Bluetongue in Cattle: Diagnosis and Virus Isolation

Guillermo Liendo, *D.V.M., Graduate Student,*
Department of Veterinary Parasitology, Microbiology &
Public Health, College of Veterinary Medicine,
Oklahoma State University, Stillwater, OK 74078
ánd
Anthony E. Castro, *D.V.M., PhD.,*
Oklahoma Animal Disease Diagnostic Laboratory,
Oklahoma State University, Stillwater, OK 74078

Summary

In certain cattle herds infected with bluetongue virus (BTV) and located in Oklahoma, abortions and developmental anomalies have been observed. Arthrogryposis, scoliosis and kyphosis were present in neonates and fetuses from several bluetongue-infected cattle. Bluetongue virus has been isolated from bone marrow and washed red blood cells from several of these fetuses. Bluetongue serotypes 13 and 17 were identified by serum-virus neutralization from these affected herds. Field isolates of BTV have been adapted to cell cultures following isolation in embryonating chicken eggs. Bluetongue virus was found to be highly membrane-associated, which added to the complexity in isolation attempts. However, increased yield of BTV in cell cultures was obtained by continuous gentle rocking during adsorption of virus to cellular monolayers.

Serologic data obtained from cattle during a 3-year period from July 1, 1978 to June 30, 1981 by complement fixation (CF) and agar-gel immunodiffusion (AGID) tests indicated an infection rate of 68% by CF from July 1, 1978 to June 30, 1980 and 49.5% by CF and AGID from July 1, 1980 to June 30, 1981. Recommendations for handling cattle infected by BTV are also presented.

Introduction

Bluetongue disease was first described in sheep in South Africa in the 1800's and subsequently worldwide (9, 10, 17). In the United States Bluetongue virus (BTV) was first isolated and identified in 1952 from affected sheep in California (15, 16). But clinically, the disease in sheep had been recognized earlier in Texas and described as "soremuzzle" (17). Bluetongue in cattle was first reported between 1889 and 1904 and called "Mycotic stomatitis" (17). In 1975, BTV was initially isolated from *Culicoides* gnats in Australia. More recently in Brazil, BTV was isolated from domestic cattle.

Bluetongue as a clinical disease in cattle is usually inapparent (17). Clinically, however, bluetongue in cattle is characterized by lameness or stiffness and inflammation with subsequent ulceration of the oral mucosa. A necrosis of the epithelium of the muzzle may occur producing a burnt appearance. Laminitis that may lead to sloughing of the hoof is a common finding in bluetongue-infected cattle. Infertility, abortions and developmental anomalies have been seen in bluetongue-infected herds (5).

The etiological agent of bluetongue is an Orbivirus which contains double-stranded RNA and is 60 to 80 nm in diameter (11). There are 20 known serotypes of BTV distributed worldwide (17). In the United States, only 4 serotypes of BTV (10, 11, 13, and 17) are found. The virus is membrane-associated (8,22) and found primarily within red blood cells (1), bone marrow and in the intima of blood vessels (22). The virus is transmitted by an insect vector, *Culicoides variipennis*, also known as "biting midges, punkies or no-see-ums" (21) (Fig. 1).

Bluetongue infection can be diagnosed by serology using the modified complement fixation (CF) test and by agar-gel immunodiffusion (AGID) (4). Isolation of BTV from clinical specimens is done in embryonating chicken eggs and viral isolates are identified by either serum-virus neutralization or a fluorescent antibody test (4).

In this report we present information about the clinical disease as observed in field cases, methods used in the isolation of BTV from malformed fetuses, and the results of 3 years of serologic testing for the disease.

Materials and Methods

The data presented is from two cattle herds whose history suggested BTV infection.

Herd No. 1 - A herd of 280 beef cattle located in Canadian county, Oklahoma, was composed of Limousin and Angus bulls and a mixture of Hereford, Hereford-Angus, and Charolais cows. A Pinzgauer bull from Montana was introduced into the herd. Infertility was very high and 6 to 7

LIVING PROOF YOU CAN PREVENT CALF SCOURS*

Calf #62 and thousands more like him are alive today because their dams were vaccinated with VICOGEN prior to calving.[1]

Whether used to protect beef herds in Montana or dairy calves in New York, VICOGEN is the only clinically proven vaccine to prevent calf scours due to enteropathogenic strains of *E. coli* with the unique K99 antigen.

