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VIRUS ISOLATION FROM SEMEN AND
SEROLOGY OF YOUNG BULLS AT THE
KANSAS BULL TEST STATION OF BELOIT

by

David John Rademacher

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Major Professor

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TABLE OF CONTENTS

	Page
Introduction	1
Materials and Methods	
Animals and Specimens	8
Agar Gel Immunodiffusion (AGID) Test for Bluetongue .	9
Serum Neutralization	11
Viral Isolation	13
Results	16
Discussion	19
References	23

INTRODUCTION

A large percentage of the important pathogenic bovine viruses may be transmitted through infected semen. Viruses isolated from bull semen include infectious bovine rhinotracheitis (IBR), bovine enteroviruses, bovine viral diarrhea (BVD), foot-and-mouth virus, parainfluenza III virus, paravaccine virus, ephemeral fever, and bluetongue virus (BTV) (3,5,6,7,8,16,17,18,22,30,31,34). Some viruses such as bluetongue virus and infectious bovine rhinotracheitis (IBR) virus become latent and may be spread in the semen for many years even in bulls with serum antibody titers to these viruses (22,31). It has been shown that a bull carrying the bluetongue virus in his semen for eight years was able to infect 14 of 14 susceptible heifers (21,22). The infection caused a viremia in all heifers, but produced antibodies in only one. Abortion was noted in one heifer and the other 13 heifers had malformed calves that were viremic at birth. IBR virus has also been shown to be present in semen of infected bulls for long periods of time and could have been responsible for infertility (31).

Bulls at the Kansas Test Station are brought to the station from many farms. Mixing bulls from many localities may introduce pathogenic viruses to the entire group. Examining the semen of these bulls just prior to their sale would indicate the presence of virus in the semen at that time.

IBR is the most commonly reported viral contaminant in semen (17,25,34). In addition to respiratory disease, conjunctivitis, abortion and occasionally encephalitis, IBR virus causes infectious

pustular vulvovaginitis (IPV) and infectious balanoposthitis (13,17, 27). IBR, a herpes virus, can lay dormant whether a clinical infection is observed or not. Infectious virus may later be shed due to increased levels of corticosteroids from exogenous or endogenous sources (19). Reactivations have been shown to be associated with stress from disease, socialization, movement, transport, estrus, and parturition (19,32).

Cattle are thought to be the principal reservoir of IBR virus which is widely distributed in cattle populations world wide. Transmission of IBR virus has been demonstrated from respiratory, ocular, and reproductive secretions. IBR infected bulls have been shown to shed IBR virus sporadically in semen for several years (30,31,32). Conversely, Schultz, using the "Cornell Semen Test" (28) which entailed testing large numbers of semen samples together in an in vivo procedure showed that semen collected from bulls which became serologically positive to IBR as a result of subclinical infection and thereafter vaccinated intranasally with an IBR-PI₃ vaccine every 4 to 6 months, did not have IBR virus in their semen. It could not be postulated whether the IBR virus was not shed in the semen because of the vaccination program, or if the strain was of a different type than previously recognized. Schultz later removed several of the bulls involved in the natural subclinical infection, treated them with corticosteroid, and the virus was recovered from nasal mucosa as well as from the semen and prepuce (27). IBR may contaminate semen as a result of genital infection.

BVD is a ubiquitous, easily transmitted, RNA virus of the genus Pestivirus in the family Togaviridae which is enzootic throughout the

the world. Although most infections are inapparent, clinical cases range from the mild febrile disease to an acute fatal syndrome. The virus initially infects the reticuloendothelial system (having the capacity of being immunosuppressive) with subsequent invasion of oral and gastrointestinal mucosal tissue, characterized by fever, nasal and ocular discharges, anorexia, diarrhea, lameness and oral erosions or ulcerations (13). Infection of pregnant cattle may result in abortion, stillbirths, mummified fetuses, or birth of calves with various congenital defects including cerebellar disorders. Calves which survive in utero infections may exhibit immune tolerance and have persistent viremia (29).

A non-cytopathogenic strain of BVD was demonstrated by cellular interference and immunofluorescence from a bull with a sub-clinical chronic persistent infection, apparently acquired in utero (6). Antibodies against BVD virus were not detectable in the serum of this bull. The bull had acceptable fertility but poor sperm quality. Virus was detected at 15 and 16 months of age and again around 3 years of age. Seroconversion occurred in 5 heifers bred to this bull. Four of them delivered normal calves and one aborted due to unknown causes (23).

