Effect of Natural Breeding of Heifers to a Bluetongue Virus Carrier Bull

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Summary

Fourteen nulliparous heifers were impregnated over a period of one year by a bluetongue virus carrier bull known to shed bluetongue virus in his semen. All 14 heifers became viremic, but no readily apparent clinical sig s of bluetongue disease were seen. Two heifers did not conceive after inital insemination and were rebred twice before they were impregnated. One heifer aborted between 55 and 62 days after conception and was subsequently anestric for 300 days. This heifer was the only one of the 14 heifers to develop precipitating antibody to bluetongue virus, first detectable at 100 days after insemination. Thirteen of the 14 heifers calved after gestation periods ranging from 281 to 297 days (median = 288 days)/ Twelve calves were born alive and all exhibited varying degrees of congenital anomalies including excessive gingival tissue, crooked legs, macula on the skin of their noses and tilted jaws. Five calves were characterized as weak and ataxic at birth. One calf was dead at parturition and had to be removed by caecarean section. All live calves were viremic at birth.

Introduction

When bluetongue virus (BTV) was isolated by sheep inoculation from the semen of a latently infected Hereford bull at our laboratory, studies were initiated to determine whether BTV was present in semen of other infected bulls. Subsequenly, 4 mature bulls were infected serially by bites of BTV-infected *Culicoides variipennis* flies, and BTV was isolated and confirmed in 10 blood and semen specimens collected concurrently from each bull through 106 days after insect transmission (1). Successful isolation of BTV from the semen specimens in African green monkey kidney cells (Vero MARU[a]) was attributed later to the quality of the deionized distilled water used in the preparation of the cell culture inoculum (2). Also, BTV seropositive bulls in the field and BTV carrier bulls had a positive correlation between the presence of BTV in semen specimens and abnormalities (concavities) and BTV-like particles in the heads of affected spermatozoa (3).

The BTV carrier bull in the present study was produced by infection of his dam at 60 days' gestation by bites of BTV-infected *C. variipennis* (4, 5). An overwintering mechanism for perpetuation of BTV was discovered when the virus was biologically transmitted by the bites of *C. variipennis* from this bull to recipient sheep (6). The present study was initiated to determine whether a known BTV carrier bull that sheds BTV in his semen could transmit BTV to his offspring through natural breeding.

Materials and Methods

Animals--Twelve nulliparous Hereford heifers, 16 to 18 months old, were purchased from a closed, BTV-free herd that was isolated from other cattle. Two BTV-free nulliparous Hereford-Holstein crossbred heifers of similar age born and raised at the laboratory were included. BTV-susceptible Warhill sheep, 1 to 2 years old, were used for isolation of virus. The Hereford bull (1A) was born 5 June 1970 (4) and was 5 to 6 years old during this experiment.

Virus--A total of 45 confirmed BTV isolates were obtained from bull 1A from the time of his birth until the natural breeding of heifers. These isolates included 36 from blood and 9 from semen samples. Several of these BTV isolates were serotyped later as serotype 11 (7). However, a retrospective evaluation of the original experiments from which this bull was produced (4) confirmed that although serotype 13 had been used to infect his dam, only serotype 11 was found in the samples obtained from bull 1A. The BTV used for sheep challenge inoculation was the 2nd and 3rd sheep passage of BTV (serotype 11) obtained from the blood of bull 1A at 5 years of age and was stored at 4°C as blood preserved in OPG.[b]

Viral assay--Sheep, embryonating chicken eggs (ECE), and Vero cell cultures (VCC) were inoculated as previously described (1, 4).

Whole blood to which an equal volume of OPG solution was added and blood with heparin (heparinized blood) for washed erythrocyte samples were collected routinely from heifers after breeding. These samples were assayed by intravascular inoculation of 12 to 18 of the 11-day-old ECE

at 0, 28, and 50 days after natural breeding exposure (DAE) and every 50 days thereafter through parturition.

At parturition, 3 kinds of samples were obtained from the dams for viral assay. 1) 200 ml. of heparinized blood: for erythrocytes to be washed once before inoculation; 2) 10 ml. of heparinized blood to be washed once with 15 = 2 mg. lipase c added followed by incubation at 37°C for 1 hour and storate at 4°C in an equal volume of OPG; and 3) colostral whey samples as a pool from each quarter of the udder.

