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Cell culture adapted bluetongue virus in dogs

by

Linn Arthur Wilbur

A dissertation submitted to the graduate faculty

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CHAPTER 1. GENERAL INTRODUCTION

Introduction

Modified live virus (MLV) vaccines containing attenuated strains of virus are administered to provide protection against many different diseases. Modified live viral vaccines elicit both humoral and cell mediated immune responses and usually provide long term protection against disease. Inactivated viral (killed virus, KV) vaccines induce primarily humoral immune response that provide disease protection of a shorter duration than MLV vaccines. Killed virus vaccines are considered to be safer to use than MLV. With MLV vaccines there exists the risk that the attenuated viruses present in the vaccines may revert to virulence or that viral contaminants may be inadvertently introduced into the vaccines. Those risks are not present in KV vaccines that contain only killed components, as all ingredients are inactivated during the manufacturing process.

The U.S. veterinary vaccine manufacturers are regulated by the United States Department of Agriculture (USDA) and follow high quality standards to assure that veterinary vaccines are pure, safe, potent, and effective. Those standards apply to all veterinary vaccines produced in the U.S. whether KV or MLV or produced for inter or intra state shipment. As part of the veterinary regulatory licensure process, strict guidelines for use of each vaccine are established and incorporated as label claims. The restrictions noted on the label claim include the species, age, and breeding status. It has been recognized that pregnant animals may be more susceptible to MLV vaccines and unless proven otherwise, label claims specifically exclude pregnant animals from the recommended use. In addition, there have been multiple recommendations from the scientific community to not use MLV

vaccines in pregnant animals, but often MLV vaccines are used as boosters to pregnant animals during middle to late gestation to induce high antibody titers so that colostral antibody transfer to neonates is optimized.

The objectives of these studies is to describe the investigation of the virus isolated from several field cases of pregnant bitches that aborted following vaccination with a modified live, four component USDA licensed canine vaccine and the subsequent discovery of a bluetongue virus (BTV) contamination in that vaccine. This is the first association of BTV with disease in dogs. The association of abortion and death with BTV is further evaluated in pregnant bitches and evidence of cell culture adapted BTV as the cause of abortion is determined. The final study investigates the role of cell culture adapted BTV on non pregnant dogs and non pregnant sheep. That study determines if there are any acute consequences to administration of cell culture adapted BTV to non pregnant dogs and if this cell culture adapted bluetongue virus could potentially be spread to and cause infection or disease in sheep.

Dissertation Organization

This dissertation is organized in the style of separate manuscripts. Three manuscripts are included and represent chapters 2 through 4. Separate references are therefore included for the literature review and the 3 chapters. The first manuscript, chapter 2, "Abortion and death in pregnant bitches associated with a canine vaccine contaminated with bluetongue virus," has already been published and was written as a case report published in the Journal of the American Veterinary Medical Association and therefore does not include an abstract. The subsequent papers, Chapters 3 and 4, are intended as research reports for submission to

the American Journal of Veterinary Research and include abstracts.

Literature Review

Family *Reoviridae*

The viral family *Reoviridae* consists of nine genera based on a similarity of structure, genome, and replication patterns. The genera in the family include, *Cypovirus* (cytoplasmic polyhedrosis viruses), *Phytoreovirus* (plant reovirus group 1), *Fijivirus* (plant reovirus group 2), *Oryzavirus* (plant reovirus group 3), *Aquareovirus* (golden shiner virus), *Orthoreovirus* (reoviruses), *Orbivirus* (orbiviruses), *Rotavirus* (rotaviruses), and *Coltivirus* (coltiviruses).[1,2] Of these, the latter 5 are of human or animal disease importance. Viruses in the genus *Aquareovirus* are associated with viral diseases of fish. The reoviruses have been isolated from humans and animals[3] and have been associated with disease in poultry and mice but not in other mammals.[4] The orbiviruses are the cause of African horse sickness, epizootic hemorrhagic disease of deer, equine encephalosis, and bluetongue disease in ruminants. Diseases caused by the rotaviruses include diarrhea in human infants and enteritis in animals. The *coltivirus* genus includes the virus causing Colorado tick fever.[3]

The viruses in this family are characterized by a viral icosahedron shape, spherical, 60-80 nm in diameter, and non enveloped. The outer shell consists of either a single or double outer protein coat and an inner protein capsid core. The double stranded RNA (dsRNA) genome consists of 10 to 27 segments, with the number of segments being characteristic for each genera. Replication occurs in the cytoplasm with inclusion body formation.[3,5] Intact virions enter via receptor-mediated cell endocytosis while core viral particles without the outer coat (produced by enteric protease breakdown) may directly enter

the cytoplasm. In either case, the virus is degraded to a core particle prior to replication. Capping and transcription occurs on the 5' mRNA molecule via virion associated transcriptase and capping enzymes. Initially only certain genes are transcribed, with the remaining genes being suppressed until early viral protein expression. Minus sense RNA is synthesized, forming dsRNA which is further transcribed forming additional dsRNA, but non capped. Translation of viral structural proteins occurs followed by viral particle self assembly. Release of virions occurs via cell lysis.[4]

The family was initially named in 1959 based on select members of the echovirus 10 group that had been isolated from respiratory and gastrointestinal tracts, but had not been associated with any known diseases. The family was named based on the perception that these were "respiratory, enteric, orphan" (reo) viruses. Later the rotaviruses and orbiviruses were added to the group.[3]

Orbivirus genera

The orbivirus genus is characterized by 10 segments of dsRNA. Virions have a poorly defined outer protein capsid when observed by electron microscopy using negative staining techniques,[2,6] and are transmitted to animals in blood from arthropod vectors. Viral particles characteristically are 65-80 nm in diameter, disassociate the outer and inner capsid at moderate pH (< pH 5), are inactivated at low pH (< pH 3.0), are non infective at higher temperatures (> 60 C), and have a decreased infectivity following ether treatment.[2,7] The orbiviruses have 7 structural proteins, identified as VP1 to VP7 based on molecular weight on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).[6] The outer shell consists primarily of VP2 and VP5. VP2 is the primary antigen eliciting neutralizing

antibodies. The orbiviruses inner shell consists of 32 capsomers composed of 2 of the 7 structural proteins, VP3 and VP7. VP1, VP4, and VP6 are found in the inner core in close association with the dsRNA. Antibody against VP7 is used to differentiate serogroups within the orbiviruses and VP5 has been used for type specificity.[7,8] The virus has 4 non structural (NS) proteins, NS1, NS2, NS3, and NS3A.[7,8] These NS proteins are associated with viral mRNA, are the most abundant component of the granular viral inclusion bodies found in orbivirus infected cells, and are believed to be involved in initial virion assembly. NS3 and NS3A are part of the viral release mechanism of orbivirus infected cells.[8] Bluetongue virus 1 is the type species.[2]

The 10 segments of BTV are divided into 3 large RNA segments (L1 to L3), 3 medium (M4 to M6), and 4 small (S7 to S10) based on migration patterns in PAGE gels.[2] VP1 is encoded by genome segment L1, VP2 by L2, and VP3 by L3. VP4 is encoded by M4 genome segment, VP5 by M5, VP6 by S9, and VP 7 by S7. NS1 is encoded by M6, NS2 by S8, and NS3 and NS3A by S10.[8]

There are 14 major serogroups recognized historically for the orbiviruses based on soluble antigens in complement-fixation, immunofluorescence, and/or agar-gel precipitation tests. Each of these serogroups have from 1 to 24 serotypes differentiated by serum neutralization. Recent genetic hybridization and sequencing studies have compared the homology of the various serogroups and have shown the previous serological groupings to be supported at the genomic level.[9] Similar studies done at the serotype level within several serogroups have shown that genetic reassortment has been a major factor in the evolution of distinct serotypes, but has not supported that reassortment occurs between serogroups.[6]

The serogroups of economic importance in animals are bluetongue and African horse

sickness viruses. Bluetongue virus is endemic in North and South America, Africa, Middle East, Asia, and Australia and causes disease in sheep, cattle, goats, and deer. African horse sickness virus is endemic in Africa and there have been viral incursions into Spain and Portugal. African horse sickness causes disease in horses, zebras, and dogs.[9] Other orbiviruses of disease importance in animals are epizootic hemorrhagic disease virus (wild ruminants and cattle), Ibaraki virus disease (cattle), and equine encephalosis (horses). Orbivirus infection in humans include Changuinola virus, Kemerovo complex viruses, Lebombo virus, and Orungo virus.[2,9]

Transmission of the viruses occurs in various arthropod vectors including gnats, mosquitoes, phlebotomi (sand flies), or ticks. Replication of certain of the orbiviruses occurs in both the arthropod vector and in the vertebrate host. Normally no adverse effects are noted in the orbiviruses arthropod vectors, while in vertebrate hosts, disease can be inapparent to life threatening.[2]

Bluetongue virus

Bluetongue virus, as the prototype species for the orbivirus genus, has been extensively studied. The outer capsid has been elucidated by cryoelectron micrographs and has been shown to have a well defined icosohedral symmetry of 860 Å diameter. This is in contrast to the polymorphic symmetry depicted by conventional negative staining electron micrographs. The outer capsid proteins, VP5 and VP2, have globular and "sail like" shapes, respectively. VP5 proteins reside on each of the VP7 trimers that form the six-member rings of VP7 in the BTV inner core. VP2 proteins form spikes that sit above 180 of the 260 VP7 trimers of the inner core and form 60 triskeletal-type motifs. VP2 trimers are believed to be

the serological hemagglutinating and neutralization antigens.[10,11]

The inner capsid is 690 Å in diameter, of icosohedral symmetry, and has 260 knob like structures consisting of 780 VP7 units.[11] The structure is organized into pentameric and hexameric units with intervening channels. The 132 resulting channels occurring at the three-fold axes have dimensions of approximately 70 Å in depth and 80 Å wide.[12] Additional work has shown that the inner capsid consists of 2 layers, an outer layer composed of VP7 trimers and an inner layer composed of VP3 monomers held to each other by hydrophobic interactions. At the three-fold axes small pores of 7 Å diameter exist, while 9 Å pores exist at the five-fold axes. The smaller pores are believed to be capable of enlargement by conformational change of the side chains, but are blocked by VP7. The larger pore at the five-fold axes can also be enlarged by conformational changes and may be the mechanism for movement of metabolites into the inner core for viral mRNA transcription and export of nascent mRNA from the core.[8,13]

A subcore composed of VP1, VP4, and VP6 forms a lattice within the VP3 based inner layer. The NS proteins and the dsRNA are associated within the lattice and anchored to the VP3 inner layer.[12,13] The NS proteins are believed to elicit cell mediated responses.[14]

In vertebrate erythrocytes, binding of BTV is to specific sialic acid containing, serine-linked, oligosaccharides in the glycophorins. This site is believed to be a receptor for both erythrocytes and other vertebrate animal cells.[15] Binding to vertebrate cells is believed to be mediated via VP2 of the outer capsid. Binding to insect cells is believed to occur via VP7 following uncoating of the outer capsid.[16] Entry into the cell occurs via receptor mediated endocytosis. Within an hour of entry the viral outer capsid is removed, and large translucent vesicles are formed and migrate close to the nucleus.[17] Entry into the cytoplasm

occurs by penetration of the endosomal membrane and the binding to the cell cytoskeleton prior to initiation of viral transcription.[18] The viral cores, composed of the inner capsid, non structural proteins, and viral dsRNA, initiate viral translation and transcription. Those mRNA and proteins move through the viral pores into the cytoplasm, followed shortly by cellular synthesis inhibition.[19] Virus specific polypeptides are detected within 2 to 4 hours of infection, peak at 11 to 13 hours, and slowly decrease until cell lysis occurs.[20,21] NS1 forms tubules within the cytoplasm. Bluetongue virus sub cores are initially formed (10 dsRNA segments, VP1, VP3, VP4, and VP6), VP7 is added to form the inner core, and finally the outer core is added. A matrix forms around the core particles, composed of viral mRNA and virus specific proteins. Virus inclusion bodies are formed of BTV particles. The number and size of the viral inclusion bodies is dependent on the stage of infection, with inclusions bodies being maximized as viral replication peaks.[20] The first two stages occur within the inclusion body. Final virion assembly occurs with the addition of the outer shell (VP2 and VP5) and occurs in close proximity to the inclusion body. The virion associates with the tubules, is transported to the cell membrane, associates with NS3/NS3A containing extra virion vesicles, and is released via cell budding, extrusion, or lysis.[22-24]

Presently 24 serotypes of BTV are recognized world wide. Serotypes of BTV isolates are determined based on serum neutralization (SN) studies using antiserum produced against whole virus. The SN techniques currently accepted by the Office International des Epizooties (OIE), the world organization for animal health, include plaque reduction, plaque inhibition, and microtiter neutralization.[25]

Bluetongue disease is historically a disease of ruminants. Sheep (especially European breeds), antelope, white-tailed deer, African antelope, and other artiodactyls are

the predominant species affected with clinical signs. Cattle and goats may occasionally show clinical signs.[26-28] Bluetongue was first recognized in South Africa over a hundred years ago and was described separately by D. Hutcheon and by J. Spreull in 1902.[26] The disease was subsequently described in 1943 in Cyprus, but probably had occurred there since 1924. Additional outbreaks in the Mediterranean region occurred in Israel in 1951, in Pakistan in 1959, and in India in 1963. Soremuzzle of sheep was described in 1952 in the United States of America (USA) and is believed to have originated in Texas as early as 1948. This was subsequently confirmed as being caused by BTV. Bluetongue virus was discovered in Portugal in 1956 and in Spain in 1957.[27] Subsequently the virus has been found in most tropical and subtropical regions of the world. The initial concerns with BTV reflected a belief that BTV occurred only in epidemics and caused major economic hardships on the sheep industry. In the 1960s and 1970s BTV was considered an emerging disease, and international trade restrictions were placed on animal movement. Subsequently it has been shown that in sheep and cattle bluetongue is an endemic disease that occurs throughout the world without overt clinical disease.[29]

Transmission of bluetongue virus

Bluetongue virus is not horizontally transmitted and vertical transmission via semen is rare. The primary means of infection is by bites of BTV infected midges of the *Culicoides* species. *Culicoides* spp. are found year round in tropical areas of the world. In sub tropical and areas adjacent *Culicoides* spp. are found on a seasonal basis.[26] In sub tropical and adjacent areas the presence of *Culicoides* spp. varies and is dependent on temperature, wind, and moisture conditions. Many of the areas adjacent to sub tropical areas are free of

Culicoides spp. except from the middle of summer until first frost. Presence of *Culicoides* spp. and emergence of disease caused by BTV in the subtropical and adjacent areas are highly correlated to these factors. Disease is associated with increasing midge populations, and bluetongue has been reported to occur when frost free days exceed 100 days.[30] Introduction of *Culicoides* spp. into areas normally free is believed to occur via wind borne movement of the insects.[31]

Midges become infected by ingesting blood of clinical or non clinical infected animals. Viral replication occurs in the salivary glands and highest concentration of virus occurs 6-8 days after ingestion. Subsequent biting of susceptible animals results in transmission.[31] Midges remain infected indefinitely, but no transovarian passing of the virus to the insect's offspring occurs. Bluetongue virus is thought to be maintained in an endemic area by overwintering of the *Culicoides* spp. or by the subsequent reintroduction of the virus to midges by long term infected animals.[32]

Bluetongue virus can be isolated from semen. Intrauterine transmission of BTV has been reported from semen from bulls infected naturally or experimentally.[32-35]

Bluetongue virus in the world

Bluetongue virus serotypes 2, 10, 11, 13, and 17 have been found in North America and are principally transmitted by *C. variipennis*. Serotypes 1, 3, 4, 6, 8, 12, and 17 are found in the Caribbean basin and transmitted by C. *variipennis* and *C. insignis*.[26] Australia has 8 serotypes, serotypes 1, 3, 9, 15, 16, 20, 21, and 23 and the potential vectors are *C. wadai, C. brevitarsis, C. actoni,* and *C. fulvis*.[36,37] Africa has 17 serotypes including serotypes 1, 2, 3, 4, 6, 10, 12 and 16 and the predominant vectors are *C. imicola*

(*C. pallidennis)*, and *C. milnei*.[38] The Middle East and Asia have reoccurring outbreaks, information on serotypes is sketchy at best, but serotypes 1 to 4, 7, 9, 10, 12, 16, 17, 20, 21 and 23 are believed to be present from all countries from Greece through Japan.[29] Israel has reported serotypes 2, 4, 6, 10, and 16 and *Culicoides* spp. *C. imicola* and *C. obsoletus* are the most important vectors. Malaysia has reported serotypes 1, 3, 9, 15, 16, 20, 21, and 23 and up to 42 species of *Culicoides* spp. are potential vectors.[39]

Presently Europe is believed to be free of BTV but the presence of *C. imicola* provides for a potential vector if the virus is reintroduced.[40]

Distribution of bluetongue virus in North America

Bluetongue virus is endemic in 30 states of the USA but is found rarely or not at all in the 18 northern and northeastern states, Alaska, and Hawaii. Serotypes 10, 11, 13, and 17 are found in those areas. Serotype 2 has not been found since 1986 and was only isolated in Florida and Alabama.[41] This distribution follows closely the climatological data for the USA, where incidence of BTV follows occurrence of 100 frost free days or more.[30]

Bluetongue virus in Canada has occurred only as incursions of BTV 11 from the USA and no endemic BTV is known to exist.[42]

Clinical disease and virulence mechanisms of bluetongue virus

Bluetongue virus infection can occur in most ruminants, but disease typically occurs primarily in sheep and wild ruminants. Cattle become infected, have a prolonged viremia, but do not usually show clinical signs. Cattle play a significant role in the continuation of the disease and the endemic nature of BTV. Bluetongue virus can be detected in blood from

cattle for up to 38 days post infection by embryonated chicken egg inoculation and greater than 100 days using polymerase chain reaction (PCR) based detection.[43] Most sheep also become infected without showing clinical signs. Sheep show both individual and breed susceptibilities. Breeds originating in the temperate regions are more susceptible than those of the tropical regions and older sheep are more susceptible than younger sheep.[29,44] In addition there are differences in the virulence of different serotypes of BTV and, at least for one serotype, difference in the virulence of strains of the same serotype when administered to cattle, although this study has not be replicated.[45] Epizootics of disease occur in endemic areas, at the interfaces of endemic areas, and in areas where the virus is more episodic.[29,44]

When clinical disease does occur in sheep, it is characterized initially by difficult breathing and panting, hyperemia of the lips, muzzles, and ears, fever (up to 42 C), and depression. The dental pad becomes ulcerated and lameness may occur due to inflammation of the coronary band. Uncommonly, the tongue may turn cyanotic (hence the name for the disease, bluetongue). More progressive disease includes muscle necrosis, torticollis, vomiting, pneumonia, conjunctivitis and alopecia.[28] Death normally occurs by pulmonary insufficiency due to exudate and hemorrhage that fills the lungs. Mortality in the USA is less than 10% compared to higher mortality reported for African serotypes.