A cytopathic strain of BVD virus (NADL strain) was isolated by cell culture method from 4 of 98 semen samples collected from 9 bulls following BVD virus inoculation (34). Three of the 4 bulls were seropositive and the other seronegative prior to inoculation. Kahrs suggests that the exposure may have been massive enough to overwhelm the resistance conferred by serum antibody (17). Whitmore indicated that the risk for virus transmission via the semen would be greatest during the period of active viremia (34).

Bluetongue virus is an infectious, noncontagious, arthropod-borne disease of domestic and wild ruminants. It is placed in the genus Orbivirus of the family Reoviridae. While other vectors have been identified, Culicoides variipennis is the primary vector of BTV (20). In the United States, BTV has been isolated in 32 states and serologically evident in 48 states (15). Twenty serotypes of BTV are known, four of which have been found in the United States (serotypes 10, 11, 13 and 17).

Sheep are generally affected with the acute syndrome of BTV while cattle tend to have inapparent infections. Only 2-5% of infected cattle show signs, but 25% of cattle in so-called blue-tongue areas have positive antibody titers (24). Biological transmission by C. variipennis has been demonstrated from sheep to sheep, sheep to cattle, cattle to cattle, and cattle to sheep (20). Significant economic losses are being felt by the cattle industry due to the restrictions and requirements placed on the export of cattle and cattle semen.

Bluetongue may develop as an acute clinical syndrome in cattle, characterized by severe inflammation of mucous membranes of the mouth, nasopharynx, esophagus and rumen, fever, pityriasis, emaciation and lameness.

After introduction to peripheral circulation, localized multiplication takes place in the cytoplasm of vascular endothelial cells. The prime pathogenic mechanism of BTV seems to be disseminated intravascular coagulation associated with degenerative changes and necrosis of the blood vessel walls as a result of viral injury to endothelial

cells during replication (12). A cell-associated, primary erythrocytic viremia follows and may persist even after the development of viral-specific antibodies (12). In utero transmission occurs in cattle and can result in abortion, hydroencephally, congenital deformity, and immunologically tolerant calves (11). The virus can produce pathological changes in the reproductive tract of bulls such as focal degeneration of seminiferous tubules, hemorrhage and hyperemia in the colliculus seminalis (11).

Duration of immunity is hard to ascertain due to variability in antigenicity of the different serotypes. Recovery from an attack of BTV sets up a solid immunity which lasts for a period of months to further infection of that serotype (11). Partial cross immunization has been shown against some of the different strains (11).

Isolation work on bluetongue virus from cattle specimen, has been performed through sheep inoculations and embryonated chicken eggs. Inoculation of embryonated chicken eggs by the intravenous route is believed to be more sensitive than the chorioatlantoic membrane or yolk-sac routes in isolating from samples with low viral titers (33). Recent emphasis has been on the detection of BTV by cell culture techniques. Bluetongue virus was isolated by sheep inoculation and in cell cultures from semen of a bull with a non-clinical infection up to 300 days after exposure (22). Isolation of BTV in vero (MARU) cell culture, with confirmation by the indirect immunofluorescence test, was successful from 10 erythrocyte and 10 semen specimens from 4 bulls (5). It was suggested that the quality of distilled water was an important factor in the isolation

of BTV in cell culture indicating that the need for optimal attachment of some arboviruses is dependent on specific monovalent or divalent cations (5).

Many techniques have been employed for the detection of viruses from cattle semen. Each technique has its own inherent problems, and when searching through the literature there is a wide variety of results. As told by Kahrs (17), the true test of viral transmission via semen is recognition of clinical disease, infection, or seroconversion in inseminated susceptible females. Due to the expense and variabilities in the present methods, a more practical and efficient method is sought. Problems such as cytotoxicity of semen and loss of virus in sample preparation whether from exposure or dilution to undetectable levels are possibilities for some of the variability observed in virus isolation techniques.

Abnormalities are commonly observed in spermatozoa, and virus-like particles have been associated with various of these; such as nuclear vacuoles and inclusion bodies in spermatozoa with crater defects (9,17). Using the electron microscope, Foster (9) reported abnormalities that ranged from a small cavity between the acrosome and nucleus with some involvement of the nucleus, to an enlargement of the cavity accompanied by vesiculation that could affect the entire acrosome. A positive relationship was observed between infectivity in cell cultures of semen samples, and observation of abnormalities and virus-like particles in heads of affected spermatozoa (9).