Samples obtained from the calves at birth were: 1) 50 ml. of heparinized blood for washed erythrocytes; 2) a 10-ml. sample of whole blood with added OPG, and 3) a 10-ml. heparinized blood sample treated with lipase as above. Tissue samples obtained from stillborn calves included major organs and femoral bone marrow to which an equal amount of OPG was added.

Antibody Assay--Blood samples for serum were obtained from the calves at birth (precolostral) and from the dams at 0, 28, and 50 DAE, at 50 day intervals thereafter, and at parturition. Whey samples were obtained from colostral milk collected from each quarter of the udder. Sheep serum samples were obtained at 0 and 42 days after inoculation (DAI) and at 21 days after challenge (DAC).

The agar gel precipitin (AGP) test was used to detect precipitating (P) serum antibody to BTV (8). Serums were heated at 56°C for 30 minutes for the plaque neutralization (PN) tests. Vero cells were used in the PN test as previously described (6) with reference serotype 11 viral antigen (7). All BTV isolates were identified by an indirect fluorescent antibody test in VCC (9).

Blood Collection and Hematology--Cattle were bled at least once a week for leukocyte counts and packed cell volumes (PCV). Sheep were bled daily for leukocyte counts and PCV through 10 DAC. Blood samples for complete hemograms were obtained from all newborn calves. Hemoglobin concentration, PCV, and total and differential leukocyte counts were determined by standard methods and leukocytes and erythrocytes were counted with an electronic cell counter.

Clinical Observations--Rectal temperatures were recorded in the morning and afternoon. Animals were observed daily for other clinical signs of disease.

General procedures--Beginning in May when the heifers were 2 years old, the bulls was allowed to naturally breed them 3 times while they were in estrus; after being bred each heifer was placed in an insect-secure isolation room. The bull inseminated all 14 heifers over a period of a year; most of the heifers were bred during the summer-fall bluetongue vector season. Heifers that did not become impregnated or that aborted were rebred 3 times at each succeeding estrous period.

Pregnancy was determined by rectal palpation beginning about 35 days after breeding. Stages of gestation were determined at least once each week. Ten of the 14 heifers were bred between May 22nd and November 11th of the first year, and the other four heifers were bred between March 11th and May 3rd of the next year (Table I). Calves were numbered the same as their dams with the addition of the letter A.

Results

Twelve of the 14 heifers were impregnated at the first breeding by the BTV carrier bull (Table I). Heifer No. 1 was confirmed pregnant from 35 to 55 DAE, but on 62 DAE she was diagnosed as not pregnant. No external evidence of reproductive failure was noted during this time. The heifer remained anestric through 300 days at which time she was treated for anestrus and subsequently was rebred and impregnated at 363 DAE. Two of the 14 heifers were rebred twice. Heifer No. 2 was impregnated at the 2nd rebreeding or 77 DAE. Heifer No. 7 apparently was inpregnated at the 1st rebreeding or 54 DAE (Table I) although she was in estrus at 103 DAE and rebred a 2nd time. The gestation period for heifer No. 7 conceiving at 54 DAE was 287 days. Both heifers were noted as questionable for abortion (Table I) because neither of the heifers could be confirmed as pregnant between 35 to 54 DAE.

BTV was isolated from all 14 heifers by the use of a combination of the 3 virus assay systems (Tables I and II). BTV was readily isolated in ECE in replicates from the washed erythrocyte sample obtained at 100 DAE in OPG from heifer No. 1; this was the first heifer bred and the first blood sample found positive for BTV.

Heifer No. 3 was the only heifer to develop clinical signs of disease. She developed a fever and leukopenia on 64, 65, and 69 DAE. All the other 12 pregnant heifers remained clinically normal throughout the experiment.

Heifer No. 1 was the only heifer with BTV antibodies detectable by the AGP and PN tests. These antibodies were first detected in the serum samples obtained at 100 DAE, and they remained detectable through 363 DAE when she was rebred.

