Epizootic hemorrhagic disease virus in Montana deer and cattle
by Timothy Jay Feldner

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Veterinary Science
Montana State University

Abstract:
A cytopathic agent was recovered from the spleen and blood of a white-tailed deer that exhibited gross lesions consistent with what has been called hemorrhagic disease (HD) in white-tailed deer. An experimental white-tailed deer developed similar symptoms and gross lesions five days, post-inoculation with a 10% spleen suspension from the original white-tailed deer.

The viral isolate was propagated in VERO cells and produced a cytopathic effect at 72 hours post-infection consistent with that produced by bluetongue virus (BTV) or epizootic hemorrhagic disease virus (EHD) when grown in the same cell system.

Electron microscopy allowed for a determination of viral morphology and size. The isolate was approximately 56.5 nm in diameter with large doughnut shaped capsomeres consistent with orbivirus morphology.

Results of the indirect fluorescent, antibody test indicated that the viral isolate was EHDV. Further verification of identity was obtained in the plaque reduction neutralization test which indicated that the isolate was EHDV of the Alberta serotype.

Serum samples were collected from free-ranging deer and from bovine populations in eastern Montana to determine the activity of both EHDV and BTV in the two populations. Results indicated that 79% of the bovine samples collected from areas in which HD had been reported within the last three years were seropositive to EHDV.

On the other hand, only 3% of the same samples were BTV seropositive. Of the deer serum samples collected from HD areas, 73% of the mule deer were found to be EHDV seropositive while only 5% of the whitetailed deer were EHDV seropositive. The low percentage of EHDV seropositive white-tailed deer was considered to be a result of the high mortality rate (90%) reported for EHDV infection in white-tailed deer. Approximately 15% of the deer serum samples, obtained from HD areas were also BTV seropositive.

Results of this study indicate that EHDV was the etiologic agent responsible for the recent HD outbreaks in eastern Montana. These findings also suggest that EHDV may have been responsible for the previous HD outbreaks that have occurred in the same area. The high EHDV exposure rate detected in the bovine population indicated their active role in the epizootiology of EHDV in eastern Montana.

STATEMENT OF PERMISSION TO COPY

In presenting this thesis in partial fulfillment of the requirements for an advanced degree at Montana State University, I agree that the Library shall make it freely available for inspection. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by my major professor, or, in his absence, by the Director of Libraries. It is understood that any copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Signature Timothy Jay Ackley

Date 5/14/80

EPIZOOTIC HEMORRHAGIC DISEASE VIRUS IN MONTANA DEER AND CATTLE

by

TIMOTHY JAY FELDNER

A thesis submitted in partial fulfillment
of the requirements for the degree

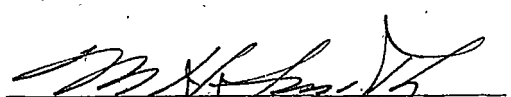
of

MASTER OF SCIENCE

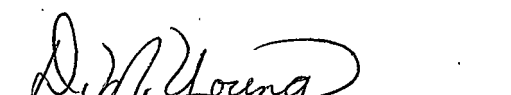
in

Veterinary Science

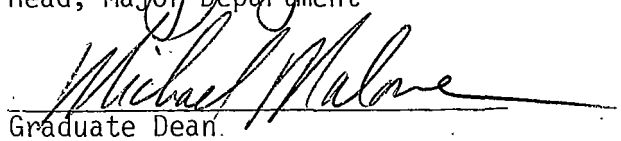
Approved:



Chairman, Graduate Committee



Head, Major Department



Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

May, 1980

ACKNOWLEDGEMENTS

The author would like to thank Dr. M. H. Smith for his support, guidance, and, above all, his daily wisdom throughout the course of this study. Thanks also go to his graduate committee and especially to Dr. Richard Mackie for his help in arranging for the collection of field samples. The interest and support of members of the Montana Department of Fish, Wildlife, and Parks, including Mr. Jon Swenson, Mr. Gary Dusick, and Mr. Ken Walcheck, helped to get this study off the ground. Appreciation also goes to the National Animal Disease Center and the Arthropod Borne Animal Disease Research Center for their generous donation of test reagents and reference viral strains and especially to Ruth Breckon of the ABADRC for her help and suggestions.

A special thanks go to the author's wife, Karen, and to his children, Jarid and Lindsie, for their patience and understanding.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

A cytopathic agent was recovered from the spleen and blood of a white-tailed deer that exhibited gross lesions consistent with what has been called hemorrhagic disease (HD) in white-tailed deer. An experimental white-tailed deer developed similar symptoms and gross lesions five days post-inoculation with a 10% spleen suspension from the original white-tailed deer.

The viral isolate was propagated in VERO cells and produced a cytopathic effect at 72 hours post-infection consistent with that produced by bluetongue virus (BTV) or epizootic hemorrhagic disease virus (EHD) when grown in the same cell system.

Electron microscopy allowed for a determination of viral morphology and size. The isolate was approximately 56.5 nm in diameter with large doughnut shaped capsomeres consistent with orbivirus morphology.

Results of the indirect fluorescent antibody test indicated that the viral isolate was EHDV. Further verification of identity was obtained in the plaque reduction neutralization test which indicated that the isolate was EHDV of the Alberta serotype.

Serum samples were collected from free-ranging deer and from bovine populations in eastern Montana to determine the activity of both EHDV and BTV in the two populations. Results indicated that 79% of the bovine samples collected from areas in which HD had been reported within the last three years were seropositive to EHDV. On the other hand, only 3% of the same samples were BTV seropositive. Of the deer serum samples collected from HD areas, 73% of the mule deer were found to be EHDV seropositive while only 5% of the white-tailed deer were EHDV seropositive. The low percentage of EHDV seropositive white-tailed deer was considered to be a result of the high mortality rate (90%) reported for EHDV infection in white-tailed deer. Approximately 15% of the deer serum samples obtained from HD areas were also BTV seropositive.

Results of this study indicate that EHDV was the etiologic agent responsible for the recent HD outbreaks in eastern Montana. These findings also suggest that EHDV may have been responsible for the previous HD outbreaks that have occurred in the same area. The high EHDV exposure rate detected in the bovine population indicated their active role in the epizootiology of EHDV in eastern Montana.

CHAPTER 1

INTRODUCTION

Epizootics of what has been referred to as hemorrhagic disease (HD) have occurred in white-tailed deer (Odocoileus virginianus) populations in various regions of the United States for quite some time. The earliest reports of HD in white-tailed deer date back to 1890 (89). Isolation of etiologic agents from natural outbreaks as well as work done with experimental infections in white-tailed deer have indicated that two very closely related viruses, bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV), are the most likely pathogens involved in these fatal HD outbreaks. Both of these viruses have been shown to be transmitted by the same arthropod vector, Culicoides variipennis. The clinical disease and the pathologic lesions caused by both viruses in white-tailed deer, as well as the pathogenesis of both diseases, is indistinguishable (93, 101, 103, 105).

While EHDV is considered, at this time, to affect only a few species of wild ruminants, BTV was first recognized as a serious pathogen in the ovine species. BTV is now known to cause morbidity and mortality in the bovine as well as in various species of wild ruminants. EHDV has recently been shown to have widespread activity in bovine populations resulting in ill defined consequences (1). The host range of the two viruses and the ever increasing amount of contact occurring between wild and domestic ruminants in the

United States establishes the essential nature of the identification of the etiologic agent involved in any HD outbreak in a white-tailed deer population.

