

FAILURE OF EMBRYOS FROM BLUETONGUE INFECTED CATTLE TO TRANSMIT VIRUS TO SUSCEPTIBLE RECIPIENTS OR THEIR OFFSPRING

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ABSTRACT

Sixty heifers were infected with bluetongue virus (BTV) by the bites of the vector and by inoculation with insect origin virus. During the acute and convalescent stages of the infection, embryos were collected nonsurgically from these animals and washed according to the recommendations of the International Embryo Transfer Society (1). No BTV was isolated from 77 of these embryos when they were inoculated onto cell culture and into embryonating chicken eggs. There was no evidence of lateral BTV transmission when 231 of these embryos were transferred into susceptible recipients, nor was there evidence of vertical BTV transmission to the 88 calves resulting from these transfers.

Another six donors that were assumed to have recovered from a natural infection of BTV, were added to the study to increase the probability of obtaining embryos from a persistently infected BTV carrier. However, it was determined later that these animals had not been infected with BTV but with the closely-related epizootic hemorrhagic disease virus (EHDV). Embryos were collected from these donors and washed as above. Neither BTV nor EHDV was isolated from 26 of these embryos by the inoculation of cell culture and embryonating chicken eggs. There was no evidence of lateral BTV or EHDV transmission to recipients of 15 of these embryos or of vertical BTV or EHDV transmission to the resulting 7 calves. However, two recipients of embryos from one of these donors developed antibodies to BTV 6 to 9 months after transfer. Passive antibodies to BTV were also detected in their calves. There is good evidence that these two recipients acquired BTV from natural exposure to infected insect vectors and not from the transferred embryos.

Key words: bluetongue, bovine, embryo transfer, virus transmission

INTRODUCTION

Washed embryos obtained from cattle infected with bluetongue virus (BTV) have been transferred to susceptible recipients without transmitting the agent horizontally to the recipients or vertically to the offspring (2-6). However, the cumulative number of transfers accomplished in these studies is small, and all the

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embryos were collected from artificially infected, viremic donors. Therefore, the data needed to estimate the risk of introducing BTV by importing bovine embryos from countries where the virus is enzootic is not available (7). Accordingly, this study was initiated to 1) provide the trial replications needed to estimate the probability of BTV transmission by the transfer of embryos from an infected donor, 2) mimic as closely as possible the natural infection of embryo donors, and 3) collect the embryos at various periods during the donors' host-agent interaction with the virus.

MATERIALS AND METHODS

Facilities

All virus isolation attempts in cell culture and embryonating chicken eggs as well as all serological tests were conducted at the National Veterinary Services Laboratories (NVSL), USDA, APHIS, in Ames, Iowa.

The Roman L. Hruska U.S. Meat Animal Research Center (MARC), Clay Center, Nebraska, was the site of the donor infection, embryo collection and most embryo transfers. During the BTV vector season at MARC, the embryo recipients were at pasture on the U.S. Dairy Forage Research Center (DFRC) at Madison, Wisconsin, which is located in an area free of competent BTV vectors.

Donor Infection and Insemination

Sixty sexually mature, healthy, nonpregnant, nonlactating, BTV-susceptible heifers 2 to 5 days post estrus, were infected with BTV serotype 11 strain CO75B300 (BTV11) by the bites of *Culicoides variipennis* (Coquillett) ssp. *sonorensis* and by the intradermal inoculation of a suspension of homogenized, infected, *C. variipennis sonorensis*. *C. variipennis* are biting midges and the principal vectors of BTV in North America.

The midges used in this study came from the AK colony maintained at the Arthropod-borne Animal Disease Research Laboratory at Laramie, Wyoming. At 1 to 4 days after emergence, they had fed through a chick skin membrane on defibrinated blood from a sheep that was infected with BTV11. After 14 to 17 days of extrinsic incubation, these midges, in lots of 25 to 100, were given access to shaved areas on the backs of the donor heifers for 30 minutes. During this time 37.5% of the midges took a blood meal, and it was determined later that 42.5% of those feeding were infected with BTV11.