The purpose of this report was to study the prevalence of some viral diseases at a Bull Test Station and the likelihood of trans-

mission of viral diseases in cattle semen. More emphasis was placed on bluetongue virus due to its limitation on international semen trade. However, it was our intention to establish a technique which could be used for viral isolation in general.

MATERIALS AND METHODS

1

Animals and Specimens

One hundred sixty bulls from the Kansas Bull Test Station (KBTS) were used in this study. These bulls came from multiple owners located throughout Kansas. Bulls arrived at the test station at an approximate age of 6 months; at this time each bull was processed, which included vaccinations from IBR and BVD and placed in pens with a capacity of approximately 50 bulls per pen. Bulls remained at the station for 6 months after which time they were dispersed. It was just prior to the dispersals that the samples for this study was taken.

Semen was collected by means of electroejaculation and serum collected from each bull. Eighty bulls were collected on 2 different dates. At the time of collection of the first 80 semen samples, slides were prepared for later morphologic examination for which acrosomes, incidence of cytoplasmic droplets, detached sperm heads and mid piece and tail defects were studied by phase-contrast microscopy. Remaining semen was immediately placed in liquid nitrogen storage. Eighty additional semen samples were collected in similar fashion, however at this time slides were not made. Virus isolation work was performed on all semen samples and blue-tongue virus antibodies were tested for by agar gel immunodiffusion. A serum neutralization test was performed on 120 serum samples for IBR and BVD antibodies. Group I consists of 80 serum samples from the first collection and Group II consists of 40 samples from the second collection.

Agar Gel Immunodiffusion (AGID) Test for Bluetongue

Nine-tenths percent (0.9%) solution of agarose agar^a was prepared in physiological saline (0.85% NaCl in distilled water). Agar was boiled or autoclaved until dissolved. Agar (45° C) was poured into commercially available petri dishes^b to give an agar thickness of 2.8mm (15 ml of agar in 100 x 15 mm plate). Plates were allowed to cool in a relatively dust-free environment with lids off to permit the escape of water vapor. Wells were cut in the solidified agar using a 7 well patterned template cutter.^c Agar plugs were suctioned from the wells using a metal or glass cannula drawn to a small opening (1-2 mm in diameter).

Antigen^c was placed in the center well with a capillary pipette. Positive bluetongue control serum^c was placed in wells on each side of the serum sample to be tested (i.e., three test samples can be tested with each pattern). Wells were completely filled to the agar surface, leaving no meniscus. Plates were incubated at room temperature (20-25° C) in a closed moist chamber. Tests were read between 24 and 48 hours incubation. An intense narrow beam of light against a black background was used for reading. (Figure 1)

^aAgarose type II No. A06877, Sigma Chemical Company, St. Louis, Mo.

^bFisher brand. Fisher Scientific Company.

^cSupplied by National Veterinary Service Laboratories (NVSL), Animal and Plant Health Inspection Service, USDA, Ames, IA 50010.

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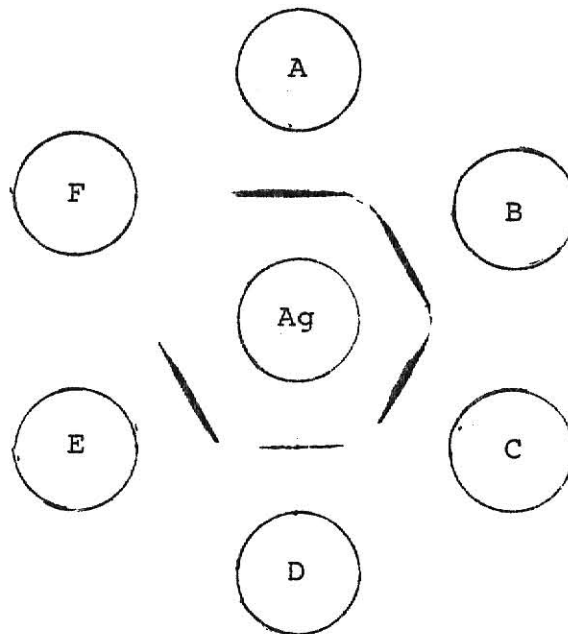


Figure 1. Immunodiffusion test which has antigen in center well; control positive serum in wells A, C and E; strong positive serum in well B; weak positive in well D; negative serum in well F.