The pathogenesis of BTV infection in sheep includes initial infection via intradermal inoculation by a *Culicoides* spp. Replication is detected initially in tonsils, spleen, and regional lymph nodes followed shortly by viremia.[46] Edema occurs due to viral damage to endothelial cells. Involvement of endothelial cells may be selective with a predilection for certain organs and for specific blood vessels.[32] Perivascular lymphocyte infiltration, microthrombi formation, and micro infarction occur throughout the body with severest

lesions occurring at the mucosal surface in striated muscle of the limbs and in the heart. The pathognomonic lesion for sheep is pulmonary artery hemorrhage.[26] In sheep, viremia is first detected 3-6 days post infection, peaks at 7-8 days and declines rapidly to day 14, and is rarely detected after day 30.[32]

In cattle, BTV infection is predominantly asymptomatic. Initial infection occurs similarly to sheep with the lymphoid tissues being the first site of viral replication. The virus infects peripheral blood mononuclear cells and platelets, and transfer of the virus occurs via the lymphatic system. Secondary viral replication can occur in the spleen and lymph nodes. Additional mononuclear cells and erythrocytes become non preferentially infected. Virus infection of the cells of the blood system persists for the life of the cell. Although a prompt neutralizing immune response is elicited, the virus appears to sequester within the circulating cells, and virus can be detected for a period similar to the life span of the individual cell type.[46-48]

Bluetongue virus has been reported with disease in pregnant sheep and cattle, however it apparently occurs very rarely. Strain differences have been noted that indicate an increased incidence for particular strains being more associated with infection of the pregnant uterus, embryo, and fetal infections, but has not definitively been shown to occur. Early embryonic deaths, abortions, and fetal malformations have been reported as resulting from BTV infections. Association of BTV with temporary sterility and infertility in rams and bulls has also been suggested.[26,49] Bluetongue virus shedding in semen has been documented and occurs while animals are viremic.[50,51] Bluetongue virus infections have not been associated with embryos collected during embryo transplant procedures if following adequate washing procedures.[52]

Diagnosis of bluetongue virus

The identification of BTV as a cause of disease in sheep is initially based on clinical signs and presence of potential vectors. Disease in cattle and other ruminants is more problematic, not only due to the typical asymptomatic state, but also due to the confusion with other disease with similar clinical signs.[53] The definitive diagnosis of BTV infection is the isolation of virus from infected tissues. The classic laboratory technique for viral isolation is the intravenous inoculation of embryonated chicken eggs (ECE),[54] although cell culture inoculation on African green monkey kidney cells (Vero), baby hamster kidney, or *Aedes* insect cell line (C6-36) is possible, but the latter cell culture techniques have lower sensitivity for field isolates than the ECE inoculation.[55] The sample of choice for viral isolation from field studies is washed erythrocytes. Washing is necessary to remove the possibility of BTV antibody being present in the serum and blocking attachment and infection.[53] Alternatively, the inoculation of sheep with large volumes via the subcutaneous route may be the most sensitive method. This technique is required for certification of imported fetal bovine serum into the USA, but has been replaced by the ECE inoculation for most field isolation studies.[54] Recently, various nested PCR based detection assays have been proposed for detection of both BTV and to serogroup samples. The PCR techniques have been shown to be more sensitive at detecting the presence of BTV than that of ECE inoculation,[43,56,57] but have the disadvantage of not resulting in an isolate of the virus.

Detection of BTV in tissues sections is done by fluorescent antibody staining,[58] immunoperoxidase methods,[59,60] or by *in situ* hybridization.[61-63] In addition to the recent PCR based methods for serogrouping and serotyping, the classical methods for identification are based on serological techniques. Immunofluorescence, antigen capture enzyme-linked

immunosorbent assays, and immunospot are group specific tests for BTV. For serotyping, classical viral neutralization tests are used and include plaque reduction, plaque inhibition, and microtiter neutralization, and are the techniques recognized by the OIE.[25,52] Initially a complement fixation test was used to titer serum but was generally replaced by the agar gel immunodiffusion test. This latter test is now supplemented with a competitive ELISA (cELISA) test that has been shown to be more sensitive and able to differentiate serological response to BTV from epizootic hemorrhagic disease virus responses.[64]

Bluetongue virus in non ruminants

Bluetongue virus historically has not been associated with infection and disease in animals other than ruminants. Subsequent to reports of BTV causing abortion and death in pregnant bitches following administration of a modified live canine vaccine contaminated with BTV,[65-68] follow up surveys of carnivores have been conducted. A serological survey of dogs in Georgia found 1 of 40 dogs with serological BTV titers.[69] The one dog had recently been vaccinated, but it could not definitely be shown whether that was with a potentially contaminated vaccine. A survey of black bears in Florida found 3 of 61 bears to be seropositive for BTV or epizootic hemorrhagic disease virus (EHD),[70] a closely related virus to BTV.[53] A survey of carnivores in Africa found evidence of BTV antibodies.[71] The authors speculate that possible routes of infection may include ingestion as is seen in dogs for African horse sickness virus.

African horse sickness virus in dogs

African horse sickness (AHS) is a disease of *Equidae* caused by another serogroup of the orbivirus distinct from the serogroup including BTV and also transmitted by *Culicoides* spp.[72,73] Morphologically the virus is similar to BTV in size, shape, organization, and genomic segments.[74]

African horse sickness is considered endemic to Africa, primarily the sub Sahara region,[38,75] with outbreaks occurring in Spain, Portugal, Yemen, and the Middle East.[76,77] The disease was first reported in South Africa in the 1700's and subsequently spread to other parts of Africa.

The disease in horses presents in up to 4 different clinical syndromes, described as pulmonary, cardiac (or edema), fever, and a mixed form that is a combination of pulmonary and cardiac. The pulmonary form is characterized as being an acute, febrile disease with horses showing depression and anorexia which rapidly progresses to respiratory distress and death. The cardiac form is a subacute disease that presents as horses with fever, but lower than the pulmonary form, and swelling of the anterior-dorsal regions. Death occurs 4-8 days after initiation of clinical signs. The fever syndrome is a mild to subacute disease with a low grade fever presenting biphasically, typically in the afternoon. No other clinical signs are noted. The mixed form of AHS is found primarily at necropsy when both cardiac and pulmonary lesions are noted. Mortality for the pulmonary form is usually 100%, for the cardiac form 50-70%, and usually no mortality is associated with the fever syndrome. The mixed form has mortality between the pulmonary and cardiac forms.[78,79]

At necropsy, diffuse pulmonary edema with or without pleural effusion is found for the pulmonary form. Hydropericardium and petechial and ecchymotic hemorrhages of the

epi and endo cardium are found in the cardiac form along with edema of the affected portion of the body. No lesions are found in horses with the fever form.[78]

The pathogenesis of AHS is not well understood, but is believed to closely follow that of BTV with initial replication occurring in lymphoid tissues followed by dissemination via a viremic phase. A predilection for the endothelial cells and concurrent endothelial cell damage has been demonstrated,[80] but replication is not at a high enough level to account for the AHS virus noted in horses.[78] Isolation from erythrocytes is possible, but classical techniques of cell culture isolation or newborn mice intracerebral inoculation[72] can be depended upon to isolate virus only during the early clinical, viremic time period.[81] Recent studies using reverse transcription PCR have demonstrated virus association with erythrocytes from horses vaccinated with a subunit vaccine and then subsequently challenged with AHS. African horse sickness nucleic acid in these horses was detected by PCR for up to 22 weeks.[82]

African horse sickness virus antibodies have been demonstrated to occur in multiple African mammals including cheetahs, lions, African wild dogs, jackals, hyenas, large-spotted genets, and domestic dogs. Lions and hyenas had the highest percent of animals positive.[83] The route of infection is believed to be via eating meat from animals infected with AHS. African horse sickness virus has been isolated from dogs in Egypt,[84] and dogs experimentally infected with AHS via various routes, including ingestion, have been demonstrated to have AHS antibodies.[85] Early reports at the start of the twentieth century reported death in dogs from ingestion of AHS infected meat.[37,86] A more recent study demonstrated that 13 of 17 dogs consuming meat from a horse that had died from AHS died within 12 days. Two of the dogs were necropsied and showed generalized congestion and cyanosis. Those dogs had

severe diffuse pulmonary congestion and edema. The lungs were filled with exudate and 350 ml of transudate fluid was recovered from the thoraxes. Virus consistent with AHS was recovered from both dogs.[87]

Viruses Associated with Abortion in Dogs

Infective agents associated with abortion and still birth in dogs include brucellosis, salmonellosis, streptococcal infection, toxoplasmosis, canine distemper, canine herpesvirus, and canine parvovirus-1 (minute virus of canine).[88] Of the viral causes, canine herpesvirus infection[89-91] and canine distemper[92] have most closely been associated with transplacental infection and subsequent abortion in the dog. The remaining virus, canine parvovirus-1, has been associated with transplacental infection and abortion in experimental infection but has not been reported as a cause of abortion in clinical cases.[88,93] Of the orbiviruses, only African horse fever has been historically associated with causing infection and there is no record of that disease being associated with abortion in dogs.

Historical Viral Contaminants of Human and Veterinary Vaccines

The possibility and source of viral contamination of vaccines is dependent on the method of production of the vaccine. When produced by either *in vivo* or *in vitro* methods, the starting virus (master seed) must be free of extraneous agents to assure that the final product is not contaminated. If produced *in vivo* from eggs or directly in or from animals each lot must be tested free from viral contamination or the herd or flock must be of known virus-free status. For veterinary poultry products this is assured by using eggs from flocks that are routinely monitored, tested, and known to be specific pathogen free. For processes

using mammalian animals, tissues, or fluids such a certification is also possible, but more typically products from such production methods are limited to product where inactivation of the viral product occurs. When producing vaccines using primary cells or continuous cell lines, contamination of the cells from the original tissue source, from nutrient supplements, or from animal origin components is possible. Testing to assure that these ingredients are free of extraneous agents is part of production and quality control methods. In the USA specific requirements for testing are subject to governmental regulations and the required testing codified in the Code of Federal Regulation chapters 9 or 21 respectively, for veterinary and human vaccines.[94,95] Certainly there is ample evidence of cell lines being found contaminated with viruses[96-103] that could potentially be a source for contamination of viral vaccines.

Historically, failure to totally inactivate virus in killed viral products has occurred with killed rabies vaccines, killed foot and mouth vaccines, and inactivated polio vaccines. Contamination of *in vivo* produced vaccination materials has also occurred during small pox vaccination when using human lymph contaminated with syphilis.[104]

In more recent times the contamination of oral polio vaccines with simian virus 40 from 1955 to 1961 resulted in the potential exposure of 98 million people in the USA to the virus. Studies are still ongoing to determine if adverse effects of the SV40 exposure have occurred in this population.[96,105] Other human vaccines reported to contain contaminating viruses are live polio, measles, mumps, and rubella vaccines contaminated with bacteriophages[106] and yellow fever vaccine contaminated with avian leukosis virus.[107]

Animal vaccines contaminated with extraneous viruses have included a 1950 outbreak of Newcastle virus from live fowl pox and laryngotracheitis vaccine, a 1951

outbreak of fowl leukosis following the use of killed Newcastle disease vaccine thought to contain leukosis virus, and a 1956 report of visceral lymphomatosis virus in live Newcastle disease vaccines.[108] Additional contamination in poultry vaccines include the presence of reticuloendotheliosis virus in a Marek's disease vaccine[109,110] and the potential contamination of multiple vaccines with chicken anemia agent virus.[111]

Additional techniques using PCR have been devised that suggest that the genome of various viruses are present in vaccines. This technique although useful for screening purposes, is sensitive to any viral genome present, whether from live virus or killed virus as would occur during processing of fetal bovine serum. Contaminants implicated have included bovine polyomavirus,[112] avian leukosis virus,[113] wild type canine parvovirus,[114] pestivirus,[115-117] and reverse transcriptase activity as an indicator for retroviral contaminants.[118]

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CHAPTER 2. ABORTION AND DEATH IN PREGNANT BITCHES ASSOCIATED WITH A CANINE VACCINE CONTAMINATED WITH BLUETONGUE VIRUS

A paper published in the Journal of the American Veterinary Medical Association[a]

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Two pregnant bitches, a 1.5-year-old Samoyed and a 2-year-old Golden Retriever, both in the last third of gestation, were vaccinated with a federally licensed, four-component, modified-live vaccine that contained canine distemper virus (CDV), canine parainfluenza virus (CPIV), canine adenovirus 2 (CAV2), and canine parvovirus (CPV) during a routine visit to a veterinary practitioner in Idaho. The vaccines were rehydrated with a killed coronavirus vaccine or a combination killed coronavirus vaccine/leptospira bacterin and were administered IM as a booster. Two days after vaccination, both bitches showed signs of depression and were febrile. Treatment with antibiotics was initiated. The Samoyed

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aborted 3 days after vaccination, and the Golden Retriever 4 days after vaccination. Both bitches continued to show signs of depression and became anorectic; the Golden Retriever remained febrile. Eight days after vaccination, the Samoyed developed dyspnea that rapidly became worse and resulted in death. The Golden Retriever failed to respond to antibiotic and supportive treatment, continued to show signs of depression, and remained anorectic and febrile. The Golden Retriever was euthanatized 9 days after vaccination at the owner's request. At necropsy, sanguineous pleural effusion, serous pericardial fluid, congested to hemorrhagic areas in the dorsal portions of the lungs, splenomegaly, enlarged mesenteric lymph nodes, and pale kidneys were observed in both dogs.[f]

Specimens, including 3 aborted fetuses from the Samoyed and selected tissues from both bitches, were submitted for histologic, bacteriologic, and virologic examination to a state veterinary diagnostic laboratory.[g] Histologic lesions were not observed in specimens obtained from the brain, lung, kidney, liver, spleen, and heart of the Samoyed fetuses. The placenta from the Samoyed had mild, focal, fibrinosuppurative vasculitis. Tissues from the Golden Retriever were observed to have severe diffuse glomerulonephropathy, moderate diffuse lymphocytic degenerative cardiomyopathy, severe centrolobular hepatocellular degeneration with centrolobular lymphocytic perivasculitis, and passive congestion of the lung. Neither specific organisms nor pathognomonic lesions were observed in any of the tissues.

Bacteriologic cultures of the 3 Samoyed fetuses were negative for pathogenic bacteria after 72 hours of incubation. Placental tissues from the Samoyed had a large number of

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[f] F. G. Hughbanks, Gooding, Idaho

[g] Washington Animal Disease Diagnostic Laboratory, Pullman, Wash

bacteria, but pathogenic bacteria were not isolated after 72 hours of incubation. Bacteriologic cultures of tissues from the Golden Retriever were negative after 72 hours.

Results of viral isolation studies performed on pooled tissue specimens from the 3 Samoyed fetuses were negative after 3 passages in Vero (African green monkey kidney) cells, Crandell feline kidney cells (CRFK), and Madin Darby canine kidney cells. Placenta and mesenteric lymph nodes from the Samoyed were found to contain a cytopathogenic agent after 3 passages in Vero and CRFK cells. Coronavirus-like agents were observed, by means of electron microscopy, in the intestinal contents from the Golden Retriever. Viruses that produced a cytopathic effect were isolated in Vero and CRFK cells from the lung and mesenteric lymph nodes of the Golden Retriever.

The viral isolates were amplified by passage in Vero and CRFK cells. To aid in identification, the viruses were tested for chloroform susceptibility[l] and CPV immunofluorescence and infected cells were stained with H&E. The viruses were chloroform resistant, did not react with CPV reagents, and produced variously sized eosinophilic cytoplasmic inclusion bodies. The viral isolates were tentatively identified as members of the family *Reoviridae*.

Because clinical signs developed following vaccination, the practitioner initiated a veterinary biologics consumer report with federal veterinary biologics regulatory officials.[h] Identification of the isolates from the Samoyed and the Golden Retriever was requested from the laboratory supporting the federal veterinary biologics program.[i] Results of serum-neutralization tests as well as direct and indirect fluorescent antibody examination of the

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[h] Veterinary Biologics Field Operations, Ames, Iowa.

[i] National Veterinary Services Laboratories, Ames, Iowa.

isolates were negative for viruses routinely associated with disease in dogs. The isolates had minor cross-reaction with a reovirus 1, 2, and 3 polyvalent conjugate. Examination of electron micrographs of the isolate from the placenta of the Samoyed revealed a 60- to 70-nm diameter, spherical, nonenveloped viral particle with 1 apparent capsid (Figure 1), tentatively identifying the isolate as an orbivirus of the *Reoviridae* family. Nucleic acids from this isolate were extracted with a chloroform-phenol procedure and compared to similar chloroform phenol extracted preparations of other representative members of the *Reoviridae* family, canine rotavirus,[j] reovirus 1,[k] and bluetongue virus (BTV),[l] by means of 10.0% polyacrylamide gel electrophoresis (Figure 2).[2] The 10 gene segments and the genome migration pattern were consistent with the *Orbivirus* genus. The isolates reacted intensely with a fluorescent antibody conjugate for BTV. This conjugate is known to have limited cross-reaction with epizootic hemorrhagic disease of deer virus.[m]

Samples of the four-component, modified-live viral vaccine used in vaccinating the bitches were examined for viral contamination. Vaccine tested was from samples retained by the federal veterinary biologics regulatory agency. For all veterinary biologics, each production lot has a unique serial number and is individually tested by the manufacturer. Samples of each serial must be submitted to the federal veterinary biologics regulatory agency for storage in a veterinary biologics repository prior to the serial being approved for sale. Repository samples are randomly tested to confirm the manufacturers' test results and

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[j] American BioResearch, 12621 N Milton Rd. Milton, Tenn.

[k] ATCC VR-230, American Type Culture Collection, 12301 Parklawn Dr. Rockville, Md.

[l] Bluetongue serotype 17, courtesy of the Diagnostic Virology Laboratory, Ames, Iowa.

[m] Diagnostic Virology Laboratory, Ames, Iowa

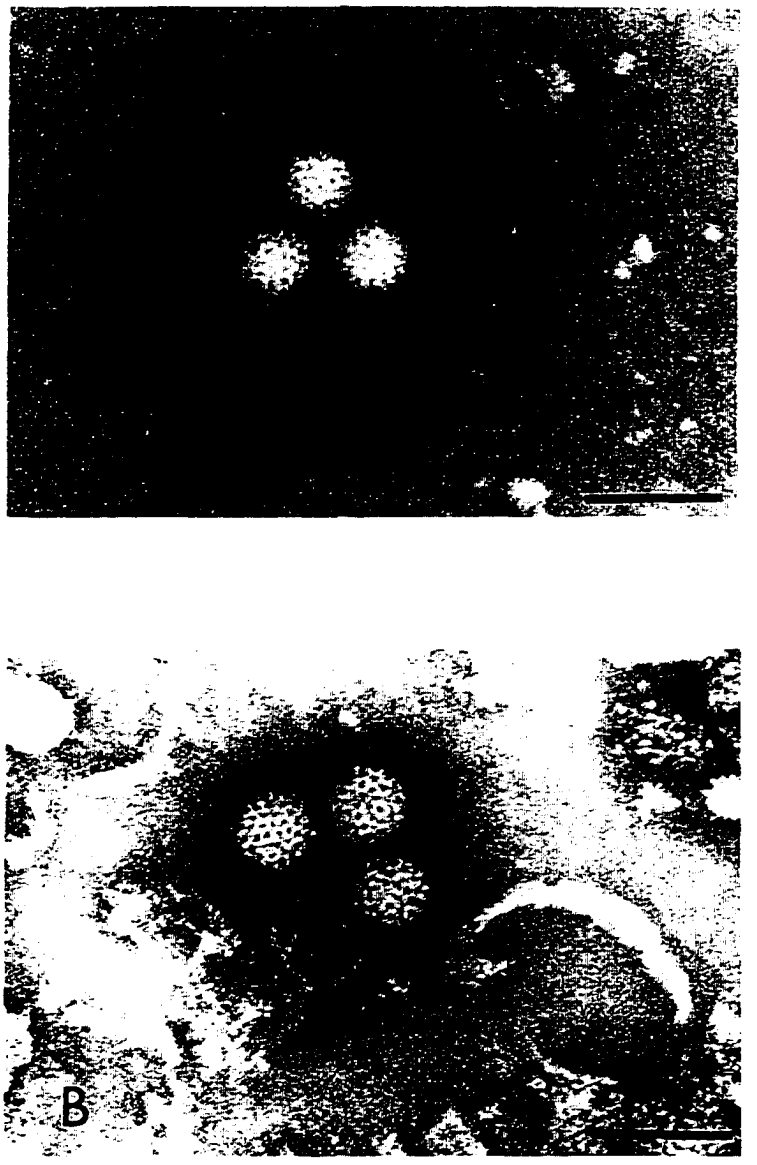

Figure 1. Electron micrograph of virus isolated from the placenta of a dog that aborted and died after receiving a modified-live vaccine during late-term pregnancy (A) and of a virus isolated from a sample of the vaccine the dog received (B). Negatively stained with 1.0% phosphotungstic acid; bar = 100 nm.

