AN INVESTIGATION ON THE LABORATORY DIAGNOSIS OF BLUETONGUE IN CATTLE

By

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DIAGNOSIS OF BLUETONGUE

IN CATTLE

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CHAPTER I

INTRODUCTION

The Clinical and Diagnostic Problem

Bluetongue (BT) disease is found worldwide in ruminant species (6). Bluetongue has been clinically recognized in the United States in sheep since 1948 and in cattle since 1889 and has been found primarily in the South and Southwest (46). In North America, four serotypes of bluetongue virus (BTV) have been found; three of these serotypes (10, 11, and 13) have been isolated in other continents; however, serotype 17 has been found only in the United States.

In cattle, infection in utero by BTV prior to the 7th month of gestation has produced calves which remain viremic for life and lack antibodies to BTV (30). These calves are able to transmit the virus to their progeny; thus, perpetuating BTV infection of the herd (30).

Laboratory diagnosis of bluetongue virus infection is done by serologic methods (e.g. complement fixation and agar-gel immunodiffusion tests). Complement fixing antibodies appear in the serum of the animal early in the disease and subsequently disappears within a 3-month period. Precipitin-type antibodies are not detectable in animals acutely infected but appear days after infection and then persist for 3 years or longer.

Diagnosis of BT infection by virus isolation is a lengthy, laborious process because field strains of BTV do not replicate directly in

cell culture. For isolation from clinical specimens, embryonating chicken eggs are inoculated by the intravascular route. Following isolation of BTV in eggs, adaptation of virus to cell culture usually requires 3 to 4 blind passages before a cytopathic effect (CPE) develops. Identification of each of the 20 antigenic types of BTV is difficult due to the presence of a group specific antigen and cross-reactions between the different serotypes (50). Also, BTV cross reacts in virus neutralization tests with epizootic hemorrhagic disease virus of deer (50). Bluetongue virus is highly membrane-associated (14) which appears to interfere with the isolation of the virus from clinical specimens. The affinity of BTV for cellular membranes also interferes with the ability of BTV to agglutinate red blood cells (26,27,57). The hemagglutinating capability of the virus can be employed in serologic identification of the various serotypes of BTV (57).

Diagnosis of bluetongue infection in cattle is difficult, time-consuming, and laborious, however, because of the clinical disease produced in cattle, its diagnosis is important to the livestock industry. Prevention and control of bluetongue infection in cattle cannot proceed until the understanding of the pathogenesis of BTV is acquired. Paramount to the preceding objective is the requirement for reliable methods for the diagnosis of bluetongue and the identification of the various antigenic types of the virus.

The objectives of the present investigation were to explore certain of the laboratory methods for the isolation of BTV and to describe the clinical signs associated with infection by BTV in cattle and newborns. The distribution of BT infection in cattle in Oklahoma as determined by serology was also reviewed (32). Finally, the immunoelectrophoresis

(IEP) procedure was investigated as a possible method to identify the various serotypes of BTV.

CHAPTER II

REVIEW OF LITERATURE

Bluetongue, also called "Catarrhal fever" of sheep, "sore muzzle" of sheep, "range stiffness" in lambs, is an infectious viral disease of ruminants which is transmitted by an insect vector Culicoides variipennis. Bluetongue in sheep and cattle is clinically characterized by inflammation and congestion of the mucous membranes, emaciation, and lameness due to inflammation of the coronary band and sensitive laminae of the hoof (6,24,45).

Bluetongue was first described in South Africa by the 1800's, but apparently the disease was recognized in that country since the early 1700's (22,24). From Africa, bluetongue has spread to other parts of the world. The disease has been reported in Iran (1), Cyprus (17), Palestine (21), West Pakistan (53), Turkey, Spain, Portugal and India (24,45,56). In 1975, Bluetongue virus (BTV) serotype 20 was isolated in Australia from Culicoides gnats and the serotype reported as avirulent for nonpregnant adult cattle and mildly pathogenic for sheep (27). In a recent report (1981), BTV has been found in Brazil (27).

In North America, BTV was first isolated in 1952 in California, but the disease previously had been recognized in Texas in the 1900's (45). Bluetongue virus has been isolated in 30 states within the United States. Furthermore, there is serologic evidence of Bluetongue infection in cattle in 49 states with the exception of Alaska and Rhode Island (45).

In the genus Orbivirus, within the family Reoviridae, BTV is the type species. The virus has icosahedral symmetry and contains 32 capsomeres each of circular configuration (42). The ribonucleic acid (RNA) of BTV is segmented into 10 pieces each with molecular weight of 0.3 to 2.7×10^6 daltons; total molecular weight of the RNA is approximately 12×10^6 daltons (40). Studies of various bluetongue virus serotypes by nucleic acid hybridization analysis have found that certain RNA segments hybridize very extensively while others hybridize poorly (42). The virus contains a RNA polymerase enzyme which is activated only upon removal of the outer viral shell (42). Bluetongue virus loses its infectivity at pH 3.0 and lipid solvents have reduced the infectivity of the virus by 10-fold (42).

Bluetongue virus has remained infective in preserved defribrinated blood for 25 years at ambient temperature (48), and also, BTV is very stable at refrigerator temperature. Decreases in the infectivity of the virus occur at freezing or at temperatures below -30 C (45).

Bluetongue has 20 known serotypes found world wide (23). In the United States, only serotypes 10, 11, 13 and 17 are found. A survey in the United States among isolates of BTV demonstrated a greater prevalence of serotypes 11 and 17 than serotypes 10 and 13 (4). Serotype 17 has, at present, only been found in the United States (5).

The infection of cultured cells with BTV produces proliferation of membranous structures along with the production of 2 types of inclusion bodies (9,10). The Type I inclusion body occurs in the nucleus and the Type II inclusion body is located in the cytoplasm. The cytoplasmic inclusion bodies fluoresce specifically when reacted with fluorescein-labelled immunoglobulins specific for BTV (10). Bluetongue virus is

released from the host cell by budding through the plasma membrane as single virions or in viral aggregates. Viral particles are highly associated with cell membranes in either intact or disrupted cells (14). The affinity of BTV for endothelial cells of blood vessels has been demonstrated by use of the fluorescent antibody test (55).

Clinical specimens for the isolation of BTV from either domestic or wild ruminants include placenta (25), bone marrow (2), semen (11), spleen, and red blood cells. The addition of metal ions (e.g. Zinc or Strontium) appears to enhance virus isolation from hemolysed blood (11). The virus has also been isolated from insects of the genus Culicoides (2,28).

The disease of bluetongue in experimentally infected sheep has an incubation period of 3 to 7 days (24,34). Elevation of the body temperature and/or increases in the respiration rate are early clinical signs observed in infected sheep (34). The temperature of affected sheep has reached a peak of 40 C to 41 C or higher on day 7 or 8 after virus inoculation. Hyperemia of the oral and nasal mucosa develops subsequent to the elevation of body temperature. A hyperemia also may be observed on the skin where the wool is thin. Salivation and ocular discharge do occur with a nasal discharge which is mucopurulent and occasionally bloody. Petechial hemorrhages are present in the oral cavity, nose, and conjuntiva. The hyperemia (of the oral cavity) progresses to cause necrosis of the gums, tongue, and cheeks. The tissue erodes due to necrosis and leaves ulcers of 2 to 4 cm in size and irregular shape. The tongue acquires a characteristic bluish discoloration thereby the name "Bluetongue". Due to the severe oral lesions, sheep become anorexic and depressed. A severe coronitis usually occurs with subsequently

sloughing of the corneal structure of the hoof and consequently sheep will not move and will stand with arched backs. Muscular lesions consisting of necrosis of muscle fibers are responsible for loss of condition in severely affected animals (24). Vomiting that leads to aspiration pneumonia has been seen in animals infected with BTV (24,34, 47). Sporadic abortion and fetal malformations are associated with the disease in pregnant ewes. In sheep, the disease has a seasonal incidence being more prevalent during late spring, summer, and early fall, which coincides with the peak of the insect vector population (45).

Early clinical signs noticed in BTV-affected cattle include lameness or stiffness when the animals are moved. Affected cattle are depressed and will usually withdraw from the herd. Rectal temperature in cattle may increase to 40 or 41 C and the presence of lesions on the oral mucosa will coincide with the temperature spike (45). Oral lesions described in cattle consist of swelling of the lips, gums, and tongue with an excessive salivation (45). The swelling and hyperemia of the oral mucosa may progress to necrosis and ulceration of the anterior portion of the tongue, dental pad, and lips. A "burnt" appearance of the muzzle occurs in affected cattle. Cracks in the skin of the muzzle may develop which eventually leads to sloughing of the skin. A serosanguineous exudate or secretion occurs on the muzzle in the acute phase of disease which later becomes mucopurulent and crusty. A finding which is rare in occurrence is the ulceration of the teats and udder. A severe coronitis which causes sloughing of the hoof is a frequent finding in affected animals (45). The accompanying abortions and fetal malformations which occur following infection of cattle are typified by a hydranencephaly (37,43) and arthrogryposis which is a

permanent contraction of the joint. Bluetongue virus infection which occurs during the first or second trimester of pregnancy often leads to fetal anomalies (38). Bluetongue virus is usually non-pathogenic for bovine fetuses in the last trimester of gestation (30).

Clinical signs in cattle experimentally-infected with bluetongue virus include a transitory anorexia lasting for 1 to 3 days with an increased body temperature and associated leukopenia (45). Severe clinical signs similar to those observed under field conditions have not been described in experimentally-infected cattle. Although cattle may develop severe clinical signs of bluetongue infection, the disease in most cattle is usually inapparent. However, cattle can become carriers and remain viremic for long periods of time (8,41). Latently-infected cattle are thus able to transmit the virus to noninfected arthropod vectors (36). A viremia can exist in animals with neutralizing antibodies to BTV (33,39). The prolonged viremic periods in cattle and the persistence of virus in the presence of neutralizing antibodies can explain the "overwintering" mechanism of BTV (36). Certain of the viral strains isolated from latent bluetongue-infected cattle have low pathogenicity and immunogenicity for sheep (36). Bluetongue virus has been transmitted experimentally from cattle to sheep and between cattle (39).

Infection of sheep and cattle by BTV has been diagnosed by either a modified complement fixation (CF), agar-gel immunodiffusion (AGID), or virus neutralization test and by a fluorescent antibody technique. The modified CF and AGID tests detect group specific antigens while the virus neutralization test is serotype specific (7,50,51), thus, this test is used for identification of serotypes. The modified CF test

detects IgG antibodies in cattle recently infected by BTV, but antibodies measured by CF are transient (7,50). Precipitin type antibodies which are measured by AGID are of the IgG type and these persist for periods up to 3 years (29).

