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CONSERVATION OF GERM PLASM FROM BISON INFECTED WITH *BRUCELLA ABORTUS*

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ABSTRACT: Reproductive procedures for cattle were adapted to American bison (*Bison bison*) to evaluate the potential preservation of germ plasm from bison infected with *Brucella abortus* without transmission of the pathogen to the recipient or offspring. Two of four experimentally inoculated bison bulls excreted *B. abortus* in the semen. Four healthy calves were produced from non-infected, un-vaccinated bison cows by natural breeding with a bison bull excreting *B. abortus* in the semen. There was no seroconversion of the cows or their calves. Two culture negative bison calves were produced by superovulation of infected bison donor cows followed by artificial insemination and embryo transfer without transmitting *B. abortus* to recipient cows or calves. These limited data indicate that embryo manipulatory procedures and natural breeding in bison may facilitate preservation of valuable germ plasm from infected bison while reducing the risk of transmission of *B. abortus* to recipients and progeny.

Key words: Bison, *Bison bison*, *Brucella abortus*, brucellosis, domestic bovine reproductive procedures, embryo transfer, reproduction.

INTRODUCTION

Brucellosis is a bacterial disease of many animal species, including humans and is caused by *Brucella* spp. In most natural host species, the usual clinical manifestation is abortion and/or reduced fertility. The principal means of transmission of *Brucella abortus* in domestic cattle is by ingestion of placental fluids or membranes at the time of abortion or parturition. The national brucellosis eradication program operates under the guidelines provided in the United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Uniform Methods and Rules (UM&R), Brucellosis Eradication program (Anonymous, 1992). Accordingly, current federal regulatory guidelines require that infected cattle or bison (*Bison bison*) must either be neutered or removed from susceptible populations to eliminate brucellosis. With the continuing debate over proposed brucellosis management practices in the Yellowstone National Park (Wyoming, USA) and adjacent areas which result in large annual losses of bison (Lawler, 1997), development of methods to preserve bison with superior or unique genotypes would be a welcome alternative to mitigate the loss of valuable germ plasm from this region of the USA. Thus, it is important to know if the germ plasm of potentially unique bison can be salvaged by adapting reproductive techniques used in other species, including natural breeding and/or superovulation, artificial insemination, and embryo transfer. A potential disadvantage of this approach for use of infected bison would be transmission of *B. abortus* to the recipient of the germ plasm and/or to the resulting offspring. To be an acceptable method, the germ plasm of *B. abortus* infected bison should be preserved without the future potential for transmission of *B. abortus*.

An objective of our study was to determine if experimentally infected bison bulls excreted *B. abortus* in the semen and if *B. abortus* semen positive bison bulls were

capable of infecting susceptible bison cows by natural breeding. Orchitis caused by *B. abortus* in bison was first documented by Creech (1930) from the National Bison Range (Moiese, Montana, USA). It is unknown if *B. abortus* is venereally transmitted by bison bulls. Tunnicliff and Marsh (1935) reported isolating *B. abortus* from bison bulls located at the National Bison Range and Yellowstone National Park in 1932 and 1933. Their observations of the rate of orchitis and epididymitis varied considerably. In one group of 26 pairs of testicles obtained randomly at slaughter in 1932, only one testicle had gross lesions, from which *B. abortus* was isolated, yet 16 of the 26 bulls were serologically positive. In 1933, ten bulls were sampled at random of which six had testicular lesions and one testicle was culture positive for *B. abortus*. Corner and Connell (1958) reported that enlarged testicles in a pendulous scrotum were common in bison bulls from the Elk Island National Park (Alberta, Canada). Choquette et al. (1978) found testicular enlargement in 22 (4%) of 496 males over a 4 yr period at Elk Island, and 13 (76%) of 17 were positive on multiple tests for *Brucella* antibodies. Testes from seven of these bulls were culture negative for *Brucella* spp.

