THESIS

VERTICAL TRANSMISSION IN THE CULICOID VECTOR AS A POSSIBLE MECHANISM FOR BLUETONGUE VIRUS TRANSEASONALITY

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY SUSAN MARIE DEINES ENTITLED "VERTICAL TRANSMISSION IN THE CULICOID VECTOR AS A POSSIBLE MECHANISM FOR BLUETONGUE VIRUS TRANSEASONALITY" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT

VERTICAL TRANSMISSION IN THE CULICOID VECTOR AS A POSSIBLE MECHANISM FOR BLUETONGUE VIRUS TRANSEASONALITY

Bluetongue virus (BTV) is transmitted between vertebrate hosts by arthropod vectors in the genus *Culicoides*. In tropical and sub-tropical climates the virus is maintained in a continual cycle between the culicoid vector and the vertebrate host; however, bluetongue disease is also enzootic in temperate regions, such as northeastern Colorado. The mechanism by which BTV overwinters in these regions, where the adult culicoid vector does not survive cold winter months, is unknown. The primary objective of this study was to determine if vertical transmission of BTV in the culicoid vector is the mechanism for BTV transeasonality in regions of temperate climate.

Culicoid larvae were collected from two sites near Brighton, Colorado, where BT disease is known to be enzootic. A light-trap was also set at one of these sites for the collection of adult culicoid flies.

Processed pools of field-collected culicoid larvae, culicoid flies allowed to emerge in the laboratory from collected larvae and culicoid flies collected in the light-trap were first assayed for the presence of BTV antigen using an antigen capture enzyme-linked immunosorbent assay (ELISA). A total of 49,521 larvae, 11,221 emergent flies, and 901 light-trap collected flies were assayed. The resulting minimum field infection rates (MFIRs), based on the presence of BTV antigen, were 0.20% for larvae, 0.59% for emergent flies and 1.56% for light-trap collected flies ($P<0.001$).

Isolation of infectious BTV was then attempted from the putatively positive emergent and light-trap collected fly pools. Three different

isolation systems were used: 1) intravascular inoculation of embryonated chicken eggs (ECE), 2) baby hamster kidney (BHK-21) cell culture and 3) intrathoracic inoculation of *Aedes triseriatus* mosquitoes.

The culicoid pools putatively positive for BTV by ELISA yielded a total of 12 BTV isolates. The MFIRs for emergent flies and light-trap collected flies, based on virus isolation, were 0.10% and 0.11%, respectively (P=0.66). All of the isolates were found to be BTV serotype 17, except for one isolate from an emergent fly pool, which was determined to be BTV serotype 11.

C. variipennis, the only known vector for BTV in the United States, was not the only vector from which BTV was isolated. BTV isolates were obtained from pools composed of *C. variipennis* (MFIR=0.07%), *C. crepuscularis* (MFIR=0.18%) and culicoids belonging to the *selfia* group (MFIR=0.22%) No statistically significant differences (P<0.05) were observed among the three species.

The results of this study support the hypothesis that BTV is vertically transmitted in the culicoid vector and that this may be the mechanism by which the virus overwinters in temperate regions. Additionally, it appears that *C. variipennis* is not the only culicoid species in which the virus is maintained. *C. variipennis* is the epidemic vector for the disease, but other culicoid species, *C. crepuscularis* and members of the *selfia* group in particular, may be endemic vectors and reservoir hosts for the virus.

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I. LITERATURE REVIEW

A. Historical Background

Bluetongue disease (BT), an affliction of domestic and wild ruminants, was first described as a disease of South African sheep by Hutcheon in 1881. Most likely the etiologic agent was indigenous to South Africa, but serious outbreaks of BT disease were not observed until the introduction of susceptible European sheep in the late 1800's. Spreull renamed the disease, then called malarial catarrhal fever, bluetongue in 1905 and suggested that it was a viral disease. Shortly thereafter, in 1906, Theiler proved that the causative agent of BT was filterable and, most likely, a virus.

Originally thought to infect only sheep, bluetongue virus (BTV) was shown to also cause a disease in cattle, then called pseudo foot-and-mouth disease, by Bekker in 1934. In 1944, DuToit proved that the biting gnat *Culicoides* was the vector for the virus.

BT was thought to exist only on the African continent until 1943 when the first confirmed case of BT occurred outside of Africa in Cyprus (Gambles, 1949). In 1948 BT appeared in the United States, causing an outbreak of a disease in Texas called "soremuzzle of sheep" (Hardy and Price, 1952). Another outbreak of the disease occurred in Texas and California in 1952, at which time the virus was isolated and the disease was confirmed to be BT (McKercher et al., 1953).

Today, BTV is found throughout the world in Africa, Asia, India, the Middle East, the Caribbean, South America, Japan, Mexico, Australia and the United States (Ozawa, 1985; Mellor, 1990). Clinical BT disease is not observed in countries such as Australia, Mexico and Japan, despite the presence of the virus (Gibbs, 1983; Ozawa, 1985; Stott et al., 1989); the European Community is considered to be free of BT disease (Dexter, 1985).

B. The Bluetongue Virion

1. Serological Classification

BTV is the prototype virus of the genus *Orbivirus* in the family Reoviridae. The orbiviruses are separated into 12 antigenically related serogroups and 1 ungrouped set of viruses (Knudson and Monath, 1990; Gorman, 1992). Important orbiviruses belonging to other serogroups include epizootic hemorrhagic disease of deer virus (EHDV) and African horse sickness virus (AHSV).

There are at least 25 BTV serotypes world-wide (Davies et al., 1992). BTV serotypes 2, 10, 11, 13, and 17 exist in the U.S. (Collisson and Barber, 1985).

2. Stability

BTV is resistant to lipid solvents such as ether, sodium deoxycholate and chloroform, as are reoviruses (Svehag, 1966). Unlike reoviruses, which are stable over a wide pH range, BTV is active in a narrow pH range of 6.3-8.0 (Owen, 1964; Svehag, 1966). Limited digestion with trypsin, chymotrypsin and thermolysin enhances viral infectivity due to proteolytic cleavage of the outermost capsid protein, but extended digestion greatly reduces infectivity (Svehag, 1966; van Dijk and Huismans, 1980; Cowley and Gorman, 1990). BTV is relatively stable at 4°C, but purified virus is extremely unstable at higher temperatures in the absence of all extraneous protein (Howell et al., 1967). Snap-freezing the virus at -70°C in a stabilizer, such as buffered lactose peptone or bovine serum albumin, maintains viral infectivity over long periods of time, whereas slow freezing at -20°C is detrimental to the virus (Howell et al., 1967). The viral transcriptase operates optimally at 28°C as opposed to 45°C for other dsRNA viruses such as reoviruses (Verwoerd and Huismans, 1972).

3. Morphology

Like other members of the Reoviridae family, BTV is non-enveloped, has a segmented dsRNA genome, and is composed of two concentric protein

shells. The virion is composed of three minor core proteins (VP1, VP4, and VP6), two major core proteins (VP3 & VP7), 2 outer capsid proteins (VP5 & VP2) and ten genomic segments of dsRNA. The three minor core proteins, VP1, VP4 and VP6, are enclosed within the innermost shell along with the genomic segments. This shell is icosahedral and composed almost entirely of the major core protein VP3 (Huismans and van Dijk, 1990). Together, these four proteins and the genome make up the subcore of the virion. VP7, the other major core protein, exists as trimers on the subcore surface and with the subcore makes up the virion core particle, which measures approximately 69 nm in diameter (Huismans et al., 1987b, Prasad et al., 1992; LeBlois and Roy, 1993). The viral capsid, composed of proteins VP5 and VP2, surrounds the viral core protein and the complete virion measures approximately 81 nm in diameter (LeBlois and Roy, 1993).

4. Genomic Coding Assignments

The BTV genomic segments range in M_r from 0.5 X 10^6 to 2.7 X 10^6 resulting in a total M_r of 13 x 10^6 (Fukusho et al., 1989). Except for S10, all of the genomic segments are monocistronic (Mertens et al., 1984; Roy, 1992). There are three large genomic segments, L1, L2 and L3, which code for core proteins VP1, VP2 and VP3, respectively. Segments M4 and M5 code for proteins VP4 and VP5, respectively. Proteins VP6 and VP7 are coded for by S9 and S7, respectively. Four non-structural proteins, NS1, NS2, NS3 and NS3A, are synthesized in BTV infected cells. Their synthesis is virally induced and they are coded for by the viral genome segments M6, S8 and S10, respectively. S10 codes for both the NS3 and NS3A proteins.

5. Proteins

a. Minor core proteins-VP1, VP4, and VP6

VP1, the largest of the BTV proteins, is present in a low molar ratio in the virion (Urakawa et al., 1989, Huismans and van Dijk, 1990). This protein is the putative viral RNA polymerase and may also mediate inclusion of other viral components into viral cores and play an important structural role (Urakawa et al., 1989, Louden and Roy, 1991).

BT viral mRNA is capped at the 5′ end during transcription. The VP4 core protein is the putative RNA capping enzyme, guanylyl transferase (Roy, 1992; LeBlois et al., 1992).

The third minor core protein, VP6, is thought to also be associated with the mRNA polymerase complex. It has been shown to be capable of binding dsRNA, ssRNA transcripts, and other nucleic acid species (Roy et al., 1990a). The sequence of this protein is similar to other helicases, indicating that it may be involved in the unwinding of the positive sense, dsRNA viral genome (Roy, 1992).

b. Major Core Proteins-VP3 and VP7

VP3 is a major structural core protein that is highly conserved among BTV serotypes and other *Orbivirus* serogroups (Huismans and Erasmus, 1981; Roy et al., 1990b; Roy, 1992). The other major core protein, VP7, occurs in greater abundance than VP3 and is the major BTV serogroup specific protein (Huismans and Erasmus, 1981; Oldfield et al., 1990). The VP7 protein contains a single lysine residue that is conserved among all BTV serotypes sequenced and shared by the VP7 protein of the closely related EHD virus (Kowalik and Li, 1991; Iwata et al., 1992).

c. Outer Capsid Proteins-VP5 and VP2

Of all the BTV proteins, the outer capsid proteins VP2 and VP5 are the least conserved among BTV serotypes (Mecham et al., 1986). VP2 is the major determinant of serotype specificity. It is also proposed to be the viral hemagglutinin and induces neutralizing antibody production (Huismans and Erasmus, 1981; Cowley and Gorman, 1987; Roy et al., 1990b). VP2 is required for attachment of the virus to vertebrate cells (Cowley and Gorman, 1990; Huismans and van Dijk, 1990).

VP5 is more conserved among serotypes than VP2 (Oldfield et al., 1991; Roy, 1992). It is theorized that VP2 obscures VP5 on the virion surface (Oldfield et al., 1991). Therefore, VP5 has less exposure to specific antibody elicited in an immune response and a less varied antigenic structure.

Both VP2 and VP5 may be essential for activation of an effective immune response (Roy et al., 1990c).

d. Non-structural proteins-NS1, NS2, NS3 and NS3A

The four non-structural proteins encoded by the viral genome are synthesized in BTV infected cells. NS1 and NS2 are synthesized by the infected cell in abundant amounts while NS3 and NS3a are synthesized in minuscule amounts (Roy, 1992).

The NS1 protein is the major protein synthesized in BTV infected cells and is the major component of tubules found in the cytoplasm of these cells (Huismans and Els, 1979; Eaton et al., 1988; Urakawa and Roy, 1988).

NS2 appears to be associated with the production of viral inclusion bodies (VIB) within the infected cell (Eaton et al., 1990). This is the only BTV protein to be phosphorylated and it is able to bind ssRNA (Huismans et al., 1987a; Wade-Evans, 1992). Both the VIB and tubules are attached to the intermediate filament component of the cell cytoskeleton and are presumed to be involved in the viral assembly process (Eaton et al., 1987).

NS3 and NS3A are both encoded by genome segment 10. NS3A is not a cleavage product of NS3, but is initiated at a separate codon downstream from the initiation codon for the NS3 protein (Wu et al., 1992). These are glycosylated, integral membrane proteins localized at the surface of the infected cell (Wu et al., 1992). NS3 and NS3A appear to be necessary in the release of virus from the infected cell (Hyatt et al., 1993).

6. Replication

The details of BTV replication are not all known, but the virus replicates in a manner similar to other reoviruses (Schiff and Fields, 1991). The first step in replication is adsorption to the cell, which occurs within 20 minutes (Howell et al., 1967). The virus then enters the cell by endocytosis into a phagocytic lysosome. Within this lysosome, the proteins VP2 and VP5 are removed, probably by proteolysis (Cowley and Gorman, 1990). Uncoating of the virion generates a subviral particle (SVB) containing an active RNA dependent RNA polymerase (van Dijk and Huismans, 1980). This SVB

is released into the cell cytoplasm by crossing the lysosomal membrane and a complementary positive sense mRNA strand is transcribed from each negative sense RNA template (Roy, 1992). Viral mRNA, which is thought to be capped at the 5′ end and methylated during transcription, is utilized both for protein translation and as the positive sense strands in progeny genomes (Roy, 1992)

Translation of the viral proteins appears to be temporally regulated as the VP7 protein is first detected in an infected cell 3 hours after inoculation, NS1 and NS2 proteins are detected 5 hours post inoculation, and the VP2 protein is not detected until 8 hours after infection (Whetter et al., 1990). Viral morphogenesis is associated with perinuclear viral inclusion bodies (VIB). Recent studies show the presence of virion cores and virus-like particles within and at the periphery of the VIB matrix. These cores and particles contain proteins VP5 and VP7 but not VP2. VP2 is found near the outside edge of the VIB. These findings suggest that BTV particles are synthesized and assembled within the VIB matrix and upon release from the VIB acquire the VP2 protein (Brookes et al., 1993).

Viral mRNA serves as a template for the synthesis of new negative sense RNA strands. Presumably, the same virus-coded polymerase associated with virus cores carries out the replication and negative sense strands are synthesized from these transcripts, resulting in the dsRNA segments encapsidated in progeny virus (Acs et al., 1971; Urakawa et al., 1989). Release of the virus from the cell is associated with the NS3 and NS3A proteins, possibly by the following mechanism: BTV binds to the filaments of the cell cytoskeleton by both of the outer capsid proteins and NS3/NS3A, contained in smooth-surfaced vesicles, can then interact with the bound BTV. The vesicles are transported to the plasma membrane where fusion occurs and the virus is released by extrusion through the cell plasma membrane (Cromack et al., 1971; Hyatt et al., 1993).

C. Vertebrate Hosts

The host range for BTV is limited to members of the mammalian suborders Ruminantia and Tylopoda of the order Artiodactyla (Metcalf et al., 1985).

Although primarily a disease of sheep, other domestic ruminant hosts include cattle, goats and water buffalo. Serologic evidence of BTV infection has been recorded in a number of species of wild ruminants including elk, antelope, bighorn sheep, barbary sheep, moose and several species of deer (Hoff and Hoff, 1976; Trainer and Jochim, 1969; St. George, 1985). BTV has been isolated from naturally infected bighorn sheep, deer, mountain gazelle, a Reeve's muntjac, and an East African greater Kudu (Hoff et al., 1973; Hoff and Hoff, 1976).

D. Clinical Signs

BTV infection in sheep is generally subclinical and mild but may be acute and severe. Mortality rates range from 2-50% in field outbreaks with a morbidity rate of 10-75% (Erasmus, 1975; Hardy and Price, 1952; McKercher et al., 1953; Hourrigan and Klingsporn, 1975b). The severity of the disease is dependent on the pathogenicity of the virus, the susceptibility of the animal and environmental conditions. Generally, the disease manifests itself in sheep in the following progression (Erasmus 1975; Uren and Squire, 1982; Parsonson 1990): Fever is usually the first indication of disease, occurring approximately 8 days post infection and lasting an average of 6-7 days. This may be accompanied with an increase in respiration rate. Early clinical signs appear 1-2 days later, including hyperemia of the skin, especially in hairless regions such as the muzzle, axilla, and inner thighs, inflammation and swelling of the oral mucosa and edema of the face, lips, and ears. Frothy salivation may be observed and rarely the tongue becomes swollen, cyanotic and protrudes from the mouth; hence the name "bluetongue". Lesions of the skin then appear, most commonly in and around the oral cavity. If lesions occur beneath the pelt of the animal, loss of wool, or "wool break", may occur. In acutely infected sheep, depression, anorexia, and coronitis may be observed.

BTV infection may be directly responsible for death due to cardiac failure resulting from widespread lesions in the cardiac muscle (Mahrt and

Osburn, 1986) but, more commonly, death is due to secondary bacterial infections such as bilateral bronchopneumonia (Erasmus, 1975).

