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# A COMPARISON OF THE FLUOROMETRIC ASSAY (FIAX) WITH TWO OTHER SEROLOGICAL TESTS IN EXPERIMENTAL AND NATURAL BLUETONGUE VIRUS INFECTED SHEEP

By

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A COMPARISON OF THE FLUOROMETRIC ASSAY (FIAX)

WITH TWO OTHER SEROLOGICAL TESTS IN

EXPERIMENTAL AND NATURAL BLUETONGUE

VIRUS INFECTED SHEEP

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# CHAPTER I

## INTRODUCTION

Five of the 23 known serotypes of bluetongue virus (BTV) found worldwide have been reported in the United States. These serotypes appear in sheep, goats, cattle, and wild ruminants (1, 2, 3). Laboratory methods for the detection of BTV in an infected individual can be difficult because of difficulty in isolation of the agent from clinical cases (4).

First passage of clinical material for virus isolation on appropriate cell cultures usually does not reveal the presence of the virus. Therefore, tissue culture or intravenous inoculation into embryonated chicken eggs (ECE) is necessary. Also three to four blind passages of BTV on cell cultures are frequently required for adaption of BTV from ECE to a cell culture system (5, 6).

In addition to the virus isolation technique, several serological methods have been used to detect the presence of the infection. The most widely used method in the United States is the agar gel immunodiffusion test (AGID) (8). This technique is simple, rapid, and requires less technical laboratory procedures than other methods available for serological surveys. The test, however, only detects common antigens that are shared by numerous serotypes; therefore, it cannot distinguish between different serotypes that may be prevalent in an area (6, 9, 10).

Another common serological method used is the serum neutralization (SN) test; this method will not only quantitate the neutralizing antibodies present in the animals but will also distinguish between the antibodies against different serotypes. This procedure requires specific viral antigens of the known serotype, specific serotype antisera, and cell culture. This method is reliable for the detection of exposed animals due to its sensitivity (10, 11).

Some other serological techniques that have been used include: hemolysis-in-gel (HIG); modified complement fixation (MDCF) test; plaque neutralization (PN) test; and enzyme-linked immunosorbent assay (ELISA) test. These serological tests have limitations. They are not only time-consuming but also require special complicated techniques which make them unsatisfactory for herd surveys (12, 13, 14, 15, 16, 17, 18, 19).

The purpose of this study is to adapt the FIAX to bluetongue serology and compare it to several of the serological techniques used presently for identification of antibody against BTV and to modify virus isolation techniques, currently in use in various diagnostic and research laboratories, in an attempt to make the procedures faster and more efficient.

# CHAPTER II

# REVIEW OF LITERATURE

## Introduction

The literature reviewed relative to the problem of this research consists of five parts: (a) history of bluetongue disease; (b) causative agents; (c) insect vectors and susceptible hosts; (d) pathogenesis; and (e) selected laboratory methods for the detection of antibodies against BTV infection. The first part deals with different names that have been given to bluetongue. The second section contains the classification of the virus, morphological structure, and serotypes present in the United States. The third part lists several insect vectors and susceptible hosts. The fourth part deals with the consequence of the disease, beginning with the incubation period, clinical signs, and pathological findings. Some laboratory methods dealing with virus isolations and serological tests for BTV infection are summarized in section five.

## History of Bluetongue

The disease was first recognized in 1700 in South Africa. The affected hosts were sheep imported from Europe. Bluetongue has been given several different names through the ages. The disease has been called pseudo-foot and mouth disease in South African cattle. It was termed mycotic stomatitis in the United States in cattle in 1948.

Bluetongue was also called sore muzzle when it was detected in Texas sheep in 1948. The virus was first isolated from sheep in California in 1952 (20). The virus was isolated from cattle in Oregon in 1959 (20, 21).

## Structure and Biochemical Properties

Bluetongue virus is classified in the Family Reoviridae Genus Orbivirus (22, 23, 24).

The genome of BTV consists of 10 segments of double-stranded ribonucleic acid (RNA). The total molecular weight of the nucleic acid is 15 million daltons (23).

The capsid of BTV contains 32 capsomeres arranged in icosahedral symmetry. The diameter of the hollow cylindrical shape capsomere is 8 to 11 millimicrons (mμ) in diameter (23).

There are several structural polypeptides in each virus particle. Two polypeptides, designated as P2 and P5 with molecular weights of 100,000 and 600,000 daltons, respectively, are located in the diffuse, structureless outer capsid layer (23, 24). These two polypeptides are required for virion infectivity (25). Two major polypeptides, P3 and P7, and three minor polypeptides are located in the core region (24). Polypeptide P2, with the greatest variation in size, is responsible for the serotype specificity (24). The neutralizing antibody titers from bluetongue convalescent sheep sera were correlated with precipitation of P2 (5). Polypeptide P5, which also showed a variation in size among the different serotypes, was not reported to confer serotype specificity. A third polypeptide P7 was responsible for the cross-immune precipitation of all investigated serotypes (24).

Monoclonal antibody directed against polypeptide P2 neutralized BTV

17 and protected neonatal mice against BTV 17 (26). Gumm and Newman (27) isolated polypeptide 7 (molecular weight of 38,000 daltons) from BTV infected BHK cells. This group antigen was noninfectious when injected intravenously in BTV susceptible Dorset horn sheep.

Bluetongue virus, like other members of the reoviridae, is capable of exchanging pieces of genome. The genetic reassortment phenomenon explained the new serotype isolated from areas with mixed serotypes of bluetongue infection (23, 28).

The virion of BTV has a diameter of 60 to 70 mμ. With fine hair like structures extending from the capsomeres, the overall diameter is 70 to 80 mμ (22, 23). The function of the hair-like structures was believed to increase the stability of the virus (12). A form of BTV encased in a membrane was also observed. The last form of BTV contained a membrane surrounding the core particles (22). Multiplication of virus in the cell lines was reported to reach a maximum yield after 12 hours with a latent period of four hours (23). Two to four hours after infection, the first virus-specific protein in BTV infected cells was demonstrated. The rate of protein synthesis increased and reached maximum at 11 hours after infection. The synthetic rate was then stable for 15 hours (23). During the synthetic phase, all 10 genomes of BTV were transcribed into 10 pieces of messenger RNA. The new virions were released by a cell lysis process (23). The complex morphology together with the cell associated properties could be the possible explanation why both virus isolation and antibody response are present at the same time in experimental sheep. Because the virus acquired the envelope from the host cell of origin, they were protected from the effect of neutralizing antibodies which circulate around the particles (22).

Bluetongue virus in the blood is stable at ambient laboratory temperatures. There was a significant lowering of the infectivity titer when the virus was frozen slowly at -10 C and -20 C (22). Bluetongue virus was stable when stored at -70 C for three years. One-half of the tissue culture 50% infectivity titer (TCID50) was lost when the virus was stored at 5 C or 22 C to 25 C for a month. Virus particles were released from the infected blood by chemical or mechanical treatment such as digestion with lipase or sonification. When equal volumes of the stabilizer buffered lactose peptone (BLP) and the virus solution were used, the virus remained stable even during lyophilization.

The virus was inactivated at 46 C to 50 C probably due to RNA inactivation. Virus was also inactivated in the presence of a low concentration of trypsin. The virus was inactivated within a minute when the pH of the media was not between 6.0 and 8.0. However, BTV was stable in the presence of chloroform and Genesolv-D (22).

## Serotypes and Antigenic Variation

Twenty-three serotypes of bluetongue have been reported worldwide. Five serotypes have been reported in the United States. Serotypes 10, 11, and 13 were reported not only in the United States but also in some other countries. Serotype 17 was discovered in the United States and has not been recognized in other countries (1). In 1983, serotype 2, a new serotype, was isolated from cattle in Florida (1, 2, 3). Each antigenic type of virus conferred solid immunity to the homologous serotype but not to different ones. Bluetongue virus showed antigenic drift due to the recovering of different antibody responses as a result of an infection (22).

The four BTV isolates (10, 11, 13, and 17) were studied by the oligonucleotide fingerprint analysis technique (20). The corresponding individual RNA segments of BTV were comparable and the same serotype of virus when collected at different time intervals had a high potential for genetic drift. The possibility that BTV serotype 17 evolved from BTV serotype 11 was also noted in the study (20). The only commercially available vaccine contains only a single strain (serotype 10) which stimulates antibody responses in vaccinated animals against only this serotype (22).

## Susceptible Hosts

Sheep are a primary host of BTV. European breed sheep are more susceptible to the disease than the African or Asiatic breeds. Young lambs, as well as older sheep, are susceptible to the disease. However, in some experiments, older sheep showed more severe clinical disease. Cattle and buffalo are also susceptible to BTV infection. The disease in cattle is usually sporadic, mild, and inapparent. Goats also are susceptible to BTV infection (22, 31, 32, 33). Several species of wild ruminants are susceptible to BTV such as the desert bighorn sheep, mule deer, blesbok, white tailed deer, bighorn sheep, barbary sheep, black tailed deer, pronghorn antelope, and elk (22, 34, 35). Bluetongue has also been reported in wild rodents (22). Embryonated chicken eggs, new born mice, and suckling hamsters have all been used for BTV studies (22).