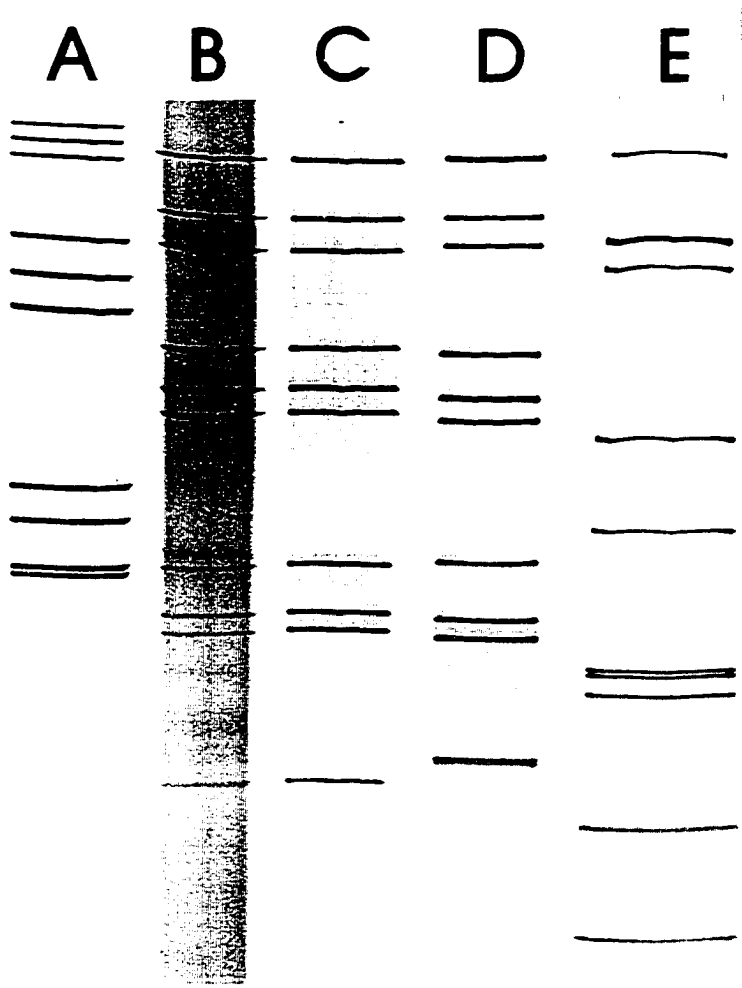

Figure 2. Migration pattern of viral genome segments on silver stained 10% polyacrylamide gel electrophoresis. Lane A = reovirus 1; Lane B = virus isolated from contaminated vaccine Lane C = virus isolated from a pregnant bitch that aborted and died after receiving the contaminated vaccine Lane D = bluetongue virus serotype 17; Lane E = canine rotavirus (second band is a doublet that resolved into 2 distinct bands when subjected to further electrophoresis).

are maintained to allow additional testing in the event of a consumer complaint. Two serials were known to have been purchased by the veterinary practitioner, but it was not known which of the serials was used to vaccinate the 2 bitches. Repository samples of each of these serials were reconstituted with sterile diluent, treated with chloroform to inactivate CDV and CPIV, neutralized with an equal volume of CAV2-specific canine antisera, and inoculated onto 75-cm^2 flasks of Vero cells, known to be nonpermissive for growth of CPV. Flasks with cytopathic effect similar to that produced by the isolates from the Samoyed and Golden Retriever bitches were identified. Bluetongue virus was identified by means of further passage and fluorescent antibody examination. Virus was isolated from 1 of the 2 serials implicated by the veterinary practitioner. The cytopathic effect, ultrastructural characteristics on electron microscopy (Figure l), genome electrophoretic pattern (Figure 2), and fluorescent pattern of the vaccine isolate were consistent with those expected with BTV and comparable to those of the isolates from the Samoyed and Golden Retriever. Further passages of the vaccine-inoculated cell cultures were negative for CDV, CAV2, CPIV, and CPV when examined by means of direct or indirect fluorescent antibody tests.

Additional consumer reports were received from an Iowa veterinary practitioner. This practitioner first reported the death of 2 pregnant bitches, the abortion in and subsequent death of 3 other bitches, and the death of 5 pups and their dam following administration of vaccine by the dogs' owner. This practitioner later reported abortion in and death of 2 additional bitches following vaccination by the veterinary practitioner. The vaccine associated with these reports was the same manufacturer's CDV, CPIV, CAV2, and CPV vaccine as those identified in the Idaho cases, but had a different serial number. The Iowa

practitioner submitted tissue specimens to 2 different diagnostic laboratories,[n] and samples were subsequently submitted to the veterinary biologics regulatory laboratory.[i] Each specimen was suspended with an equal volume (weight/volume) of tissue culture medium, homogenized with a tissue homogenizer,[o] and centrifuged at 1400 X g for 20 minutes. The supernatant was chloroform treated and used to inoculate Vero cells. Flasks with cytopathic effect were passaged, and virus was identified as described for the other isolates. Isolates consistent with BTV were obtained from maternal liver, small intestine, large intestine, kidney, and spleen, but not from aborted fetal material. Repository samples of the associated vaccine serial were examined as described and found to be contaminated with BTV.

Four additional cases have been reported. Three of these involved abortion and death, and the fourth, vaginitis. Viruses consistent with the previous isolates were obtained by methods similar to those described. These isolates were submitted to the veterinary biologics regulatory laboratory[i] by the state diagnostic laboratory[g] and reacted with the BTV fluorescent antibody conjugate. In each instance, the vaccines used were different serials of the same manufacturer's vaccine, and repository samples were found to be contaminated with BTV.

The viral isolate obtained from the repository sample corresponding to one of the vaccines implicated in the Iowa cases has been serotyped as BTV 11 with a plaque inhibition method (Figure 3).[3] Serotype 11 is a domestic strain of BTV.[4]

The veterinary biologic manufacturer was informed of the isolation of a viral

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[n] Animal Disease Research and Diagnostic Laboratory, South Dakota State University, Brookings, SD, and Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa.

[o] Kinematica Model 10-35, Brinkmann Instrument, Wesbury, NY.

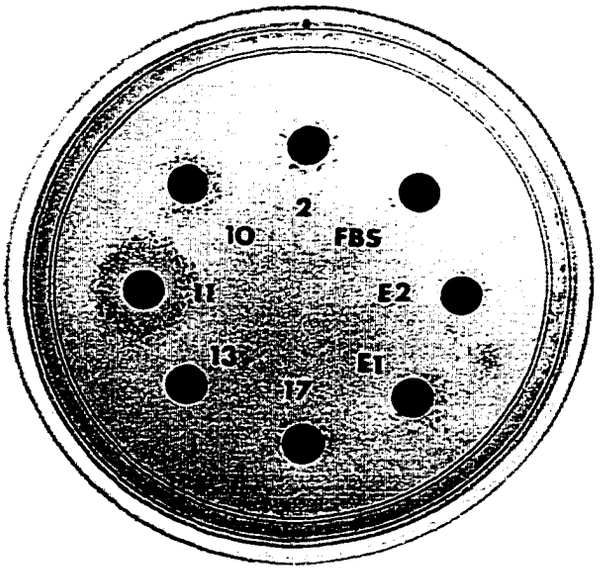

Figure 3. Plaque inhibition test on McCoy cell line for serotyping viruses isolated from a contaminated vaccine. Serotype is indicated by viable cells (stained with neutral red) surrounding a disk following 3 days of incubation. Antisera directed against domestic strains of bluetongue virus (BTV) and epizootic hemorrhagic disease of deer virus (EHDV), another orbivirus known to cross-react with BTV, were used. Vaccine isolate was used to infect monolayer cell culture; paper disks were saturated with antiserum (counterclockwise) to BTV 2, BTV 10, BTV 11, B1V 13, BTV 17, EHDV 1, EHDV 2, and fetal bovine serum.

contaminant. The manufacturer voluntarily recalled all vials of the vaccine with serial numbers the same as those associated with these cases.

Bluetongue virus has not previously been associated with disease in dogs, but has been considered a disease of ruminants.[5,6] African horsesickness is caused by an orbivirus not found in the United States that is serologically distinct from BTV and is the only orbivirus previously associated with naturally occurring infection in dogs. African horsesickness virus has not been associated with abortion in bitches, but rather has been characterized as causing viremia with subclinical signs.[7,8]

In the present report, a BTV contaminant given with a historically safe canine vaccine apparently caused disease when administered to late-term pregnant bitches. Recommendations to not vaccinate pregnant animals with modified-live vaccines, unless the vaccine is specifically approved for that use, have previously been made,[9,10] and may be indicative of an increased sensitivity of pregnant animals and their fetuses to viruses.[11] Additional case reports have been received implicating serials of BTV-contaminated vaccine with various disease syndromes in nonpregnant dogs, but viral isolation studies on tissue submissions, to date, have been BTV negative.

Several veterinary practitioners associated clinical disease in dogs with recent vaccination and a nontypical virus was isolated from tissue specimens collected from affected dogs. The USDA's Veterinary Biologics Consumer Information Hotline (515/ 232-5789) was used to inform the regulatory agency of adverse reactions associated with a licensed veterinary vaccine. That information was used to identify a suspect product and confirm contamination in associated vaccine serials; this resulted in these serials being recalled. Practitioners, diagnostic laboratories, the federal regulatory agency, and the veterinary biologic manufacturer worked together to remove a contaminated product from the market.

Acknowledgements

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results; and the staff and faculty of the Washington Animal Disease Diagnostic Laboratory for their technical and investigative help.

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CHAPTER 3. ABORTION IN PREGNANT BITCHES FOLLOWING CHALLENGE WITH CELL CULTURE ADAPTED BLUETONGUE VIRUS

A paper to be submitted to the American Journal of Veterinary Research

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Abstract

A cell culture adapted bluetongue virus (BTV), isolated from a contaminated commercial modified-live 4-component canine vaccine, was administered by the intramuscular route to four pregnant bitches in mid gestation. The bitches were monitored and observed for 10 days. Elevated temperatures, vomiting, anorexia, depression, and vaginal discharge were observed. Three of the 4 bitches were observed to have aborted on days 4 to 6 after challenge. The remaining bitch was determined to have aborted when necropsied at the conclusion of the study. One bitch exhibited respiratory distress, another had a rapidly falling temperature after aborting and subsequently died within 24 hours.

Bluetongue virus was isolated from blood samples collected on days 1 to 9, with

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maximum viral titer obtained on days 3 to 5. Bluetongue virus was isolated from tissues from the 4 bitches and from fetuses from 2 of 3 bitches. The most consistent BTV isolation came from lung, spleen, and placenta of the bitches. One of 4 of the bitches seroconverted based on a commercial cELISA diagnostic kit. The remaining 3 had rising cELISA inhibition percentages, indicative of the early stages of an immune response.

Following the administration of a cell culture adapted BTV, bitches became clinically ill, BTV was recovered during clinical illness, 3 of 4 of the bitches were observed to abort, and BTV was isolated from both maternal and fetal tissues. This study demonstrates that cell culture adapted BTV can cause clinical illness and abortion in pregnant bitches when iatrically administered.

Introduction

Bluetongue virus (BTV), the prototype virus for the genus *Orbivirus*[1] is naturally transmitted to animals by various *Culicoides* spp. midges, and has historically infected sheep, cattle, and wild ruminants.[2,3] Clinical disease occurs primarily in sheep. Cattle and wild ruminants, although infected, normally remain subclinical. Bluetongue virus has not been reported to be associated with infection and disease in non ruminants.

Recently, abortion and death in pregnant bitches following use of a commercial modified live vaccine contaminated with a BTV have been reported.[4-6] In those studies, the same serotype BTV was isolated from a multivalent vaccine and from various tissues of both bitches and aborted fetuses. Based on these isolations, an association with the clinical disease in these bitches was made. This study details the experimental infection of pregnant bitches with a cell culture adapted BTV obtained from the contaminated vaccine to show that

cell culture adapted BTV iatrically administered can conclusively be demonstrated as the cause of infection, viremia, and abortion in pregnant bitches.

Materials and Methods

Dogs

Four purebred female beagles were used in this study. Two bitches were obtained from a commercial dog colony and had only been vaccinated with a killed rabies product. Bitches were supplied as being in mid to late gestation based on date of last observed breeding. Two bitches were obtained from the National Veterinary Services Laboratories (NVSL) SPF dog colony and had never received any vaccinations. Bitches were housed in a climate controlled environment in separate pens, 2 bitches per room. The floor of the pen was washed twice daily and the bitches were fed a commercial dry dog food supplemented with commercial wet food. Bitches within each room had fence line contact. Bitches from the commercial colony were wormed and treated for fleas upon arrival and a 21-day acclimation period was observed prior to initiation of the experiment. Bitches from the NVSL colony were locally moved and allowed to acclimate for 3 days. The experiment was conducted with the approval of the NVSL Animal Care and Use Committee in accordance with the NIH guidelines and the Animal Welfare Act.

Challenge

Bluetongue virus was isolated as previously described from a modified live, multivalent canine distemper, canine parvovirus, canine parainfluenza, and canine adenovirus-2 vaccine that had been associated with abortion and death in pregnant bitches.[5] One additional passage in a baby hamster kidney cell line was used to expand the isolate

from the previous two passages in a Vero cell line. The challenge was examined for other contaminating viruses and bacteria by the methods described in Section 9 of the Code of Federal Regulations. The virus was diluted 1:10 in MEM without fetal bovine serum. The 4 bitches received 1.0 ml of the challenge virus in 1 site on the rear leg by the intramuscular route using a 22-gauge 1 inch needle and syringe.

Observation and sample collection

Each bitch was observed twice daily starting a day prior to challenge (day -1) through day 10 post challenge. Rectal temperatures were taken and blood collected daily in tubes containing EDTA. Blood for serum was collected on days 0, 4, 7, and 10 post challenge. Frequency of observation and recording of rectal temperatures was increased for all bitches as animals exhibited clinical signs. Clinical signs were recorded and bitches observed for abortion. Any aborted fetuses or bitch dying during the 10-day study was immediately necropsied and samples collected and frozen at -70 C for viral isolation. All surviving animals were humanely euthanatized, necropsied, and samples for viral isolation collected and frozen at -70 C on day 10 post challenge.

Blood samples

Serum was separated from clotted blood by centrifugation at 1,800 X *g* for 10 minutes and stored at 4 C. A commercial BTV competitive ELISA (cELISA) diagnostic kit was used to determine BTV antibody levels.[c]

EDTA blood samples were centrifuged at 750 X *g* for 10 minutes and the serum removed. The RBCs were washed by suspending the RBC pellet in a volume of 4 C sterile

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[c] Bluetongue antibody test kit, cELISA, Veterinary Diagnostic Technology, Inc.,Wheat Ridge, CO 80033

PBS equal to the removed serum volume and then centrifuged at 750 X *g* for 15 minutes at 4 C. The procedure was repeated for a total of 3 washes. The final washed RBC pellets were suspended with an equal volume of buffered lactose peptone with 375 μg/ml gentomycin, 50 μg/ml kanamycin, and 50 μg/ml amphotercin B. The RBC suspension was maintained in an ice bath and sonicated for 3-1 minute cycles at 60 watts in a cup horn to disrupt the cells and release virus.

RBC BTV titers

Two fold dilutions of the sonicated RBC suspension, starting at 1:5 were made in MEM with 750 units/ml penicillin, 75 units/ml streptomycin, and 50 μg/ml gentamycin. These were inoculated, 5 wells per dilution, onto confluent Vero cell culture using a 96-well plate format. Undiluted sonicated RBC solution had previously been shown be toxic to cells. The plates were observed daily under low power magnification for 7 days, wells exhibiting CPE recorded, and the end point titer determined by the method of Spearman and Kärber as modified by Finney.[7]

Viral isolation from tissues

Tissues were treated as previously described [5] substituting MEM media containing 75 units/ml penicillin, 225 units/ml streptomycin, and 150 μg/ml gentamycin. Kidney, lung, spleen, liver, uterus, and placenta were individually examined from each bitch and each aborted fetus. Four hundred microliters (400 μl) of supernatant was inoculated onto 25 cm^2 flasks of confluent Vero cell culture and 400 μl onto a 25 cm^2 flask of embryonic bovine kidney (EBK) primary cells for each supernatant. Flasks were incubated at 36 C in a 5% CO_2 incubator. Flasks were observed daily for CPE and frozen at -70 C when CPE was observed. Flasks not exhibiting CPE were subpassaged 2 additional times at 4-6 day

intervals. Flasks not exhibiting CPE at the end of the 2nd passage were considered negative. Positive CPE flasks were thawed at room temperature, centrifuged at 200 X *g* for 10 minutes, and the supernatent individually collected. Vero cell culture in 8 chamber glass slides, 24 hours after inoculation and incubation at 36 C in 5% CO_2, were inoculated with 0.1 ml per chamber of the supernatent and further incubated at 36 C in a 5% CO_2 incubator. Slides were examined daily and fixed with acetone if CPE was evident or fixed at 7 days if no CPE was observed. Cells were examined using a direct fluorescent antibody BTV conjugate[f] and an ultraviolet light microscope and considered a positive isolate if specific BTV fluorescence was observed.

Results

Challenge

The BHK passaged BTV titered 7.3 $\log_{10}$ tissue culture infective dose 50 ($TCID_{50}$) in the Vero cell culture by CPE, resulting in a challenge of 6.3 TCID50 $\log_{10}$ for the 4 experimental bitches after the 1:10 dilution. The challenge virus was shown to be free of any contaminating virus.

Observations

Clinical signs observed included vomiting, anorexia, depression, respiratory distress, vaginal discharge, and abortion (Table 1). Abortion was observed in 3 of the 4 bitches and on necropsy there was evidence that the remaining non aborting bitch (bitch 3) had been pregnant and recently aborted pups. Based on the cotyledon size these were judged to be

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[f] Bluetongue fluorescent antibody conjugate, National Veterinary Services Laboratories, Ames, IA

Table 1. Days post inoculation at which clinical signs were observed for experimental bitches.

	Bitch 1	Bitch 2	Bitch 3	Bitch 4
Abortion	6	4	n.o.	5
Vaginal discharge	6-8	4-5	n.o.	5
Respiratory distress	8-10	6-8	n.o.	5
Depression	7-10	n.o.	n.o.	n.o.
Anorexia	5-10	5-10	n.o.	4-5
Vomiting	6, 8	2	0, 1, 9	3

n.o = not observed

early term fetuses. No aborted fetuses were observed from bitch 3, nor were clinical signs other than vomiting observed. One bitch (bitch 4) died on day 5, a day after aborting. Initially 1 pup was aborted and 4 were found to be still retained on necropsy for bitch 4.

The bitches had clinically normal temperatures until day 3 post challenge, when 2 of the 4 bitches had temperatures higher than normal (Figure 1). By day 4 post challenge all 4 bitches had elevated temperatures. One of the bitches had a rapid decline in temperature from the time of abortion to death 24 hours post abortion. By day 8 post challenge 2 of the 3 remaining bitches (bitch 2 and 3) had returned to within the normal range. The remaining bitch (bitch 1) remained febrile until the end of the 10 day post challenge observation period.

Blood samples

The percent inhibition in a cELISA increased during the 10 day observation period (Figure 2). Only one bitch was positive at 10 days post inoculation when evaluated by the 50% threshold value for ruminants.