In cattle and goats BTV infection is usually inapparent, the only clinical signs being transient, mild fever, leukopenia, viremia and the production of neutralizing antibody (Luedke and Anakwenze, 1972; Erasmus, 1975; MacLachlan et al., 1987). However, clinical BT has been reported in cattle and goats (Bowne et al., 1968; Hourrigan and Klingsporn, 1975a). Clinical disease is observed in less than 5% of an infected adult cattle population (Metcalf et al., 1980 and Luedke and Walton, 1981). Vesicular stomatitis, infectious bovine rhinotracheitis, foot-and-mouth disease, rinderpest, bovine viral diarrhea, and mycotic stomatitis are some of the diseases that can easily be mistaken for BTV (Bowne et al., 1966b; Hourrigan and Klingsporn, 1975a). Epizootic hemorrhagic disease of deer (EHD), a related orbivirus that affects cattle and deer but not sheep, produces clinical signs in cattle identical to those observed in sheep infected with BTV and can also be mistaken for BT disease (Jones et al., 1981).

E. <u>Pathogenesis</u>

BTV is introduced into the vertebrate host intradermally via the bite of an infected culicoid vector. The virus undergoes primary replication in the lymph nodes, and can usually be first isolated from the lymph nodes of the head, cervical area, tonsils and spleen (Pini, 1976). The presence of BTV in the spleen shortly after infection suggests that a low titer, primary viremia occurs after initial infection of draining lymph nodes resulting in the infection of other lymph nodes and the spleen (Pini, 1976). After an incubation period of 6-7 days, the virus enters the general circulatory system, resulting in a high titer, secondary viremia. Endothelial and periendothelial cells of the vascular system, as well as target organs, are infected and secondary viral replication occurs (Stair, 1968). Clinical manifestations, such as lesions of the oral cavity and edema of the face, coincide with maximum titers of virus, leukopenia and the presence of high titers of neutralizing antibody (Pini, 1976). This co-existence of high

coincide with maximum titers of virus, leukopenia and the presence of high titers of neutralizing antibody (Pini, 1976). This co-existence of high virus titers and neutralizing antibody suggests a mechanism by which the virus is protected from antibody (Luedke 1970a; Alstad et al., 1977). Recent *in vivo* studies have demonstrated that BTV persists in the cell membrane invaginations of bovine erythrocytes, affording protection to the virus from specific neutralizing antibody (Brewer and MacLachlan, 1994).

F. Economic Importance

BTV is a veterinary pathogen of great economical significance to the livestock industry. The sheep industry is most severely impacted by clinical BT disease due not only to high fatality rates but also due to indirect losses such as marked loss of condition, increased susceptibility to secondary infections, protracted convalescence, impaired wool-growth and cost of treatment (Gibbs, 1983).

The cattle industry is also affected by BT disease. In 1979, direct losses attributed to BT in cattle in an epizootic in Mississippi and four surrounding states were estimated to exceed $12 million (Metcalf, 1980). However the major impact of BT on the cattle industry is as a constraint on exports. If an animal tests seropositive for BTV antibody, all other animals from that area, as well as germplasm from those animals, may be restricted from import into other countries. Over $4 million was estimated to have been lost the year BTV was first identified in Australia despite the fact that clinical BT was not reported (Gibbs, 1983). In the U.S., $24 million is estimated to be lost annually due to bans on importation of bull semen to the United Kingdom, Australia, and New Zealand (Gibbs, 1983).

G. Virus-Vector Associations

1. The Culicoid Vector

Observation that the occurrence of BT disease was seasonal and the incidence was greater in areas of heavy biting insect populations led to early speculation that the disease was transmitted by insect vectors. In

South Africa, DuToit (1944) incriminated tiny, biting gnats belonging to the genus *Culicoides* (Diptera:Ceratopogonidae) as vectors of BTV after demonstrating transmission of BT disease from infected to susceptible sheep by *C. imicola* (pallidipennis). In the U.S., the only proven vector of BTV is *C. variipennis* (Coulliquett), although *C. insignis* (Lutz) has been implicated as the primary vector of BTV serotype 2 in south Florida (Greiner et al., 1985). *C. variipennis* has been shown to be capable of biological transmission of BTV in both sheep and cattle (Luedke et al., 1967). The range for *C. variipennis* extends from mid-Florida and mid-Mexico into parts of Canada, but it is rarely found in the far northeastern and southeastern U.S. (Jones and Foster, 1978b). *C. variipennis* prefers a habitat that is heavily polluted with cattle manure, but may also be found in fresh, salt and alkaline habitats. *C. variipennis* readily attacks cattle, horses, swine, sheep and deer (Jones et al., 1981; Jones and Foster, 1978b).

Culicoids have four life cycle stages: egg, larva, pupa and adult. The eggs are laid in batches of 30-250, depending on species, and hatch into the first of four larval stages. The larvae move with a distinctive writhing motion. Pupation follows the fourth instar larvae stage. Pupae are comma shaped, 6-7 mm in length and do not feed during the 3-7 days before emerging into adult flies. Adult culicoids measure 1-5 mm in length and only the female feeds on blood (Burgess and Cowan, 1993). *C. variipennis* has a short generation time, as little as two weeks in Colorado. Seven generations may occur in one year, providing abundant vectors of BTV (Barnard and Jones, 1980).

2. Virogenesis

C. variipennis is the only BTV vector for which detailed virogenic data exist. Ingestion of a viremic blood meal by a female fly causes the muscles of the diverticula to contract, resulting in the diversion of most of the blood meal to the hind portion of the mid-gut (Megahed, 1956). The virus infects the mid-gut cells and replicates within them (Chandler et al., 1985; Ballinger et al., 1987; Siebruth et al., 1991). Over the first 24 hours virus titer decreases, presumably as attachment, penetration and uncoating of

the virus occurs (Foster and Jones, 1979). The virus then escapes from the mid-gut, enters the haemocoel and is disseminated to secondary target organs, primarily the fat-body and salivary glands, where it again replicates (Bowne and Jones, 1966; Chandler et al., 1985; Ballinger et al., 1987). Virus titer peaks and then plateaus 7-9 days post infection (PI), maximum titers reaching 4-5 log_{10} $TCID_{50}$ per insect (Jochim and Jones, 1966, Jennings and Boorman, 1980; Mellor, 1990). Virus titers remain constant 26-35 days PI (Mellor, 1990; Jochim and Jones, 1966). Transmission of the virus is possible 10-14 days PI and the bite of a single fly may be sufficient to cause infection of a vertebrate host (Foster et al., 1963; Foster et al., 1968b).

3. Vector Competence

Most species of culicoids are refractory to oral infection with BTV. Although there are over 1000 species of culicoids worldwide, less than 20 have been associated with BTV and only 6 have been proven to transmit the virus, although additional species are suspected to be competent vectors (Mellor, 1990).

Different populations of *C. variipennis* contain variable proportions of individuals that are refractory to oral infection with BTV, even though 100% of the population can be infected parenterally (Jones and Foster, 1966; Jones and Foster, 1974). Furthermore, field populations of *C. variipennis* that are susceptible to BTV infection are heterogeneous in susceptibility to different BTV serotypes and different populations vary in susceptibility to a single BTV strain (Jones and Foster, 1978a; Mecham & Nunamaker, 1992; Tabachnick, 1991). This variability appears to be due to a mesenteron infection barrier in some *C. variipennis*, which is under the control of two genetic factors (Jones and Foster, 1974; Jones and Foster, 1978a). The first is a single gene with a dominant allele conferring resistance and the second is a gene(s) controlling the degree of susceptibility if the dominant gene is absent or inoperative. Genetic susceptibility to BTV infection does not guarantee vector competence. *C. variipennis* infected with low titers of BTV (<2.5 log_{10} $TCID_{50}$) but unable to transmit virus exhibit a mesenteron escape barrier (Jennings and Mellor,

1987). The virus is restricted to the mid-gut cells and does not disseminate to secondary target organs, including the salivary gland, preventing the infected fly from transmitting the virus.

In the U.S. there are 5 sub-species of *C. variipennis*: *C. v. albertensis*, *C. v. australis*, *C. v. occidentalis*, *C. v. sonorensis*, and *C. v. variipennis* (Wirth and Jones, 1957). The geographic distribution of these sub-species supports the hypothesis that genetic variation between sub-species determines vector competence. *C. v. sonorensis* is found in the southwestern and western U.S. where BT is most prevalent; *C. v. variipennis* is found in the northwest, north central, and mid-west U.S. where BT is rare, suggesting that *C. v. sonorensis* is a genetically competent vector of BTV and *C. v. variipennis* is not. Genetic studies have demonstrated that a single gene locus, the *blu* locus, controls oral susceptibility to BTV in a laboratory strain of the sub-species *C.v. sonorensis*, although it is likely that there are additional controlling loci in populations of *C. variipennis* (Tabachnik, 1992).

H. Diagnosis

The diagnosis of BTV infection in vertebrates is done either by isolation and identification of the virus or by detection of viral antigen-specific antibodies from the serum of infected animals. Serologic tests are either serogroup or serotype specific. Serogroup specific tests include complement fixation (CF), immunodiffusion (ID), immunofluorescence (IF), hemolysis-in-gel (HIG) and enzyme linked immunosorbent assay (ELISA). BTID (Pearson and Jochim, 1979) has been the standard test used by diagnostic laboratories in the U.S. to test animals for export (Jochim, 1985). However, there may be cross-reactivity with antibody from the antigenically related EHD virus using BTID (Jochim, 1985). Recently, a competitive ELISA (cELISA) was approved for use in the U.S. to replace, or be used in combination with, the BTID test (A.D. Alstad, National Veterinary Services Laboratories, personal communication). The cELISA is more specific than BTID for BTV antibody and does not cross-react with EHDV (Afshar et al., 1987).

Serotype specific tests such as plaque inhibition and plaque neutralization procedures are the primary tests used for serotyping the virus. Often there is extensive cross-reactivity between the closely related BTV serotypes, making interpretation of the results difficult (Della-Porta et al., 1985). Animals with multiple infections of BTV may produce antibody which will cross-react with orbiviruses to which there has been no exposure (Thomas, 1985; Della Porta et al., 1985).

Isolation of BTV is most often done by inoculation of embryonated chicken eggs with blood or semen samples from animals potentially infected with BTV. Inoculation of susceptible sheep with blood or semen samples is considered to be a more sensitive isolation system, but this method is obviously expensive and often impractical (Jochim, 1985).

Intravascular inoculation of embryonated chicken eggs is the "gold-standard" for BTV isolation today. BTV will propagate in embryonated chicken eggs but not in other animals commonly used for laboratory isolation of viruses such as guinea pigs, rabbits, and chicks (Mason et al., 1940).

Because this isolation technique is expensive, laborious and time-consuming, alternative methods are being examined. Direct isolation in cell culture is much less sensitive than egg inoculation, although cattle pulmonary endothelial (CPAE) cells have been demonstrated to approach the level of sensitivity obtained by virus isolation in eggs (Wechsler and McHolland, 1988)

Research is being directed toward new rapid diagnostic tests for BTV, including nucleic acid hybridization (Schoepp et al., 1991; Venter et al., 1991; Venter et al., 1993), polymerase chain reaction (Gould et al., 1989; Wade-Evans et al., 1990; Dangler et al., 1990), ELISA (El Hussein et al., 1989; Mecham, 1993), dot immunoperoxidase assay (Afshar, 1991), and reverse target capture hybridization (Raich et al., 1994). To date, virus isolation is still the most sensitive technique, as problems exist with most of the other methods in detection of low-titer, field-strains of BTV in animal sera or tissue (Schoepp et al., 1991; de la Concha-Bermejillo et al., 1992; Mecham, 1993).

I. Prevention and Control

Control of the vector population and vaccination of susceptible hosts are two effective methods for controlling arbovirus diseases. Sanitation control and treatment of culicoid breeding sites with larvicides has been shown to be effective in reducing vector populations, and consequently BT disease, under experimental conditions (Holbrook, 1985). However, due to the density of larval populations, it is difficult to ensure that all larvae are treated and don't persist in sufficient numbers to continue the disease cycle.

Ivermectin may be a possible method for control of the adult culicoid vector. *Culicoides brevitarsis*, feeding on cattle that had been intravenously inoculated with Ivermectin within the previous ten days, experience mortality rates of 99% 48 hours after feeding (Standfast, 1985).

Prevention of BT disease by vaccination would seem to be the most effective form of control, but there are complications. Because there are numerous serotypes of BTV, it is difficult to produce a vaccine providing complete protection against all serotypes. In the U.S. the only BTV vaccine approved for use is a cell-culture adapted, modified-live vaccine against BTV serotype 10 (Stott et al., 1981; Stevens et al., 1985; Roy et al., 1990c). This vaccine is not effective in regions of the U.S. where BTV serotypes other than BTV-10 are prevalent (Jones et al., 1981). In South Africa a series of three modified-live BTV pentavalent vaccines are administered at three week intervals resulting in exposure to fifteen BTV serotypes over a six week period (Stevens et al, 1985). Due to the segmented BTV genome, the use of a modified-live vaccine may potentially result in reversion to a virulent strain by multiplicity reactivation. Reassortment of BTV genome segments has been demonstrated in both the vertebrate host and the culicoid vector (Samal et al., 1987; El Hussein, 1989). The use of an egg-adapted, modified-live BT-10 vaccine in the U.S. resulted in high-titer viremia, clinical disease and teratogenic effects in vaccinated sheep. In addition, there was evidence of vector transmission of BTV from vaccinated to susceptible sheep (Foster et al., 1968b). Similar results were observed in

Australia when a modified-live BTV-1 vaccine was administered to pregnant ewes at 35-42 days gestation (Johnson et al., 1992)

A recombinant BTV vaccine may solve these problems. Genomic segments expressing either the BTV VP2 and VP5 proteins or the BTV VP3 and VP7 proteins are inserted into baculovirus vectors. Insect cells co-infected with these vectors produce non-infectious, double-shelled, virus-like particles (VLP). These VLPs resemble authentic BTV in size, appearance and biochemical composition, and they are potent immunogens that are incapable of replication due to the absence of the genome (Roy et al., 1990c; Pearson and Roy, 1993).

J. **Transeasonality of BTV**

In tropical and neo-tropical regions, BTV is maintained in nature in a continual cycle between culicoid vector and vertebrate host. The adult vector ingests a viremic bloodmeal from a BTV infected vertebrate host and the virus replicates within the vector. After viral replication in the salivary glands, the vector is capable of transmitting the virus by biting a susceptible vertebrate host, in which the virus again replicates.

This cycle is broken in temperate regions due to the absence of the adult culicoid vector during cold winter months. The overwintering mechanism of BTV is unknown. Several mechanisms exist for the overwintering of arboviruses including: 1) re-introduction of infected vectors, 2) the presence of other, unrecognized vectors, 3) persistence in the vertebrate host, 4) overwintering in the infected adult vector and 5) vertical transmission in the vector (Reeves, 1974).

BTV may be mechanically re-introduced into an enzootic area each year by migratory birds or mammals or by BTV infected culicoids carried on wind currents. It appears that the Ona A strain of BTV-2 found in Florida was introduced by infected culicoids carried on wind currents from Cuba (Sellers and Maarouf, 1989). Similarly, BTV-11 may have been introduced into British Columbia from Washington, U.S.A. by wind currents, resulting in epizootic outbreaks of BT in 1987 and 1988 (Sellers and Maarouf, 1991).

Other unrecognized vectors of BTV may harbor the virus during cold winter months. The sheep ked, *Melophagus ovinus*, is capable of mechanical transmission of BTV (Luedke et al., 1965), and the argasid tick, *Ornithodoros coriaceus*, has been shown to be capable of biological transmission of BTV under laboratory conditions (Stott et al., 1985). What role these arthropods may play in the natural maintenance of BTV is not clear.

Research regarding persistence of BTV in the vertebrate host has produced controversial results. Cattle, which are usually sub-clinically infected with BTV, have been suggested to be chronic reservoir hosts for the virus. Transplacental transmission of BTV in cattle resulting in latent or persistent infection of the offspring has been demonstrated by some researchers (Bowne et al., 1968; Luedke et al., 1970b, Luedke et al., 1977a, 1977b, 1977c, 1977d; Luedke et al., 1982; Luedke and Walton, 1981). However, more recent studies have been unable to reproduce any of these findings and dispute the natural occurrence of transplacental transmission of BTV as well as latent and persistent infections (Bowen et al., 1985; Parsonson et al., 1987; Gard et al., 1989; Acree et al., 1991; Roeder et al., 1991).