In one study samples of sera with CF titers of 1:5 or greater tested by the CF and AGID tests were found to have a correlation of results of 96% (50). Serologic results showed that 35% of serum samples which were anticomplementary by the CF test were positive by the AGID test (50). Twenty-three percent of the cattle sera and 7.3% of sheep sera previously diagnosed as negative by the CF test were found positive by the AGID test (50). The differences found between the two tests can be explained by differences in the half-life of the types of antibodies measured by both tests (50). It has been reported (29,44) that the AGID test is less accurate in detecting animals which have been recently infected, but will detect animals that have been infected and have recovered from the disease. Serologic cross reactions between BTV and other agents that clinically resemble BTV have not occurred using the AGID test (44).

Immunofluorescence has been used to detect the antigens of BTV in tissues of infected animals. Stair et al.in 1968 demonstrated the viral antigens of BTV in the endothelium (intima) of blood vessels (55); however, the immunofluorescence procedure was not deemed conclusive for bluetongue because viral antigen was randomly distributed producing a dust-like fluorescence (52). The immunofluorescent test has been reported to be more reliable when experimentally-infected C. variipennis was used and the head of the mosquito was found to be the best source of antigen (28).

Inoculation of susceptible sheep or 9 to 12 days embryonating chicken eggs (ECE) have been reported as sensitive methods for the isolation of bluetongue virus (15,19,20). Bluetongue virus-infected Culicoides variipennis can be assayed by the same methods (16). The intravascular (IV) route for inoculation of specimens for BTV isolation in ECE has been shown to be more sensitive than the yolk sac route (16, 20). Antigens of BTV have been seen in membranes of chicken embryos infected with the virus using the immunofluoresence technique (19). Chicken embryos affected by the virus die within 3 to 5 days after inoculation and have a characteristic cherry-red discoloration, edema, and petechial or ecchymotic hemorrhages on the chorio-allantoic membrane (32).

There are two commercially available bluetongue vaccines which are prepared in eggs or tissue culture for use in sheep. These vaccines are effective against the homologous vaccine serotype (e.g. BTV serotype 10) in the prevention of BT in vaccinated sheep. Egg-adapted vaccines have produced higher indexes of virus neutralizing antibodies in sheep than the tissue culture-adapted vaccines (35). However, the egg-adapted vaccines produced abortion when pregnant ewes were vaccinated with the greatest susceptibility in ewes who were in the 5th or 6th month of gestation (54).

An inactivated BTV vaccine has been recently developed (49). This experimental vaccine has induced the production of precipitating antibodies; however, induction of virus-neutralizing antibodies in the host has not been detected. This inactivated vaccine has protected inoculated animals upon challenge with virulent homologous BTV (49). Current data indicates that once bluetongue is present in an area or herd it is very difficult to eradicate (12). Management's only defense against

BTV appears to be by limiting the access of susceptible animals into areas where potential insect vectors are present. The lack of cross-protection between the different antigenic types of BTV argues for the development of diagnostic tests that identify the various antigenic types. The identification of a specific serotype in an area where bluetongue is endemic would be a major step for the control of bluetongue in ruminants.

CHAPTER III

MATERIALS AND METHODS

Specimen Submission and History of Cattle Herds

During 1980 and 1981 a total of 200 field cases were submitted for BTV isolation. Of these submissions, 90% came from cases where reproductive problems had occurred in the cattle herds. The clinical history obtained from most cattle herds were similar to the history presented in the following two herds. In both herds, a confirmation of infection with BTV was substantiated by the isolation of the virus.

Virus Isolation

Herd No. 1

A herd of 280 beef cattle, located in Canadian County in Oklahoma, was composed of two bulls, of Limousin and Angus breeds, and a mixture of Hereford, Hereford-Angus, and Charolais cows. A Pinzgauer bull from Montana was later introduced into the herd. During the 1980 calving season, a total of 6 to 7 fetal malformations occurred and herd infertility was very high. Blood samples obtained from 3 bulls and 6 cows ranging in age from 4 to 7 years, were sent to our laboratory for virus isolation and serology.

Herd No. 2

In 1981, a group of 35 Hereford heifers located in Major County in Oklahoma, were bred to a Longhorn bull from Colorado recently introduced into the herd. In a previous 15 year period no other introduction of cattle had occurred in this herd. In the first 3 parturitions of heifers bred to the Longhorn bull, two deformed calves were born. Specimens of tissues from the calves were submitted for virus isolation and sera from several animals were collected for serology for BTV.

Clinical Specimens

Heparinized blood was usually submitted to the laboratory for isolation of BTV. Bone marrow obtained from the long bones (usually the femur) was removed and processed for isolation of virus. Pools of tissues (e.g. liver, spleen, lung and kidney) were also used as 10% tissue homogenate for inoculation into embryonating chicken eggs (ECE). Blood was processed by lysing erythrocytes in sterile distilled water and after three washes with PBS the lysed cells were inoculated into ECE. Bone marrow and tissue homogenates were sonicated for one minute prior to the inoculation of ECE.

Embryonating Chicken Eggs

Specific pathogen-free (SPF) embryonating chicken eggs[a] at 9 to 12 days of embryonation were used to isolate BTV by intravascular inoculation. Following inoculation of the specimen, eggs were incubated at 33 C. Early death of the embryos, due to trauma caused by excessive

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[a]Larson Laboratories Egg Inc., Gowrie, Iowa 50543.

bleeding, was decreased from 30% to 5% by immediately sealing inoculated ECE. For diagnosis by the fluorescent antibody test, the head, wings, and legs of embryos with visible hemorrhages and edema were removed and the body of the embryo was then bisected. The head, anterior part of the body, and the chorionallanitoic membrane were placed onto a chuck with mounting fluid[b] then quick frozen and thin-sectioned (6 μm) in a cryostat.[c] Sections of the embryonic tissues were then reacted with fluorescein-labeled antibody to BTV. The posterior portion of the body of the embryo was homogenated and inoculated as a 10% suspension onto cell cultures.

Cell Cultures

Baby hamster kidney (BHK-21)[d], clone 13, and green monkey kidney, clone Maru (Vero-M)[d], were grown in Eagle's modified minimal essential medium[e] containing 0.1 mg/ml of gentamicin with 10% horse serum for BHK-21 and 10% bovine fetal serum for Vero-M cells.

Preparation of Bluetongue Virus (BTV) Pools

Serotypes 10, 11, 13 and 17 of BTV were obtained from the National Veterinary Services Laboratories (NVSL).[f] Each serotype available was propagated in monolayers of BHK-21 which were incubated at 37 C in a CO_2

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[b]Lab-Tek Products, Naperville, Illinois 60540.

[c]Damon/IEC Dividion, Needham Hts., Massachusetts 02194.

[d]American Type Tissue Culture Collection, Rockville, Maryland 20852.

[e]Flow Laboratories, McLean, Virginia 22102.

[f]Kindly provided by Dr. J. E. Pearson at NVSL, Ames, Iowa 50010.

incubator. Prior to inoculation with the virus, flasks (75 cm^2) containing monolayers of cells were washed with medium without serum, then inoculated with 5 ml of the virus suspension. Adsorption of the virus onto the monolayers of cells was done by incubation of inoculated flasks at 37 C for 1 hour on a rocker plate.[g] Following virus adsorption, the virus inoculum was removed and cells were refed with maintenance medium (Eagle's) which contained 2% horse serum and 0.1 mg/ml of gentamicin then incubated at 37 C in a 5% CO_2 incubator. When the cytopathic effect (CPE) of BTV completely destroyed the monolayer of cells, the virus-cell suspension was transferred into 50 ml centrifuge tubes and then frozen and thawed 3 times. To release membrane-associated virus, cells were sonicated[h] for one minute at a maximum intensity setting and followed by centrifugation at 340xg to remove cell debris in a IEC HN-S benchtop centrifuge.[c]

Electron Microscopy

Bluetongue virus serotype 10 and 17 obtained from infected monolayers of Vero-M cells was concentrated by centrifugation of 50,000 xg for 1 hour in a SW41 rotor in a L-G5 preparative centrifuge.[i] The pellet of virus was then resuspended in 0.5 ml of buffered normal saline. The virus suspension was sprayed onto three 200 mesh parlodion, carbon-coated grids with a nebulizer.[j] Grids were subsequently stained for

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[g]Bellco Glass Inc., Vineland, New Jersey 08360.

[h]Biosonic IV. VWR Scientific, San Francisco, California 94119.

[i]Bechman Instruments, Inc., Houston, Texas 77036.

[j]Pellco All-glass, Ted Pella Company, Tustin, California 92680.

20 seconds with 2% phosphotungstic acid (PTA) at pH 7.1. To visualize the membrane-associated virus, sonicated BHK-21 cells, previously infected with BTV, were fixed in fresh 2% glutaraldehyde, embedded in resin, and stained with 5% uranyl acetate and 0.3% lead citrate (32,58).

Agar-gel Immunodiffusion (AGID) Test

A solution of Agarose[k] agar (0.9%) was dissolved in 0.85% saline prepared in distilled water. The agar was boiled until it completely dissolved and then autoclaved at 15 lbs pressure for 10 minutes. The agar was then cooled to 45 C and 6 ml of agar was dispensed per 60X15 mm Petri plate. A thickness of 2.8 of agar was poured into each plate and cooled with lid off to permit the escape of water vapor. Wells for the immunodiffusion reagents were cut using a 7 well mold (Figure 1). Virus antigens were placed in the center well and BTV-positive serum was placed in alternate wells from the test serum samples. Plates injected with reagents were then incubated to room temperature (25-27 C) for 24 hours in closed humid chamber. Positive antiserum to BTV was provided by NVSL.[f]

Preparation of Soluble Antigens From BTV for Immunodiffusion and Immunoelectrophoresis

The soluble antigens of BTV were extracted according to the method described by Kanitz (31). Cell cultures of Vero-M in 490 cm^2 roller flasks[l] were inoculated with 10 ml of each of the 4 serotypes of BTV.

