus, phlebovirus and hantavirus genuses.[50,58-61,66,76,77,139] In most viral infections, the Mx protein acts by interfering with transport of viral components to the target compartments in infected cells. In a model proposed by Haller and Kochs (2002),[59] MxA forms large, membrane-associated self-assemblies that store monomers of this protein. With infection, monomers bind to viral

target structures, forming new assemblies involving viral intermediates and leading to mislocalization of viral components and consequent viral inhibition.[59] The human MxA has been shown to inhibit replication of La Crosse virus by binding to the viral nucleocapsid protein and forming large viral copolymers that accumulate in the perinuclear region.[76] In humans, two interferon regulated Mx genes have been identified: MxA, that has antiviral activity and MxB, that has no antiviral activity. Previous studies have demonstrated antiviral activity of bovine Mx2 in cattle infected with BVDV.[130]

Interferon tau is a type I interferon that is expressed early in pregnancy during development of the ruminant conceptus, and unlike most interferons, is not considered to be inducible by viral infection. Expression of interferon tau in the ovine conceptus occurs only between 11 to 18 dg and is essential for recognition of pregnancy.[9,10]

Significance of pathogen in human medicine

Two cases of encephalitis caused by CVV have been described in humans in the United States.[25,128] One patient developed severe meningoencephalitis, and died after 4 months.[128] The other infected patient developed a mild encephalitis and had a full recovery within a few weeks after infection.[25]

In one serologic study, Bunyawera virus antibodies were associated with congenital defects in humans. A significant correlation was shown between macrocephalic or microcephalic babies born to women that had antibodies for CVV and/or Tensaw virus; however, in this report, the data was not shown, and it is not clear

that cross neutralization tests were done to confirm the specificity of the antibodies detected and that cross reaction between CVV and Tensaw viruses did not occur.[23] Another study failed to identify any significant correlation between seropositivity for CVV and women that gave birth to anencephalic babies in Texas.[41] No other bunyaviruses in the *Bunyaviridae* family have been associated with congenital malformations in humans. Further studies should validate if CVV may be associated with specific human congenital malformations.

Some causes of human arthrogryposis include: mutagenic agents, chromosomal abnormalities, toxic chemicals or drugs, hyperthermia, neuromuscular blocking agents and mechanical immobilization.[55,141] Despite significant differences between the placentation in humans and that in ruminants, CVV-induced arthrogryposis in ovine fetuses could serve as a model to study the development of arthrogryposis in human patients.

CHAPTER II

CACHE VALLEY VIRUS INFECTION OF THE OVINE FETUS: TARGET CELLS AND INFECTION SEQUENCE

Introduction

Cache Valley Virus (CVV) is a mosquito-borne Bunyavirus in the *Orthobunyavirus* genus of the Bunyamwera group endemic in the United States that causes abortion, fetal death and malformations in small ruminants.[29-31,39,43,44] The *Orthobunyavirus* genus also includes Akabane virus and Aino virus that are associated with abortions and fetal malformations in calves and lambs.[11,126,127] CVV also infects a variety of animal species including horses,[24,79,96] deer,[16,103] goats, pig, and caribou.[34] In humans, it can cause encephalitis and meningitis.[25,128] Outbreaks of CVV with dramatic fetal lamb losses have been reported.[29-31,39,44]

Experimental ovine fetal infections with CVV and Akabane virus occur during a narrow window of susceptibility before development of ovine fetal immunocompetency, which occurs at approximately 70-75 dg,[94,113] with the severity of the fetal lesions depending on the fetal age when ovine fetuses are infected. Akabane virus causes severe necrosis of fetal brain, skeletal muscle and placenta.[26,81,107] Fetal malformations are unlikely to occur after 40 dg,[107] and if the fetus survives the infection, lambs are born with malformations of central nervous system (CNS) and musculoskeletal (MSK) malformations.[26,81,107] Similarly with CVV, the ovine fetus is susceptible if inoculated

before 48 dg, and CVV infection can result in fetal death, stillbirths and congenital malformations such as hydrocephalus, hydranencephaly, microencephaly, porencephaly, torticollis, scoliosis, and arthrogryposis.[29,30,34,39,44] Experimentally, embryonic mortality and mummification occur if the virus is inoculated before 28 dg, and if the virus is inoculated after 48 dg, no malformations have been observed.[30,39]

Although previous studies have reproduced CVV-induced malformations in ovine fetuses;[30,39] those studies did not characterize the early histologic lesions and did not identify the cells targeted by the virus during early infection. The objective of this study was to determine the early lesions and infection sequence of cells infected by CVV in the ovine fetus in order to correlate the early lesions with the CNS and musculoskeletal malformations seen in spontaneously affected lambs.

Materials and methods

Experimental animals and viral inoculation

All procedures in this study were conducted using protocols approved by the University Biosafety and Animal Use Committees. Fifteen, seronegative Rambouillet ewes were synchronized and bred naturally as previously described.[30] Pregnancies were confirmed ultrasonically by 33 dg. On day 35, the pregnant ewes were transferred to a BSL2 confinement building, and the amniotic cavity of each fetus was inoculated with a 1ml inoculum containing 10^5 50% tissue culture-infective doses (TCID50) of CVV that had been isolated from the allantoic membrane of an experimentally infected animal[30]

(infected group) or 1ml of minimum essential medium (MEM) (mock-infected/control group), as previously described.[29,30,43] Using standard virologic techniques, CVV was cultured in Vero cells for preparation of the virus inoculum.[89] The virus-infected and mock-infected ewes were housed in separate, confinement buildings. Ewes were monitored three times per day for clinical disease, and heparinized blood samples were drawn from ewes every 12 hours after infection for the first 4 days and immediately frozen at -80°C for virus isolation. Serum samples were collected from the ewes at day 0 (pre-infection) and on the day of the necropsy for CVV-serum neutralization testing.

Necropsy, sample collection, tissue preparation and light microscopic tissue examination

At 7, 10, 14, 21, 28 days post infection (dpi), three ewes (one mock-infected and two virus-infected) were humanely euthanized with an overdose of pentobarbital. Macroscopic lesions were noted, and fetal tissues, amniotic and allantoic fluids and placenta were harvested for testing. One set of fresh samples of brain, spinal cord, skeletal muscle, cotyledons, amniotic and allantoic fluids and membranes were frozen at -80ºC for viral isolation, and a separate set of fresh tissues were placed in an RNA stabilization solution (RNA later, Ambion Life technologies, Calsbard, CA) for PCR assays. Fetal tissues and fetal membranes were fixed overnight in 4% paraformaldehyde and in Davidson's AFA (acetic acid, formalin, alcohol) fixative to compare each fixative's performance in immunohistochemistry (IHC) and in situ hybridization (ISH) techniques. Tissues were paraffin-embedded and sectioned at 5µm for routine light

microscopy using hematoxylin and eosin staining (H&E), IHC, and ISH. Sections stained with H&E were evaluated for histologic changes. The distribution of the changes observed in the brain was based on previously described ovine fetal brain anatomy.[8]

Development of polyclonal antibody and immunohistochemical analysis for CVV

Purified viral proteins prepared from Vero cell culture[118] were submitted to the Proteintech Group, Inc (Chicago, IL) for development of a purified, rabbit polyclonal antibody against CVV. Briefly, after development of cytopathic effect, the flasks were frozen and thawed 3 times, followed by sonication at 42 watts for 5 min to enhance release of cytoplasmic viral particles. The viral solution was clarified by centrifugation at 1500 x g for 15 min. The virus was pelleted at 64000 x g for 1 hour in a 20% sucrose cushion (Beckman SW27). The formed pellet was resuspended in Tris-HCl and NaCl buffer and added to a continuous 20% to 60% sucrose gradient at 94000 x g for 3 hours. All formed fractions were collected and concentrated at 80000 x g on a 10% sucrose cushion. All purification steps were conducted at 4ºC. Viral protein content was determined by the bisinchinic acid (BCA) protein assay, and the proteins fractionated on a polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS), according to the manufacturer's recommendations (Thermo Scientific, Rockford, IL). To establish sensitivity of the CVV antibody and its approximate working range, a western blot using the CVV antibody at a concentration of 100µg/ml on cell lysates of CVV-infected cells, was done according to the manufacturer's recommendations (R&D systems, Minneapolis, MN). The purified antibody was used for immunohistochemical evaluation

of CVV antigen on deparaffinized sections mounted on positively-charged, silanized slides using an automated staining system for immunohistochemistry (DakoCytomation Autostainer®, Dako, Carpinteria, CA). Briefly, endogenous enzyme was blocked with hydrogen peroxide. Samples were pre-treated with unconjugated avidin and biotin. Nonspecific epitopes were blocked with Background Sniper (Biocare Medical, Concord, CA). Slides were incubated for 30 min with the CVV polyclonal antibody (1:300), followed by incubation for 20 min with the secondary antibody (MACH 2, Biocare Medical). Sections were stained with 3.3-diaminobenzidine tetrachloride (DAB, Dako) and counterstained with hematoxylin.

Tissues were evaluated to determine percentage of infected cells in each examined organ and graded as follows: less than 3% of cells positive (+); between 3-15% of cells positive (++); more than 15% of cells positive (+++). To evaluate cross reaction between the anti-CVV antibody and other bunyaviruses, IHC testing also was done on deparaffinized sections of stored, formalin fixed tissue blocks known to contain La Crosse, Main Drain or San Angelo virus from a previous study.[27]. Similarly, additional antigen testing was conducted on blocks from calves infected with Akabane, and Rift Valley Fever virus kindly provided by Dr. Makoto Yamakawa (National Institute of Animal Health, Japan) and Dr. Sophette Gers (Western Cape Department of Agriculture, South Africa), respectively.

In situ hybridization for CVV

A CVV-specific, digoxigenin (DIG)-labeled, single-stranded cDNA probe was generated as previously described.[74,83,117] Briefly, RNA was extracted and purified from CVV-infected cell culture fluid using a RNeasy mini kit (Qiagen, Valencia, CA). A unique, 530 bp segment from a conserved region of the CVV-M gene glycoprotein 1 (GenBank AF082576.1) was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) using CVV specific primers (F: AGC CTA GATTGT ATA GAC TGT GGA CCA, R: TTG GAT CAA TTG ATA AAA TAA GGA TTC). To generate a primarily single-stranded, labeled product, a second PCR reaction was conducted on the resulting amplicon using only the reverse primer in combination with a DIG DNA labeling mix (Roche Diagnostics, Manheim, Germany) according to the manufacturer's instructions.

The ISH assay was conducted on deparaffinized tissue sections mounted on positive-charged, silanized slides (ProbeOn Plus; Fisher Scientific, Pittsburg, PA) as previously described.[146] Slides were initially inserted in a slide holder before being exposed to a series of solutions utilizing the capillary gap refill technology of the manual MicroProbe system (Fisher Scientific) with minor modifications to the described protocol. Briefly, following serial hydration steps, the sections were digested with Proteinase K (100 µg/ml in phosphate-buffered saline) for 8 min at 37°C, followed by post-fixation in 0.4% paraformaldehyde for 10 min at 4°C. The hybridization step was performed using 50ng of the CVV-specific, DIG-labeled probe in the hybridization cocktail solution (Fisher Scientific, Pittsburg, PA) with an initial 10min incubation at

100°C followed by a 2 hour incubation at 42°C. Unbound probe was removed by decreasing salt washes (2X to 0.2X standard saline citrate buffer). To detect bound, DIG-labeled probe, slides were incubated for 1 hour at 37°C in an alkaline phosphatase-labeled, anti-DIG antibody solution (Roche Diagnostics) followed by a 30 min incubation in a development solution containing the chromagen substrate, nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate (NBP/BCIP), Lastly, slides were dehydrated and counterstained with Bismarck Brown prior to microscopic examination. Evaluation of the distribution of the signal in infected organs was performed as described for the IHC. In order to evaluate the specificity of our CVV DIG-labeled probe, ISH was also performed on deparaffinized tissue sections from animals infected with the non-CVV bunyaviruses described above.

Virus isolation

Viral isolation from blood and tissues was performed as described previously.[30] Inocula included: 1:10 MEM suspensions of either fresh fetal tissues, membranes, cotyledons, ewe blood, amniotic or allantoic fluids. The inocula were layered onto drained monolayers of Vero cells, incubated for 1 hour at 37°C, and subsequently flooded with MEM containing 10% FBS and incubated at 37°C in 5% CO_2 for 5 days with daily evaluation for cytopathic effect.

Serum neutralization

Ewe serum samples were tested for CVV neutralizing antibodies in triplicates using a microtiter, serum neutralization test in 96-well plates as previously described.[30] Serum samples were inactivated at 56°C for 30 min and diluted 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256 with MEM. Prior to inoculation, the diluted sera were incubated at 37°C for 60 min with an equal volume of 100 $TCID_{50}$/ml of CVV. After incubation, Vero cell suspensions diluted in MEM were added to virus-antibody mixtures, and the plates were incubated at 37°C in 5% CO_2 for 5 days. Monolayers were evaluated daily for cytopathic effect, and titers were calculated according to the Spearman Karber method.[75,138] Titers above 1:4 were considered positive.

Results

Gross findings

Three fetuses were collected from CVV-infected ewes at 7 and 10 dpi and four fetuses were collected at 14, 21 and 28 dpi (Table 1). Of the harvested fetuses at 7 dpi, one recovered fetus was grossly normal and two fetuses were diffusely dark red with pale red amniotic fluid (non-viable). Two, grossly normal fetuses and one non-viable fetus were recovered at 10 dpi. At 14 dpi, one recovered fetus was dead in early stages of mummification (not included in Table 1), and three fetuses were viable, but one had the amnion closely apposed (oligohydramnios) and compressing and bending the body. All four fetuses at 21 dpi were viable. One of these fetuses had marked oligohydramnios,

torticollis, arthrogryposis and scoliosis (Fig. 1.B), and another fetus had mild kyphosis. Three viable fetuses and one mummified fetus (not included in Table 1) were recovered at 28 dpi. One of these fetuses had moderate oligohydramnios with torticollis and hypercontracted flexed frontlimbs (arthrogryposis).