Serum Neutralization

Each serum sample was tested for IBR and BVD antibodies using the microtitration neutralization test. Twenty-five μ l of Eagle's minimal essential medium^d was dispensed into each well of a microtiter plate^e using a microdropper.^f Serum samples were inactivated at 56°C for 30 minutes, then 0.025 ml was placed into the first well of each column in duplicate. Serial two-fold dilutions were made using 0.025 ml diluter loops.^f A plastic lid was placed over each plate between interim periods to minimize contamination and evaporation.

The virus (IBR or BVD) was added in 0.025 ml amounts to all the wells. The antigen was pretitered then diluted to 100-500 TCID₅₀. Virus controls, 10^{-1} - 10^{-4} in Eagle's MEM, were each transferred to 4 wells of the microtiter plate using 0.025 ml of diluent and 0.025 ml of the proper virus dilution. The plates were agitated and incubated at 37°C for one hour.

Passage one embryonic bovine kidney cell cultures were trypsinized and a cell suspension was made using MEM media containing 10% BVD antibody free bovine fetal serum^g, 100 units of penicillin, 100 mg streptomycin and 2.5 mg/m. amphotericin B. The cell culture suspension was counted using a hemocytometer and diluted to 100,000 cells/ml. The plate was removed after the one hour incubation and

^dGibco Laboratories, Grand Island, N.Y. 14072

^eCostar, 96-well tissue culture clusters, Cambridge, MA 02139.

^fDynatech Laboratories, Alexandria, VA 22314.

^gSterile System, Logan, UT 84321.

0.1 ml of cell suspension was dispensed into each of the wells used in the test. The plates were incubated at 37°C in a 5% CO₂ humidified incubator.

The IBR test were examined at 48-72 hours when the cytopathogenic effects (CPE) in the virus controls indicated that 100-500 TCID₅₀ were present in the virus test dose. The BVD test plates were examined at 72 and 96 hours for CPE.

Viral Isolation

The following cell cultures were used in this study; Vero MARU (African green monkey kidney), VM149 (monkey kidney-Panama cell line), BK (Solorzano cell line of bovine kidney) and EBK (embryonic bovine kidney). The EBK's are a primary cell culture propagated from the kidney of fetuses obtained from slaughtered pregnant cattle. The fetal kidneys were shown to be free of non-cytopathogenic strains of BVD virus by passing the cultures three times and examining each passage by immunofluorescent antibody technique.

The inoculum was prepared by combining 0.1 ml of the raw semen, 0.1 ml of an antibiotic solution (penicillin, 10,000 IU; streptomycin, 10,000 ug/ml; amphotericin B, 2.5 ug/ml) and 0.8 ml of double distilled water (5). The semen suspension was sonicated^h on ice, twice for 15 seconds. A 10 second interval was allowed between sonications (5,9).

Monolayered VM149, vero, and BK and EBK cell cultures grown in 25 cm² plastic flaskⁱ were washed with CMF-PBS pH 7.2 then 0.2 ml of the inoculum was added to each cell type. The samples were absorbed for one hour at 37°C. Maintenance media (medium 199^j for VM149's and vero's/Eagle's MEM for BK's and EBK's) were added to each flask and changed every 48 hours.

The inoculated cell culture flasks were incubated at 37°C and 5% CO₂ in a humid chamber until 50-70% of the cells showed CPE.

^hFisher-Sonic Dismembrator-Model 300, Farmingdale, N.Y. 11735.

ⁱCostar Tissue Culture Flask, Cambridge, MA 02139.

^jGibco Laboratories, Grand Island, N.Y. 14072.

If no CPE was noted, the cultures were observed for 10 days and harvested at 4°C. A blind subpassage was made of each of these cell cultures by sonicating the sample as above and inoculating cell cultures of the respective cell type.

If no CPE was noted after another 10 days the cultures were harvested, sonicated and inoculated on VM149 and EBK cell cultures grown on cover slips in Leighton tissue culture tubes.^k All samples were tested for the presence of bluetongue and BVD antigens by the use of immunofluorescent antibody technique after an incubation of 72 hours. The VM149 cultures were used for bluetongue test and EBK cultures for the BVD FA test.

The cover slips were removed from the tubes, washed twice by immersion in two separate coplin jars of phosphate-buffered saline solution (PBS), pH 7.2. The excess solution was removed and the coverslips immersed in cold acetone (4°C). The coverslips were fixed for 10 minutes, drained, and air dried. Slides were placed on a flat surface, flooded with appropriate fluorescein-conjugated antiserum, IBR, BVD and BT^l and placed in a humid chamber at 37°C for 30-45 minutes. The conjugate was removed by washing in 2 changes of PBS 0.01 M, pH 7.2-7.4. The coverslips were immersed for 10 seconds in Evans blue solution^m (0.02% in distilled water), rinsed in distilled water and allowed to air dry. The coverslips were mounted on microscope slides, using 50% glycerin in PBS.