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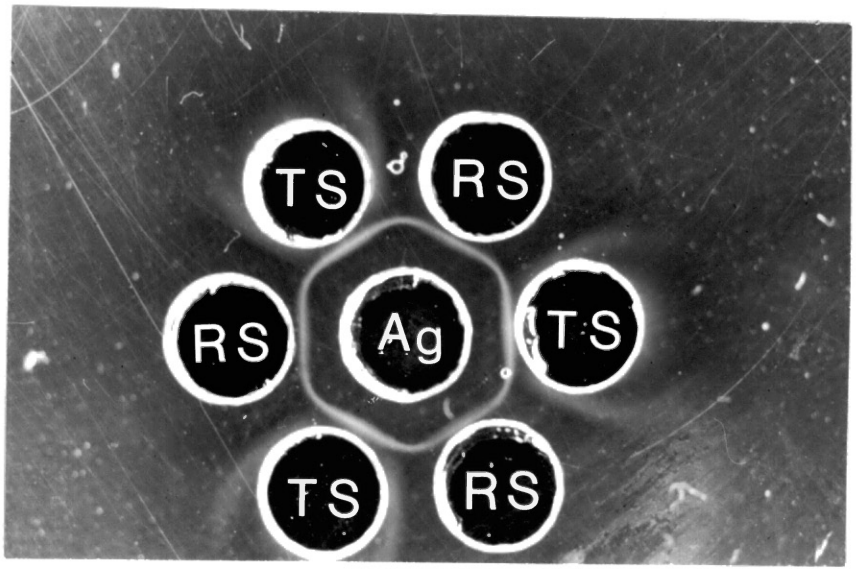
[k]Marine Colloids, Inc., Rockland, Maine 04841.

[l]Corning Glass Works, Corning, New York 14830.

Figure 1. Agar-gel Immunodiffusion Test for the Detection of Group Specific Antibodies to Bluetongue Virus. Arrow Indicates Continuous Line of Identity of Test Serum (TS) From a Cow and Bluetongue Positive Reference Sera (RS).
Ag = Bluetongue Virus Antigen.


TS
RS
RS
Ag
TS
TS
RS

When viral CPE was complete, 1/2 volume of 3% Tween-80[m] in 0.002 M phosphate buffered saline (PBS), pH 7.3 was added the virus suspension. The roller flasks were then incubated for an additional 2 hours at a speed of 3 revolutions per minute and following incubation, the mixture was frozen and thawed once. Supernatant fluids and disrupted cells were then transferred into 250 ml centrifuge tubes. These suspensions were centrifuged at 1,300 xg for 30 minutes and the supernatant fluids transferred into 500 ml beakers. The pellets were discarded. Cold, saturated ammonium sulfate[n] in 1/2 volume of virus suspension was then added slowly with constant stirring. The mixture was then transferred into 250 ml centrifuge tubes and allowed to equilibrate at 4 C for 30 minutes. The mixture was centrifuged at 16 C for 1 hour at 1,300 xg. The virus antigen was located at the surface of the suspension and the virus-containing layer had a "butter-like" appearance. The layer with virus was transferred into a 50 ml centrifuge tube and spun at 800 xg for 20 minutes. The salt layer located underneath the layer of virus was then discarded. The "butter-like" virus antigen was dissolved in equal volumes of CHES[o] (0.02 M CHES, 0.14 NaCl, pH 9.5) and 1 volume of PBS, sodium azide was added to a final concentration of 0.02%. The extracted virus was then stored at -20 C.

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[m]Fisher Scientific Company, Fair Lawn, New Jersey 07410.

[n]Sigma Chemical Company, St. Louis, Missouri 63178.

[o]Cyclohexylaminoethanosulfonic Acid, Sigma, St. Louis, Missouri 63178.

Immunoelectrophoresis

Immunoelectrophoresis was performed using an immunoelectrophoresis kit.[p] The barbital buffer used for electrophoresis was composed of 32.1% w/w Trimethamine (Tris), 13.7% w/w barbital, 54.2% w/w sodium barbital (ionic strength 0.031) at a pH of 8.6. For the preparation of each immunoframe, agar[q] was washed 3 times in distilled water and once in reagent grade acetone then dried for 12 to 24 hours. A volume of 62.5 ml of the barbital buffer was mixed with 2.5 g of washed agar and 187.5 ml of distilled water. Sodium azide, to prevent bacterial growth, was added to the buffer in a final concentration of 0.02%.

Six glass slides (25 mm x 75 mm) were placed on each leveled immunoframe. Agar was used as a sealant for glass slides on the immunoframes then 10 ml of agar were added to each prepared immunoframe. The agar-coated slides were placed in a humid, 4 C, chamber where they solidified overnight. Well patterns of 1.5 mm in diameter were cut on each slide using a commercial cutter.[p] The agar plugs were then removed and the wells filled immediately with extracted soluble antigen of BTV. The immunoframes were then placed on the electrophoresis apparatus in which the chambers were previously filled with 900 ml cold barbital buffer. Paper wicks[p] were placed in contact with the agar and buffer. A voltage of 300 or 400 volts was applied using a commercial power source for varying periods of time, namely 10, 20, or 30 minutes. Following each electrophoretic run, a trough (1 mm in width and 65 mm long) was cut with a razor blade on each side at a distance of 1.6 mm

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[p]Gelman Instruments Co., Ann Arbor, Michigan 48106.

[q]Noble Agar, Difco Laboratories, Detroit, Michigan 48200.

from the antigen well. Antiserum to each serotype of BTV was then placed into the trough. The slides were placed in a closed humid chamber at 4 C. After a 24 hour period, the immunoframes containing the slides were washed several times with 0.3 NaCl over a period of 24 to 48 hours to remove nonspecific precipitin lines.

Slides which were air-dried were stained with either light green or acid fuchsin. The acid fuchsin stain was prepared using 5 g acid fuchsin, 500 ml of absolute methyl alcohol, 100 ml of acetic acid, and 400 ml of distilled water. Stained slides were then decolorized for 45 minutes in a solution of 500 ml of absolute methyl alcohol, 100 ml of concentrated acetic acid, and 500 ml of distilled water. The light green stain was composed of 5 g light green, 700 ml of absolute methanol, 100 ml of glacial acetic acid, and 200 ml of distilled water. The slides were decolorized similar to the slides stained with acid fuchsin.

Preparation of Antiserum to Serotypes of BTV

Sixteen guinea pigs weighing between 400 to 800 grams were pre-bled prior to the inoculation of BTV. Four animals were used for the inoculation schedule with each serotype of BTV.

The inocula consisted of supernatant fluids obtained following sonication (1 minute) of BTV-infected BHK-21 cells. Viral fluids were emulsified with an equal volume of complete Freunds adjuvant.[r] Each total inoculum consisted of 0.1 ml injected subcutaneously (SQ) in alternated foot pads or in the flank. A volume of .10 ml was injected at weekly intervals during a 4 week period. Booster doses of identical

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[r]Difco Laboratories, Detroit, Michigan 48200.

material was SQ inoculated at the 6th and 8th after the initial injection. The guinea pigs were euthanitized by the 10th week of the inoculation schedule. Following clotting of blood collected, serum was separated by centrifugation at 1,300 xg. Sera were then stored at -20 C. The antisera produced in the guinea pigs against each serotype were tested for antibody to BTV by the AGID test.

CHAPTER IV

RESULTS

Clinical Observations in Calves Born Into Herds Infected With BTV

Developmental anomalies were observed in newborn or stillborn calves from bovine herds suspected of infection by BTV. The anomalies seen in a stillborn small-sized calf (Figure 2) from a herd where the history was unavailable included Kyphosis or a backward curvature of the vertebral column with deformities of the skull. A newborn calf from herd No. 2 was born with a shortened neck, due to scoliosis or lateral S-shaped curvature of the cervical vertebra and the first 8 thoracic vertebrae (Figure 3). Futhermore, this latter calf had permanent contracture of the carpal joints and over-extension of the fetlock joints or an arthrogryposis. The cleft palate with an accompanying hydrocephalus (Figure 4A, B) was seen in a third calf from another BTV-seropositive herd where "unthrifty or weak" calves had been born.

Serologic Tests on Cattle Herds Infected With BTV

From herd numbered 1, 8 of 9 animals were positive for antibodies to BTV by the complement fixation test. In this herd, BTV was isolated from 6 of 9 bovines. In herd numbered 2, the Longhorn bull and the 2 cows, each of which had a deformed calf, were seronegative for anti-

Figure 2. Neonatal Deformities Associated With Infection by Bluetongue Virus: A Stillborn Calf With Kyphosis and Domed Head.