Table 1. Gross findings observed in fetuses infected with CVV

Ovine Fetus #	dpi/dg	Gross Findings
1	7/42	NF
2	7/42	Dark red fetus and fluids (dying)
3	7/42	Dark red fetus and fluids (dying)
4	10/45	NF
5	10/45	NF
6	10/45	Dark red fetus and fluids (dead)
7	14/49	NF
8	14/49	Oligohydramnios, kyphosis
9	14/49	NF
10	21/56	NF
11	21/56	Mild concave spinal flexion
12	21/56	Oligohydramnios, torticollis, arthrogryposis and kyphosis
13	21/56	NF
14	28/63	Oligohydramnios, arthrogryposis, torticollis
15	28/63	NF
16	28/63	NF

NF: No gross findings.

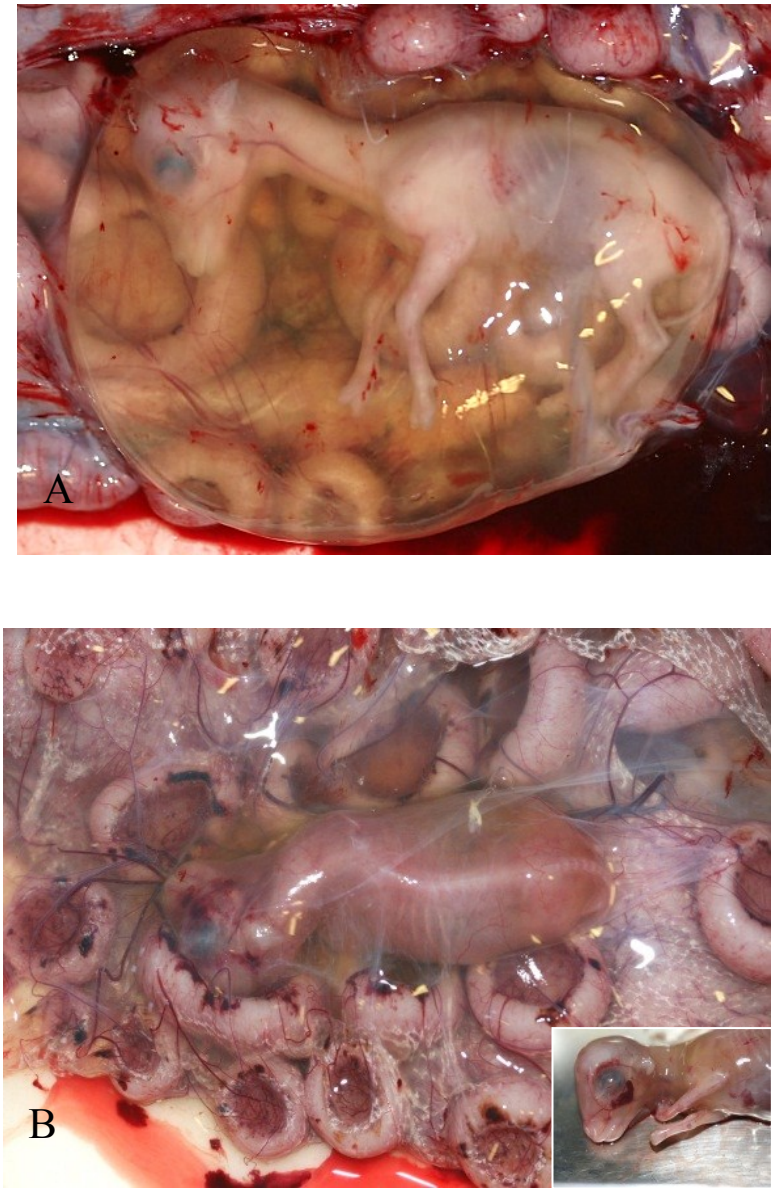

Figure 1. Gross findings in control and infected ovine fetuses. A. Control fetus at 56 dg. The fetus is floating freely within the amniotic cavity. B. Ovine fetus infected with CVV, 21 dpi (56 dg). Severe oligohydramnios results in fetal membranes apposed to the body of the fetus. The fetus has scoliosis at the level of cervical and thoracic vertebrae. Inset: Severe arthrogryposis is manifested by hypercontraction of the limbs.

Histologic findings

Most histologic lesions in infected fetuses were limited to the CNS and musculoskeletal system (MSS). Multifocal, severe areas of necrotic debris without inflammation were observed in the brain (BRA), spinal cord (SPC) and skeletal muscle (SKM) at 7 dpi. The areas of severe necrosis affected primarily the matrix zone and mantle layers of the telencephalon with moderate scattered necrotic areas affecting the brainstem and gray matter of the spinal cord, especially in the dorsal horns. No vessel lesions or thromboses were noted. The appendicular and dorsal SKM had multifocal, moderate to mild necrotic areas.

At 10 dpi, areas of necrosis were observed affecting the matrix and intermediate zones of the telencephalon and brain stem. Moderate necrosis was seen in the dorsal horns of the SPC. Necrosis similar to that observed at 7 dpi was seen in the SKM. In addition, SKM had multifocal, mild clusters of infiltrates of granulocytes within and among the SKM fibers along with multifocal, mild hemorrhage. Perivascular accumulations of cellular debris were observed in the fetal membranes of the non-viable fetuses.

At 14 dpi, multifocal, moderate areas of necrosis were in all layers of the ventricular walls of the telencephalic wall and in the brain stem resulting in hydrocephalus ex vacuo (Fig. 2.B1). The necrosis noted in the SPC was more extensive and severe than observed at 7 and 10 dpi (Fig. 3.B1). An infiltrate of mononuclear cells was observed in the meninges of the BRA and SPC. No significant necrosis was observed in the SKM; however, small aggregates of mononuclear cells and granulocytes

were within the SKM (Fig. 4.B1), focally in the tongue and in the smooth muscle of the intestine. Marked perivascular accumulation of necrotic cellular debris was observed in the amniotic membrane of the mummified fetus.

At 21 dpi, severe necrosis, loss of neuropil, meningeal inflammatory infiltration (meningitis) and hydrocephalus ex vaccuo were observed in the BRA. The SPC of three infected fetuses had marked parenchymal loss and micromyelia. BRA and SPC had multifocal areas of mineralization. Focal mononuclear cell myositis was also observed along with reduction in the thoracic and lumbar dorsal muscle mass in two fetuses. Findings at 28 dpi were similar to those observed in the SPC, BRA and SKM of fetuses at 21 dpi, with less necrotic tissue and severe hydrocephalus (Fig. 2.B2) and micromyelia (Fig. 3.B2). Moderate reduction in the dorsal SKM mass was observed in one of the fetuses at 28 dpi (Fig. 4.B2). Additionally, one fetus had mild, focal necrosis in the tubular epithelial cells of the kidneys. Hypercontraction of myotubules and mild mononuclear perivascular and interstitial infiltrates were observed in the SKM of both control and infected fetuses at 21 and 28 dpi; however, most infected fetuses had slightly greater numbers of mononuclear cells in meninges.

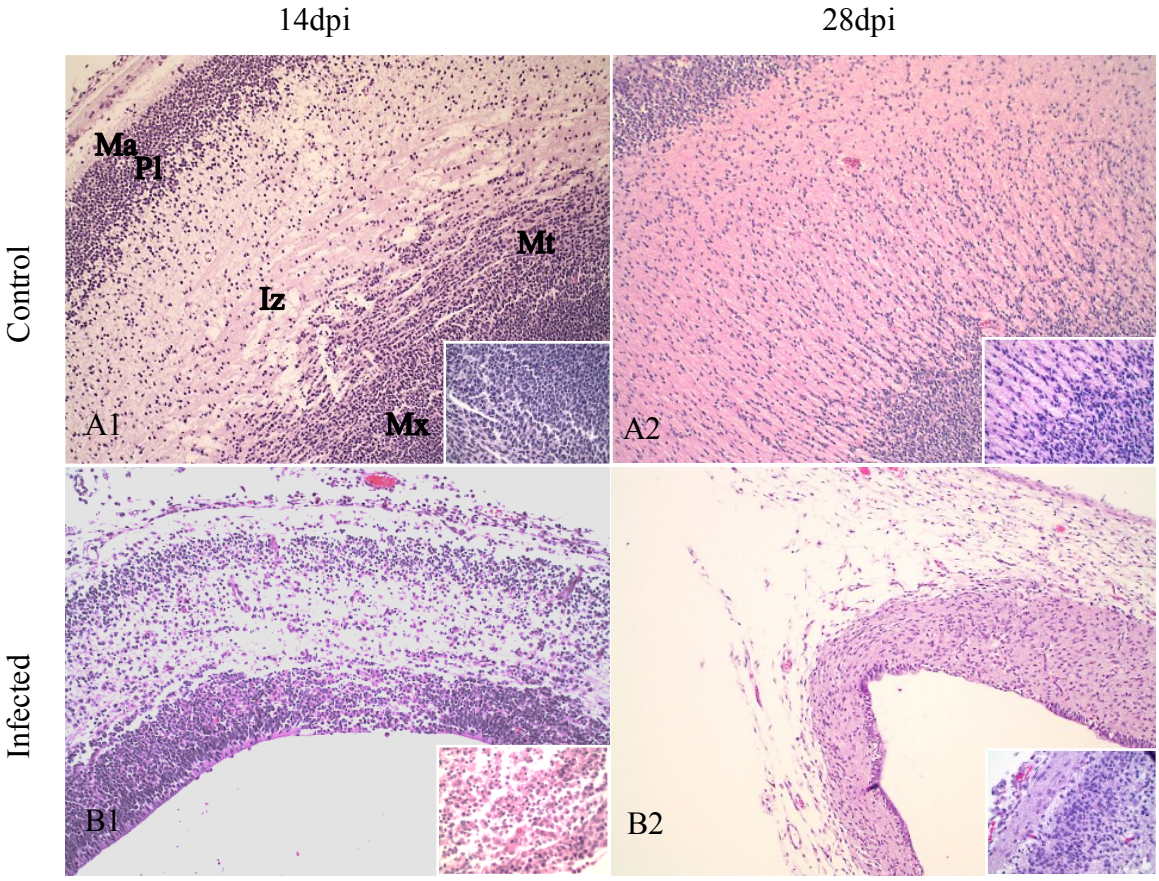

Figure 2. Histology of telencephalic wall of control (A) and CVV-infected fetuses (B). Regions of the telenchalic wall are marked as: Ma, marginal layer; Pl, primordial layer; Iz, intermediate zone; Mx, matrix layer; Mt, mantle zone, which is the most external layer of the matrix layer. Multifocal areas of necrosis characterized by accumulation of necrotic and karyorrhectic debris are seen at 14 dpi (B1). The telencephalic wall was markedly thinner due to cellular loss at 28dpi (B2). H&E; 100X mag; inset 400X mag.

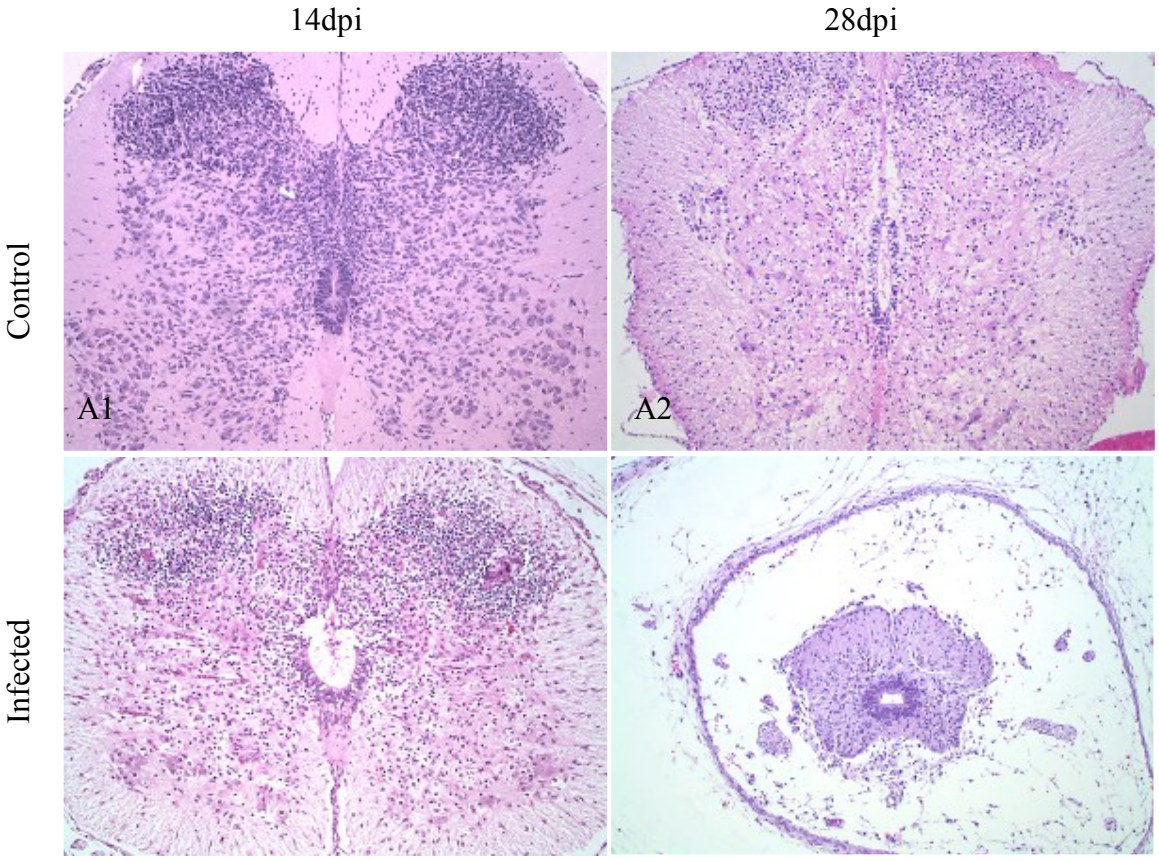

Figure 3. Histology of spinal cord at the thoracic level of control (A) and CVV-infected fetuses (B). Multifocal areas of necrosis with accumulation of necrotic and karyorrhectic debris are seen in the gray matter (B1). Progressive cellular and parenchyma loss with micromyelia is noted at 28 dpi (B2). H&E; 100X mag.