In sheep, vaccination of pregnant ewes between 28-56 days gestation has been shown to result in abortion and congenital abnormalities of the fetus (Schultz and DeLay, 1955) and BTV has been isolated from lambs during the first week of life that were born to ewes inoculated with BTV at 60-90 days gestation (Gibbs et al., 1979). However in a later study, in which ewes were inoculated at 35-42 days gestation, there was no evidence of transplacental transfer of BTV (Flanagan et al., 1982). Another similar study demonstrated that transplacental infection of the fetus occurred only if pregnant ewes were inoculated with the vaccine strains of BTV (Johnson et al., 1992). In ewes inoculated with field strains of BTV there was no evidence of transplacental infection of the fetus.

The consensus of expert opinion is that there is no convincing experimental evidence that field strains of BT viruses, which have not been manipulated in the laboratory, cross the placenta and infect the fetus, resulting in a persistently infected, seronegative adult animal (Parsonson, 1992).

some mosquitoes (Bailey et al., 1978). It has been shown that *Culicoides* can survive for 53 days at 4°C and for 51 days outdoors in cold temperatures (Nevill, 1971). However, *C. variipennis* is only known to overwinter as fourth instar larvae in Colorado (Barnard, 1980).

Several studies were unable to demonstrate transovarial transmission (TOT) of BTV in the culicoid vector (Jones and Foster, 1971; Chandler et al., 1985; Ballinger et al., 1987; Nunamaker et al., 1990). However, in all of these studies flies from laboratory colonies were used that may have lost the capability for TOT through years of colonization or may have been derived from a strain genetically incapable of TOT.

Vertical transmission in culicoids need not be transovarial. In mosquitoes infected with St. Louis encephalitis virus and dengue viruses, vertical transmission occurs during oviposition. Presumably, eggs are infected by insemination during oviposition, which could also occur in culicoids (Francy, et al., 1981; Rosen, 1987; Pelz and Freier, 1990).

K. Purpose of Study

The following studies were conducted to investigate the hypothesis that BTV is maintained transeasonally in Colorado by vertical transmission in the culicoid vector. This was accomplished by first assaying field collected culicoid larvae for the presence of BTV antigen, and then isolating BTV from those samples testing positive for BTV antigen. In addition, potential vectors of BTV, other than *C. variipennis*, were identified.

II. DETECTION OF BLUETONGUE VIRUS ANTIGEN IN POOLS OF FIELD-COLLECTED CULICOIDS

A. Introduction

The primary objective of this portion of the study was to determine if vertical transmission in the culicoid vector is the overwintering mechanism for BTV. A secondary objective was to identify putative overwintering vectors for the virus. Culicoid larvae were collected from two field sites, where BT disease is known to be enzootic, and assayed for BTV. Culicoids overwinter as fourth instar larvae and detection of BTV in these culicoids would support the hypothesis that BTV is vertically transmitted in the culicoid vector.

Since BTV is very difficult and expensive to isolate, it was more feasible in terms of time, money and labor to first assay collected culicoid samples for the presence of BTV antigen. Virus isolation could then be attempted from samples testing positive for the presence of BTV antigen.

A system was needed that would allow for the rapid assay of numerous samples at minimum cost. Enzyme linked immunosorbent assay (ELISA) is a method that is widely used for the detection of viral antigen. Antigen capture ELISAs, in particular, have been developed for the detection and identification of some arboviruses, such as La Crosse virus, eastern equine encephalomyelitis virus and Highlands J virus, from insect vectors (Hildreth et al., 1982; Hildreth and Beaty, 1984).

The antigen capture ELISA utilizes a virus specific antibody to "capture" viral antigen from a sample. The wells of a 96-well microtiter plate are coated with the capture antibody. Sample is added to these coated wells and any viral antigen present in the sample is bound to the capture antibody. Addition of a secondary antibody, produced in an animal species

other than that from which the capture antibody was derived, "detects" the bound viral antigen. An enzyme-conjugated, anti-species, indicator antibody is then added which binds to the secondary antibody. Finally, a substrate solution is added which reacts with the enzyme bound to the indicator antibody to produce a colored product. BTV antigen present in positive sample wells can be quantitated with a spectrophotometer.

An antigen capture ELISA was used in this study to assay all samples for the presence of BTV antigen. The ELISA used was a slightly modified version of an ELISA previously developed in this laboratory for the detection of BTV antigen in *C. variipennis* (El Hussein, 1989). These modifications included the use of an alternate substrate solution and capture antibody, which enhanced both sensitivity and specificity.

B. Materials and Methods

1. Positive and Negative Controls

a. Stock Virus

Positive controls consisted of BTV infected viral cell culture lysates. Wild type BTV serotypes 2 (Ona B strain), 10 (strain 8), 11 (station strain, 1962), 13 (strain 67-41B) and 17 (strain 62-455) were obtained from the Arthropod-Borne Animal Disease Research Laboratory (USDA-ABADRL), Laramie, WY,. These viruses had previously been adapted to grow in embryonated chicken eggs (ECE), passed in vertebrate cell culture lines, plaque purified and then propagated in baby hamster kidney (BHK-21) cells (El Hussein, 1989).

BHK-21 monolayers in $75cm^2$ polystyrene tissue culture flasks (Corning Glass Works, Corning, NY) were inoculated with 100 microliters of these stock viruses and incubated at 34^0C. When 50% of the infected cells demonstrated cytopathic effects (CPE), a rubber policeman was used to scrape the cells from the flask into Leibovitz growth medium (L-15 growth medium [Sigma Chemical Co., St. Louis, MO] with 10% FBS and antibiotics). The cell suspension was sonicated for 15 seconds (Fisher Sonic Dismembrater, Fisher Scientific, Springfield, NJ), aliquoted and stored at -70^0C.

The titers of the stock viruses were determined by quantal infectivity assay. Serial, 10-fold dilutions of the stock virus were made in L-15 maintenance medium (L-15 with 2% FBS and antibiotics). BHK-21 monolayers in a 96-well microculture plate (Corning Glass Works, Corning, NY) were inoculated with fifty microliters of virus dilution. Seven replicate wells were inoculated with each of the virus dilutions. One hundred microliters of L-15 maintenance medium were then added to each well. The plates were incubated at 34°C for 5-7 days and observed for CPE. Titers were calculated using the Spearman-Karber Method (Dougherty, 1964) and expressed as dilutions required to infect 50% of cell culture wells ($TCID_{50}$).

All stock virus serotypes were used to determine the sensitivity of the ELISA. BTV-10 was the only serotype used as a positive control in the assay of samples for BTV antigen.

b. *Culicoides variipennis*

Adult and larval forms of *C. variipennis* used as controls were obtained from the AK colony (Bruneau strain) maintained at the USDA-ABADRL, Laramie, WY. This colony originated from field material collected in 1973. Adult *C. variipennis* infected with different BTV serotypes were provided by Dr. F.R. Holbrook (USDA, ARS, Laramie, WY) and used as positive controls. These flies were intrathoracically inoculated with BTV, held alive for 10 days at 26°C and 40% relative humidity, sacrificed and stored at -70°C.

Uninfected *C. variipennis* adults and larvae used as negative controls were provided by Mr. Lee Thompson (USDA, ARS, Laramie, WY).

2. Antibodies

a. Bovine Antibodies

Bluetongue Reagent Serum, purchased from Veterinary Diagnostic Technologies, Inc. (Wheat Ridge, CO) was used as the bovine capture antibody. The antibody had been produced in cattle inoculated with BTV infected cell culture lysates, although the exact method of preparation was not disclosed (Dr. Michael Jochim, personal communication).

b. Guinea Pig Antibodies

Guinea pig antibodies to BTV had previously been prepared by hyperimmunizing guinea pigs with purified BTV-10 proteins and purifying the serum IgG by DEAE-Affi Gel Blue (Bio-Rad, Richmond, CA) chromatography. IgG antibodies were quantitated using the Bio-Rad protein assay, aliquoted and stored at -20°C (El Hussein, 1989).

c. Mouse Antibodies

The mouse antibodies used were provided by Dr. Robert Lanciotti (Division of Vector-borne Diseases, Centers for Disease Control, Fort Collins, CO). These antibodies were prepared by hyperimmunizing adult mice with brain suspensions from suckling mice infected with BTV-10 as described by Brandt et al. (1967). The ascitic fluids were collected by paracentesis 38 days after the first injection and stored at -20°C.

d. Secondary Antibody

The secondary antibody, purchased from Jackson Immuno Research Laboratory (West Grove, PA), consisted of goat anti-mouse antibodies (heavy & light chains) conjugated with horseradish peroxidase (HRP).

3. Sample Collection and Processing

a. Sample Sites

Culicoid larvae were collected at two permanent breeding sites for *C. variipennis* near Brighton, CO. These two sites were: the Ausman farm, a working dairy farm of approximately 200 head of dairy cattle and the Red Barn farm, a farm with approximately 200 head of beef cattle. Both sites have areas of pasture with poor drainage contaminated with various waste and sewage effluent. Scientists from the USDA-ABADRL (Laramie, WY) had previously demonstrated BTV endemicity at both of these sites by either virus isolation or seroconversion.

b. Collection of Samples

Culicoid larvae were collected from poorly drained sites contaminated with organic matter. Two types of mud samples were collected at each site, one at the water line of the waste effluent and the other immediately above the water line or from hoofprint depressions of cattle. Larvae samples were collected at each site once a month from April 1993 through October 1993. A light trap was set at the Ausman farm twice a month from June through October, to collect adult culicoids.

c. Processing of Samples

Light-Trap Flies

Adult culicoid flies obtained from the light-trap were sorted by species into pools of not more than 50. The species was determined by the pattern of wing-pigmentation (Fig 2.1). Flies were triturated in sterile mortars and pestles in 1.0 ml of grinding medium (2% FBS, 3,000 U penicillin, 3000 mg streptomycin and 67 ng fungizone per 1 ml PBS, pH adjusted to 7.2-7.4 with 7.5% $NaHCO_3$) and stored at 4°C. The samples were sonicated for 30 seconds just prior to testing by ELISA.

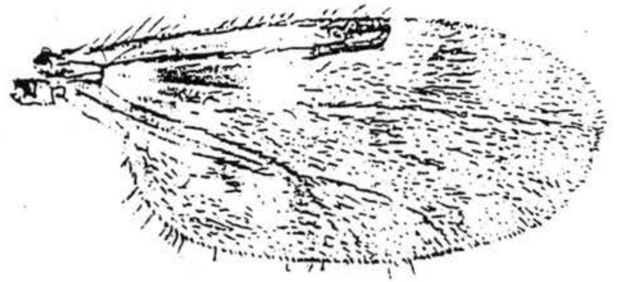
Larvae

In the laboratory, mud samples were placed into large plastic pans. Distilled water was added to the pans and the pans were placed under heat lamps (~30°C) to encourage the culicoid larvae to emerge from the mud. Larvae emerged within 3-5 days and were collected from the surface of the water using Pasteur pipets, counted, washed in distilled water, and sorted into pools of 50.

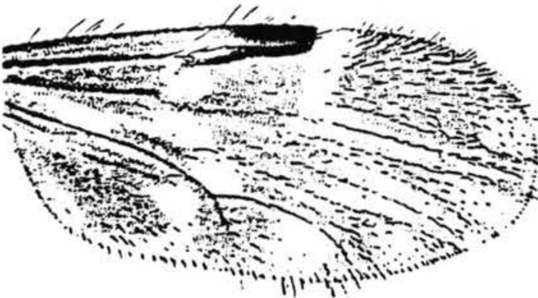
Emergent Flies

Culicoid pupae were removed from the pans and maintained at room temperature (~22°C) until adult culicoid flies emerged. Emergent flies were held for a minimum of 14 days before being processed. The flies were identified as above and sorted into pools of not more than 50 according to species.

C. variipennis

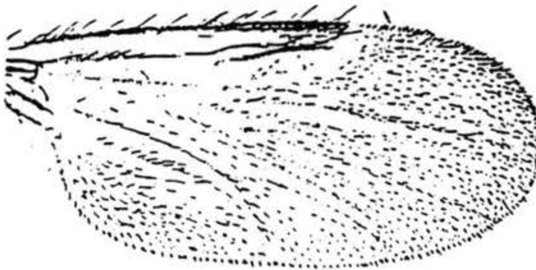


C. crepuscularis



C. haematopotus

C. stellifer



Selfia group

C. cockerellii

Figure 2.1

Wing Pigmentation Patterns Used
To Identify Culicoid Flies
(Taken from *Arthropods of Florida* by F. Blanton and W. Wirth)

Larvae and emergent fly pools were triturated as above in 1.5 ml grinding medium. One third of the ground sample (0.5 ml) was frozen at -70°C for future use. The remaining 1 ml of sample was stored overnight at 4°C and sonicated for 30 seconds before testing by ELISA the following day.

4. Enzyme Linked Immunosorbent Assay (ELISA)

Except for two modifications, the ELISA was performed as previously described (El Hussein, 1989). These modifications included the use of a bovine capture antibody and a tetramethylbenzidine (TMB) substrate (Sigma Chemical Company, St. Louis, MO). Checkerboard titrations were performed to determine the optimal amount of bovine antibody to use; the optimal concentration was that giving maximum absorbance and minimum background noise.

The bovine capture antibody was diluted 1:700 in carbonate-bicarbonate buffer, pH 9.6. One hundred microliters of the diluted antibody were added to each well of a 96 well plate (Immulon-2, Dynatech Lab, Inc., Alexandria, VA) and the plate was incubated at room temperature for 18-24 hours. The plate was then washed 5 times with PBS, pH 7.2, containing 0.05% Tween-20. Two hundred ul of blocking buffer (4.0 gm dry skim milk, 20 ml 10X Dahlberg's blotto block [Archambault, 1988], and 176 ml dd H_20) were added to each well, and the plate was incubated at 36°C for 1 hour. After incubation, the plate was washed as above and 50 ul of sample were added to each of three replicate wells. The plate was again incubated for 1 hour at 36°C and then washed as above. The mouse anti-BT-10 virus ascitic fluid was diluted 1:5000 in blocking buffer and 50 ul of mouse antibody were added to each well. After 1 hour incubation and 5 washes, 50 ul of goat anti-mouse antibody conjugated to HRP diluted 1:10,000 in blocking buffer were added to each well. The plate was incubated for 1 hour at 36°C. After 5 final washes, 100 ul of TMB substrate (3,3',5,5' tetramethylbenzidine diluted in citrate-acetate buffer containing 15 ul 30% H_2O_2/100ml buffer) were added to each well, and the plate was held in the dark at room temperature for 10 minutes. Wells containing BTV antigen turned blue. Upon addition of 50 ul 2N H_2SO_4, wells positive for BTV antigen turned yellow. Absorbance was measured at

450nm with a Bio-Rad Model 450 Microplate Reader. The three values obtained for each sample were averaged. The mean ELISA absorbance value of the sample was considered positive if it exceeded the mean absorbance value plus three standard deviations of the respective negative control (uninfected BHK-21 cells, *Culicoides* flies or larvae).

C. Results

1. Absolute Sensitivity of ELISA

The modified ELISA (bovine capture antibody) detected all 5 of the US BTV serotypes (Table 2.1). The ELISA detected 2.5 log_{10} of the 50% tissue culture dose ($TCID_{50}$) per ml of BTV-2, 2.9 logs of BTV-10, 3.9 logs of BTV-11, 2.9 logs BTV-13 and 2.4 logs of BTV-17. The diagnostic criterion for uninfected BHK cells had an absorbance value of 0.076; the limit of sensitivity for BTV infected cell culture was 2-4 logs of virus, depending on serotype. The ELISA also detected 1 infected culicoid fly in a pool of 49 uninfected flies. The absorbance value for this pool was 0.095 compared to an absorbance value of 0.085 for the uninfected, negative fly pool. The titer of this pool was 2.3 $log_{10}TCID_{50}/ml$, indicating that the test was equally sensitive in detecting viral antigen in fly pools.

The results for the ELISA using a bovine capture antibody compared to those obtained using a guinea pig capture antibody are given in Table 2.2. The limit of sensitivity for detecting antigen in infected cell cultures was approximately 3-4 logs of virus using the guinea pig capture antibody. The ELISA detected 3.5 log_{10} of the 50% tissue culture dose ($TCID_{50}$) per ml of BTV-2, 2.9 logs of BTV-10, 3.9 logs of BTV-11, 3.9 logs of BTV-13 and 3.4 logs of BTV-17. These results are similar to those obtained in previous experiments, in which the limit of sensitivity was approximately 3 logs of virus using a guinea-pig capture antibody (El Hussein, 1989).