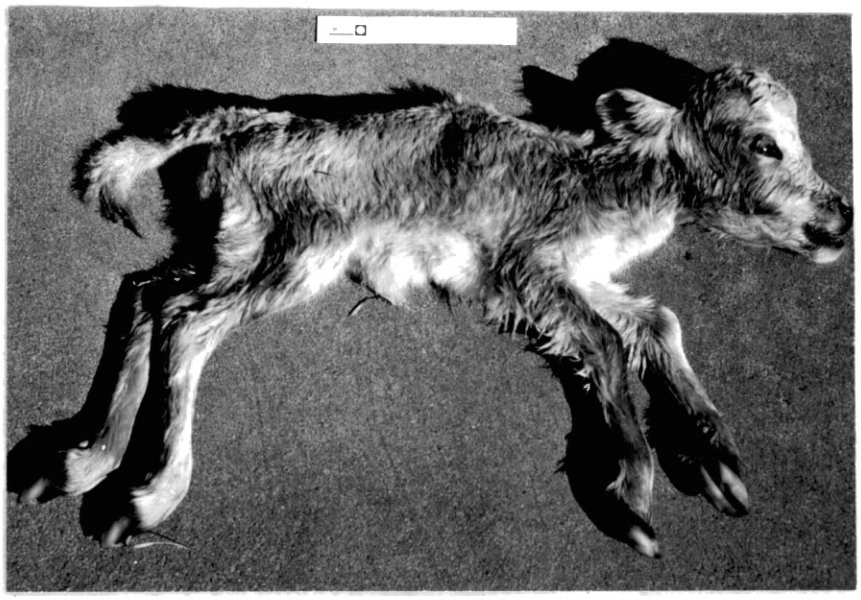
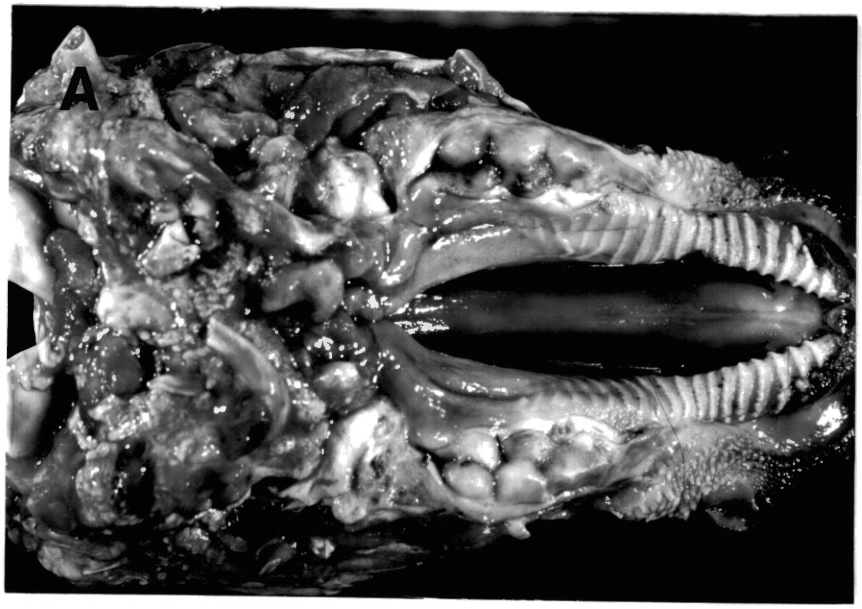
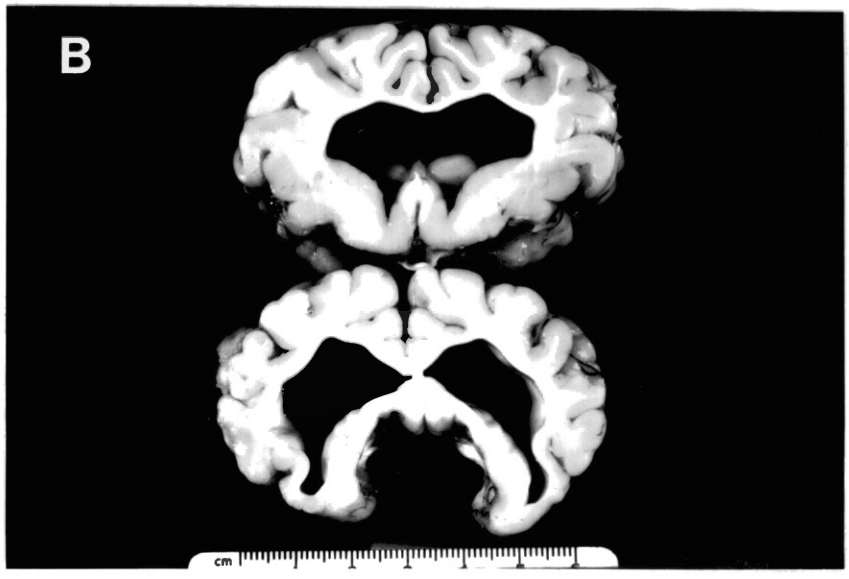


Figure 3. A Newborn Calf From Which Bluetongue Virus Was Isolated With Scoliosis of the Vertebral Column and Arthrogryposis of the Carpal and Contracture of Fetlock Joints.

Figure 4. Developmental Anomalies Seen in a BTV-infected Newborn Calf. A) Cleft Palate, B) Hydrocephalus.


A


B
cm

bodies to infectious bovine rhinotracheitis (IBR) and Bovine Viral Diarrhea (BVD) virus. The bull and one of the two cows tested from herd No. 2 were seropositive to BTV. Several bulls in the herd, which were in contact with the heifers when tested, produced one seropositive animal.

Clinical Histories and Virus Isolation Attempts From Field Cases

Bluetongue virus has been isolated and identified in our laboratory from 12 field cases during a two year period. In most submissions for the isolation of BTV, reproductive problems were present in the herd. In these herds, infertility was the primary complaint which in most cases was diagnosed as embryonic death by the veterinarian. Cattle which were detected as pregnant by rectal palpation were subsequently found open 60 to 90 days later. Several of these early embryonic deaths were confirmed as due to BTV by subsequent isolation of BTV. In 3 cases, further confirmation of a recent infection with BTV was suggested by high complement fixation titers to BTV and positive AGID tests. All the cows tested which had early embryonic deaths were positive for antibodies to BTV by the AGID test. Abortions and "unthrifty or weak calves" were often found in these cows in subsequent calvings. These "weak" calves usually did not nurse and died shortly after birth. Fetal malformations were also reported by the veterinarians to have occurred in cattle herds with the preceding clinical history.

Bluetongue virus was isolated in ECE from washed and lysed red blood cells and sonicated bone marrow tissue from the newborn calf from herd No. 2 (Figure 3) and the stillborn calf seen in Figure 2. The

developmental deformities in a third calf associated with infection by BTV are presented in Figure 4. These gross anomalies included a cleft palate and a hydrocephalus of the cerebral hemispheres. Bluetongue virus was isolated from tissues from the calf which had the gross anomalies presented in Figure 4. Embryonating chicken eggs inoculated with the fluid of erythrocytes from each of the 3 calves died within 3 to 5 days. The dead embryos had a characteristic "cherry-red" discoloration due to hemorrhages with an associated edema (Figure 5). Thin-sections of tissues from the BTV-infected embryos had fluorescence specific for BTV when reacted with fluorescein-conjugated antibody to BTV (Figure 6). A greater number of fluorescent foci specific for BTV were observed in the brain tissue of the dead embryos. Subsequently, BTV isolated in ECE was inoculated onto flasks which contained monolayers of BHK-21 and Vero-M cells. Cytopathic effects of BTV were usually seen after 2 or 3 blind passages in cell culture. The CPE of BTV in cellular monolayers of Vero-M was characterized primarily by rounding of the cells (Figure 7). The CPE was slow in developing and usually required 5 to 7 days before the entire cell monolayer was affected. Several isolates of BTV were sent to NVSL for the determination of the serotype of the virus. Previous results by a virus-neutralization assay in ECE, indicated that serotype 13 and 17 of BTV were present in cattle herds in Oklahoma.[s]

Experimental Studies on the Growth of BTV in Cell Culture

Due to the difficulty in isolating BTV directly in cell cultures

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[s]Dr. A. E. Castro, Oklahoma Animal Disease Diagnostic Laboratory, personal communication.

Figure 5. Chicken Embryos Infected With BTV; Two Normal Chicken Embryos Are Seen on Right and Two BTV-infected Hemorrhagic Embryos (Dark Areas) Are Presented on the Left.

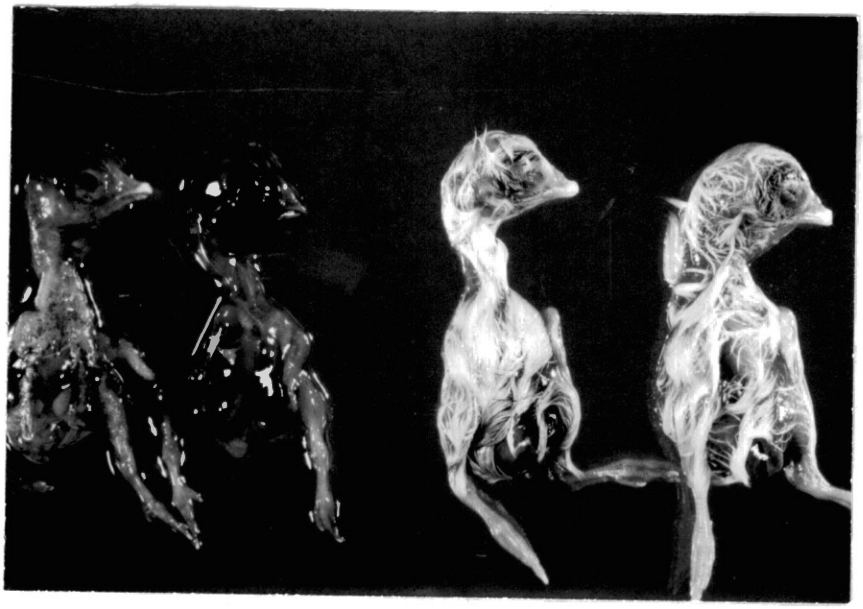


Figure 6. Specific Immunofluorescence (Circles) of the Antigen of BTV in Tissue Sections of a BTV-infected Chicken Embryo.

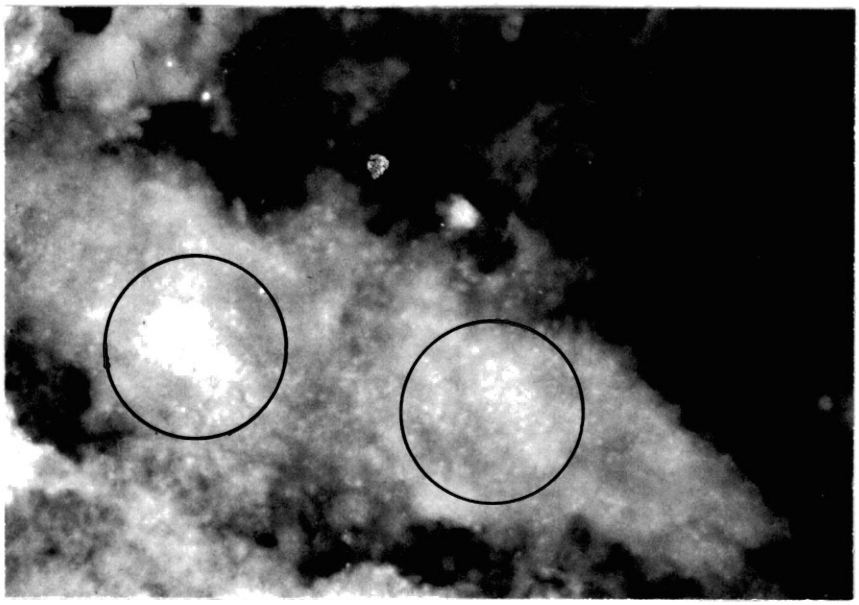
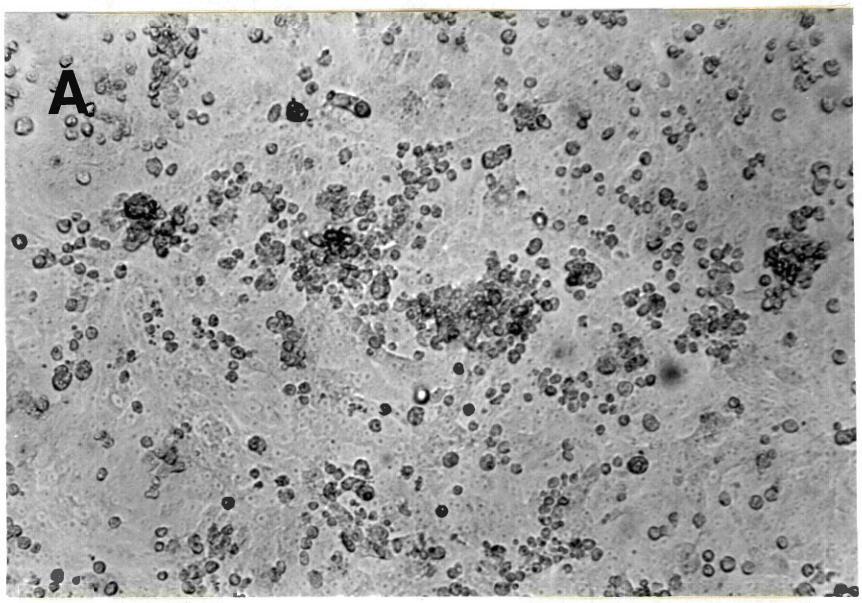
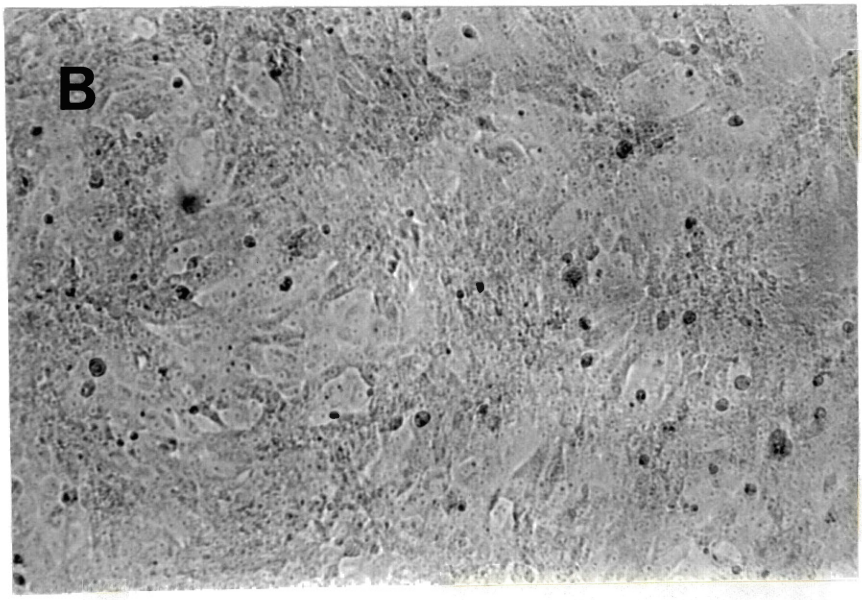