In retrospect, the above exposure would have infected most of the heifers. However, if the insect feeding alone had failed to infect sufficient donors, the necessary number of embryo transfers would not have been obtained, and resources invested in qualifying the large number of recipients would have been wasted. Therefore, following the insect exposure, each heifer was inoculated intradermally with an infected midge suspension containing about 6×10^5 median chicken embryo intravascular lethal doses (CEIVLD50) of BTV11. This suspension was made up of midges that had fed, when 1 to 4 days old, on a BTV11-infected sheep. After 16 days of extrinsic incubation, the midges were pooled, homogenized, and stored in

phosphate-buffered saline solution (PBSS) with antibiotics and 0.5% bovine serum albumin. Virus isolation and antibody titers confirmed that all 60 heifers became infected with BTV-11; however, one was too small to use in the study.

Between 5 and 8 days post infection, the 59 remaining donors were given a superovulatory treatment (8). They were artificially inseminated at 12, 24 and 36 hours after the onset of estrus (Day 0) with semen from bulls that had no evidence of exposure to BTV. Embryos were collected on Day 7 or 8 and, at the same time, a blood sample from the donor was submitted to the laboratory for virus isolation. If BTV was isolated, the embryos were considered to be from an "acute" donor.

At 1 1/2 and 3 months post infection, second and third embryo collections from these donors were attempted. Blood taken from each heifer at the time of embryo collection was submitted for virus isolation and serological testing. Animals that were no longer viremic were considered "convalescent" donors.

In March 1987, six donors that had not been artificially infected were added to the study. It was assumed that these animals had acquired a natural BTV infection during a previous vector season because they were positive to the BTV AGID test in December 1986. They were considered to be "recovered" donors, and it was believed that their embryos would expand the spectrum of donor host-agent interaction represented in the study to include a population that might contain persistent virus carriers.

Embryo Collection and Treatment

Using nonsurgical procedures, embryos were flushed from the uterus with 0.5 to 1 l of sterile Dulbecco's phosphate buffered saline containing bovine serum albumin (BSA) fraction V. The effluent was allowed to stand for one hour in a sterile, siliconized, graduated cylinder. A 100-ml aliquot of the sediment was then divided among three search dishes and examined microscopically. All embryos were placed in holding dishes. The remaining sediment solution was frozen and stored at -70°C until it could be tested for the presence of BTV.

The embryos were washed 10 times in 2-ml aliquots of fresh sterile medium. They were agitated throughout the wash volume, and a new pipet was used to carry them to the next wash in 20 μ l of fluid. All embryos (up to 10) from a single donor were washed together.

Washed embryos were examined microscopically at 50x magnification. Only Grade 1 and 2 morulae and blastocysts whose zonae pellucida appeared to be intact were selected for transfer. Each selected embryo was identified to its dam and sire and was either placed in a recipient within a few hours of collection or frozen for later transfer. Freezing and thawing was done according to a standard protocol (8).

Embryos from a given collection that were not selected for transfer were frozen in one vial and submitted to NVSL for virus isolation attempts in cell culture and embryonating chicken eggs. Also, fluids from the last two washes were combined, frozen, and submitted to NVSL for virus isolation.

Recipient Qualification and Isolation

Embryos from infected donors had to be the only possible source of BTV infection for the recipients or their offspring. Therefore, all candidate recipients with prior BTV exposure were rejected, and the remaining recipients were isolated from BTV vectors for the duration of the study.

To do this, a large number of candidate recipients were tested for antibodies against BTV and EHDV using the respective AGID tests. Negative animals were retested twice more at 30-day intervals. At the time of the second test, blood cells from the candidates were washed with PBSS and inoculated into sheep susceptible to BTV. The inoculated sheep underwent the blood autograph procedure and were monitored for pyrexia and leukopenia for 4 weeks (9). Serum specimens collected from the sheep 40 to 45 days after inoculation were tested for the presence of BTV antibodies. Only heifers with negative results on all serologic tests and on the sheep inoculation test were used in the trial.