## Insect Vector and Transmission

The bluetongue virus has been isolated from pools of Culicoides pallidipennis collected in the United States; from C. pallidipennis, C. milnei, and C. tororoensis in Kenya; and from C. pallidipennis in Israel. Foster and Luedke et al. (22) conducted experiments to demonstrate that C. variipennis was a biological vector of bluetongue. A minimum of 10 days was found to be required before a vector could biologically transmit BTV disease from an infected animal to a susceptible one (22, 31, 32). Following an inoculation of BTV in C. variipennis, the virus multiplied and reached a peak after 6 to 7 days and the virus concentration remained high through the 26 days of the experiment. The salivary glands of the fly undergo pathologic changes after infection with the virus. The virus was found to multiply in the salivary glands of the vector to a greater yield than in the mammalian cell cultures (22).

In a study of the geographic distribution and whether species of Culicoides in Australia were possible vectors of BTV, only one species, C. brevitarsis, was concluded to be a vector of BTV (32).

Many other insects that feed on sheep and cattle can also act as mechanical vectors for BTV transmission; BTV was isolated from Aedes lineatopennis in South Africa. Melophagus ovinus were infected with the Cyprus strain (22).

## Pathogenesis of Bluetongue

The incubation period following BTV injection varied from 2 to 15 days with an average of 6 days in sheep (31). There was no report on the incubation period following natural infection (22, 31).

Clinical diseases which has been reported in sheep varied from an

inapparent condition to an acute infection. The first sign was the elevation of the rectal temperature to about 41 C to 42 C. The average duration of the fever was 6 to 7 days (3, 22, 31). The febrile response may be the only indication of infection in some sheep (31). The degree of febrile response cannot be correlated with the severity of other clinical signs. The peak of temperature response did, however, correlate with the peak of viremia and the peak of leukopenia. In more acute forms, there was an increase in the respiratory rate before the elevation of the temperature. The elevated temperature lasted from 2 to 4 days. The sheep then showed characteristic clinical signs of smacking of the lips and shaking of the head (22).

In a severely infected sheep, the hyperemic tongue may turn blue. The disease received its name from the cyanotic tongue. Due to the excessive swelling of the head, two similar diseases, photosensitization and grub, are sometimes misdiagnosed. Some acute infected sheep die from foreign body pneumonia within 2 days after the swelling of the pharnyx as a result of edema of the epiglottis which causes difficulty of eructation (3, 22).

Prostration and torticollis may occur as a result of the severe muscular weakness. Coronitis, may affect all 4 feet. Sheep show severe lameness in 12 to 21 days after infection. Breaking of wool fibers or casting the entire fleece may also occur (22). In bluetongue infected sheep, intramuscular connective tissue and fascia become gelatinous and have a moist appearance. This is caused by the exudation of serous fluid. Following the acute febrile response, the muscular fibers undergo necrosis. Muscle fibers at the neck region are damaged; torticollis may be observed. Muscle fibers in the thighs, shoulders,

back, and sternum of sheep show petechial or ecchymotic hemorrhages. Because of the lack of blood supply in certain muscular groups due to thrombosis, necrosis of these regions may be observed (22).

The most prominent lesions are in the digestive tract and include excoriations of the lips, tongue, dental pad, and buccal mucosa. Necrotic ulcers are frequently observed. The entire digestive tract is covered with vascular congestion and hemorrhage. The large intestine may contain catarrhal lesions as the disease progresses. Infected animals die because of the complications created by the disease (22).

In an experimental vaccination with a modified live BTV vaccine during the first 4 to 8 weeks of pregnancy, deformed or dead lambs (dummy lambs) were born at full term. The major lesions were noticed in the brain. Both cerebral and cerebellous tissue were affected and resulted in hydranencephaly (36).

Another experiment in the pregnant ewe was conducted by vaccination with a modified live virus vaccine at 19 to 29 and 40 days following conception. Lesions in the uterus and placenta were caused by the inflammatory reaction of the arteries that supply those areas. Half of the experimental ewes had focal or diffuse hemorrhages and necrosis in the placenta (36).

The pathologic process was initiated in the fetus shortly after vaccination. Viruses were recovered from the liver of the fetus or from the central nervous system of the newborn lambs. The cerebellum was atrophic. Cavitation in the cerebrum and brain stem was also noticed. The development of the brain was abnormal. Vascular damage was suggested as a cause of the brain defects due to the presence of the lesion along the course of the developing blood vessels in the cerebral cortex and

symmetrically in portions of the superficial brain stem. The skeletal system of the fetus was affected also by the infection, for example, shortening of the long bones. Some experiments suggested that the approximate fetal mortality rate was 10% (36).

Cattle infected with BTV showed pathological changes in the digestive tract similar to those of sheep. Coronitis was reported in the later stages. Diffuse hemorrhagic lesions occurred in the myocardium (22). Three distinctive features in infected calves were reported. These were: deformed head, curvature of the vertebral column, and arthro-gryposis (37).

An experiment to recover the virus from semen of bulls during and after the course of infection was conducted and BTV was isolated from the ejaculates collected between 10 and 28 days after the bulls had been innoculated. The viral shedding period in the semen corresponded to the viremic period. No virus was isolated from the semen after the termination of viremia (38).

Virus was not transmitted to uninfected embryo recipient cattle when embryos were transferred from viremic, high titer infected donors (30). Infected preimplantation embryos from experimentally infected cattle resulted in the termination of embryo development and degeneration of the cells. After 24 hours of infection by BTV, tubular structures of a specific nonstructural viral protein were accumulated in the cells. The replication of virus had a lethal effect on the preimplantation embryo. As a result of this experiment, the induction of the persistent infection by the early infected embryo was not possible in natural infection (40).

The disease in deer can be inapparent or acute. The main affected

organ in deer was the tongue. Hemorrhage, degeneration, and necrosis of the tissues occurred as a consequence of vascular obstruction due to the presence of thrombi (22). An inapparent infection with serotypes 13 and 17 was found in free-ranging white tailed deer (41).

When pregnant North American elk were infected in the first, third, and fourth months of gestation with serotype 11, the infected calves were stunted, had some degree of difficulty in breathing and opacity of the cornea and had latent infection (42).

## Selected Laboratory Methods

Serological responses against BTV were studied in 137 serum samples from both experimental animals (sheep, cattle) and suspected naturally infected animals (cattle, deer). Three serological methods were compared to demonstrate the level of antibody (11). When compared with the MDCF or the AGID, the PN proved to be the most sensitive and reliable. The titers of antibody measured by the PN were detected earlier and were higher than those detected by the MDCF test. At one to two weeks following exposure, the serum antibodies were detected by PN but not by MDCF or AGID. Serum antibody levels measured by the PN peaked between 2 and 3 weeks. However, the MDCF and the AGID detected positive antibodies at 2 to 3 weeks post-infection and peaked between 4 to 6 weeks. The plaque neutralization test detected all the positive cases but the MDCF and AGID had several false negatives (11).

In another study of serological methods, MDCF, AGID, fluorescent antibody (FA), and PN tests were conducted to detect antibodies to BTV infection. The group specific antibodies were detected by MDCF, AGID, and FA. The levels of antibody detected by MDCF lasted at least 1 year

and up to 3 years. The results of the AGID test were closely paralled to those of the MDCF test. However, MDCF has a disadvantage of anti-complementary activities due to the contamination and hemolysis of the red blood cells (9, 14).

The assay of BTV had been conducted in sheep in the past until 1940 when Mason et al. discovered that this virus can be cultivated in ECE under special conditions (5). This recovery opened the opportunity for many researchers to study BTV isolation. Luedke compared the yolk sac and intravascular routes of virus isolation from infected blood and concluded that the intravascular assay method gave 2 to 4 log10 higher than the yolk sac assay method (5). Luedke also suggested the use of sonification of blood samples before inoculation into the ECE (5).

Osburn (6) studied the isolations of BTV in 4 western states. His study included 1,295 animal blood samples from cattle, sheep, goats, and antelopes. He found that the prevalence of infection with serotype 11 was the highest (51.8%), followed by serotype 17 (25.7%), and serotype 13 (19.2%). The lowest prevalence was for serotype 10 which was only 3.2%. Half of the survey sera from animals which BTV was isolated were test positive by AGID. Multiple serotype infections in cattle were found also. This study suggested a possibility that cattle may be a reservoir for infection of sheep (6).

An indirect immunofluorescent technique (FIAX™) has been used for detection of Immunoglobulin (Ig) G, Ig A, Ig M, Complement C3, Complement C4, ultralow levels Ig G, low levels of albumin, anti-DNA antibodies, antinuclear antibodies, and antitoxoplasma antibodies (43).

The fluorescent immunoassay has been employed also to detect and measure antibodies to cytomegalovirus and herpes simplex virus (43).

The viral antigens were immobilized on one side of the StiQ™ samplers. The fluorescein isothiocyanate (FITC) labeled antibodies on the surface were measured by inserting StiQ™ samplers in the FIAX fluorometer. The delta fluorescence signal unit (D-FSU) was obtained by subtracting the fluorescence (non-specific factors) on the opposite side. This D-FSU is the net amount of fluorescence caused by antigen-antibody reaction. The magnitude of the antibodies is determined by using a calibration curve formed by serum with known amounts of positive antibodies determined from hemagglutination inhibition tests (HAI). The total process required only two hours for 50 serum samples. The FIAX microcomputer does curve fitting and computation for the test.