Figure 7. Cytopathic Effect of BTV in Living Vero-M Cell Cultures. A) Infected Cell Monolayer After 4 Days, B) Control Cell Monolayer.


A


B

and the low yield of BTV from field cases, attempts to increase the yield or concentration of BTV in cell culture were made. Serotypes of BTV 10 and 17 were inoculated onto BHK-21 cells and gently rocked during the adsorption of the virus. An increased yield of virus of up to 1000-fold was obtained with both serotypes tested when compared to the BTV yields from stationary cell cultures (Table 1).

To determine if virulence for cell culture was predominant with one serotype, duplicate cell cultures of BHK-21 and Vero-M each received an equal inoculum of a virus pool containing 10^4 TCID (50% tissue culture infectious doses) 50/ml. Serotype 17 of BTV was found to be more virulent as measured by CPE, than either serotypes 10, 11, or 13. The CPE produced by serotype 17 of BTV developed within 12 hours following inoculation of virus and the cellular monolayer was completely destroyed within 48 hours. The CPE seen with the other serotypes of BTV developed more slowly and CPE was observed by 24 hours with complete destruction of the cellular monolayer occurred by 60 hours.

Electron Microscopy of BTV-infected Cell Cultures

Attempts to purify virus from supernatant fluids obtained following sonication or several freeze-thaw cycles of BTV-infected cells were discouraging due to the low yields of virus. Therefore, pellets of BTV-infected cells disrupted by sonication, fixed, and stained were examined by electron microscopy to acertain the location of the virions. Virions of bluetongue were observed primarly associated with the membranous structures of the infected cells (Figure 8). Virions were observed attached to partially disrupted cellular membranes (Figure 8A, B). Viral

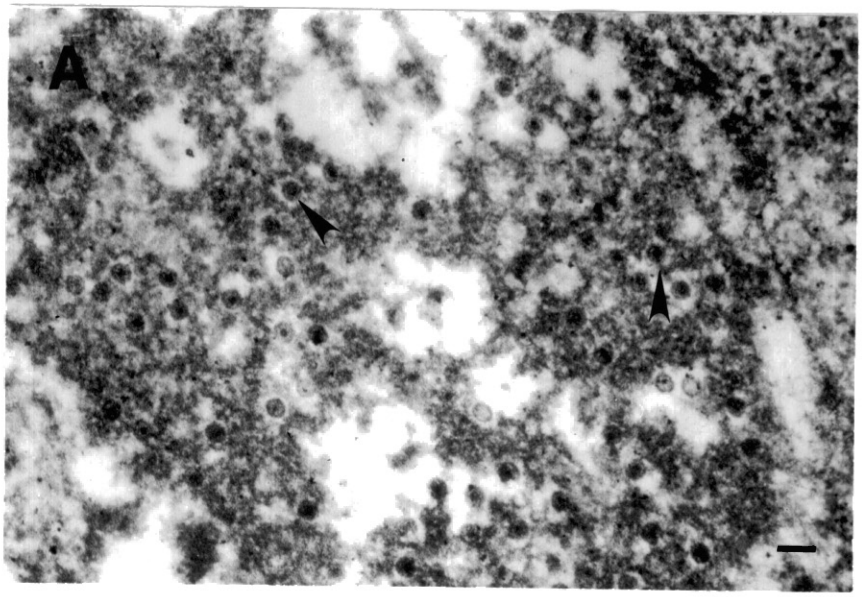
TABLE I

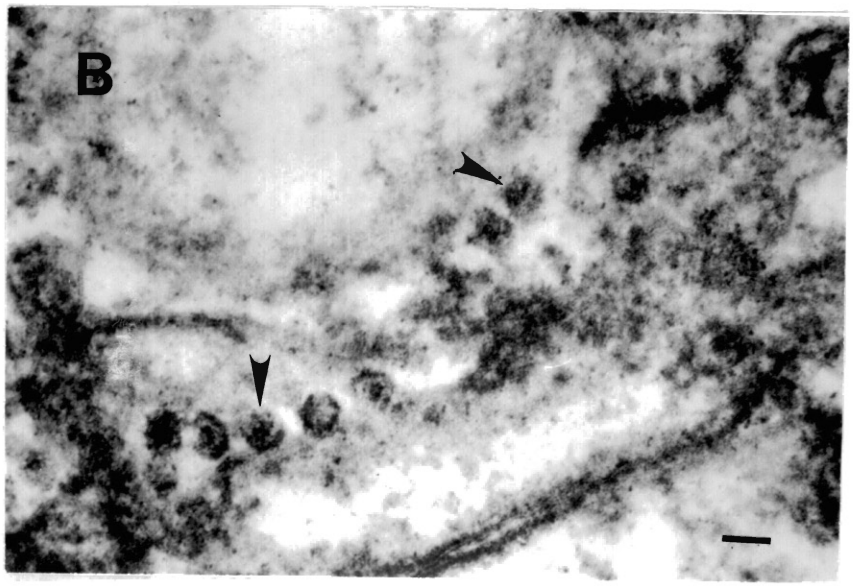
EFFECTS ON THE YIELDS OF BTV FOLLOWING ADSORPTION ONTO CELL CULTURES OF BHK-21 WITH ROCKING MOTION

Experiment No.	Bluetongue Virus Serotypes	Virus Titer After Adsorption Onto Cell Culture	
		Stationary	Rocked
1	10	5.4*	8.1
2	17	6.8	9.3

*Titer of Virus log 10 TCID 50/ml.

Figure 8. Electron Micrographs of BTV (Serotype 17) in Sonicated Cell Cultures of BHK-21 Cells. A and B) Numerous Viral Particles (Arrow Heads) Firmly Attached to Dissociated Membranes.


A


B

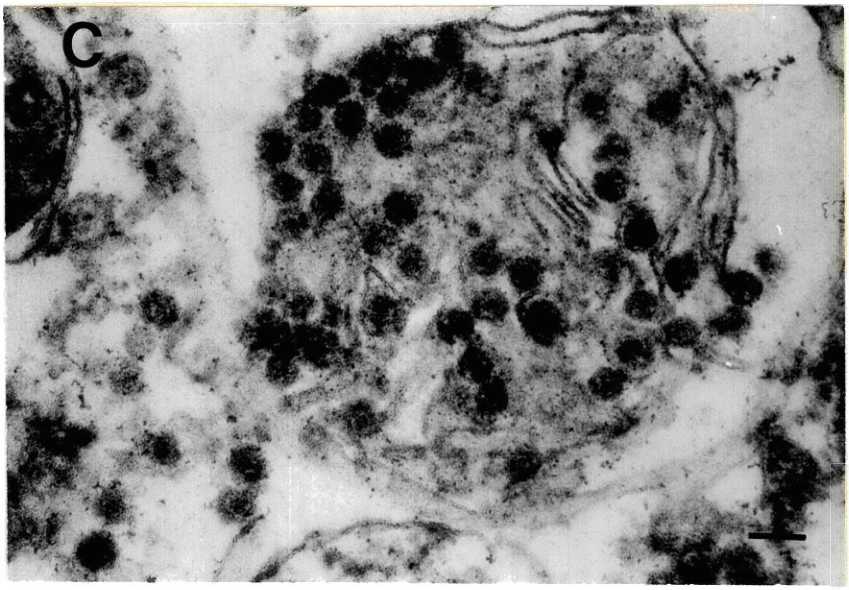
particles were also found in large aggregates inside vesicles (Figures 8C, D). Most of the virions located in these cells were either membrane-bound or within cellular vacuoles or vesicles and had an uniform size of 70 nm. Based on these electron microscopy observations, purification of BTV for extraction of soluble antigens was done on disrupted pellets of BTV-infected cells.

Electrophoretic Studies With Soluble Extracts From BTV-infected Cells

Attempts to determine if the electrophoretic pattern of the various serotypes of BTV were different was investigated using extracted soluble antigens of BTV.