Sporadic outbreaks of HD in white-tailed deer have been recorded in the state of Montana since 1961 (113). Although these outbreaks have seemingly caused morbidity and mortality in free ranging deer only, the etiologic agent involved and its interrelationship with domestic ruminants within the state has never been elucidated.

BTV has the potential for having a great economic impact in a state such as Montana where beef production is a multi-million dollar industry. Presently, absolute embargos of United States livestock are in effect in many countries due to the activity of BTV while other countries will not allow importation of United States livestock without expensive testing first being done. This, along with the direct effect of BTV in sheep and cattle and the congenital effects shown to be associated with BTV infection, are cause for concern.

Similarly, EHDV and BTV could have an obvious direct effect on a second economic resource in the state of Montana; its wildlife population. The very close association, including antigenic similarities, between EHDV and BTV, raises questions as to the effects EHDV, although not previously recognized, may have on

domestic livestock. Indeed, Ibaraki virus, a virus which causes a bluetongue-like disease in Japanese cattle, is now thought to be a strain of EHDV (1,13).

In order to further understand the HD problem as it occurs in Montana, two major objectives were developed as a part of this study. First, to identify the etiologic agent involved through viral isolation from natural HD cases collected in the field and through the collection of serological data from free-ranging deer in areas where HD was or had been active; and second, to estimate the involvement of domestic ruminants in the HD outbreaks through a serological survey of the bovine populations in and around areas of HD outbreaks for antibody to both EHDV and BTV.

This thesis is a report on the results from studies designed to achieve these aforementioned objectives.

CHAPTER 2

LITERATURE REVIEW

For at least the last two decades, a highly fatal disease has been occurring periodically in the Montana white-tailed deer (Odocoileus virginianus) population. The disease is sporadic in its appearance and occurrs only in the late summer and early fall suggesting transmission by an arthropod vector. Deer observed during the terminal stages of the disease have been noted to have a loss of alertness, drooping ears, and extended necks indicating dyspnea (89). Some of the diseased deer have also been observed with blood flecked foam near the muzzle area.

During these outbreaks, many of the deer carcasses have been found near water resulting in three possible implications. First, the deer may be febrile and be instinctively trying to cool their bodies in water. One observer watched a disoriented white-tail submerge itself up to the neck before falling dead in the water (113). Second, the weakened condition of the affected animals may cause them to choose downhill trails which, in most cases, eventually lead to water. Finally, the affected deer may simply be seeking protective cover from the heat of the sun and find such cover along streambeds and riverbeds (35). Almost all carcasses found during the outbreaks have been in good flesh indicating that the animals died from an acute disease.

Post-mortem examinations of white-tailed deer found during

the disease outbreaks have yielded fairly consistent results. Among the findings are small subpericardial and myocardial hemorrhages, massively edematous lungs with hemorrhages over the surface, excessive amounts of serous fluid in the thoracic cavity and pericardial sac, and petechial hemorrhages on the lymph nodes, thymus, and the gastrointestinal tract (83). All of these lesions are indicative of experimental bluetongue virus (BTV) or epizootic hemorrhagic disease virus (EHDV) infection in white-tailed deer (23, 54, 103, 105, 108). Without isolation and identification of the etiologic agent involved, these outbreaks are simply referred to as hemorrhagic disease (HD).

The first documented cases of HD in Montana occurred in 1961 in primarily the northeastern section of the state. There were scattered reports of deer mortalities during the summer months of 1962, 1970, and 1971. In 1976, a large scale die-off occurred with mortalities reported in 34 areas in eastern Montana, primarily McCone and Powder River counties (113). Again, in 1977, there were heavy white-tailed deer losses during the summer months along the Yellowstone River drainage in Richland and Dawson counties. In one study area, consisting of approximately 16 miles of white-tailed deer habitat along the Yellowstone River, it was estimated that 190 white-tails, about one third of the existing population in that area, had died of HD (93). Finally, in 1978, white-tailed deer

mortalities occurred in the southeastern section of Montana near the town of Ekalaka. Calculations using known population data in the Long Pines area, 30 miles south of Ekalaka, indicated that one third of the area's white-tail deer population, or approximately 500 deer, may have died of HD (19). Many of the dead deer in this area were reported by hunters as being antlered males in velvet indicating that death occurred sometime during July or August.

In an attempt to identify the causative agent of the 1976 HD outbreak, blood and tissue samples from deer mortalities were sent to virologists in Alberta, South Dakota, and Ohio; to the National Animal Disease Center in Ames, Iowa; and to the University of Wisconsin's Veterinary Science Laboratory. None of these laboratories were able to isolate the causative agent of the HD from tissues received. Workers from Wisconsin, however, using a macro agar gel precipitin test and employing tissue suspensions as antigens and reference BTV and EHDV antisera, indicated that either BTV, EHDV, or both, were involved in the outbreak (118).

Characteristics of the Viruses

BTV and EHDV are very closely related viruses of the orbivirus genus. Ten distinct orbivirus subgroups are recognized based upon serological results indicating shared antigens among certain members (6, 47). The presence of shared antigens, or cross reacting antigens, between BTV and EHDV has warranted their placement in the

same serological subgroup (72).

The orbivirus genus is included with the reovirus and probable rotavirus genera in the reoviridae family since all members possess double stranded RNA as their nucleic acid type (69). Like the reoviruses, BTV and EHDV have been shown to have a segmented genome consisting of ten segments of three distinct size ranges (44, 56, 107, 111).

BTV and EHDV are morphologically indistinguishable, precluding differentiation by electron microscopy. The capsids of both range in diameter from 55 to 65 nanometers and are single layered structures composed of 32 capsomeres (21, 73, 99, 106, 109). The capsomeres are relatively large (10.5 to 11.5 nanometers in diameter) with an axial hole 4.5 nanometers in diameter resulting in a "doughnut like" appearance (106). Although neither virus has a true envelope, confusion resulted from early reports of enveloped particles in purified virus preparations. These structures are now known as "pseudo envelopes" and are actually cellular membranes which occasionally enclose one or more virions. The pseudo envelopes may be removed through further purification procedures without affecting infectivity and are not considered to be part of the virion (21, 99).

BTV and EHDV are also very similar in terms of their physiochemical properties. Both have been reported to be relatively

resistant to lipid solvents and sodium deoxycholate with only slight losses in infectivity occurring after incubation with solvent for 16 to 20 hours (6). Unlike the reoviruses, BTV and EHDV are heat labile as well as very acid labile with complete loss of infectivity occurring below a pH of 6.4 within 30 minutes (24, 43, 57).

Cells infected with either BTV or EHDV respond with the production of dense cytoplasmic inclusion bodies. With BTV infection, some of these cytoplasmic inclusions contain specific BTV antigen as demonstrated by the direct fluorescent antibody technique (11). With EHDV infection, the inclusions appear as viral matrices containing more and more viral progeny as infection progresses (106). These viral progeny become associated with the newly formed vesicular membranes within the cytoplasm resulting, perhaps, in the formation of pseudo envelopes around groups of viral particles (99). BTV and EHDV conclude their replication cycle with cytolysis which results in their release from the infected cell.

EHD History

EHD was first described by Shope et al. (88) in 1955 after an outbreak in New Jersey white-tailed deer. They pointed out the fact that a similar disease had occurred as early as 1890 and at irregular intervals since that time. The outbreaks, usually reported by hunters and woodsmen of the area, had been variously diagnosed as blackleg, blacktongue, mycotic stomatitis, or hemorrhagic septicemia.