Another objective of our study was to determine if embryos collected from *B. abortus* infected, superovulated bison cows which are artificially inseminated with semen from noninfected bison bulls can be transferred to susceptible recipient bison without transmitting *B. abortus*. Use of embryo transfer for the conservation of bovine germ plasm from *B. abortus* infected cattle has been investigated for several years. Voelkel et al. (1983) cultured uterine flushings and embryos from superovulated, seropositive cows with negative results; however, embryos from cows inoculated in utero with *B. abortus* strain 2308 were culture positive. Stringfellow et al. (1982) did not isolate *B. abortus* from embryos of superovulated cows with culture positive lymph nodes. Stringfellow et al. (1984) incubated bovine embryos in various concentrations of *B. abortus* inoculated media, serially washed in sterile media 10 times, and no brucellae were cultured from any washes beyond the sixth serial wash and no washed zona pellucida-intact embryos were culture positive. Stringfellow et al. (1985) and Stringfellow and Wright (1989) also studied the effects of superovulation on *B. abortus* infection of the bovine uterus and found six of 11 cows had culture positive flushings when superovulated and flushed 21 to 34 days after *B. abortus* induced abortion. Two subsequent flushings at 60 to 90 days after abortion were culture negative. Barrios et al. (1988) did not find *B. abortus* when infected cattle were superovulated and flushed for ≥96 days after abortion or calving.

MATERIALS AND METHODS

Experimental infection of bison bulls

Four unvaccinated bison bulls (numbers 13, 137, 904, 907), aged 3.5 to 4.5 yr, were inoculated by bilateral conjunctival inoculation of 50 μl containing 1×10^{10} colony forming units (CFU) of *B. abortus* biotype 1 which was originally isolated from a Yellowstone National Park bison. Two bulls (numbers 13 and 137) were from a non-infected herd while the other two bulls (numbers 904 and 907) were from a herd naturally infected with *B. abortus* and were seropositive. All bulls were examined by standard breeding soundness evaluations and were found to be fertile with regard to quantity, morphology and motility of spermatozoa. All semen samples were collected by electrojaculation. The two seropositive bison bulls, which had culture negative semen, were injected intramuscularly with 0.2 mg/kg of dexamethazone (Western Veterinary Supply, Porterville, California, USA) on Monday, Wednesday and Friday for 3 wk after inoculation to induce immunosuppression and potentially enhance *B. abortus* excretion in the semen (Kuttler and Adams, 1977). Jugular blood was collected monthly from each bull, and serum was harvested by centrifugation for detection of anti-*B. abortus* antibodies. Semen samples were collected approximately monthly for seven times from bulls 137 and 13 and 11 times from bulls 904 and 907 for semen evaluation and bacteriologic culture for *B. abortus* between October 1992 and May 1993.

Semen samples were frozen at −20 C until cultured by streaking 600 μl on three 150 mm plates of Farrell's medium (Farrell, 1974). After Gram staining, suspicious colonies were grown on tryticase soy agar (TSA) (Difco Laboratories, Detroit, Michigan, USA) plates. Typing of isolates was based on standard methods (Alton et al., 1988; Anonymous, 1965b). Serum samples from the bulls and cows were tested serologically with the card test (CT) (Anonymous, 1965a), complement fixation (CF) (Jones et al., 1963), rivanol (RIV) (Anonymous, 1965a), indirect enzyme linked immunosorbent assay (iELISA) (Byrd et al., 1979), competitive enzyme linked immunosorbent assay (cELISA) (D-Tec Brucella-A, Synbiotics Corp., San Diego, California, USA) (Adams and Mia, 1991), and particle concentration fluorescence immunoassay (PCFIA) (Snyder et al., 1990).

