Bovine Herpesvirus Type 1 in the Sperm of a Bull From a Herd With Fertility Problems

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SUMMARY

A herd of 125 Holstein cows manifested fertility problems for two years. The number of services per pregnancy was 2.97, conception rate was 33% after the first service, and the average number of open days was 127. Abortions occurred in four cows over the last 12 months. The herd was not vaccinated against any disease. Natural service by a bull and artificial insemination were used for breeding the cows. Bovine herpesvirus type 1 was demonstrated in sperm heads from the bull by direct and indirect fluorescent antibody techniques, and the virus was isolated on cell cultures. The virus was also isolated from the uterine secretions of some cows and from two aborted fetuses.

RÉSUMÉ

Présence de l'herpèsvirus bovin du type 1 dans le sperme du taureau d'un troupeau qui connaissait des problèmes de fertilité

Un troupeau de 125 vaches Holstein connut des troubles de fertilité, pendant deux ans. Le nombre de saillies par gestation s'élevait à 2.97, le taux de conception n'atteignait que 33% après la première saillie et le nombre moyen de jours entre le vêlage et la gestation ultérieure atteignait 127. Quatre vaches avortèrent au cours de la deuxième année. Ce troupeau n'avait reçu aucune vaccination et on y utilisait les saillies naturelles et l'insémination artificielle. L'immunofluorescence directe et indirecte permit de démontrer la présence de l'herpèsvirus bovin du type 1, dans le sperme du taureau de ce troupeau, et on l'isola sur cultures cellulaires. Les sécrétions utérines de certaines vaches et deux avortons recelaient aussi ce virus.

INTRODUCTION

Bovine herpesvirus type 1 (BHV-1) attacks the respiratory and genital tracts of cattle causing rhinotracheitis, vulvovaginitis and balanoposthitis. The virus can also cause conjunctivitis, encephalitis and enteritis. Infection can result secondarily in abortion or infertility (12). BHV-1 is spread from infected cattle through nasal, vaginal and preputial discharges, aborted fetuses and semen (10,14).

Like many other herpesviruses, BHV-1 is capable of establishing a latent infection in cattle. The mechanism of latency and reactivation of the virus remains unknown, and the tissues in which BHV-1 establishes latency subsequent to clinical disease are still to be identified. Snowdon (18) demonstrated recrudescence of BHV-1 in cattle up to 578 days postinfection. Other workers reported sporadic virus reexcretion by naturally or experimentally infected cattle with and without a serum antibody response (4, 11, 16). Reactivation of BHV-1 in cattle was also observed following treatment of latently infected animals with corticosteroids (6, 16).

MATERIALS AND METHODS

Case history

A herd of 125 Holstein cows experienced fertility problems over two years. All the cows developed postpartum metritis. The cows in the herd were bred by artificial insemination (AI), but after three or four unsucessful AI, open cows were served by a bull. All heifers were bred naturally by the same bull. The average number of services per pregnancy was 2.97, conception rate after first service was 33% and the average number of open days was 127. More than 18% of cows had at least four services and four cows aborted within the last 12 months. The herd had not been vaccinated against any disease.

Most of the cows had estrus within 30 days after calving, and palpation of the genital tract revealed evidence of cyclicity. In many cows, there was a yellow, sticky vulvar discharge on the external genitalia. At estrus, the secretion became white and cloudy. Close examination of the vestibular, urethral and clitoral areas revealed granular spots.

Bovine herpesvirus type 1 has been demonstrated in the semen of experimentally or naturally infected bulls but the source of virus in the semen was not identified (19). A few other viruses have been shown to infect sperm. Foster et al (9) demonstrated by electron microscopy the presence of blue-tongue virus-like particles in the heads of spermatozoa in semen from bulls latently infected with the virus. Brackett et al (5) inoculated rabbit spermatozoa with simian virus 40 (SV40) DAN, and subsequently recovered the virus genome from one-cell and two-cell embryos fertilized by these spermatozoa.

This study reports a natural infection of a bull with BHV-1, and subsequent abortion and infertility in cows covered by the bull. Viral antigen in the spermatozoa was demonstrated by fluorescent antibody test, and the virus was isolated in cell cultures. BHV-1 was also isolated from uterine secretions of cows covered by the bull.