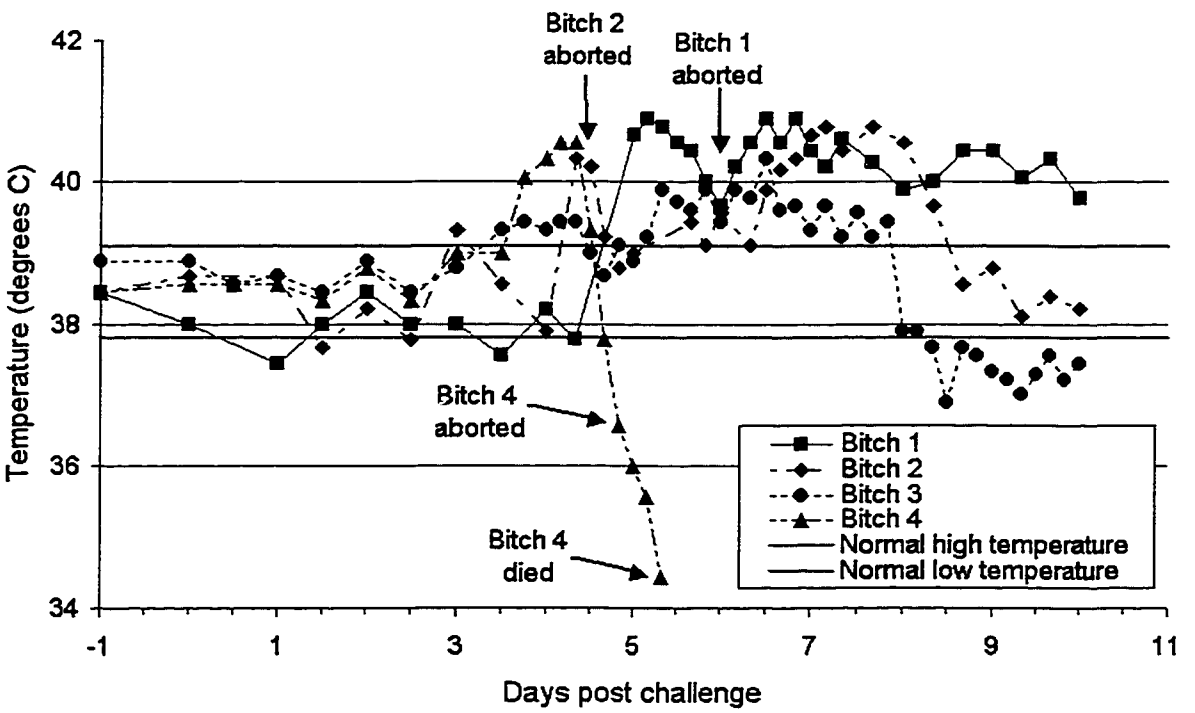

Figure 1. Temperature of BTV challenge bitches for days -1 to 10 post challenge with time of observed abortions and death indicated.

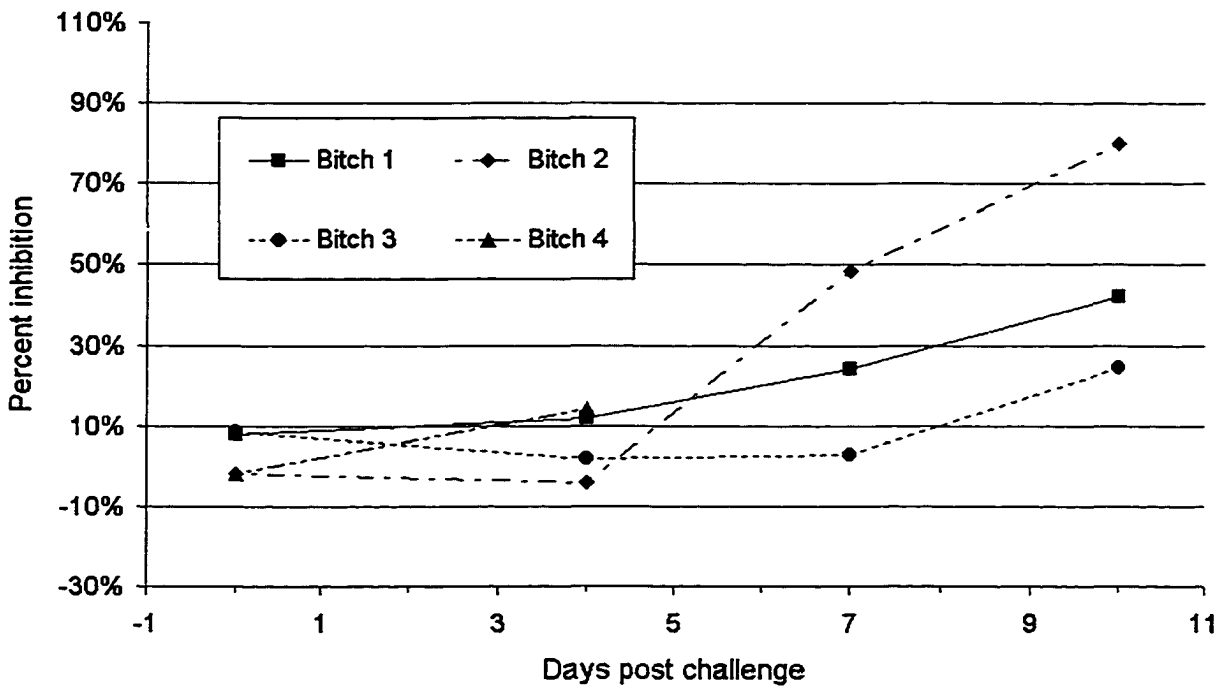

Figure 2. Percent inhibition of serum from challenged bitches by a cELISA commercial diagnostic test kit for days 0, 4, 7, and 10. Bitch 4 died on day 5. Greater than 50% inhibition is considered a positive antibody titer for bovines. No inhibition level has been determined for dogs.

RBC BTV titers

Bluetongue viral titers obtained in Vero cell culture are reported in Figure 3. Viremia was evident from day 1 to day 9, with all 4 of the bitches being viremic by day 3. Peak titers occurred on day 4 and 5. Titers declined starting day 5 with the three surviving bitches being below detection levels by day 9.

Viral isolation from tissues

Bluetongue virus was isolated from all four of the bitches and from 2 of the 3 bitches aborting fetuses (Table 2). Bluetongue virus isolation was most consistent from lung, placenta, and spleen samples of the dams with the remaining tissues having sporadic isolation of BTV.

Table 2. Viral isolation of BTV by tissue for challenged bitches and their aborted fetuses

	Bitch 1*		Bitch 2*		Bitch 3*		Bitch 4**	
	Dam	Fetus	Dam	Fetus		Fetus	Dam	Fetus
Lung	+	+	+	+	+	n/a	+	-
Liver	-	-	-	+	-	n/a	-	-
Spleen	+	-	+	-	+	n/a	+	-
Kidney	-	-	-	+	+	n/a	+	-
Uterus	-	n/a	+	n/a	+	n/a	+	n/a
Placenta	+	n/a	+	n/a	+	n/a	+	n/a

n/a = not applicable
* Euthanatized at 10 days post challenge
** Died 5 days post challenge

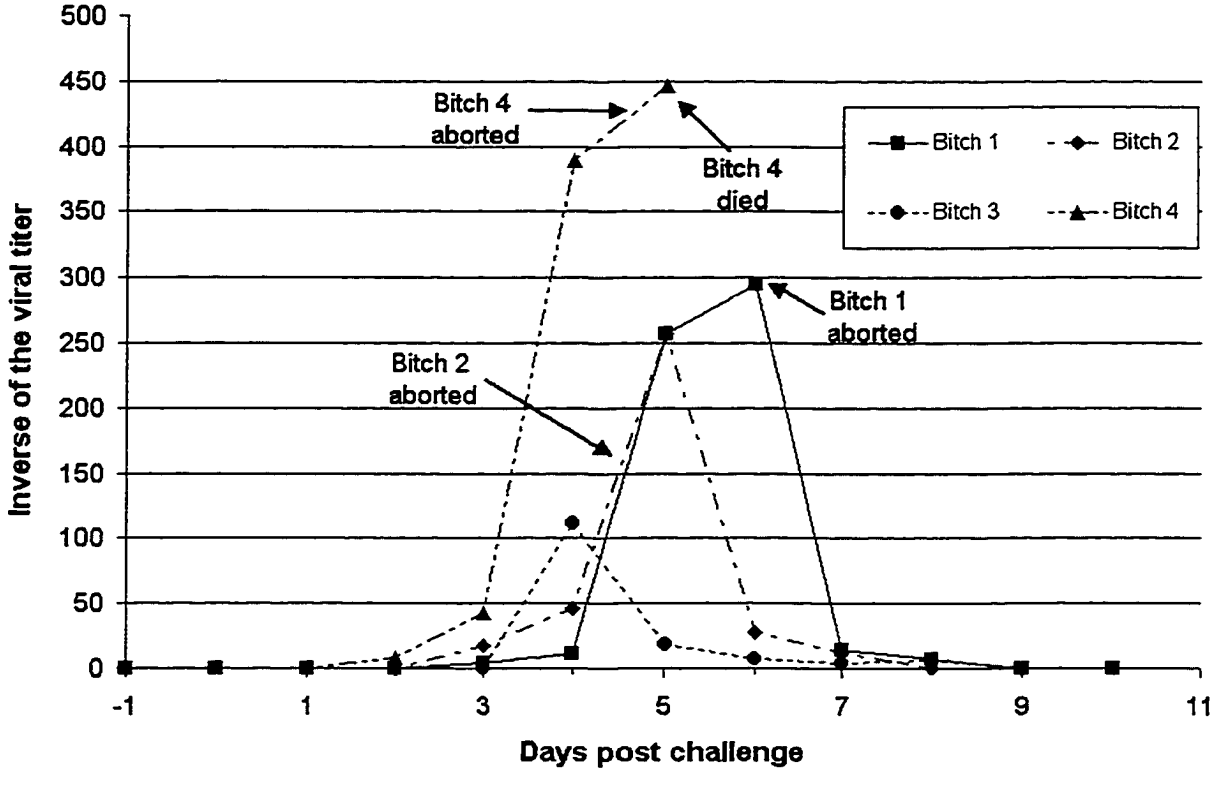

Figure 3. Bluetongue virus endpoint titers from RBCs obtained from BTV challenged pregnant bitches. Reciprocal of the dilution by days post challenge. Bitch 4 died on day 5.

Discussion

Results of this study show that cell culture adapted BTV can cause infection, viremia, and abortion in pregnant bitches. The virus used was derived from a commercial modified live 4-component canine vaccine contaminated with BTV. Three of the 4 bitches were observed to abort and necropsy evidence indicated that the fourth bitch also aborted. The challenge administered to the bitches was derived from cell culture adapted BTV. Bluetongue virus was recovered from blood prior to and following abortion, and was isolated from maternal and fetal tissues.

Clinical signs for the BTV challenged bitches were not unique or pathognomonic. Abortion due to BTV could not be definitively determined by the clinical signs shown by these animals. Vomiting and a febrile response initially occurred, followed by depression and anorexia. Only in one bitch was there respiratory distress similar to that reported in field cases.[4,5] This is in contrast to the histopathology and *in situ* hybridization findings of pulmonary edema and lung involvement in the study by Brown et. al. The tissues examined were representative tissues from bitches in this study.[8] Abortion occurred 4 to 6 days after administration of the challenge, slightly longer than some of the observations in the field cases. In each of the observed abortions in this study, a rising fever and viremia preceded abortion. The death of the one bitch could not be conclusively linked to the BTV infection, but occurred within a day of aborting.

Viremia during days 1 to 9 days post BTV challenge is in contrast to the viral persistence lasting for 100 to 160 days and 38 to 100 days noted respectively in cattle and sheep for BTV.[9,10] The clearing of the BTV viremia is more characteristic of African horse sickness virus (AHSV) infection, another Orbivirus, in bitches.

Historically BTV isolation has required isolation via intravenous inoculation of embryonated eggs, with cell culture being considered less sensitive. In these cases, as the BTV isolate in this study is of cell culture adapted origin, isolation in cell culture was as sensitive as embryonated egg isolation when evaluated in our preliminary studies (data not shown).

The lack of seroconversion for 3 of the 4 bitches as measured by the cELISA may be due to the short, 10 day period for this study. The 2 surviving bitches that did not seroconvert by the end of the study had a rising inhibition percentage. Whether a different threshold would be more appropriate than the 50 percent inhibition threshold established for ruminants is of interest. Based on the 1 positive bitch and the assumed continued response if the 2 other surviving bitches had been allowed to develop a humoral immune response, the cELISA appears to be an appropriate method for determining seroprevelence to BTV in bitches.

This study provides definitive evidence that cell culture adapted BTV administered by a route similar to that used for vaccines, causes abortion in pregnant bitches. Bluetongue virus was recovered from all 4 of the bitches during clinical illness, 3 of 4 of the bitches were observed to abort, and BTV was reisolated from various tissues of the bitches. The origin of the cell culture adapted BTV was from a contaminated vaccine that had been associated with abortion or abortion and death in pregnant bitches. This study emphasizes the need for adequate veterinary biological testing during the manufacturing of vaccines to assure that vaccines are free of BTV or any contaminates.

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CHAPTER 4. SUSCEPTIBILITY OF NON PREGNANT DOGS AND SHEEP TO CELL CULTURE ADAPTED BLUETONGUE VIRUS

A paper to be submitted to the American Journal of Veterinary Research

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Abstract

A cell culture adapted bluetongue virus (BTV), isolated from a contaminated commercial modified live 4-component canine vaccine, and the contaminated canine vaccine, were administered to various ages of young dogs (6 and 12 week old), to juvenile dogs, and to pregnant bitches. No adverse clinical signs or febrile response were observed in the non pregnant dogs. In a second experiment, washed red blood cells (RBC) from BTV isolate-challenged pregnant bitches that were collected during the time of abortion was given to non pregnant sheep and pregnant bitches. The pregnant dogs in both the first and second experiment died, aborted and died, or aborted and were euthanatized. Sheep exhibited a febrile response and mild clinical signs of 4 days duration and subsequently recovered.

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Seroconversion occurred in all of the 12 week old dogs and in a portion of the 6 week old dogs, but did not occur until at least 14 days post challenge. All the sheep receiving the RBC challenge seroconverted. Bluetongue virus was isolated from tissues collected at necropsy from non pregnant dogs at 10 days post challenge but not from non pregnant dogs at 24 days post challenge. Virus was isolated from multiple tissues from the pregnant bitches but not from the bitches' fetuses.

These studies demonstrate that no acute clinical disease occurred in non pregnant dogs, though virus could be isolated and there was a detectable BTV serological response following administration of a BTV contaminated modified live vaccine or a BTV isolate from the contaminated vaccine. No transfer of BTV could be detected from non pregnant dog to dog. Mild clincial disease occurred in sheep from the cell culture adapted BTV isolate and the pregnant bitches are a theoretical possible source for infection of BTV *Culicoides* spp. vectors.

Introduction

Bluetongue virus occurred as a contaminant in a modified live, multivalent canine distemper, canine parvovirus, canine parainfluenza, and canine adenovirus-2 vaccine and was distributed for use in the veterinary community and by animal owners.[1,2] Dogs of all ages and pregnancy status received the BTV contaminated vaccine prior to recognition that it was contaminated. Abortion and death was reported as occurring in pregnant bitches following vaccination.[3] Administration of modified live vaccines to pregnant animals is not a recommended procedure.[4,5] Multicomponent, modified live vaccines are recommended for administration to dogs as young as 6 weeks of age by veterinary manufacturers and may be

practiced in even younger age dogs when colostrum deprived.[6] No acute disease was reported from non pregnant dogs following field administration of the contaminated vaccine. The first part of this study examines the effect of cell culture adapted BTV alone and in combination with a modified live viral vaccine on non pregnant dogs.

Bluetongue virus is primarily transmitted to ruminants via various biting midges of the *Culicoides* spp. that have ingested BTV contaminated blood from viremic ruminants.[7] Historically, BTV was considered to be a disease confined to ruminants.[8-10] With the demonstration of viremia in pregnant bitches following administration of a BTV isolate obtained from a BTV contaminated vaccine, there exists the possibility that these dogs could serve as a point source for spread of BTV. The infectivity of cell culture adapted BTV for sheep injected with blood from viremic dogs is examined in the second experiment.

Materials and Methods

Non pregnant dogs

Twelve 6 week old, mixed sex, purebred beagles, seronegative to BTV, were obtained from a commercial dog colony where dams were routinely vaccinated with commercial canine vaccines. Four 6 week old, four 12 week old, and four juvenile, BTV seronegative purebred beagles, mixed sex, were obtained from the National Veterinary Services Laboratories (NVSL) specific pathogen free (SPF) dog colony. The NVSL dog colony is maintained unvaccinated. Non pregnant dogs were housed in climate controlled environments on commercial, plastic coated, expanded metal pads in separate groups based on age and treatment and allowed to co-mingle within their group. Supplemental zoned heat was supplied based on age and animal condition. The floor of each pen was washed twice

daily and the dogs were fed a commercial dry dog food supplemented with commercial wet food.

Pregnant dogs

Eight pregnant bitches were obtained from commercial purebred beagle kennels at various times and were all in at least second parturition. Bitches were observed to have been mated and all animals were believed to be in mid to late gestation. Bitches had been routinely vaccinated with various manufacturers multicomponent vaccines against common canine diseases and rabies. All bitches were seronegative to BTV. Pregnant bitches were housed in a climate controlled environment in separate pens, in treatment groups of 2 bitches per room. The floor of the pen was washed twice daily and the bitches fed a commercial dry dog food supplemented with commercial wet food. Bitches had fence line contact within each room, but no contact with other dogs.

Sheep

Four mixed breed, but predominantly wool breed, sheep were obtained from a commercial sheep source. Vaccination history of the sheep was unknown. All sheep were seronegative to BTV. Sheep were maintained in climate controlled rooms, 2 sheep per room. The pens were washed daily. Animals were fed a commercial pelleted feed and alfalfa cubes.

Experiment 1 challenge

Bluetongue virus was isolated as previously described from a modified live, multivalent canine distemper, canine parvovirus, canine parainfluenza, and canine adenovirus-2 vaccine that had been associated with abortion and death in pregnant bitches.[1] One additional passage in a baby hamster kidney cell line was used to expand the isolate

from the previous two passages in a Vero cell line (African green monkey kidney cell line). The contaminated BTV vaccine isolate was end point titrated by cytopathic effect (CPE) in Vero cells prior to being diluted for challenge. The titer was calculated by the method of Spearman and Kärber as modified by Finney.[11] The isolate was diluted 1:16.3 in MEM without fetal bovine serum and designated as the BTV isolate challenge. The challenge, as used, was examined for other contaminating viruses by the methods described in Section 9 of the Code of Federal Regulations.[12]

Sufficient vials originating from the same lot of the contaminated viral vaccine were rehydrated with sterile water on each day of challenge, pooled, and designated as the BTV contaminated vaccine challenge. The BTV virus content of representative vials of the BTV contaminated vaccine were previously titered in Vero cells as described above, except the canine vaccine was chloroform treated to inactivate the canine distemper and canine parainfluenza virus and neutralized with antiserum specific to canine adenovirus 2 prior to titering. Vero cells were not permissive for viral replication of canine parvovirus.

Multiple vials of one serial of commercial vaccine from the same manufacturer, containing the same modified live viruses, but known to be free of BTV contamination, was rehydrated with sterile water on the day of challenge, pooled, and designated as the non contaminated vaccine challenge.

The twelve 6 six week old dogs from the commercial colony were housed in 2 rooms, 6 dogs per room. Four of the first group of 6 (3 males, 1 female) received 1.0 ml of the BTV isolate challenge by the intramuscular route in one site on the hind leg with a 22-gauge 1 inch needle and syringe. Four of the second group of 6 dogs (3 males, 1 female) received 1.0 ml of the BTV contaminated vaccine challenge by the same route. The remaining 2

dogs in each group (2 male, 1 male and 1 female, respectively) were not inoculated and served as contact controls. Dogs were observed, rectal temperatured, and clinical signs recorded daily from the day prior to challenge to 24 days post challenge. Blood was collected for serum on days 0 and every 3 to 4 days post challenge. On day 10 post challenge, half of the dogs in each challenge group and contact control group were humanely euthanatized, necropsied, and samples collected for viral isolation. On day 14 post challenge, the remaining dogs that were previously challenged were administered 1.0 ml of the same challenge by the same route. On day 21 all remaining dogs were humanely euthanatized, necropsied, and samples collected for viral isolation.

The four 6 week old dogs (4 males) from the NVSL SPF colony were housed as a group as were the four 12 week old NVSL SPF dogs (4 females). Two dogs in each group received 1.0 ml of the BTV isolate challenge and the other two, 1.0 ml of the BTV contaminated vaccine challenge by the same intramuscular route. Dogs were rechallenged 14 days after the first challenge. Dogs were observed and blood sampled at 3 to 4 day intervals as previously described for 21 days and 35 days respectively. The dogs were humanely euthanatized, necropsied, and samples collected for viral isolation at the conclusion of the observation period.

The four juvenile dogs (3 female, 1 male) were housed as a group. Two dogs received 1.0 ml of the BTV isolate challenge and the other 2, 1.0 of the BTV contaminated vaccine challenge as previously described. Dogs were observed and blood sampled as the other groups, but the dogs were euthanatized, necropsied, and samples collected on day 14 post challenge without receiving a second challenge.