The rubella antibody detection by the FIAX test was compared to several other methods of rubella antibody detection. There was a high correlation of the FIAX results with those of the hemagglutination and with the ELISA tests. The agreement of the tests were 99.7% and 98.6%, respectively (44). Brody et al. (45) reported on a comparison of the commercial rapid screening tests "RUBACELL" and the FIAX test. Both methods were reported as a highly specific, rapid, and sensitive test. In addition, both tests also showed a high predictive value of a positive result. However, the "RUBACELL" had an advantage over the FIAX with the time cost analysis (45). Cremer et al. (46) tested for rubella antibodies by the FIAX method. The results from their study also showed good agreement with FIAX at high HAI titers. However, at low or negative titers, the comparison was only fair.

Serological methods for cytomegalovirus and herpes simplex virus were also compared. When FIAX was compared to the complement fixation test (CF), 97% of the results were in agreement. The levels of antibody

to herpes simplex virus and cytomegalo virus determined by the FIAX test were as sensitive as those determined by the CF test. However, at low levels of antibody to cytomegalovirus, the results did not correlate well (46). Another comparison of both tests revealed specificity of FIAX was 95% for cytomegalo virus and 92% for herpes simplex virus. When paired samples were tested for the significance of the rising titers, the results for the cytomegalo virus were 96%, but the herpes simplex virus results were only 80% (43).

# CHAPTER III

## MATERIALS AND METHODS

### Cell Cultures

Baby hamster kidney (BHK-21) and African green monkey kidney, Clone Maru (Vero-M), were used for virus propagation. Cells were grown in a minimal essential media (MEM) or RPMI-1640 or F-15 which contained 0.1 mg/ml of gentamycin and 10.0% fetal bovine serum (FBS). Cells were grown in a 5.0% carbon dioxide ($CO_2$) atmosphere in a humidified chamber at 37 C.

### Preparation of BTV

Bluetongue virus serotypes 10, 11, 13, and 17 from the National Veterinary Service Laboratories (NVSL), Ames, Iowa, were used in this study. BHK-21 cells were grown until a monolayer was formed. Just before inoculation, each monolayer (75 $cm^2$) was washed once with RPMI without serum and then inoculated with 5.0 ml of 10.0% virus suspension of each serotype of BTV used in this study. Adsorption was facilitated at 36 C on a rocker plate (Bellco Glass Inc., Vineland, New Jersey) for 2 hours. The unattached virus suspension was removed by washing the monolayer culture twice with RPMI. Infected monolayers were then fed with media containing 2.0% FBS and 0.1 mg/ml of gentamycin. When a 75% cytopathic effect (CPE) was demonstrated, an equal volume of BLP was added. The virus-cell suspensions were then frozen at -70 C for

2 to 5 days. The frozen virus-cell suspensions were then thawed prior to virus harvest. The thawed virus-cell suspension was sonicated for 1 minute by a Bio-Sonic IV sonicator (VWR Scientific, San Francisco, California) at the maximum intensity setting. The suspension was then centrifuged at 340 X G for 15 minutes to remove cell debris (IEC HN-S benchtop centrifuge Damon/IEC Davidson, Needham Heights, Massachusetts). Stock viruses were stored frozen at -70 C in 1 ml volumes in plastic containers (Nunce, Roskilde, Denmark).

For further virus propagation, Vero-M cell cultures were used rather than the BHK-21 cells. Inoculations of the monolayer cultures, washings, and feedings by F-15 were performed as described above, except that incubation was done at 32 C. Viruses were harvested as described above.

## Experimental Animals

Six five-month-old Suffolk and Suffolk-crossed ram lambs were used for experimental infections. The rams were obtained from a local serologically negative flock when tested for antibody to BTV infection by the AGID. The animals were observed daily for 4 months to insure the absence of clinical signs of BTV infection. The serum was collected from each sheep one day prior to inoculation to confirm the absence of a BTV antibody. During the experiment, the sheep were separated into 3 groups of 2 animals each. One group was used as a contact and temperature control. Each group was housed in an isolation unit. The sheep were numbered 86, 87, 88, 89, 94, 96 for identification.

## Experimental Infections

Sheep nos. 86 and sheep 96 were injected subcutaneously in the neck regions with 3.0 ml of BTV serotype 13 suspension (passage one) grown on BKH-21 cell culture. The titer of the virus suspension was determined by titration on Vero-M cells in a 96-well tissue culture plate. The titer was found to be $10^{4.2}$ TCID 50/0.025 ml as computed by the Reed and Muench Method (47). The bluetongue virus serotype 13 were neutralized by serotype specific antiserum provided by the NVSL. Sheep nos. 87 and 89 were infected by subcutaneous injections in the neck regions with 3.0 ml of BTV serotype 17, prepared as described above. The virus titer was $10^{3.8}$ TCID 50/0.025 ml. Before being used, BTV serotype 17 was neutralized by serotype specific antiserum as described above.

Sheep nos. 88 and 94 were used as temperature and contact controls throughout the experiment.

Three weeks after the initial inoculation, the sheep were reinoculated by a subcutaneous injection with 5.0 ml of the animal's own whole blood which had been collected in a sterile vacutainer containing 15.0 mg of EDTA (Becton-Dickinson, Rutherford, New Jersey).

Nine weeks post inoculation, sheep nos. 86 and 96 were inoculated with a heterologous virus serotype by subcutaneous injection with 3.0 ml of BTV serotype 17 suspension.

## Clinical Observations

The sheep were observed daily during a four-month period prior to initial inoculation. Normal rectal temperatures were established before the experiment began. After virus inoculation, the observations for clinical signs were continued once or twice daily throughout the

five-month period of the experiment. Fever, ocular and nasal discharges, ocular cloudiness, and moist rales were criteria observed for this experimental disease. The presence or absence of oral and foot lesions were also determined.

Rectal temperatures were recorded daily for 28 days after the first injection. Temperatures were also measured daily for 10 days following the injections of the animal's own blood. Five days after the second injection with the heterologous serotype of BTV, rectal temperatures of sheep nos. 86 and 96 was measured daily for 8 additional days.

All of the sheep were bled at one-week intervals. At each bleeding, about 10 ml of blood was collected. Approximately 10 ml of additional blood were collected in EDTA.

Blood samples were stored for 1 to 3 days at 5 C before being processed. Sera were collected by centrifugation at 500 X G in a bench top centrifuge (Clay Adams, Parsippany, New Jersey) for 5 to 10 minutes. Sera were stored at -20 C until tested.

### Virus Isolation From Blood of BTV Experimentally Infected Sheep

All of the blood samples collected from experimentally infected sheep were stored at 4 C until tested, except for the blood samples from sheep that were bled on week 17 which were processed and inoculated into ECE on the same day. Packed blood cells were prepared by centrifugation of the whole blood collected in EDTA at 200 X G at 4 C for 15 minutes. The packed blood cells were washed 3 times in sterile phosphate buffered saline (PBS), pH 7.2. The cells were lysed in sterile distilled water at 4 C. The lysates were then injected intravenously into ECE or were inoculated onto a monolayer of Vero-M cells.

## Embryonated Chicken Egg Inoculation

Specific pathogen free eggs (Larson Laboratories Egg, Inc, Gowrie, Iowa) were incubated at 36 C for 11 days. Eggs were candled twice before inoculation with virus to find and eliminate the dead embryos. The first candling was done 8 days after incubation and the second just before blood-lysate inoculations. Non-fertile and dead embryos were discarded. The medium-sized veins in the embryos were then selected and marked. A portion of the shell over the marked side was removed. About 0.1 ml of blood lysate was injected intravenously into ECE. Two to four eggs were used for each sample. Holes in the shell were sealed with a mixture of acetone and butylacetate containing oil of mustard (Ducocement, Dupont Co., Wilmington, Delaware) immediately following the inoculations to decrease the chance of embryonic death. Inoculated eggs were incubated at 33 C and candled on 8 and 16 hour intervals up to 144 hours after inoculation. Embryos that died were refrigerated until they could be opened.

Dead embryos with cherry red discoloration or petechial hemorrhage were decapitated and washed 3 times in sterile PBS, pH 7.2. Heart, liver, and spleen were removed and washed in PBS. Sterile F-15 media with antibiotics and an equal volume of BLP were added at approximately 10 volumes for each volume of the embryo. The media mixture and embryos were then homogenized using the Stomacher (Teckar Company, Cincinnati, Ohio) for 1 minute before being centrifuged at 500 X G. Supernatants were collected and refrigerated at 4 C until inoculated onto a monolayer of cells.

## Cell Culture Viral Isolation

Monolayers of Vero-M in 25 $cm^2$ tissue culture flasks were used for virus isolations. A mixture of 2.5 ml of infected chicken embryo supernatant, 20 mM of hepes, 0.25 mg of gentamycin, 2.0% fetal bovine serum, and F-15 media was added onto a monolayer. Viral adsorption was done on a rocker plate at 36 C. After 14 to 16 hours, the embryo mixture was discarded and the monolayer was washed twice with fresh media. Subsequently, the monolayer was fed with new F-15 media and incubated at 32 C. Two to six flasks were used for each isolation. To isolate virus from the blood, the red blood cell lysate was used in place of chicken embryo supernatant. Following inoculation, the Vero-M cells were examined daily for visible CPE.

Monolayers which showed no visible CPE within 5 to 7 days were blind passaged onto a second monolayer, and again cells were observed daily for 1 week for signs of cytopathology.