Shope was able to isolate a "filterable virus" from a naturally infected deer and transmit the disease to a healthy deer. He called the "new" virus epizootic hemorrhagic disease virus because of the salient clinical and pathological features of the disease (88, 89). An almost simultaneous outbreak of HD in Michigan white-tailed deer was reported by Fay et al. (22) as being caused also by a "filterable virus".

During the late summer and early fall of 1956, an epizootic of HD occurred in southeastern South Dakota (80). A virus isolated during this outbreak was reported to induce lesions similar to those produced by the New Jersey isolate. Using a series of neutralization tests in white-tailed deer, Mettler (71) came to the conclusion that the South Dakota strain and the New Jersey strain of the EHDV were immunologically related but not identical. Epizootics of EHD have been reported periodically in South Dakota since 1956. In 1969, serological evidence of EHDV in deer was detected in 14 South Dakota counties while evidence of EHDV in porcine or bovine species was detected in 34 counties (78).

EHD occurred in both western North Dakota and southeastern Alberta in the summer and fall of 1962. Tissues collected from a diseased white-tailed deer in North Dakota yielded EHDV serologically identical, as determined by neutralization tests in mice, with the New Jersey strain of the virus (116). During the outbreak, 200 white-

tailed deer, 18 mule deer (Odocoileus hemionus), and 11 antelope (Antelocapra americana) mortalities were attributed to EHD (84). In 1970, an epizootic in the same area of western North Dakota caused an estimated 2000 white-tailed deer deaths. During this epizootic, EHDV was again isolated and was considered to be the etiologic agent responsible for the outbreak. Serological data collected in 1970 and 1971 in North Dakota indicated that while only 6% of the white-tailed deer tested were seropositive for exposure to EHDV, 43% of the mule deer tested were EHDV seropositive (35). These data suggest the lethality of EHDV for white-tailed deer and the greater resistance of mule deer to the disease. During the 1962 Alberta outbreak, 450 white-tailed deer, 20 mule deer, and 13 antelope mortalities were reported (14). The EHDV isolated in Alberta was said to be identical to the New Jersey strain but the testing procedures used were not mentioned (18).

Comparison of the various EHDV isolates was greatly facilitated in 1962 when Mettler (71) was able to successfully propagate EHDV in suckling mice and in HeLa cells. Taking advantage of these new methods, Trainer and Wilhelm (116), using a mouse neutralization test and hyperimmune rabbit antisera, compared the EHDV isolates from five geographical areas. They concluded that the New Jersey, South Dakota, Michigan, and two North Dakota strains were all of the same serotype. Hence, the work of Mettler and the work of Wilhelm and

Trainer was in disagreement as to the existence of more than one serotype of EHDV. Further advances in diagnostic techniques, however, allowed Barber and Jochim (5) to establish the existence of two distinct EHDV serotypes using the plaque reduction neutralization test in BHK-21 cells. They then proposed the present nomenclature for the EHDV serotypes as EHDV type I, with the New Jersey strain as the prototype, and EHDV type II, with the Alberta strain as the prototype. The original South Dakota strain, which Mettler first proposed as being a different serotype than the New Jersey isolate, was lost through storage in the laboratory and was never adequately serotyped.

Epizootics of HD in deer have occurred in Washington state in 1946, 1953, and 1967 (24). During the 1967 epizootic 227 deer mortalities, the majority of which were white-tails, were reported. EHDV has since been isolated from the spleen of one of the 1967 mortalities but has not been serotyped.

Serological surveys conducted in Idaho and Oregon have indicated the previous exposure of black-tailed deer and mule deer to EHDV. In Idaho, 4 of 80 serum samples tested were positive for EHDV antibody while 12 of 524 samples were seropositive in a study conducted in Oregon (55, 94).

EHDV has been isolated from white-tailed deer during HD outbreaks in Wyoming, Kentucky, and North Carolina (39). During a

1971 HD outbreak in the southeastern United States, EHDV seropositive deer were identified in Florida and Georgia. At the same time, deer seropositive for BTV were identified in Florida, North Carolina, and South Carolina (81). Isolation of both BTV and EHDV from one deer mortality during this 1971 outbreak, as well as strong serological evidence indicating the activity of both viruses, has implicated both BTV and EHDV in the outbreak (102).

Further serological surveys have indicated the activity of EHDV in Nebraska, Texas, and Wisconsin (39, 115). It should be noted that all EHD outbreaks, or suspected EHD outbreaks, seem to have been confined to the North American continent. Also, most of these outbreaks have occurred west of the Mississippi River. The only evidence of EHDV outside of the North American continent was the isolation of EHDV from pooled collections of Culicoides spp. in Ibadan, Nigeria (58).

BT History

BT has been recognized as a disease entity for nearly a century since susceptible European sheep were introduced into South Africa (42). The first description of the disease was made by Hutcheon in 1881 who called it "epizootic catarrh" (45). In 1906, Theiler (98) showed that the causative agent was capable of being filtered through a Berkfeld filter and was probably a virus.

BTV seemed to be confined to the African continent until 1943

when an outbreak in sheep on the island of Cyprus caused up to 70% mortality in some areas (30). The disease quickly spread to Palestine and Syria and eventually reached the United States where it was diagnosed in Texas in 1948 (33). BT then appeared in California in 1952 where a viral isolate was identified as BTV (68). Since that time, BTV has become a widespread problem in domestic and wild ruminants throughout the world and has now been diagnosed in at least 30 states in the United States (3).

Host Range

Although BT was first recognized as a disease of sheep, cattle, and goats, BTV is now known as a widespread pathogen in North American and African wild ruminants (9, 103). Serological evidence of BTV has been found in North American moose, barbary sheep, elk, antelope, white-tailed deer, black-tailed deer, and mule deer (104). BTV has also been isolated from natural outbreaks in captive white-tailed deer and desert bighorn sheep (93). Isolation of BTV has been reported as well from two species of African wild rodents, a field mouse (Rhabdomys pumilio) and a swamp rat (Odomys irroradus) (103).

EHDV, on the other hand, was long believed to cause disease in white-tailed deer only. EHDV has since been isolated from diseased mule deer and antelope (14, 84). The susceptibility of a variety of domestic and laboratory animals to EHDV has been tested under

experimental conditions resulting in negative or inconclusive results (31, 57). The relationship of the bovine to EHDV, however, seems unclear. Serological data gathered by various investigators indicates that as much as 75% of the bovine population in some areas of the United States are EHDV seropositive (1, 55, 78). Furthermore, EHDV has been isolated from natural outbreaks in cattle in at least two instances (5, 28). Experimental infections in the bovine, however, while resulting in a viremia which may last for up to 50 days post infection, have not been shown to cause any apparent clinical disease (31). A similar situation is seen in experimentally infected elk (Cervus candadensis) where a cell associated viremia may be detected for up to 30 days post infection although no clinical disease is evident (37). Presently, both the bovine and the elk are considered as possible reservoir hosts for EHDV due to the long lasting viremia associated with experimental infection.