Six of the pregnant bitches were divided into 3 groups, 2 dogs per room. One group

received 1.0 ml of the BTV isolate challenge , the second group 1.0 ml of the BTV contaminated vaccine challenge, and the third group 1.0 ml of the non contaminated vaccine. All challenges were administered in one site in the rear leg. Bitches were observed twice daily starting one day prior to challenge and blood samples for serum collected on the day of challenge and at 3 to 4 day intervals. Bitches were observed more frequently, up to hourly intervals, as they developed clinical signs. All abortions and live births were recorded and fetal and placental tissues collected.

Experiment 2 challenge

Blood was collected from bitches who had received the BTV isolate from the contaminated vaccine in a previous experiment. The challenge used was from a pool of blood from dogs demonstrated to be viral positive (viremic) and from days that coincided with the time of observed abortions. The EDTA treated blood sample was centrifuged at 750 X *g* for 10 minutes and the serum removed. The RBC were washed by suspending the RBC pellet in sterile PBS at 4 C, equal to the removed serum volume and then centrifuging at 750 X *g* for 15 minutes at 4 C. The procedure was repeated for a total of 3 washes. The final washed RBC pellets were suspended with an equal volume of buffered lactose peptone with 375 µg/ml gentamycin, 50 µg/ml kanamycin, and 50 µg/ml amphotercin B and stored at 4 C until used. The RBC BTV challenge was administered undiluted.

The BTV blood challenge was titrated in 10 day old chicken egg embryos and the embryos observed for 7 days. The buffered lactose peptone-stored RBC's were centrifuged and 0.2 ml of packed cells lysed in 4 ml of sterile distilled water and then QS to 10 ml in buffered lactose peptone with antibiotics for a final dilution of 1 to 20. Additional 1 to 5 dilutions were made in buffered lactose peptone with antibiotics. The chorioallantoic vessels

of the embryos were visualized by candling, a portion of the egg shell removed, and 0.1 ml intravenously administered.[c] Embryos dying from day 2 through 7 were used to determine the titer by the methods of Spearman and Kärber as modified by Finney.[11]

Two pregnant bitches and 4 sheep (2 female, 2 male) received a total of 2.0 ml, divided equally in each hind leg, of the RBC BTV challenge administered with a 23 g 1 inch needle and syringe. Bitches were observed and rectal temperature recorded at least twice daily starting on the day prior to challenge through day 10 or as long as the bitches survived. As bitches developed clinical signs they were observed more frequently, up to hourly intervals. Blood samples for serum were collected on the day of challenge and at 3 to 4 day intervals. All abortions and live births from the bitches were recorded and fetal and placental tissues collected as appropriate. Sheep were observed and rectal temperature recorded daily, and twice daily as clinical signs occurred, from the day prior to challenge to day 20 post challenge. Blood samples for serum were collected on the day of challenge and at 3 to 4 day intervals. All surviving sheep were necropsied on day 20 and tissue samples collected for viral isolation.

Animal care and approval

The experiments were conducted with the approval of the NVSL Animal Care and Use Committee and in accordance with the NIH guidelines and the Animal Welfare Act. Animals were acclimated prior to initiation of all studies.

Blood samples

Serum was separated from clotted blood by centrifugation at 1,800 X *g* for 10 minutes and stored at 4 C. A commercial BTV competitive ELISA (cELISA) diagnostic kit

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[c] Protocol courtesy of the Diagnostic Virology Laboratory, National Veterinary Services Laboratory, Ames, IA

was used to determine BTV antibody levels.[f]

Viral isolation from tissues

Tissues were treated as previously described[l] substituting MEM media containing 75 units/ml penicillin, 225 units/ml streptomycin, and 150 μg/ml gentamycin. Brain, spinal cord, heart, muscle, kidney, lung, spleen, liver, bone marrow, mesenteric lymph node, aorta, colon, tonsil, and small intestine were examined when possible. Uterus, uterine fluid, and placenta tissues were individually examined from each bitch where possible. Four hundred microliters (400 μl) of supernatant was inoculated onto 25 cm^2 flasks of confluent Vero cell culture and 400 μl onto a 25 cm^2 flask of embryonic bovine kidney primary cells for each supernatant. Flasks were incubated at 36 C in a 5% CO_2 incubator. Flasks were observed daily for CPE and frozen at -70 C when CPE was observed. Flasks not exhibiting CPE were subpassaged 2 additional times at 4 to 6 day intervals and if no CPE observed were considered negative. Flasks with CPE were thawed at room temperature, centrifuged at 200 X *g* for 10 minutes, and the supernatant individually collected. Vero cell cultures in 8 chamber glass slides, 24 hours after seeding and incubating at 36 C in a 5% CO_2 incubator, were inoculated with 0.1 ml per chamber of the supernatant and further incubated at 36 C in a 5% CO_2 incubator. Slides were examined daily and fixed with acetone if CPE was evident or fixed at 7 days if no CPE was observed. Cells were examined using a direct fluorescent antibody BTV conjugate[g] and an ultraviolet light microscope and considered a positive isolate if specific BTV fluorescence was observed.

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[f] Bluetongue antibody test kit, cELISA, Veterinary Diagnostic Technology, Inc., Wheat Ridge, CO 80033

[g] Bluetongue fluorescent antibody conjugate, National Veterinary Services Laboratories, Ames, IA

Results

Experiment 1

Challenge

The viral titer of the BTV isolate titered 6.8 $\log_{10}$ tissue culture infective doses fifty percent end point ($10^{6.8}$ $TCID_{50}$) per ml, which after dilution resulted in a challenge of $10^{5.6}$ $TCID_{50}$ per ml. The BTV viral titer of a representative sample of the contaminated vaccine titered $10^{5.5}$ $TCID_{50}$ per ml. No viral contaminants were found in the BTV isolate challenge.

Observations

Non pregnant dogs. No significant clinical signs attributable to BTV were observed in any of the 6 week old, 12 week old, or juvenile non pregnant dogs following either the initial challenge or the second challenge. Intermittent loose stools in the majority of the 6 week old dogs occurred following the second challenge of the BTV isolate and the BTV contaminated vaccine, but also was observed in the contact controls and was attributed to the diet. Rectal temperatures in all of the non pregnant dogs for the observation period were within the normal range (37.8 C to 39.1 C),[13] except for 3 dogs in the juvenile group that had temperatures exceeding normal, on days 3, 3, and 5 to 7 post initial challenge, respectively, with a maximum temperature of 39.6 C. These represented a BTV isolate dog and 2 BTV contaminated vaccine dogs.

Pregnant bitches. The 2 bitches receiving the BTV isolate remained normal through day 4 post challenge. Starting on day 5 post challenge both bitches had elevated temperatures, yellowish feces, dark mucoid vaginal discharge, and appeared generally restless. The bitches receiving the BTV isolate aborted on day 5 and 6 respectively. The bitch aborting on day 5 aborted 5 fetuses, the one aborting on day 6, 1 fetus. Both bitches

continued to show clinical signs after abortion including vaginal discharge, listlessness, rattled breathing, and coughing. The second aborting bitch died on day 8 post challenge. The first aborting bitch was euthanatized on day 8 post challenge due to a general decline in health and difficult breathing. Both bitches exhibited an elevated temperature at the time of abortion followed by a gradual temperature decline. The bitch that was euthanatized had a subnormal temperature at the time of euthanasia.

The 2 bitches receiving the BTV contaminated vaccine remained normal through day 2 post challenge. On day 3 one bitch exhibited yellowish mucoid stool and both animals had distended abdomens. One bitch on day 4 had a rapidly declining temperature and mucoid diarrhea and died. No abortion was noted. The second bitch on day 4 exhibited a febrile response followed by a rapidly declining temperature, which by day 5 was subnormal. On day 5 that bitch was observed to vomit, had a greenish vaginal discharge, and aborted 2 fetuses. The bitch died on day 5. At necropsy the first bitch had 8 retained fetuses and the second bitch 5 fetuses.

The 2 bitches receiving the non contaminated vaccine remained normal. The first bitch whelped 7 live puppies 10 days post challenge and the second bitch 7 live puppies 14 days post challenge.

Serum samples

The earliest response in the dogs from dams who had been routinely vaccinated was on day 10 post challenge when 1 of the dogs receiving the BTV isolate had a 52 percent inhibition cELISA reaction, indicative of a positive serological titer. Subsequently one of the BTV contaminated vaccine group and another of the dogs receiving the BTV isolate had a positive cELISA reaction, both on day 17. The contact controls remained negative through

the study (Figure 1 to 3). Both of the dogs from non vaccinated dams receiving the BTV isolate were positive on day 20, while the dogs from non vaccinated dams receiving the BTV contaminated vaccine remained seronegative through the study (Figures 4 and 5). Combining the results from routinely vaccinated and non vaccinated dams showed a slightly greater average response for dogs receiving the BTV isolate than the BTV contaminated vaccine. Only on day 17 and 20 could the average percent inhibition be considered positive for the BTV isolate group. For all other days, dogs from all groups were negative (Figure 6). The number of positive dogs by days post challenge for the groups of 6 week old dogs is summarized in Table 1.

One of the dogs that received the BTV isolate in the group of 12 week old dogs Became cELISA positive on day 14 post challenge and the remaining dog in that group and the BTV vaccine dogs were positive by day 21 (Figures 7 and 8). A summary of the seroconversions among 12 week old dogs is depicted in Figure 9 and Table 2.

Table 1. Sero conversion of 6 week old dogs for the 5 treatment groups. A positive was any dog whose serum tested ≥50% inhibition for any bleeding date within the specified time.

Treatment group	0 to10 DPC	14 to 24 DPC	0 to 24 DPC
BTV isolate, vaccinated dam	1/4	1/2	2/4
BTV contaminated vaccine, vaccinated dam	0/4	1/2	1/4
BTV isolate, non vaccinated dam	0/2	2/2	2/2
BTV contaminated vaccine, non vaccinated dam	0/2	0/2	0/2
Contact controls, vaccinated dam	0/4	0/2	0/4

DPC = day post challenge

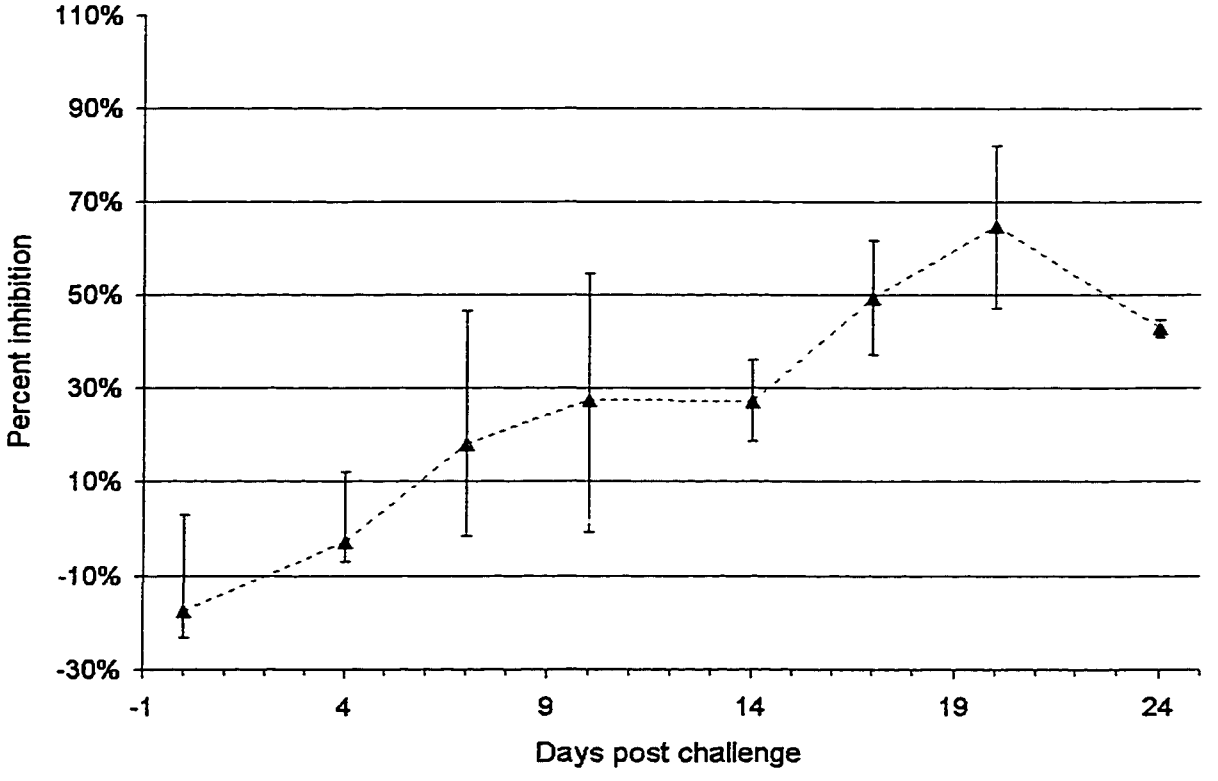

Figure 1. Serological response of 6 week old dogs from routinely vaccinated dams following administration of $10^{5.6}$ $TCID_{50}$ of a BTV isolate recovered from a canine modified live vaccine. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs. N = 4 for day 0 to 10 post challenge and n = 2 for days 14 to 24 post challenge.

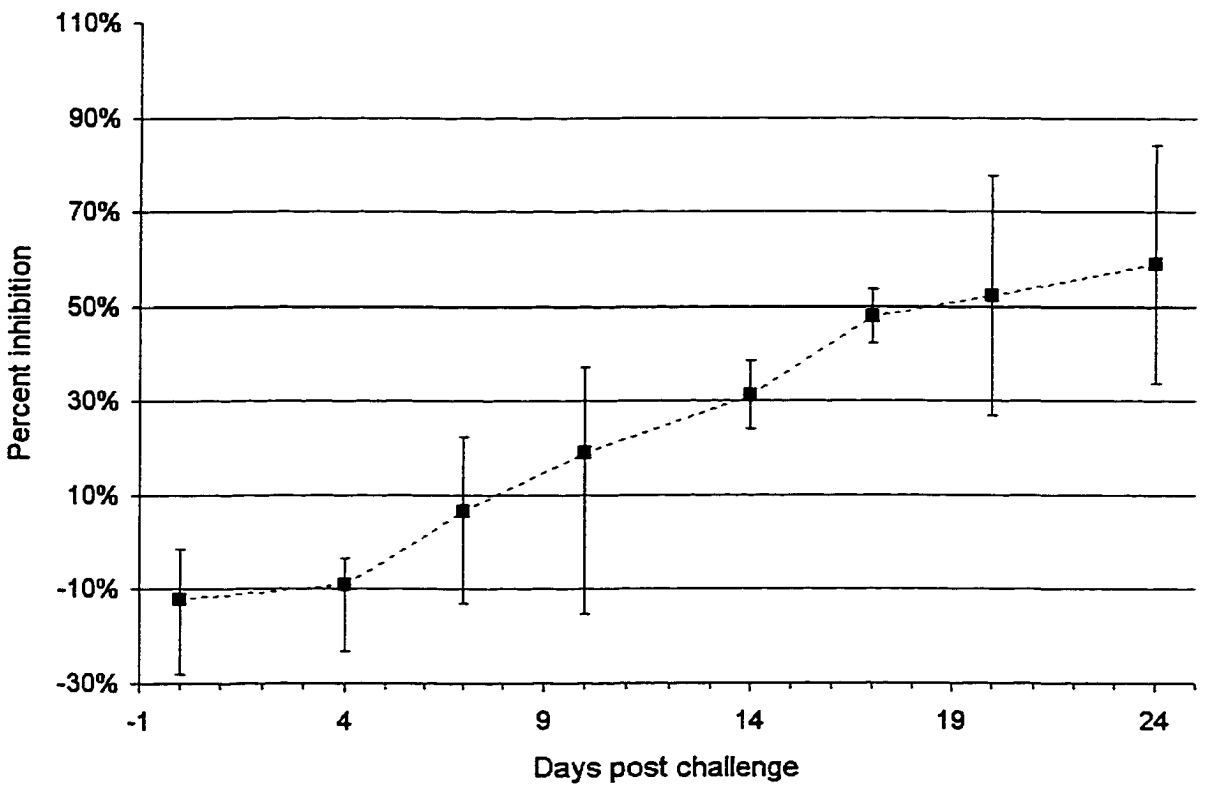

Figure 2. Serological response of 6 week old dogs from routinely vaccinated dams following administration of a contaminated vaccine containing $10^{5.5}$ $TCID_{50}$ of BTV. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs. N = 4 for day 0 to 10 post challenge and n = 2 for days 14 to 24 post challenge.

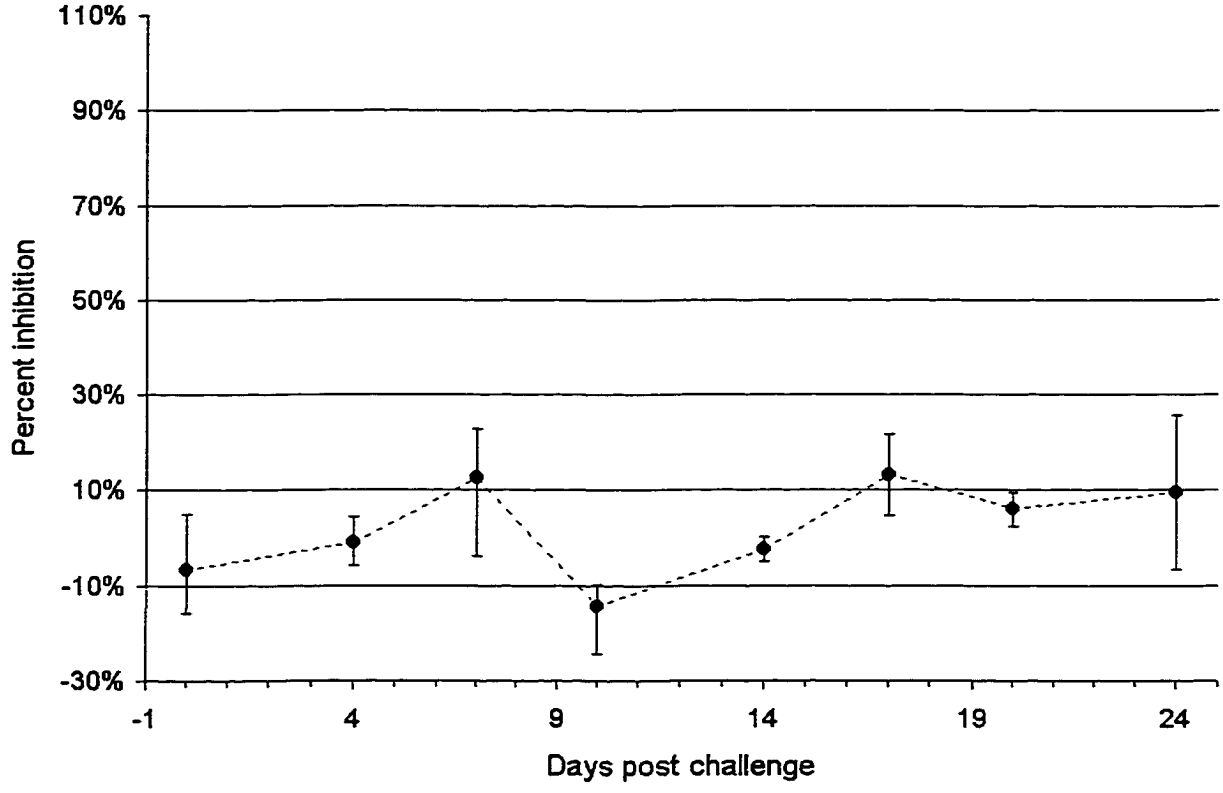

Figure 3. Serological response of 6 week old dogs from routinely vaccinated dams that were not challenged and served as contact controls in the routinely vaccinated dams BTV isolate and BTV contaminated vaccine groups. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs. N = 4 for day 0 to 10 post challenge and n = 2 for days 14 to 24 post challenge.