In the past, animals at MARC have become infected with BTV during the vector season, so the recipients had to be moved to a BTV-free area from early May until late October (10). The DFRC was selected as a summer holding area because epidemiological evidence indicated that BTV is not transmitted in Wisconsin (J.E. Pearson, personal communication, 1987; 11). To verify that the DFRC was a safe holding area, blood was collected on March 31, 1987, from 279 animals that had been exposed to flying insects at the DFRC during one or more vector seasons. Tests were negative for BTV group-specific antibody, confirming the absence of BTV transmission during those preceding seasons. Additionally, on March 31, 1987, 150 calves that had never been exposed to vectors but were scheduled to occupy summer pastures adjacent to those of the study recipients, were tested and found free of group-specific BTV antibody. One year later, most of these 150 animals were included in a group of 293 cattle tested for BTV antibody at the DFRC. The results of these tests were uniformly negative, indicating that BTV was not being transmitted at DFRC during the time the study recipients were at pasture there.

New Jersey light traps were operated at MARC from March 5 to November 14, 1987. No *C. variipennis* were caught in these traps prior to the recipients removal on May 4, 1987. The highest number of vectors was trapped on August 13, but the last female *C. variipennis* was caught on October 29, one day after the recipients were returned to MARC.

Embryo Transfer and Recipient Testing

The fresh or thawed embryos were transferred nonsurgically into recipients that had been closely observed for standing estrus. The recipient's estrous cycle at transfer was within one day of the donor's cycle at collection. Each recipient received one embryo per transfer, but recipients returning to estrus after receiving an embryo were occasionally used for later transfers. Additionally, each time a recipient received an embryo, her blood was tested for BTV or antibodies to BTV.

After receiving embryos, the recipients were observed for signs of disease and were periodically tested for BTV group-specific antibodies. If the recipient did not become pregnant as a result of a transfer but remained free of signs of or antibodies to BTV for at least 60 days after receiving the embryo, it was assumed that there had been no lateral transmission from infected donor to recipient via the embryo. If the recipient became pregnant but remained free of signs of or antibodies to BTV for at least 60 days after abortion or parturition, it was assumed that there had been no transplacental exposure to a viremic fetus that had been infected vertically via the embryo. If the offspring remained free of signs of or antibody to BTV for at least 60 days after its birth, it was assumed that there had been no vertical transmission from infected donor to offspring via the embryo.

All recipients were tested for the presence of BTV antibodies at the end of April 1987, before they were transported to the DFRC. They were again tested on July 30, 1987, while they were at the DFRC and, at this time all recipients were examined for pregnancy. Open animals were removed from the study and pregnant animals continued to be observed at the DFRC until October 28, 1987, when they were returned to MARC. Blood was collected from the pregnant recipients on October 29, 1987, and tested for the presence of BTV antibody. All recipients were tested for antibody and virus on the day that they gave birth and again more than 60 days later.

Virus Isolation and Serology

Blood cells, embryos, wash fluids, and flush fluids were sonicated and inoculated into embryonating chicken eggs by the intravenous route and onto Vero-M cell culture. Two serial passages were made in cell culture and suspensions of tissues from chicken embryos that died were inoculated by the yolk sac route into other embryonating chicken eggs as a second passage. Suspensions of embryos that died from the second passage were inoculated onto Vero-M cell cultures, which were then stained with a BTV fluorescent antibody conjugate and examined microscopically under ultraviolet illumination. The BTV and EHDV AGID and serum neutralization tests were done using previously described techniques (12,13).

RESULTS

Acute Donors

There were 169 embryos collected from 34 of the 59 viremic donors. Virus was not isolated from 57 embryos submitted to the NVSL. Recipients of 108 fresh and 2 frozen-thawed embryos remained free of evidence of BTV infection for more than 60 days post transfer. The 36 calves resulting from these transfers had no evidence of BTV infection when at least 60 days of age; their surrogate dams were also free of evidence of BTV infection at that time. Two of the embryos collected were not used in the study.