## Agar Gel Immunodiffusion Test

The soluble BTV antigens and the positive control antisera used in this experiment were obtained from NVSL. A solution of 0.9% Agarose Agar (Marine Coloids, Inc., Rockland, Maine) was dissolved in 0.85% saline in distilled water and autoclaved at 15 pound per $inch^2$ for 30 minutes. The agar solution was allowed to cool and was then stored at 4 C. Just before being used, the agar was heated and 15 ml were transferred to a 100 X 15 mm plastic petri dish. The lid was partially opened to allow the moisture to escape while the plates were cooling. Wells were cut in solidified agarose using a template with 1 center well and 6 peripheral wells. The agarose plugs were removed from the wells

by using a suction pipet. The soluble antigen was placed in the middle well; reference antisera to various serotypes and serum samples were placed in alternate peripheral wells. The AGID plates were kept at room temperature in a humidifier and the results were recorded at 24 and 72 hours. Sera with a white precipitin line between the serum and antigen wells were considered positive. The tests were repeated when results appeared questionable.

### Virus Titration and Serum Neutralization

Sterile 96 well tissue culture plates were used for titration of BTV and neutralization of serum samples. For virus titration, each viral serotype was diluted ten-fold dilution up to 1:100,000. Sixteen wells were used for each dilution series. Each well contained 0.025 ml of BTV dilution, 0.025 ml of media, and 0.05 ml of Vero-M cells. The plates were incubated at 32 C in a humidified incubator for 6 days. The plates were examined daily for CPE.

Similar methods were conducted for the SN, except for the presence of sheep sera in the wells. Serum samples were diluted two-fold starting from 1:5 up to 1:320. Two to four wells were used for each serum dilution series. A constant amount of BTV at 30 to 100 TCID 50/0.025 ml were used. BTV and antisera were allowed to neutralize for 2 hours at 32 C before Vero-M cells were added.

### Effects of Incubation Temperatures on the Infectivity Titer for BTV

BTV serotypes 13 and 17 were compared for the titer yields in this study. Sixteen wells were used for each BTV dilution. Two plates of

each serotype were incubated at 36 C and 32 C and examined daily for CPE for 6 days. The infectivity titers of each serotype at the 2 incubation temperatures were compared.

## Antigen Preparations for the FIAX Test

Several methods were used to prepare the BTV antigens for FIAX serology.

1. BTV was grown on BHK-21 cells maintained in RPMI-1640 media. The cells were incubated in a humidified incubator with 5% $CO_2$ until CPE was observed in 75% of the cells. An equal volume of BLP was added and the antigen was kept frozen at -70 C in a 25 $cm^2$ tissue culture flasks for 7 to 14 days. The frozen cells were thawed, sonicated, then centrifuged for 15 minutes. Supernatants were stored and used as antigens for the FIAX tests.

2. Second passages of BTV were propagated on Vero-M cells and F-15 media. Cells showing CPE were harvested as described above and used as antigens.

3. Antigens were processed as in method 2 on Vero-M cells and further processed by sonification and ultrafiltration. Virus-cell preparations were filtered through an anisotropic membrane (Amicon XM 100, Amicon Scientific Systems Division, Danver, Massachusetts) using nitrogen gas pressure of 15 pounds/$inch^2$. Particles of 100,000 molecular weight or greater were retained. The ultrafiltrates were diluted 1:2 with PBS pH 7.3 before being used as antigens.

4. BTV antigens were also produced from infected chick embryos as described in the virus isolations. Chorioallantoic fluid was harvested and stored at -70 C until used as antigens.

## Fluorescein-Conjugated Antisera

An IgG fraction of fluorescein-conjugated rabbit antisheep IgG, heavy and light chains specific (lot number 2180 Cooper Biomedical, Inc., Malvern, Pennsylvania) was used to detect antigen bound antibody from the sera of test animals. The conjugate contained a total protein of 6 mg/ml. This product was reconstituted with sterile distilled water to 2.0 ml and further diluted with 48 ml of PBS at pH 7.3. The product was in 1.0 ml aliquots and stored at -70 C. Before each test, a portion of conjugate was thawed and further diluted to a final concentration of 1:200 in PBS, pH 7.3 with 0.15% polyoxyethylene sorbitan monolaurate (Tween 20).

## StiQ™ Samplers

The FIAX StiQ™ samplers were purchased from International Diagnostic Technology (IDT) Santa Clara, California. The dual antigen binding and control surfaces were cellulose-acetate-nitrate.

## Buffer

The PBS at pH 7.3 contained polyoxyethylene sorbitan monolaurate (Tween 20) at a concentration of 0.15% and was used as a washing and reaction buffer throughout the test procedures.

## FIAX Testing Procedure

BTV antigens in 25 microliter amounts (diluted or undiluted) were placed on the round flat cellulose-acetate-nitate surface on one side of the FIAX StiQ™ samplers. Two to four StiQ™ samplers were used for each serum sample. The antigens were allowed to dry overnight on a flat

surface at room temperature. Using an automatic diluter, sheep sera were diluted to a working solution of 1:75 or 1:100. About 0.5 ml of diluted sera were transferred to a glass tube (12x75 mm). A StiQ™ sampler was placed into each tube containing test serum. Reactions were done at room temperature for 30 minutes on a shaker. The StiQ™ samplers were then transferred to washing buffer with 0.15% Tween 20 for 10 minutes. The StiQ™ samplers were then reacted with conjugate (0.5 ml of 1:100 or 1:200 dilution) for 20 minutes. The excess conjugates were removed with a second 10 minute wash. The StiQ™ samplers were retained in the final washing buffer until the amount of fluorescence on the StiQ™ was measured using a FIAX 100™ Fluorometer (International Technology, Walsh Ave., Santa Clara, California). After all pregain signals were obtained, the StiQ™ with the highest reading was used to adjust the instrument to read 160 fluorescence signal units (FSU). All the StiQ™ samplers were then read again and the FSU values were recorded. Sera obtained from non-inoculated sheep were processed also in a similar manner as for the inoculated sheep.

## Pre-treatment of the StiQ™ Samplers

In one experiment, StiQ™ samplers were dipped in 2.0% rabbit serum in PBS. The treated StiQ™ samplers were allowed to dry at room temperature before proceeding to a regular FIAX testing procedure.

## Serological Surveys of Sheep Antibody to BTV in Payne County, Oklahoma

Eight flocks of sheep located in Payne County, Oklahoma were chosen for this survey. An alphabetical letter was assigned to each flock as

flock A to flock H. Breeds of the sheep were Suffolk, Suffolk mixed, Dorset, and Dorset mixed and they were commercial grade. None of the sheep in these flocks were previously tested for the presence of BTV infection. All mature sheep in flock A to F, both male and female with the age ranges from 5 months to 5 years, were bled during the month of August to September, 1983. Because flock G and H contained a large number of mature sheep, 52 and 12 sheep, respectively, animals were randomly selected and bled. A total of 170 serum samples were obtained: flock A was 5, flock B was 10, flock C was 17, flock D was also 17, flock E was 19, flock F was 38, flock G was 52, and flock H was 12. All sera were tested for antibodies against BTV by the AGID. Sera from flock F containing both positive and negative sheep were chosen for comparison of the serological methods used in this study.

## CHAPTER IV

## RESULTS

### Clinical Observations in Experimental Sheep

After a subcutaneous injection with BTV serotype 13, sheep no. 86 had an elevated temperature from 38.3 C (before inoculation) to 40.2 C on day 6 and 40.5 C on day 7. However, sheep no. 96 had an earlier febrile response; on day 5, the temperature increased to 40.0 C and on day 6 the temperature elevated to 41.1 C. Then on day 7, the temperature decreased slightly to 40.5 C. Both sheep showed clinical signs of ocular and nasal discharges from day 5 to day 7 (Table I). The discharges from sheep no. 96 were watery, but the discharges from sheep no. 86 were mucopurulent.

Sheep infected with serotype 17 also exhibited elevated temperatures. Accumulations of serous and purulent oculonasal discharges were also observed. Sheep no. 87 had a temperature of 40.2 C and 40.6 C on day 6 and day 7, respectively. During the same time of observation, sheep no. 89 had a temperature of 41.3 C and 40.6 C. Nasal and ocular discharges were obvious in both sheep. Sheep no. 87 had serous discharges during day 5 through 7. Sheep no. 89 also exhibited similar discharges on days 4 to 7.

Temperatures obtained from the non-inoculated control group (nos. 88 and 94) were lower than 40.0 C during the duration of observation.

On the eighth day of the experiment, sheep no. 86 continued to

TABLE I

CLINICAL OBSERVATIONS IN SHEEP EXPERIMENTALLY INFECTED WITH BLUETONGUE VIRUS SEROTYPE 13 AND 17

| Sheep Number | BTV Serotype | Temperature Measured | | Clinical Signs | |
|---|---|---|---|---|---|
| | | Day* | Temp** | Day* | Sign |
| 86 | 13 | 6 | 40.2 | 5 to 7 | Purulent nasal and ocular discharges |
| | | 7 | 40.5 | | |
| | | 8 | 40.0 | | |
| | | 12 | 40.0 | | |
| | | 13 | 40.1 | 28 to 31 | Purulent nasal and ocular discharges, coughing |
| 96 | 13 | 5 | 40.0 | 5 to 7 | Serous nasal and ocular discharges |
| | | 6 | 41.1 | | |
| | | 7 | 40.5 | | |
| | | 10 | 40.2 | | |
| | | 11 | 40.1 | 11 to 14 | Cloudy and swollen right eye |
| | | 23 | 40.0 | | |
| 87 | 17 | 6 | 40.2 | 5 to 7 | Serous nasal and ocular discharges |
| | | 7 | 40.6 | | |
| 89 | 17 | 6 | 41.3 | 4 to 7 | Serous nasal and ocular discharges |
| | | 7 | 40.6 | | |
| | | 28 | 40.0 | | |
| 88 | - | - | - | - | - |
| 94 | - | - | - | - | - |

*post inoculation day

**in degree C

maintain a temperature higher than 40.0 C, but the temperature decreased slightly on the ninth, tenth, and eleventh days. On the twelfth and thirteenth day after BTV injection, the temperatures increased to 40.0 C and 40.1, respectively. Sheep no. 96 also had an elevated temperature of 40.2 C and 40.1 C on day 10 and 11. During days 11 through 14, a swelling of the right eye with corneal opacity of sheep no. 96 was observed. The sheep appeared blind in the right eye during this entire period. After four days, the swollen eye and the clouded cornea returned to normal. No hyperemia of the ocular blood vessels or abrasive lesions of the eyes were observed.