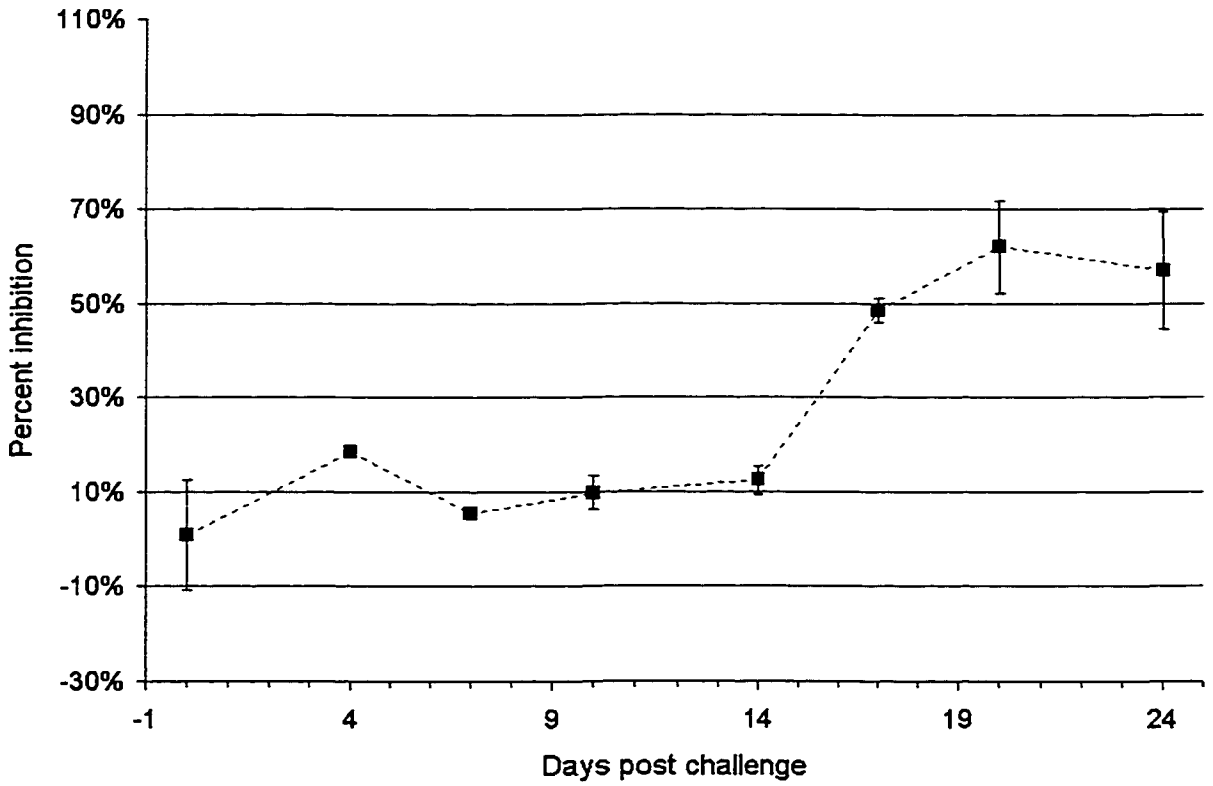

Figure 4. Serological response of 6 week old dogs (n = 2) from SPF, non vaccinated dams following administration of a $10^{5.6}$ $TCID_{50}$ of a BTV isolate recovered from a canine modified live vaccine. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs.

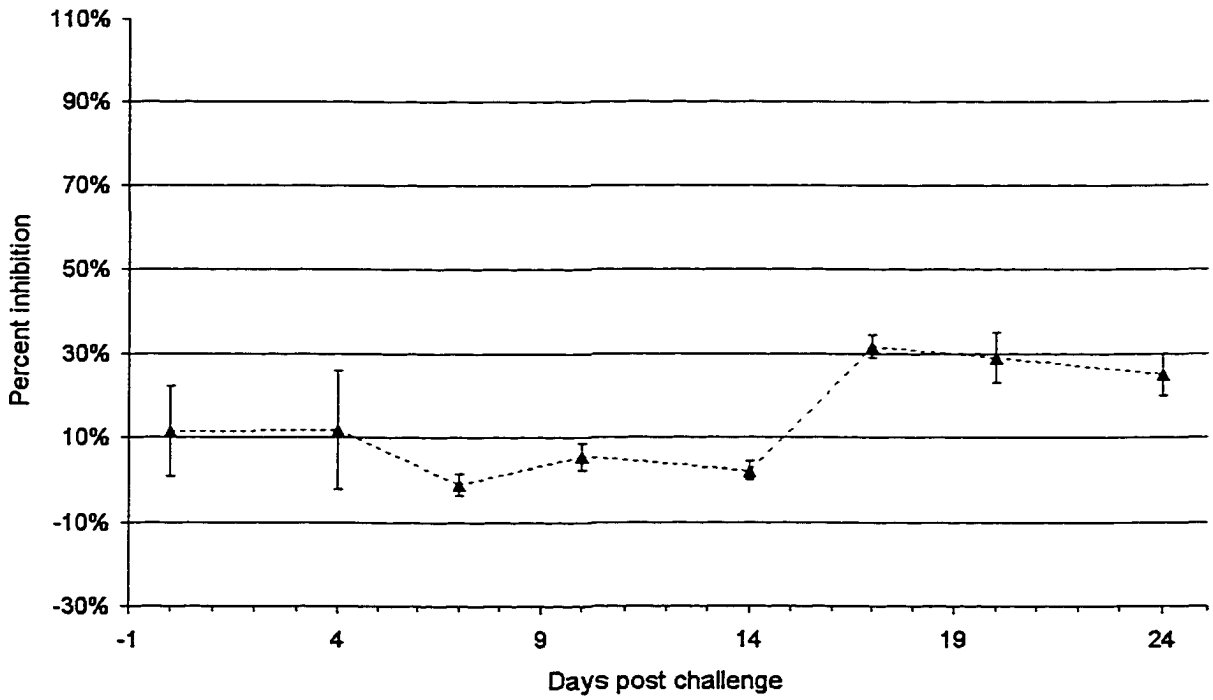

Figure 5. Serological response of 6 week old dogs (n = 2) from SPF, non vaccinated dams following administration of a contaminated vaccine containing $10^{5.5}$ $TCID_{50}$ of BTV. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs.

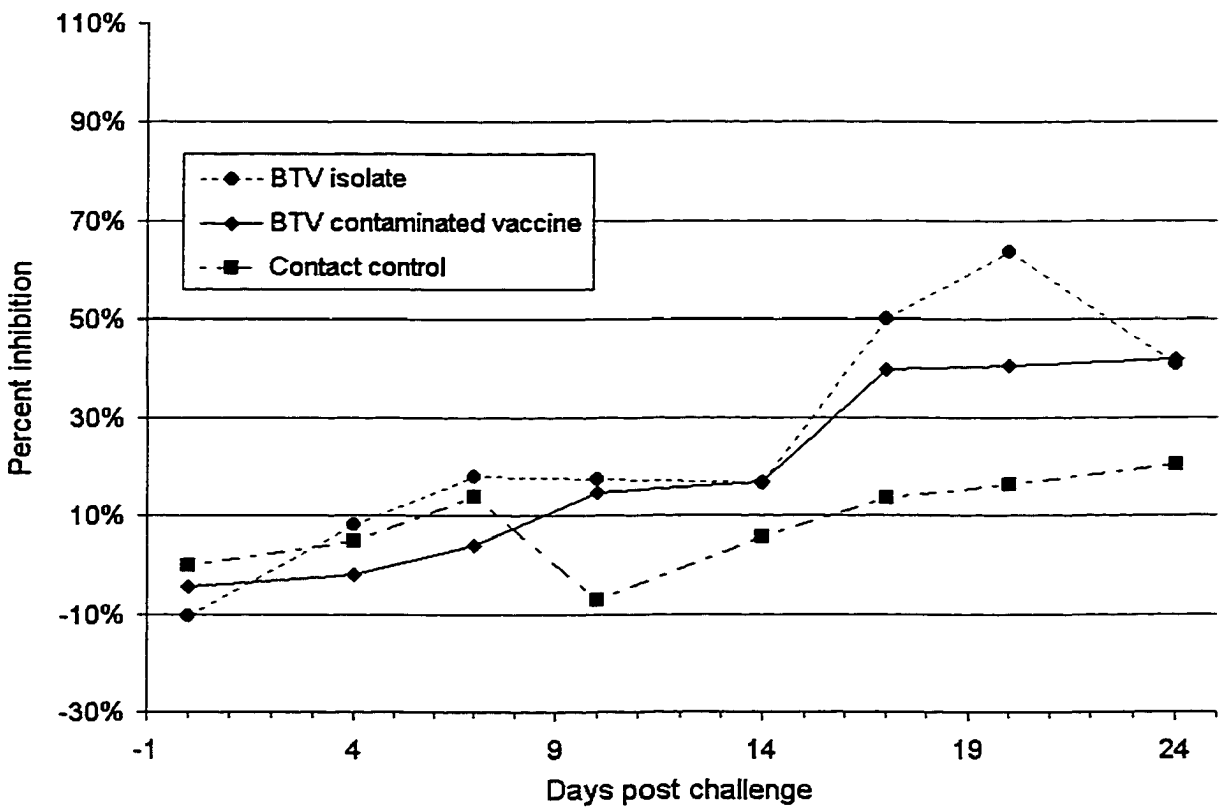

Figure 6. Serological response by treatment for all 6 week old dogs. The BTV isolate group received $10^{5.6}$ $TCID_{50}$ of a BTV isolate recovered from a canine modified live vaccine. The BTV contaminated vaccine group received a commercial vaccine containing $10^{5.5}$ $TCID_{50}$ of BTV. The contact controls received not treatment. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs. N = 6 for days 0 to 10 post challenge and n = 4 for days 14 to 25 for both the BTV isolate and BTV contaminated vaccine groups; n = 4 for days 0 to 10 post challenge and n = 2 for days 14 to 25 for the contact controls..

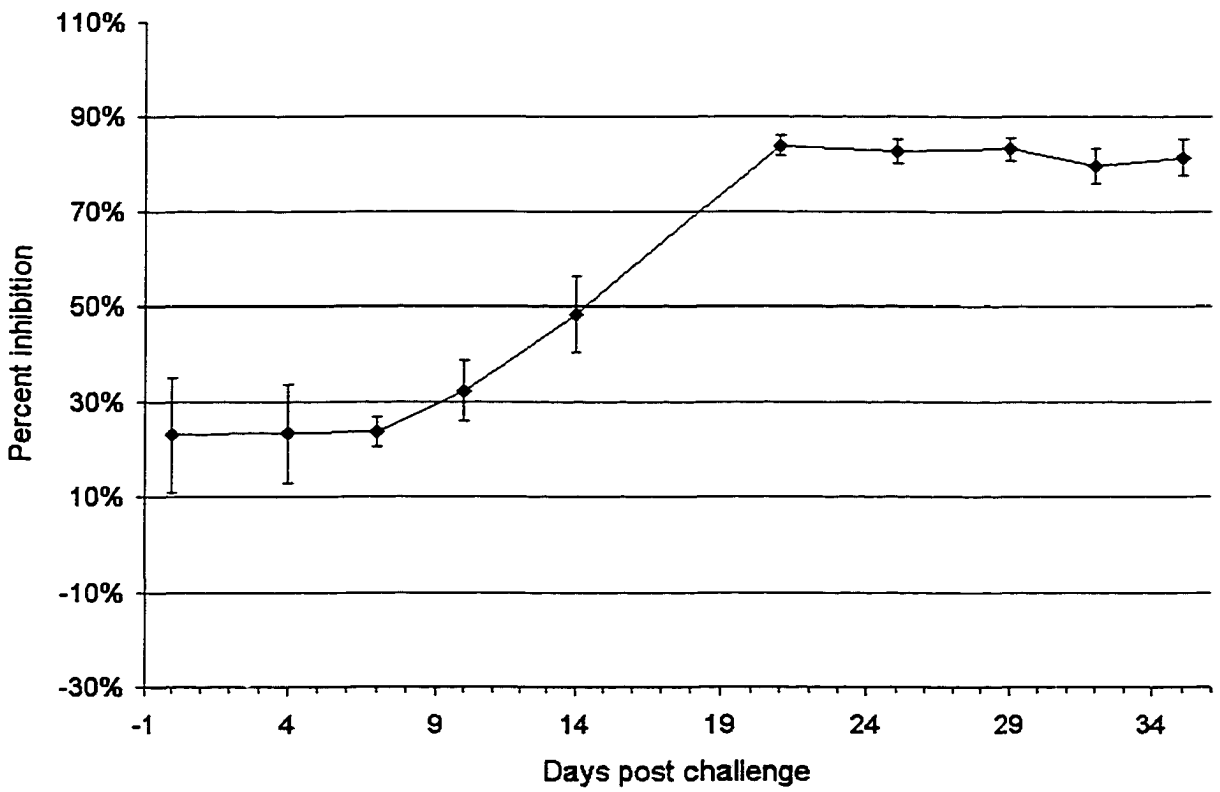

Figure 7. Serological response of 12 week old dogs (n = 2) from SPF, non vaccinated dams following administration of $10^{5.6}$ $TCID_{50}$ of a BTV isolate recovered from a canine modified live vaccine. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs.

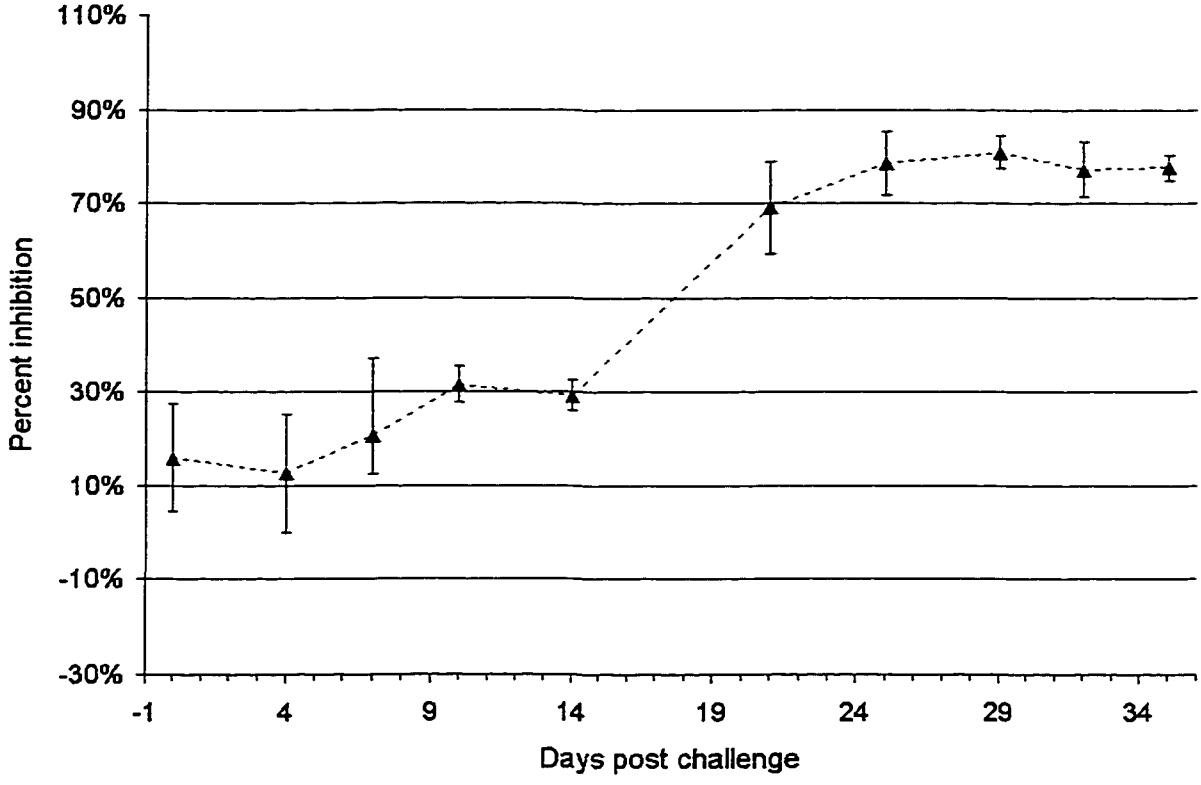

Figure 8. Serological response of 12 week old dogs (n = 2) from SPF, non vaccinated dams following administration of a contaminated vaccine containing 10^{55} $TCID_{50}$ of BTV. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs.

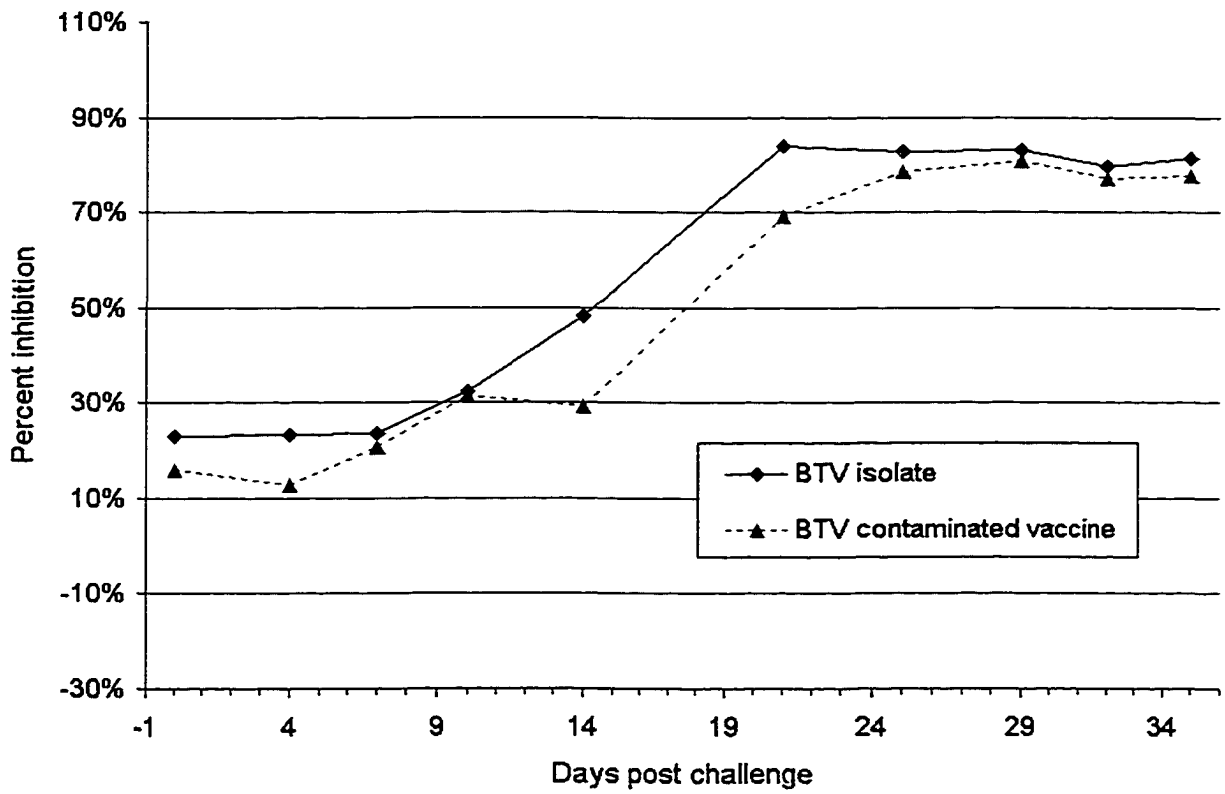

Figure 9. Serological response by treatment for the 12 week old dogs. The BTV isolate group (n = 2) received $10^{5.6}$ $TCID_{50}$ of a BTV isolate recovered from a canine modified live vaccine. The BTV contaminated vaccine group (n = 2) received a commercial vaccine containing $10^{5.5}$ $TCID_{50}$ of BTV. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs.

Table 2. Numbers of 12 week old dogs from non vaccinated dams considered serologically positive over total number of dogs for the 2 treatment groups. A positive was any dog whose serum tested ≥50% inhibition for any bleeding date within the specified time.

Treatment group	0 to10 DPC	14 to 25 DPC	25 to 35 DPC
BTV isolate	0/2	2/2	2/2
BTV contaminated vaccine	0/2	2/2	2/2

DPC = day post challenge

Both treatment groups of the juvenile dogs remained serologically negative through day 10 post challenge when they were humanely euthanatized and necropsied (Figures 10 to 12).