VICOGEN is an inactivated bacterial vaccine made from a concentrated culture of *E. coli*, containing high levels of K99 antigen. As you may know, only those strains of *E. coli* with the K99 pili (antigen) cause calf scours.[2] The VICOGEN-vaccinated
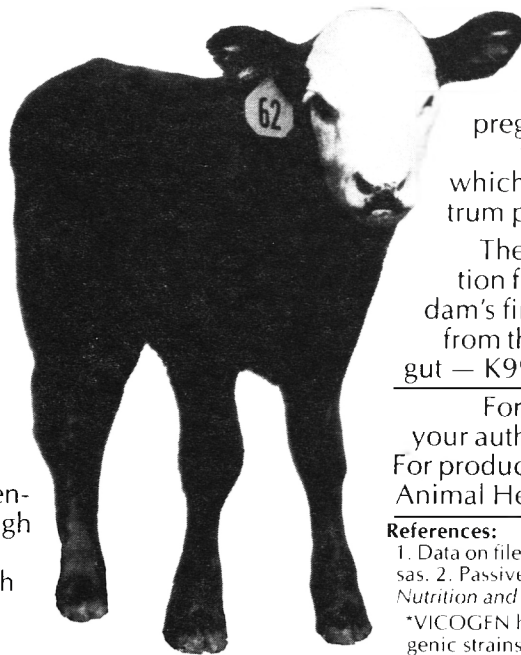
pregnant dam produces antibodies to the K99 antigen in her blood which then concentrate in the colostrum prior to calving.

The newborn calf receives protection from maternal antibodies in the dam's first milk. It's as simple as that — from the cow's colostrum to the calf's gut — K99 protection you can count on.

For ordering information, contact your authorized Connaught distributor. For product information, call Connaught Animal Health (toll-free) 800-255-3056.

References:
1. Data on file, Connaught Animal Health, Lenexa, Kansas. 2. Passive immunity for neonatal diarrhea. *Animal Nutrition and Health:* 30-32, March 1980.

*VICOGEN has proven effective against enteropathogenic strains of *E. coli* bacteria

Escherichia Coli Bacterin

VICOGEN™

Because K99 protection is all that counts in the prevention of E. coli calf scours

CONNAUGHT
ANIMAL HEALTH, INC.
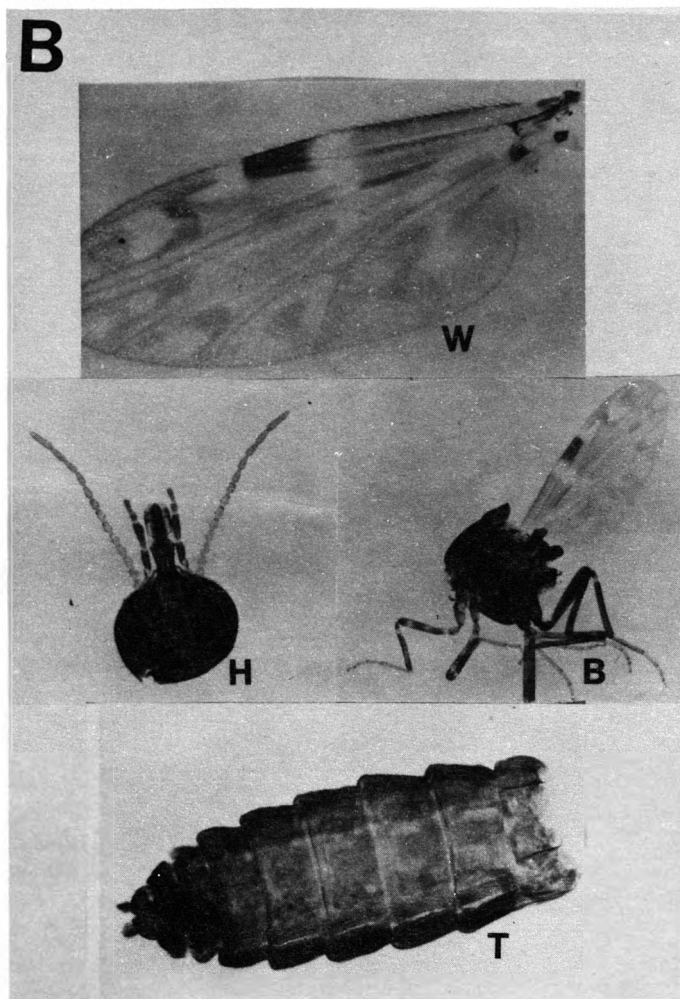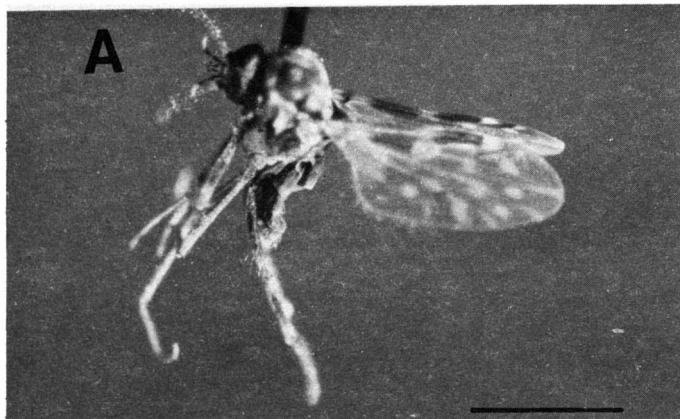8365 Quivira Rd.
Lenexa, Kansas 66215

fetal malformations occurred during 1980. Blood samples from 9 animals, 3 bulls and 6 cows with an age range of 4 to 7 years, were sent to our laboratory.