Because BTV was not isolated by ECE inoculation from heifers No. 11, 12, 13, and 14 through parturition (Table II), additional sheep were used to detect the virus in these 4 heifers. A pool of 150 ml. of stored washed erythrocyte samples (50 ml.) collected at 28, 50 and 100 DAE from each of the 4 heifers was inoculated into a sheep. Two of the 4 inoculums were positive for BT by the sheep assay. The 2 sheep that were negative for isolation of BTV through 42 DAI were reinoculated with another pool of 150 ml. of stored washed erythrocytes from the same heifers. These 2 inoculums were the 150, 200, and 250 DAE samples from heifers 11 and 12; and both inoculums were positive for BTV by the sheep assay.

Twelve sheep were inoculated with 100 ml. of the washed erythrocyte samples obtained from 12 of the heifers at parturition. BTV was not isolated in ECE from blood samples collected from these sheep although 5 of the 12 sheep developed mild bluetongue-like clinical responses. Two of the 5 sheep were AGP-test positive for BTV on 42

Table I

Summary of Breedings and Rebreedings in Chronological Order, Abortions, and Day after Exposure (DAE) of Bluetongue Virus (BTV) Isolations from Heifers Naturally Bred by BTV Carrier Bull

Heifer No.	Month and day bred	DAE rebred	Abortion	DAE of BTV isolation	
1*	May 22nd	363+	yes	100,150,250	
2	June 17th	55,77	?‡	100,200	
3	July 1st	....	....	64,69,100,200,287	
4	July 28th	....	....	50,100,200,287	
5	August 13th	....	....	50,150,281	
6	August 25th	....	....	50,150	
7	September 5th	54§,103	?‡	28,50,150	
8	September 5th	....	....	28,50,150	
9	November 11th	....	....	28,50	
10	November 11th	....	....	50,100	
11	March 11th	....	....	(150,200,250)‖	296
12	April 5th	....	....	(150,200,250)‖	
13	April 7th	....	....	(28,50,100) ‖	100
14	May 3rd	....	....	(28,50,100) ‖	

* Only dam with BTV antibodies; antibodies first detected at DAE 100.
\+ Long period of anestrus.
‡ Never confirmed pregnant after first breeding.
§ Impregnation on this DAE as obviously pregnant on DAE 124.
‖ BTV isolated in sheep inoculated with a pool of 150 ml washed erythrocytes (50 ml for the 3 days listed) obtained from each heifer.

DAI and were completely protected when their immunity was challenged with homologous virulent BTV. Also, another 4 sheep had very severe BTV clinical responses after challenge of their immunity; these responses suggested previous sensitization with BTV.

All 12 live calves were confirmed viremic with BTV at birth. Assay of their precolostral blood samples resulted in 5 isolations in ECE of BTV from washed erythrocytes in OPG, 7 isolations in VCC from washed erythrocytes with added lipase in OPG, and 1 isolation from the 50 ml. of washed erythrocyte inoculum by sheep inoculation (Table III). A 50-ml. centrifuged liver suspension in OPG obtained from the stillborn calf No. 13A was also positive for BTV by sheep inoculation. Assay of the liver, spleen, femoral bone marrow, and brain suspension in OPG by ECE from 13A were negative for isolation of BTV. However, another sheep inoculated with a 40-ml. centrifuged femoral bone marrow suspension in OPG had mild BT-like clinical signs. Although BTV was not isolated from this sheep, it developed severe BT clinical signs after a challenge of immunity with homologous virulent virus. The 3 sheep that were inoculated with the 50 ml. of washed erythrocyte inoculums obtained from calves No. 3A, 10A, and 12A also developed very severe BT clinical signs after challenge of their immunity with homologous BTV.