^kBellco Biological Glassware and Equipment, Vineland, NJ.

^lNADL, Ames, IA.

^mEastman Kodak Co., Rochester, NY 14650.

The slides were examined by fluorescent antibody microscopy.

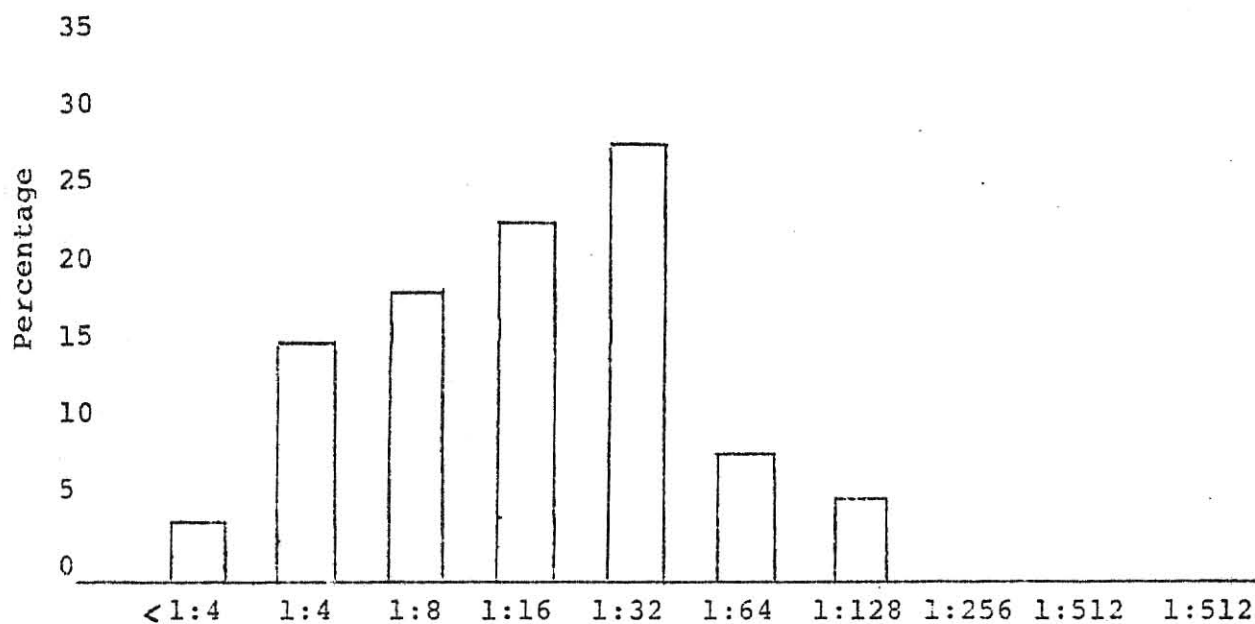
Sample slides were compared to positive and negative controls.

