Investigation of an outbreak of infectious pustular balanoposthitis in cattle breeding bulls at a frozen semen bank

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Summary

Infectious pustular balanoposthitis (IPB) is one of the reproductive disorders caused by bovine herpesvirus 1 (BoHV1) that can be transmitted through artificial insemination. A herd of 63 breeding bulls at a frozen semen bank in Odisha state in India experienced a

suspected outbreak of IPB, with 11 bulls showing clinical signs of the infection. Clinical signs were noticed in two bulls initially and a few days thereafter in the other nine animals. Serum samples from 53 bulls were examined for anti-BoHV1 antibodies using a virus neutralisation test and a competitive enzyme-linked immunosorbent assay (cELISA); the remaining ten bulls were not included in the study because it was difficult to restrain them at that time. Paired serum samples were collected 21 days apart from ten clinically affected bulls (the eleventh clinically affected bull was not included in the study for the reason stated above). In the neutralisation test, the paired serum samples showed a two- to fourfold increase in anti-BoHV1 antibody titre; in the cELISA, the paired samples were also found positive for anti-BoHV1 antibodies. Serum samples from 43 in-contact bulls were collected about day 22 after the first observation of clinical infection in the herd. Among these serum samples, a total of 30 were found positive for anti-BoHV1 antibodies in the VNT and a total of 30 were found positive in cELISA. Ten samples were positive in one test but not the other and 25 tested positive in both tests. In all, 35 serum samples from in-contact bulls tested positive in either one or both of the two types of test. An overall agreement of 76.74% was found in detection of anti-BoHV1 antibodies in the two tests. Sensitivity was higher than specificity in detection of anti-BoHV1 antibodies in the serum samples. The glycoprotein C region of the genomic DNA of BoHV1 was amplified from semen samples by polymerase chain reaction. The findings from the outbreak indicate that continuous check-up of breeding bulls at frozen semen banks is warranted to avoid the risks associated with artificial insemination.

Keywords

Bovine herpesvirus 1 – Cattle – Competitive enzyme-linked immunosorbent assay – Infectious pustular balanoposthitis outbreak – Polymerase chain reaction – Virus neutralisation test.

Introduction

Bovine herpesvirus 1 (BoHV1) infects cattle and buffalo, causing a number of economically important disease syndromes (1). These

include fever, respiratory distress with bilateral nasal discharge, increased respiratory rate, persistent harsh cough, hyperaemia of nasal mucous membrane, excessive salivation (commonly known as infectious bovine rhinotracheitis [IBR]), ocular infections causing keratoconjuctivitis and lacrymal discharge, inflammation of the genital tract (infectious pustular balanoposthitis [IPB] in males, infectious pustular vulvovaginitis [IPV] in females), abortion, mastitis and dermal signs (2, 3, 4). The first report of an outbreak of IBR in crossbred calves in India was in 1976, and since then the seroprevalence of BoHV1 and disease outbreaks in females causing abortion have been reported (5, 6, 7, 8, 9). However, reports on the genital form of the disease in bulls with clinical manifestation of IPB have been scarce in India (10). Bulls affected with IPB show hyperaemia of penile and preputial mucous membranes with vesicle formation and adhesions, annular constrictions, penile distortion and loss of libido. In recent years animal health authorities in India have paid increasing attention to screening the bovine population, including breeding bulls, to determine their status regarding sexually transmitted diseases including IBR/IPV/IPB and to take precautionary steps to maintain BoHV1-free animals at frozen semen banks and artificial insemination (AI) centres. The results of an investigation into a suspected outbreak of IPB in cattle bulls at a frozen semen bank in Odisha state in India are reported.

Materials and methods

History

Following directives from the Indian government, bulls at the frozen semen bank are regularly screened for sexually transmitted diseases (brucellosis, IBR) at six-monthly intervals by the Regional Disease Diagnostic Laboratory, Kolkata. A virus neutralisation test (VNT) is in routine use for screening serum samples for the presence of anti-BoHV1 antibodies.