Clinical examination of the genital organs of the bull showed ulcers in the sheath, but the penis, glans, prepuce, scrotum and testes appeared normal. Semen was submitted for examination four time over a four month period, and a preputial washing was submitted once. At necropsy, the genital organs of the bull were submitted for virus detection.

Fluorescent antibody technique

The fluorescent antibody (FA)

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technique was used to demonstrate virus antigen in the sperm and testes of the bull. A portion of fresh semen from the bull was centrifuged at 800 x g for ten minutes. The supernatant was discarded, and the spermatozoa were washed and centrifuged three times in phosphate buffered saline (PBS) pH 7.2. After final centrifugation the sedimented spermatozoa were resuspended in PBS to 25% of the original volume. A drop from the sperm suspension was added into each well of a multitest slide[1] and allowed to dry at room temperature. The preparations were fixed in acetone for ten minutes, air dried, washed three times with PBS, and dried again. The samples were subjected to direct and indirect FA staining.

The indirect FA technique was performed using hyperimmune rabbit antisera to BHV-1 or bovine virus diarrhea BVD and antirabbit goat fluorescein-conjugated globulin[2]. The antisera were diluted 1:25 and the antiglobulin was diluted 1:64 in PBS. Normal goat serum was included as a control during each test. The direct FA method was performed using fluorescein conjugated goat antisera to BHV-1 or BVD[2]. A check on the specificity of the tests was performed on FBS cells infected with BHV-1 or BVD, and on uninfected cells as outlined in Tables I and III. Frozen sections from several parts of the testes were stained by direct of indirect FA technique.

Virus isolation

For virus isolation, a portion of each semen sample was frozen and thawed three times and centrifuged at 500 x g for 15 minutes. The supernatant was diluted 1:16 in Eagle's minimum essential medium (EMEM) with 5% fetal calf serum (FCS) and antibiotics. The samples were inoculated onto 75% confluent monolayers of fetal bovine skin (FBS) cells at the second to fourth passage levels. After absorption for one hour at 37°C, the inoculum was removed and the cell sheet was washed at least three times with MEM. The cells were incubated in MEM with 5% FCS and examined at three and 12 hours after infection for evidence of toxicity due to the inoculum. Three blind passages were carried out after seven days incubation. Cell cultures that did not show cytopathic effect (CPE) were stained by the direct fluorescent antibody (FA) technique. Samples showing CPE were harvested for a further passage. The isolated virus was subjected to virus neutralization tests using monospecific rabbit anti-BHV-1 or anti-BVD sera. The virus was also identified by electron microscopy after negative staining. Seminal and uterine secretions, a preputial washing, and extracts of tissues from testes were inoculated onto cell cultures for similar studies.

TABLE I
OUTLINE OF INDIRECT FA TEST FOR DETECTION OF VIRUS IN BOVINE SPERMATOZOA AND FETAL BOVINE SKIN CELL CULTURES

	Antisera to BHV-1	Antisera to BVD	Normal Serum	Conjugated Antiglobulin	Fluorescent Results
Cells	+	-	-	+	positive
infected	-	+	-	+	negative
with	-	-	+	+	negative
BHV-1	-	-	-	+	negative
Cells	+	-	-	+	negative
infected	-	+	-	+	positive
with	-	-	+	+	negative
BVD	-	-	-	+	negative
Uninfected	+	-	-	+	negative
control	-	+	-	+	negative
cells	-	-	+	+	negative
	-	-	-	+	negative
Spermatozoa	+	-	-	+	positive
from	-	+	-	+	negative
infected	-	-	+	+	negative
bull	-	-	-	+	negative

+ = serum or conjugate added.
- = serum not added.

Bacterial and Mycoplasma isolation

Uterine and vaginal secretions, semen, and preputial washings were examined for mycoplasma as described (1). Vaginal secretions were submitted for routine bacteriological examination.