Herd No. 2 - A group of 35 Hereford heifers located in Major county, Oklahoma, were bred to a Longhorn bull recently introduced into the herd and purchased in Colorado. In the previous 15 years no new introduction of animals had occurred in the cow herd. Two deformed calves were born from the first 3 parturitions in the heifers bred to the Longhorn bull.

Cell culture - Baby hamster kidney cells (BHK-21) clone 13 and green monkey kidney cells clone Maru (Vero-M) (a) were grown in Eagle modified minimum essential medium (b) and 10% horse serum with 0.1 mg/ml of gentamicin.

Bluetongue virus serotypes - Bluetongue virus serotypes 10, 11, 13 and 17 were obtained from National Veterinary Services Laboratory (NVSL) (c).

Preparation of bluetongue virus pools - Bluetongue virus serotypes 10, 11, 13 and 17 were propagated in monolayers of BHK-21 cells incubated at 37°C. Cell monolayers were washed with media without serum, then inoculated with 5 ml suspension of virus. Adsorption of the virus on cell monolayers was done by incubation of 37°C for 1 hour on a rocker plate (d). 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 and incubated under CO_2 at 37°C. When cytopathic effects (CPE) of the virus completely destroyed the cell monolayer, the virus-cell suspension was transferred to 50 ml centrifuge tubes and frozen and thawed 3 times. To release membrane-associated virus, cells were sonicated (e) and the maximum intensity setting for one minute followed by low speed centrifugation (849xg) to remove cell debris.

Embryonating chicken eggs - Nine to eleven day old specific pathogen-free embryonating chicken eggs (ECE) (f) incubated at 33°C were used to isolate BTV by intravascular inoculation. Death of the embryos, due to bleeding, was diminished by sealing them immediately following inoculation. The head, wings and legs of inoculated embryos with hemorrhages and edema were removed and the embryo was bisected. Part of the embryo was thin sectioned by a cryostat for use in immunofluorescence testing and part was homogenated and inoculated onto cell cultures. Frozen section of the embryonic tissues were reacted with fluorescein-labeled antibodies to BTV.

Fig. 1. (A) *Culicoides sp.* Bar represents 1 mm.
(B) *Culicoides variipennis* vector for bluetongue virus; component parts used for identification: Photograph shows W = wing with spicules, H = head with biting parts, B = body, T = caudal portion (27x)

Electrol Microscopy - Virus obtained from infected monolayers was concentrated by centrifugation at 50,000 xg for 1 hour in a SW41 rotor and a L-65 preparative centrifuge (g). The viral pellet was resuspended in 0.5 ml of buffered saline. The virus was placed onto 200 mesh parlodium-coated grids with a nebulizer (h). Carbon-coated grids were stained for 20 seconds with 2% phosphotungstic acid (PTA) at pH 7.1. To observe membrane-associated virus, sonicated BHK-21 cells were fixed in 2% glutaraldehyde, embedded in resin and stained with uranyl acetate and lead citrate (23).

Agar-gel immunodiffusion tests (Fig. 6) were performed according to the method described by Jochim and Pearson (12).

Results

Eight of nine animals from Herd No. 1 were diagnosed positive for antibodies to BTV by CF. Furthermore, BTV was isolated from 6 of these 9 animals. The Longhorn bull and the 2 cows with deformed calves from Herd No. 2 were negative for antibodies to infectious bovine rhinotracheitis virus and bovine virus diarrhea. The bull and one of the two cows were seropositive to BTV. Several bulls that were in contact with the heifers were also tested and one was also seropositive to BTV.

Developmental abnormalities typical for BTV were observed in 2 calves submitted for necropsy (Fig. 2 A, B). The anomalies in small sized calf (Fig. 2A) where the herd history was unknown included kyphosis of the vertebral column with deformity of the skull. The other calf from herd 2 (Fig 2B) had a very shortened neck due to scoliosis of the cervical vertebra which also included the first 8 thoracic vertebrae. In addition, the latter calf had a permanent contracture of the carpal joints and over-extension of the fetlock joints.