The serological results for the pregnant bitches from the BTV isolate, the BTV contaminate and the non contaminated vaccine are provided in Figure 13. Only 1 bitch from the BTV isolate group seroconverted. Of the two groups receiving BTV (BTV isolate and BTV contaminated vaccine challenges), none of the animals survived beyond day 8. Neither of the bitches in the non contaminated group seroconverted through day 14 when they were humanely euthanatized and tissues collected.

Viral isolation from tissues

Viral isolation from the 6 week old dogs occurred only in the dogs challenged with the BTV isolate and the dogs challenged with the BTV contaminated vaccine that were euthanatized and necropsied 10 day post challenge (Tables 3 and 4). No viral isolation occurred in the contact control dogs at either 10 or 24 days post challenge or in the BTV isolate or BTV contaminated vaccine groups at 24 days post challenge. The most consistent tissue for isolation from the 10 days post challenge groups was the spleen, followed by the

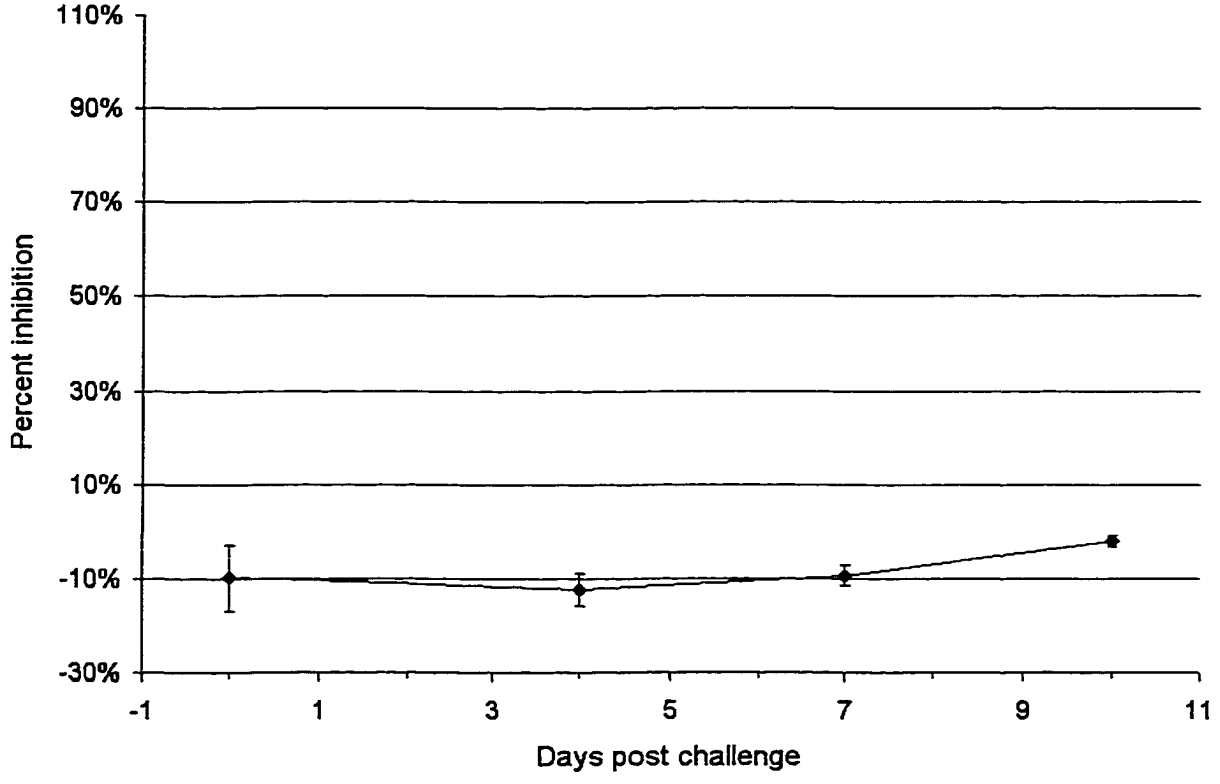

Figure 10. Serological response of juvenile dogs (n = 2) from SPF, non vaccinated dams following administration of a $10^{5.6}$ $TCID_{50}$ of a BTV isolate recovered from a canine modified live vaccine. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs.

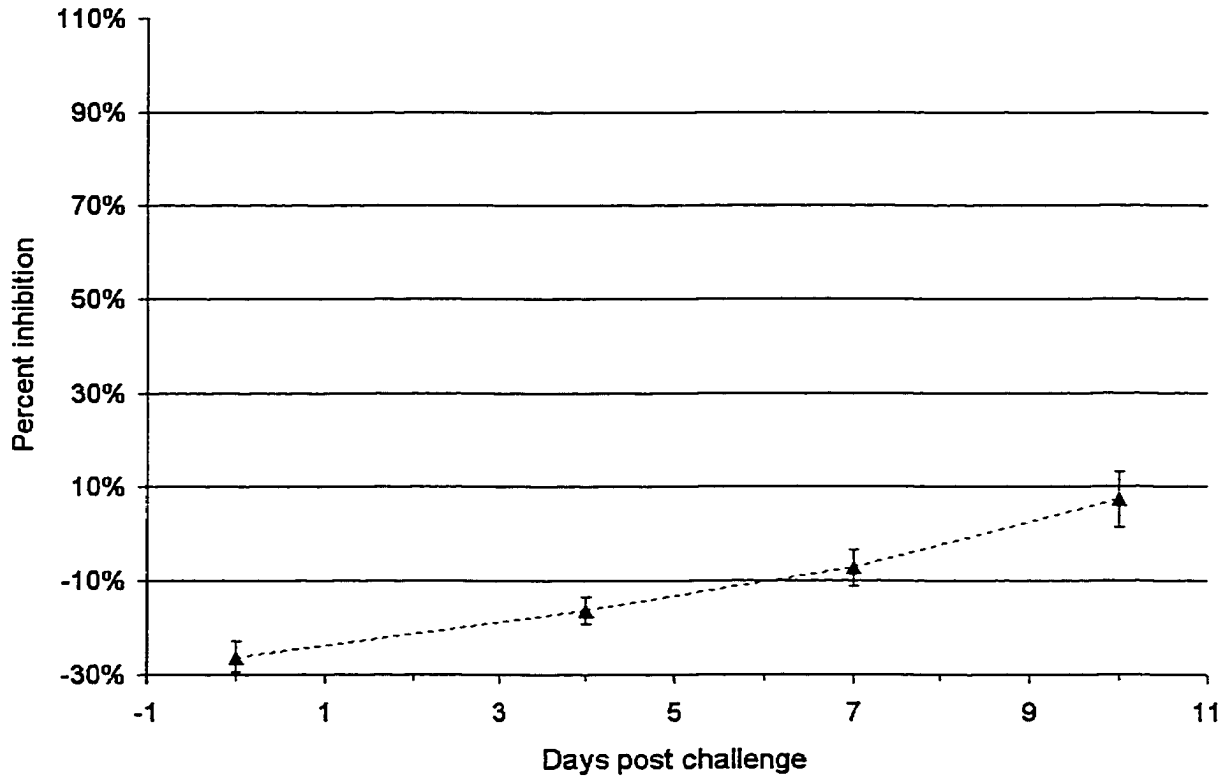

Figure 11. Serological response of juvenile dogs (n = 2) from SPF, non vaccinated dams following administration of a contaminated vaccine containing $10^{5.5}$ $TCID_{50}$ of BTV. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs.

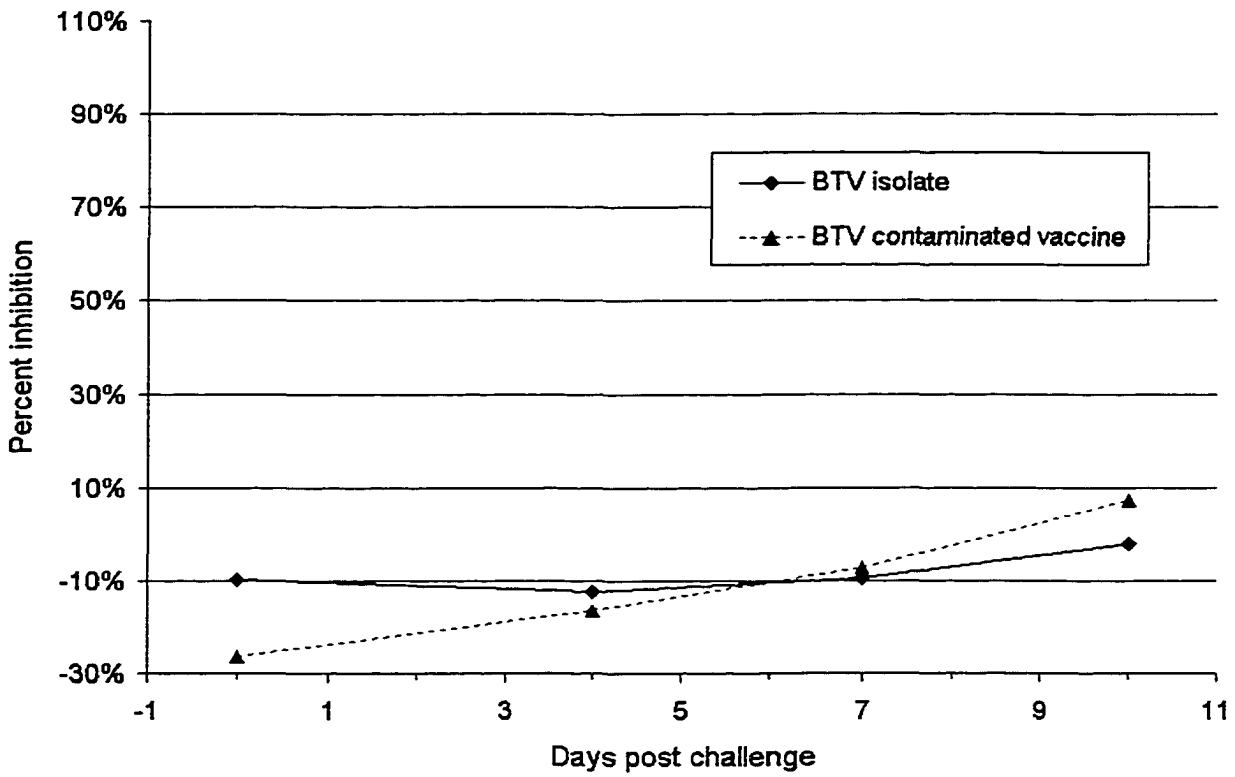

Figure 12. Serological response by treatment for the juvenile dogs. The BTV isolate group (n = 2) received $10^{5.6}$ $TCID_{50}$ of a BTV isolate recovered from a canine modified live vaccine. The BTV contaminated vaccine group(n = 2) received a commercial vaccine containing $10^{5.5}$ $TCID_{50}$ of BTV. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs.

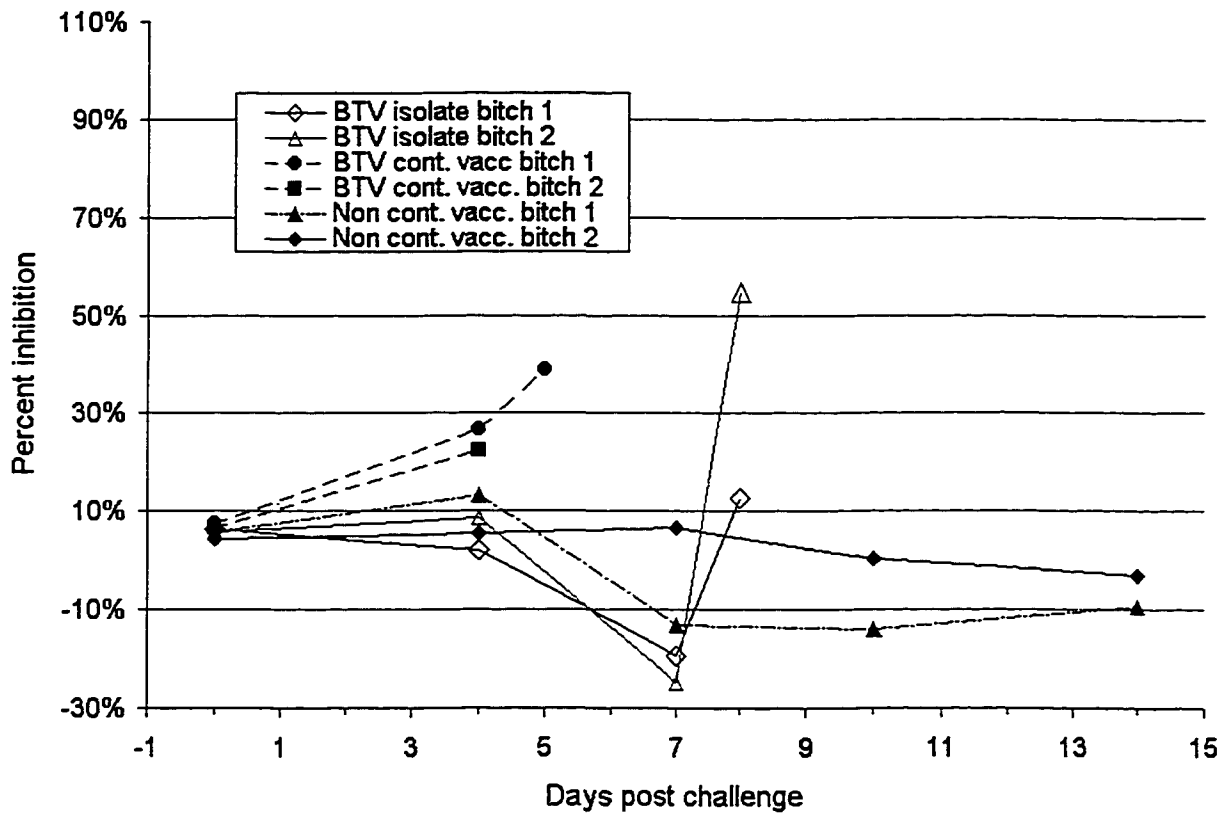

Figure 13. Serological response by treatment for the pregnant bitches. The BTV isolate group received $10^{5.6}$ $TCID_{50}$ of a BTV isolate recovered from a canine modified live vaccine. The BTV contaminated vaccine (BTV cont. vacc.) group received a commercial vaccine containing $10^{5.5}$ $TCID_{50}$ of BTV. The non contaminated vaccine (Non cont. vacc.) Group received a serial of vaccine from the same manufacturer but know to be not contaminated with BTV. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs.

Table 3. Viral isolation on Vero and embryonic bovine kidney cells from 6 week old dogs from routinely vaccinated dams. A tissue was considered positive if BTV was isolated from either cell. Results are entered as the number positive versus number tested.

	6 week old dogs from routinely vaccinated dams								
	BTV isolate			BTV contaminated vaccine			BTV contact control		
	10 DPC	24 DPC	Isolation totals	10 DPC	24 DPC	Isolation totals	10 DPC	24 DPC	Isolation totals
Brain	0/2	0/2	0/4	0/2	0/2	0/4	0/2	0/2	0/4
Spinal cord	ns	ns	ns	ns	ns	ns	ns	ns	ns
Heart	0/2	0/2	0/4	0/2	0/2	0/4	0/2	0/2	0/4
Liver	1/2	0/2	1/4	0/2	0/2	0/4	0/2	0/2	0/4
Lung	1/2	0/2	1/4	1/2	0/2	1/4	0/2	0/2	0/4
Kidney	2/2	0/2	2/4	1/2	0/2	1/4	0/2	0/2	0/4
Muscle	0/2	0/2	0/4	1/2	0/2	1/4	0/2	0/2	0/4
Spleen	2/2	0/2	2/4	2/2	0/2	2/4	0/2	0/2	0/4
Bone marrow	0/2	0/2	0/4	0/2	0/2	0/4	0/2	0/1	0/3
Mesenteric LN	1/2	0/2	1/4	2/2	0/2	2/4	0/2	0/2	0/4
Aorta	0/2	0/2	0/4	1/2	0/2	1/4	0/2	0/2	0/4
Uterus	0/1	0/1	0/2	0/1	0/1	0/2	ns	0/1	0/1
Small intestine	1/2	0/2	1/4	1/2	0/2	1/4	0/2	0/2	0/4
Colon	0/2	0/2	0/4	1/2	0/2	1/4	0/2	0/2	0/4
Tonsil	1/2	0/2	1/4	2/2	0/2	2/4	0/2	0/2	0/4

DPC = days post challenge, ns = no sample, LN = lymph node

Table 4. Viral isolation on Vero and embryonic bovine kidney cells from 6 week old dogs from SPF, non vaccinated dams. A tissue was considered positive if BTV was isolated from either cell. Results are entered as the number positive versus number tested.

	6 week old dogs from SPF, non vaccinated dams	
	BTV isolate 24 DPC	BTV contaminated vaccine 24 DPC
Brain	0/2	0/2
Spinal cord	ns	ns
Heart	0/2	0/2
Liver	0/2	0/2
Lung	0/2	0/2
Kidney	0/2	0/2
Muscle	0/2	0/2
Spleen	0/2	0/2
Bone marrow	0/2	0/2
Mesenteric LN	0/2	0/2
Aorta	0/2	0/2
Uterus	ns	ns
Small intestine	0/2	0/2
Colon	0/2	0/2
Tonsil	0/2	0/2

DPC = days post challenge, ns = no sample, LN = lymph node

mesenteric lymph node, kidney, tonsil, and liver. No isolation occurred from the brain, heart, bone marrow, or uterus.

No viral isolations occurred in the 12 week old dogs when tissues were collected at 35 days post challenge (Table 5).

Only tissues from 1 animal in each treatment group of the juvenile dogs were tested. The lung in the BTV isolate group was the only tissue positive when samples were collected at 10 days post challenge (Table 6).

Isolation results for the pregnant bitches is reported in Table 7. Tissues and placenta from the BTV isolate and the BTV contaminated vaccine group were found consistently positive for BTV. Tissues from the aborted or retained fetuses yielded sporadic virus isolation. No BTV was isolated from the bitches receiving the non contaminated vaccine.

Experiment 2

Challenge

The RBC challenge titered $10^{5.1}$ egg lethal dose 50% (ELD_{50}) for a challenge of $10^{5.4}$ ELD_{50} in the 2 ml challenge dose.

Observations

Sheep. Sheep receiving the RBC challenge exhibited no clinical signs through day 6 post challenge. On day 6 rapid respiration, lethargy, and inappetance were observed which lasted through day 10. Rectal temperatures peaked on day 7 for 4 of 4 sheep and again on day 10 for 2 of 4 sheep (Figure 14). Baseline temperatures for the sheep were consistently in the high normal to slightly above normal range for all days, but temperatures exceeded those baselines for the days noted. Baseline temperatures observed were consistent with previous observations for sheep held in climate controlled facilities when sheep had any appreciable

Table 5. Viral isolation on Vero and embryonic bovine kidney cells from 12 week old dogs from SPF, non vaccinated dams. A tissue was considered positive if BTV was isolated from either cell. Results are entered as the number positive versus number tested.

	12 week old dogs from SPF, non vaccinated dams	
	BTV isolate 35 DPC	BTV contaminated vaccine 35 DPC
Brain	0/2	0/2
Spinal cord	ns	ns
Heart	0/2	0/2
Liver	0/2	0/2
Lung	0/2	0/2
Kidney	0/2	0/2
Muscle	0/2	0/2
Spleen	0/2	0/2
Bone marrow	0/2	0/2
Mesenteric LN	0/2	0/2
Aorta	0/2	0/2
Uterus	ns	ns
Small intestine	0/2	0/2
Colon	0/2	0/2
Tonsil	0/2	0/2

DPC = days post challenge, ns = no sample, LN = lymph node

Table 6. Viral isolation on Vero and embryonic bovine kidney cells from juvenile dogs from SPF, non vaccinated dams. A tissue was considered positive if BTV was isolated from either cell. Results are entered as the number positive versus number tested.