There were 6 female and 7 male calves born and their weights varied from 28.6 to 38.2 kilograms. At birth, all 12 live calves had gross anomalies and 5 of the 12 exhibited gross dysfunctions (Table IV). Eleven of the live calves had excessive, discolored gingival tissue as previously described (4). Four calves had distinct maculae present on their muzzles; this abnormality had not been observed previously in BTV-infected calves. Some of the maculae observed at birth coalesced within days after birth, but in some affected calves, new maculae formed and coalesced before they gradually disappeared. Three calves had arthrogryposis and 3 had jaw deformities in the form of tilted mandibles (4). One calf had macrotia (abnormally large ears) that were mainly enlarged in the conchal cartilage. All anomalies disappeared from 1 to 4 months after birth. The most obvious dysfunctions observed in 2 of the 5 calves that had dysfunctions were ataxia, weakness, and opisthotonos. Several episodes of opisthotonos were observed in the 2 calves within 2 hours after birth. In the other 3 calves, ataxia and weakness were marked, and they had to be helped to nurse by bucket feeding for several days. Although the calves appeared fairly normal after 2 to 4 weeks, they were never vigorous.

The fetus of heifer No. 13 was monitored closely by rectal palpation during the last month of gestation because the fetus was almost totally unresponsive to touch during this time. Labor started on 297 DAE, but the calf was dead in utero and had to be removed by Caesarian section. The only abnormality noted was excessive gingival tissue. The liver appeared abnormal and was swollen and mottled, and the lighter areas were tan.

Table II

Number of Bluetongue Virus (BTV) Isolates Obtained from Pregnant Heifers in Embryonated Chicken Eggs (ECE). Vero Cell Cultures (VCC), and Sheep. Sheep Were Inoculated Only With Washed Erythrocyte Samples Obtained at Paturition.

Heifer No.	BTV Assay Systems		
	ECE	VCC	Sheep*
1	3	2	ND
2	2	1	ND
3	3	2	0
4	3	2	0
5	4	0	Susp.+
6	3	1	0
7	3	0	Susp.+
8	4	0	Susp.+
9	3	1	0
10	2	0	Susp.+
11‡	0	1	Susp.+
12‡	0	0	0
13‡	0	1	0
14‡	0	0	0
Totals	30	11	0

* ND = not determined

+ Sheep deevloped a suspicious bluetongue clinical response but BTV was not recovered; 2 of these 5 sheep developed BTV precipitins and were immune upon chalelnge with hemologous virus.

‡ A pool of 150 ml. of washed erythrocytes that represented 3 days of collections was positive for BTV by sheep inoculation for each of these 4 heifers.

Table III

Bluetongue Virus (BTV) Isolations in Embryonated Chicken Eggs (ECE), Vero Cell Cultures (VCC), and Sheep from Neonatal Calves, Calves were Born to Heifers Naturally Impregnated by BTV Carrier Bull

Calf No.	BTV Assay Systems+		
	ECE	VCC	Sheep
1A*	...	...	...
2A	Pos	Neg	Neg
3A	Neg	Pos	Neg‡
4A	Neg	Pos	Neg
5A	Pos	Neg	Neg
6A	Neg	Pos	Neg
7A	Pos	Neg	Neg
8A	Pos	Neg	Neg
9A	Neg	Pos	Neg
10A	Neg	Pos	Neg‡
11A	Neg	Pos	Neg
12A	Pos	Neg	Neg‡
13A	...	...	Pos§
14A	Neg	Pos	Pos
Totals	5 Pos	7 Pos	2 Pos

* Calf of this dam was aborted, and she remained anestric.

+ Neg = no BTV isolated; Pos = BTV isolated.

‡ These 3 sheep developed severe bluetongue clinical response upon challenge of their immunity with homologous BTV.

§ BTV was isolated from the sheep inoculated with the liver suspension in OPG obtained from the stillborn calf.

Discussion

Results in our previous studies with BTV in pregnant cattle showed that the virus may persist in cattle (4, 5) has an effective natural overwintering mechanism in cattle which depends upon the bites of the vector (6), and can be shed in the semen of both naturally and experimentally infected bulls (1-3). However, no information was available on venereal transmission to the dam or vertical transmission to the offspring from infected bulls. Also, no information was available on the number of natural services that might be required to demonstrate venereal transmission of the virus. We allowed 3 natural services by the bull in this study while recognizing that in natural field situation, more or fewer than 3 natural services would be likely. In contrast, we also recognized that artificial insemination with the semen of our bull would have imparted a considerable dilution factor and could have resulted in completely different data. Thus, the study first had to be carried out by the natural method of breeding.