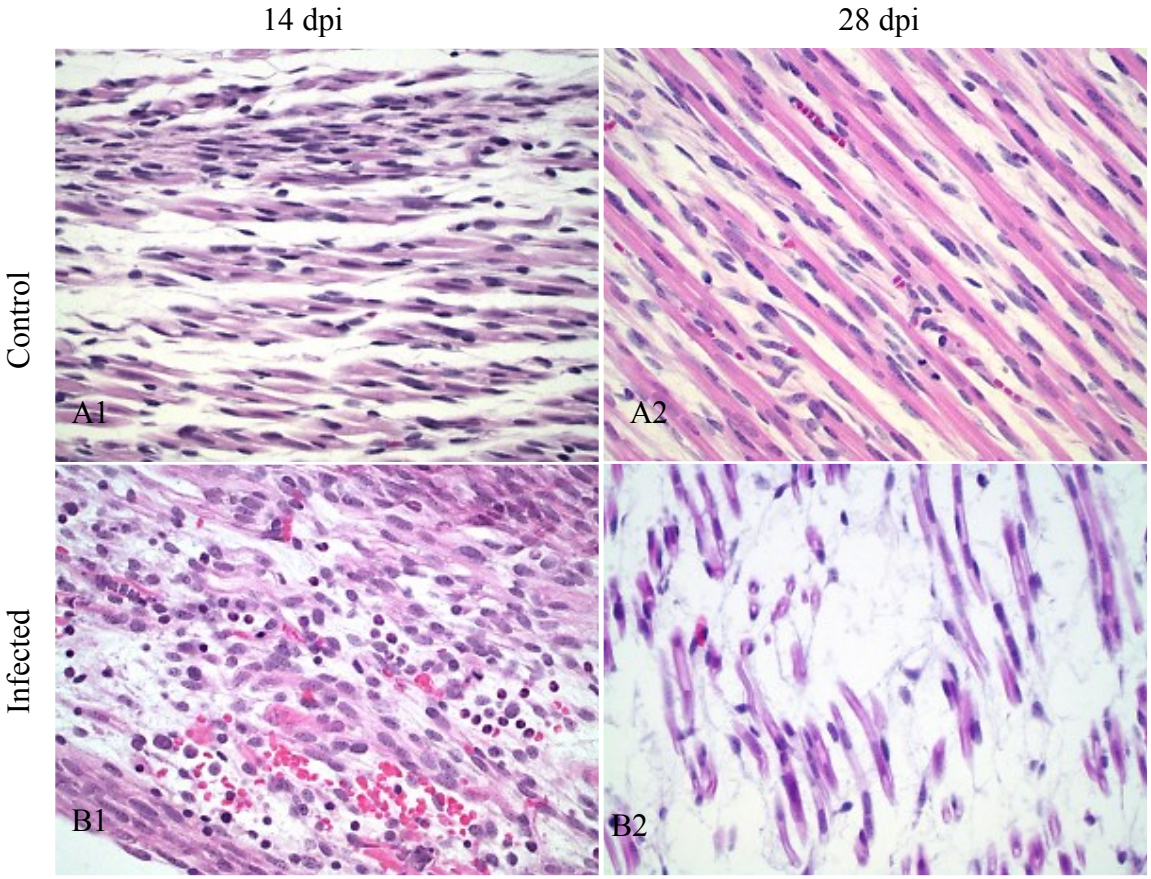

Figure 4. Histology of skeletal muscle of control (A) and infected fetus with arthrogryposis (B). Scattered inflammatory infiltrates composed of granulocytes and mild hemorrhage are within skeletal muscle fibers of a 14 dpi fetus (B1). At 28 dpi (B2) loss of skeletal muscle fibers were evident on the dorsal skeletal muscle around vertebrae. H&E; 400X mag.

Distribution of IHC viral antigenic signal in fetuses infected with CVV

The IHC CVV antigen signal in infected fetuses is shown in Table 2 and results were identical for tissues fixed in both fixatives. The tissues with abundant viral signal included the BRA, SPC and SKM with the signal primarily associated with necrotic foci. Within the BRA, the distribution of intense viral signal was diffuse within the telencephalic walls at 7 and 10 dpi, whereas it was mainly within the matrix zone at 14 dpi (Fig. 5.A2), and the mantle layer and primordial layers at 21 dpi. Only scattered foci had positive cells throughout the telencephalic walls at 21 dpi. The SPC had intense and diffuse viral signal distribution throughout all levels of the spinal cord at 7 and 10 dpi, but primarily within the dorsal aspect after 14 dpi. Viral signal was also identified in many myotubules and myofibers in the developing SKM around the vertebrae and ribs (Fig. 5.A3) with slightly fewer cells being positive within the SKM of the front and hind limbs, especially after 21 and 28 dpi. Virus-positive cells were cleared from SKM earlier than from the CNS. Scattered positive cells were observed in the neurons of the retina and ganglia, cardiomyocytes, and renal tubular and glomerular epithelial cells. Rare, positive cells were seen in the media of the aorta, as well as in the lymph nodes and spleen. Scattered mesenchymal cells in the fetal membranes were positive and the amniotic membranes were more intensely affected than the allantoic membranes. The

amniotic epithelium appeared to be rarely infected. No viral protein signal was observed in the cotyledons. A progressive decrease in viral antigen was observed in most affected tissues as the infection progressed, with rare positive cells noted in tissues at 28 dpi. IHC viral antigen signal was not observed in tissues collected from control animals and the antibody did not cross react with antigens in tissues of animals infected with other bunyaviruses.

Distribution of ISH CVV viral signal in fetuses infected with CVV

The distribution of the ISH CVV viral signal is shown in Table 2, and results were identical for tissues fixed in both fixatives. Similar to the results observed with the IHC, the tissues with most abundant viral signal were BRA (Fig. 5.B2), SPC and SKM (Fig. 5.B3). The distribution and quantity of signal in other tissues were similar to those observed with IHC, although the viral signal appeared reduced in comparison to that observed with IHC. Tissues collected in early infection had more intense signal when compared to those of later infection. Viral signal was not identified in the fetal membranes using this ISH protocol. ISH signal was not observed in tissues collected from control animals or from animals infected with other bunyaviruses.

Table 2. Detection of CVV in tissues of infected fetuses at different days of gestation

Tissues		Brain			Spinal Cord			Sk muscle			Ganglion		Heart		Kidney		Eye		Allantois		Amnion		Al Fluid	Am Fluid
Dpi	Methods Fet#	IHC	ISH	VI	IHC	ISH	VI	IHC	ISH	VI	IHC	ISH	IHC	ISH	IHC	ISH	IHC	ISH	IHC	VI	IHC	VI	VI	VI
7	1	+++	+++	+[1]	+++	+++		+++	+++	+[1]	+++	+++	+	-	++	+			++	+[1]	+++	+[1]	+	+[1]
	2	+++	+++		+++	+++		+++	+++		+++	+++	+	+	+	+	+	+	++	+[1]	++	+[1]	+[1]	+[1]
	3	+++	+++		+++	+++		+++	+++	+	++	++	-	-	++	++	-	-	+	+	+++	+	+[1]	+
10	4	+++	+++	+[1]	+++	+++	+[1]	+++	+++	+[1]	++	++	++	+	-	-	++	+	-	+	+++	+	+[1]	+[1]
	5	+++	+++	+[1]	+++	+++	+[1]	+++	++	+[1]	+	+	++		+	-	++	+		-		+[1]	+[1]	+[1]
	6			+						+											-		+	+	+
14	7	+++	+++	+	+++	+++	+[1]	++	+	+[1]	+	-	+	-	++	++	++	+	-	+[1]	+	+[1]	+	+[1]
	8	+++	+++	+[1]	+++	+++	+[1]	+++	+	+[1]	++	+	++	+	++	++	++	+	+	-	+	+[1]	+	+
	9	+++	+++	+[1]	+++	+++	+[1]	+++	+++	-	+	+	+	-	+	+	++	++	+	+[1]	++	+[1]	+[1]	+[1]
21	10	+++	+++	+	++	++	+	+	-	-	+	-	-	-	+	+	++	+	+	-	+	+	+	+
	11	++	++	+[1]	++	+	+[1]	+	-	+	+	-	+	-	+	+	+		+	+	+	+		+
	12	+++	+++		++	+	+[1]	++	++	+[1]	++	+	+	-	++	+	++	+	-	+	+	+	+	+[1]
	13	++	++	+[1]	++	+	+[1]	++	+	+[1]	+	+	+	-	+	+	+	-		+	++		+	-
28	14	++	++	+[1]	+	+	+[1]	+	-	+	-	-	+	-	+	-	+	+	-	-	+	+	+	-
	15	++	++	+[1]	+	+	-	+	+	-	+	-	+	-	+	-	+	+	-	+	-	+	+	+
	16	+	+	+[1]	-	+	-	-	-	+	-	-	-	-	-	-	-		-	+	++	+	+[1]	+

Dpi: days post infection; IHC: immunohistochemistry; ISH: in situ hybridization: VI: Viral isolation; [1]: Virus was isolated at 1st passage. Blank spaces indicate samples that were not examined.

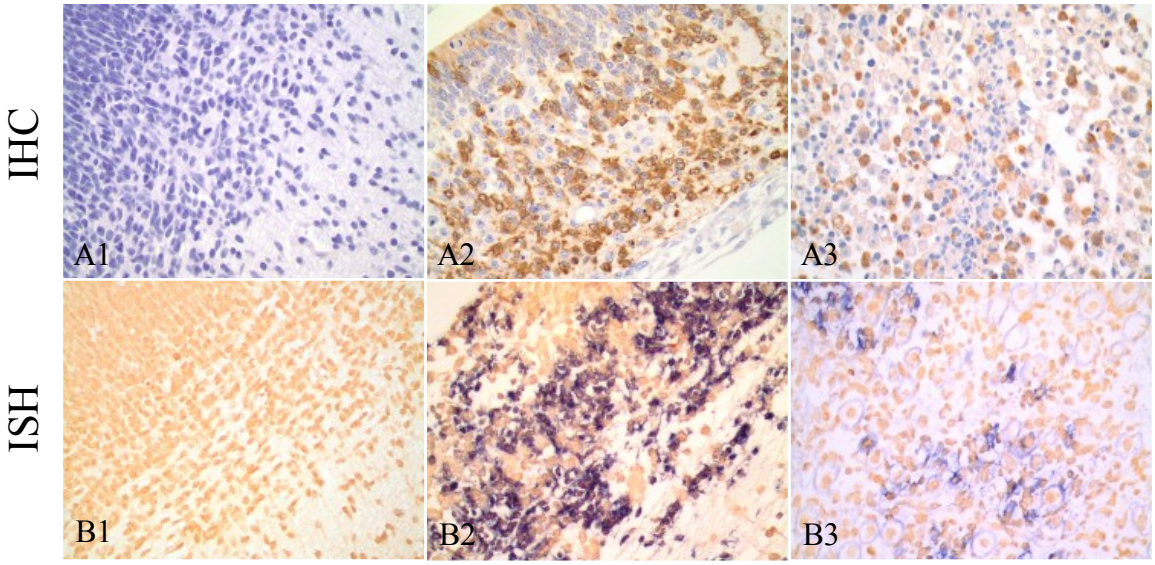

Figure 5. Immunohistochemistry and in situ hybridization in brain and skeletal muscle. A rabbit polyclonal antibody against CVV and stained with DAB chromagen (brown) was used to detect CVV protein in the brain of a control (A1) and an infected fetus (A2) and in the skeletal muscle of an infected fetus at 14 dpi (A3). In situ hybridization using a digoxigenin labeled DNA probe complementary to a conserved region of the CVV M gene glycoprotein 1 and labeled with BCIP/NBT (blue) was performed in brain from control (B1) and infected fetuses (B2) and skeletal muscle from an infected fetus at 14 dpi (B3). No viral signal was detected in the brain of control fetuses (A1, B1) Numerous cells in the brain (A2, B2) and skeletal muscle (A3, B3) are positive for CVV viral protein (A2, A3) and for the CVV M glycoprotein 1 (B2,B3). 400X mag.

Viral isolation

The virus isolation results are summarized in Table 2. Virus was isolated from most examined tissues, except for the cotyledons, where virus was isolated only from one 7-day infected fetus on 3rd passage. Virus was isolated on 1st passage mainly from BRA and SPC, as well as from amniotic fluids and membranes. No virus was isolated from blood collected from infected ewes. No virus was isolated in any tissue collected from control animals.

Serum neutralization

All infected ewes seroconverted to CVV, except for one ewe euthanized at 7 dpi. On the day of euthanasia, the serum neutralizing antibody titer for infected ewes were: 1:16 at 7 dpi, 1:8 and 1:32 at 10dpi, 1:16 and 1:32 at 14 dpi, 1:32 and 1:64 at 21 dpi, and 1:64 and 1:128 at 28 dpi. No control animals seroconverted for CVV.

Discussion

This study focused on CVV-induced, gross and histologic lesions and the distribution of the virus during infection of ovine fetuses from 35 to 63 dg (28 dpi). The brain and skeletal muscle lesions noted in this experimental infection have a distribution and degree of involvement that correlates to those observed in spontaneous natural infections.[29,31,39] Although previous experimental infection studies using the intrauterine route of inoculation with CVV have reproduced the syndrome seen in ovine fetuses in

field outbreaks.[30,39,43,67] the sequence of changes in the infected cell populations and their distribution in tissues has not been done. Success of experimental infection of ovine fetuses using the intravenous inoculation of pregnant ewes with CVV is unpredictable even with high doses of low passage isolates.[42] Low transplacental fetal infection rates have occurred in experiments with Akabane virus as well.[81,110] The reason for the low success of some arbovirus transplacental infection by intravenous infection remains a fertile area of research and is speculated to involve ability of effector molecules in vector saliva injected with the virus into the mammalian host to amplify the primary viremia.[42] In order to avoid failure of transplacental infection in this study and reduce the number of animals, the intrauterine route of inoculation was used.