RESULTS

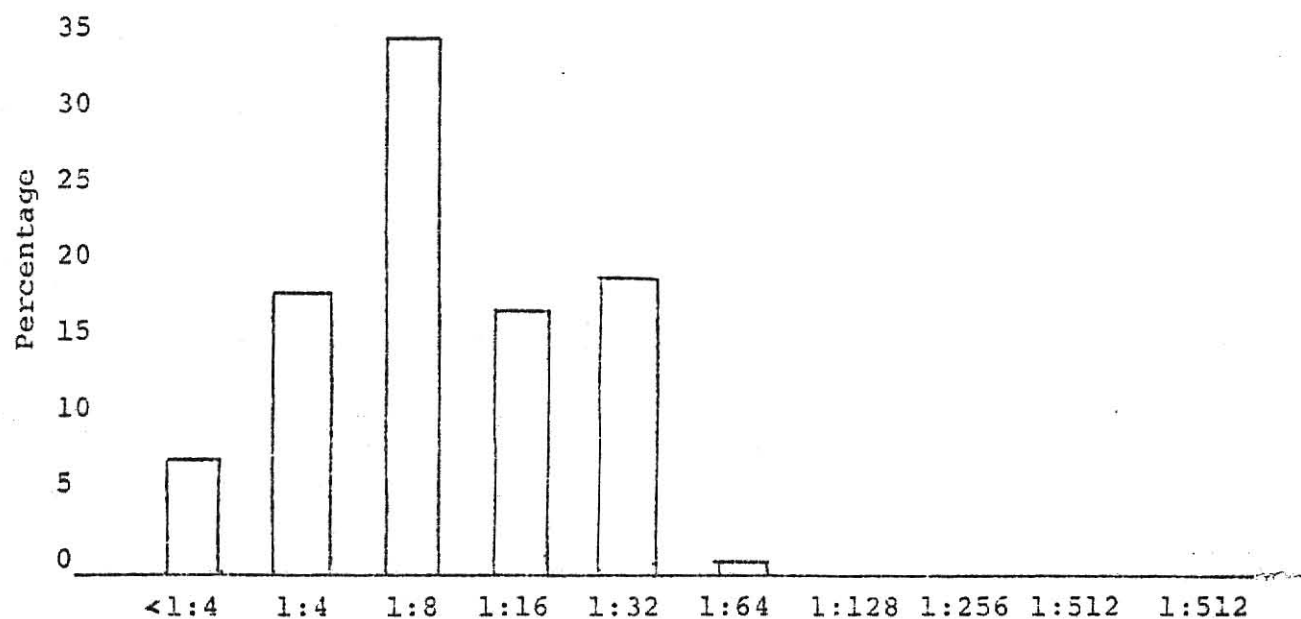
Serum neutralization antibody titers for both BVD and IBR indicated a high degree of exposure to these viruses. There was a wide range of antibody titers; BVD ranged from 1:4 to 1:256, IBR ranged 1:8 to 1:512. Bovine viral diarrhea antibody titers equal to and greater than 1:32 were observed in 21.25% of the bulls in Group I and 85% of the bulls in Group II. (Graphs 2 and 4). Infectious bovine rhinotracheitis antibody titers equal to and greater than 1:32 were observed in 41.25% of the bulls in Group I and 87.5% of the bulls in Group II. (Graphs 1 and 3).

Agar gel immunodiffusion was performed on serum samples of 160 bulls of which 19 were serologically positive, indicating a 13% incidence.

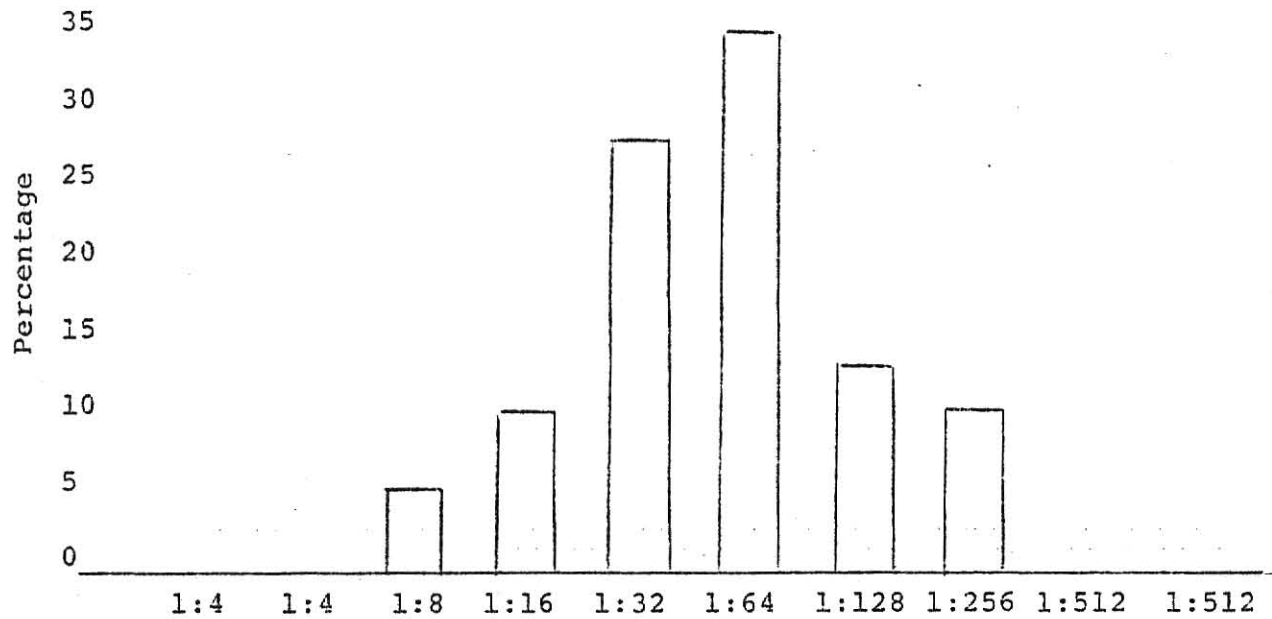
Results of virus isolation by cell culture inoculation revealed the detection of a non-cytopathogenic BVD virus in 2 of the 160 semen samples. This was identified by the use of immunofluorescent antibody technique.