2. Absolute Specificity of ELISA

There was no cross-reactivity with two strains of EHD using either the bovine or guinea pig capture antibody. The absorbance values for EHD-1

Table 2.1

Detection of Bluetongue Viral Antigen by ELISA
Using a Bovine Capture Antibody

	ELISA Mean Absorbance Value						
	BT-2 (5.5)[a]	BT-10 (5.9)	BT-11 (5.9)	BT-13 (5.9)	BT-17 (5.4)	EHD-1 (6.0)	EHD-2 (6.1)
Virus Dilution							
10^{-1}	0.328	0.387	0.349	0.568	0.530	0.051	0.052
10^{-2}	0.169	0.226	0.154	0.213	0.236	-----	-----
10^{-3}	0.079	0.083	0.074	0.087	0.099	-----	-----
10^{-4}	0.066	0.069	0.062	0.068	0.066	-----	-----
1/50 flies (2.3)[b]	0.095						
Uninfected BHK Cells	0.076[c]						
Uninfected Larvae	0.067[c]						
Uninfected Flies	0.085[c]						

[a] Numbers in parentheses indicate stock virus titers ($log_{10}TCID_{50}$/ml).
[b] Virus titer ($log^{10}TCID^{50}$/ml) of pool containing 1 BTV-17 infected fly and 49 uninfected flies
[c] Diagnostic criterion (mean absorbance value plus 3 standard deviations)

Table 2.2

Detection of Bluetongue Viral Antigen by ELISA
Using a Guinea Pig Capture Antibody

	ELISA Mean Absorbance Value						
	BT-2 (5.5)[a]	BT-10 (5.9)	BT-11 (5.9)	BT-13 (5.9)	BT-17 (5.4)	EHD-1 (6.0)	EHD-2 (6.1)
Virus Dilution							
10^{-1}	0.246	0.288	0.291	0.629	0.636	0.067	0.061
10^{-2}	0.122	0.143	0.107	0.159	0.180	-----	-----
10^{-3}	0.078	0.086	0.062	0.076	0.069	-----	-----
10^{-4}	0.079	0.082	0.069	0.070	0.063	-----	-----
1/50 flies (2.3)[b]	0.086						
Uninfected BHK Cells	0.085[c]						
Uninfected Larvae	0.078[c]						
Uninfected Flies	0.128[c]						

[a] Numbers in parentheses indicate stock virus titers ($\log_{10}TCID_{50}$/ml
[b] Virus titer ($\log^{10}TCID^{50}$/ml) of pool containing 1 BTV infected fly and 49 uninfected flies
[c] Diagnostic criterion (mean absorbance value + 3 standard deviations)

(New Jersey) and EHD-2 (Alberta) using the bovine capture antibody were 0.051 and 0.052, respectively. The diagnostic criterion for uninfected BHK cells had an absorbance value of 0.076 (Table 2.1). When guinea pig capture antibody was used, the absorbance values were 0.067 and 0.061 for EHD-1 and EHD-2, respectively; the absorbance value for the diagnostic criterion was 0.085 (Table 2.2). The non-specific background noise was reduced using the bovine capture antibody as compared to the guinea-pig antibody, particularly with respect to absorbance values obtained for uninfected control flies. The absorbance value used for the diagnostic criterion for uninfected flies was 0.085 using bovine antibody compared to 0.128 using guinea-pig antibody (Tables 2.1 and 2.2).

3. <u>ELISA Results</u>

<u>Light-Trap Fly Pools</u>

A total of 901 culicoid flies were collected in the light trap and sorted by species into 70 pools. Fourteen of these pools tested positive for BTV antigen by ELISA. The minimum field infection rate (MFIR) was determined to be the percentage of the total population collected divided by the number of putatively positive flies. This percentage was calculated by dividing the total number of flies tested by the number of pools testing positive for BTV antigen, assuming that each positive pool contained at least one BTV infected fly. The MFIR for all light-trap collected flies was 1.56%

Of the 14 positive pools, 10 pools were *C. variipennis,* 1 pool was *C. crepuscularis*, 1 pool was of the *selfia* group, 1 pool was *C. stellifer*, and the species in the remaining pool was unknown (Table 2.3).

<u>Larvae Pools</u>

A total of 1001 larval pools, consisting of 49,521 larvae, were processed and tested by ELISA. The resulting MFIR was determined to be 0.20%, as 99 of these pools tested positive for BTV antigen (Table 2.4).

Table 2.3

Detection of BTV Antigen in Light-Trap Culicoid Fly Pools

Pool No.	Pool Size	Date	Site	Culicoid Species	EIA Absorbance Pool[1]	EIA Absorbance Control[2]
13	25	6/15/93	Ausman	*variipennis*	0.137	0.126
14	25	6/15/93	Ausman	*variipennis*	0.170	0.126
15	25	6/15/93	Ausman	*variipennis*	0.178	0.126
18	25	6/21/93	Ausman	*variipennis*	0.105	0.089
34	25	8/10/93	Ausman	*variipennis*	0.092	0.089
39	1	8/24/93	Ausman	*selfia group*	0.131	0.087
40	8	8/24/93	Ausman	*stellifer*	0.170	0.087
41	6	8/24/93	Ausman	*crepuscularis*	0.088	0.087
42	16	8/24/93	Ausman	unknown	0.160	0.087
43	25	8/24/93	Ausman	*variipennis*	0.110	0.087
44	25	8/24/93	Ausman	*variipennis*	0.123	0.087
45	25	8/24/93	Ausman	*variipennis*	0.137	0.087
46	15	8/24/93	Ausman	*variipennis*	0.109	0.087
47	25	8/24/93	Ausman	*variipennis*	0.082	0.077

[1] ELISA Mean Absorbance Value
[2] Mean of Negative Control Plus 3 Standard Deviations

Table 2.4: Detection of BTV Antigen in Culicoid Larval Pools

Pool No.	Pool Size	Date	Site	EIA Absorbance Pool[1]	EIA Absorbance Control[2]	Pool No.	Pool Size	Date	Site	EIA Absorbance Pool[1]	EIA Absorbance Control[2]
3001	50	4/20/93	Ausman	0.059	0.057	3225	50	6/07/93	Ausman	0.057	0.052
3014	51	4/20/93	Red Barn	0.059	0.057	3227	50	6/07/93	Ausman	0.055	0.052
3050	50	5/17/93	Ausman	0.010	0.008	3228	50	6/07/93	Ausman	0.060	0.052
3051	50	5/17/93	Ausman	0.009	0.008	3229	50	6/07/93	Ausman	0.054	0.052
3054	50	5/17/93	Ausman	0.009	0.008	3232	50	6/07/93	Ausman	0.053	0.052
3055	50	5/17/93	Ausman	0.009	0.008	3234	50	6/07/93	Ausman	0.053	0.052
3056	50	5/17/93	Ausman	0.009	0.008	3235	50	6/07/93	Ausman	0.053	0.052
3057	50	5/17/93	Red Barn	0.010	0.008	3236	50	6/07/93	Ausman	0.055	0.052
3058	50	5/17/93	Ausman	0.010	0.008	3237	50	6/07/93	Ausman	0.054	0.052
3059	50	5/17/93	Ausman	0.009	0.008	3238	50	6/07/93	Ausman	0.056	0.052
3060	50	5/17/93	Ausman	0.009	0.008	3239	50	6/07/93	Ausman	0.054	0.052
3065	50	5/17/93	Red Barn	0.010	0.008	3267	50	6/07/93	Ausman	0.065	0.061
3066	50	5/17/93	Red Barn	0.009	0.008	3268	50	6/07/93	Ausman	0.074	0.061
3067	50	5/17/93	Red Barn	0.009	0.008	3269	50	6/07/93	Ausman	0.065	0.061
3111	50	5/31/93	Red Barn	0.070	0.066	3270	50	6/07/93	Ausman	0.063	0.061
3112	50	5/31/93	Red Barn	0.074	0.066	3271	50	6/07/93	Ausman	0.065	0.061
3113	50	5/31/93	Red Barn	0.070	0.066	3272	50	6/07/93	Ausman	0.067	0.061
3114	50	5/31/93	Red Barn	0.071	0.066	3273	50	6/07/93	Ausman	0.070	0.061
3115	50	5/31/93	Red Barn	0.075	0.066	3274	50	6/07/93	Ausman	0.070	0.061
3117	50	5/31/93	Red Barn	0.073	0.066	3275	50	6/07/93	Ausman	0.062	0.061
3119	50	5/31/93	Red Barn	0.067	0.066	3282	50	6/07/93	Ausman	0.063	0.061
3120	50	5/31/93	Red Barn	0.067	0.066	3283	50	6/07/93	Ausman	0.063	0.061
3125	50	5/31/93	Red Barn	0.070	0.066	3290	50	6/07/93	Ausman	0.068	0.061
3133	50	5/31/93	Red Barn	0.069	0.066	3292	50	6/07/93	Ausman	0.063	0.061
3152	50	5/31/93	Red Barn	0.068	0.065	3320	50	6/28/93	Red Barn	0.070	0.063
3154	50	5/31/93	Red Barn	0.068	0.065	3321	50	6/28/93	Red Barn	0.076	0.063
3157	50	5/31/93	Red Barn	0.066	0.065	3322	50	6/28/93	Red Barn	0.064	0.063
3192	50	6/07/93	Ausman	0.074	0.071	3323	50	6/28/93	Red Barn	0.064	0.063
3210	50	6/07/93	Ausman	0.078	0.071	3327	50	6/28/93	Red Barn	0.064	0.063
3213	50	6/07/93	Ausman	0.076	0.052	3329	43	6/28/93	Red Barn	0.066	0.063
3216	50	6/07/93	Ausman	0.054	0.052	3333	50	6/28/93	Red Barn	0.064	0.063
3217	50	6/07/93	Ausman	0.058	0.052	3334	50	6/28/93	Red Barn	0.065	0.063
3218	50	6/07/93	Ausman	0.057	0.052	3335	45	6/28/93	Red Barn	0.065	0.063
3219	50	6/07/93	Ausman	0.055	0.052	3337	50	7/12/93	Ausman	0.052	0.051
3220	50	6/07/93	Ausman	0.059	0.052	3370	50	7/12/93	Ausman	0.053	0.052
3221	50	6/07/93	Ausman	0.057	0.052	3390	50	7/12/93	Ausman	0.058	0.056
3224	50	6/07/93	Ausman	0.053	0.052	3391	50	7/12/93	Ausman	0.058	0.056

Table 2.4 (continued)

Detection of BTV Antigen in Culicoid Larval Pools

Pool No.	Pool Size	Date	Site	EIA Absorbance Pool[1]	EIA Absorbance Control[2]
3398	50	7/12/93	Ausman	0.070	0.056
3399	50	7/12/93	Ausman	0.065	0.056
3417	50	7/12/93	Ausman	0.051	0.050
3418	50	7/12/93	Ausman	0.051	0.050
3420	50	7/12/93	Ausman	0.052	0.050
3421	50	7/12/93	Ausman	0.053	0.050
3422	50	7/12/93	Ausman	0.052	0.050
3423	50	7/12/93	Ausman	0.052	0.050
3424	50	7/12/93	Ausman	0.050	0.050
3425	50	7/12/93	Ausman	0.051	0.050
3429	50	7/12/93	Ausman	0.051	0.050
3430	50	7/12/93	Ausman	0.051	0.050
3431	50	7/12/93	Ausman	0.051	0.050
3433	50	7/12/93	Ausman	0.052	0.050
3437	50	7/12/93	Ausman	0.051	0.050
3439	50	7/12/93	Ausman	0.051	0.050
3440	50	7/12/93	Ausman	0.051	0.050
3442	50	7/12/93	Ausman	0.052	0.050
3663	50	8/23/93	Ausman	0.097	0.095
3677	50	8/23/93	Ausman	0.058	0.057
3763	50	8/30/93	Ausman	0.113	0.110
3764	50	8/30/93	Ausman	0.132	0.110
3819	50	8/30/93	Red Barn	0.073	0.068
3971	50	9/30/93	Ausman	0.235	0.171

[1] ELISA Absorbance Value
[2] Mean of the Negative Control Plus 3 Standard Deviations

Emergent Fly Pools

A total of 11,221 emergent flies were placed in 334 pools according to species. Sixty-six of these pools were positive for BTV antigen by ELISA, yielding a MFIR of 0.59% (Table 2.5).

Emergent fly pools positive by ELISA were composed of only three species of culicoids: *C. variipennis, C. crepuscularis,* and *C. selfia.*

The difference between the absorbance values obtained for emergent fly pools testing positive for BTV antigen and the value for the diagnostic criterion was considerably greater than the difference obtained for larval pools ($P<0.001$) (Tables 2.4, 2.5 and 2.6). Also, the MFIR for emergent flies was 0.59%, nearly three times the MFIR of 0.20% obtained for larval pools. (Table 2.6).

The prevalence of BTV in collected emergent fly pools with respect to the month collected is presented in Table 2.7 and Figure 2.2. The MFIR for the month of April was 0.063%. None of the pools collected in May tested positive for BTV antigen. In June and July the MFIR's were 0.63% and 0.56%, respectively. Again in August, none of the pools were positive for BTV antigen. In September and October the MFIR increased to 0.97% and 1.16%, respectively.

A comparison of the MFIR was made with respect to populations of culicoid species (Table 2.8 and Figure 2.3). *C. variipennis* was the most common species collected, accounting for 53.1% of the total collected population. *C. crepuscularis* was the second most common species collected, accounting for 30.5% of the total population. However, the MFIR for *C. crepuscularis* was 1.02%, more than three times the MFIR of 0.32% obtained for *C. variipennis*. Culicoids belonging to the *selfia* group accounted for only 4.1% of the collected fly population, but had a MFIR of 0.66%, which was twice as great as the MFIR obtained for *C. variipennis*. The remaining pools that were putatively positive were comprised of culicoids of unknown species, as they could not be reliably identified due to damaged wings.

Table 2.5

Detection of BTV Antigen in Emergent Culicoid Fly Pools

Pool No.	Pool Size	Date	Site	Culicoid Species	EIA Absorbance Pool[1]	EIA Absorbance Control[2]
1	51	4/20/93	Ausman	*variipennis*	0.058	0.057
9	11	4/20/93	Red Barn	*crepuscularis*	0.061	0.057
13	50	4/20/93	Ausman	*crepuscularis*	0.086	0.038
15	37	4/20/93	Ausman	*selfia group*	0.063	0.038
16	50	4/20/93	Ausman	*variipennis*	0.039	0.038
17	30	4/20/93	Ausman	*crepuscularis*	0.079	0.038
18	27	4/20/93	Ausman	*variipennis*	0.046	0.038
19	17	4/20/93	Red Barn	*variipennis*	0.041	0.038
20	41	4/20/93	Red Barn	*crepuscularis*	0.071	0.038
22	25	4/20/93	Red Barn	*selfia group*	0.051	0.038
79	53	6/08/93	Red Barn	*variipennis*	0.129	0.093
80	56	6/08/93	Red Barn	*variipennis*	0.162	0.093
83	25	6/08093	Red Barn	*crepuscularis*	0.095	0.093
104	50	6/21/93	Ausman	unknown	0.052	0.049
117	50	7/06/93	Ausman	unknown	0.081	0.077
138	17	7/12/93	Ausman	unknown	0.063	0.061
140	50	7/26/93	Ausman	unknown	0.066	0.061
143	50	7/26/93	Ausman	*crepuscularis*	0.092	0.089
154	50	7/26/93	Ausman	*crepuscularis*	0.093	0.089
159	50	7/26/93	Ausman	*crepuscularis*	0.091	0.089
210	50	7/26/93	Ausman	*crepuscularis*	0.119	0.101
213	50	7/26/93	Ausman	*crepuscularis*	0.112	0.101
218	50	7/26/93	Ausman	*crepuscularis*	0.102	0.101
230	50	7/26/93	Ausman	*crepuscularis*	0.112	0.101
233	53	7/26/93	Ausman	*crepuscularis*	0.115	0.101
246	50	7/26/93	Ausman	*crepuscularis*	0.068	0.057
247	10	7/26/93	Ausman	unknown	0.060	0.057
249	29	7/26/93	Ausman	*variipennis*	0.059	0.057
253	10	7/26/93	Ausman	*crepuscularis*	0.061	0.057
259	50	7/26/93	Ausman	*variipennis*	0.103	0.087
260	50	7/26/93	Ausman	*crepuscularis*	0.107	0.087
261	2	7/26/93	Ausman	*selfia group*	0.112	0.087
263	50	7/26/93	Ausman	unknown	0.129	0.087
265	50	7/26/93	Ausman	*crepuscularis*	0.117	0.087
267	50	7/26/93	Ausman	*variipennis*	0.090	0.087
268	50	7/26/93	Ausman	*variipennis*	0.098	0.087
269	50	7/26/93	Ausman	*crepuscularis*	0.182	0.087
270	50	7/26/93	Ausman	*crepuscularis*	0.088	0.087
271	26	7/26/93	Ausman	*variipennis*	0.111	0.087
272	50	7/26/93	Ausman	*crepuscularis*	0.165	0.087
273	50	7/26/93	Red Barn	*crepuscularis*	0.144	0.077
275	50	7/26/93	Red Barn	*variipennis*	0.081	0.077
276	23	7/26/93	Red Barn	unknown	0.121	0.077
277	50	7/26/93	Red Barn	*crepuscularis*	0.100	0.077
278	50	7/26/93	Red Barn	*variipennis*	0.084	0.077
279	50	7/26/93	Red Barn	*variipennis*	0.101	0.077
280	50	7/26/93	Red Barn	*crepuscularis*	0.139	0.077
281	10	7/26/93	Red Barn	*variipennis*	0.102	0.077
282	19	7/26/93	Red Barn	*crepuscularis*	0.101	0.077
292	50	8/30/93	Ausman	*crepuscularis*	0.097	0.067
293	50	8/30/93	Ausman	*crepuscularis*	0.069	0.067
294	50	8/30/93	Ausman	unknown	0.073	0.067
296	39	8/30/93	Ausman	*crepuscularis*	0.096	0.067
297	10	8/30/93	Ausman	*variipennis*	0.069	0.067