Bluetongue virus was isolated in ECE from washed red blood cells and sonicated bone marrow cells from the calf born in Herd 2. Chicken embryos inoculated with the specimens died within 3 to 5 days following inoculation. The infected embryos had a characteristic cherry-red discoloration due to hemorrhages and associated edema (Fig. 3A). Frozen sections of tissues from BTV-infected embryos had specific fluorescence when tested directly with fluorescein-conjugated antibody to BTV (Fig. 3B). Subsequently, BTV isolated from these infected embryos was passaged twice in BHK-21 and once in Vero-M cell cultures. A cytopathic effect, characterized by rounding of the cells, was observed in Vero-M cells at 24 hours and progressed to complete destruction within 5 to 7 days (Fig. 4).

g) *Beckman Instruments, Inc., Houston Texas 77036*
h) *Pellco All-glass, Ted Pella Company, Tustin, California 92680*
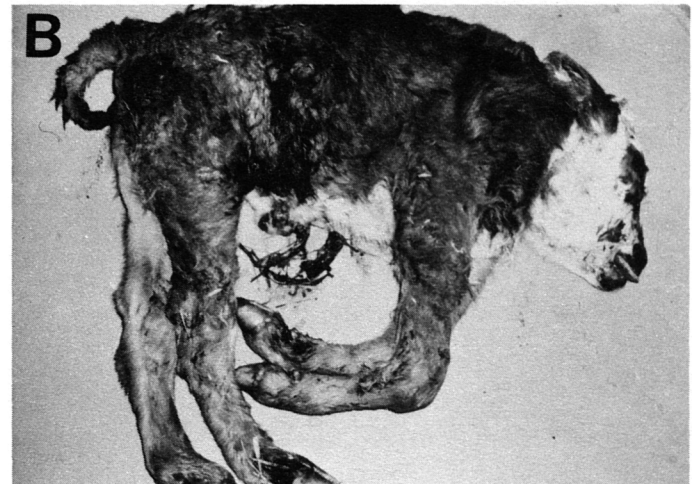

Fig. 2   Developmental anomalies in calves associated with infection by bluetongue virus. (A)   Stillborn calf with kyphosis and domed head. (B)   Newborn calf with scoliosis of the vertebral column and arthrogryposis of front and rear limbs.

Table 1

Cattle in Oklahoma Seropositive for Bluetongue Virus from 1978 to 1981*

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

[1]CF = Complement Fixation
*From July 1, 1978 to June 30, 1981
[2]AGID = Agar-gel immunodiffusion
 ND = Not Done

Fig. 4. Cytopathic effect seen by day 5 in Vero-M cell culture with bluetongue virus isolated from newborn calf depicted in Figure 2B.
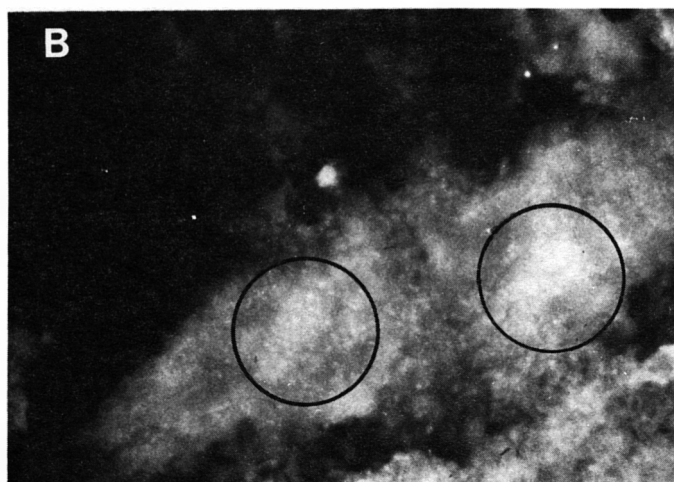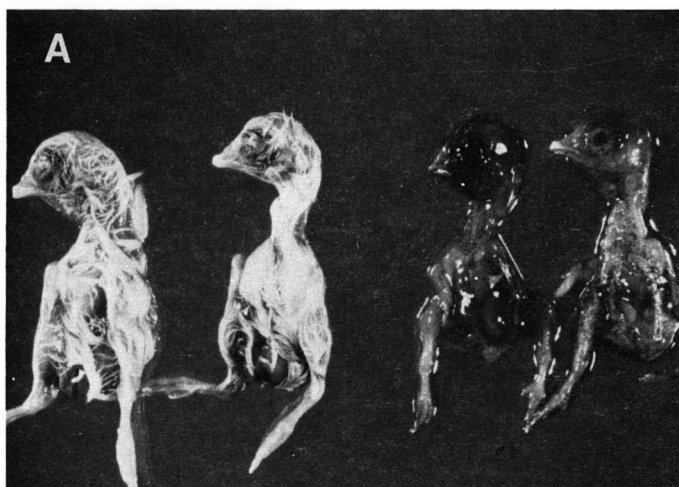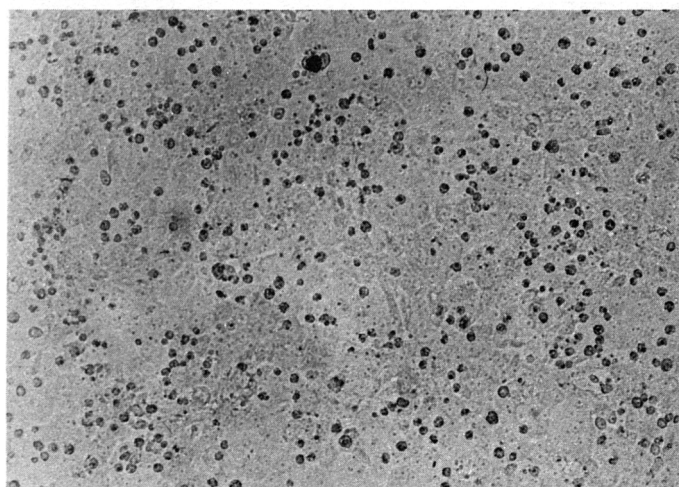
Fig. 3 Bluetongue-infected chicken embryos and immunofluorescence: (A) Two normal chicken embryos on left; two bluetongue-infected hemorrhagic (dark areas) embryos on right; (B) Specific immunofluorescence (circles) for bluetongue-antigen in frozen section of bluetongue-infected chicken embryo.