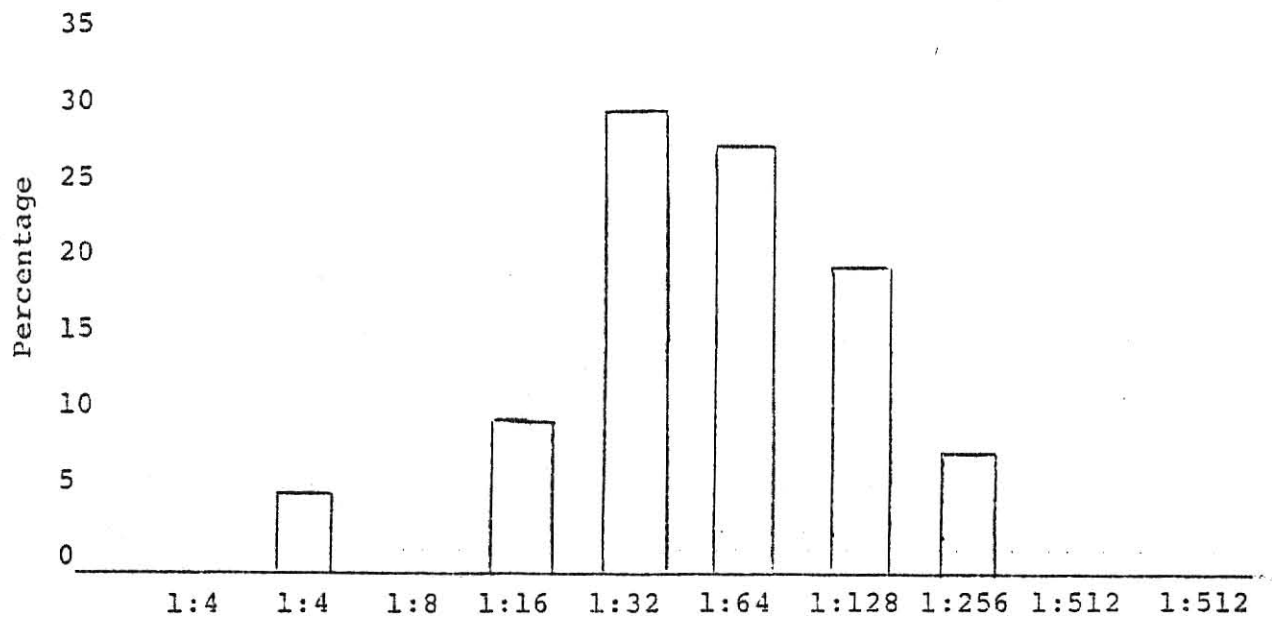
Graph 1. Distribution of serum neutralization titers for IBR in Group I bulls.



Graph 2. Distribution of serum neutralization titers for BVD in Group I bulls.



Graph 3. Distribution of serum neutralization titers for IBR in Group II bulls.



Graph 4. Distribution of serum neutralization titers for BVD in Group II bulls.

DISCUSSION

The bulls were vaccinated on arrival at the test station, and serum samples were drawn 6 months later before they were dispersed. Compared to lower titers seen with vaccinations, titers of $\geq 1:32$ for IBR and BVD were observed, indicating a more recent exposure to the wild-type virus strains. Vaccination titers generally range from 1:4 to 1:8 and occasionally 1:16 for IBR, while BVD titers range from 1:8 to 1:16.

This study shows a high prevalence of IBR and BVD which was expected in a group of animals pooled together from such a wide geographic area. With this type of exposure and re-exposure to the virus, it was felt that there was a greater chance to catch these animals in a viremic state, thus increasing the chance of transmitting the virus in semen. However, as indicated by Schultz et. al. (26), a vaccination program (vaccinations every 3-4 months) may have been enough to suppress a viremic state from developing to such an extent that it could be detected in the semen. Nineteen of the bulls were tested serologically positive for bluetongue virus showing an obvious incidence of BTV in Kansas herds.