Table 2.5 (continued)

Detection of BTV Antigen in Emergent Culicoid Fly Pools						
Pool No.	Pool Size	Date	Site	Culicoid Species	EIA Absorbance Pool[1]	Control[2]
301	50	8/30/93	Ausman	*crepuscularis*	0.108	0.091
304	24	8/30/93	Ausman	*crepuscularis*	0.125	0.091
306	26	8/30/93	Ausman	*variipennis*	0.375	0.171
307	15	8/30/93	Ausman	*crepuscularis*	0.353	0.171
310	29	10/1/93	Ausman	*crepuscularis*	0.170	0.125
315	22	10/1/93	Ausman	*crepuscularis*	0.147	0.125
319	50	10/1/93	Ausman	*crepuscularis*	0.247	0.226
324	50	10/1/93	Ausman	*crepuscularis*	0.378	0.226
326	40	10/1/93	Ausman	unknown	0.461	0.226
329	50	10/1/93	Ausman	*variipennis*	0.258	0.188
330	32	10/1/93	Ausman	*crepuscularis*	0.417	0.188
333	50	10/1/93	Ausman	*variipennis*	0.303	0.188

[1] ELISA Absorbance Value

[2] Mean of Negative Control Plus 3 Standard Deviations

Table 2.6

Comparison of Larval and Emergent Fly Pools Positive for BTV Antigen by ELISA			
	Light-Trap Flies	Culicoid Larvae	Emergent Flies
Total Number Collected	901	49,521	11,221
Number of Positive Pools	14	99	66
Minimum Field Infection Rate[1]	1.56%	0.20%	0.59%

[1] Calculated as the total number of pools testing positive for BTV antigen divided by the total number of culicoids collected.

* The absorbance values for flies and larvae were compared using a 2 sample T-test with a non-pooled standard deviation. The difference between the two sample sets was considered to be significant, as the probability that such a difference occurred due to coincidence (P) was less than 0.001. The Mann-Whitney test, a non-parametric test that doesn't assume normality in a population, was also used and the P value obtained was again < 0.001 (Devore and Peck, 1986).

Table 2.7

Emergent Culicoid Fly Pools Collected by Month

Month Collected	Total Flies Collected	Total Positive Pools	Minimum Field Infection Rate[1]
April	1586	10	0.63%
May	865	0	0.00%
June	640	4	0.63%
July	6310	35	0.56%
August	208	0	0.00%
September	924	9	0.97%
October	688	8	1.16%
Total	11,221	66	0.59%

[1] Calculated as the total number of pools testing positive for BTV antigen divided by the total number of culicoids collected.

* The minimum field infection rates for each month were compared using a chi-squared test. (Devore and Peck, 1986) The differences between the MFIR for each month were determined to be statistically significant. (X^2=12.84, P=0.045)

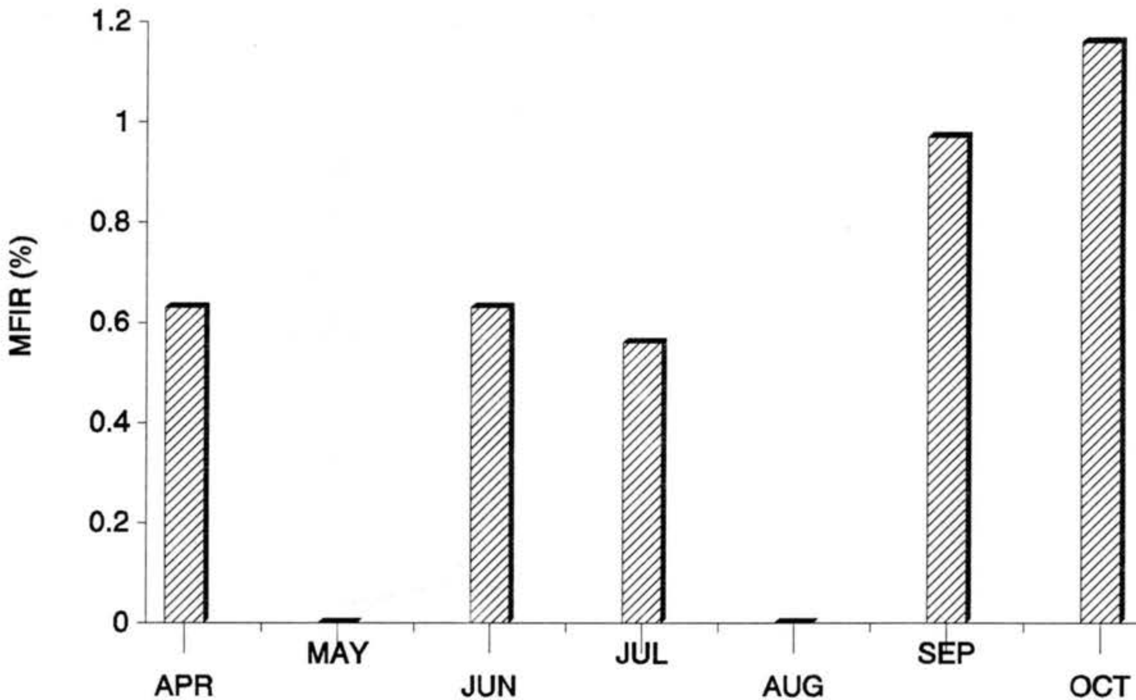


Figure 2.2

Prevalence of BTV in Culicoid Fly Pools by Month

Table 2.8

Populations of Collected Emergent Culicoid Fly Species

Culicoid Species Collected	Number of Flies Collected	Number of Positive Pools Collected	Percent Flies Collected	Minimum Field Infection Rate[1]
variipennis	5956	19	53.1	0.32%
crepuscularis	3425	35	30.5	1.02%
selfia group	454	3	4.1	0.66%
stellifer	89	0	0.8	0.00%
cockerellii	128	0	1.1	0.00%
haematopotus	36	0	0.3	0.00%
unknown	1133	9	10.1	0.79%
Totals	11,221	66	100.0	0.59%

[1] Calculated as the total number of pools testing positive for BTV antigen divided by the total number of culicoids collected.

* The minimum field infection rates for each culicoid species were compared using a chi-squared test (Devore and Peck, 1986). The differences in the MFIR for each of the species were determined to be statistically significant. (X^2=20.76, P=0.002)

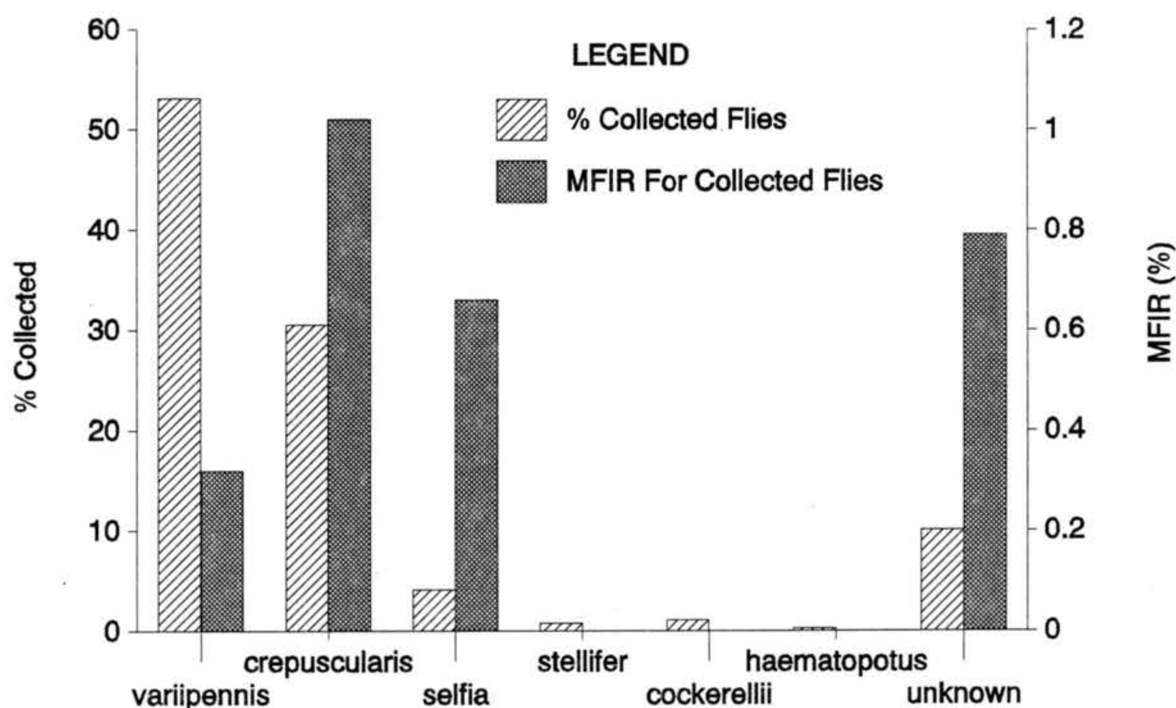


Figure 2.3

A Comparison of Collected Emergent Culicoid Fly Pools With Respect to Species

D. Discussion

The results from the assay of culicoid pools using the ELISA appear to support the theory of vertical transmission of BTV in the vector. The MFIR for light-trap culicoid fly pools was 1.56% (Table 2.6). These putatively positive flies were collected only in the months of June and August. BT disease outbreaks usually occur in late summer and early fall due to amplification of the virus in vertebrate hosts during the spring and summer months. Light-trap flies collected in August may have become infected with BTV by ingestion of a viremic blood meal from a BTV infected host; unfortunately, parity status of these flies was not determined. However, those light-trap flies collected in June testing positive for BTV antigen may have become infected with BTV by vertical transmission of the virus in the vector. These flies were collected early in the year, and if vertebrate hosts were infected with BTV in sufficient numbers to infect flies at this time, it would seem likely that BTV infected culicoids would also have been collected in the light-trap in July. As this was not observed, the putatively positive light-trap flies collected in June may represent flies that emerged in the spring from BTV infected larvae.

The MFIR for collected culicoid larval pools was 0.20%, while the MFIR for emergent culicoid fly pools was 0.59%, nearly three times that obtained for larvae. Also, the difference between the pool absorbance values and the control absorbance values was considerably less for larval pools than for emergent fly samples. Possibly BTV is present in larvae in such a small amount, or in some form which is undetectable by ELISA, becoming patent only after metamorphosis into the adult fly.

It was demonstrated that St. Louis encephalitis virus (SLE) could not be isolated from SLE infected, overwintering *Culex pipiens* until the mosquitoes had been held in the insectary for a minimum of fifteen days (Bailey, 1978). Therefore, emergent culicoid flies were held for at least fourteen days before being processed for assay and this may account for the differences observed in the detection of BTV antigen in emergent flies as compared to larvae.

The number of ELISA positive emergent fly pools per month also appears to substantiate the theory of vertical transmission of BTV (Table 2.7 and Figure 2.2). The MFIR for the month of April was 0.63%. In May the MFIR decreased to 0%, as none of the pools tested positive for BTV antigen. The MFIRs increased again to 0.63% and 0.56% in June and July, respectively. These values were similar to the MFIR value obtained for May. Again in August there were no positive pools, but in September and October the MFIR's increased dramatically to 0.97% and 1.16%, respectively. These results suggest amplification of BTV during the summer months. This amplification may be due to horizontal amplification within the vertebrate population or vertical transmission of BTV within the vector population.

A comparison of putatively positive emergent fly pools with respect to species (Table 2.8 and Figure 2.3) suggests that *C. variipennis* may not be the only culicoid species to vector BTV. *C. variipennis* was the predominant culicoid species collected, accounting for 53.1% of the collected population, with a MFIR of 0.32%. However, *C. crepuscularis* had a MFIR of 1.02% although it comprised only 30.5% of the total population collected. BTV antigen was also detected in pools of culicoids belonging to the *selfia* group. Perhaps *C. variipennis* is the epidemic vector of BTV while other culicoid species, *C. crepuscularis* and members of the *selfia* group in particular, may act as endemic or maintenance vectors of the virus.

The use of a bovine capture antibody in the ELISA proved to be more sensitive and specific in the detection of BTV antigen in infected cell culture lysates than the use of a guinea-pig capture antibody. The limit of sensitivity using the bovine capture antibody was 2-4 logs of virus as compared to 3-4 logs when the guinea pig-capture antibody was used. This increased level of sensitivity may be explained by the derivation of the capture antibody. When natural hosts for BTV, such as cattle, are infected with the virus, viral replication occurs, eliciting the production of specific antibody against all antigenic BTV proteins synthesized. However, BTV inoculation of an unnatural host, such as the guinea pig, may not result in infection of the host and replication of the virus so that only antibody specific for the immunodominant BTV protein(s) injected is produced.

Therefore, the bovine capture antibody is more sensitive than the guinea pig antibody because it contains specific antibody against both the proteins used to immunize the host and proteins synthesized during viral replication.

Cattle used for the production of the capture antibody are inoculated with different serotypes of BTV so the bovine capture antibody also contains antibody elicited by several serotypes. The guinea-pig antibody is specific for only one BTV serotype, BTV-10. For these reasons, the use of the bovine capture antibody increased the level of sensitivity by one log of virus compared to the guinea-pig antibody in the detection of cell culture infected with BTV serotypes 2, 13 and 17 (Tables 2.1 and 2.2). However, both capture antibodies were equally sensitive in the detection of cell culture infected with BTV-10. This is most likely due to the phenomenon of affinity maturation, in which increased affinity of antibody molecules for a specific antigen is attained with repeated immunization of a host (Abbas et al., 1991). Hyperimmunization of the guinea-pig with BTV-10 proteins would result in this increased affinity of specific antibody for BTV-10 antigen, rendering this capture antibody as sensitive in the detection of BTV-10 as the bovine capture antibody.

The modified ELISA, using the bovine capture antibody, was less sensitive in detecting BTV serotype 11 than the other BTV serotypes (Table 2.1). Whereas the limit of sensitivity for BTV serotypes 2, 10, 13 and 17 was 2-3 logs of virus, the limit of sensitivity for BTV-11 was approximately 4 logs of virus. Perhaps the cattle used to produce this antibody were not inoculated with BTV-11, and less specific antibody to this serotype was produced.

Neither of the capture antibodies cross-reacted with two strains of the related *Orbivirus* EHDV, indicating that these antibodies are most likely directed against the BTV serotype protein VP2, which been shown to be the BTV protein least similar to the EHDV proteins (Roy, 1992).

The values used for the diagnostic criterion for uninfected BHK-21 cells, uninfected culicoid larvae and uninfected culicoid flies were all lower using the bovine capture antibody than those obtained when the guinea-pig antibody was used. The higher absorbance values obtained for uninfected

BHK-21 cells using the guinea-pig capture antibody may be due to non-specific binding of the goat anti-mouse secondary antibody to the guinea-pig capture antibody, as both antibodies are derived from closely related species. The reason for the reduction in other non-specific background using the bovine capture antibody is unclear.

The results obtained from assay of the culicoid samples by ELISA provide presumptive evidence that BTV is vertically transmitted in the culicoid vector and overwinters within culicoid larvae.

III. ISOLATION OF BLUETONGUE VIRUS IN POOLS OF FIELD-COLLECTED CULICOIDS

A. Introduction

Field-strains of BTV are difficult to isolate, usually requiring several blind passages before becoming adapted to a particular laboratory system. Sheep were used decades ago for BTV isolation and are still considered today to be one of the most sensitive indicators for the presence of BTV in diagnostic samples. There are several disadvantages to using sheep for BTV isolation, such as the unavailability of susceptible sheep in areas where BT is enzootic, the need for insect-proof facilities to house sheep used for virus isolation and the expense incurred in purchasing and maintaining sheep.