Due to the complexity in the purification of BTV, pellets of disrupted cells were examined by electron microscopy for the presence of the virus. Virions of bluetongue were observed associated primarily with membranous structures of the infected cells (Fig 5A). Virions were observed attached to partially disrupted cellular membranes (Fig. 5 B, C) and viral particles were also found in large aggregates inside vesicles (Fig. 5D).

Further studies to determine the virulence of BTV in cell cultures of BHK-21 and Vero-M receiving equal concentrations of the virus indicated that BTV serotype 17 was more virulent, as measured by CPE, than serotypes 10, 11, or 13. The CPE induced by BTV serotype 17 developed by 12 hours following inoculation and the monolayer was completely destroyed within 48 hours. The CPE for the other serotypes of BTV developed more slowly and was seen by 24 hours with complete destruction by 60 hours.

In attempts to increase the yield of BTV from cell cultures inoculated with field isolates, serotypes 10 and 17 were inoculated onto BHK-21 cells and rocked during adsorption of virus. An increase of 1000-fold in virus yields occurred with both serotypes tested when compared to virus yields from cell culture held stationary during virus adsorption. Therefore, continuous movement of virus over the cellular monolayer was critical in enhancing virus yields.
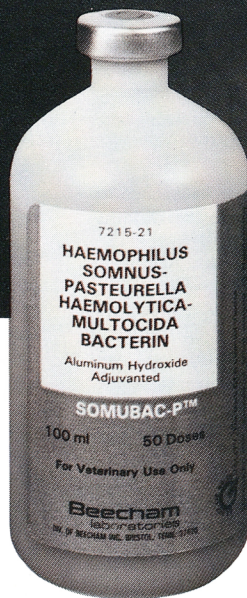
In Table 1, were present the number of seropositive Oklahoma cattle tested for BTV in our laboratory. The percent of seropositive cattle by CF was 68.6% in 1980. The CF test results are shown for sera which had a titer equal to or greater than 1:5 to BTV. The number of seropositive cattle by AGID was 56.6% during a 9-month test period extending from October 1980 through June 1981. In Fig. 7, the number of seropositive cattle in Oklahoma are shown by county. The greatest concentration of seropositive cattle came from herds located in the counties in the central part of the state.