RESULTS

FA test

Spermatozoa from the infected bull obtained on August 8, 23 and November 11 showed BHV-1 specific fluorescence after direct or indirect FA staining. The fluorescence was detected in the post-nuclear cap area of the sperm head (Figure 1). Fluorescence was not observed in spermatozoa obtained on October 10. Results of controls for specificity of the test are indicated in Tables I and II. Tissue sections from various parts of testes did not show any virus specific fluorescence.

TABLE II
RESULTS OF DIRECT FA TEST FOR DETECTION OF VIRUS IN BOVINE SPERMATOZOA AND FETAL BOVINE SKIN CELL CULTURES

	Conjugated Antisera to BHV-1	Conjugated Antisera to BVD	Fluorescent Results
Cells infected	+	-	positive
with BHV-1	-	+	negative
Cells infected	+	-	negative
with BVD	-	+	positve
Spermatozoa from	+	-	positive
infected bull	-	+	negative

+ = conjugate added.
- = not added.

[1]Flow Laboratories, Toronto, Ontario.
[2]Microbiological Associated, Bethesda, Maryland.

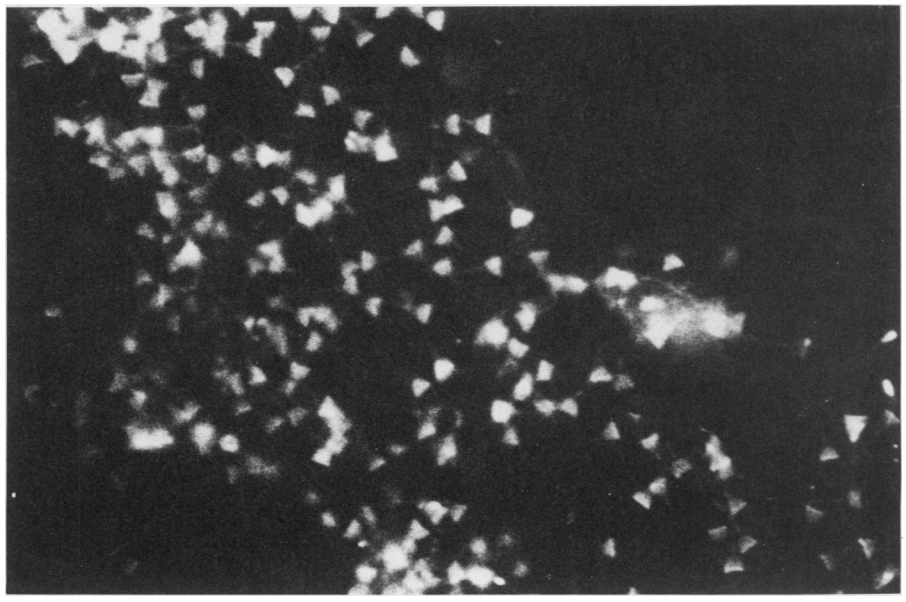

FIGURE 1. Fluorescence in the postnuclear cap area of bovine sperm infected with BHV-1 after indirect FA staining. X190.

Virus isolation

Bovine herpesvirus type 1 was isolated from semen specimens submitted on August 8 and 23, but not from those submitted on October 10 or November 11. Virus was not isolated from a preputial washing submitted on August 8 or from testes submitted at necropsy. BHV-1 was isolated from uterine secretions of eight animals examined (Table III). A neutralization test using anti-BHV-1 antiserum confirmed the identity of the virus as BHV-1. Antiserum to BVD did not neutralize the virus. Electron microscopic examination showed enveloped virus particles with the morphology of herpesvirus.

Bacterial and Mycoplasma isolation

Ureaplasma were isolated from the uterine or vaginal secretions of six cows (Table III). Nonpathogenic bacteria were isolated from the vaginal secretions of seven cows. Ureaplasma or bacteria were not detected in the semen or preputial washing. Brucella and Leptospira were not detected.

DISCUSSION

This study demonstrates the presence of BHV-1 antigen associated with the spermatozoa of a naturally infected bull. The fluorescence observed may have originated from either within or without the sperm head. Since herpesviruses replicate in the nucleus, it could be speculated that the fluorescence was of intranuclear origin. Morphological changes were observed in the same batches of spermatozoa in which the virus was demonstrated (Lamothe, unpublished). Bluetongue virus-like particles have also been detected by electron microscopy in the heads of sperm from a naturally infected bull (9), suggesting that the virus may have replicated in the sperm heads. On the other hand, in experimental attempts to infect rabbit spermatozoa with SV40, it was noted that the virus particles were adsorbed to the surface of spermatozoa without penetration. Penetration occurred only when SV40 DNA was inoculated.