Transmission

Historically, outbreaks of both EHD and BT have occurred during the late summer and early fall and have ceased abruptly with the onset of cool temperatures. These observations led early workers to speculate that BTV was transmitted by an insect vector (92, 98). This speculation was substantiated in 1934 when Nieschelz (76) found that a mosquito, Aedes lineatopennis, was capable of harboring

BTV for up to 19 days post-infection. Isolation of BTV from Culicoides species trapped around areas of BT outbreaks was accomplished in 1944 and 1954 leaving only the actual mechanical or biological transmission of the virus to be demonstrated (20, 82). DuToit made progress in that respect by transmitting BTV to susceptible sheep with Culicoides pallidipennis (20). Some doubt remained as to biological transmission, however, since the insects that DuToit used were not allowed an adequate period to digest the blood meal and because susceptible animals were not held in insect free inclosures. Final proof of biological transmission was made by Foster (27) in 1963 by using experimentally infected sheep and biologically transmitting BTV to susceptible sheep with Culicoides variipennis. Luedke (65), using the same biological vector, was able to show transmission from infected sheep to susceptible cattle and vice-versa.

After taking a meal of infective blood, a minimum incubation period of 10 days is required before Culicoides can biologically transmit BTV to a susceptible animal (27). During this incubation period, BTV has been shown to multiply to a high titer within the salivary glands of the insect vector reaching a peak titer six to seven days after infection and remaining sufficiently high to infect susceptible mammalian hosts for up to 26 days (9, 12). Culicoides are capable of taking numerous blood meals during their lifetime;

thus, each infected fly could conceivably transmit BTV to several susceptible animals.

Mechanical transmission of BTV has been accomplished experimentally using the sheep ked (Melophagus ovinus) (61). While some investigators have reported contact transmission of BTV between penned animals, the majority of investigators disagree (87, 92, 98, 101). Repeated oral exposure, however, related to the frequency and not to the amount of inoculum used, has been shown by Luedke (51) to cause clinical BT in sheep. Interestingly, five of the seven sheep that developed clinical BT in Luedke's study did not develop significant antibody titers to BTV. Urine from infected sheep does contain pathogenic BTV allowing for possible oral exposure under natural conditions.

Prompted by morphological, pathological, and clinical similarities between BTV and EHDV, Boorman and Gibbs (7) carried out studies to determine whether EHDV was capable of multiplying in Culicoides. They found that EHDV multiplies in Culicoides variipennis with virus increasing to 1000 times the amount originally ingested. If virus is inoculated directly into the hemocoele, the amount of virus present rises to almost 100,000 times the amount inoculated by seven days post inoculation. These results suggested that Culicoides variipennis could be infected with EHDV by ingestion and that the infected midges could be capable of transmitting the

virus by bite. Isolation of EHDV from wild Culicoides in Nigeria enhanced speculation that Culicoides was involved in the natural transmission cycle of EHDV.

Final incrimination of Culicoides variipennis as a biological vector of EHDV was obtained by Foster et al. (25) in 1977. In a series of trials, EHDV was transmitted between white-tailed deer by Culicoides variipennis. The insects were laboratory reared and given a blood meal, through a membrane, of infective blood. After incubation periods of 18 to 20 days, the insects were allowed to feed on a recipient host deer. Recipient host deer were then used as infective donors for further biological transmission. Using these methods, EHDV was successfully transmitted through two serial passages and caused acute disease in recipient host deer.

Evidence also exists for the transmission of EHDV through contact or oral exposure. In 1964, Ditchfield and associates (18) hypothesized that the normal route of EHDV transmission was through oral exposure and showed that pathogenic EHDV was shed in the feces of an infected animal. This oral exposure, they postulated, caused an asymptomatic illness although an occasional animal, because of marked susceptibility or because of a massive dose of the virus, succumbed to the infection. Mechanical or biological transmission by insects was thought of as an abnormal route of infection which generally culminated in death of the infected animal.

Pathology and Pathogenisis

Upon entry into a susceptible host, both EHDV and BTV require an incubation period of approximately four to twelve days before the appearance of clinical signs (23, 59). Studies done by Pini (79) indicate that BTV enters the regional lymph nodes of a susceptible animal and from there is disseminated via lymph or blood to lymphoid tissues in other areas of the body. After primary replication in these areas, a viremia develops (usually six days post-infection) and virus is carried to the majority of body tissues resulting in the appearance of macroscopic lesions by eight days post-infection. Viremia has been shown to be cell associated and contained primarily within the erythrocytic fraction of the blood in the case of both BTV and EHDV infection (4, 38, 62). The association of the virus with the cellular fraction, especially in the case of BTV, may be responsible for the ability of the virus to co-exist with neutralizing antibody leading to latent infections, particularly in the bovine (31).

During the viremic stage of experimental EHDV infections in white-tailed deer, evidence of viral replication within the vascular endothelial cells has been observed ultrastructurally (17, 106, 108). A proclivity of BTV for vascular endothelial cells has also been reported (32, 93). The hemorrhagic manifestations of EHD and BT in white-tailed deer are thought to be the result

of a major disturbance both of vascular integrity and of homeostasis of coagulative mechanisms. Interruption of the endothelial cells through direct viral action may allow contact between procoagulants of the blood and collagen, activating the intrinsic pathway of blood coagulation. At the same time, damage to endothelial cells may cause the release of tissue factor and the activation of coagulation through the extrinsic pathway (93, 108). Microthrombi, a direct result of blood coagulation, have been demonstrated histologically in both experimental BTV and EHDV infection in white-tailed deer and correspond to the thrombocytopenia observed during the course of both diseases (23, 53).

Clinical signs of EHDV or BTV infection in white-tailed deer begin four to five days post infection with a pyrexia which corresponds to peak viremia (39, 115). Shortly thereafter, the animal shows signs of anorexia, weakness, hyperemia of the conjunctiva, the tongue, and the oral and nasal mucosa, dyspnea, and, often, excessive salivation. After four to 48 hours of the acute disease, the body temperature may become subnormal preceding coma and death (29, 54, 89, 101, 112). The mortality rate for both EHDV and BTV infection in white-tailed deer has been reported to be close to 90% although BTV is the least fatal of the two (39).

Grossly, the overwhelming lesion of experimental EHDV or BTV infection in white-tailed deer is that of hemorrhage, ranging

from petechial to ecchymotic, in various locations. Several target areas may also be affected by thrombosis of small vessels and accompanying tissue necrosis. These areas include the oral and nasal mucosae, the tongue, the forestomach mucosa and submucosa, the myocardium, and the acinar cells of the mandibular salivary glands (23).

Widespread microthrombi formation throughout the microvasculature contributes to the development of edema associated with EHDV and BTV infections. Interference with normal blood circulation results in subcutaneous, intramuscular, and facial edema, as well as excess edema fluid in the pericardial, peritoneal, and pleural cavities. Massive pulmonary interlobular edema is also a very consistent finding with EHDV and BTV infections in white-tailed deer (23, 53).

Hematological studies concerning experimental EHDV infection have supported the diagnosis of disseminated intravascular coagulation (DIC), or consumptive coagulation, as a significant mechanism in the pathogenesis of the disease. Shope (89) noted in early studies that "blood remained fluid for an unusually long time after it was shed", and postulated that the infected deer had bled into their own tissues accounting for the shock that normally preceded death. Karstad et al. (54) found greatly prolonged blood coagulation times and prothrombin times that paralleled the increase in body temperature. A corresponding increase in the erythrocyte count during the terminal

stages of the disease has been attributed to hemoconcentration as evidenced by the severe edema. Although total leukocyte counts remain unchanged during EHD, a neutrophilia occurs with a corresponding lymphopenia during the terminal stages (117). Neutrophilia may occur during a number of disease conditions which include hemolytic crisis and significant blood loss (86). The utilization of fibrinogen and factor VIII late in EHDV infection further implicate DIC in the pathogenesis of EHDV infection in white-tailed deer (16). Hemorrhage, due to the utilization of coagulation factors, damage to endothelial linings of vascular beds, and the formation of microthrombi, becomes the overwhelming result of EHDV or BTV infection in white-tailed deer.