Natural breeding of susceptible bison cows by *B. abortus* infected bulls

From 15 February 1993 through 15 June 1993, two unvaccinated bison bulls with culture positive semen were bred by natural service to six brucellosis-free, unvaccinated bison cows (aged 2 to 6 yr). The cows were examined for pregnancy at 45 to 60 day intervals by rectal palpation and/or ultrasound (Pierson and Ginther, 1984) using a Tokyo Keiki LS-1000 instrument (Product Group International Inc., Boulder, Colorado, USA). Four of six cows conceived and delivered healthy calves. Immediately after parturition, samples collected from the cows (placenta, milk samples from each quarter of the mammary gland, two uterine swabs) and the calves (meconium, abomasum, lung, mediastinal lymph node) were cultured (Farrell, 1974; Alton et al., 1988).

Embryo transfer from *B. abortus* infected bison donor cows

Sixteen, unvaccinated, experimentally infected bison cows inoculated bilaterally intraconjunctivally with 1×10^7 CFU *Brucella abortus* strain 2308, and previously determined to be culture positive from milk, placenta or uterine swabs at various times post-parturition, were superovulated 3 to 6 mo post-partum using modifications of the bison embryo transfer techniques of Dorn et al. (1990). Eight series of the superovulations were undertaken during the year. Briefly, where A.M. indicates before 10:00 A.M., and P.M. indicates after 3:00 P.M., the basic protocol was scheduled as follows on (1) Day 4 (A.M.) recipients were implanted with norgestomet (Syncro-Mate-B, Sanofi Animal Health, Inc., Overland Park, Kansas, USA) subcutaneously (SQ) in the ear and injected with 2 ml estradiol (Sanofi Animal Health, Inc., Overland Park, Kansas, USA) intramuscularly (IM); (2) Day 0 (A.M.) donors were implanted with Syncro-Mate-B SQ; (3) Day 3 (A.M.) donors were injected with 2,500 IU pregnant mare serum gonadotropin (PMSG) (Intervet International B.V., Boxmeer, Holland) IM, 8 mg follicle stimulating hormone (FSH) (Schering-Plough Animal Health Corp., Kenilworth, New Jersey, USA) IM, and 25 mg prostaglandin $F_2\alpha$ ($PGF_2\alpha$) (Upjohn Kalamazoo, Michigan, USA) IM; (4) Day 5 (A.M.) recipients had the Synchromate B implant removed and were injected with 25 mg $PGF_2\alpha$ IM, and donors had the Syncro-Mate-B implant removed and were injected with 25 mg $PGF_2\alpha$ IM; (5) Day 6 (A.M. and P.M.). We observed recipients for estrus; (6) Day 7 (A.M.) recipients were injected with either 2 ml gonadorelin (GnRh) diacetate tetrahydrate (Cystorelin, Sanofi Animal Health, Inc., Overland Park, Kansas, USA) IM or 4,000 IU human chorionic gonadotropin (hCG) (Steris Laboratories, Inc., Phoenix, Arizona, USA) IM and observed for estrus, and donors were injected with 4,000 IU hCG and artificially inseminated at 36 and 48 hr post-implant removal with 0.5 ml of semen containing 1–5 $\times 10^8$ spermatozoa/ml and having motility ≥60%: (7) Day 7 (P.M.) recipients were observed for estrus and artificially inseminate donors; (8) Day 8, (A.M.), recipients were observed for estrus, and artificially inseminated donors if still in estrus on day 7 (P.M.); and (9) Day 14 (A.M.) non-surgically collected and transfered embryos to recipients. To reduce the stress of handling, the donor cows were injected intramuscularly by pole syringe with a combination of 50 mg of xylazine hydrochloride (Rompun, Miles Haver Animal Health, Kansas City, Missouri, USA), 5 mg of acepromazine (Ft. Dodge Co., Ft. Worth, Texas, USA), and 50 mg of ketamine hydrochloride (Ketaset, Western Veterinary Supply, Buda, Texas, USA) 10 to 30 min before being restrained in a squeeze chute. Fresh semen for artificial insemination was collected by electrojaculation from a seronegative semen culture negative bull. The resulting embryos were washed and subjected to 100 μg/ml of streptomycin sulfate (Gibco, Gaithersburg, Maryland, USA) according to the bovine embryo transfer standards of Stringfellow and Seidel (1990) and Stringfellow et al. (1991) and transferred to recipients with the zona pellucida intact. Nine seronegative, non-vaccinated bison cows (3 to 7 yr), obtained from a known non-infected herd, were used as embryo recipients. Serum samples were collected when pregnancy evaluations by rectal palpation and/or ultrasound were performed at 45 to 60 days after each transfer and each one