A preliminary study using gel immunoelectrophoresis to ascertain if different precipitin lines occurred with the soluble extracts of serotypes 10, 11, and 13 of BTV is presented in Table II. The number of visible precipitin lines increased as the time increased in a which a 300 volt current was applied (Figures 9, 10). Certain common precipitin lines (Figure 10, lines c, d) were present with all 3 serotypes of BTV tested. Two bands (Figure 9, 10 bands a, b) which migrated rapidly toward the anode were found only when BTV serotype 13 was reacted with antiserum to BTV type 11. Line c (Figure 9) was observed to be composed of two bands when the time was increased to 45 minutes with a current of 300 volts (Figure 10). There were no lines observed when antiserum to BTV serotype 13 was used in each of the electrophoretic runs. When a current of 400 volts was applied for similar periods of time, precipitating lines were not seen with any of the 3 serotypes tested.

Figure 8. Electron Micrographs of BTV (Serotype 17) in Sonicated Cell Cultures of BHK-21 Cells. C) Association of 70 nm Viral Particles With a Mitochondria, D) Viral Particles (Arrow Heads) Enclosed in Cellular Vesicle. Cells Were Stained With Uranylacetate and Lead Citrate. Bar Represents 100 nm.


C

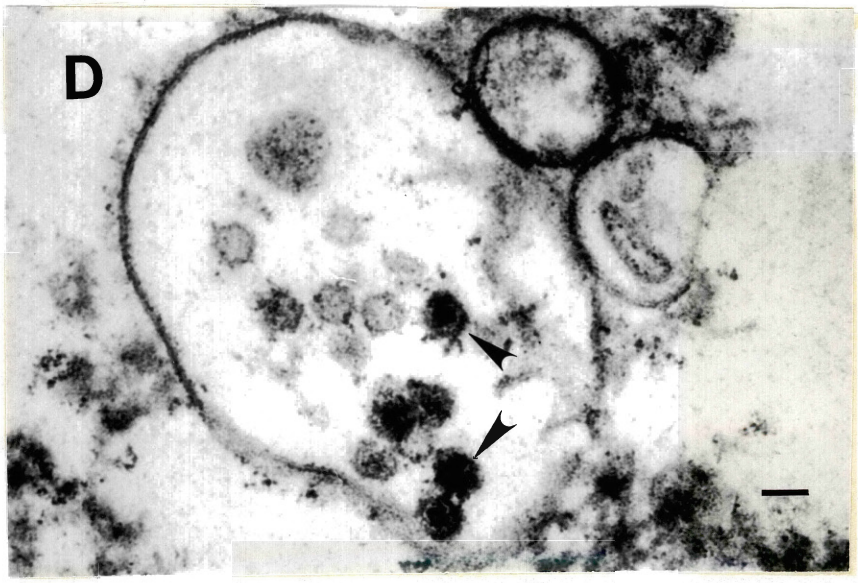

D

TABLE II

PRECIPITIN LINES OBSERVED FROM EXTRACTED SOLUBLE ANTIGENS OF 3 SEROTYPES OF BTV OBSERVED AFTER IMMUNOELECTROPHORESIS

Antiserum to BTV Serotypes	Extracted Soluble Antigens from BTV Serotypes											
	10				11				13			
	Minutes				Minutes				Minutes			
	10	20	30	45	10	20	30	45	10	20	30	45
10	c*	-	c	-	c	c	c	c,d	c	c	c	c,d
11	c	c	c,d	c,d	c	c	c	c,d	a,b,c	c	c	a,b c,d
13	-**	-	-	-	-	-	-	-	-	-	-	-
17	c	c	c	c,d	c	c	c	c,d	c	c	c	c,d

*Type of precipitin line present.

**No precipitin lines were present.

Figure 9. Immunoelectrophoretic Pattern of Extracted Soluble Antigens of Bluetongue (Serotype 13) Virus Using 300 Volts for 10 Minutes and Reacted Antiserum to Bluetongue (Serotype 11) Virus. A = Well Which Contained Soluble Viral Antigens; As = Trough in Which Antiserum Was Placed; a, b and c Were the Precipitin Lines Obtained. Agarose Gel Was Stained With Light Green. + = Anode, - = Cathode.

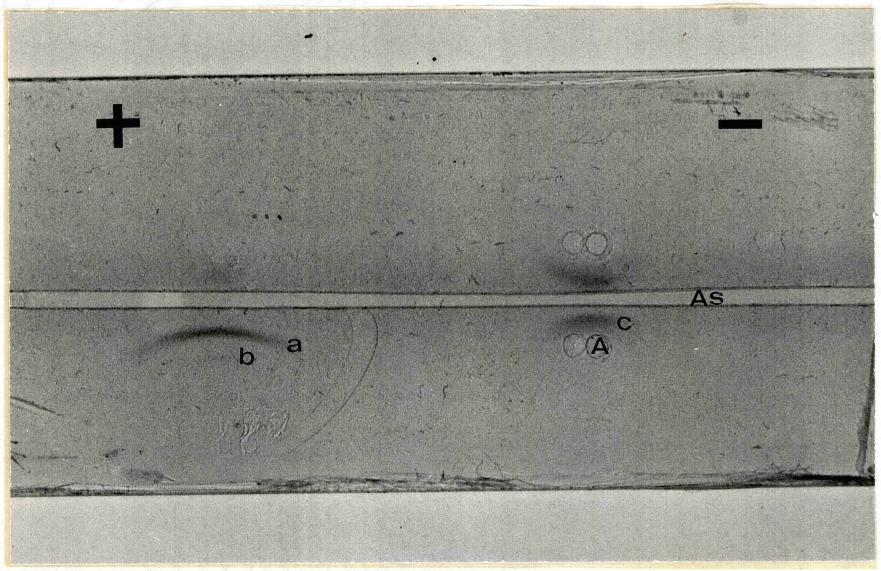
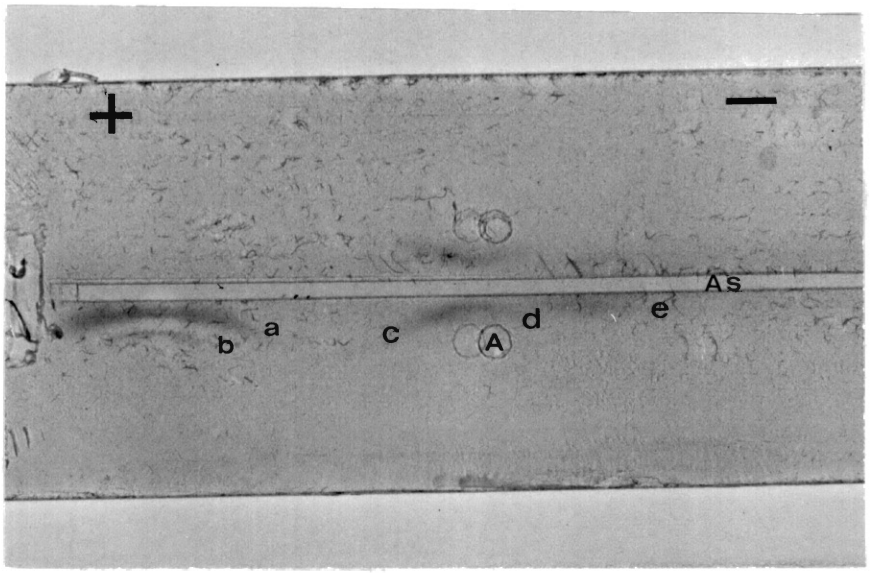

+
−
As
c
A
b
a

Figure 10. Immunoelectrophoretic Pattern of Extracted Soluble Antigens of Bluetongue (Serotype 13) Virus Using 300 Volts for 45 Minutes. Slide Was Allowed to React for 24 Hours With Antiserum to Bluetongue (Serotype 11) Virus. A = Well Which Contained Soluble Viral Antigens; As = Trough in Which Antiserum Was Placed; Letters a, b, c, and d Indicate Precipitin Lines Obtained and the Line Depicted by e Was Non-specific. Agarose Gel Was Stained With Acid Fuchsin.


+
—
As
b
a
c
A
d
e

Serologic Survey of Bovine Sera Submitted for Diagnosis of BTV

To determine the location of cattle herds in Oklahoma positive for antibodies to BTV, a serologic survey was done on cattle sera submitted for a cattle abortion profile or where reproductive problems were indicated in the clinical history. The number of seropositive cattle to BTV in Oklahoma, tested by the Oklahoma Animal Disease Diagnostic Laboratory, is presented in Table III. During 1979 and 1980, the percent of seropositive cattle for BTV which were tested by CF was 68.6%. The CF results reflect sera which had an antibody titer to BTV equal or greater than 5 or the reciprocal of the serum dilution. From October in 1980 through June in 1981, when the AGID test was first used, the percent of seropositive cattle to BTV was 56.6% (Table III). In Figure 11, the number of seropositive cattle in Oklahoma by county is presented. The greatest concentration of seropositive cattle came from herds located in the counties in the central and southern areas of the state.

TABLE III

CATTLE IN OKLAHOMA FOUND SEROPOSITIVE FOR BTV FROM 1978 TO 1981*

Results of Serology	YEAR					
	1979		1980		1981	
	CF[1]	AGID[2]	CF	AGID	CF	AGID
Number of Positives	843	ND	274	ND	50	372
Number of Negatives	387	ND	125	ND	146	284
Percentage of Positives	68.5	ND	68.6	ND	25.5	56.6

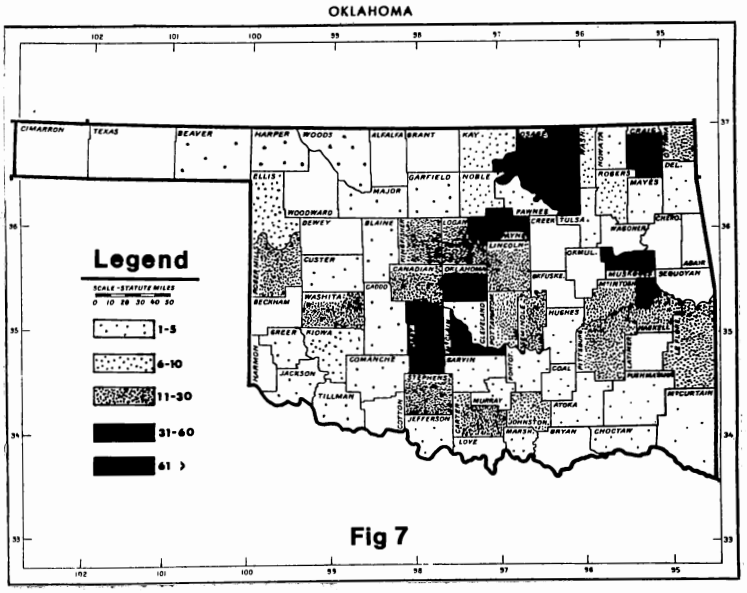
*From July 1, 1978 to June 30, 1981.