All of the BTV serotype 17 injected sheep and the control animals exhibited temperatures below 40.0 C during the second week of observation. Finally, on day 28 after inoculation, sheep no. 86 again developed purulent ocular and nasal discharges. Also, coughing developed during this period. These discharges, together with the moist rales, were observed for 3 to 4 days. After injection with their own blood on week 3, only minimum elevation of temperatures were measured. Only sheep nos. 89 and 96 had elevated temperatures on days 28 and 23, respectively.

Following necropsy, microscopic findings in sheep no. 86 showed some degree of muscular necrosis with phagocytosis; however, one of the non-inoculated control sheep (88) also revealed similar microscopic finding in the muscular tissue. Sheep no. 96 had an abscess in the lung with adjacent chronic interstitial pneumonia. A thick fibrous connective tissue band was found surrounding the necrotic, purulent material. The lung tissue was also altered. Severe peribronchial and perivascular lymphoid infiltrates were noticed. Edema and immature connective tissue

were also noted in the lung of sheep no. 96. All of the sheep were infected with Haemonchus contortus (Trichostrongylidae) parasites in the abomasum.

Virus Isolation Attempted From Experimentally Infected Sheep

The isolation of BTV was attempted from lysed blood samples (Table II). The first assay procedure, in an attempt to isolate BTV directly in the tissue culture system of Vero-M, demonstrated no observable CPE. Isolation of virus by the second method was successful. Most of the infected ECE showed typical BTV lesions as early as 1 to 2 days after inoculation. Edema of the head of the embryo was obvious. Petechial and ecchymotic hemorrhages were found on the head and body of the embryo. Hemorrhage was also observed on the chorioallantoic membrane (Figures 1, 2, and 3). Except for 3 samples, all of the isolates caused lesions in the chicken embryos. These negative samples were from sheep no. 86 of week 10, sheep no. 87 of week 8, and sheep no. 89 of week 8.

Cytopathology typical of BTV infected cells was observed after 2 to 3 days without any further passage. The cytopathology of BTV on Vero-M cells was characterized by vacuolating and rounding of the cells (Figure 4 and 5). Circular and refractive cells were observed around several foci. As the cytopathic effect progressed, some elongated cells were noticed. The centers of the infected foci were empty due to the detachment of the infected cells. The cytopathic effect caused by BTV was slow and always required 4 to 5 days to spread through the entire monolayer.

TABLE II

COMPARISON OF TWO BLUETONGUE VIRUS ISOLATION METHODS FROM LYSED BLOOD OF EXPERIMENTALLY INFECTED SHEEP

| Sheep Number | Week After Inoculation | Isolation Method I | | Isolation Method II | |
|---|---|---|---|---|---|
| | | Vero-M | Vero-M | ECE | Vero-M |
| 86 | 1-3 | -* | - | +## | +** |
| | 10 | - | - | -# | - |
| | 11-13 | - | - | + | + |
| | 17 | - | - | + | + |
| 96 | 1-3 | - | - | + | + |
| | 10 | - | - | + | + |
| | 11-13 | - | - | + | + |
| | 17 | - | - | + | + |
| 87 | 1-3 | - | - | + | + |
| | 8 | - | - | - | - |
| 89 | 1-3 | - | - | + | + |
| | 8 | - | - | - | - |

* No cytopathic effect

** Cytopathic effect

# No cherry red discoloration

## Cherry red discoloration

Figure 1. Ten-day Old Non-Inoculated Chicken Embryo Without Petechial or Ecchymotic Hemorrhage. Note the Transparent Chorioallantoic Membrane.

Figure 2. Ten-day Old Chicken Embryo Injected With BTV Serotype 13. Showing Characteristic of "Cherry Red" Discoloration.

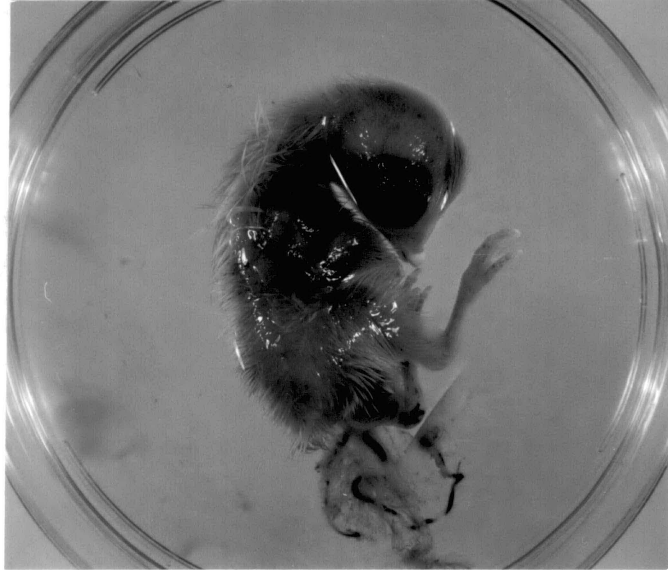
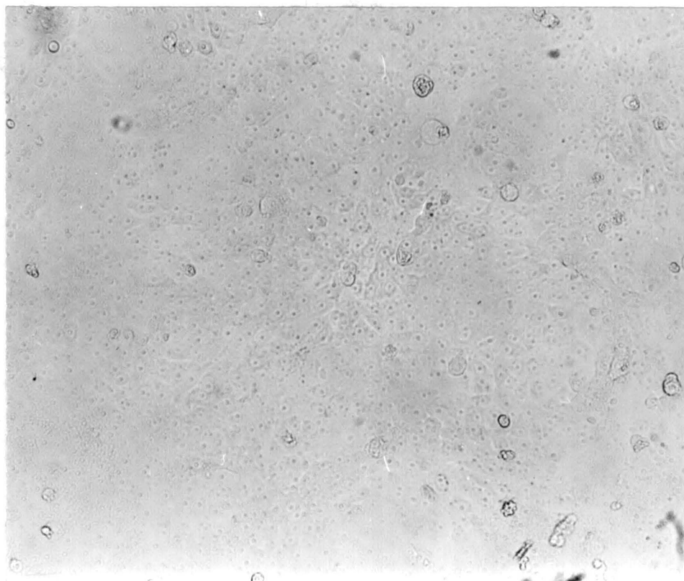
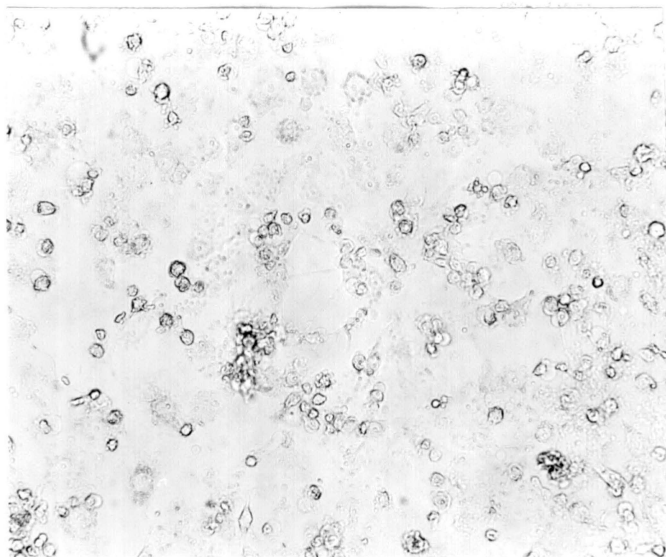


Figure 3. Two BTV Infected Chicken Embryos With Petechial and Ecchymotic Hemorrhages, Edema of Embryos, and Hemorrhage of the Chorio-allantoic Membrane.

Figure 4. Non-Inoculated 5 Day Old Vero-M Cells. Notice the Uniformity of the Cells.

Figure 5. Vero-M Cells Two Days After Inoculation With BTV Serotype 17. Notice the Vacuolated and Rounded Cells.





## The Yield of BTV at Two Different Incubation Temperatures

The effect of incubation temperature on the infectivity titer of BTV serotype 13 and 17 are summarized in Table III. At 32 C incubation, both serotypes appeared to reveal a higher infectivity titer. An increased yield of virus of up to 10 fold was obtained with both serotypes tested at 32 C when compared to the BTV yield from 36 C of incubation.