The data supports the hypothesis that the fetal central nervous system and skeletal muscle are targeted in CVV infection. Severe necrosis in stained sections of brain, spinal cord and skeletal muscle of infected fetuses was observed coincident with the presence of infected cells as seen with both IHC and ISH. Such lesions reasonably would result in the hydrocephalus, porencephaly, micromyelia and body malformations described in lambs associated with CVV outbreaks. Viral signal was also observed in other cells of the peripheral nervous system, including the ocular retinal cells and in ganglion cells in the dorsal root ganglia, but the result of these lesions have not been investigated in spontaneous cases of CVV-malformed lambs. In spontaneous and experimental cases of CVV[2] and Akabane virus,[26,80,81,107] lesions in the spinal cord affect both the dorsal and ventral horns, with slightly more severe lesions involving the ventral motor neurons. In this study, most fetuses at 7-14 dpi had more severe lesions and

intense viral signal in the dorsal horns, even though, in the fetuses at 21 and 28 dpi, the loss of parenchyma affected both the dorsal and ventral horns and caused severe micromyelia. Both direct infection of the SKM and the indirect changes associated by possible denervation atrophy probably contribute to the development of musculoskeletal malformations. In addition to the CVV tropism for the SKM, the virus also appears to target other myocytes in the fetuses. Although necrosis was not seen on light microscopy, viral signal was seen in the cardiac muscle and smooth muscle of the gastrointestinal tract of some fetuses.

Fetal membranes and placental fluids have been shown to contain virus in early infection.[29,34,39,43] In this study, CVV was isolated from and it persisted in the fetal membranes and fluids throughout infection. Using IHC, virus was seen to infect cells of the amniotic and allantoic mesenchyme, but more mesenchymal cells and only rare epithelial lining cells of the amniotic membrane were infected. With the progression of the infection, fewer cells were seen to be infected, but virus levels still persisted in fetal membranes and fluids. Although functional testing of the membranes was not done, it may be speculated that viral infection disrupts the intramembranous movement of fluid in the amnion that contributes to the oligohydramnios. In ovine fetuses, the intramembranous pathway is considered a major route of absorption of water from the amniotic cavity.[48,52-54] The ISH technique failed to identify viral signal in fetal membranes. This lack of signal is presumed to be related to degradation of viral RNA from these thin specimens, most likely during sample storage in the fixative solution

prior to testing. Use of shorter fixation times for this assay is recommended in future studies.

In the first experimental infections with CVV, virus was routinely isolated from cotyledons,[30,31] but in the present study, low viral levels were detected in the fetal cotyledon in only one infected fetus. Examination of infected tissues showed limited involvement of trophoblasts. We speculate that the previous reported success in CVV isolation from this tissue could have resulted from contamination due to the proximity of high titered fetal membranes and fluids. The limited involvement of trophoblasts in the experimental infection presumably reflects the artificial intrauterine route of fetal infection. Intravenous infection with Akabane virus has been shown to cause more necrosis with more viral antigen detected in the trophoblasts and placentomes.[110]

One cause of congenital limb and axial skeletal malformations is the decreased fetal movement (fetal akinesia)[5,55,56,100,120,129,133] associated with oligohydramnios. Spinal flexion and the resulting increased pulmonary and abdominal compression along with diaphragmatic displacement have also been described in ovine fetuses with experimentally induced oligohydramnios produced by drainage of amniotic and allantoic fluids in late gestation.[63] Based on experimental studies with ovine fetal fluid drainage and the demonstration of CVV-induced oligohydramnios,[39,41] fetal akinesia also could play a role in the pathogenesis of the musculoskeletal deformities observed in CVV infection. Many processes may affect amniotic fluid volume. For example, experiments performed in ovine fetuses have also demonstrated that severe placental insufficiency resulting in hypoxia and hypercapnia, alters the amniotic fluid composition and can be

associated with the development of oligohydramnios.[51] The association of severe viral infection with development of oligohydramnios has only been reported with ovine CVV infection. In the present study, three infected fetuses displayed severe oligohydramnios with axial and limb deformities relatively early in fetal life. Obviously, the CNS and muscle lesions induced by CVV must contribute to these lesions. Abnormalities affecting the central and peripheral nervous systems are actually considered to be the most common cause of decreased fetal movement in affected fetuses with subsequent development of arthrogryposis.[55] In this study, one infected animal at 21 dpi had kyphosis, without oligohydramnios. The combined effect of the CNS and skeletal muscle lesions, and oligohydramnios early in fetal development would explain the severe axial and limb malformations often seen in lambs born after CVV infection. Even though oligohydramnios has not been described in experimental Akabane virus infections, lung hypoplasia, a component of the "oligohydramnios tetrad" associated with oligohydramnios in human fetuses,[120,141,143] has been seen in some ovine fetuses experimentally infected with Akabane virus.[107] Considering this finding, oligohydramnios may occur with other bunyaviral infections.

Another mechanism associated with the development of oligohydramnios is renal disease. In this study it was seen that CVV-infected scattered cells of the renal tubules and glomerulae. The ovine fetal mesonephros is functional and able to produce urine that is excreted into the allantoic cavity as early as 30 dg.[35,150,151] The vast majority of the urine produced in early gestation is primarily transferred to the allantoic cavity via the urachus.[6,147,151] The origin and control of amniotic fluid during early gestation of ovine

fetuses has not been completely elucidated. However, since the amniotic fluid during early gestation is believed to be primarily an ultrafiltrate of fetal blood plasma, renal disease could possibly affect amniotic fluid volume.[28,92] If one considers the possibility that the fetal urine production also contributes to the formation of the amniotic fluid in early gestation, even mild infection of the fetal kidney could possibly affect fetal urine production and consequentially contribute to oligohydramnios. In any case, renal virus load is mild, and necrosis was only observed in one fetus at 28 dpi.

The IHC and ISH protocols developed to identify CVV in experimentally infected tissues from ovine fetal tissues, could be further used for diagnostic purposes to confirm or rule out natural cases of CVV on properly collected tissues. Both techniques tested specific for CVV, and could be used to differentiate between CVV and Akabane virus infection, since no signal was detected in specimens previously confirmed to be positive for Akabane virus by IHC. Certainly in areas where other bunyaviruses are active, it would be advisable to test the specificity of this antibody to other viruses. In terms of sensitivity, the IHC seemed more sensitive than ISH and identified viral protein in some infected tissues not identified with ISH. Additionally, IHC frequently detected a higher percentage of cells infected with CVV in different tissues. However, some sensitivity in identifying virus may be improved by reducing fixation times particularly in fetal membranes. Both IHC and ISH represent excellent tools for further investigational studies, as well as for diagnostic purposes in the detection of CVV in early abortions.

CHAPTER III

CACHE VALLEY VIRUS CENTRAL NERVOUS SYSTEM TROPISM AND TARGET CELLS IN INFECTED FETUSES

Introduction

Cache Valley virus (CVV) was first isolated from a mosquito pool collected in 1956 in Cache Valley, Utah.[69] CVV is endemic in the United States,[21,22,95] and has been established as an infectious cause of abortion and fetal malformations in small ruminants.[29-31,39,44] Serologic evidence of human infection with rare reported cases of encephalitis and meningitis are also described.[23,25,93,128,142,148]

CVV is a member of the family *Bunyaviridae*, *Orthobunyavirus* genus, Bunyamwera group. The viruses within the *Orthobunyavirus* genus are transmitted by mosquitoes, and this genus includes several viruses that infect or cause encephalitis in humans, including the members of the California group, such as: California encephalitis virus, La Crosse virus, Jamestown Canyon virus, Snowshoe hare virus. A member of the Simbu group, Akabane virus, causes abortions and fetal malformations in ruminants, similar to those caused by CVV. [11,126,127] The forms of diseases in mammalian hosts infected with orthobunyaviruses can be simplistically divided into either primary CNS infection with encephalitis in mature animals or transplacental fetal infections with subsequent fetal malformations. [109]

In sheep, CVV is responsible for embryonic and fetal death, stillbirths and congenital central nervous system (CNS) and musculoskeletal system (MKS) malformations.[29,30,34,39,44] It has been shown that CVV[30,31,39,44] and Akabane virus[26,36,108,110] infect cells primarily in the brain and spinal cord of sheep; however, no studies have determined which cells within the CNS are susceptible to viral infection. Nor has any study described changes in the resident cell populations in the CNS beyond necrosis. In order to determine specific cell populations within the CNS that are infected by CVV, as well as any changes in these cell populations that may occur, we employed indirect fluorescent microscopy using a double-labeling technique against CVV and different cell types within the CNS.

Materials and methods

The ovine fetal samples used in this study were collected from pregnant ewes infected with CVV as described on Chapter II. Briefly, CVV-seronegative ewes at 35 dg were inoculated in utero with 1ml of minimum essential medium (MEM) (mock-infected/control group) or with a 1ml inoculum containing 10^5 50% tissue culture infection doses ($TCID_{50}$) of a second passage of a CVV isolate from the infected allantoic membranes of an experimental study.[30] Three ewes (one mock-infected and two virus-infected) were humanely euthanized at 7, 10, 14, 21, 28 dpi (dpi). Samples were harvested and fetal tissues were collected and fixed in 4% paraformaldehyde or in

Davidson's AFA (acetic acid, formalin, alcohol) fixative for routine histologic processing paraffin-embedding.

Paraffinized tissue sections of the brain and spinal cord from control and infected fetuses were mounted on positive-charged, silanized slides, deparaffinized and heat treated at 120°C for 30 min in sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH6.0) for antigen unmasking and retrieval. Sections were further washed with phosphate buffered saline (PBS) and blocked with PBS-T (1X PBS, 0.1% Triton X-100) containing 5% goat serum for 1 hour at room temperature. The slides were incubated at 4°C overnight with antibodies against neurons (mouse anti-NeuN, clone A60, Millipore, Temecula, CA; diluted 1:80); astrocytes (mouse anti-glial fibrillary acidic protein (GFAP), clone GA5, Millipore; diluted 1:300); neural stem cells and macroglia (rabbit polyclonal anti-Olig2, Millipore; diluted 1:100); or microglia (rabbit polyclonal anti-Iba1, Wako, Chuo-Ku, Osaka, Japan; diluted 1:500). After several washes, the secondary antibody conjugated with Alexa Fluor 594 (Invitrogen, Eugene, OR; diluted 1:1000) was applied and incubated for 1 hour at 25°C. Following additional washes, sections were incubated overnight at 4°C with a second primary antibody, anti-CVV (diluted 1:300). After several washes, the secondary antibody conjugated with Alexa Fluor 488 (Invitrogen; diluted 1:1000) was incubated for 1 hour at 25°C. Finally, the nuclei were stained with Hoechst 33258 (Invitrogen) at a final concentration of 2 μg/mL for 1 min, and a solution containing Sudan Black was used to inhibit autofluorescence. Sections were mounted and coverslipped with Fluoromount-G (Southern Biotech, Birmingham, AL) and kept in the dark at 4°C. The sections labeled

with anti-Iba1 (rabbit) were not double-labeled with CVV. In order to do double-labeling with CVV and Iba1, additional serial sections were stained with another antibody against microglia (a goat polyclonal anti-Iba1, dilution 1:300; Abcam, Cambridge, MA) using a modified protocol using PBS-T containing 5% donkey serum to block non-specific binding. The first primary antibody used was CVV followed by binding with secondary antibody AlexaFluor 488. Sections were blocked again with 5% rabbit serum, and anti-Iba1 (goat; diluted 1:300) was used as the second primary antibody followed by binding with the secondary antibody AlexaFluor 594. In addition, the following antibodies were also tested in the brain and spinal cord of control and infected fetal tissues at 63 dg: myelin proteolipid protein (PLP) (mouse anti-PLP, clone AA3, gift from Dr. Jianrong Li, Texas A&M University; dilution 1:500), Nestin (rat anti-nestin, clone 401, dilution 1:200; Millipore), neurofilament (mouse anti-phosphorylated neurofilament, clone SMI31, dilution 1:200; Covance, Emeryville, CA), myelin associated glycoprotein (MAG) (hamster polyclonal ascitic fluid anti-MAG, not diluted; gift from Dr. Jianrong Li, Texas A&M University), myelin basic protein (MBP) (rat polyclonal anti-MBP, dilution 1:100;Millipore), Olig1 (mouse polyclonal anti-Olig1, dilution 1:100; Millipore). Slides were incubated, washed and stained with similar concentrations of Alexa Fluor conjugated antibody as above to assay for activity. The primary antibody was not used in negative control sections.

Tissue images were captured with a fluorescence microscope (Olympus IX71) equipped with an Olympus DP70 digital camera (Leeds Instruments, Irving, TX, USA). To evaluate changes in microglial cell populations and expression of GFAP in infected

and non-infected fetuses, images were obtained from ten different fields for each antibody and color intensity was measured using ImageJ (National Institute of Health (NIH), USA). The background of captured images was equally corrected for each antibody using ImageJ.

The averages of the measurements obtained for each slide were subjected to the least square analysis of variance (ANOVA) to determine differences between infected and non-infected groups. Differences between groups of different dpi were subjected to the one pair students t-test. In order to reduce heterogeneity between samples, the data was log-transformed, and *p*-values below 0.05% were considered statistically significant. The data is presented as means with overall standard error.

Results

The NeuN antibody identified the nuclei of developing neurons in the brain and gray matter of the spinal cord of control and infected fetuses at different dg. A large amount of CVV viral antigen signal was detected around the neuronal nuclei, and this was interpreted to be within the cytoplasm of neurons (Fig. 6). In addition, viral signal was detected free within the parenchyma and within the cytoplasm of NeuN-negative cells. CVV viral signal was distributed diffusely within the telencephalic walls, brainstem and spinal cord at 7, 10, 14 and 21 dpi, and only scattered foci had viral positive cells in the brain and spinal cord at 28 dpi, as shown in Chapter II.