BTV and EHDV may also cause reproductive problems in deer as well as in sheep and cattle. Congenital abnormalities due to BTV infection in pregnant sheep were first noticed by Shultz et al. (91) in 1954 when a modified live vaccine was used on pregnant ewes. It is now recognized that in utero BTV infection at an early fetal age is directly responsible for congenital encephalopathies in lambs as well as being abortogenic and teratogenic in pregnant cattle exposed during the first or early second trimester of pregnancy (62, 96). Abortogenic effects of BTV have been reported in white-tailed deer as well as transplacental passage and fetal involvement during experimental infections with

EHDV (17, 23, 100).

Immunology

The immunological aspects of both BTV and EHDV infection are as complex as they are varied. Other than the serological and epidemiological aspects, most of the research done concerns BTV infection. This work covers everything from in utero infection and resulting immunological tolerance to polyvalent vaccine production.

BTV and EHDV, like other viruses possessing segmented genomes, have the potential for antigenic variation leading to the appearance of new strains of the virus. The presence of multiple antigenic variants of BTV was first suspected when vaccinal failures occurred after using monovalent vaccines produced through viral attenuation in embryonating chicken eggs. Neitz (74) established the plurality of antigenically different strains by carrying out a series of cross protection tests in sheep. Howell (41) was able to classify the antigenic varients into 12 distinct serotypes by using an *in vitro* neutralization test. Since that time, the number of BTV serotypes has risen to 20 (3, 15). All of the serotypes appear to have a common antigenic component detectable by the complement fixation test, and an unknown number of different specific antigenic components detectable by the serum virus neutralization test (42). Recent work has indicated that polypeptide 2, a component of the

viral capsid, is responsible for the strain specificity of the various BTV serotypes. At the same time, polypeptides 3 and 7 are thought to be responsible for not only the antigenic similarities between the various BTV serotypes, but also the antigenic similarities detected between BTV and EHDV (77, 110).

To date, only two distinct serotypes of EHDV have been recognized (5). The lack of antigenic variants of EHDV may simply reflect the small number of isolations that have been made, in most cases from wild ruminants, since the initial isolation of EHDV in 1955.

As mentioned earlier; BTV and EHDV are classified within the same orbivirus subgroup according to their antigenic relationship. Evidence of this close antigenic relationship is exhibited by a cross reaction in the complement fixation test using immune mouse ascitic fluid and infected suckling mouse brain antigen (6, 72). Lack of a cross reaction in the plaque reduction neutralization test indicates that while EHDV and BTV may share a common group antigen, each also has its own serotype specific antigen.

A third member of the BTV subgroup, Ibaraki virus, causes a BT like disease of cattle in Japan. Although originally thought to be a strain of BTV, serological comparisons with BTV serotypes revealed no antigenic relationship. Recent studies have now shown that Ibaraki virus shares not only a common group antigen with both

serotypes of EHDV, but also a serotype specific antigen with the Alberta strain of EHDV as evidenced by a cross reaction in the plaque reduction neutralization test (13). Since EHDV and BTV may also share a common group antigen, an indirect antigenic linkage, or an evolutionary linkage, is apparent between BTV and Ibaraki virus.

The antigenic relationships mentioned above leave room for much exciting speculation concerning the BTV subgroup of the orbivirus classification. First, the possibility for antigenic variation through shift or drift (immunological selection) within the segmented genome becomes apparent. Dual infections with different strains of BTV or with BTV and EHDV have been reported and present the possibility for recombination of genetic information, or phenotypic mixing, resulting in strains presenting new antigenic properties (28, 95). Second, variation of the virus through passage in an uncommon host, as evidenced by the attenuation of EHDV in suckling mice, may result in subtle changes in the host range of the virus and subsequent pathological changes within a host once refractory to the disease syndrome (90).

The pathological consequences of an in utero BTV or EHDV infection have been mentioned previously. Further work on in utero BTV infection has revealed that bovine fetuses infected at 60 to 120 days of gestation may result in the birth of calves that are latently infected with BTV. These calves usually experience BT

disease within the first six months of life following the loss of maternal antibody. The calves may be immunologically incompetent (tolerant) or immunologically competent in terms of BTV and, in either case, may harbor the virus for periods up to five years (62, 63, 64). The fact that latent virus can coexist with neutralizing antibody in immunocompetent animals thus holds true for BTV as it does for lymphocytic choriomeningitis and lactic dehydrogenase virus infections in mice and Aleutian disease virus in mink (67). With BTV infection, resistance of the virus to neutralizing antibody may be a direct result of the close association of BTV, like EHDV, with the cellular fraction of the blood (50).

Epizootiology

The insect vector responsible, at least in part, for the transmission of BTV and EHDV has been identified as Culicoides variipennis. The method of survival of either virus during the interepizootic periods, however, has not been completely explained. While survival of BTV within the arthropod vector by transovarian transmission has been eliminated as an overwintering mechanism for BTV, there are no comparable reports for EHDV (39).

An accumulation of evidence has suggested that the bovine may be a reservoir host for BTV (10, 65, 75). Luedke et al. (66) reported the biological recovery of BTV from an in utero infected hereford bull for up to five years after birth. Recovery was

facilitated by the bites of non-infected Culicoides variipennis which resulted in a "showering effect" of latent virus into the bloodstream of the animal. Culicoides feeding on the bull after these stimulating bites were then infected and, after a suitable incubation period, were able to biologically transmit the virus to susceptible animals. A mechanism such as this for either BTV or EHDV would allow for not only a suitable reservoir host, but also for the reappearance of the virus at a time when the insect vector has again become active and biological transmission has once again become possible.

The bovine has also been suggested as a reservoir host for EHDV (31). EHDV, as mentioned earlier, has been isolated from natural infections in the bovine in at least two instances (5, 28). Although no clinical disease is apparent in experimentally infected cattle, viremia has been detected for as long as 50 days post infection (31). Furthermore, serological evidence of EHDV exposure in the bovine has been detected by more than one investigator and in one instance was detected in 75% of the cattle tested in a particular area (1, 55, 78).

Serological surveys in both domestic and wild ruminants have identified geographical areas which are considered to be enzootic for EHDV, BTV, or both. Antibody to both viruses, for example, has been found in a large percentage of the white-tailed

deer on the Welder Wildlife Refuge in Texas (34, 40, 115). Recognizable outbreaks of either disease, however, have not occurred in this area which may indicate the enzootic nature of the diseases. Although sub-species differences in the Texas deer may play a role in their resistance to BTV or EHDV outbreaks, the determining factor as to whether an area is enzootic or has potential for an epizootic area is probably the mode of transmission of the virus. Epizootic areas may be defined as areas in which a large susceptible population is available and appropriate conditions exist for the rapid transmission of the virus (115). Oral exposure to EHDV has been reported to cause sub-clinical disease in white-tailed deer with the resultant build up of immunity (17, 18, 115). In enzootic areas, this mode of transmission may produce immunity in a large enough proportion of the population to prevent an epizootic from occurring.