The results of this study by natural breeding suggest that BTV may be continuously present in the semen of our carrier

Table IV

Gross Anomalies and Dysfunctions in Neonatal Calves Born to Heifers Naturally Impregnated by a Known Bluetongue Carrier Hereford Bull

Calf No.	Gross anomalies	Gross dysfunctions
2A	Excessive ginigival tissue	None
3A	Macrotia, excessive gingival tissue	None
4A	Macula, excessive gingival tissue	Ataxia, opisthotonos, weakness
5A	Arthrogryposis, agnathia (tilited mandible), excessive gingival tissue	Ataxia, weakness
6A	Arthrogryposis, macula, excessive gingival tissue	Ataxia, (reverse locomotion), weakness
7A	Excessive gingival tissue	None
8A	Agnathia, excessive gingival tissue	None
9A	Arthrogryposis, excessive gingival tissue	Ataxia, weakness
10A	Agnathia, excessive gingival tissue	None
11A	Macula, excessive gingival tissue	None
12A	Excessive gingival tissue	None
13A	Excessive gingival tissue	(Stillborn)
14A	Macula	Ataxia, opisthotonos, weakness

bull. In contrast to data in this and a previous study, the isolation of BTV from our carrier bull's semen by inoculation of sheep, ECE, and VCC was not always successful. The semen samples were usually assayed at 100 day intervals and these assays were continued through the natural breeding period described in this paper. During this latter time, BTV was isolated once in sheep, 3 times in ECE, and 3 times in VCC. The data in Tables I and II suggest that BTV was not as readily isolated from pregnant heifers No. 11 through 14 that were bred in March, April, and May of the succeeding year as from those heifers that were bred primarily during the vector season of the preceding year.

The 3 assay systems used to detect BTV were not used to compare their respective sensitivity. They were used because results in previous work (4) had shown that multiple assay systems and multiple blood samples appeared to be necessary to determine BTV persistence in cattle. The ECE assays stayed relatively current to the work load, and most samples assayed by this method were completed within 1 to 3 months. In comparison the samples assayed directly in VCC were often delayed and backlogged because of the necessity of making 2 subpassages. Therefore, emphasis was placed on obtaining assays of samples collected at parturition within 3 to 4 months after their collection. Sheep were less sensitive than the other two assay systems for detecting BTV from the specified inoculated samples obtained at parutrition. However, sheep did detect BTV in pooled washed erythrocyte samples obtained from pregnant heifers No. 11 to 14 whereas the ECE BTV assays were negative. In this situation, we believe the amount of inoculation was probably important.

Previous experience with lipase and sonication in the direct titration of virulent BRV in ECE inoculated by the yolk-sac procedure with blood samples obtained from infected sheep (10) had revealed a more consistent pattern of BTV deaths in embryos than that observed in embryos inoculated with the same samples after sonication without added lipase. A 1 $\log_{10}$ increase in the titer of detectable virus also was noted by using lipase with sonication. Lipase was believed to enhance the release of the cell-associated virus. Addition of lipase to the washed erythrocyte samples facilitated the isolation of BTV directly in VCC from the calves at birth but not from their dams.

The failure of most infected heifers to develop P and PN antibodies was unexpected. Previous experience suggested that BTV originating from the semen of our bull was pathogenic, virulent, and immunogenic for sheep. This fact was also demonstrated with the homologous sheep challenge virus used in this study. However, we had no information as to what characteristics the virus might demonstrate under natural breeding conditions. We believe that the development of P and PN antibodies in heifer No 1 was probably associated with the abortion and accompanying host response. The apparent low-level but long-duration viremia in heifers No. 3 and 4 should have been sufficient to cause antibody development. We can only speculate that either no antibodies were produced in 13 of the 14 heifers, that the antibodies produced were not detectable by the tests used, or that the antibodies produced may have been relatively short lived.

The present study duplicated natural field conditions and demonstrated that veneral transmission of BTV by natural breeding by a bull known to shed the virus in his semen is an effective, nonvector-mediated mechanism for the perpetuation of BTV in cattle.

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