Discussion

Developmental anomalies such as arthrogryposis, hydranencephaly, and abnormalities of the vertebral column have been described in cattle experimentally-infected with BTV and in field cases (13, 14). Furthermore, breeding problems such as infertility, repeat breeding and abortions have also been seen in BTV-infected cattle. In a 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 bluetongue and BTV was isolated from deformed calves
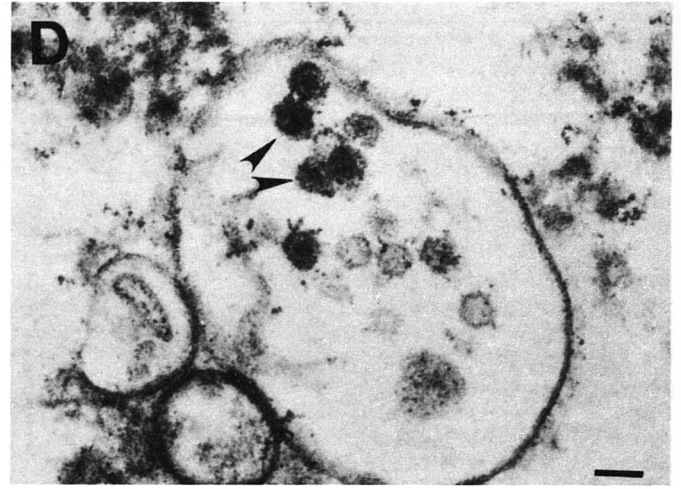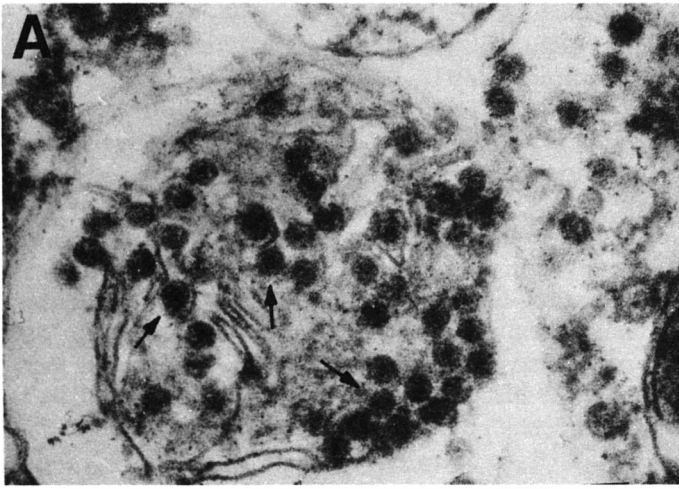
Fig. 5.   Electron micrographs of bluetongue virus (serotype 17) in sonicated cultures of BHK-21 cells: (A) Association of 70 nm viral particle (arrows) with mitochondria; (B and C) Numerour viral particles (arrowhead) firmly attached to dissociated membranes; (D) Viral particles (arrowhead) enclosed in cellular vesicle. Bar represents 100 nm.
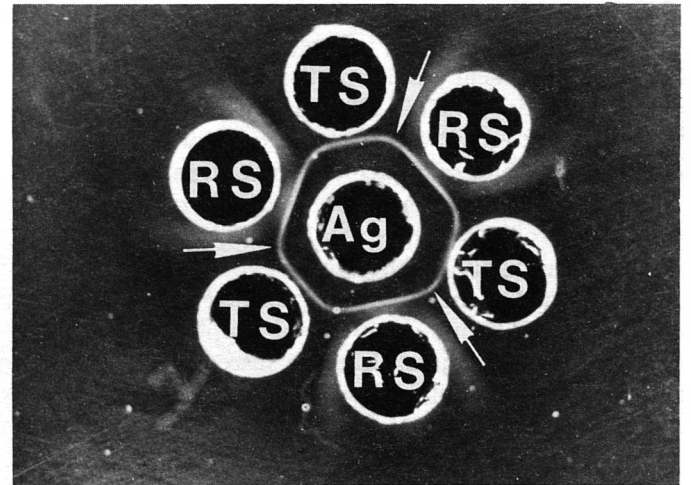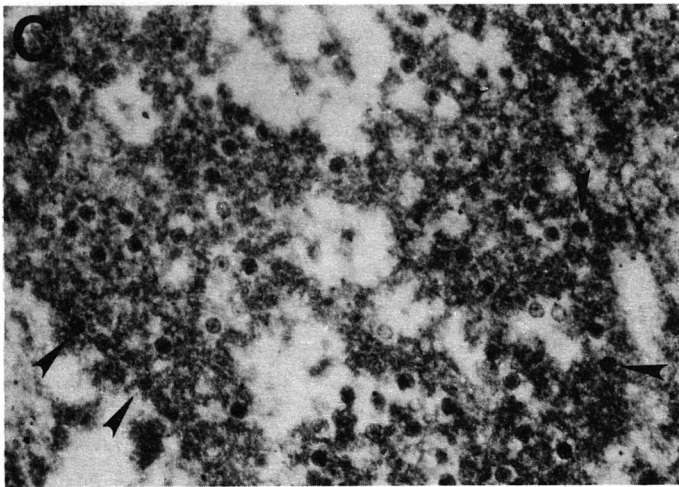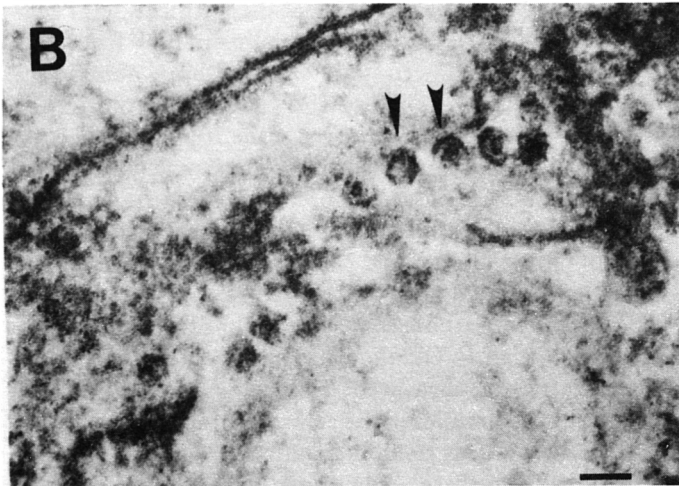
Fig. 6.   Agar-gel immunodiffusion test for the detection of group specific antibodies to bluetongue virus. Arrows indicate continuous line of identity of test serum (TS) and bluetongue positive reference sera (RS). Ag = Bluetongue virus antigen.
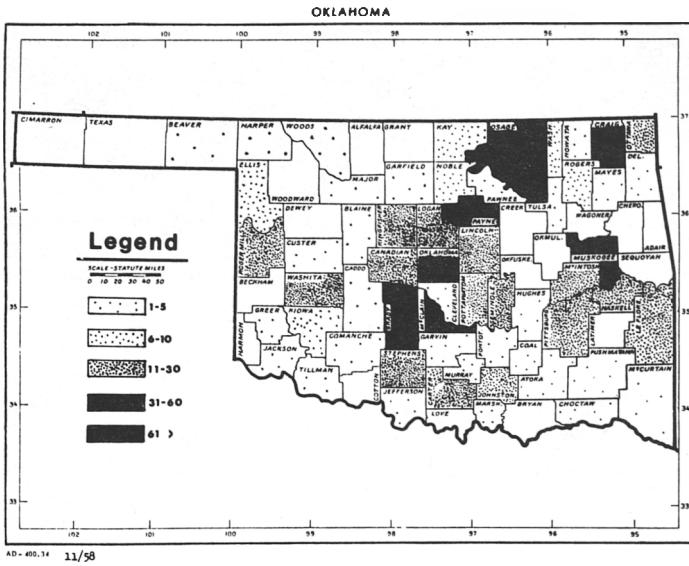
Fig. 7. A map of Oklahoma which depicts the distribution by county of seropositive cattle for bluetongue virus from sera submitted to the Oklahoma Animal Disease Diagnostic Laboratory from July 1978 through June 1981.