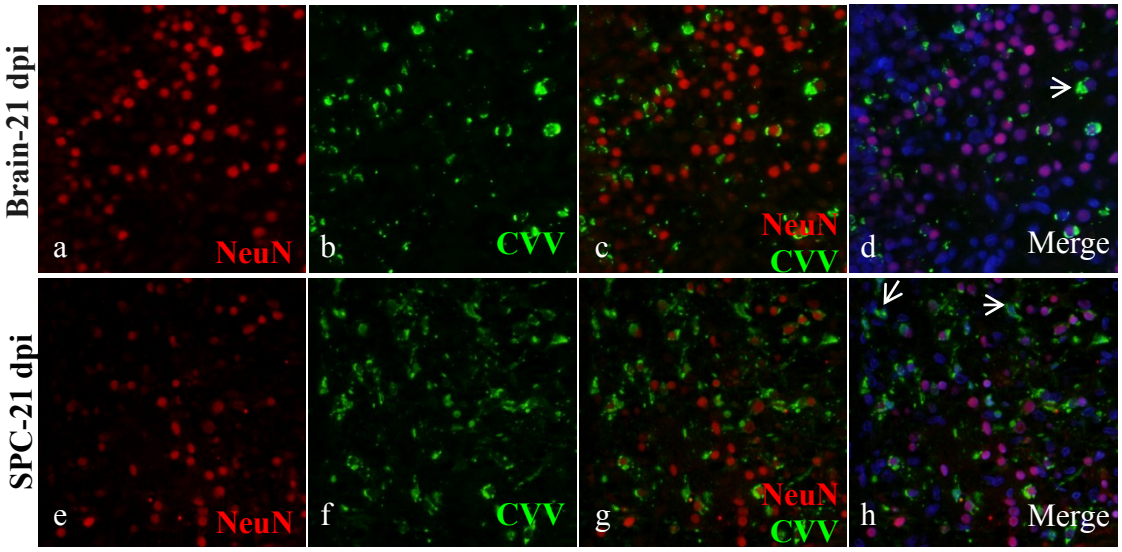

Figure 6. Immunofluorescence for NeuN and CVV in the brain and spinal cord of infected fetuses. Sections of CNS labeled with NeuN (a,e), CVV antigen (b,f), double labeled with CVV (c,g) and double-labeled merged with Hoechst nuclear staining (d,h). Large numbers of NeuN-positive cells in the brain (c,d) and spinal cord (g,h) have intracytoplasmic viral signal. Viral signal is shown in the cytoplasm of NeuN-negative cells (d,h) (arrows).

GFAP was weakly expressed in the fetal brain after 56 dg (21 dpi); whereas, GFAP expression was stronger and more consistent in the spinal cord after 49 dg (14 dpi). In the brain, GFAP expression was mainly seen in periventricular regions. The spinal cord had more diffuse and intense GFAP expression. Usually, CVV viral signal did not colocalize with astrocytic processes and cytoplasm. No significant difference was observed in the expression of GFAP in sections of brain and spinal cord of infected versus non-infected fetuses (Fig. 7).

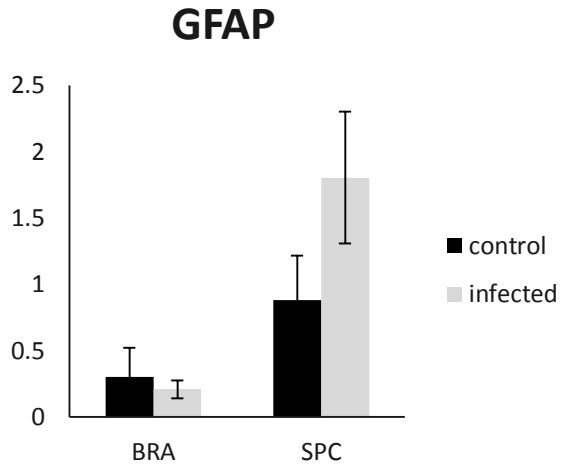

Figure 7. ImageJ measured intensity of GFAP in the brain and spinal cord of control and CVV-infected fetuses. No significant difference was detected in brain and spinal cord labeled with GFAP. $p > 0.05$

Both Iba1 antibodies used in this study detected microglia in the brain and spinal cord of control and infected animals. The goat anti-Iba1 was used for double-labeling with CVV; whereas, the rabbit anti-Iba1, due to its better cellular definition, was used to determine microglial cell numbers in the CNS. No colocalization between CVV and the cytoplasm of microglial cells was observed in examined sections from the brain and spinal cord of infected or control fetuses. Even though viral signal was not observed in microglial cells, a significant increase in the expression of the microglial marker (Iba1) was observed in sections of brain (p-value <0.05) and spinal cord (p-value <0.005) in infected versus non-infected fetuses, especially around areas with abundant viral signal (Figs. 8 and 9). The data was pooled into two groups, infected and control, because no significant difference was observed between infected fetuses comparing each dpi, or control fetuses comparing each dg. Increased expression of the microglial marker were in infected fetuses as early as 42 dg (7 dpi) and even though a significant difference was not observed between different days of infection, on average, the highest expression of the microglial marker was observed in the brain and spinal cord at 14 dpi in infected fetuses followed by a progressive reduction in the expression of the microglial marker in the brain at 21 and 28 dpi. A constant reduction of the expression of the microglial marker was noted in the spinal cord at 21 and 28 dpi.

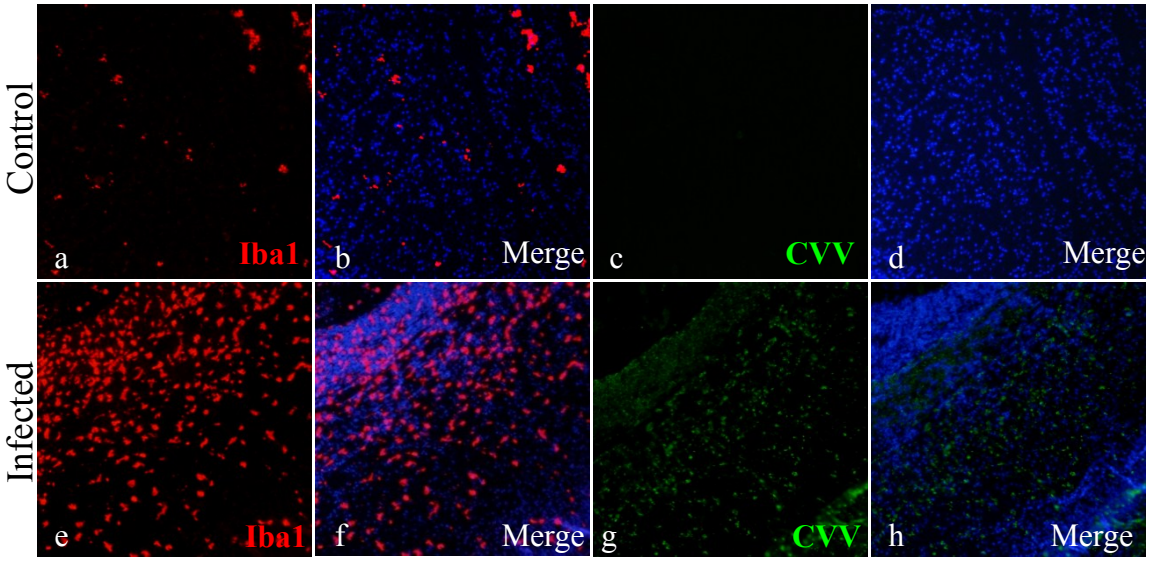

Figure 8. Immunofluorescence for Iba1 and CVV in the brain. Serial sections from the cerebrum of a control (a-d) and an infected (e-h) fetus at 63 dg/28dpi. The control fetus has few Iba1-positive cells (a,b), and there is no viral signal (c,d). The infected fetus has a marked increase in the number of Iba1-positive cells (microglia) (e,f). The same region has strong viral signal (g,h).

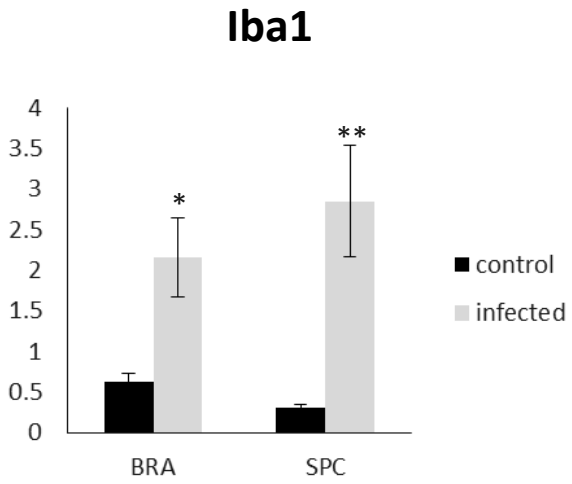

Figure 9. ImageJ measured intensity of Iba1 in the brain and spinal cord of control and CVV-infected fetuses. A significantly greater intensity of Iba1 was in the brain and spinal cord of infected versus control fetuses. *$p < 0.05$, **$p < 0.005$.

Olig2-positive cells were identified multifocally in the brain of control and infected animals after 45 dg (10 dpi) and scattered cells were identified in the spinal cord after 49 dg (14 dpi). In the brain, these scattered Olig2-positive cells were identified in areas that corresponded to regions with large numbers of NeuN-positive cells. Scattered viral signal was observed around Olig2-positive cells in the brain of infected animals at 14, 21 or 28 dpi, and only rare Olig2-positive cells in the spinal cord had intra-cytoplasmic viral signal.

Antibodies against myelin proteolipid protein (PLP), Nestin, Neurofilament, MAG, Olig1 and MBP failed to detect cellular and/or parenchymal expression in the brain and spinal cord of tested samples.

No immunofluorescence was seen in sections where the primary antibody was omitted.

Discussion

This study attempted to identify specific cell populations within the CNS that may be infected by CVV and how these populations change during and after CVV infection in ovine fetuses. This study also focused on evaluating changes in residing CNS cell populations in response to CVV infection. The major findings were that CVV primarily targets neuronal cells and not glial cells and that the infection is associated with a severe microgliosis in the brain and spinal cord of 42 to 63 dg infected fetuses.

NeuN, a monoclonal antibody that detects a neuron-specific nuclear protein, has been previously studied and used to detect neurons in fetal and adult human and rodent brain,[101,125] as well as neurons of middle to late gestation ovine fetuses.[91,135] CVV viral signal was detected primarily in neurons and, rarely, was detected in the cytoplasm of astrocytes and Olig2-positive cells. Areas with extracellular viral signal corresponded to areas of necrosis and likely represent areas with breakdown of cells and release of viral antigen. Even though the largest amount of viral signal was detected in the cytoplasm of neurons, viral signal was identified in the cytoplasm of numerous cells not labeled with NeuN. Although these were morphologically similar to NeuN-positive cells, their identity remains unknown. Human fetal brain NeuN-positive cells are expressed early in gestation, and increased numbers are seen in different regions of the brain with the progression of gestation.[125] For this reason, we believe that the NeuN-negative cells were likely primitive neurons or neuronal cells not expressing NeuN at the evaluated time points. Conceivably, these cells could represent premature glia as well.

Ionized calcium binding adaptor molecule 1 (Iba1) is a macrophage specific protein, highly expressed in microglia. Iba1 genes are conserved between species, and identical cDNA sequences have been identified in homolog proteins AIF, MRF-1, ITR-1 and BART-1in humans and rodents. Iba1 is expressed in resting and activated microglial cells.[70,86] and injured neurons release factors that activate microglia.[140] A significant microgliosis was identified in infected fetuses as early as 42 dg. The microgliosis observed in the brain and spinal cord of infected animals could be linked to an early fetal development of an immune response to viral infection. In fact, upregulation of

proinflammatory cytokines associated with innate immune response are observed in CVV-infected fetuses (shown in Chapter IV). Some of these cytokines, such as IL-1, IL-6 and TNF-α, are known to be produced by activated microglia in response to neuronal injury.

Iba1 antibodies identified microglial cells in the brain of control and infected animals; however, the rabbit Iba1 gave better definition of the microglial cytoplasm and cellular membranes. Therefore, this antibody was used for determination of microglial expression in the CNS. Because we were unable to do double-labeling with two antibodies from the same host species (the anti-CVV antibody was a polyclonal antibody made in rabbits), the goat Iba1 was used for double-labeling with CVV. No colocalization of CVV with microglial cells was detected in examined specimens.

Microgliosis induced by neuronal damage is usually associated with astrocyte activation in mature brains. Activated microglial cells release proinflammatory cytokines that stimulate development of astrogliosis and CNS scar formation.[140,155] In the present study, no significant increase in GFAP-positive cells or astrocytic processes was observed in infected fetuses compared to control fetuses. GFAP expression was low in earlier control and infected fetuses, but increased expression was noted in all fetuses at 56 and 63 dg (21 and 28 dpi), primarily in the spinal cord. The most likely reason for the lack of astrogliosis in infected fetuses is the presumed immaturity of astrocytes in early developing fetuses. Previous studies evaluating ovine fetal brain at 60 dg identified GFAP-positive cells only after 80 dg.[68] In human fetuses, low numbers of GFAP-positive cells are identified mainly around the ventricles at/after 12 weeks of gestation,

which corresponds to 45 dg in ovine fetuses, even though vimentin-positive cells, presumed to be immature astrocytes, have been identified in 5 weeks old human fetuses.[144]

Olig2 is a basic helix-loop-helix transcription factor that was initially described to be specific for oligodendrocytes. Further studies revealed that this transcription factor is expressed throughout the CNS also being expressed in neural stem cells that will differentiate into neurons and astrocytes.[104] In the ventral spinal cord motor neuron areas, a population of Olig2-positive neural progenitor cells give rise to motor neurons and then to oligodendrocytes.[84] The differentiation of Olig2-positive cells has been investigated.[84,85,104] Even though complex and not completely elucidated at this point, it appears that Olig2-positive cells differentiation in fetuses depends on the anatomical location of these cells and up/downregulation of the Olig2 gene, with some Olig2-positive neural stem cells becoming neurons, astrocytes or oligodendrocytes. In the forebrain, large numbers of neuron progenitors are Olig2-positive. Late Olig2-positive cells in the telencephalon will likely differentiate into astrocytes, whereas Olig2-positive cells in the diencephalon, spinal cord and hindbrain most likely give rise to oligodendrocytes.[85,104] Similar studies using Olig2 have not been conducted in the brain of ovine fetuses during early development; however, if the known differentiation and distribution of Olig2-positive cells in the brain and spinal cord of fetal mice and humans applies in sheep, the few Olig2-positive cells with cytoplasmic viral signal identified in this study could be neuronal progenitor cells. This is reasonable considering that these Olig2-positive cells also were found in areas that have large numbers of NeuN-positive

cells. If some of the detected Olig2-positive cells were being programmed to develop into oligodendrocytes or astrocytes, that would support our conclusions that CVV does not target these cell lineages, as reflected by the low amount of viral signal observed in the cytoplasm of the differentiated cells. Further studies to determine the specific distribution and differentiation of these cells in the brain and spinal cord of ovine fetuses await new fetal markers before final conclusions can be made regarding all early targets of CVV.