Diagnosis and Control

The similarities that exist in the gross and histological lesions produced in white-tailed deer by not only BTV and EHDV, but also mucosal disease virus, malignant catarrhal fever virus, and a number of bacterial pathogens, emphasize the need to isolate and identify the etiologic agent involved in any HD outbreak. Isolation of EHDV and BTV may be accomplished most easily by the inoculation of susceptible deer or sheep with tissue suspensions obtained from diseased animals. Virus may be recovered from a

variety of tissues with spleen and the cellular fraction of the blood being the two best sources (71, 85, 89).

While BTV may also be isolated through the inoculation of embryonating chicken eggs, there are conflicting reports concerning the success of cultivating EHDV in avian systems (25, 36, 89). Use of embryonating chicken eggs or natural susceptible hosts for viral isolation is advantageous in that preliminary adaptation of the virus to the system is generally not required.

BTV and EHDV may both be propagated in suckling mice or hamsters. In this system, however, serial passage is necessary for the adaptation of the virus. The same adaptation problems are encountered when isolation is attempted directly onto cell culture. In most cases, a series of blind passages must be carried out before cytopathic effect (CPE) becomes visible. Cell culture systems suitable for the propagation of BTV include primary newborn ovine kidney, Marmosa mitis embryo fibroblasts, BHK-21 (clone 13), and African green monkey kidney (VERO) cells. Systems which have been suitable for the propagation of EHDV include primary fetal white-tailed deer spleen, HeLa, BHK-21 (clone 13), and African green monkey kidney (VERO) cells (2).

The most substantial problem in isolating the etiologic agent from a HD outbreak in white-tailed deer lies in obtaining appropriate samples from field cases. Peak viremia, in both BTV and EHDV

infections, is reported to correspond to the temperature spike seen in the diseased animals (39, 116). Hence, samples collected from animals which have not yet succumbed to the infection are the most fruitful for isolation attempts. The difficulty of obtaining field samples from a wildlife population during a specific stage of a disease is apparent.

A number of tests may be applied in the identification of BTV or EHDV once they have been isolated. Group specific tests, which will not differentiate between the various BTV serotypes and which, in some cases, may cross react with a common antigenic component of EHDV include the modified direct complement fixation test and the agar gel precipitin (AGP) test (8, 46). The indirect fluorescent antibody test (IFAT) is a group specific test but may be used to differentiate between BTV and EHDV provided that cross reactions are eliminated by dilution of the respective antisera (47). BTV and EHDV may be differentiated by the use of the serum virus neutralization test (SN) or the plaque reduction neutralization (PRN) test (49). The SN and PRN tests are both serotype specific and will distinguish not only between EHDV and BTV, but also between the various serotypes of each with the use of serotype specific antisera.

A number of serological tests have been employed for the detection of group specific or type specific antibody to EHDV and

BTV in the serum of wild and domestic ruminants. The PRN and SN tests, being serotype specific, have limited value in BTV serological surveys or in the certification of BT disease free animals due to the strain variations of BTV (9). The highest titers of neutralizing antibody to BTV, as determined by the SN test, are reached at about four weeks post-infection and are reported to persist for as long as two years (65).

Group specific tests, on the other hand, may detect antibody to any of the BTV serotypes. Because of the group specificity of these tests, however, cross reactions may occur between BTV and EHDV antibody. The modified direct complement fixation test is now the official and most commonly used test to detect antibody to BTV. This test, however, is relatively insensitive for the detection of antibody and, in some instances, may not be able to distinguish between EHDV and BTV antibody (70, 72). Complement fixing antibody peak titers are reached at five weeks post infection but disappear from the circulation after six to eight weeks further inhibiting the meaningfulness of the complement fixation test (42).

The AGP test, adapted for the detection of BTV antibody by Jochim and Chow (48), is also a group specific test. This test is a modification of the Ouchterlony test and employs a non-infectious soluble antigen placed in a central well while serum samples to be tested for precipitating antibody are placed in

peripheral wells. Improved sensitivity may be obtained by placing reference BTV antiserum in peripheral wells adjacent to the serum samples to be tested (46). BTV soluble antigen was reported by Wang (114) as being derived from the virion surface. The actual composition of the soluble antigen has been disputed and may, in fact, be a metabolic by-product of viral replication within the infected cell (48). Although cross reactions may occur in the AGP test, only high titering EHDV antiserum will cross react with BTV soluble antigen and even then form only a faint precipitin line (46). BTV antiserum, on the other hand, does not cross react with the major component of EHDV soluble antigen but may cross react with what is termed a minor component of the soluble antigen (52). Precipitating antibody can be detected in experimentally infected sheep and cattle three to four weeks post infection and persists for at least two years (48).

Control of either BT or EHD in domestic or wild populations is a very difficult problem. Vaccines using attenuated strains of the virus have been produced for both BTV and EHDV. The existence of multiple strains of BTV, however, makes identification of the specific strain active in a particular geographic area essential in order for a vaccine to be effective. BTV vaccine available in the United states is produced using the BTV 8 (type 10) strain of the virus (3). There are at least four strains or antigenic types

of BTV active in the United States (3, 60). These antigenic types exhibit varying degrees of cross protection in sheep cross immunity challenge tests (9). The strain variation, along with the fact that vaccination of pregnant ewes may cause congenital abnormalities, exemplify the problems encountered in a vaccination program for BTV. To complicate matters further, Foster et al. (26) has reported that BT vaccine virus may not only cause clinical disease in sheep, but also may revert back to its more virulent form through further passage in hemophagous insects.

Immunization studies done by Shope and associates (90) indicate that EHDV may be attenuated by serial passage in suckling mouse brain and will produce a solid and durable immunity in white-tailed deer as shown by challenge with homologous virus. Again, however, the problems encountered with BTV vaccines are encountered with EHDV vaccines (87). Additionally, the problems of vaccinating a wild population make vaccination for EHD an improbable method of control in the future. Some hope may reside in the development of an oral vaccine that could be distributed in a palatable form in areas where serological data and vector presence may indicate the possibility of future EHD epizootics. Identification of the reservoir host for EHDV may lead to future possibilities for the control of EHD in free-ranging deer populations.

CHAPTER 3

MATERIALS AND METHODS

Cell Culture and Culture Media

African green monkey kidney (VERO) cells [1] were used exclusively for virus isolation, propagation, and titration procedures. VERO cell cultures were grown in 90% medium 199 (M-199), pH 7.2, and 10% fetal calf serum and maintained in serum free Eagle's basal medium in Earle's balanced salt solution (EBME). One ml of a cell suspension containing approximately 150,000 cells per ml was used to seed each 16 mm diameter well in tissue culture cluster plates of 24 [2]. Four chambered slides [3] were seeded with 0.5 ml of the same suspension for the production of monolayers. Monolayers were usually complete after one day of incubation at 37°C in an humidified atmosphere of 5% CO_2 in air.

Virus

Reference viral strains used were EHDV-New Jersey, EHDV-Alberta, and BTV type 13. All three strains were obtained from the Arthropod Borne Animal Disease Research Center in Denver, Colorado where they had been adapted previously to VERO cell culture.

Stock virus pools were produced from infected VERO monolayers grown in 250 ml plastic tissue culture flasks with a surface area

---

[1] Arthropod Borne Animal Disease Research Center, Denver, Colo.

[2] Costar, Division Data Packaging Corp., Cambridge, Mass.