[1]CF = Complement Fixation.

[2]AGID = Agar-gel Immunodiffusion.

ND = Not Done.

Figure 11. A Map of Oklahoma Which Depicts the Distribution by County of Seropositive Cattle for BTV From Sera Submitted to the Oklahoma Animal Disease Diagnostic Laboratory From July, 1978 to June, 1981.


OKLAHOMA
Legend
SCALE - STATUTE MILES
0 10 20 30 40 50
1-5
6-10
11-30
31-60
61 >
CIMARRON
TEXAS
BEAVER
HARPER
WOODS
ALFALFA
GRANT
KAY
GARFIELD
NOBLE
MAJOR
ELLIS
WOODWARD
DEWEY
BLAINE
CUSTER
CANADIAN
OKLAHOMA
LOGAN
PAWNEE
CREEK
TULSA
OSAGE
ROGERS
MAYES
DELAWARE
CHEROKEE
WASHINGTON
NOWATA
CRAIG
OTTAWA
MUSKOGEE
SEQUOYAH
WAGONER
PITTSBURG
HUGHES
OKMULGEE
MCINTOSH
BECKHAM
WASHITA
CADDO
GREER
KIOWA
JACKSON
COMANCHE
GARVIN
COAL
ATOKA
TILLMAN
JEFFERSON
MURRAY
JOHNSTON
MARSHALL
BRYAN
CHOCTAW
LOVE
Fig 7

CHAPTER V

DISCUSSION AND CONCLUSIONS

The diagnosis of bluetongue infection in cattle and sheep is a laborious, time-consuming process. Until recent studies by Luedke (40) in cattle, little was known concerning the clinical disease in the bovine and the accompanying reproductive problems. This investigation was done to, a) explore possible methods in the laboratory of enhancing the isolation of virus from field cases in cattle and b) to describe certain of the associated fetal anomalies in BTV-infected cattle.

Developmental malformations such as arthrogryposis, hydrancephaly, and abnormalities of the vertebral column have been described in cattle experimentally-infected with BTV and also from field cases (43,45). The deformities observed in calves (Figures 2, 3 and 4) born to cows infected with bluetongue virus were distinctive and therefore, could be easily recognized by the veterinarian. Major gross anomalies observed were, a) domed head, b) a curvature of the vertebral column (e.g. Kyphosis or scoliosis), and c) arthrogryposis or an abnormal permanent contracture of the joints. By histopathology examination, porencephaly or cavities in the brain cortex, a characteristic feature was used in a tentative diagnosis of bluetongue infection when observed in aborted fetuses. Furthermore, reproductive problems such as infertility associated with early embryonic death (40) have also been seen in BTV-infected cattle.

In a 1981 field outbreak of bluetongue in Oklahoma cattle, a Longhorn bull newly introduced into the herd appeared to have been the carrier of BTV. The bull was seropositive for antibodies to BTV and the virus was isolated from a deformed calf sired by him. Viral isolation attempts from the bull were precluded by the early slaughter of the animal. A recent report indicated the presence of reproductive problems in 18 heifers bred to a BTV-carrier bull which shed the virus in the semen (13,40). When infertility was seen in cattle infected with BTV, early embryonic death was proposed as the cause. Furthermore, embryonic deaths have recently been observed in breeding experiments with a BTV-carrier bull (40).

In BTV-infected herds, calves that become infected in utero and are born normal can become carriers of the virus for their lifetime (38). These animals are usually seronegative, perhaps due to immunologic tolerance, but they harbor the virus and can transmit BTV to their offspring (38). Therefore, calves that are infected in utero should not be kept as replacement animals in the herd.

Transmission of BTV is primarily by the bite of an infected arthropod vector, Culicoides variipennis, which is a crepuscular (twilight) and noctural feeder (32). In Oklahoma, the midge probably overwinters in the larval form in the soil. The larvae of Culicoides requires a very wet environment and tolerates highly polluted waters which contain a high level of organic materials. The size of these insects is relatively small (2 mm in length) and they are difficult to see during their feeding on an animal. Keeping susceptible cattle away from the breeding areas of this insect and adequate protection in the evening for cattle may prevent transmission of BTV from animal to animal by the Cullicoides midge.

There are 20 known serotypes of BTV for cattle and sheep (45) and a variation in both immunogenicity and pathogenicity for animals has been demonstrated (36). In the present study, serotype 17 of BTV was found to be more virulent for cell cultures of BHK-21 and Vero-M cells than serotypes 10, 11, and 13 as measured by the extent and rapidity of CPE. This increased virulence for cell cultures by serotype 17 may reflect the prevalence and virulence of this serotype in the field, since serotype 17 has been identified in 40% of the cases where BTV has been isolated in the United States (4).

The diagnosis of BTV from field specimens required adequate concentrations of virus in the specimen used. By inoculation of blood samples into ECE followed by passage in cell culture, BTV has been successfully isolated from 12 of 200 submitted samples. Confirmation of the viral isolates as BTV was done by a direct immunofluorescence test on thin sections of BTV-infected embryos; however, by this technique the serotype of the virus could not be determined. This diagnostic procedure required 7 to 10 days to complete; however, it was the most reliable method for the isolation of BTV because field strains of BTV rarely propagate directly onto cell culture.

Electron microscopy studies indicated that BTV was highly membrane-associated as previously reported by other investigators (14). The close association of BTV with cellular components could explain, a) the difficulty in the isolation of BTV from field specimens and b) the failure to find high yields of cell-free virus from supernatant BTV-infected cells. Therefore, for the isolation of BTV from field specimens, both supernatant and cellular debris from lysed erythrocytes were inoculated into ECE. This isolation method for BTV, using cellular

stroma appeared to enhance the success in the isolation of virus from clinical specimens.

Attempts to pass BTV isolated in ECE onto cell cultures usually produced no virus or low levels of virus and required several passages in cell culture. A cell culture rotary tube method has been shown to increase the frequency of BTV isolates from field specimens (3), hence, when BTV was isolated in ECE and subsequently passed into cell cultures, the flask containing cells and virus was placed on a rocker plate for 1 hour to enhance the uptake of virus. This method of adsorption has increased the yield of BTV from inoculated cells up to 1000-fold (Table I).

Identification of numerous viral antigens has been accomplished by their ability to move in an electrical field (18). Preliminary results using this technique to identify differences in the antigens of the various serotypes of BTV were slightly encouraging. In most immunoelectrophoretic tests done, a consistent precipitating component was found adjacent to antigen-well with each of the serotypes tested (Table II). This precipitin line, which occurred as a single or double line, probably reflects the group-specific antigen for BTV. This group-specific antigen of BTV produces the line also detected by AGID (50). Two other fast-migrating precipitin lines were found when viral serotype 13 was reacted with antiserum to serotype 11 of BTV. These additional lines could reflect other viral antigens associated specifically with serotype 13. Because these were preliminary experiments, no attempts were made to quantify the protein concentration in each of the prepared viral extracts. An increased protein concentration in these viral extracts might have revealed other lines or minor antigens which

could identify specific for a serotype of BTV; however, this will require large volumes of BTV. Though, initial experiments using IEP revealed slight differences in one of the 4 serotypes examined, future investigations by IEP might be productive in describing unique antigens to each serotpye thereby, enhancing the accuracy of identifying field strains of BTV.

The number of cattle seropositive for antibodies to BTV is dramatically high in Oklahoma which reflects a high exposure rate to BTV. The distribution of BTV-infected cattle, as measured by serology, appears to be concentrated in counties in the central and southern areas of the state. These serologic results in cattle may reflect, a) a greater number of submissions from these areas or b) a greater concentration of cattle in these counties exposed to the virus. The CF and AGID tests measure primarily immunoglobulin G_1 in the bovine. Thus, the results by each of these serologic tests are probably comparable. However, with the AGID test there was a slight increase in the percentage of total seropositive cattle during fiscal 1981. In a recent survey in cattle, a high percentage of BTV-infected cattle occurred in the southern part of the United States (46), which is the area where the susceptible vector is primarily found. Until additional information is available concerning the distribution of BTV and its transmission in the field, valid recommendations for its control are tenuous.

The inherent difficulty in the identification of bluetongue in cattle remains a problem primarily due to the nominal clinical signs in affected cattle and low mortality in adult cattle. The major problems reported in the field in cattle herds affected with BTV are reproductive. Therefore, if abortions or infertility is detected in a herd,

bluetongue should be a prime consideration. Further investigations into the method of propagating virus in cell culture thus enhancing virus isolations will accelerate the diagnosis of BTV. Finally, a rapid diagnosis procedure is required to identify the various serotypes of BTV to ascertain the prevalence of BTV in the field and guard against the introduction of new serotypes of increased virulence or possible viral recombinants. This study has attempted to increase the knowledge of BTV infection in cattle and its laboratory diagnosis so that an effective and accurate diagnosis of bluetongue can be achieved.

BIBLIOGRAPHY

1. Afshar, A., H. Kayranfar. "Occurrence of Precipitating Antibodies to Bluetongue Virus in Sera of Farm Animals in Iran". Vet. Rec., 94 (1974), 233-235.

2. Alstad, A. D., D. Burger, F. W. Frank, C. S. McCain, T. Moll, E. S. Stauber. "Localization of Bluetongue Virus in Red Blood Cells of Experimentally Infected Sheep". Am. Assoc. Vet. Lab. Diag. Proc., 20th Ann. Mtg. (1977), 273-290.

3. Bando, B. M. "Isolation of Bluetongue and Epizootic Hemorrhage Disease Viruses in Cell Culture". Am. Assoc. Vet. Lab. Diag. Proc., 18th Ann. Mtg. (1975), 163-174.

4. Barber, T. L. "Temporal Appearance, Geographic Distribution, and Species of Origin of Bluetongue Virus Serotypes in the United States". Am. J. Vet. Res., 40 (1979), 1654-1656.

5. Barber, T. L., M. M. Jochim. "Serotyping of Bluetongue and Epizootic Hemorrhagic Disease Viral Strains". Am. Assoc. Vet. Diag. Proc., 18th Ann. Mtg. (1975), 149-162.

6. Blood, D. C., J. A. Henderson, D. M. Radostitis. Veterinary Medicine, 5th ed., Lea & Febiger, Philadelphia, 1979.

7. Boulanger, P., J. F. Frank. "Serological Methods in the Diagnosis of Bluetongue". Aust. Vet. J., 51 (1975), 185-194.

8. Bowne, J. G., M. M. Jochim. "Cytopathological Changes and Development of Inclusion Bodies in Cultured Cells Infected with Bluetongue Virus". Am. J. Vet. Res., 28 (1962), 1091-1105.