Several other antibodies against mature or immature oligodendrocytes (Olig1, MBP, MAG) were used in sections of the brain and spinal cord of ovine fetuses in this study in an attempt to specifically identify oligodendroglial cells. Unfortunately, the antibodies used failed to identify cells in the ovine fetus. Similarly, antibodies to identify neuronal progenitor cells (Nestin) and neuronal cells (Neurofilament) also failed to detect cells in the CNS of the ovine fetus. For the most part, these antibodies have not been tested in young ovine fetuses. Because these were developed for other species, they may not cross react with ovine tissues, or the cells of the immature fetuses studied here have not expressed the markers yet.

CHAPTER IV

OVINE FETAL INNATE IMMUNE RESPONSE TO CACHE VALLEY VIRUS INFECTION

Introduction

Cache Valley virus (CVV) is a mosquito-borne bunyavirus of the family *Bunyaviridae*, *Orthobunyavirus* genus, Bunyamwera group,[127] and endemic in the North America.[39] Serologic studies have shown that unlike most bunyaviruses, the vectors of CVV also infect larger mammals with virus being isolated from sheep, cattle and horses, and it causes reproductive losses in small ruminants.[29-31,34,39,40,44,45] Rarely, the virus has been associated with meningoencephalitis in humans.[25,128] "In utero" ovine fetal infection causes abortion and fetal malformations, mainly affecting the musculoskeletal (MSS) and central nervous systems (CNS).[29-31,34,39,44]

Previous studies describing and experimentally reproducing CVV-induced malformations in ovine fetuses showed that the development of fetal lesions depends on the fetal age when infected. If the virus is inoculated between 28 and 48 dg, fetal death and abortion or MSS and CNS malformations occur. No apparent malformations are observed if the virus is inoculated after 48 dg.[30,39] Virus isolation only can be made from early infected fetuses, and viral recovery from tissues of term abortions and malformed lambs is uniformly unsuccessful. The virus is cleared from infected tissues within a few

weeks after infection and before the presumed age of fetal immunocompetency, at approximately 70-75 dg.[39,94,113,127]

The gestation period of the ewe is approximately 145 days. Ovine fetuses develop erythropoiesis, myelopoiesis and megakaryopoiesis in the yolk sac and liver at approximately 17 dg. At approximately 20 to 25 dg, lymphocyte production begins in the thymus, and lymphocytes are in the bloodstream at 48-50 dg.[99] At 45-50 dg, T and B lymphocytes and cells with surface immunoglobulins are seen in the spleen and lymph nodes.[87,88] The lymph nodes become integrated into a lymphatic system around 65 dg.[99] Establishing the time when fetuses are able to respond to antigens is difficult because available data is based on use of assays such as serum neutralization that were established for use in mature animals. Additionally, gestation assay points used in experiments are somewhat arbitrary. Ovine fetal antibody response to viral infection at titers greater than 1:2 has been detected after 76-78 dg.[106] Because the ruminant syndesmochorial placenta prevents passage of immunoglobulins from the ewe to the fetus,[145] antibodies in fetuses and precolostral newborns are those produced by the fetus. For this reason, *in utero* viral infections can be diagnosed in aborted fetuses and stillborn ruminants using serum neutralization tests. Such testing is necessary with CVV because the fetus clears the virus long before the end of gestation.[34,39]

In Chapter II, it was demonstrated that fetuses infected with CVV early in gestation (around 35 dg) have low viral signal in tissues around 56 dg and are able to clear the infection before development of an adaptive immune system at 75 dg.[39] Similar, age-based findings have been described in Akabane virus-infected

fetuses.[26,94,106] Because no effective serum neutralization antibody (an adaptive immune response) has been shown in ovine fetuses at the time when CVV is cleared from fetuses, it may be that the ovine fetus mounts an innate immune response for early viral clearance. To test this hypothesis, expression of selected genes associated with the innate immune response were compared in tissues of CVV-infected and non-infected ovine fetuses, and CVV viral mRNA was quantitated in selected tissues to correlate the measured innate response with viral clearance. In addition, fetal MX protein, an interferon-stimulated GTPase previously associated with antiviral activity against bunyaviruses,[50,66,76,124,139] was quantitated in fetal allantoic and amniotic fluids. Expression of the interferon stimulated gene 15 (ISG15), that has been shown to be produced by fetuses in response to viral infection in utero,[27,130,134] was evaluated in CVV-infected fetal tissues. Finally, distribution of B and T lymphocytes and immunoglobulin-positive cells was evaluated in infected and non-infected, ovine fetal tissues in early gestation.

Materials and methods

Virus inoculation and sample harvesting

A group of fifteen, seronegative, pregnant Rambouillet ewes were housed in BSL2 confinement buildings according to protocols approved by The Institution Animal Use and Biosafety Committee. At 35 dg, ewes were inoculated in utero with a 1ml inoculum containing 10^5 50% tissue culture infection doses ($TCID_{50}$) of CVV (infected group) [3] or

1ml of minimum essential medium (MEM) (mock infected/control group) as previously described in Chapter II. The viral inoculum was derived from the second passage of an isolate from allantoic membrane from an experimentally infected fetus.[30] At 7, 10, 14, 21 and 28 dpi, three ewes (one mock-infected and two virus-infected) were humanely euthanized. Selected fetal tissues and their fluids were harvested and immediately frozen at -80ºC or placed in an RNA stabilization solution (Ambion, Life Technologies, Carlsbard, CA) and frozen at -80ºC. The remaining fetal tissues and placenta were fixed in 4% paraformaldehyde and in Davidson's AFA (acetic acid, formalin, alcohol) fixative.

Real time quantitave polymerase chain reaction (qPCR)

To quantitate relative numbers of CVV viral copies, and genes of interest, real time qPCR was performed on samples of brain and muscle harvested from CVV-infected or mock-infected ovine fetuses according to a previously published protocol with minor modifications.[37] Briefly, RNA was isolated from harvested samples and homogenized in Trizol reagent (Gibco BRL, Bethesda, MD) according to the manufacturer's recommendation. In order to eliminate contamination with genomic DNA, the extracted RNA was treated with the DNAse1 amplification reagent (Invitrogen, Carlsbard, CA), and the RNA concentration was quantified by spectrophotometry. RNA quality was determined by denaturing samples in an agarose gel electrophoresis. The cDNA was synthesized from 500ng of total RNA combined with primer mix containing oligodT primer (0.2μg/ml), random hexamer primer (300

μg/ml; Invitrogen), dNTPmix (10mM each) and incubated at 65ºC for 5 min. After incubation, SuperScript II reverse transcriptase (Invitrogen) was added to the reaction according to manufacturer's recommendations and reverse transcribed under the following conditions: 25°C for 10 min; 42°C for 60 min and 70ºC for 5 min. To test for genomic DNA contamination, control reactions were prepared without reverse transcriptase.

Specific oligonucleotide primers of interest (Table 3) were obtained from Oligo 5 program (Molecular Biology Insights, Inc) for the following genes: M segment of glycoprotein 1 of CVV, interferon-α (IFN-α), IFN-β, TNF-α, IL-1, IL-6, Mx1, Mx2, ISG15, TLR7 and TLR8. The real-time qPCR reaction was performed using the ABI prism 7900HT system (Applied Biosystems, Foster, CA) with power SYBR green PCR Master Mix (Applied Biosystems). Primer specificity and efficiency (-3.6>slope>-3.1) were confirmed using a test amplification run. The data was normalized using cycle threshold (Ct) values for the ovine glyceraldehyde 3-phosphate dehydrogenase (GADPH) gene in each sample. Semi quantitative analysis was calculated with the ΔΔCT method, and expressed as relative fold changes compared with the lowest value for mock-infected samples. To calculate relative amounts of viral mRNA, relative fold changes were compared with the infected sample with the lowest ΔΔCT.

Table 3. Primer sequences used for RT-qPCR

Gene name	Forward	Reverse
CVV	CAC CAG CGA AAT CCC AAT CAC CA	CTC CAG ACA TAG CAC CCA CCA
TNF-α	CGG CGT GGA GCT GAA AGA CAA	CTG CGA GTA GAT GAG GTA AA
IL-1	AGT GAT GGC TTG CTA CAG T	CCG AGG TCC AGG TGT T
IL-6	GAG GGA AAT CAG GAA ACT GT	CTC GTT TGA GGA CTG CAT CT
TLR7	ACT CCT TGG GGC TAG ATG GT	GCT GGA GAG ATG CCT GCT AT
TLR8	TCC ACA TCC CAG ACT TTC TA	GTT CTT GTC CTC ACT CTC TT
MX1	GTA CGA GCC GAG TTC TCC AA	ATG TCC ACA GCA GGC TCT TC
MX2	CAT CCA TAA ATC GCT CCC CTT GT	GCT CCT CTG TCG CCC TCT GGT
ISG15	TGA CGG TGA AGA TGC TAG GG	ACT GCT TCA GCT CGG ATA CC
IFN-α	ACC CAG CAC ACC TTC CAG CTC TT	CCT CGC AGC CCC TCC TC
IFN-β	TGG TTC TCC TGC TGT GTT TCT C	CGT TGT TGG AAT CGA AGC AA

Protein slot blot

The protein concentration in the allantoic and amniotic fluid was determined using the Bradford Assay as recommended by the manufacturer (Bio-rad Laboratories). According to the protein concentration, samples were prepared and diluted to 12µg of protein for the amniotic fluid and 50µg of protein for the allantoic fluid in a final volume of 200µl of Tris-Buffered saline (TBS). The volume was deposited onto a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA) in a Bio-dot SF micro-filtration apparatus (Bio-Rad Laboratories). Nonspecific binding sites were blocked by immersing the membrane in 5% skim milk in TBS-Tween (TBST) for 1 hour at room temperature on an orbital shaker. The membranes were incubated overnight at 4°C in 2.5% milk-

TBST containing a rabbit anti-ovine Mx1 polyclonal antibody (gift from Dr. Troy Ott, Penn State University)[73] at a concentration of 1:10000. The membrane was then incubated for 1 hour with goat anti-rabbit IgG (1:10000 dilution in 2.5% milk-TBST) and further incubated for 1 min with Bio-Rad chemiluminescence reagent. The concentration of each protein in the samples was determined quantitatively in relative light units using Bio-Rad chemidoc imager.

Immuhistochemistry

Immunohistochemistry was performed on 5µm, deparaffinized sections of paraformaldehyde-fixed tissues mounted onto positively-charged, silanized slides using an automated system for immunohistochemistry (DakoCytomation Autostainer®, Dako, Carpinteria, CA) with minor modifications to the IHC protocol described in Chapter II. Antigen retrieval was performed in a decloaker chamber (Biocare Medical, Concord, CA) with slides soaked in citrate buffer pH 6.0. Sections were incubated with anti-ISG15 polyclonal antibody (diluted 1:500) for 30 min (Dr. Thomas Hansen, Fort Collins, CO),[10] CD3 (diluted 1:400) polyclonal antibody for 35 min (Dako), anti-CD79a antibody, clone JCB117 (diluted 1:200) for 30 min (Dako), anti-ovine IgG (diluted 1:500) for 35 min and anti-ovine IgM (diluted 1:2000) for 30 min (KPL, Inc, Gaithersburg, MD). The primary antibody was followed by incubation with the

secondary antibody (MACH 2 anti-rabbit Biocare Medical [CD3, IgG, IgM]; or ImmPress anti-Mouse Ig, Vector Labs [ISG15, CD79a]) for 20 min. A chromagen complex, 3.3-diaminobenzidine tetrachloride (DAB, Dako), was used to detect the targeted antigens, and sections were counterstained with hematoxylin. Slides were coverslipped with permount mounting solution. Tissues were evaluated to determine percentage of infected cells in each examined organ and graded as follows: less than 3% of cells positive (mild); between 3-15% of cells positive (moderate); more than 15% of cells positive (marked).

Statistical analysis

The quantitative data for mRNA expression and protein levels on fetal fluids were subjected to the Wilcoxon signed rank test to determine differences between infected and non-infected groups. Differences between groups at different dpi were subject to the one pair students t-test. In order to reduce heterogeneity between samples, the data was log-transformed if necessary, and *p*-values below 0.05% were considered statistically significant. The data are presented as means and overall standard error.

Results

Real-time qPCR

No significant difference in gene expression was observed either between infected animals at each different dpi or between control animals at different dg. Therefore, the data for each gene for each evaluated tissue was pooled into two groups: control and infected animals (Fig. 10). The following genes were significantly upregulated (p-value <0.05) in the brain and skeletal muscle of CVV-infected fetuses versus noninfected fetuses: IL-1, IL-6, Mx2, ISG15, TLR7 and TLR8. Only in the brain was TNF-α significantly upregulated. Except for ISG15 and Mx1, significantly higher upregulation of these genes was observed in the brain compared to the skeletal muscle. Similarly, the relative number of copies of CVV was significantly higher in the brain when compared to the skeletal muscle (Fig. 11). Progressively lower means of relative number of copies of CVV were observed in both brain and skeletal muscle with the progression of the infection. A statistically significant difference was observed in the brain of fetuses between 14-28 dpi (CVV in the brain of 10dpi fetuses were excluded from analysis due to high variability in those samples), and in the SKM when comparing 10-14 dpi, 10-21 dpi, and 10-28 dpi.