TABLE III

EFFECT OF THE YIELD OF BTV FOLLOWING INCUBATION AT TWO DIFFERENT TEMPERATURES

| BTV Serotype | Virus Titer Incubation Temperatures 36 C | 32 C |
|---|---|---|
| 13 | 3.85* | 4.61 |
| 17 | 2.50 | 3.77 |

* Titer of Virus Log 10 TCID 50/0.025 ml

## Agar Gel Immunodiffusion Test

Experimental sheep infected with BTV serotype 13 and serotype 17 demonstrated positive reactions when tested by the AGID method on the

second week after inoculation (Table IV and V). The precipitating antibodies against the BTV infection remained positive in all the infected animals to the end of the experiment. The sera collected from sheep nos. 86 and 96 during week 12 to 16 showed weak positive reactions in the test.

## Virus Neutralization Test

The neutralizing antibody responses to BTV injection in experimental sheep are shown in Figures 6 to 9. Sheep no. 86, when compared with sheep no. 96, had lower neutralizing antibody responses to BTV serotype 13 and 17 injection. Both sheep nos. 87 and 89 showed high levels of neutralizing antibody against BTV 17.

## Comparison of Various Antigens for the FIAX Test

Both of the diluted and undiluted NVSL soluble antigens used for AGID tests did not reveal any possible promising results indicating a usefulness in FIAX tests (Table VI). After a comparison of 7 methods of antigen preparation, antigen prepared from BTV serotype 10 on BHK cells and antigen prepared from BTV serotype 11 on Vero-M cells revealed the highest responses against sera from sheep nos. 89 and 86, respectively (Table VII).

Pre-treatment of the StiQ™ samplers by dipping in 2.0% rabbit serum in PBS pH 7.3 showed lower FSU values (Table VIII) and did not improve the test.

TABLE IV

ANTIBODY RESPONSES IN SHEEP EXPERIMENTALLY INFECTED WITH BTV AS DETECTED BY THE AGID DURING THE FIRST EIGHT-WEEK AFTER INOCULATION

| BTV Serotype | Sheep Number | Week after Inoculation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 13 | 86 | -[a] | - | + | +[b] | ++[c] | ++ | ++ | ++ |
| 13 | 96 | - | - | + | + | + | ++ | ++ | ++ |
| 17 | 87 | - | - | + | + | + | + | + | + |
| 17 | 89 | - | - | ++ | ++ | ++ | ++ | ++ | ++ |
| - | 88 | - | - | - | - | - | - | - | - |
| - | 94 | - | - | - | - | - | - | - | - |

[a] Negative, no precipitin line (-)

[b] Moderate positive reaction (+)

[c] Strong positive reaction (++)

TABLE V

ANTIBODY RESPONSES IN SHEEP EXPERIMENTALLY INFECTED WITH BTV AS DETECTED BY THE AGID DURING THE SECOND EIGHT-WEEK AFTER INOCULATION

| BTV Serotype | Sheep Number | Week after Inoculation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| 17 | 86 | +[b] | + | + | ±[d] | ± | ± | ± | ± |
| 17 | 96 | + | ++[c] | + | ± | ± | ± | ± | ± |
| - | 88 | -[a] | - | - | - | - | - | - | - |
| - | 94 | - | - | - | - | - | - | - | - |

[a] Negative, no precipitin line (-)

[b] Moderate positive reaction (+)

[c] Strong positive reaction (++)

[d] Weak positive reaction (±)

Figure 6. Comparison of Antibody Levels to BTV in Serially Collected Sera From Sheep 96 Inoculated With BTV Serotypes 13 and 17. Measured by the Fluorometric Assay (FIAX) and the Serum Neutralization (SN) Test.

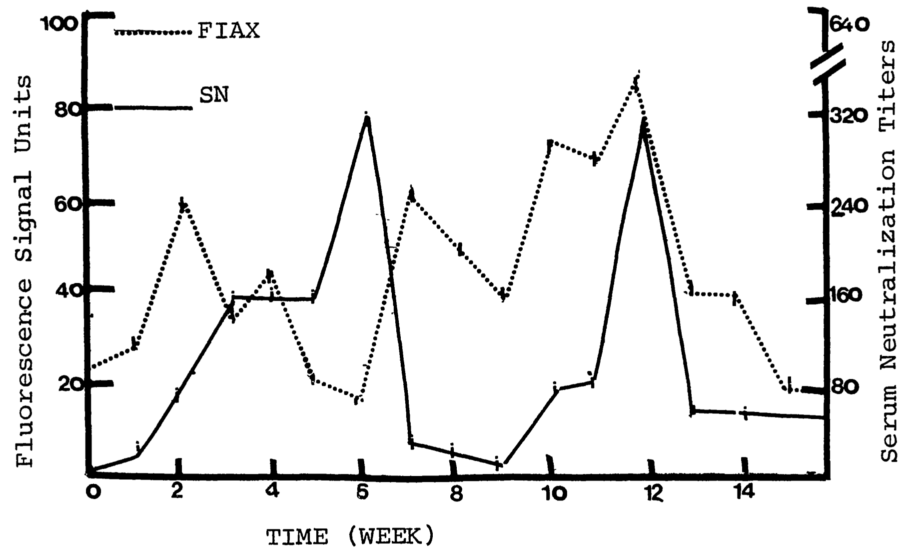

FIAX
SN
100
80
60
40
20
640
320
240
160
80
Fluorescence Signal Units
Serum Neutralization Titers
0
2
4
6
8
10
12
14
TIME (WEEK)

Figure 7. Comparison of Antibody Levels to BTV in Serially Collected Sera From Sheep 86 Inoculated With BTV Serotypes 13 and 17. Measured by the Fluorometric Asay (FIAX) and the Serum Neutralization (SN) Test.

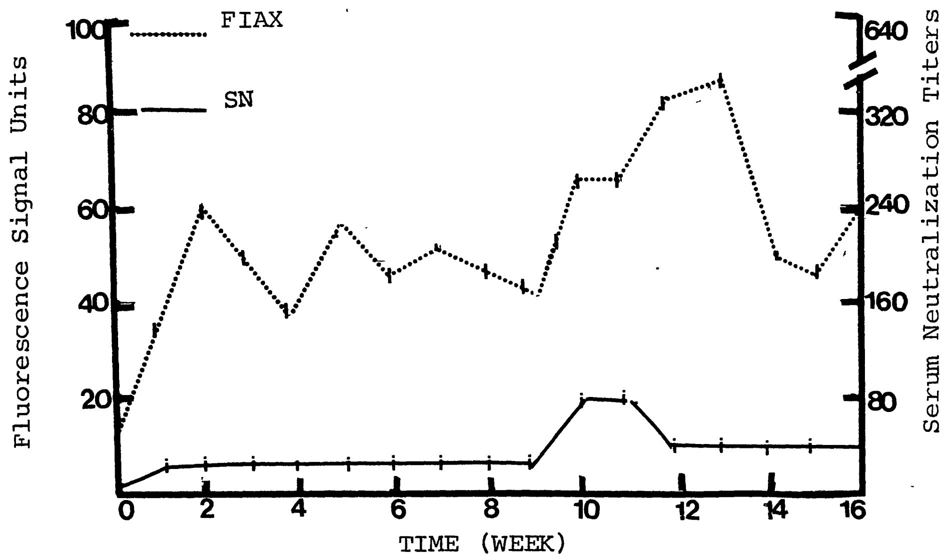

FIAX
SN
Fluorescence Signal Units
100
80
60
40
20
640
320
240
160
80
Serum Neutralization Titers
0
2
4
6
8
10
12
14
16
TIME (WEEK)

Figure 8. Comparison of Antibody Levels to BTV in Serially Collected Sera From Sheep 87 Inoculated With BTV Serotype 17. Measured by the Fluorometric Assay (FIAX) and the Serum Neutralization (SN) Test.

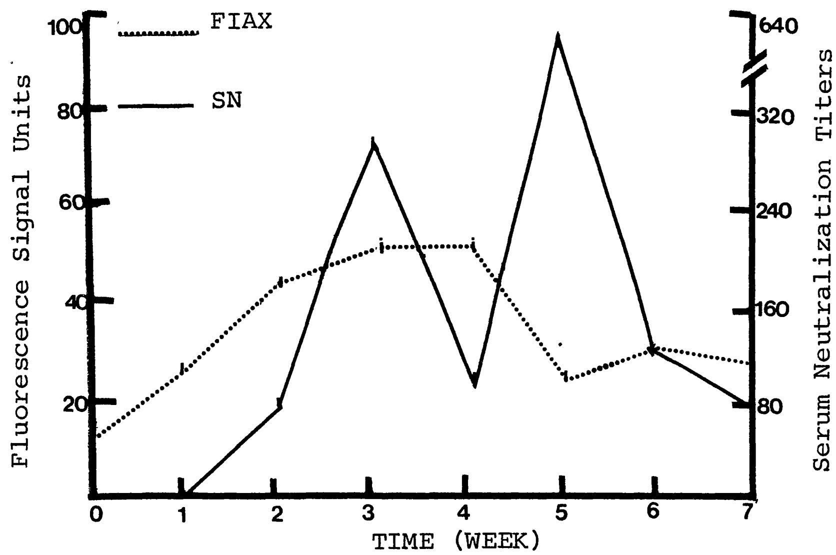

FIAX
SN
100
80
60
40
20
640
320
240
160
80
Fluorescence Signal Units
Serum Neutralization Titers
0
1
2
3
4
5
6
7
TIME (WEEK)

Figure 9. Comparison of Antibody Levels to BTV in Serially Collected Sera From Sheep 89 Inoculated With BTV Serotype 17. Measured by the Fluorometric Assay (FIAX) and the Serum Neutralization (SN) Test.