In 1940, it was discovered that BTV could be propagated in embryonated chicken eggs (ECE) inoculated by the yolk-sac method (Mason, 1940). Later experiments demonstrated that the important consideration for propagation of BTV in eggs was the temperature of incubation, as it was found that a test temperature of 33.6^{0}C was optimal for adapting and attenuating BTV in eggs (Alexander, 1947). Twenty years later, a method for the intravascular (IV) inoculation of ECE with field samples suspected to contain BTV was described (Foster and Luedke, 1968a; Goldsmit and Barzilai, 1968). Chicken embryos were shown to be 100-1000 times more sensitive to BTV infection when inoculated by the IV method as opposed to the yolk-sac method. Intravascular inoculation of ECE is the method most commonly used today for isolation of BTV from diagnostic samples.

Direct isolation of BTV in cell culture is not as sensitive as isolation in either sheep or ECE (Wechsler and McHolland, 1988). Isolation

of BTV has been tried in a number of vertebrate cell lines, including baby hamster kidney (BHK-21), African green monkey kidney (Vero), rabbit kidney, bovine kidney, canine kidney, bovine nasal turbinate, bovine endothelium (CPAE), bighorn sheep tongue, equine dermis, gekko lung, rainbow trout gonad, and mouse fibroblast cell lines. BHK-21 cells were demonstrated to be more susceptible than any other cell line tested except for CPAE cells, which were only slightly more sensitive than BHK-21 cells (Wechsler and McHolland, 1988). Two invertebrate cell lines, *Aedes albopictus* (C6/36) and *C. variipennis* (CuVa) were also shown to be less sensitive to BTV infection than BHK-21 cells. Therefore, BHK-21 or CPAE cells are routinely used for isolation of BTV from diagnostic samples following passage in ECE. Upon death of the chick embryo due to BTV infection, the embryo is homogenized and passed onto cell culture. The cell culture can then be observed for viral CPE and tested for the presence of BTV antigen.

Propagation of a number of arboviruses by parenteral inoculation of a variety of arthropods has also been demonstrated (Hurlbut and Thomas, 1960; Rosen and Gubler, 1974). Intrathoracic inoculation of these arthropods with arboviruses avoids the mid-gut barrier, allowing replication of an arbovirus in an arthropod that is not a natural vector. Generally arboviruses do not have an adverse effect on the arthropod host, necessitating the use of fluorescent antibody staining or other indirect assay to demonstrate the presence of the virus.

Several reports cite instances of BTV detected in one system but not in others (Gibbs, 1983; Sawyer et al., 1986; Wechsler and McHolland, 1988). Therefore, multiple systems are recommended for isolation of field-strains of BTV (Jochim, 1985). Since the use of sheep was not practical in this study, three alternate methods were chosen for the isolation of BTV from pools of collected culicoids: 1) intravascular inoculation of ECE, 2) direct isolation in BHK-21 and CuVa cell lines and 3) a novel technique, intrathoracic inoculation of *Aedes triseriatus* mosquitoes.

Aedes albopictus mosquitoes have been used for the propagation of dengue virus (Rosen and Gubler, 1974) and *Toxorhynchites* mosquitoes have been used for the propagation of dengue, St. Louis encephalitis, and Japanese

encephalitis viruses (Rosen, 1981). Therefore, intrathoracic inoculation of *Aedes triseriatus* mosquitoes was chosen as an isolation technique in this study. It was known that the C6/36 cell line, derived from *Aedes albopictus* mosquitoes, is susceptible to BTV infection, suggesting that BTV propagation might be possible by parenteral inoculation of a temperate zone mosquito such as *A. triseriatus* (Sawyer et al., 1986; Wechsler et al., 1989).

B. Materials and Methods

1. Stock Virus

BTV serotype 17 (strain 62-455), obtained from the Arthropod-Borne Animal Disease Research Laboratory (Laramie, WY) after passage twice in sheep, once in ECE and 5 times in BHK-21 cell culture was used for all trials. The stock virus titer was 5.4 $\log_{10}$ ($TCID_{50}$/ml) in BHK-21 cells.

2. Culicoid Samples

BTV isolation was attempted from light-trap fly pools that had not been frozen, but were stored at 4°C (Chapter 2, page 22). Aliquots (0.5 ml) of the triturated emergent culicoid fly pools, previously frozen at -70°C (Chapter 2, page 22), were quick-thawed in a 37°C water bath and then held at 4°C prior to attempted isolation of BTV.

3. Eggs

Fertile chicken eggs certified to be salmonella-and other pathogen-free (SPF) were purchased from Hy-Vac Laboratory Eggs Company (Adel, Iowa).

4. Cells

Established lines of BHK-21 and CuVa cells were used. The CuVa cell line, derived from embryos of *C. variipennis*, was obtained from Dr. Melody Jensen (USDA-ABADRL, Laramie, WY). Both cell types were grown in L-15 medium containing L-glutamine, 10% tryptose phosphate broth, 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin

(100 ug/ml). BHK-21 cells were propagated at 36°C and CuVa cells at 32°C in humid incubators without added CO_2.

Maintenance medium, used in cultures inoculated with BTV or culicoid pools suspected to contain BTV, was prepared as above except that it contained 2% FBS, 300 U/ml penicillin, 300 ug/ml streptomycin, and 40 ug/ml gentamicin. The pH of the medium was adjusted to 7.2-7.4 with 7.5% $NaHCO_3$. Due to its toxicity for CuVa cells, fungizone (60 ng/ml) was added only to the maintenance medium used for BHK-21 cells.

5. Mosquitoes

Female *Aedes triseriatus* mosquitoes were obtained from the Arthropod-borne and Infectious Diseases Laboratory (Ft. Collins, Colorado).

6. Monoclonal Antibody

The monoclonal antibody used in the indirect immunofluorescent technique (IFA) was supplied by Dr. James Collins (CSU Veterinary Diagnostic Laboratory, Ft. Collins, Colorado). Hybridomas were produced by the fusion of a myeloma cell line (SP2/0-Ag14) with spleen cells from BALB/c mice inoculated with BTV-10 (Jochim and Jones, 1983). Primed adult BALB/c mice were injected intraperitoneally with cells from the cloned hybridomas. Mouse ascites fluid was collected 2-3 weeks later. Cells were removed from the fluid by centrifugation and the fluid was diluted 1:2 in glycerol, aliquoted and stored at -70°C.

7. Fluorescein Conjugated Secondary Antibody

A commercially prepared fluorescein-conjugated goat immunoglobulin G anti-mouse IgG used was purchased from Organo Teknika Cappel (Durham, North Carolina).

8. Egg Inoculations

Culicoid pools positive for BTV by ELISA were diluted 1:2 in an antibiotic solution (200 ul sample diluted in 200 ul of solution containing 7 U penicillin, 7 mg streptomycin and 21 ng fungizone and adjusted to pH

7.2-7.4 with 7.5% $NaHCO_3$) and stored overnight at 4°C. Diluted samples were again diluted 1:5 in PBS the following day, resulting in a final 1:10 dilution of the sample.

Eleven day old ECE were candled and those containing dead embryos were discarded. The location of a blood vessel lying near the surface of the egg, toward the air sac end, was marked on the shell. A square of about 0.5 cm was cut through the shell with a small electric drill fitted with a 1 mm scoring drill bit. The piece of shell was carefully removed so as not to rupture the underlying shell membrane, and the exposed membrane was swabbed with mineral oil to make it translucent.

Ten replicate eggs were inoculated for each pool. A 30-gauge needle attached to a 1 ml tuberculin syringe was used to deliver 100 ul of sample intravascularly into each egg. Following inoculation, the membrane was sealed by swabbing the area with melted wax. Inoculated eggs were incubated at 33.6°C in a stationary, humid egg incubator. Trypticase soy and Emmon's agar plates were streaked with each inoculum to test for bacterial and fungal contamination.

Eggs were candled daily for seven days. Embryos dying within the first twenty-four hours were discarded; death during this time is usually due to contamination or other non-specific causes. Embryos dying days 2-7 post inoculation were removed from the egg and examined for evidence of BT infection. BT infection generally produces a swollen and hemorrhagic embryo. The embryos were then decapitated and placed in 50 ml conical tubes with an equal volume of buffered lactose peptone. The tubes were shaken vigorously, vortexed for approximately 30 seconds and frozen at -70°C.

The embryo homogenate for each sample was quick-thawed and 1 ml of the thawed homogenate was used to inoculate the BHK-21 monolayer in a $25cm^2$ tissue culture flask. The cells were observed for seven days for CPE. If after seven days no CPE was observed, the flask was frozen at -70°C and a second passage in cell culture was performed. The cells were quick-thawed and the BHK-21 monolayer in another $25cm^2$ tissue culture flask inoculated with 1 ml of the thawed, cell lysate. If CPE was not observed within seven days of this second passage, the pool was considered to be negative for BTV.

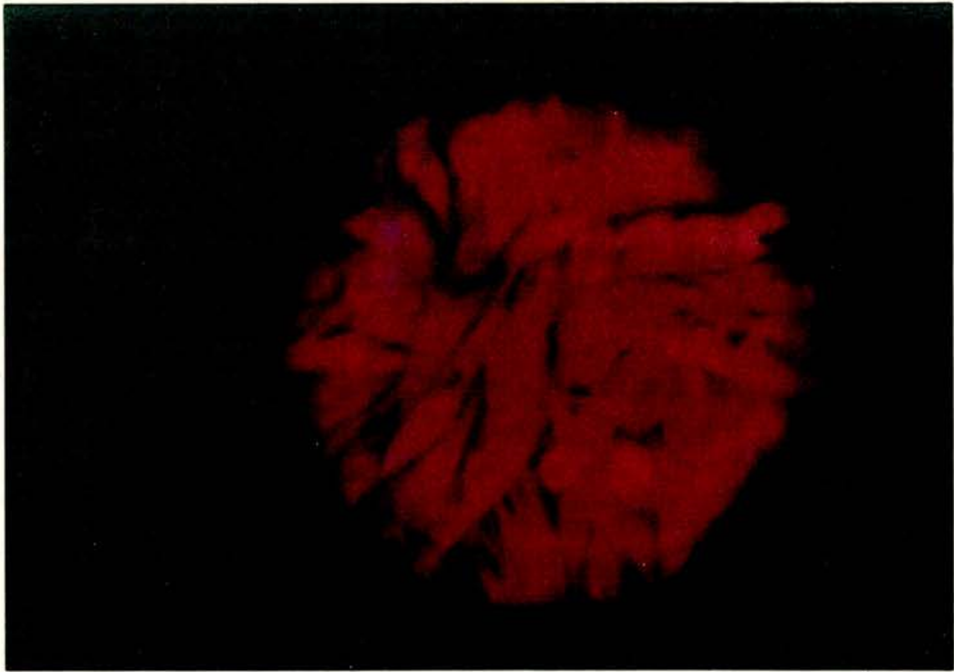
If CPE was observed in either of the two cell culture passes, the cell culture supernatant was tested for the presence of BTV antigen by ELISA, as previously described (Chapter 2, pgs. 24-25), and by indirect immunofluorescent antibody technique (IFA). Only culicoid pools testing positive for BTV antigen by both ELISA and IFA were considered to be positive for BTV.

9. Indirect Fluorescent Antibody Technique

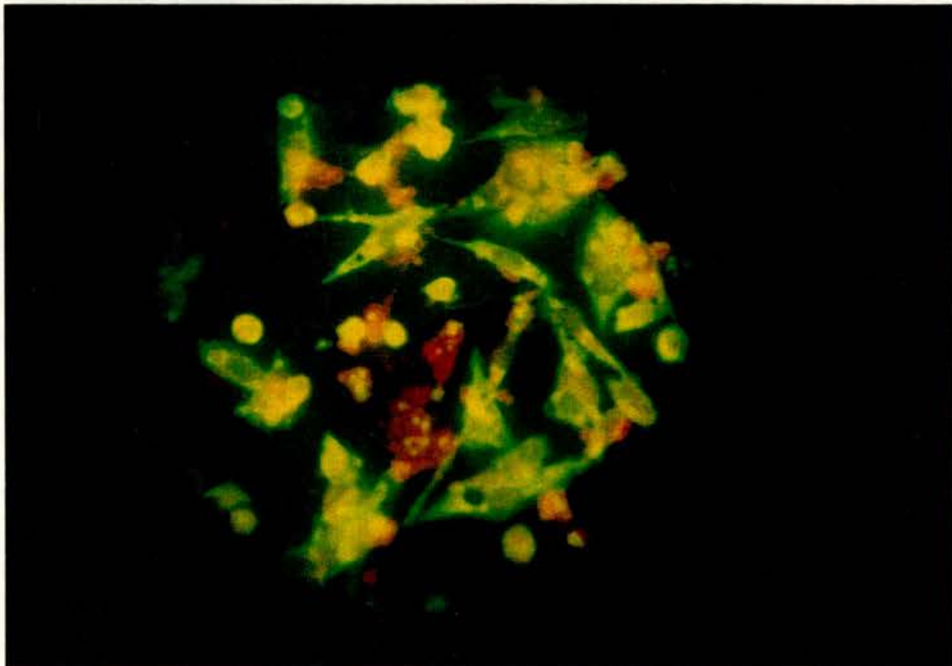
To perform IFA, BHK-21 monolayers grown in 8 well Lab-Tek chamber slides (Nunc, Inc, Naperville, Illinois) were inoculated with 50 ul of cell culture supernatant per chamber. After the addition of 150 ul maintenance medium to each well, the chambers were incubated at 34°C for 3-5 days. Following incubation, the cell culture medium was decanted, the chamber frame removed, the slides rinsed with PBS and the cells fixed with acetone for 10 minutes. Prior to staining, the cells were rehydrated by placing the slides in PBS for 10 minutes. A 1:200 dilution of the anti-BTV monoclonal antibody in PBS was used to stain the cells and the slides were then incubated for 40 minutes in a humidified chamber at 36°C. This was followed by two 10 minute rinses in PBS and one 1 minute rinse in ddH_20. After air-drying, the cells were stained with the fluorescein conjugated goat anti-mouse antibody diluted 1:200 in PBS. Evan's blue dye was added to the conjugate to counterstain the cells. The slides were incubated for 40 minutes at 36°C and then washed as above. Three drops of a 50% PBS and 50% glycerol solution were placed between the slide and a 24x60 mm glass coverslip. The cells were observed using an epifluorescence microscope (Olympus model BH-2) equipped with an HBO 100-W high pressure mercury burner and an IF-490 exciter filter. BTV infected cells exhibit perinuclear, viral-inclusion bodies in the cytoplasm that fluoresce when viewed under the microscope (Figure 3.1).

10. Cell Culture Inoculations

Culicoid pools were diluted 1:2 in antibiotic solution as previously described and stored overnight at 4°C. BHK-21 and CuVa monolayers in $25cm^2$ tissue culture flasks were inoculated with 100 ul of the diluted pool



BHK-21 Control Cells
(Magnified 1000X)



BHK-21 Cells 72 Hours Post Infection
With BTV-17
(Magnified 1000X)

Figure 3.1

delivered to each flask in 900 ul of maintenance medium. The flasks were incubated at 34°C for 1.5 hours and rocked every 15 minutes during the incubation period. The cell monolayers were then rinsed with saline A and 5 ml of fresh maintenance medium was added to each flask. Flasks were incubated for 7 days at 34°C for BHK-21 cells and 32°C for CuVa cells.

BHK cell monolayers were observed for CPE. A pool was considered to be negative for BTV if CPE was not observed after three passages in cell culture. If CPE was observed in any of these three passages, ELISA and IFA were used to confirm the presence of BTV antigen.

Since CuVa cells do not demonstrate CPE when infected with BTV, three blind cell culture passages were made in CuVa cells and the final passage tested for BTV antigen by ELISA and IFA.

11. Mosquito Inoculations

Culicoid pools were diluted 1:10 in PBS. Each of 10 replicate *Aedes triseriatus* mosquitoes was intrathoracically inoculated with 0.5 ul of the diluted pool. Inoculated mosquitoes were maintained at 22°C for a minimum of 14 days before being chilled and decapitated. Individual mosquito heads were squashed onto glass microscope slides and assayed for BTV antigen using the IFA technique.

The mosquito bodies were pooled for each sample, frozen at -70°C, quick-thawed, and triturated in a mortar and pestle in 1.0 ml of sterile PBS. BHK-21 cells in 25cm^2 tissue culture flasks were inoculated with 100 ul of the sample delivered in 900 ul of maintenance medium. The cells were incubated at 34°C and observed for CPE for 7 days.