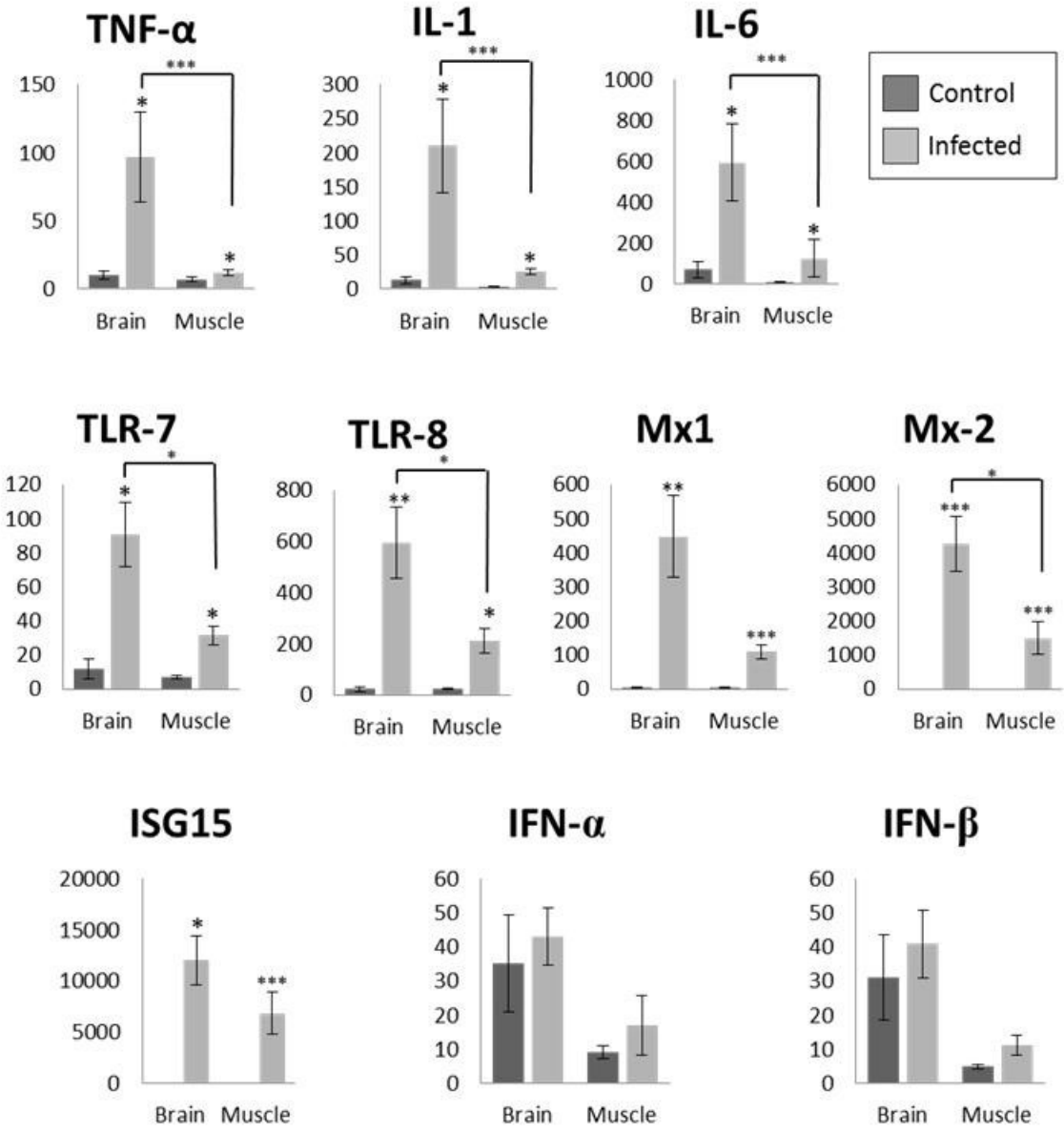

Figure 10. Relative expression of genes related with immune response in brain and skeletal muscle of control and CVV-infected animals. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

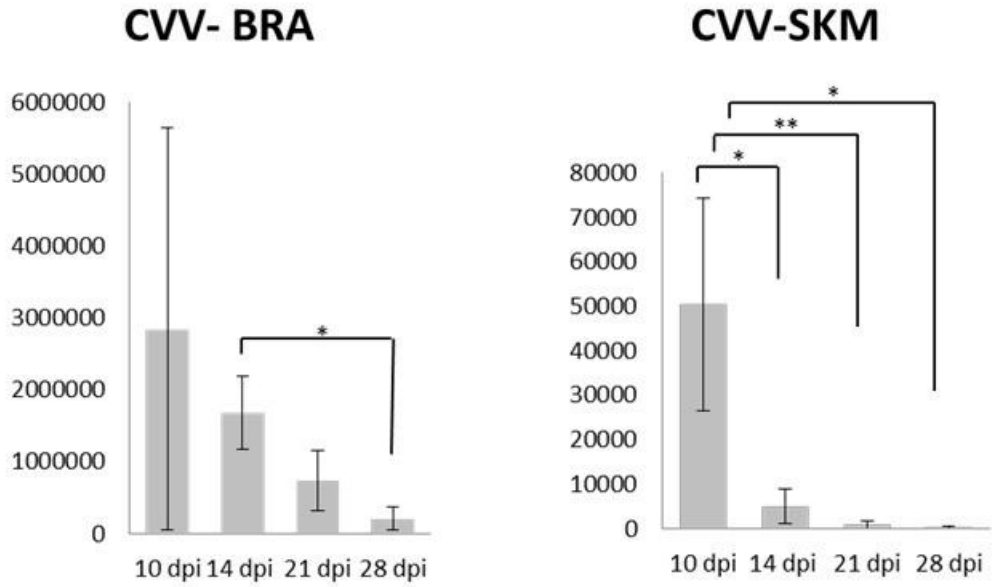

Figure 11. Relative number of CVV viral copies in main target tissues, brain (BRA) and skeletal muscle (SKM) after 10, 14, 21 and 28 dpi. *$p < 0.05$, **$p < 0.01$.

Protein slot blot

A significant (p-value <0.01) increase in the amount of Mx1 protein in the allantoic and amniotic fluid was observed in infected fetuses versus non-infected fetuses at each time point examined (Fig. 12). Similar to the quantitative data collected with the real time qPCR, no effect of day or interaction of day versus infection was seen in evaluated samples.

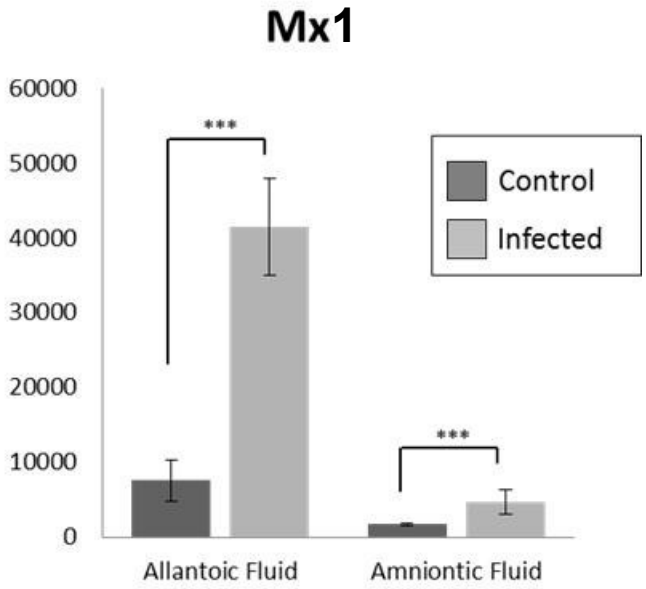

Figure 12. Amount of ovine Mx1 protein in allantoic and amniotic fluid in control and infected animals. $p < 0.005$

Immunohistochemistry for ISG15

ISG15 was detected in several tissues of all infected fetuses, but no ISG15 immunolabeling was observed in control fetuses (Fig. 13). In infected fetuses, a strong signal was observed in numerous cells of the cerebral parenchyma and meninges, and fewer cell numbers were positive in the spinal cord. Most fetuses at 7-21dpi had marked ISG15 signal in the brain, with moderate signal observed at 28 dpi. The ISG15 signal in the spinal cord of infected fetuses varied between mild to moderate at all test points. Multifocal clusters of cells with positive immunolabeling were observed in the SKM of infected fetuses through 21 dpi with only rare cells with positive signal at 28 dpi. Other

tissues with clusters of cells with positive signal included: heart, smooth muscle of intestine, tongue, fibroblasts in the subcutaneous tissue, wall of large arteries, around small vessels, spleen (one fetus at 21dpi), and rarely in the lungs, tonsils and thymus of earlier infected fetuses.

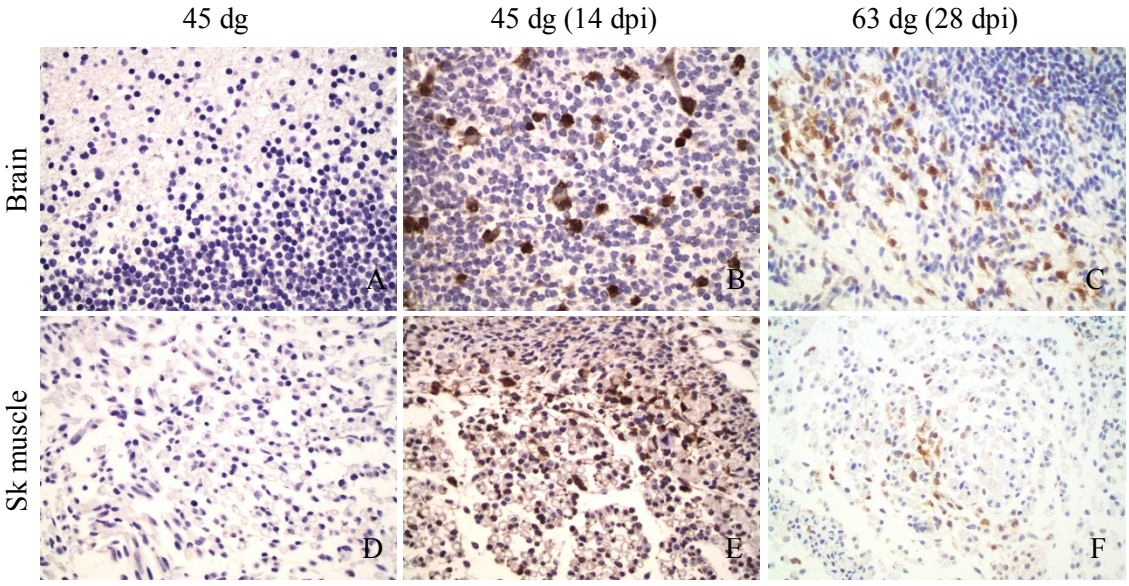

Figure 13. Immunohistochemistry for ISG15 in brain and skeletal muscle. A rabbit polyclonal antibody against ovine ISG15 protein was stained with DAB chromagen (brown) on the brain (A,B,C) and skeletal muscle (D,E,F) from 45 dg control fetuses (A,D) and from infected fetuses at 14 dpi (45 dg) (B,E) and 28 dpi (63 dg) (C,F). Numerous cells in the brain and skeletal muscle of infected fetuses are positive for ISG15. No positive cells were observed in tissues of control fetuses.

Immunohistochemistry of B and T lymphocytes, IgG and IgM

At 7 dpi/42 dg, scattered CD79a positive cells were observed in the dorsal abdominal cellular lymphoid aggregates around the abdominal aorta, which correspond to sites of development of rudimentary renal or mesenteric lymph nodes, and rarely in the hepatic sinusoids in both control and infected animals. At 10 dpi/45 dg, slightly increased numbers of CD79a, IgG and IgM-positive cells were observed in the same locations in infected and control animals (Fig. 14, A-C). Rare IgM-positive cells were observed in the meninges of one infected animal. At 14 dpi/49 dg, slightly increased numbers of CD79a, IgG and IgM-positive cells were observed in the sites described above, and at this timepoint, CD79a and IgM-positive cells were in the spleen of one of the infected animals (Fig. 14, D-F). Surprisingly, another infected animal also had a marked infiltration of CD79a, IgG and IgM-positive cells in the meninges of the brain and spinal cord and in rare cells within the parenchyma (Fig. 14, G-I). Increased numbers of CD79a, IgG and IgM-positive cells were arranged around splenic arterioles in both infected and control animals at 21 dpi/56 dg and 28 dpi/63 dg, besides also being in the previously described sites. Numerous IgG and IgM-positive cells were observed in the meninges of the spinal cord of two infected fetuses at 21 dpi, and rare immunoglobulin-positive cells were observed in one fetus at 28 dpi. Moderate to marked numbers of CD3 positive cells, evaluated only in sections of brain and transversal sections of the abdomen including the lumbar spinal cord of control and infected fetuses at 21 and 28 dpi, were detected in the spleen, renal and mesenteric lymph nodes in all

fetuses. Scattered CD3 positive cells were in the meninges of the BRA and SPC of one infected fetus at 14dpi and in the meninges of the SPC in one fetus at 21 dpi.

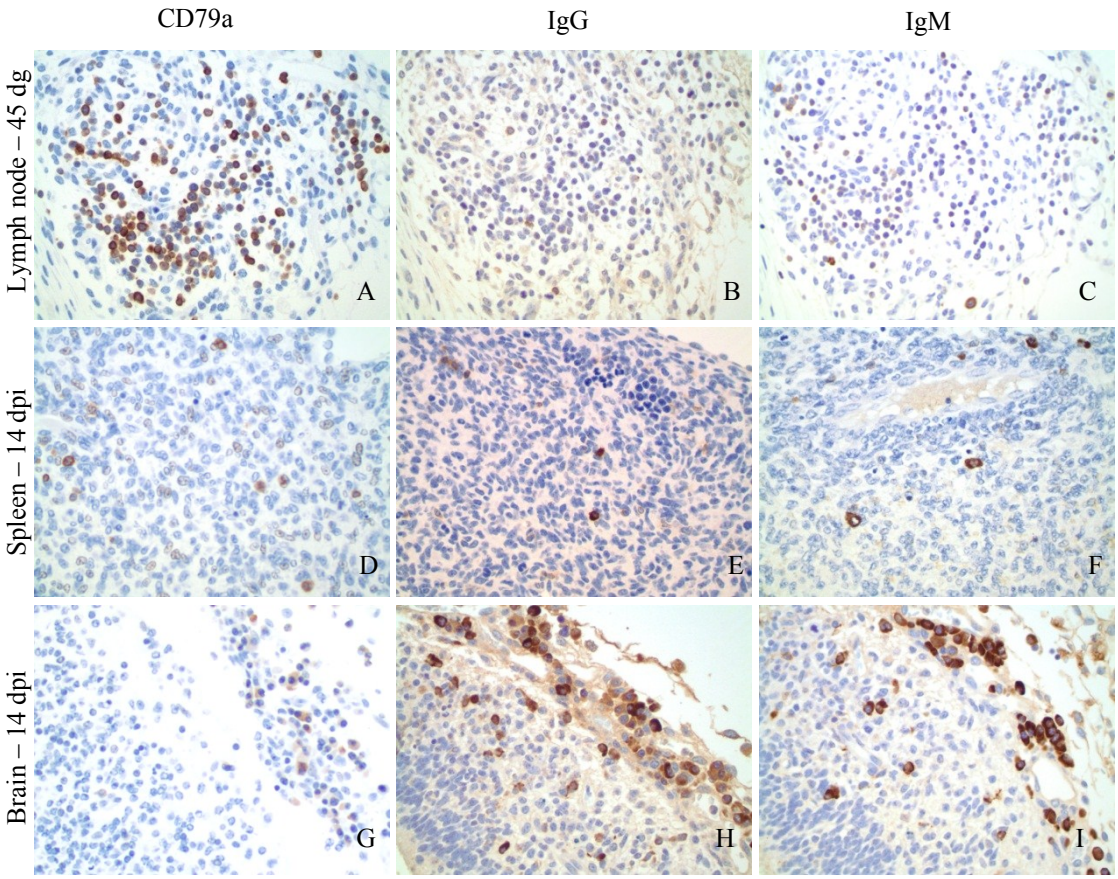

Figure 14. Immunohistochemistry for B cells, plasma cells and ovine IgG and IgM in lymph node, spleen and brain. A polyclonal antibody against B cells and plasma cells (CD79a), ovine IgG and ovine IgM was stained with DAB chromagen (brown). CD79a (A), IgG (B) and IgM (C) positive cells were observed in rudimentary lymph nodes at 42-45dg in control fetuses. CD79a (D), IgG (E) and IgM (F) positive cells were observed in the spleen of infected fetuses. A remarkable number of CD79a (G), IgG (H) and IgM (I) positive cells were seen in the leptomeninges of an infected fetus at 14 dpi.