[3] Lab-Tek Products, Division Miles Lab. Inc., Kankakee, Il.

of 75 cm^2. When cytopathic effect (CPE) reached 70% to 100% in the infected monolayers, media and cell debris were harvested by low speed centrifugation (150 X g). The cell debris was sonicated in a small amount of supernate, added back to the total supernate, and centrifuged again at 1000 X g for one hour. Cell debris was then discarded and the supernate mixed with an equal volume of buffered lactose peptone and stored in one ml aliquots at $-70^{o}C$.

Antibody

Reference EHDV and BTV antibody of bovine origin were obtained from the National Animal Disease Center in Ames, Iowa. Homologous antibody against the viral isolate was prepared in African pygmy goats. Two goats were initially infected through subcutaneous inoculation of five ml of infective deer blood in OPG [4]. At 50 days post inoculation (DPI), the goats were re-inoculated with five ml of a tissue culture prepared virus suspension containing approximately 10^7 plaque forming units (PFU). Hyperimmune sera was collected 14 days later at 64 DPI.

Virus Isolation

Blood and tissue samples for virus isolation attempts were obtained with the cooperation of the Montana Department of Fish, Wildlife, and Parks. Department personnel were contacted so that

---

[1]Anticoagulant preservative solution consisting of potassium oxalate, 5 g; phenol, 5 g; glycerin 500 ml; and distilled water, 500 ml.

field samples from any suspected HD mortality would be shipped, on ice, to the Veterinary Research Laboratory in Bozeman, Montana.

Isolation was attempted both directly in cell culture and through inoculation of an experimental white-tailed deer. Inocula for experimental infection consisted of a 10% spleen suspension prepared in EBME containing 5000 units of penicillin and 5 mg of streptomycin per ml of suspension. After subcutaneous inoculation with eight ml of the spleen suspension, the temperature and clinical signs of the experimental animal were monitored daily. Heparinized blood samples for isolation attempts in cell culture were collected from the experimental animal when clinical signs indicated an ongoing viremia.

Virus isolation in cell culture was done according to the method of Bando (2) with slight modifications. Whole blood from natural HD cases, or heparinized blood from an experimental animal, was first mixed with an equal volume of sterile water. The mixture was sonicated at a setting of 4 kc/second for one minute and diluted 1:5 with M-199 containing penicillin (200 units/ml) and streptomycin (200 mcg/ml). The diluted blood preparation was adsorbed in two ml quantities onto drained VERO monolayers that had been established in 250 ml tissue culture flasks. After a one hour adsorption period at 37^{o}C, the inocula were removed, the cell sheets were rinsed with a calcium and magnesium free saline solution,

and the maintenance media was replaced.

Infected flasks were observed daily for the appearance of CPE. CPE negative samples were taken through three consecutive blind passages at five day intervals before being considered negative. CPE positive samples were harvested when 70% to 100% of the monolayer was affected and processed in the same manner as the stock virus pools.

Viral Titrations

A modification of the plaque assay method described by Jochim et al. (49) was used for viral titrations. After establishing VERO monolayers in 24 well plates, growth medium was removed and 0.2 ml of five-fold virus dilutions made in serum free M-199 were added to appropriate wells. Adsorption was allowed for one hour at 37°C, the dilutions were removed, and the monolayers were overlaid with 0.5% agarose in M-199. After incubation for six days in humid air-tight containers at 37°C, the monolayers were fixed for 30 minutes at room temperature by the addition of 0.5 ml of 10% buffered neutral formalin to each well. Agar plugs were then removed and the monolayers were stained with crystal violet. Plaques in each well were counted to determine the number of plaque forming units (PFU) and calculations made to determine the number of PFU per ml of stock virus pool.

Electron Microscopy

Although differentiation between BTV and EHDV cannot be achieved by morphological examination, electron micrographs were produced of viral isolates to assure the presence of an orbivirus. VERO cells were grown in 250 ml tissue culture flasks and infected with stock pools of the viral isolate. Monolayers were freeze-thawed when CPE approached 50% monolayer destruction and the culture fluid and cellular debris harvested. Cellular debris was pelleted by low speed centrifugation and the resulting supernate discarded. The pellet was resuspended in five ml of distilled water and two drops of the suspension added to a spot plate well containing three drops of 4% phosphotungstic acid (PTA), 15 drops of distilled water, and two drops of a freshly prepared bovine serum albumin solution. Contrast and droplet spreading were optimized by varying the final concentrations of PTA and albumin. After mixing, the preparation was sprayed onto a carbon coated formvar-filmed grid with a Pelco [5] all glass nebulizer. Grids were examined immediately with a JEOL 100CX transmission electron microscope operated at 80 kv.

Indirect Fluorescent Antibody Technique (IFAT)

The identity of the viral isolate was investigated using the IFAT as described by Jochim et al. (44). VERO monolayers were grown

[5] Ted Pella Inc., Tustin, Calif.

in four chambered slides and infected with the viral isolate or with reference virus strains. Slides were fixed at 24 or 48 hours post-infection by removing one half of the media in each chamber and replacing it with cold acetone. After 15 minutes at 4°C, the acetone media mixture was replaced with pure acetone for a 30 minute fixation period at -70°C. The acetone was then removed and the slides stored at -70°C until stained and examined.

The staining procedure was conducted at room temperature. Monolayers were rinsed with carbonate-bicarbonate buffer (pH 9.0) and 0.15 ml of a five-fold EHDV antibody dilution, prepared in carbonate-bicarbonate buffer, applied to appropriate chambers. The slides were held for 30 minutes, serum dilutions were discarded, and the monolayers were rinsed with four changes of buffer for one, three, three, and three minutes. After rinsing, 0.15 ml of a 1:16 dilution of fluorescein conjugated anti-bovine IgG [6] was added to the chambers. After 30 minutes, the monolayers were rinsed once again using the same procedure. Plastic chambers and gaskets were removed and coverslips were mounted using Wellcome's medium (pH 8.9). The slides were examined using a Leitz Orthoplan microscope equipped with a darkfield condensor and a 200-W mercury vapor lamp.

---

[6] Miles Lab. Inc., Research Division, Elkhart, In.

Serological Study Areas

Four separate study areas were included in the serological survey for BTV and EHDV antibody in the serum of free ranging deer. The Long Pines, Intake, and Missouri Breaks areas had experienced, or were presently experiencing, HD outbreaks in the white-tailed deer population. A fourth study area, the Bridger Mountain area, had no history of HD problems in the free ranging deer population.

Long Pines is located approximately 30 miles southeast of Ekalaka in Carter county, Montana. The area is part of Custer National Forest and consists of pine covered hills with an elevation of 4000 feet. Elevation drops adjacent to the Long Pines area onto agricultural lands involved in both beef and wool production. Cattle are also grazed in the Long Pines area during the spring and summer under lease agreement. White-tailed deer mortalities were reported in this area in the summer and fall of 1961, 1970, 1976, and 1978 (113).

The Intake area, consisting of about 80 square miles of white-tailed deer habitat, is located 10 miles northeast of Glendive in Dawson county, Montana. The area is bordered on one side by the Yellowstone River and includes both riverbottom and agricultural lands with an elevation of approximately 3000 feet. The estimated population density of 43.5 white-tailed deer per square mile fell

to 31 per square mile after a HD outbreak that occurred the summer and fall of 1977 (97). Similar outbreaks were reported in nearby areas along the Yellowstone River in 1975 and 1976.

The Missouri Breaks area is in Garfield and Phillips counties and consists of high bluffs bordering the Missouri River as well as dense thickets along the riverbottom. The area supports both mule deer and white-tailed deer and is grazed by lease agreement during the spring and summer. HD outbreaks have been reported in the Missouri Breaks area in 1961 and in 1976 (113).