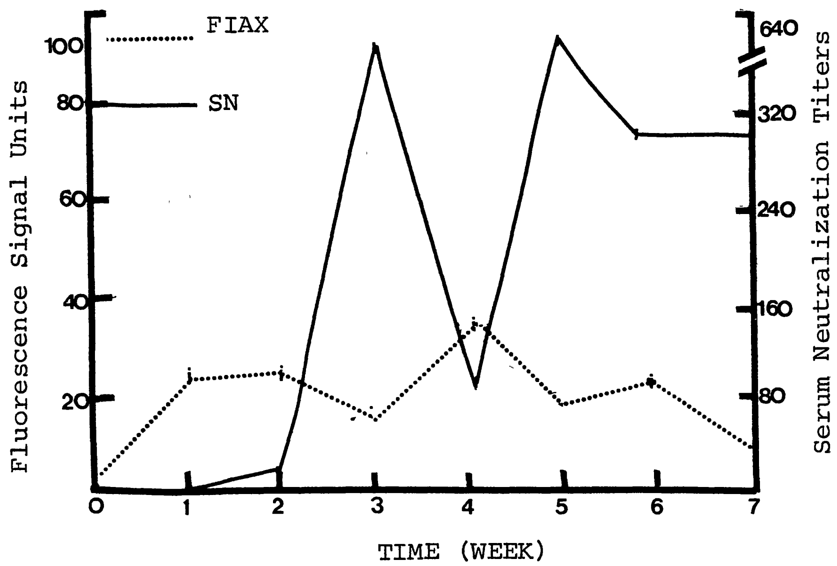

FIAX
SN
Fluorescence Signal Units
100
80
60
40
20
640
320
240
160
80
Serum Neutralization Titers
0
1
2
3
4
5
6
7
TIME (WEEK)

TABLE VI

COMPARISON OF DILUTED AND UNDILUTED BLUETONGUE SOLUBLE ANTIGEN (AGID ANTIGEN) USING FIAX TEST

| Sheep Number | Infected With BTV Sterotype | Fluorescence Signal Unit | |
|---|---|---|---|
| | | Concentrated Antigen[a] | Diluted Antigen[b] |
| 94 | - | 42 | 29 |
| | | 42 | 26 |
| 86 | 13 | 42 | 28 |
| | | 41 | 30 |
| 89 | 17 | 45 | 32 |
| | | 48 | 32 |

[a] Concentrated antigen obtained from the National Veterinary Service Laboratory

[b] Diluted 1:2 in PBS, pH 7.3

TABLE VII

COMPARISON OF BTV SEROTYPE 10, 11, 13, AND 17 ANTIGENS FOR THE FIAX TEST FROM VERO-M, BHK CELL CULTURES AND EMBRYONATED CHICKEN

| BTV Serotype | Sheep Number | Serological Responses* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | BTV10 BHK[a] | BTV11 BHK[b] | BTV11 VERO-M[c] | BTV13 BHK[d] | BTV13 CAF[e] | BTV13 CE[f] | BTV17 CE[g] |
| 13 | 86 | 5 | 1 | 4 | 1 | 1 | 1 | 3 |
| | 86 | 6 | 1 | 3 | 0 | 0 | 1 | 2 |
| | 96 | 6 | 2 | 2 | 3 | 1 | 3 | 4 |
| 17 | 89 | 7 | 1 | 5 | 1 | 1 | 2 | 3 |
| | 89 | 9 | 2 | 5 | 1 | 1 | 2 | 4 |
| - | 94 | 5 | 2 | 1 | 1 | 0 | 1 | 2 |

* Pregain fluorescence signal unit

[a] BTV serotype 10, first passage on BHK 21

[b] BTV serotype 11, first passage on BHK 21, BHK 21 applied on opposite side of StiQ™

[c] BTV serotype 11, second passage on Vero-M

[d] BTV serotype 13, first passage on BHK 21

[e] BTV serotype 13, second passage from chorioallantoic fluid

[f] BTV serotype 13, second passage on chicken embryo

[g] BTV serotype 17, second passage on chicken embryo

TABLE VIII

COMPARISON OF VARIOUS BTV SEROTYPE 17 ANTIGENS PREPARATIONS USING FIAX TEST

| | Serological Response (Fiax Value) | | | |
|---|---|---|---|---|
| | Conjugate 1:200 | | Conjugate 1:100 | |
| Antigen Preparation | Serum Dilution 1:75 | 1:100 | Serum Dilution 1:75 | 1:100 |
| Ultra-concentrated# | 40.5+3.5## | NT§ | 44.0±4.0 | NT |
| Ultrafiltrate | -1.0±3.0 | NT | -10.5±4.0 | NT |
| Crude | 38.5±2.5 | 40.5±0.5 | 38.0±9.0 | 42.5±11.5 |
| Crude* | 13.5±3.5 | 22.0±5.0 | 11.5±10.5 | 18.0±19.0 |

# Diluted 1:2 in PBS, pH 7.3

## Mean±standard deviation from 6 StiQ™ samplers

§ No Test

* StiQ™ sampler pre-treated with 2.0% rabbit sera
Positive sera obtained from a BTV experimentally infected sheep

## Antibody Responses to BTV Infection Measured by the FIAX Method

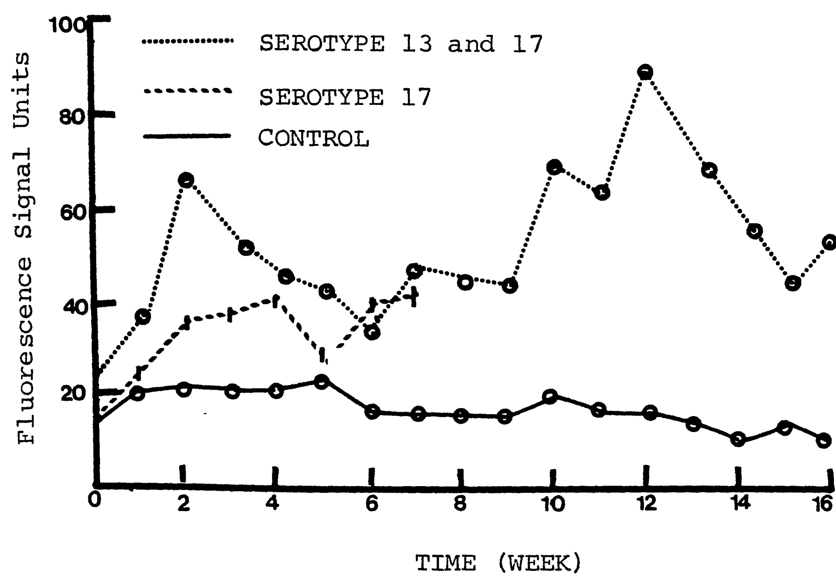
The antibody responses to BTV serotypes 13 and 17 in experimentally infected sheep (86, 87, 89, 96) are represented in Figures 6 to 9. The antibody responses detected by the FIAX method were different from those measured by SN. The average antibody response, as measured by the FIAX test, for both sheep in the same group and infected by the same serotypes was calculated (Figure 10). The antibody responses from the controls were also included in this study. The antibody responses from sheep nos. 86 and 96 were the highest for the entire 16 weeks of the experiment with 1 exception. The antibody levels at week 6 were lower than those of sheep nos. 87 and 89 detected at the same period of time. The control sheep demonstrated the lowest response.

## Serological Surveys of BTV Infections in Local Sheep

Table IX shows the results of the serological survey for BTV in selected sheep in Payne County, Oklahoma. Fifty-three sheep (31.2%) showed a positive reaction, 22 sheep (12.9%) showed a strong positive reaction, and 31 sheep (18.23%) showed a weak positive. One hundred and seventeen of the serum samples (68.8%) were negative. The percentage of positive sheep was highest in flock D (52.9%) and was the lowest in flock E (36.8%). Antibodies were not found in flocks A, C, and H. The number of weak and strong positive reactions were similar in flocks B and G. Flock D contained the highest percentage of sheep showing a weak-positive reaction to the test (88.9%).

The data collected relative to the number of weak positive and

Figure 10. Comparison of Antibody Levels in Serially Collected Sera From 3 Groups of Sheep. Measured by the Fluorometric Assay (FIAX). Average of Antibody Levels from 2 Sheep Inoculated With the Same Serotypes of BTV.



SEROTYPE 13 and 17
SEROTYPE 17
CONTROL
Fluorescence Signal Units
100
80
60
40
20
0
2
4
6
8
10
12
14
16
TIME (WEEK)

TABLE IX

A SEROLOGICAL SURVEY OF BTV INFECTIONS IN PAYNE COUNTY, OKLAHOMA, USING AGID TEST

| Date | Flock | No. of Serum Sample | AGID | | | |
|---|---|---|---|---|---|---|
| | | | Negative | Positive | Weak Positive | Strong Positive |
| 8-08-83 | A | 5 | 5 | 0 | 0 | 0 |
| 8-10-83 | B | 10 | 6 | 4 | 2 | 2 |
| 8-13-83 | C | 17 | 17 | 0 | 0 | 0 |
| 8-17-83 | D | 17 | 8 | 9 | 8 | 1 |
| 8-18-83 | E | 19 | 12 | 7 | 3 | 4 |
| 8-19-83 | F | 38 | 23 | 15 | 9 | 6 |
| 8-25-83 | G | 52 | 34 | 18 | 9 | 9 |
| 9-12-83 | H | 12 | 12 | 0 | 0 | 0 |
| TOTAL | 8 | 170 | 117 | 53 | 31 | 22 |

strong-positive indications were analyzed using a chi-square test at the 5% level of significance to answer the following null hypothesis:

> $H_o$: There is no significant difference between the number of weak positive and strong positive indications detected by using the AGID test.