Discussion

Ovine fetuses are able to mount an immune response associated with CVV infection earlier in gestation than previously described.[64,94,106,113] Although the fetal immune response was not able to completely clear virus by the end of the study, at 63 dg, as shown in Chapter II, infected ovine fetuses were able to reduce the viral load from the CNS and MKS, tissues targeted by CVV. Viral signal was also markedly reduced or absent in the ganglion, retina, kidney and heart.

As shown in Chapter II, CVV targets mainly cells within the CNS and musculoskeletal system, and viral signal was markedly reduced in the brain and almost completely cleared in the skeletal muscle after 21-28 dpi in fetuses infected at 35 dg. Similarly, a progressive reduction in viral load, as shown by mean relative amounts of viral copies was observed in the brain and SKM in this study, with significant differences observed between most days of infection in the SKM, and in the brain between 14 and 28 dpi. In addition, the numbers of relative viral copies in the brain were significantly higher than in the skeletal muscle.

The Mx protein, an interferon stimulated GTPase involved in intracellular trafficking, membrane remodeling and fusion processes, is capable of restricting growth of viruses, including influenza virus, measles virus and bunyaviruses within the orthobunyavirus, phlebovirus and hantavirus genuses.[50,58-61,66,76,77,139] In viral infections, the Mx protein acts by interfering with transport of viral components in infected cells. In a model proposed by Haller and Kochs (2002),[59] MxA forms large, membrane-

associated self-assemblies that store monomers of this protein. With infection, monomers bind to viral target structures, forming new assemblies involving viral intermediates that lead to mislocalization of viral components and consequent viral replication inhibition.[59] Human MxA has been shown to inhibit replication of La Crosse virus by binding to the viral nucleocapsid protein, and forming large copolymers that accumulate in the perinuclear region.[76] Previous studies have demonstrated antiviral activity of bovine Mx2 in cattle infected with BVDV.[62,130] Because Mx has a demonstrated antiviral effect against bunyaviruses, this study measured expression of the Mx molecule in infected tissues. A significant upregulation of ovine Mx1 and Mx2 was observed in evaluated tissues from infected fetuses. Increased secretion of Mx1 was identified in fetal fluids in infected ovine fetuses. Thus, this protein reasonably could contribute to clearance of CVV infection.

Another gene that was significantly upregulated in both brain and skeletal muscle and highly expressed in tissues in infected animals was ISG15. Similar to the Mx protein, ISG15 is induced by type I interferon, and has been associated with antiviral activity.[134] In recent studies, ISG15 has been shown to conjugate to proteins in a manner similar to ubiquitin.[4,156] Modifications and interference of antiviral signaling pathways involving ISG15 are mechanisms used by Crimean-Congo hemorrhagic fever virus, a bunyavirus, to evade the innate immune response.[4] Upregulation of numerous ISG, including ISG15, and continuous stimulation of the innate antiviral response has been demonstrated in blood and tissues of bovine fetuses and cattle infected with BVDV.[62,130]

No significant difference was observed in the expression of selected sequences of type I interferon genes, despite the fact that the interferon induced genes, ISG15 and ovine Mx1 and Mx2, were markedly upregulated in infected fetuses. Previous studies have demonstrated that ovine fetuses within the second and third trimester of gestation are able to produce levels of interferon similar or greater to those produced by adult animals in response to a viral infection.[121] Since type I interferon induced genes were upregulated, it is presumed that type I interferon genes were upregulated at some time point, and due to its short half-life, upregulation was not detected at the time points evaluated. Even though less likely, one may have to consider the possibility of both ISG15 and ovine Mx being upregulated by an interferon-independent pathway or that the selected sequences amplified in this study were not representative of the type I interferon production status in infected fetuses.

CD79a-positive cells (B lymphocytes and plasma cells) were observed in the spleen of CVV-infected fetuses at 49 dg. This is similar to results in previous studies, that have identified B lymphocytes in ovine fetal lymph nodes and spleen as early as 47-48 dg.[87,88,114] Both infected and non-infected fetuses had scattered CD79a-positive, IgM and IgG-positive cells in lymphoid aggregates in the dorsal aspect of the abdominal cavity, sites of development of rudimentary renal and/or mesenteric lymph nodes, as early as 42 and 45 dg, respectively. The identification of CD79a-positive cells in regional lymph nodes earlier than observed in the fetal spleen, supports the previously proposed idea that extrasplenic sites have the potential of producing B lymphocytes,[7,114]

and potentially explains the fact that splenectomized fetuses are also capable of colonizing Peyer's patches with B lymphocytes.[114,115]

Besides the presence of CD79a, IgM and IgG-positive cells in the spleen and lymph nodes of both infected and non-infected fetuses, a marked infiltration of CD79a, IgM and IgG-positive cells was also observed within the meninges with fewer cells within the parenchyma of the brain and spinal cord of infected fetuses at 49 dg. It appears that the ovine fetus not only mounts an innate immune response to a viral infection, but is also able to stimulate an adaptive immune response. Unfortunately, blood samples were not available from the fetuses during these experiments and fetal neutralizing antibodies could not be assayed. In ovine fetuses infected with Akabane virus, the earliest day of gestation where IgM and IgG-positive cells were observed in tissues was 59-60 days gestation. Titers >1:4 were detected only after 100 dg.[70,149] Two other studies detected neutralizing antibodies after 76-78 dg, with titers ranging from 8-64 in one of these studies.[94,106]

CHAPTER V

SUMMARY AND CONCLUSIONS

CVV is a viral pathogen with a tropism for the ovine fetal CNS, skeletal muscle and fetal membranes. The study of early infection demonstrated a tropism that correlated well with the CNS and musculoskeletal malformations observed in spontaneous CVV disease. With CVV, the development of arthrogryposis probably has a multifactorial pathogenesis involving effects on developing neurons, myocytes, and fetal membranes (Fig. 15). CVV is the only viral infection shown to cause oligohydramnios. The virus is cleared from most infected fetal tissues by approximately 65 dg, before the presently accepted onset of ovine immunocompetence.

In the CNS of ovine fetuses, CVV targets primarily fetal neurons and rarely glial cells. The viral infection is associated with and presumably induces a marked microgliosis in fetuses; however, astrocytosis is not identified. The fetus is able to mount an immune response associated with CVV infection earlier in gestation by upregulating genes that participate in innate immune response. Possibly the intense microgliosis in the brain is an attempt of the fetus to control the virus infection, by releasing antiviral cytokines that contribute to viral clearance in the CNS. The infected fetus upregulates Mx genes that have been shown to restrict growth of bunyaviruses. In addition, cells of the adaptive immune response, CD79a-positive, IgM and IgG-positive cells, are found in large numbers in early infected tissues and also could contribute to viral clearance.

Future studies should evaluate even earlier dpi to determine which tissues are initially infected and how does the virus spreads to all infected tissues. The features that control the intravenous route of inoculation and role of mosquito effector molecules in the natural route of inoculation of CVV in ovine fetuses.

Furthermore, studies should be conducted to better understand the functional development of innate and adaptive immune response in ovine fetuses exposed to CVV and to other viral infections. For example, to better understand the role of Mx in viral clearance, an Mx knockout mouse model could be tested to evaluate if Mx is indeed a key player in innate immunity against CVV. In addition, future studies should determine the specificity of early CD3, CD79a, IgG and IgM-positive cells in infected tissues

Finally, the ovine fetus infected with CVV is a model for development of CNS and MSK fetal malformations and oligohydramnios.

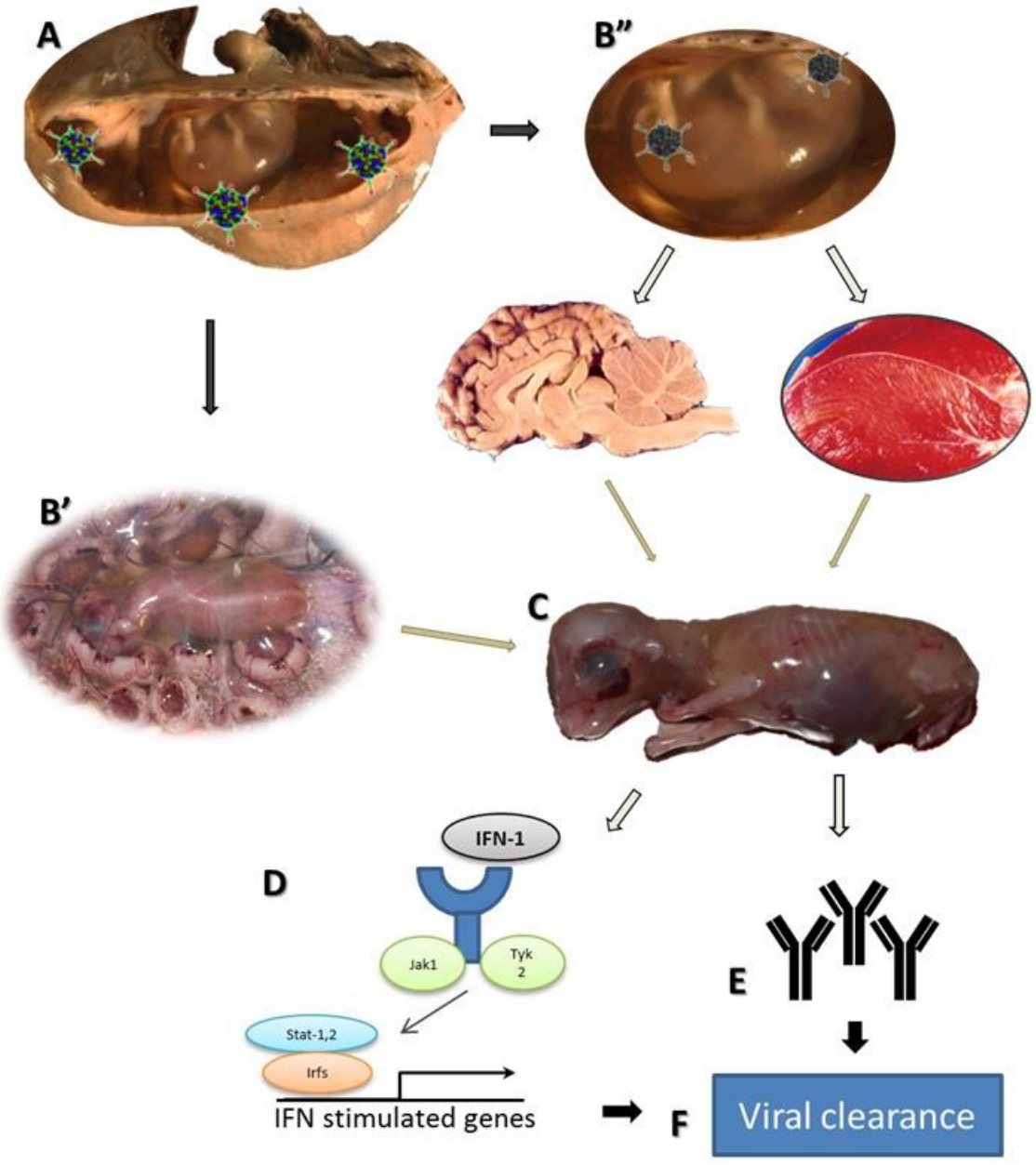

Figure 15. Proposed pathogenesis of CVV infection in the ovine fetus. CVV infects the fetal membranes (A), which results in oligohydramnios (B’) and subsequent fetal akinesia. After penetrating fetal membranes, CVV infects fetal target tissues (B”), mainly the CNS and SKM. The infection of the fetal membranes, CNS and SKM results in fetal malformations (C). The fetus stimulates the innate immune response (D) as well as the adaptive immune response by producing antibodies (E) which will further result in viral clearance from infected tissues (F).

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APPENDIX A

List of antibodies

Antibody	Application	Host	Catalog #	Concentration
CD3	T lymphocytes	Rabbit	Dako: A045201	1:400
CD79a	B Lymphocytes	Mouse	Dako: M705001	1:200
CVV	CVV antigen	Rabbit	--	1:300
GFAP	Astrocytes	Mouse	Millipore: MAB360	1:300
Iba1	Microglia and macrophages	Rabbit	Wako: 019-19741	1:500
Iba1	Microglia and macrophages	Goat	Abcam: ab5076	1:300
IgG	Ovine IgG	Rabbit	KPL: 01-23-02	1:500
IgM	Ovine IgM	Rabbit	KPL: 01-23-03	1:2000
ISG15	ISG15	Mouse	Gift from Dr. Hansen	1:500
MAG	Mature oligodendrocytes	Hamster	Gift from Dr. Li	1:1
MBP	Mature oligodendrocytes	Rat	Millipore: MAB386	1:100
Nestin	Neuron progenitor cells	Rat	Millipore: MAB353	1:200
NeuN	Differentiated neurons	Mouse	Millipore: MAB 377	1:80
Neurofilament	Axons	Mouse	Covance: SMI-31R	1:200
Olig1	Oligodendrocyte lineage	Mouse	Millipore: MAB5540	1:100
Olig2	Neural progenitor cells and mature oligodendrocytes	Rabbit	Millipore: MAB9610	1:100
PLP	Mature oligodendrocytes	Rat	Gift from Dr. Li	1:500

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