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Convalescent Donors

There were 141 embryos collected from 44 convalescent donors. Virus was not isolated from blood taken from the donors at the time of embryo collection or from 20 washed embryos submitted to the NVSL. Recipients of 59 fresh and 62 frozen-thawed embryos remained free of evidence of BTV infection for more than 60 days post transfer. The 52 calves resulting from these transfers had no evidence of BTV infection when at least 60 days of age; their surrogate dams also remained free of evidence of BTV infection.

Recovered Donors

There were 52 embryos collected from six donors that were thought to have recovered from BTV infection but had actually recovered from EHDV infection. Virus was not isolated from blood taken from these donors at the time of embryo collection or from the 25 washed embryos submitted to NVSL. Ten frozen embryos were not used in the study. Of the remaining embryos, 11 were transferred fresh and 6 were frozen and thawed before transfer. The recipients of 15 of these embryos remained free of evidence of BTV or EHDV infection as did their 7 calves for more than 60 days after parturition. However, two recipients that had remained free of signs and antibodies to BTV for more than 5 months after transfer developed antibodies to BTV serotype 11 by 9 months after transfer. Calves from these two recipients also had antibodies to BTV at the time of their birth.

DISCUSSION

There was no evidence of lateral BTV transmission when 231 embryos were collected from donors in the acute or convalescent stages of BTV infection and transferred into susceptible recipients. Also, there was no evidence of vertical BTV transmission to any of the 88 offspring resulting from these transfers. The failure to isolate BTV from 77 additional embryos collected from these donors confirms earlier studies. No virus was isolated from flush fluids or wash fluids, even from the viremic donors. This may have been because very few of the collections contained appreciable amounts of blood.

The two recipients developing antibodies to BTV serotype 11 had both received one embryo from a collection of 13 obtained on May 1, 1987. These embryos came from a donor considered to be BTV "recovered" because serum collected from it in November 1986 was positive on a BTV AGID test. However, serum neutralization tests on serum taken from this donor at the time of embryo collection on May 1, 1987, revealed no neutralizing antibodies to BTV. Instead, neutralizing antibodies to EHDV was present at a 1:640 dilution. Therefore, the November 1986 response on the BTV AGID test was a cross-reaction due to high EHDV antibody levels (12-16). Because this donor had recovered from an EHDV infection and not a BTV infection, the BTV serotype 11 acquired by the recipients could not have come from the embryos they received.

Most probably, the two recipients acquired the BTV11 from exposure to infective *C. variipennis* after their return to MARC in October 1987. This infection

had to have occurred very late in the year because one recipient received its embryo on May 1 and the other on May 20, 1987; yet both recipients were negative on serological tests conducted July 30 and October 29, 1987. The first serum samples containing antibodies were collected at calving, on February 2 and on February 18, 1988. *C. variipennis* were active at MARC after October 28, 1987. Climatic data indicate that October temperatures in the Clay Center area were below average, but November temperatures were above average (17). Light traps maintained at MARC from March 5, 1987, through November 14, 1987, were capturing *C. variipennis* until October 29, 1987, even though the first freeze had occurred on October 9, 1987. Other cattle at MARC, not involved in this study, acquired BTV11 antibodies in the fall of 1987 (G.S. Ross, personal communication, 1988).

In summary, this study found no evidence of BTV11 transmission from viremic or convalescent donors to susceptible recipients or their offspring by Day 7 to 8 embryos that had been washed according to the recommendations of the International Embryo Transfer Society. No virus was recovered when washed embryos from the same donors were assayed in Vero-M cell culture and embryonating chicken eggs. This information will enable the assessment of the risk associated with importation of embryos obtained from similar donors in countries where BTV is enzootic.

Unfortunately, the "recovered" donors selected in our study had been infected with EHDV instead of BTV. Therefore, our results provide no information about the risks associated with importation of embryos obtained from recovered donors in countries where BTV is enzootic.

The only two recipients developing BTV antibodies in this study received embryos from one of the EHDV-recovered donors. Therefore, their exposure to BTV was not due to the transferred embryo. Most likely they acquired the BTV serotype 11 from the bites of naturally infected vectors in Nebraska.

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