In this study, the virus antigen was consistently present in the postnuclear cap area and not in the entire sperm head. A similar observation in which labelled SV40 DNA was present only in the postacrosomal area of rabbit spermatozoa was reported (5). Sullivan (21) described the nuclear material in the sperm as homogenous in distribution except in the posterior portion where it appears to be more aggregated. Whether the aggregation affects the virus distribution remains to be discovered.

The preputial mucosa is frequently quoted as the tissue from which BHV-1 is isolated from the male genital organs. In this study, the virus was detected only in the semen. It was noted that on one occasion (October 10) the virus was not detected in the sperm, indicating an intermittent shedding. Intermittent shedding of BHV-1 has previously been reported (4, 16, 18). Latency in herpes simplex virus has been associated with the persistence of the virus in the ganglia (2, 3, 20). A similar observation remains to be demonstrated in BHV-1 latency.

Although the spermatozoa carried the virus, it is still not clear whether these spermatozoa were capable of penetrating the zona pellucida of the ova causing fertilization, and whether the resulting embryos became infected.

TABLE III
RESULTS OF LABORATORY EXAMINATION OF VAGINAL AND UTERINE SECRETIONS FROM 15 COWS WITH INFERTILITY

Cow #	Clinical Condition	Bacteria Vagina and Uterus	Virus Uterus	Mycoplasma Vagina	Mycoplasma Uterus
1	diseases[a]	-	BHV-1	-	Ureaplasma
2	diseased	-	BHV-1	-	Ureaplasma
3	diseased	-	BHV-1	-	-
4	diseased	-	BHV-1	-	-
5	diseased	-	BHV-1	-	-
6	diseased	-	BHV-1	-	-
7	diseased	*Proteus* sp. *E.* coli	BHV-1	-	-
8	diseased	-	BHV-1	-	-
9	diseased	*Bacillus* sp.	-	-	-
10	diseased	*Bacillus* sp.	-	Ureaplasma	
11	diseased	*Staph.* non-h.	-	Ureaplasma	
12	normal	*Bacillus* sp.	-	Ureaplasma	
13	normal	*Staph.* non-h.	-	Ureaplasma	
14	normal	*Staph.* non-h.	-	-	-
15	normal	*Bacillus* sp.	-	-	-

[a]The animals had vulvovaginitis, vaginal discharge and in some cases endometritis. All animals were repeat breeders.

The low conception rate (33%) and the high number of services per pregnancy (2.97) could be due to the virus infection of the embryo resulting in its death and absorption in the early stage of pregnancy. Embryonic death due to parvovirus was reported in pigs (22) and infection of embryos by other viruses has recently been reviewed (8).

Fertility problems as a result of natural infection with BHV-1 have been reported (13, 15, 17). In this study the virus was associated with fertility problems in most of the cows examined and in two aborted fetuses. However, in a few other animals ureaplasma alone or together with BHV-1 may be incriminated for some of the problems observed. Ureaplasma infection has been reported to cause bovine vulvovaginitis syndrome and fertility problems (7).

Finally, we would like to recommend that detection of BHV-1 by isolation in cell culture be accompanied by demonstration of the virus antigen in the spermatozoa by FA technique. It is important to emphasize that the specificity of the test requires a variety of controls as indicated, if negative control sperms are not available.

ACKNOWLEDGMENTS

We thank Mr. Robert Champlin and Miss Christiana Koolen for their competent technical assistance.

ADDENDUM

Since this report was prepared the herd has been vaccinated against infectious bovine rhinotracheitis and all the cows are bred by AI. Ureaplasma was treated by local infusion of lincomycin and spectinomycin preparations within ten days postpartum. Conception rate six months later was 62% after the first service, and the number of services per conception was 1.87. Valvular discharges have completely disappeared.

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