sired by him. Viral isolation attempts were precluded by the early slaughter of the bull. Thus, in this field outbreak, deformed calves were observed and by serology the animals tested were seropositive for antibodies to BTV.

In BTV-infected herds, calves that become infected *in utero* and are born normal can become carriers of the virus for their lifetime. These animals are usually seronegative, perhaps due to immunologic tolerance, but they can transmit BTV to their offspring.* In breeding experiments, bluetongue-infected bulls were able to shed virus in the semen and infect susceptible heifers and their offspring (7). 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 insect vector, *Culicoides variipennis* (Fig. 1), which are crepuscular (twilight) and nocturnal feeders. In Oklahoma, the insect probably overwinters in the larval form in the soil. The larvae require a very wet environment and tolerate highly polluted waters which contain a high level of organic materials. The size of these insects is relatively small (2 mm), thus they are difficult to see during their time on an animal. Keeping susceptible cattle away from breeding areas of this insect and adequate protection in the evening may prevent transmission of the virus from animal to animal by the *Culicoides* midge.

There are 20 known serotypes of BTV for cattle and sheep (17) and variation of BTV in both immunogenicity and

*Metcalf, H. Denver Federal Center, Denver, Colorado 80425 (personal communication)

pathogenicity for animals has been shown (13). In our studies, we found that BTV serotype 17 was more virulent for cell cultures of BHK-21 and Vero-M cells than serotypes 10, 11, and 13. This virulence in cell culture may be a reflection of the prevalence of serotype 17 which has been identified as 40% of the BTV isolated in the United States (3).

The diagnosis of BTV from field specimens is a laborious time-consuming procedure which requires adequate concentrations of virus in the specimen used. By inoculation of blood samples into ECE followed by passage in cell culture, we have been successful in isolation of virus from several BTV-infected herds. We were able to diagnose BTV using a direct immunofluorescence test on frozen sections of BTV-infected embryos, however, this technique cannot identify the serotype of the virus.

Our results indicated that BTV is highly membrane-associated as reported by other investigators (8, 22). The close association of BTV with cellular debris may account for the difficulty in the isolation of virus from field specimens and in the complexity of the purification of cell-free virus (22). Therefore, for the isolation of BTV from field specimens, both supernatant and cellular debris are inoculated into ECE. The use of a cell culture rotary tube method has been shown to increase the frequency of BTV isolated from field specimens (2). Thus, when the virus isolated in ECE are passaged in cell cultures, the virus-cell flask is placed on a rocker plate for 1 hour to enhance the uptake of virus. This method has increased the yield of BTV from cells by a 1000 fold.