Virus isolation from semen has met with varying results. Most isolations have been from animals during the active viremia as compared to the latent infections. Detection of unadapted or latent BTV is especially difficult in cell cultures which has made it necessary to continue isolation in sheep and embryonated chicken eggs.

Different procedures, and use of cell cultures more susceptible to a specific virus in question, have made virus isolation on cell

cultures possible. Vero (MARU) cell cultures have been used in the detection of BTV from semen and blood (1,5). Preliminary trials using primary EBK's showed the same sensitivity to adapted bluetongue virus as compared with vero (MARU) cells by immunofluorescent microscopy. Bando (1) also reported susceptibility of primary EBK's. Four different cell cultures were used throughout this test, with at least three different cell cultures used on each semen sample.

Transition metal ions have been reported to enhance isolation of certain virions, BTV (5) and Semliki Forest virus (35), possibly due to a higher absorption rate of the virus to cell surface. Breckon's method of using sonication and distilled water treatment of semen was used in this study to enhance isolation (5).

Problems encountered were cytotoxicity of the bovine semen on cell cultures (17,30) and the possible dilution of the virus to undetectable levels. Undiluted bovine semen may cause cytotoxicity to the cell cultures making the test unreadable; kaolin, trypsin-inhibiting-substance, pooling of samples, and dilution with distilled water or saline. All of these methods reduce the viral concentration of the sample.

BVD was the only virus isolated from semen in this study which was identified by immunofluorescent antibody technique. No indication of CPE in the cell cultures was observed in both instances where BVD was isolated. Others have reported BVD in semen of infected bulls and from healthy bulls on routine testing

for export (26,34). More than 95% of the bulls had antibody titers to BVD virus with approximately 21% of the bulls in Group I and 85% of the bulls in Group II having titers greater than 1:32, indicating recent exposure.

With such prevalence of IBR and BVD virus and the apparent trend for BTV to increase in distribution, more trade restrictions may be placed on the cattle industry. Bulls shown to be sero-negative gives no assurance that they are not undergoing primary viral infection (19), or that the animal is not immuno incompetent (6,22,29).

Most of the abnormalities seen, broken tails, curled tails, free heads and tails, were probably from handling. The major variability observed in the samples was the quantity of sperm present. No correlation was made between the morphologic abnormalities and presence of virus.

Exposure to various infectious agents, through the intermingling of animals, can induce a viremic state which may last for the first few weeks after exposure, if no complications develop. Luedke et. al. (20,22) observed that during this viremic state the semen becomes infected, after which time the body's defenses control the viremic condition. In certain cases, a virus may lay dormant only to be activated later from a stress situation. This has been proven to be true with IBR and BVD (6,27,30,32,34). Bluetongue virus seems to have been isolated beyond a normal viremic state in only one instance. It is assumed that this bull was immuno tolerant from a possible in utero exposure (22).

In this research, an attempt was made to isolate viruses beyond the viremic state from bulls naturally exposed, in order to identify the status of these bulls before dispersal. An advantage of placing bulls in test stations is the exposure to various common viruses and the chance to recover before being dispersed. One disadvantage may be the chance for latent infections.

A program of study that should be looked into is the isolation from known infected, seropositive animals, after a series of steroid treatments.

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VIRUS ISOLATION FROM SEMEN AND
SEROLOGY OF YOUNG BULLS AT THE
KANSAS BULL TEST STATION OF BELOIT

by

David John Rademacher

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ABSTRACT

Semen and serum samples from 160 young bulls (80 each at 2 different collection dates) were collected prior to their dispersal from the Kansas Bull Test Station. Virus isolation was performed on all semen samples using three of four different cell culture types; Vero MARU (African green monkey kidney), VM149 (monkey kidney - Panama cell line), BK (Solorzano cell line of bovine kidney) and EBK (embryonic bovine kidney). A non-cytopathogenic BVD virus was detected in 2 of the 160 semen samples by immunofluorescent microscopy. Antibody titers determined for BVD and IBR by serum neutralization were higher than normally seen from immunization. Bovine viral diarrhea antibody titers $\geq 1:32$ were observed in 21% of the bulls in Group I and 85% of the bulls in Group II. Infectious bovine rhinotracheitis antibody titers $\geq 1:32$ were observed in 41% of the bulls in Group I and 87.5% of the bulls in Group II. Agar gel immunodiffusion for BTV antibodies was also performed on the serum samples of which 19 were serologically positive, indicating a 12% incidence of infected bulls.