9. Bowne, J. G., A. J. Luedke, M. M. Jochim. "Bluetongue of Sheep and Cattle: Past, Present, and Future". J. Am. Vet. Med. Assoc., 151 (1967), 1801-1803.

10. Bowne, J. G., A. J. Luedke, M. M. Jochim, H. E. Metcalf. "Bluetongue Disease in Cattle". J. Am. Vet. Med. Assoc., 153 (1968), 662-668.

11. Breckon, R. D., A. J. Luedke, T. E. Walton. "Bluetongue Virus in Bovine Semen: Virus Isolation". Am. J. Vet. Res., 41 (1980), 439-442.

12. Erasmus, B. J. "The Control of Bluetongue in an Enzootic Situation". Aust. Vet. J., 51 (1975), 209-210.

13. Foster, N. M., M. A. Alders, A. J. Luedke, T. E. Walton. "Abnormalities and Virus-like Particles in Spermatozoa from Bulls Latently Infected with Bluetongue Virus". Am. J. Vet. Res., 41 (1980), 1045-1048.

14. Foster, N. M., R. D. Breckon. "Bluetongue Virus Maturation in Cultured Cells". E. M. S. A. Proc., 38th Ann. Mtg. (1980), 482-483.

15. Foster, N. M., A. J. Luedke, H. E. Metcalf. "Bluetongue in Sheep and Cattle: Efficacy of Embryonating Chicken Eggs in Viral Isolation." Am. J. Vet. Res., 33 (1978), 77-81.

16. Foster, N. M., A. J. Luedke. "Direct Assay for Bluetongue Virus by Intravascular Inoculation of Embryonating Chicken Eggs". Am. J. Vet. Res., 29 (1968), 749-753.

17. Gambles, R. M. "Bluetongue of Sheep in Cyprus". J. Comp. Path. Therap., 59 (1949), 176-190.

18. Garvey, J. S., N. E. Cremer, D. H. Sussdorf. Methods in Immunology, 3rd ed., W. A. Benjamin Inc., Massachusetts, 1977.

19. Gleiser, C. A., E. L. Stair, L. O. McGill. "Diagnosis of Bluetongue in Cattle by Intravascular Inoculation of Chicken Embryos and Immunofluroescence". Am. J. Vet. Res., 30 (1969), 981-986.

20. Goldsmit, J., E. Barzilai, A. Tadmor. "The Comparative Sensitivity of Sheep and Chicken Embryos to Bluetongue Virus and Observation of Viremia in Experimentally Infected Sheep". Aust. Vet. J., 51 (1975), 190-196.

21. Goor, S. "Bluetongue of Sheep in Palestine". Refush. Vet., 7 (1950), 147-148.

22. Howell, P. G. Bluetongue, in Emerging Diseases of Animals. F.A.O. Agri. Studies No. 61, F.A.O. of U.N., Rome, 1963.

23. Howell, P. G. "The Antigenic Classification and Distribution of Naturally Occurring Strains of Bluetongue Virus". J. South Afr. Vet. Med. Assoc., 41 (1970), 215-223.

24. Howell, P. G., D. W. Vermored. "Bluetongue". Virology Monographs, 9 (1971), 37-74.

25. Hubbert, W. T., J. H. Brynes, P. C. Estes, J. W. Foley. "Bovine Bluetongue: Viral Isolation from Placentoma and Serologic Survey in Pregnant Cows". Am. J. Vet. Res., 33 (1972), 1879-1882.

26. Hubschle, O. J. B. "Applying ELISA and Hemagglutination Inhibition Techniques for Detection of Bluetongue Virus Antibodies". Vet. Lab. Diag. Proc., 2nd Int. Symp. (1981), 244-247.

27. Hubschle, O. J. B., R. J. Lorenz, H. D. Matheka. "Enzyme-linked Immunosorbent Assay for Detection of Bluetongue Virus Antibodies". Am. J. Vet. Res., 12 (1981), 61-65.

28. Jennings, M. J. Booman. "Use of Indirect Fluorescent Antibody Technique for Detection of Bluetongue Virus Antigen in Tissue Smears from Culicoides variipennis". Vet. Micro., 5 (1981), 13-18.

29. Jochim, M. M., T. L. Chow. "Immunodiffision of Bluetongue Virus". Am. J. Vet. Res., 30 (1969), 33-41.

30. Jochim, M. M., A. J. Luedke, T. L. Chow. "Bluetongue in Cattle: Immunogenic and Clinical Responses in Calves Inoculated in Utero and After Birth". Am. J. Vet. Res., 35 (1974), 517-522.

31. Kanitz, C. L. "Immunodiffusion Test for Bluetongue and Epizootic Hemorrhagic Disease: A Rapid, Simple Method for Preparing Soluble Antigens". Am. Assoc. Vet. Lab. Diag. Proc., 20th Ann. Mtg. (1977), 291-302.

32. Liendo, G. E., A. E. Castro. "Bluetongue in Cattle: Diagnosis and Virus Isolation". The Bovine Practitioner, 16 (1981), 87-95.

33. Luedke, A. J. "Bluetongue in Sheep: Viral Assay and Viremia". Am. J. Vet. Res., 30 (1969), 499-509.

34. Luedke, A. J., J. G. Bowne, M. M. Jochim, C. Doyle. "Clinical and Pathological Features of Bluetongue in Sheep". Am. J. Vet. Res., 25 (1964), 963-970.

35. Luedke, A. J., M. M. Jochim. "Clinical and Serologic Responses in Vaccinated Sheep Given Challenge Inoculation with Isolation of Bluetongue Virus". Am. J. Vet. Res., 29 (1968), 841-851.

36. Luedke, A. J., M. M. Jochim, J. G. Bowne, R. H. Jones. "Observations on Latent Bluetongue Virus Isolation in Cattle". J. Am. Vet. Med. Assoc., 156 (1970), 1871-1899.

37. Luedke, A. J., M. M. Jochim, R. J. Jones. "Bluetongue in Cattle: Effects of Vector Transmitted Bluetongue Virus on Calves Previously Infected in Utero". Am. J. Vet. Res., 38 (1977), 1697-1700.

38. Luedke, A. J., M. M. Jochim, R. J. Jones. "Bluetongue in Cattle: Effects of Culicoides variipennis-transmitted Bluetongue Virus on Pregnant Heifers and Their Calves". Am. J. Vet. Res., 38 (1977), 1687-1695.

39. Luedke, A. J., R. H. Jones, M. M. Jochim. "Transmission of Bluetongue Between Sheep and Cattle by Culicoides variipennis". Am. J. Vet. Res., 28 (1967), 457-460.

40. Luedke, A. J., T. E. Walton. "Effect of Natural Breeding of Heifers to Bluetongue Virus Carrier Bull". The Bovine Practitioner, 16 (1981), 96-100.

41. Mason, J. J., W. D. Neitz. "The Susceptibility of Cattle to the Virus of Bluetongue". J. Vet. Sci., 15 (1940), 149-157.

42. Matthews, R. E. F. "Classification and Nomenclature of Viruses". Intervirology, 12 (1979), 200-203.

43. McKercher, D. G., J. K. Saito, K. V. Singh. "Serologic Evidence of an Etiologic Role of Bluetongue Virus in Hydranencephaly of Calves". J. Am. Vet. Med. Assoc., 156 (1970), 1044-1047.

44. Metcalf, H. E., M. M. Jochim. "Bluetongue in Cattle: Efficacy of Agar-gel Precipitin Test". Am. J. Vet. Res., 31 (1970), 1743-1749.

45. Metcalf, H. E., A. J. Luedke. "Bluetongue and Related Diseases". The Bovine Practitioner, 15 (1980), 188-193.

46. Metcalf, H. E., J. E. Pearson, A. L. Klingsporn. "Bluetongue in Cattle: A serologic Survey of Slaughtered Cattle in the United States". Am. J. Vet. Res., 40 (1981), 1057-1061.

47. Moulton, J. E. "Pathology of Bluetongue of Sheep in California". J. Am. Vet. Med. Assoc., 138 (1961), 493-498.

48. Neitz, W. O. "Immunologic Studies on Bluetongue in Sheep". J. Vet. Sci. Anim. Ind., 23 (1948), 93-118.

49. Osburn, B. I., J. L. Stott, T. L. Barber, M. Sawyer. "Development of an Inactivated Bluetongue Virus Vaccine". Fed. Proc., 38 (1971), 1159.

50. Pearson, J. E., D. R. Dowing, A. Gustafson, M. R. Swamson, H. E. Metcalf. "A Comparison of the Immunodiffusion Test and the Modified Direct Complement Fixation Test for the Detection of Antibody Against Bluetongue Virus in Cattle and Sheep". Am. Assoc. Vet. Lab. Diag. Proc., 22nd Ann. Mtg. (1979), 147-156.

51. Robertson, A., M. Appel, G. L. Bannister, G. M. Ruckerbauer, P. Boulanger. "Studies of Bluetongue. II. Complement Fixing Activity of Ovine and Bovine Sera". Can. J. Comp. Med., 29 (1965), 113-117.

52. Ruckerbauer, G. M., D. P. Gray, A. Girard, G. L. Bannister, P. Boulanger. "Studies in Bluetongue Virus. Detection of Virus in Infected Materials of Immunofluorescence". Can. J. Comp. Med. Vet. Sci., 31 (1967), 175-181.

53. Sarwar, M. M. "A Note on Bluetongue in Sheep in West Pakistan". Pak. J. Anim. Sci., 1 (1962), 1-2.

54. Shultz, G., P. D. DeLay. "Losses in Newborn Lambs Associated with Bluetongue Vaccination of Pregnant Ewes". J. Am. Vet. Med. Assoc., 127 (1955), 224-228.

55. Stair, E. L. "The Pathogenesis of Bluetongue in Sheep: A Study by Immunofluorescence and Histopathology". (Unpublished Ph.D. Thesis. Texas A&M University, (1968).

56. Tamer, Y. "Bluetongue Disease of Sheep in Hatay (Turkey) and Losses Over Five Years". Turk. Vet. Hekembler Dergisi., 19 (1949), 542-548.

57. Van Der Walt, N. T. "A Haemagglutination and Haemagglutination Inhibition Test for Bluetongue Virus". Onderstepoort. J. Vet. Res., 47 (1980), 113-117.

58. Venable, J. H., R. Coggeshall. "S Simplified Lead Citrate Stain for Electron Microscopy". J. Cell. Biol., 25 (1965), 407-408.

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