The number of cattle seropositive for antibodies to BTV is dramatically high in Oklahoma. The distribution of BTV-infected cattle, as measured by serology, appears to be concentrated in counties in the central and southern parts of the state (Fig. 7). This result may reflect a greater number of submissions from these areas or a greater concentration of cattle exposed to the vector. The CF and AGID tests both measure primarily immunoglobulin G_1 (6). Thus, our results by both serologic tests used probably reflect comparable distribution data on seropositive animals with a slight increase in the percentage of total seropositive cattle during fiscal 1981. In a recent survey in cattle located in Mississippi, 34% of the cattle tested were positive for antibodies to BTV and the AGID test (20). Therefore, a high percentage of BTV-infected cattle occur in the southern part of the United States (18). Until additional information is available concerning the distribution of BTV and its transmission in the field, valid recommendations for its control are tenuous.

Acknowledgement

References

1. Alastad, A. D., Burger, D., Frank, F. W., McCain, C. S., Moll, T., Stauber, E. S.: Localization of bluetongue virus in red blood cells of experimentally infected sheep. Proc. Am. Assoc. Vet. Lab. Diagnosticians 20th Ann. Mtg: 273-290, 1977. - 2. Bando, B. M.: Isolation of bluetongue and epizootic hemorrhagic disease virus in cell culture. Proc. Am. Assoc. Vet. Lab. Diagnosticians 18th Ann. Mtg: 149-162, 1976. - 3. Barber, T. L.: Temporal appearance, geographic distribution, and specis of origin of bluetongue virus serotypes in the United States. Am. J. Vet. Res. 40: 1654-1656, 1979. - 4. Boulanger, P., Frank J. F.: Serological Methods in the Diagnosis of bluetongue virus. Aust. Vet. J. 51: 185-194, 1975. - 5. Bowne, J. G., Luedke, J. A., Jochim, M. M., Metcalf, H. E.: Bluetongue disease in cattle. J.A.V.M.A. 153: 662-668, 1968. - 6. Butler, J. E.: Synthesis and distribution of immunoglobulins. J.A.V.M.A. 163: 795-800, 1973. - 7. Foster, N. M., Alders, M. A., Luedke, A. J., Walton, T. E.: Abnormalities and virus-like particles in spermatozoa from bulls latently infected with bluetongue virus. Am. J. Vet. Res. 41: 1045-1048, 1980. - 8. Foster, N. M., Breckon, R. D.: Bluetongue virus maturation in cultured cells. Proc. E.M.S.A. 38th Ann. Mtg: 482-483, 1979. - 9. Howell, P. G.: Bluetongue in Emergin disease of animals, F.A.O. agri. studies N: 61, F.A.O. of U. N., Rome 1963. - 10. Howell, P. G., Vermored, D. W.: Bluetongue. Virology monographs 9: 37-74, 1971. - 11. Jawetz-Melnick, E. L., Adelberg, E. A., Review of medical microbiology, 14th ed., page 329, 1980. - 12. Jochim, M. M., Pearson, J. E.: Protocol for the immunodiffusion test for bluetongue, Proc. Am. Assoc. Vet. Lab. Diagnosticians 22nd Ann. Mtg: 463-471, 1979. - 13. Luedke, A. J., Jochim, M. M., Bowne, J. G., Jones, R. H.: Observations on latent bluetongue virus infection in cattle. J.A.V.M.A. 156: 1871-1879, 1970. - 14. Luedke, A. J., Jochim, M. M., Jones, R. H.: Bluetongue in cattle: Effects of *Culicodes variipennis* - transmitted bluetongue virus on pregnant heifers and their calves. Am. J. Vet. Res. 38: 1687-1695, 1977. - 15. McGowan, B.: An epidemic resembling soremuzzle or bluetongue in California sheep. Cornell Vet. 43: 213-216, 1953. - 16. McKercher, D. G., McGowan, B., Horarth, J. A., Saito, J. K.: A preliminary report on the isolation and identification of bluetongue virus from sheep in California. J.A.V.M.A. 122: 300-301, 1953. - 17. Metcalf, H. E., Luedke, A. J.: Bluetongue and related diseases. The Bovine Practitioner, 15: 188-193, 1980. - 18. Metcalf, H. E., Pearson, J. E., Klingsborn, A. L.: Bluetongue in cattle: A serologic survery of slaughter cattle in the United States, Am. J. Vet. Res. 40: 1057-1061, 1981. - 19. Soulsby, E. J. L.: Helminths, arthropod, and protozoa of domesticated animals, 6th ed., pp. 392-394, 1974. - 20. Stair, E. L.: The pathogenesis of bluetongue in sheep: A study in immunofluorescence and histopathology. Dissertation, Texas A & M University, College Station, 1968. - 21. Venable, J. H., Coggeshall, R.: A simplified lead citrate stain for electron microscopy. J. Cell Biol. 25: 407-408, 1965. - 22. Vermored, D. W.: Purification and characterization of bluetongue virus, Virology 38: 203-212, 1969.