Fifty-three of 63 bulls (Jersey, Red Sindhi, Hariana, crossbreeds) reared at the frozen semen bank in Odisha state and all unvaccinated against IBR were investigated. The remaining ten bulls were not

included in the study because it was difficult to restrain them at that time. Before the outbreak, all the bulls had tested negative for anti-BoHV1 antibodies. Eleven of the bulls became affected with a disease similar to IPB: initially two bulls developed pustules and reddish eruptions on penile mucous membrane, together with yellowish white discharge and mild rise in body temperature (up to 39.4°C); subsequently, nine other bulls contracted the disease. The eleventh clinically affected bull was not included in the study for the reason mentioned above. The disease course ran to approximately 16 days and all bulls recovered. The bulls were treated with parenteral enrofloxacin (5 mg/kg body weight for five days) and underwent preputial lavage with 30 ml 5% povidone iodine solution every day for seven days. In-contact bulls exhibiting no clinical signs of IPB in the outbreak were categorised as non-clinically affected bulls, not ruling out their seropositive status and infection with a latent form of BoHV1.

Collection, transportation and processing of clinical samples

Blood samples, nasal swabs, preputial washings and semen samples were collected (11). Blood (serum) samples were collected using Vacutainers® with sterile precautions from a total of 53 breeding bulls: 7 Jersey, 2 Hariana, 12 Red Sindhi, 32 crossbreeds. Paired serum samples were also collected 21 days apart from the ten bulls with clinically overt signs of IPB (Fig. 1). The first of the paired samples was collected 1 to 3 days after the appearance of clinical signs; at the time of collection of the second serum samples the bulls were clinically recovered. Serum samples from 43 in-contact bulls were collected on day 22 after the first observation of clinical infection in the other bulls. Serum samples were clarified by centrifugation at $500 \times g$ for 10 min to remove traces of red blood cells and then inactivated at 56°C for 30 min, together with known positive and negative serum samples as controls. All serum samples were stored at –40°C until further use.

Nasal swabs and preputial washings were collected in transport medium containing minimum essential medium (MEM, Sigma) with 2% fetal calf serum (Gibco) and antibiotics (benzyl penicillin 100 IU/ml, streptomycin sulphate 100 µg/ml). After clarifying the samples by centrifugation, the supernatants were used for virus isolation without filtration.

Fresh raw and extended frozen semen straws were collected from the semen bank. The straws were coded by the authorities at the semen bank and the coding format was not made available to the authors, therefore it was not possible to relate semen straws to a particular breeding bull in the polymerase chain reaction (PCR) study. Samples were transported to the laboratory under cold chain.

Bovine herpesvirus 1 standard virus

Standard BoHV1 maintained at the virus laboratory at the Centre for Animal Disease Research and Diagnosis in Izatnagar (India) was used in the study.

Cell culture

A Madin–Darby bovine kidney (MDBK) cell line was procured from the National Centre for Cell Science, Pune (India), and cultured in MEM with 10% fetal calf serum and antibiotics (gentamicin and nystatin, 50 mg/l each).

Propagation and titration of virus

The BoHV1 virus was propagated in MDBK cells at 0.1 multiplicity of infection as stated in the standard protocol. Briefly, log phase MDBK cells were washed with serum-free MEM, inoculated with virus suspension and allowed to adsorb for 1 h at 37°C with intermittent gentle shaking. Residual inoculum was discarded and the cell monolayer was washed with MEM. The infected culture was fed with serum-free MEM and incubated at 37°C. Cultures were observed daily and harvested when 90% of the cells were showing a cytopathic effect (CPE). Infected cell culture supernatants were freeze-dried, titrated and stored at –20°C.

Positive and negative reference serum samples

Fetal calf serum that tested negative for anti-BoHV1 antibodies in the VNT was used as negative reference serum. Positive reference serum was raised in buffalo bull calves infected with BoHV1and freeze-dried for use.

Virus isolation from nasal swabs, preputial washings and semen (fresh raw and extended frozen semen)

Processed clinical samples were used for isolation of BoHV1 on MDBK cells as described in the standard test protocol (11). Cell monolayers were grown in 24-well culture plates and then fed with 200 µl of processed supernatants from clinical samples (nasal swabs, preputial washings). Thereafter, plates were incubated at 37°C for 1 h to allow adsorption of virus, if any. The monolayers were rinsed and 2 ml of maintenance medium was added to each well.

For isolation of virus from semen samples, six-well culture plates were used. Monolayers of MDBK cells were fed with diluted semen samples and the plates were incubated at 37°C for 1 h. Wells were then washed and layered with 5 ml of maintenance medium. Positive and negative virus controls were included. The inoculated plates were observed daily for presence of CPE.