The fourth study area, the Bridger Mountain area, had no history of HD and was included because of the availability of samples and use as a "negative control" area. The area is part of the Gallatin National Forest and is located approximately 25 miles north of Bozeman in Gallatin county, Montana. Elevation varies from 5000 to 9000 feet and livestock are grazed on both forest land and on private holdings adjacent to the Gallatin National Forest.

Bovine serum samples were collected from counties in eastern Montana where HD had been reported within the last three years. These study areas included Carter, Custer, Dawson, Fallon, McCone, Powder River, Prairie, and Richland counties. A small sample set was also obtained from Lake county in western Montana for use as a "negative control" area.

Serum Collection

Sera of free ranging deer were collected by jugular venipuncture from animals trapped during the 1978-79 live trapping program conducted by the Montana Department of Fish, Wildlife, and Parks. Serum samples were also obtained from hunter killed animals during the 1978 Montana big game season. Using these methods, 135 serum samples were collected from both mule deer and white-tailed deer.

Bovine serum samples were obtained from the serology section of the Montana State Diagnostic Laboratory. Portions of samples submitted to the laboratory for routine evaluation were collected and stored, like all serum samples in this study, at -20^{o}C. Approximately 340 bovine samples were collected from the study areas in question.

Plaque Reduction Neutralization Test (PRN)

Serum samples were screened at a 1:50 dilution in the PRN test for EHDV-Alberta neutralizing antibody. Homologous antibody prepared against the viral isolate was also reacted in a PRN test against reference EHDV strains for identification purposes.

A modification of the PRN test described by Jochim et al. (49) was conducted on VERO monolayers grown in 24 well plates. Heat inactivated serum dilutions were mixed with an equal volume of virus suspension calculated to contain approximately 80 PFU per 0.1 ml of suspension and incubated at 37^{o}C for one hour. After incubation,

0.2 ml of each serum virus mixture was applied to drained monolayers in appropriate wells and adsorbed for one hour at 37°C. The serum virus mixtures were then removed and the monolayers were overlaid with 0.5% agarose in M-199 containing penicillin (200 units/ml) and streptomycin (200 mcg/ml). Plates were incubated on moistened absorbent paper toweling in air tight containers at 37°C for six days. Monolayers were fixed and stained as previously described for the plaque assay titration and plaque counts were made.

Serum dilutions causing an 80% reduction in the number of PFU per well were considered positive for EHDV specific neutralizing antibody. All samples were tested in duplicate and the actual target number of PFU used in the test was calculated as described by Jochim et al. (49).

Agar Gel Precipitin Test (AGP)

All deer and bovine serum samples obtained during 1978 and 1979 were tested for BTV precipitating antibody using the micro AGP test as described by Jochim and Chow (46,48). BTV soluble antigen was provided by the National Animal Disease Center in Ames, Iowa and the Arthropod Borne Animal Disease Research Center in Denver, Colorado.

The AGP test is a modification of the Ouchterlony double diffusion test. Briefly, one inch square plexiglass templates were prepared by drilling six wells set at 60° to and 4 mm away, center

to center, from a middle well. Pre-cleaned three inch by one inch microscope slides were coated with a 0.2% solution of Bacto-agar [7] and three strips of double thickness electrical tape placed vertically down the width of each slide. The templates were then coated lightly with silicone stopcock grease and placed on the slides using the electrical tape for support. The area between the surface of the slide and the template was filled with 0.9% agarose in isotonic saline using a three ml syringe and a 26 gauge needle. Serum samples to be tested were alternated with appropriate reference antiserum in peripheral wells and soluble antigen placed in the center well. After incubation in humid, air-tight containers for 72 hours at room temperature, the templates and electrical tape were removed and the slides were immersed in isotonic saline for 24 hours. The slides were then stained with thiazine red for seven minutes and de-stained in two seven minute changes of a 1.0% acetic acid solution. Slides were examined both before and after the staining procedure and samples recorded as either positive or negative for BTV precipitating antibody.

---

[7] Difco Laboratories, Detroit, Michigan.

CHAPTER 4

RESULTS

Virus Isolation

During the course of this study, blood and tissue samples were collected from three field cases that were HD suspects. Two of the cases were mule deer which had been found in Garfield county in October of 1978. One of these mule deer, a yearling female, was alive when located. She was found hunched over, convulsive, foaming at the mouth, feverish, and diarrheic, and was euthanized. A field necropsy revealed hemorrhages in the skeletal muscle, abdominal wall, large and small intestine, and cardiac muscle. The second mule deer was a male fawn that had apparently died in a convulsive state, evidenced by signs of hyperactivity surrounding the carcass, but showed no gross lesions on post-mortem examination.

The third suspected HD case involved a three and one half year old female white-tailed deer. The animal had been recumbent and was accidentally run over and killed in Rosebud county on Septemper 11, 1978. Foam was observed around the muzzle area and at necropsy subcutaneous petechial hemorrhages as well as hemorrhages on the surface of the lung, liver, and kidney were evident. Straw colored fluid was present in the thoracic and abdominal cavities and in the pericardial sac. Blood vessels were engourged and, although the field necropsy was conducted 12 hours after death, visceral blood still remained unclotted.

An experimental white-tailed deer, seronegative to EHDV, was inoculated with spleen suspension prepared from the Rosebud county white-tailed deer mortality. At five days post inoculation, the experimental deer was found recumbent and in a shock-like state. The animal was experiencing respiratory difficulty and convulsive movements and had reddened conjunctiva and a cyanotic tongue. Blood samples collected before sacrificing the animal took approximately 25 minutes to clot as opposed to the normally observed clotting time of two to five minutes. At necropsy, hemorrhages were noted at the apex of the heart and on the serosal surfaces of the gastrointestinal tract. There was abundant straw colored fluid in the pleural cavity as well as in the pericardial sac. The lungs were involved with a very distinct interlobular edema. No bacterial pathogens were cultured or identified from blood samples taken from the experimental animal at necropsy.

A cytopathic agent was isolated in cell culture from the initial passage of treated, heparinized blood from the experimental white-tailed deer. A cytopathic agent was also isolated from the whole blood of the original white-tailed deer after one blind passage in VERO cells. No isolations were made from tissue samples collected from either of the Garfield county mule deer mortalities after three blind passages in VERO cells.

Cytopathic effect was observed in VERO monolayers infected

with the viral isolates within 48 to 72 hours post-infection. Cells appeared more granular at that time and began to detatch from the surface of the culture flask. As infection progressed, usually by 72 hours post-infection, the media became cluttered with cellular debris and only about 50% of the monolayer remained attatched to the flask surface. By 96 hours post-infection, CPE usually approached the maximum of approximately 70% monolayer destruction.

IFAT

EHDV specific fluorescence was observed in VERO monolayers both 24 and 48 hours after infection with the viral isolates. Irregularly shaped aggregations of viral antigen, usually perinuclear in distribution, were easily visualized after fluorescent staining (figure 1). No specific fluorescence was seen in non-infected monolayers or in monolayers infected with BTV type 13 (figure 2). The two viral isolates were considered identical and were designated EHDV-M78.

Electron Microscopy

Electron micrographs of the viral isolate allowed for a determination of morphology and size. Negatively stained particles were calculated to be 56.5 nm in diameter. Individual virions possessed cubic symmetry and relatively large, doughnut shaped capsomeres consistent with orbivirus morphology (figure 3).