In addition, a second passage in mosquitoes was made by inoculating another ten replicate mosquitoes with a 1:10 dilution of the bodies from the first passage as previously described. These mosquitoes were also held for 14 days at 22°C and then decapitated. The heads were stained and the bodies pooled, frozen, and passed onto cell culture as above.

Mosquito head squashes were examined by IFA for the presence of BTV antigen, but the pool was determined to be either positive or negative for BTV based on the results of virus isolation attempts in cell culture. The

sample was considered to be negative for BTV if CPE was not observed in either cell culture passage. If CPE was observed in either passage, infection of the cell culture with BTV was confirmed by ELISA and IFA.

12. Serotyping of Isolates

BTV isolates obtained from culicoid pools were serotyped by Dr. James Mecham (USDA, Laramie, WY) using a microneutralization technique. Briefly, each pool was diluted in 199E medium with 2% FBS to contain 1000 $CCID_{50}$/ml. Serial two-fold dilutions of BTV antibody were made in 199E medium containing 2% FBS and 100 ul of the diluted antibody and 100 ul of the diluted virus were added to each well of a microtiter plate. The plate was incubated for 1 hour at 4°C and then 75 ul of a Vero cell suspension, containing 7×10^5 cells/ ml, were added to each well. The cells were incubated at 37°C for 6 days, fixed, stained and examined.

C. Results

1. Sensitivity Assay

A comparison was made of the relative sensitivity of each of the three BTV isolation systems. Serial, ten-fold dilutions of BTV-17 stock virus were titrated in ECE, BHK-21 cells and *Aedes triseriatus* mosquitoes. BTV-17 titers obtained by inoculation of ECE in three separate trials were 6.8, 8.0 and 6.3 if embryo deaths were used to calculate titers, but were 6.1, 6.1 and 5.1 if CPE observed following passage of dead embryos on cell culture was used to calculate the titer (Table 3.1). One of two explanations may account for this discrepancy in titers: 1) either the titer using the number of dead embryos for calculation is artificially high, as some of these deaths were due to non-specific causes and not BTV infection, or 2) the titer using CPE observed in cell culture is artificially low, due to the failure to isolate BTV that was actually present in the embryo.

The titers obtained for BTV-17 by direct isolation in BHK-21 cell culture were 5.0, 5.6, and 3.6 (Table 3.2).

Intrathoracic inoculation of mosquitoes resulted in BTV-17 titers of 6.0, 9.2 and 6.2 based on the number of inoculated mosquito heads positive

Table 3.1: Sensitivity of Embryonated Chicken Eggs For Isolation of BTV

	Assay A			Assay B			Assay C		
BTV-17 Dilution	Embryo Deaths[a]	CPE 1P[b]	CPE 2P[c]	Embryo Deaths	CPE 1P	CPE 2P	Embryo Deaths	CPE 1P	CPE 2P
10^{-1}	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
10^{-2}	3/4	4/4	4/4	3/3	1/3	1/3	2/4	2/4	2/4
10^{-3}	3/4	3/4	3/4	4/4	3/4	3/4	2/3	2/3	2/3
10^{-4}	3/3	3/3	3/3	4/4	3/4	3/4	2/3	2/3	2/3
10^{-5}	4/4	4/4	4/4	3/3	2/3	2/3	1/3	1/3	1/3
10^{-6}	1/3	1/3	1/3	3/3	2/3	2/3	2/3	2/3	2/3
10^{-7}	0/4	0/4	0/4	1/3	1/4	1/4	1/3	0/3	0/3
10^{-8}	1/4	0/4	0/4	0/3	1/3	1/3	0/3	0/3	0/3
10^{-9}	0/3	0/3	0/3	1/4	0/4	0/4	1/4	0/4	0/3
10^{-10}	1/4	0/4	0/4	0/3	0/3	0/4	1/2	0/2	0/2
negative control[d]	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4	0/4
Titer	6.8[e]	6.1[f]	6.1[f]	8.0	6.1	6.1	6.3	5.1	5.1

[a] Total number of embryo deaths/Total number of eggs inoculated
[b] Total number of flasks in which CPE was observed in 1st passage in BHK cells/Total number of flasks inoculated in 1st passage
[c] Total number of flasks in which CPE was observed in 2nd passage in BHK cells/Total number of flasks inoculated in 2nd passage
[d] Inoculated with PBS diluent
[e] ELD_{50}/ml
[f] $TCID_{50}$/ml

Table 3.2: Sensitivity of BHK Cell Culture For Isolation of BTV

	Assay A			Assay B			Assay C		
BTV-17 Dilution	CPE 1P[a]	CPE 2P[b]	CPE 3P[c]	CPE 1P	CPE 2P	CPE 3P	CPE 1P	CPE 2P	CPE 3P
10^{-1}	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^{-2}	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^{-3}	3/3	3/3	3/3	3/3	3/3	3/3	2/3	2/3	2/3
10^{-4}	1/3	1/3	1/3	2/3	2/3	2/3	1/3	1/3	1/3
10^{-5}	0/3	0/3	0/3	1/3	1/3	1/3	0/3	0/3	0/3
10^{-6}	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
10^{-7}	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
negative control	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Titer	5.0[d]	5.0	5.0	5.6	5.6	5.6	3.6	3.6	3.6

[a] Total number of flasks in which CPE was observed in 1st passage in BHK cells/Total number of flasks inoculated in 1st passage

[b] Total number of flasks in which CPE was observed in 2nd passage in BHK cells/Total number of flasks inoculated in 2nd passage

[c] Total number of flasks in which CPE was observed in 3rd passage in BHK cells/Total number of flasks inoculated in 3rd passage

[d] $TCID_{50}$/ml

for BTV antigen by IFA, but were 6.8, 6.8 and 6.8 following passage of inoculated mosquitoes on cell culture (Table 3.3). The mosquito head squash preparations did not exhibit discrete areas of BTV antigen as seen in BHK cells stained by IFA. However, in the majority of the heads derived from mosquitoes inoculated with low dilutions of stock virus the presence of BTV antigen was clearly demonstrated (Figure 3.2). Non-specific staining was observed in several of the negative control mosquito heads examined by IFA. Therefore, titers obtained following cell culture passage of the inoculated mosquito bodies were considered to be more valid, and mosquitoes that were positive for BTV antigen by IFA were only considered to be positive for BTV if the virus was also isolated in cell culture.

An increase in viral titer following subsequent cell culture passages was not observed in any of the assays, as no additional viral isolates were obtained in either second or third passages.

The mean titer obtained by ECE inoculation was 7.0 ELD_{50}/ml, if first passage in the system resulting in egg deaths was used as the criterion, or 5.8 $TCID_{50}$/ml, if CPE following passage of the embryo on cell culture was the criterion.

Intrathoracic inoculation of mosquitoes resulted in a mean titers of 7.0 IFA_{50}/ml, if first passage in the system resulting in mosquito heads positive for BTV antigen by IFA was used as a criterion, or 6.8 $TCID_{50}$/ml if CPE following passage of inoculated mosquitoes was the criterion.

The mean titer for direct inoculation of cell culture was 4.7 $TCID_{50}$/ml for both the first and second passage.

A comparison of the mean titer obtained in the three isolation systems is presented in Table 3.4. The titers of the three systems were not significantly different ($P=0.108$) on the first passage in each particular system using a one way anaylsis of variance. The system effect was significant ($P=0.28$) on second passage in each system. Pairwise comparisons of the second passage means between the three systems showed that the cell titer mean (4.7) was significantly lower ($P=0.010$) than the mosquito titer mean (6.80). Although the systems were not significantly different in the first passage, the means showed the same order as the second passage.

Table 3.3: Sensitivity of *Aedes triseriatus* Mosquitoes For Isolation of BTV

	Assay A			Assay B			Assay C		
BTV-17 Dilution	IFA 1P[a]	CPE 1P[b]	CPE 2P[c]	IFA 1P	CPE 1P	CPE 2P	IFA 1P	CPE 1P	CPE 2P
10^{-1}	5/5	3/3	0/3	2/2	3/3	1/3	3/3	3/3	0/3
10^{-2}	3/5	3/3	0/3	4/5	3/3	0/3	3/3	3/3	0/3
10^{-3}	3/5	3/3	0/3	3/5	3/3	0/3	3/3	3/3	0/3
10^{-4}	0/5	0/3	0/3	2/4	0/3	0/3	1/4	0/3	0/3
10^{-5}	0/5	0/3	0/3	3/4	0/3	0/3	1/3	0/3	0/3
10^{-6}	0/4	0/3	0/3	2/3	0/3	0/3	1/3	0/3	0/3
10^{-7}	0/5	0/3	0/3	3/5	0/3	0/3	2/4	0/3	0/3
10^{-8}	0/5	0/3	0/3	1/4	0/3	0/3	0/3	0/3	0/3
10^{-9}	0/5	0/3	0/3	2/4	0/3	0/3	1/4	0/3	0/3
10^{-10}	0/5	0/3	0/3	0/5	0/3	0/3	0/3	0/3	0/3
negative control[d]	0/4	0/3	0/3	1/4	0/3	0/3	1/4	0/3	0/3
Titer	6.0[e]	6.8[f]	0	9.2	6.8	0	6.2	6.8	0

[a] Total number of mosquito heads positive for BTV antigen by IFA/Total number of heads examined by IFA

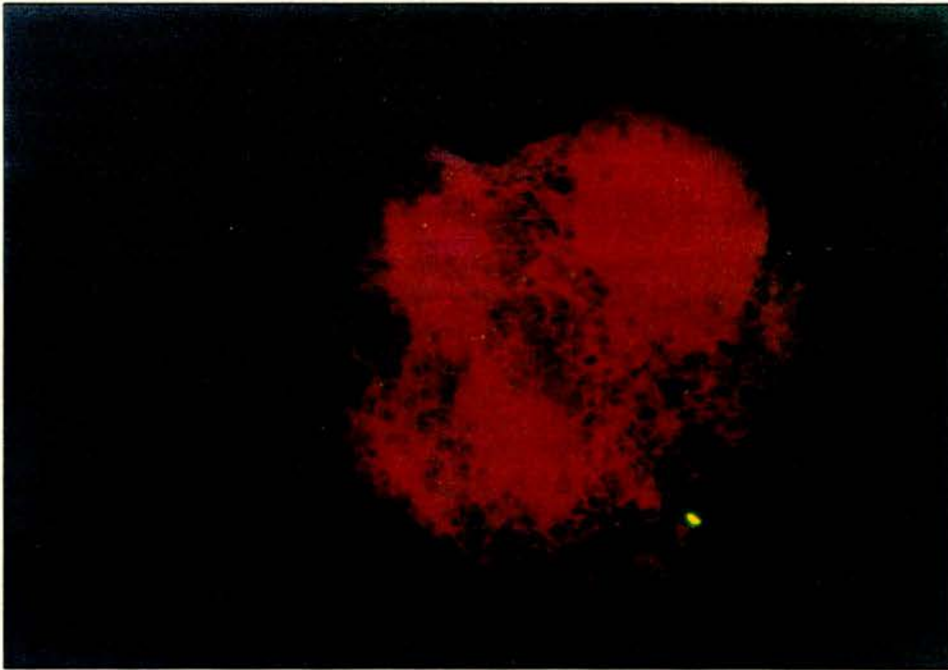
[b] Total number of flasks in which CPE was observed in 1st passage in BHK cells/ Total number of flasks inoculated in 1st passage

[c] Total number of flasks in which CPE was observed in 2nd passage in BHK cells/ Total number of flasks inoculated in 2nd passage

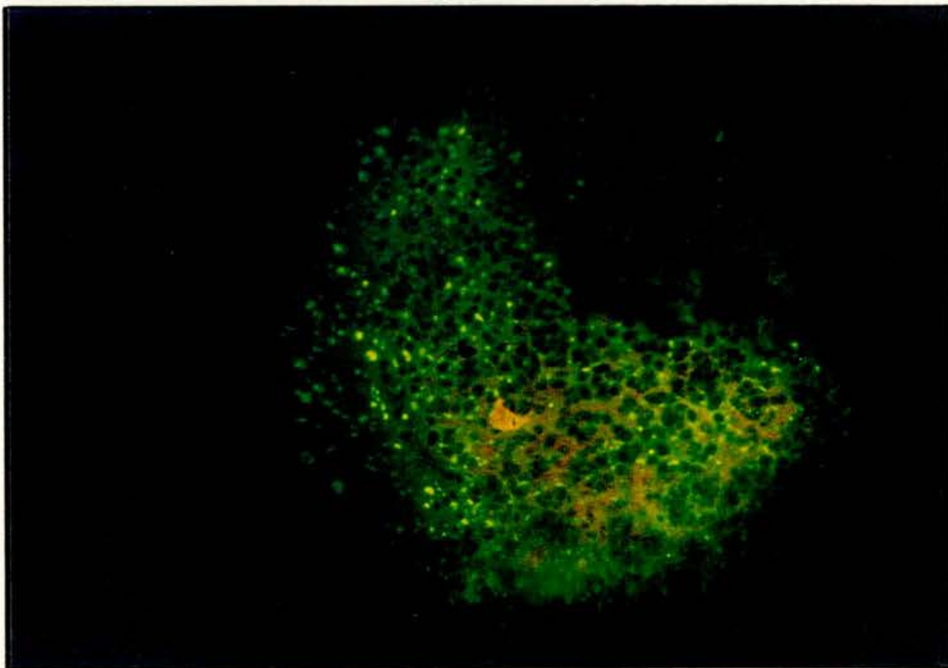
[d] Inoculated with PBS diluent

[e] IFA_{50}/ml

[f] $TCID_{50}$/ml



Head Squash of Uninfected, Control Mosquito
(Magnified 1000X)



Head Squash of Mosquito 14 Days Post Infection
With BTV-17
(Magnified 1000X)

Figure 3.2

Table 3.4

A Comparison of BTV Isolation in Embryonated Chicken Eggs (ECE), BHK-21 Cell Culture and *Aedes triseriatus* Mosquitoes

	ECE		BHK-21	Mosquitoes	
	Embryo Deaths	CPE		IFA	CPE
Trial A	6.8[a]	6.1[b]	5.0[b]	6.0[c]	6.8[b]
Trial B	8.0	6.1	5.6	9.2	6.8
Trial C	6.3	5.1	3.6	6.2	6.8
Mean Titer[d]	7.0	5.8	4.7	7.0	6.8
Standard Error[e]	0.5	0.3	0.6	1.0	0.0

[a] Titer BTV-17 (ELD_{50}/ml)
[b] Titer BTV-17 ($TCID_{50}$/ml)
[c] Titer BTV-17 (IFA_{50}/ml)
[d] Mean of the titers obtained in three trials
[e] Calculated as the standard deviation divided by the square root of $n=(3)$

2. BTV Isolation From Culicoid Pools

BTV isolation was attempted from the 66 emergent fly pools and 14 light-trap fly pools that were putatively positive for BTV by ELISA. All three of the isolation methods described above were used to assay each pool. Eleven BTV isolates were obtained from the putatively positive emergent fly pools and one isolate was obtained from a putatively-positive light trap fly pool (Table 3.5).

Egg Inoculations

BTV was isolated from six emergent culicoid fly pools, #79, 306, 315, 319, 324 and 333, using the ECE isolation method. All of the embryos from which BTV was isolated were severely hemorrhagic and contamination did not appear to be the cause of death. The inoculum had previously been determined to be sterile.

Viral CPE was observed in BHK-21 cells infected with the embryo homogenate from each of the 6 pools 5-7 days after inoculation of the first cell culture passage. The cell culture supernatant from this first passage tested positive for the presence of BTV antigen by ELISA and BHK-21 cells subsequently infected with the supernatant stained positive for BTV antigen by IFA.

Three of the pools, #79, 306 and 333, were composed of *C. variipennis* and three pools, #315, 319, and 324, were composed of *C. crepuscularis*. All of these pools were collected from the Ausman farm except for pool #79 which was collected at the Red Barn farm. Four of these six pools, #315, 319, 324 and 333, were collected in October of 1993. The remaining two pools, #79 and 306, were collected in June and August of 1993, respectively. The serotype of all these BTV isolates was BTV-17.