to 2 mo thereafter. Samples from the cow and her calf were collected immediately after parturition for culture and serological tests.

RESULTS

Experimental infection of bison bulls

Of the four inoculated bison bulls, *B. abortus* biotype 1 was cultured only once from the semen of bull 137 (1/27/93) and bull 13 (5/13/93). Extensive contamination of the semen samples made detection and quantitation of *B. abortus* difficult. The serologic results for each of the bulls are given in Table 1. The elevated and prolonged antibody responses confirm the exposure to *Brucella abortus* in all four bulls. The persistently elevated levels of anti-*Brucella* specific antibodies detected by the panel of tests in bull 137 and bull 13 for 17 mo post-inoculation strongly suggest persistent infection of the experimentally inoculated bulls.

Natural breeding of brucellosis-free bison cows by *B. abortus* infected bulls

Culture positive bull 137 was selected to breed six *B. abortus*-free cows (nos. 3, 10,

TABLE 1. Results of card, particle concentration immunofluorescence immunoassay, complement fixation, rivanol, competitive ELISA, and indirect ELISA serologic tests of bison bulls inoculated with an isolate of *Brucella abortus* from bison at Yellowstone National Park.

Bison bull	Date	Serologic tests[a]					
		Card[b]	PCFIA[c]	CF[d]	Rivanol[e]	cELISA[f]	iELISA[g]
13	2/17/94	Pos	0.28	20	2	40.75	1.130
	5/13/93[h]	Pos	0.29	20	2	45.35	1.290
	4/12/93	Pos	0.24	10	4	82.59	1.442
	1/27/93	Pos	0.20	20	2	30.21	1.334
	9/23/92[i]	Neg	0.89	00	0	13.38	0.024
	8/28/92	Neg	0.93	00	0	1.99	0.009
137	2/17/94	Pos	0.08	40	8	101.36	1.094
	5/13/93	Pos	0.07	40	8	99.73	1.560
	4/12/93	Pos	0.07	40	8	98.96	1.840
	1/27/93[h]	Pos	0.08	80	8	98.96	1.536
	11/30/92	Pos	0.09	40	8	103.34	1.342
	9/23/92[i]	Neg	0.84	00	0	0.21	0.081
	8/28/92	Neg	0.84	00	0	−7.28	0.058
904	2/17/94	Neg	0.08	40	6	100.94	1.234
	5/13/93	Pos	0.06	40	6	99.73	1.010
	4/12/93	Pos	0.15	40	4	98.80	1.539
	1/27/93	Pos	0.06	20	4	99.09	1.481
	11/30/92	Neg	0.06	40	8	106.07	1.363
	9/23/92[g]	Pos	0.10	40	2	105.56	1.464
	4/30/92	Pos	0.05	80	6	103.00	1.249
907	2/17/94	Neg	0.05	20	8	101.66	1.183
	5/13/93	Pos	0.05	10	8	99.92	1.256
	4/12/93	Pos	0.05	10	8	99.34	1.447
	1/27/93	Pos	0.05	80	8	98.83	1.425
	11/30/92	Pos	0.06	80	ND[j]	98.52	1.305
	9/23/92[g]	Pos	0.06	80	8	93.18	1.339