	Juvenile dogs from SPF, non vaccinated dams	
	BTV isolate 10 DPC	BTV contaminated vaccine 10 DPC
Brain	0/1	0/1
Spinal cord	ns	ns
Heart	0/1	0/1
Liver	0/1	0/1
Lung	1/1	0/1
Kidney	0/1	0/1
Muscle	0/1	0/1
Spleen	0/1	0/1
Bone marrow	0/1	0/1
Mesenteric LN	0/1	0/1
Aorta	0/1	0/1
Uterus	0/1	0/1
Small intestine	0/1	0/1
Colon	0/1	0/1
Tonsil	0/1	0/1

DPC = days post challenge, ns = no sample, LN = lymph node

Table 7. Viral isolation on Vero and embryonic bovine kidney cells from pregnant bitches and fetuses. A tissue was considered positive if BTV was isolated from either cell. Results are entered as the number positive versus number tested.

	Challenge group					
	BTV isolate		BTV contaminated vaccine		Non contaminated vaccine	
	Bitches	Fetuses	Bitches	Fetuses	Bitches	Fetus
Brain	1/2	1/6	2/2	13/15	0/2	ns
Spinal cord	2/2		2/2		0/2	
Heart	1/2		0/2		0/2	
Heart/aorta		1/6		0/15		ns
Liver	2/2	0/6	2/2	4/15	0/2	ns
Lung	2/2	0/6	2/2	1/15	0/2	ns
Kidney	2/2	0/6	2/2	4/15	0/2	ns
Muscle	2/2		2/2		0/2	
Spleen	2/2	0/6	2/2	2/15	0/2	ns
Bone marrow	2/2		2/2		0/2	
Mesenteric LN	2/2		2/2		0/2	
Aorta	2/2		2/2		0/2	
Uterus	2/2		2/1		0/2	
Small intestine	2/2		2/2		0/2	
Colon	2/2		2/2		0/2	
Intestine		0/6		1/15		ns
Tonsil	2/2		2/2		0/2	
Placenta		6/6		14/15		ns
Uterine/ Placental fluid		2/2		8/12		

ns = no sample, LN = lymph node

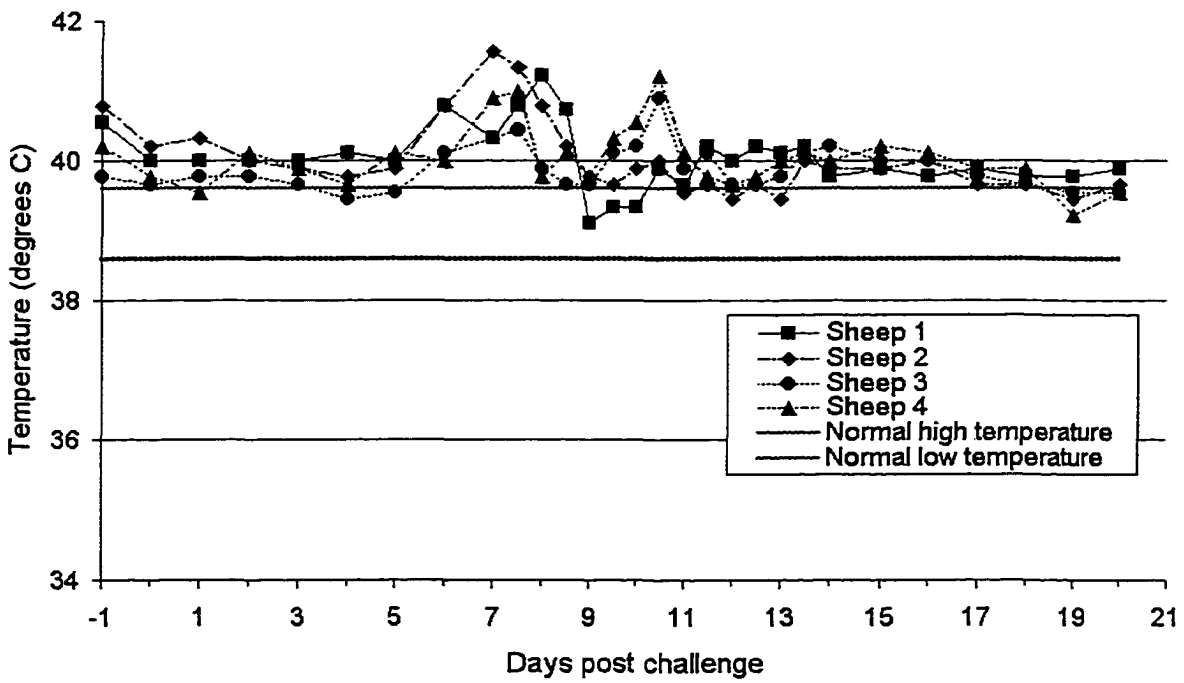

Figure 14. Rectal temperature of sheep challenged with dog RBC from bitches aborting after challenge with a BTV isolate from a contaminated canine vaccine.

amount of wool. Sheep returned to baseline temperatures following day 10 and no additional signs were noted.

Pregnant bitches. The pregnant bitches remained normal through day 4. Vomiting, inappetance, increased activity, and a general "ill at ease" behavior were noted on day 5. Vomiting, depression, and rapidly falling temperatures were observed on day 6. The first bitch died on day 6. The second bitch continued to be depressed and the bitches temperature declined further. The second bitch aborted 8 pups and was euthanatized on day 7 upon becoming listless along with a temperature below 35 C. At necropsy the first bitch had 7 retained fetuses and the second bitch 2 retained fetuses.

Serum samples

Three of 4 sheep had greater than 50% inhibition by day 10 and all had greater than 50% inhibition by day 17 (Figure 15). Neither of the pregnant bitches had significant percent inhibition by the time of their death or when they were euthanatized (Figure 16).

Viral isolation from tissues

Virus was isolated from all 4 of the sheep from tissues collected at necropsy at day 20. No one tissue was found positive in all sheep. The spleen was positive in 3 of 4 sheep (Table 8). The majority of the tissues from the pregnant bitches were found positive but the aborted fetuses or retained fetuses were only sporadically positive.

Discussion

This study found no acute disease or clinical signs elicited by either an initial or a second challenge of cell culture adapted BTV in non pregnant female dogs or male dogs, regardless if from routinely vaccinated or unvaccinated dams. No difference was noted by

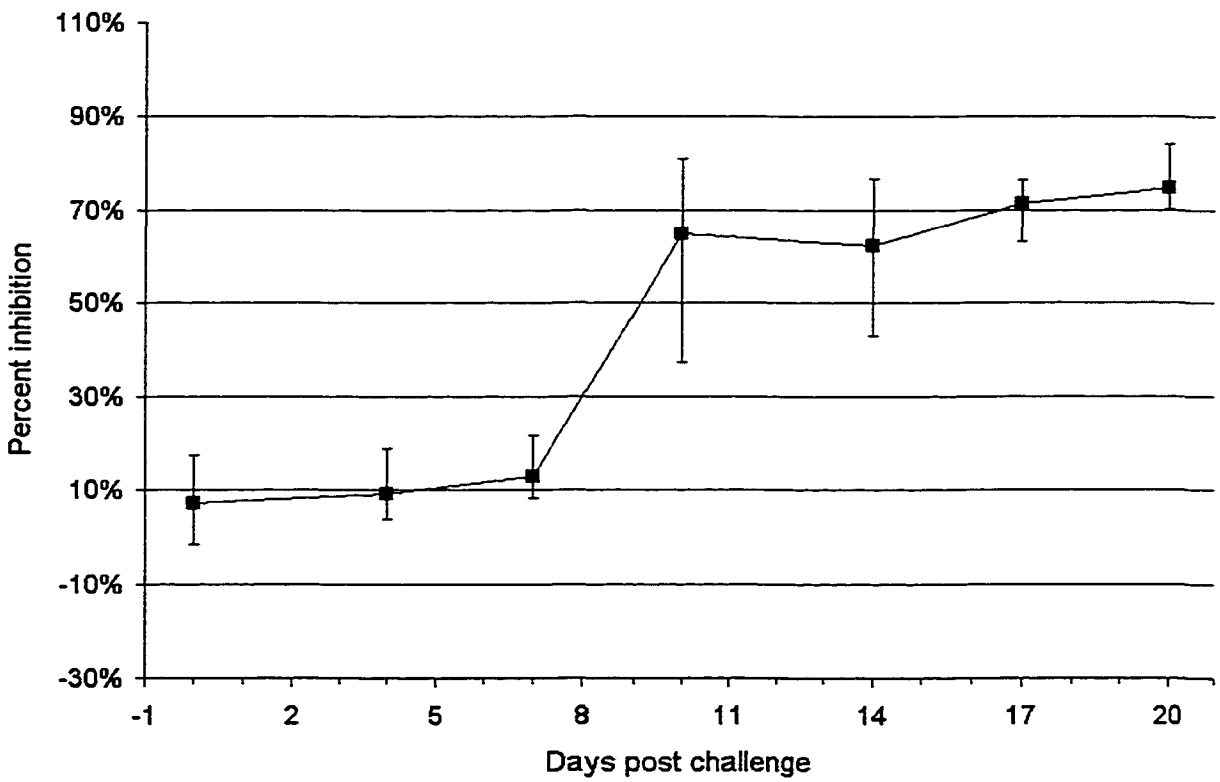

Figure 15. Serological response of sheep (n = 4) following administration of dog RBC from aborting bitches that had received a BTV isolate from a contaminated vaccine. Greater than 50% inhibition is considered a positive antibody titer in ruminants.

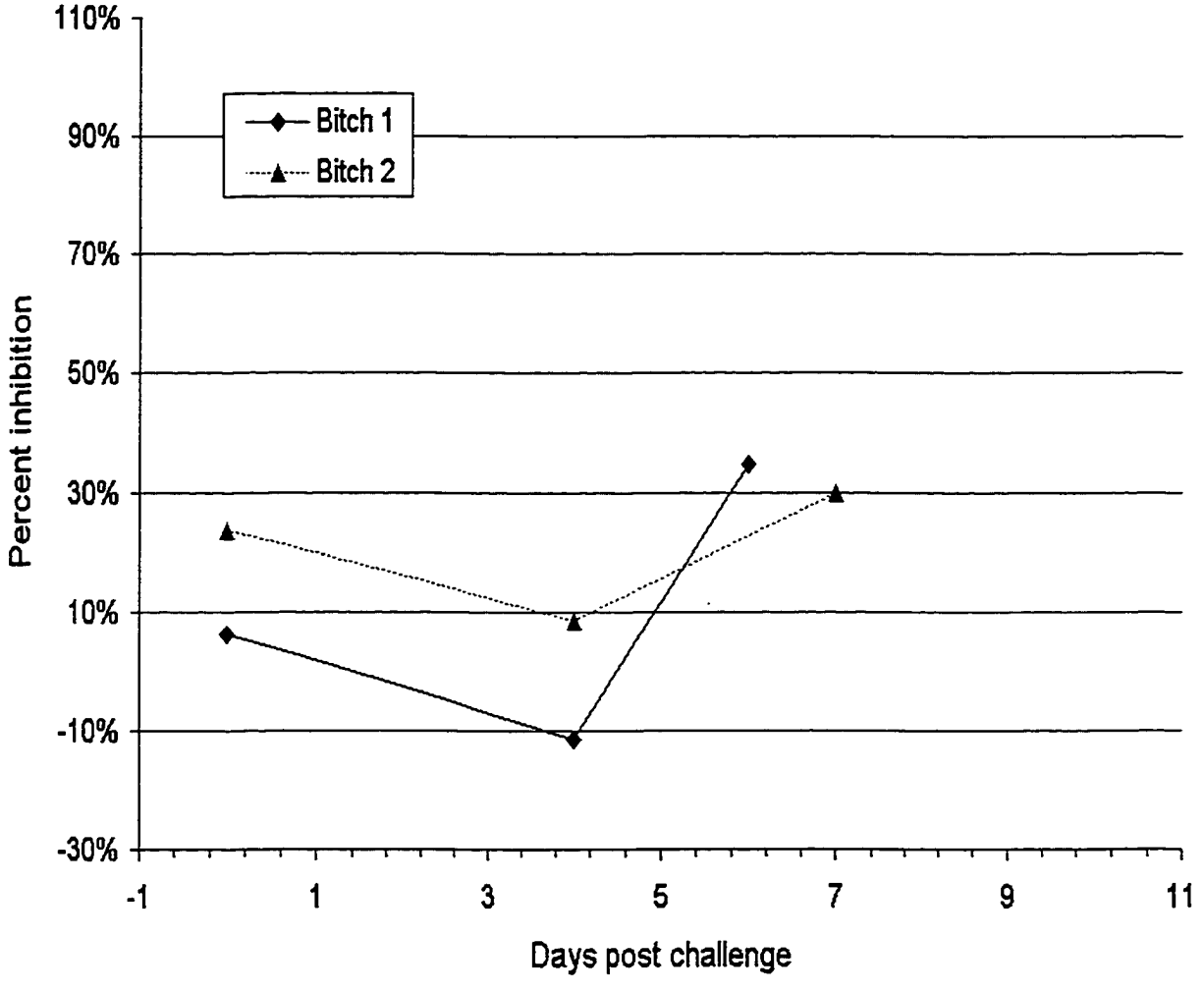

Figure 16. Serological response of pregnant bitches following administration of dog RBC from aborting bitches that had received a BTV isolate from a contaminated vaccine. Greater than 50% inhibition is considered a positive antibody titer in ruminants. No threshold inhibition level has been determined for dogs

Table 8. Viral isolation on Vero and embryonic bovine kidney cells from non pregnant sheep, pregnant bitches, and their fetuses, after receiving dog RBC from aborting bitches who had received a BTV isolate from a contaminated vaccine. A tissue was considered positive if BTV was isolated from either cell. Results are entered as the number positive versus number tested.

	Sheep 21 DPC	Bitches	Fetuses[1]
Brain	1/4	2/2	6/8
Spinal cord	0/4	1/1	
Heart	0/4	1/2	
Heart/aorta			1/8
Liver	0/4	2/2	2/8
Lung	0/4	2/2	1/8
Kidney	0/4	2/2	1/8
Muscle	0/4	2/2	
Spleen	3/4	2/2	1/8
Bone marrow	0/4	2/2	
Mesenteric LN	0/4	1/1	
Aorta	1/4	1/1	
Uterus	0/2	2/2	
Small intestine	0/4	2/2	
Colon	0/4	2/2	
Intestine			2/8
Tonsil	1/4	1/1	
Placenta			15/15
Uterine/ Placental fluid			14/17

DPC = days post challenge, LN = lymph node

[1] Represents 7 fetus from 1 bitch and a pool of multiple fetus from a 2nd bitch for all tissues except the placenta and uterine fluids. Tissues were tested as a pool due to the size of the fetuses.

age of animals, whether administered to young dogs or juvenile dogs. No acute adverse effects were noted for either the cell culture adapted BTV isolate obtained from a contaminated canine modified live vaccine or from the contaminated canine vaccine itself. As expected, no spread of the BTV was observed in contact controls. Conversely, those same challenges when administered to pregnant bitches produced death or abortion and death in both bitches in each group. This is in contrast to another control group who received a non contaminated vaccine similar to the contaminated vaccine and from the same manufacture. Both bitches in that group whelped normal pups. There was evidence that the BTV contaminated vaccine-challenged pregnant bitches had shorter duration to clinical signs and died more acutely than did the BTV isolate challenged dogs. Both challenges contained equivalent amounts of BTV.

Most of the dogs which received either the BTV isolate challenge or the BTV contaminated vaccine became seropositive. Contact controls remained seronegative. Seroconversion typically did not reliably occur in dogs until 14 to 17 days after initial vaccination. The younger 6 week old dogs, characteristic of the earliest age for first vaccination, had lower and more inconsistent serological responses than older dogs. Although 6 week old dogs are considered immunocompetent there is evidence that puppies who have nursed and have maternal antibodies are less responsive to vaccination, even when no antibodies to the vaccine antigen are passively transferred, than older dogs after the passive antibody has waned. This is believed to be due to a central suppression elicited by self regulation of immunoglobulin concentrations or may be due to nonimmunoglobulin immunosuppressive factors in colostrum and may account for the lack of response of this group.[6]

There was no difference between the immune response of the BTV contaminated vaccine groups and the BTV isolate groups. No effect on serological response due to potential immunosuppressive vaccine component administration was evident.

Based on these findings, it would be expected that a percentage of dogs in the U.S. population would be seropositive for BTV if they had received a vaccination with a BTV contaminated vaccine. Any retrospective study to determine if natural infection in dogs with BTV occurs would have to be done with serum samples collected prior to the occurrence of this contamination event.

Bluetongue virus could be isolated from non pregnant dogs 10 days after challenge with either the BTV isolate or the BTV contaminated vaccine. No differences in isolation rate between the two groups were evident. Virus was more consistently isolated in the younger dogs than in older dogs. No virus could be isolated in dogs at 24 days post challenge or later. Tissues of choice for viral isolation were consistent with recommendations for tissue isolation in ruminants, with spleen being the best tissue for isolation.

Previously it had been shown that there was a viremic phase in pregnant bitches that coincided with the time of abortion. Blood from pregnant bitches that had aborted after receiving a cell culture adapted BTV was demonstrated to be infective for sheep and capable of inducing abortion in other pregnant bitches. Although a potential source for infecting the *Culicoides* spp. vector, the short viremic phase demonstrated in previous experiments and the infectivity shown here makes this an unlikely but theoretical possibility.

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CHAPTER 5. GENERAL CONCLUSIONS

The reporting of disease and death of dogs following administration of a canine vaccine, historically safe and effective, lead to the discovery of a viral contaminate and the recall of vaccine. Because there was disease caused by an agent in a species not previously reported, studies were conducted to determine if the contaminating virus truly was the cause of disease. As disease was reported for a very specific portion of a species, based on pregnancy status, the question raised was if there was any effect on the rest of that population who received the contaminated vaccine. Finally, because of the known method of spread of this disease in the normal host by an insect vector, was it possible that a new disease reservoir could contribute to spread of the disease to other historically recognized susceptible species.

Bluetongue virus was isolated from a canine modified live vaccine that had been previously safe and effective. The association of disease with BTV in dogs was an unexpected occurrence as previously BTV was considered only a disease of ruminants. Regulatory requirements associated with production and testing of veterinary vaccines had theoretically been designed to preclude accidental contamination of vaccines and normally it was expected that wild type BTV had, at best, a very low chance of replicating in cell culture. To determine if this cell culture adapted BTV could be the cause of disease in dogs, both pregnant bitches and non pregnant dogs of various ages were challenged with an isolate of the BTV contaminated vaccine or with the BTV contaminated vaccine. Pregnant bitches died, aborted, or aborted and died from both of the cell culture adapted BTV sources. A shorter period of viremia for the BTV challenged pregnant bitches than for ruminants was

demonstrated. Pregnant dogs receiving a non contaminated vaccine remained well and whelped live pups. None of the non pregnant dogs showed any clinical signs nor was there any evidence that there was transmission from challenged dogs to contact controls. Younger dogs had a low rate of seroconversion, while older dogs uniformly seroconverted when held for a period normally adequate to elicit an immune response. Virus could be recovered in the non pregnant dogs at 10 days post challenge but not at 24 days post challenge.

Sheep receiving washed red blood cells from aborting bitches showed clinical signs and seroconverted. Pregnant bitches aborted and died when receiving the same challenge.

The isolation of BTV from pregnant bitches and non pregnant dogs raises the possibility that dogs could have been a source for transmission of the BTV to other animals if bitten by appropriate *Culicoides* spp.

Additional experimental or retrospective studies are needed to answer several questions. Although these studies demonstrated that seroconversion in non pregnant dogs is possible, adequate studies to determine if the general dog population reflected this occurrence have not been conducted. Sufficient doses of the vaccine were administered that this should be detectable, based on these findings. To further clarify the issue of possible transmission via *Culicoides* spp and the relationship to virus levels in dogs following challenge, additional studies using pregnant bitches, midges, and susceptible ruminants should be conducted

The studies reported here confirm that cell culture BTV can cause disease in pregnant bitches, can replicate and elicit an antibody response in non pregnant dogs, and was still infective in at least one of the natural ruminant hosts. No evidence presently exists for the natural transmission of BTV to dogs, but BTV can not now be excluded from disease

consideration from iatrogenic sources. Additional studies are needed to determine if there is any evidence that natural BTV infection occurs in dogs. At the minimum, the studies conducted serve to confirm the need for continued and enhanced testing of all biologicals to prevent contamination and assurance of the purity and safety of veterinary vaccines.

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