Cell Culture Inoculations

Isolation of BTV by direct inoculation of cell culture proved to be problematic. The CuVa cell line appeared to be persistently infected with BTV which became patent only after several passages of the cell line in the laboratory. BTV antigen could not be detected by ELISA or IFA in the original cells, but after five cell culture passages, BTV antigen was

Table 3.5

BTV Isolates Obtained from Pools of Field-Collected Culicoids by Inoculation of Embryonated Chicken Eggs (ECE), BHK-21 Cell Culture and *Aedes triseriatus* Mosquitoes

Pool No.	Date Collected	Culicoid Species	First Isolated In: ECE	BHK	Mosq.	Second Isolation In:[a]	Serotype
				BHK-21			
18EF[b]	4/20/93	*variipennis*	(-)	(+)	(-)	ND[c]	17
20EF	4/20/93	*crepuscularis*	(-)	(+)	(-)	ND	17
22EF	4/20/93	*selfia* group	(-)	(+)	(-)	ND	17
79EF	6/08/93	*variipennis*	(+)	(-)	(-)	ND	17
				BHK-21 clone 15			
270EF	7/26/93	*crepuscularis*	(-)	(-)	(+)	ND	11
306EF	8/30/93	*variipennis*	(+)	(-)	(-)	ECE, Mosq.	17
310EF	10/01/93	*crepuscularis*	(-)	(-)	(+)	ND	17
315EF	10/01/93	*crepuscularis*	(+)	(-)	(-)	ECE, Mosq.	17
319EF	10/01/93	*crepuscularis*	(+)	(-)	(-)	ECE, Mosq.	17
324EF	10/01/93	*crepuscularis*	(+)	(-)	(-)	ECE, Mosq.	17
333EF	10/01/93	*variipennis*	(+)	(-)	(-)	ECE	17
34LT[d]	8/10/93	*variipennis*	(-)	(-)	(+)	ND	17

[a] BTV re-isolated from embryo homogenate in ECE and/or mosquitoes
[b] emergent fly pool
[c] not done
[d] light-trap fly pool

detected in the majority of the cells by both of these methods. Similar results were observed at the USDA-ABADRL in Laramie, Wyoming, and scientists there are conducting studies to determine the molecular basis for the persistence of BTV in CuVa cells (Dr. Melody Jensen, personal communication). Due to this persistent BTV infection the use of CuVa cells was discontinued very shortly after this study began and only BHK-21 cells were used for subsequent attempts to isolate BTV.

The BHK-21 cells were briefly contaminated with BTV during the time CuVa cells were in the laboratory. Another line of BHK-21 cells, clone 15, which had not previously been used in the laboratory was substituted for the original cell line and used for all subsequent cell culture (Table 3.5).

Three BTV isolates, from emergent fly pools #18, 20 and 22, were obtained by direct inoculation of BHK-21 cells. All of these pools were collected early in the year (April) of 1993. Pool #18 was collected at the Ausman farm and pools #20 and #22 were collected at the Red Barn farm. Each of the pools was composed of a different culicoid species. Pool #18 was composed of *C. variipennis*, pool #20 was composed of *C. crepuscularis* and pool #22 was composed of culicoids belonging to the *selfia* group. Pools #20 and #22 demonstrated CPE six days after the first passage in BHK cells, but CPE was not observed until six days after inoculation of the second cell culture passage for pool #18. All three of these isolates were serotyped and found to be BTV-17.

Mosquito Inoculations

BTV was isolated in mosquitoes from two emergent fly pools, #270 and #310. Both pools were collected at the Ausman farm on 7/26/93 and 10/1/93, respectively, and both were composed of *C. crepuscularis*. One light trap pool, LT-34, also yielded a BTV isolate. This pool was collected at the Ausman farm on 8/10/93 and was composed of *C. variipennis*. Pools #310 and LT-34 were found to be BTV serotype 17 and pool #270 was found to be serotype 11.

Table 3.6

Minimum Field Infection Rates for Collected Culicoids Based on Isolation of BTV

	Total Collected	Positive Pools	MFIR[a]
Light-Trap Collected Flies	901	1	0.11%
Emergent Flies	11,221	11	0.10%

* The minimum field infection rates were compared for light-trap collected and emergent flies using a chi-squared test (Devore and Peck, 1986). There was not sufficient evidence for statistical significance. (X^2=0.19, P=0.66)

(Emergent Flies)			
C. variipennis	5956	4	0.07%
C. crepuscularis	3425	6	0.18%
Selfia group	454	1	0.22%

* The minimum field infection rates were compared for these culicoid species using a chi-squared test (Devore and Peck, 1986). There was not sufficient evidence for statistical significance. (X^2=2.77, P=0.25)

[a] Calculated by dividing the number of pools positive for BTV isolation by the total number of flies collected.

Minimum Field Infection Rates (MFIR)

The MFIR for light-trap collected and emergent flies, based on the isolation of infectious virus from pools putatively positive for BTV antigen by ELISA, was 0.11% and 0.10%, respectively ($P=0.66$) (Table 3.6). The MFIR for *C. variipennis* was 0.08%, for *C. crepuscularis* was 0.18%, and for culicoids belonging to the *selfia* group was 0.22% ($P=0.25$) (Table 3.6).

D. Discussion

Twelve BTV isolates were obtained in this study from field-collected culicoid fly pools that had tested positive for BTV antigen by ELISA. None of the isolates was obtained in more than one isolation system, a phenomenon reported in other studies in which isolation of field-strains of BTV was attempted (Gibbs, 1983; Sawyer et al., 1986; Wechsler et al., 1988).

BTV was isolated from one of the fourteen putatively positive light-trap fly pools by inoculation of *Aedes triseriatus* mosquitoes. This isolate was significant because it established that BTV, serotype 17, was circulating in the test area this year. It was expected that more than one BTV isolate would be obtained from these light-trap pools, based on the ELISA results. Aliquots of the processed light-trap pools were not frozen for future use, however, as was done for emergent fly and larval pools, but were held at 4°C for the duration of this study. Unfortunately, the majority of the pools became desiccated during this time and, although BTV was probably present in a number of these pools, viral infectivity was lost.

Eleven BTV isolates were obtained from the sixty-six antigen positive emergent culicoid fly pools. Six of these isolates were obtained by inoculation of ECE, three by direct inoculation of BHK-21 cell culture and two by inoculation of *Aedes triseriatus* mosquitoes. All of these isolates were found to be BTV serotype 17 except for isolate #270, which was found to be BTV serotype 11. In a study done by the USDA from 1976-1991, BTV 17 was the only serotype to be isolated from sheep and cattle in Colorado in 1980, 1986, and 1989 and BTV-11 and BTV-10 were isolated in 1981 (Pearson et al., 1992). The fact that all but one of the isolates obtained in this study from

the light-trap and emergent fly pools were BTV-17, and that the one remaining pool was BTV-11, is consistent with the results of the USDA study.

Because a laboratory strain of BTV-17 was used as the control for all of the isolation assays, laboratory contamination can not be completely ruled out as a possible source for the BTV-17 isolates. However, this does not seem to be the case for most of the isolates. Due to the observation that the CuVa cell line appeared to be persistently infected and the BHK-21 cell concurrently contaminated with BTV, steps were taken to determine the actual source of six of the BTV isolates (#79EF, 306EF, 315Ef, 319EF, 324EF & 333EF) obtained by inoculation of ECE. The embryo homogenate for each of these isolates was again inoculated into ECE and also into *Aedes triseriatus* mosquitoes. BTV was again isolated from all six of these pools by ECE and from all but one of the six pools by inoculation of mosquitoes (Table 3.5). As BTV was not isolated from any of the negative, uninfected ECE and mosquito controls, the isolates were derived from the embryo homogenates and not from cell culture contaminated with BTV.

Furthermore, BTV serotype 11 was not used in this laboratory during the course of the isolation assays, making laboratory contamination of pool #270EF with this serotype unlikely.

The isolates obtained by direct inoculation of cell culture, #18EF, 20EF and 22EF, are somewhat suspect in origin as the BHK-21 cell line being used at this time was later found to be contaminated with BTV. This may explain the inordinate number of isolates obtained using this technique. However, all other isolates were obtained using a new cell-line, BHK-21 clone 15, which was never demonstrated to be infected with BTV.

The problems that were encountered with respect to a persistent BTV infection in the CuVa cell line may demonstrate the mechanism by which BTV is maintained in culicoids in nature. The CuVa cells were maintained in this laboratory at 32°C. Previous studies had determined that the highest viral titers in CuVa cells infected with exogenous BTV were obtained when the cells were incubated at 25°C or 32°C as compared to incubation at 20°C or 37°C (Wechsler, 1989). BTV was not detected in the CuVa cells in this laboratory until they had been passed five times, suggesting that BTV may be present in

the original cell line in such low titer as to be undetectable by either ELISA or IFA. Perhaps incubation of these persistently infected CuVa cells at 32°C combined with repeated passage "reactivated" the virus, resulting in a productive infection of the cell culture and amplification of the virus, which could then be detected by the above mentioned techniques.

This reactivation of BTV in the laboratory may simulate *in vitro* the mechanism by which the virus is reactivated *in vivo*, as temperature appears to be critical in the reactivation of other arboviruses. In one study, a marked reduction in replication of Japanese encephalitis virus (JEV) in hibernating mosquitoes was observed when the mosquitoes were maintained at a low temperature and incubation of the mosquitoes at an elevated temperature was necessary for the detection of JEV. (LaMotte, 1963). Moreover, it has been suggested that if the metabolic rate of a BTV infected culicoid falls below a critical level, the insect will survive but BTV replication does not occur (Metcalf and Luedke, 1980). Results from a recent study regarding temperature and virogenesis of BTV in *C. v. sonorensis*, suggest that an increase in temperature may be necessary to allow latent BTV infections in the culicoid vector to be expressed (Mullens, et al., 1995). These studies support the theory that overwintering culicoid larvae are latently infected with BTV, which is not detectable by laboratory techniques. The virus may be reactivated as the outside temperature rises in the spring and increased cell division and replication within the culicoid vector occurs due to metamorphosis of the larva into the adult fly. At this time the virus becomes detectable by laboratory techniques.

A comparison of the BTV-17 titers obtained in the sensitivity assay showed inoculation of *Aedes triseriatus* mosquitoes followed by passage on to cell culture to be more sensitive than direct isolation in BHK-21 cell culture (P=0.028) The mean titer for inoculated ECE, based on viral CPE following passage of dead embryos on cell culture, was 7.0 ELD_{50}/ml. Intrathoracic inoculation of *Aedes triseriatus* mosquitoes followed by passage of inoculated mosquitoes on cell culture resulted in a mean titer of 6.8 $TCID_{50}$/ml. Direct isolation in cell culture resulted in a titer of 4.7 $TCID_{50}$/ml.

There are several advantages to using inoculation of mosquitoes that might make this technique particularly useful for the isolation of field-strains of BTV from culicoids. First, the mosquito provides a model similar to the natural culicoid host in which BTV replicates. BTV present in culicoids infected with the virus by vertical transmission, and therefore not adapted to a vertebrate host, might be more likely to propagate in an insect model than in ECE or vertebrate cell lines. Secondly, mosquitoes are much more resistant to bacterial and fungal infection than ECE or cell culture, an important consideration when assaying field-collected culicoids that are often grossly contaminated with both. BTV isolates #270EF and #310EF, isolated by inoculation of mosquitoes, would not have otherwise been obtained, as both the ECE and BHK-21 cells inoculated with these pools succumbed to bacterial and/or fungal contamination. Finally, BTV isolation in mosquitoes requires only 0.5 ul of inoculum per mosquito as compared to 100 ul of inoculum per replicate for ECE and cell culture. This is of particular importance when attempting BTV isolation from small, unique, field-collected culicoid samples.

Interestingly, additional BTV isolates were not obtained following a second passage of BTV in mosquitoes. In fact, BTV was not isolated from any mosquitoes in the second passage. These results were unexpected, as it was assumed that amplification of the virus would occur upon subsequent passage in an arthropod host. However, similar results, in which the second passage of arboviruses in arthropods was either negative or the concentration of virus yielded was lower or approximately equal to the first passage, have been reported (Hurlbut and Thomas, 1960). Perhaps virus is more frequently recovered from the first passage due to a higher concentration of the virus in the initial inoculum or due to the inability of the virus to establish secondary foci for replication within the arthropod.

Based on the sensitivity assay of the isolation systems, it would be expected that the majority of BTV isolates from field-collected culicoids would be obtained by inoculation of mosquitoes and inoculation of ECE and that a minority of isolates would be obtained by direct inoculation of cell culture. However in this study, six of the isolates were obtained in ECE

while three isolates were obtained by direct inoculation of cell culture and three by inoculation of mosquitoes.

It is not clear why more isolates were not obtained by intrathoracic inoculation of *Aedes triseriatus* mosquitoes. Possibly, field-strains of BTV are less likely to replicate in mosquitoes than the laboratory adapted strain used in the sensitivity assay. More likely, any BTV present in the culicoid pools used for inoculation of the mosquitoes was somehow inactivated. Due to constraints on the availability of mosquitoes for inoculation, many of these pools were maintained at 4°C for as long as 72 hours before mosquito inoculation, compared to 24 hours or less for pools used in ECE and cell culture inoculations. Although BTV has been shown to become only slightly less infectious when held at 4°C for extended periods of time, there may have been such a small amount of virus present in these pools that this reduction in infectivity was sufficient to prevent replication within an unnatural, arthropod host.

Since BTV was isolated from field-collected larval culicoid fly pools, it appears that the virus is transmitted vertically in the culicoid vector. As suggested by the ELISA results in the first part of this study, *C. variipennis* does not appear to be the only culicoid vector in which BTV is maintained. Of the eleven BTV isolates obtained in this study from emergent flies, four were *C. variipennis* (MFIR=0.07%), six were *C. crepuscularis* (MFIR=0.18%) and one was from the *selfia* group (MFIR=0.22%). *C. variipennis* may be the epidemic vector of BTV, because it is the predominant culicoid species in the area and readily feeds on livestock. Other culicoid species, *C. crepuscularis* and members of the *selfia* group in particular, may serve as endemic reservoir hosts and be of great importance in the transeasonal maintenance of BTV in nature.

IV. SUMMARY

The primary objective of this study was to determine if vertical transmission in the culicoid vector is the mechanism by which BTV is maintained transeasonally in temperate regions.

Field-collected culicoid larvae, culicoid flies allowed to emerge in the laboratory from collected larvae, and culicoid flies collected in a light-trap were assayed by ELISA for the presence of BTV antigen. Isolation of BTV was then attempted from culicoid pools that were putatively positive for BTV by ELISA. Twelve BTV isolates were obtained from emergent and light-trap fly pools by intravascular inoculation of ECE, direct isolation in BHK-21 cell culture or intrathoracic inoculation of *Aedes triseriatus* mosquitoes. Eleven of these isolates were found to be BTV serotype 17 and one isolate was BTV serotype 11, the two predominant BTV serotypes circulating in Colorado in the last fifteen years. These isolates were obtained from pools composed of *C. variipennis*, *C. crepuscularis* and culicoids belonging to the *selfia* group, suggesting that *C. variipennis* is not the only vector of BTV in Colorado. The MFIR for these species, based on isolation of BTV, were: 0.07% for *C. variipennis*, 0.18% for *C. crepuscularis* and 0.22% for members of the *selfia* group (P=0.25)

Early in this study the CuVa cell line was found to be persistently infected with BTV. As the BTV infection became apparent only when the cells were held at 32°C and passed a number of times, it appears that these conditions "reactivated" BTV present in these cells either in very low titer or existing in a latent condition. This infection of the CuVa cells with BTV may provide a laboratory model which simulates the mechanism by which BTV is maintained in the culicoid vector in nature. The molecular basis for the

persistence of BTV in the CuVa cell line warrants further study; understanding the mechanism of viral persistence in CuVa cells may well be involved in persistence in culicoid larvae.

The use of a novel technique for the isolation of BTV, intrathoracic inoculation of *Aedes triseriatus* mosquitoes, proved to be successful. This technique was shown to be more sensitive than direct isolation in BHK-21 cell culture for isolation of a laboratory strain of BTV. Although more BTV isolates were obtained from field-collected pools of culicoids by inoculation of ECE than mosquitoes, this was most likely due to improper handling of pools prior to inoculation of mosquitoes, which resulted in a reduction of virus infectivity.

Parenteral inoculation of mosquitoes may be particularly useful in the isolation of field-strains of BTV from culicoids because the mosquito is similar to the natural culicoid vector in which BTV replicates, mosquitoes are much more resistant to bacterial and fungal contaminants often associated with field-collected culicoids, a much smaller inoculum is required to infect a mosquito as compared to ECE or cell culture, and mosquitoes are less expensive to obtain and maintain than either ECE or cell culture.

The results of this study support the hypothesis that vertical transmission of BTV in culicoids does occur and that BTV overwinters by persistently or latently infecting culicoid larvae. These results would suggest that BT disease might be best controlled through reduction in the number of culicoid larvae overwintering in areas where BT is enzootic.

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