[a] USDA, APHIS, Brucellosis UM&R.
[b] Card test; negative = no agglutination, positive = agglutination.
[c] Particle concentration immunofluorescence assay; negative > 0.6, suspect 0.6 to 0.3, reactor < 0.3.
[d] Complement fixation, reciprocal titer; negative ≤ 1:5, suspect-1+ @1:5, positive-1+@1:10.
[e] Rivanol; negative ≤ 1, positive ≥ 2.
[f] Competitive enzyme-linked immunosorbent assay; negative < 40, suspect 40 to 70, positive > 70.
[g] Indirect enzyme-linked immunosorbent assay; negative 0 to 0.6, suspect 0.61 to 0.99, positive > 1.0.
[h] Isolation of *B. abortus* from semen.
[i] Day of conjunctival inoculation with 1×10^{10} colony forming units of Yellowstone National Park bison isolate of *B. abortus*.
[j] Not done.

TABLE 2. Generation of viable embryos, degenerate embryos, unfertilized ova, corpora lutea and ovarian follicles stimulated by superovulation and artificial insemination of bison cows[a].

Bison cow	Viable embryos[b]	Degenerate embryos[b]	Unfertilized ova[b]	Corpora lutea[b]	Ovarian follicles[b]
806	NOS[c]	NOS[c]	NOS[c]	0	1
809	1	1	1	4	2
811	1	0	2	3	2
813	NOS[c]	NOS[c]	NOS[c]	0	1
814	4	1	15	9	3
815	0	0	0	1	0
818	NOS[c]	NOS[c]	NOS[c]	NOS[c]	NOS[c]
820	1	2	1	3	1
825	0	1	0	2	1
829	0	0	1	1	3
830	2	1	0	2	1
831	0	0	0	1	0
835	0	0	0	1	0
911	0	0	0	1	2
920	0	0	0	0	0
923	0	0	1	2	2

[a] See text for completion superovulation and artificial insemination procedures.
[b] Mean of 2 or more procedures.
[c] No ovarian stimulation.

16, 18, 27, 29) by natural service from 15 February 1993 through 15 April 1993. Monthly pregnancy evaluations revealed that none of the cows were pregnant. Because of inadequate libido, bull 137 was subsequently replaced with culture positive bull 13 from April 19, 1993 through June 15, 1993. Pregnancy evaluations in late-July, 1993 revealed that four of six cows were pregnant. All cows and their calves were culture negative and remained serologically negative on the panel of tests until 60 days post-partum.

Embryo transfer from *B. abortus* infected bison donor cows

Table 2 lists the average yields for each embryo collection for the *B. abortus* infected bison donor cows. There were 33 attempts at superovulation of 16 cows of which 28 attempts provided adequate ovarian stimulation to warrant proceeding with embryo collection. From these 28 collections, 20 viable embryos, 11 degenerate embryos, and 63 unfertilized ova were harvested. Viable embryos obtained from superovulation were directly transferred to nine susceptible recipients. Due to the lack of detectable estrus by the donor cows, bison bulls were never used for natural service during any of the regimens, thus making the use of timed artificial inseminations essential. Thirteen viable embryos were transferred which resulted in two pregnancies for a pregnancy rate of 15%. Recipient bison cow 28 calved with a breech presentation. The calf was born without veterinary assistance but was dead apparently due to hypoxia created by the presentation. At necropsy, neither the placenta nor the calf had lesions compatible with those caused by *B. abortus*. Cultures of the meconium, abomasum, lung, mediastinal lymph node as well as quarter milk samples, placenta and uterine swabs from the dam were negative for *B. abortus*. Recipient bison cow 22 produced a normal calf. Calf fecal cultures collected 24 hr after birth as well as quarter milk samples, placenta and uterine swabs from the dam were negative for *B. abortus*. Serology for *B. abortus* was negative on all tests (data not shown) for all recipient cows and the embryo transfer calf when the last samples were collected at 6 mo post-parturition. From the superovulations and embryo transfers from *B. abortus* infected donors, two *B. abortus* culture negative bison calves were produced and none of the recipients had detectable *B. abortus* antibodies.