The null hypothesis was accepted as shown in Table X.

Table XI shows a comparison of the antibody responses in sheep using FIAX, AGID and SN tests. The highest FSU value in this herd was 65 and the lowest FSU value was 11. Intervals of 10 FSU were arranged into 1 group. There were 6 groups ranging from 11 to 70. All of the AGID and SN results were potentially similar. At FSU 11-20, all of the AGID and SN results were negative. In the 21 to 30 group, only 1 out of 9 samples (11.1%) showed positive in the SN and AGID. In the third group, there were 3 positive AGID samples (37.5%) and 5 positive SN samples (62.5%). As the FSU increased, the number of positive AGID and SN samples also increased. At the 41-50 range, there were 3 times (75%) more positive AGID and 7 out of 8 (87.5%) samples were positive on the SN. As the FSU increased above 51, all the samples (100%) showed positive in both test systems.

The number of sheep from flock F that had antibodies against single and multiple serotypes of BTV are presented in Table XII. There were 3 sheep (15.8%) in this flock that carried antibodies to only 1 serotype; 1 sheep had antibodies to serotype 11 and 2 sheep had serotype 13 antibodies. There were 10 sheep (52.6%) that had antibodies to 2 serotypes: one of these sheep had antibodies to both serotype 10 and serotype 11. Seven sheep in this group antibodies to serotypes 11 and 13. The last 2 sheep in this group had antibodies to serotypes 11 and 17. There were 5 sheep (26.3%) that had antibodies to 3 serotypes. Three of them

## TABLE X

## CHI-SQUARE ($\chi^2$) TABLE - DIFFERENCE BETWEEN THE STRONG POSITIVE AND WEAK POSITIVE REACTIONS FROM THE AGID TEST

| Positive Reaction | Sheep Herd | | | | | Degrees of Freedom | Calculated $\chi^2$ | $\chi^2_{0.05}$ |
|---|---|---|---|---|---|---|---|---|
| | B | D | E | F | G | | | |
| Weak | 2 | 8 | 3 | 9 | 9 | | | |
| Strong | 2 | 1 | 4 | 6 | 9 | 4 | 3.07 | 9.49 |
| Total | 4 | 9 | 7 | 15 | 18 | | | |

Since $\chi^2$ 0.050 3 df = 9.49 and the calculated $\chi^2 = 3.07 < 9.49$ the null hypothesis of there being no significant difference between the strong positive and weak positive AGID test is accepted.

Herd A, C, and H contained all negative serum samples.

TABLE XI

COMPARISON OF FIAX, AGID, AND SN TESTS FOR BTV ANTIBODIES IN FLOCK F

| FIAX[a] | Range of FSU | 11-20 | 21-30 | 31-40 | 41-50 | 51-60 | 61-70 |
|---|---|---|---|---|---|---|---|
| AGID[b] | No. positive | 0 | 1 | 3 | 6 | 5 | 1 |
| | No. negative | 5 | 8 | 7 | 2 | 0 | 0 |
| SN[c] | No. positive | 0 | 1 | 5 | 7 | 5 | 1 |
| | No. negative | 5 | 8 | 5 | 1 | 0 | 0 |
| | TOTAL | 5 | 9 | 10 | 8 | 5 | 1 |

[a] Antigen BTV serotype 17, passage 2 on Vero-M cells

[b] Antigen from NVSL

[c] Antigen from BTV passage 2 on Vero-M cells

had antibodies to serotypes 10, 11, and 13. Two sheep had antibodies to serotypes 11, 13, and 17. Only 1 sheep (5.3%) in this group revealed antibodies to all 4 serotypes.

TABLE XII

SINGLE AND MULTIPLE SEROLOGICAL RESPONSES OF SHEEP SERUM SAMPLES FROM FLOCK F

| Type of antibody* Response to BTV | Number of Serum** | BTV Serotypes # |
|---|---|---|
| 1 Serotype | 1 | 11 |
| | 2 | 13 |
| 2 Serotypes | 1 | 10, 11 |
| | 7 | 11, 13 |
| | 2 | 11, 17 |
| 3 Serotypes | 3 | 10, 11, 13 |
| | 2 | 11, 13, 17 |
| 4 Serotypes | 1 | 10, 11, 13, 17 |

* Serum neutralization test

** From sheep in Payne County, Oklahoma

# BTV from NVSL

# CHAPTER V

## DISCUSSIONS AND CONCLUSIONS

Clinical findings of BTV infected sheep revealed only ocular and nasal discharges, mild elevation of temperature and coughing. The right eye of one of the infected sheep became opaque and swollen. A similar corneal lesion was noticed in calves from infected pregnant North American elk (41). There was no congestion or hyperemia of the buccal mucosa or coronitis as described in previous studies (3, 22). This finding is not unusual because frequently signs of BTV infection are not obvious except for slight elevations in temperature which are generally not measured in a sheep rearing operation.

The isolation of BTV in ECE and subsequent passage on Vero-M is an efficient method (4). Seventy-five percent of samples processed by this method revealed CPE on Vero-M as early as 6 to 8 days. Most of the previously described methods required approximately 19 to 28 days (4). The virus isolation had a potential to be more effective by this method if more ECE were included in the experiment. Six or more embryos are recommended for this experiment to minimize the possibility of losing some embryos due to traumatic bleeding during the procedure. All inoculum which revealed negative CPE on Vero-M cells were obtained from the embryos which died within 16 hours after infection. The exact cause of death could not be attributed to the BTV infection because these embryos did not appear "cherry red".

Incubation temperatures played an important role in BTV adaptation and isolation in eggs (5). Higher yields of BTV were obtained when the virus was incubated at 32 C rather than at 36 C. The CPE at 32 C was also more evident than the CPE at 36 C.

The results of the AGID test were similar to published studies (48). Antibodies in experimentally infected sheep with BTV serotypes 13 or 17 were detected as early as 2 weeks after infection. The AGID test is quick and simple to perform but one of the disadvantage is that the serotype of the infecting BTV cannot be determined (20, 21, 24).

The fact that SN was positive as early as 1 to 2 weeks after the infection and remained positive throughout the experiment may be beneficial for BTV identification. However, there is a possibility of cross reaction in certain serotypes, such as serotypes 4, 7, and 20 (25). Another problem with SN testing is that the SN assay recognized only a fraction of protein encoded by 1 or 2 of the RNA segments. It is possible that any genotypic variations among closely related BTV isolates cannot be detected by this procedure (1, 30).

The presence of precipitating and neutralizing antibodies together with the BTV in blood may be explained in two ways. First, some of the neutralizing antibodies and precipitating antibodies were not capable of neutralizing circulating BTV. Secondly, the virus escaped the neutralizing effect by becoming highly cell associated.

Ultraconcentrated BTV antigen proved to be the most efficient antigen for this study. Fox also suggested the use of more concentrated type antigens to increase sensitivity (49). Antigen prepared from 1 serotype in this study did not react selectively to only certain serotype antibodies. This finding ruled out the possibility of serotype

specificity, detected by this test, which was suggested by Fox in his study (49).

The question of which serological method is more practical was stated by Thomas (11) as follows:

> When selecting the most suitable serological test for any particular application, considerations must include efficiency, practicability, reliability and sensitivity. Depending on the application, the relative importance of these factors varies and rarely would any one test be better than all others for all the criteria (p. 295).

The FIAX method measured the primary binding reaction between BTV and the antibody directly. But the SN test (the secondary binding reaction) measured the CPE on Vero-M cells caused by non-neutralized BTV. Therefore, the FIAX test is much more sensitive than the SN test (50).

In addition to this study, SN against all serotypes of BTV should be further conducted in experimentally infected sheep to determine the possibility of the genetic resortment nature of the Orbivirus.

Serological surveys of antibodies against BTV infection in sheep in Payne County, Oklahoma, revealed the presence of antibodies to all 4 serotypes. However, only 2 serotypes (11 and 17) of BTV have been isolated from sheep samples in Oklahoma (51, 52). The predominant antibody responses are to serotype 11, followed by serotypes 13, 17, and 10. The result of the highest distribution of antibodies to serotype 11 was in agreement with a previous study (6). However, there was some difference noted in antibody responses to serotypes 13 and 17.

When the antibodies to BTV in the survey samples were measured by the FIAX method, the FSU obtained were ranked into 6 groups. The numbers of the sera that were positive to AGID and SN test increased in a higher FSU group with the critical FSU group at 31 to 40. With the FSU

higher than 41, it is predictable that the samples have a tendency to be positive rather than negative in the AGID or SN tests. A further investigation with more samples should be performed to obtain more predictable results. When the standard ranges are established, the survey from serum samples in the field will be more reliable. Finally, it is possible that in the near future, the FIAX test can be an alternative way for the survey of BTV because the test is simple to perform and the results are easy to obtain. The only disadvantage of this test is the initial investment in the expensive instrument and the cost of the StiQ™ samplers.

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# VITA 2

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