DISCUSSION

Although no evidence of venereal transmission was found, we recognize that our results are limited due to small numbers of infected bulls. Furthermore, we were unable to experimentally induce bison bulls to continuously secrete *B. abortus* in semen, although both bulls were culture positive at least once during the period of natural breeding to the six bison cows. Furthermore, quantitation of *B. abortus* of bulls 13 and 137 semen samples was not possible due to excessive preputial con-

tamination. In spite of culturing at least seven semen samples, *B. abortus* was isolated only once from each of the two bulls. We expected to obtain positive semen cultures from bull 904 or 907, because both bulls had high anti-*B. abortus* antibody titers from previous natural infection, in addition to being inoculated with 1×10^{10} CFU of the Yellowstone *B. abortus* isolate, and being immunosuppressed by 3 wk of corticosteroid treatments after inoculation. Bulls 137 and 13 developed antibody titers on all serological tests by 8 and 16 wk after the inoculation and remained serologically positive for the next 16 and 10 wk respectively. Because neither the six cows nor their four calves produced by breeding to infected bulls shedding *B. abortus* in the semen ever developed antibody responses and were culture negative at parturition, it was concluded that under the conditions of these experiments, *B. abortus* was not venereally transmitted from males to females. Thus, these observations suggest that *B. abortus* infected bison bulls could be used for breeding non-infected bison cows where loss of unique germ plasm is at risk. These findings suggest that infected bison bulls, like *Bos* spp., are not important in venereal transmission of *B. abortus*. A small percentage of infected *Bos* spp. bulls secrete the organism in their semen (Lubbehusen and Fitch, 1926) but are not considered to disseminate the disease to cows or heifers by venereal means (Schroeder and Cotton, 1916; Crawford et al., 1990). Experimental efforts to infect *Bos* spp. cows or heifers by natural breeding with infected *Bos* spp. bulls secreting the organism yielded negative results (Thomsen, 1943). Infection of *Bos* spp. cows by artificial insemination with raw infected semen has resulted in disease with the usual manifestations (Manthei et al., 1950). The difference in the transmission of *B. abortus* by artificial insemination and rare transmission by natural service in cattle is due to the site of semen deposition (Manthei et al., 1950). Artificial insemination requires that the semen be placed in the uterus, which is an ideal environment for *B. abortus* (Manthei et al., 1950). With natural breeding, the semen is deposited in the vagina which is a harsh environment for survival of the organism due to the pH of the vagina and other immunologic factors. Comparing the results of our limited venereal transmission experiments in bison with those reported in cattle, it appears that neither infected, seropositive *Bos.* spp. nor bison bulls pose a serious threat of venereal transmission.

Two culture negative bison calves were produced by superovulation of *B. abortus* infected bison donor cows and subsequent artificial insemination and embryo transfer without transmitting *B. abortus* to the recipient bison cows or calves for a pregnancy rate of 15%. Additionally, transfer of 13 other embryos from infected donors to nine recipient bison cows with these same procedures without seroconversion of the recipients further indicates that embryo transfer could be used to preserve germ plasm with minimal risk of transmitting *B. abortus*. These data also indicate that further refinement of bison-specific superovulation procedures will be necessary to improve the efficiency of embryo transfer.

In conclusion, two of four experimentally inoculated bison bulls excreted *B. abortus* in semen and failed to transmit *B. abortus* to the dam or calf. Similarly, *B. abortus* culture negative bison calves were produced by superovulation of infected bison donor cows and subsequent artificial insemination and embryo transfer without transmitting *B. abortus* to the recipient bison cows or calves. Thus, under the conditions described herein using brucellosis-free recipients, these data tend to support the concept that use of embryo manipulatory procedures and natural breeding in bison may facilitate preservation of valuable germ plasm while reducing the risks of *B. abortus* transmission to recipients and progeny.

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