Virus neutralisation test

For the VNT, the World Organisation for Animal Health (OIE) recommended procedure was followed, with slight modifications (11). Serial twofold dilutions of inactivated serum samples, including positive and negative control serum samples, were made up to 1:1,024 in cell culture medium in 24-well plates. A 50 µl portion of each serum dilution was then placed in triplicate wells in a 96-well microtitre plate, together with undiluted test serum in a single well for toxicity control. Fifty microlitres of BoHV1 virus suspension (100 median tissue culture infective doses, $TCID_{50}$) was added to each serum well and virus control wells, excluding the cell control and serum toxicity control wells. Plates were incubated at 37°C for 2 h,

followed by the addition of 100 µl MDBK cell suspension containing 3×10^4 cells/ml. An additional 50 µl MEM was added to the toxicity control and virus control wells and 100 µl to cell control wells. Culture plates were incubated at 37°C for 3 to 5 days and observed daily for development of CPE. This procedure for testing unknown serum samples is in routine use in the laboratory, therefore the virus stock was not back-titrated. Results were expressed as the reciprocal of the dilution of serum that neutralised the virus in half the wells.

Competitive enzyme-linked immunosorbent assay

Serum samples were tested in a cELISA procured from the Institut Pourquier (France). Briefly, after adding 50 µl volumes of dilution buffer to all wells (which were precoated with viral antigen), equal amounts of positive, negative and test serum samples were added and plates were incubated for 30 min at 37°C. After washing the plates three times, 100 µl volumes of revelation solution were added to all wells. Plates were held at room temperature for 20 min and the reaction was then stopped with 100 µl 0.5 M sulphuric acid. Optical densities (OD) were read at 450 nm after blanking in air. Percentage inhibition was calculated as:

(OD analysed serum / mean OD negative control serum) × 100.

Percentage inhibition values that were more than 55%, 50–55%, and less than 50% were considered to be negative, doubtful and positive, respectively.

Genomic extraction and detection of BoHV1 by PCR

DNA was extracted for PCR analysis from 15 semen samples and positive and negative control samples (1 each) (12). Reaction mixtures comprising 200 µl lysis buffer (0.15 M sodium chloride, 0.75% sodium dodecyl sulphate, 1.5 mg/ml proteinase K, 10 µg/ml sheared salmon sperm DNA) and 100 µl semen sample were incubated at 60°C for 1 h. The mixtures were centrifuged at 12,000 × g for 30 s, then equal volumes of 6M sodium iodide were added to the supernatants and incubated for 5 min at room temperature. Nucleic

acid was extracted with 1.4 volumes of chloroform and precipitated with 0.6 volumes of isopropanol. The DNA pellets obtained after centrifugation at 12,000 × *g* were resuspended in 100 µl TE buffer (10 mM Tris-HCl, pH 7.5 + 1 mM ethylenediaminetetraacetic acid [EDTA]) and re-extracted with ten volumes of n-butanol. The final DNA pellets were dissolved in 50 µl TE buffer. In the PCR, the forward primer 5′-ACT GGT TCC GCA ACG GCT AC-3′ and reverse primer 5′-AGG ACG GGG CTT CCG ATT AG-3′ based on the glycoprotein C (gC) gene sequence of BoHV1 were used to amplify a specific product of 520 bp. A total reaction volume of 50 µl included 5 µl denatured DNA template, 200 µM each dNTP, 10% glycerol, 15 pmol each forward and reverse primer and 3 U Taq DNA polymerase in 1× Taq DNA polymerase buffer with 1.5 mM Mg^{++}. The PCR was as follows: 1 cycle at 94°C for initial denaturation, then 35 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 1 min, plus a final extension at 72°C for 5 min. The PCR products were run on 1% agarose gel.

Results

Isolation of BoHV1 from nasal swabs, preputial washings and semen on MDBK cell culture taken from clinically affected and in-contact bulls was unsuccessful. No characteristic CPE was observed after continuous blind passages (three passages for nasal swabs and preputial washings, two passages for semen).

Virus neutralisation titres and cELISA results of paired serum samples from ten clinically affected bulls are shown in Table I. The titres of serum samples on day 0 of appearance of clinical signs in the ten bulls varied from undetectable to 1:4, except in one crossbreed bull, which had a higher titre of 1:32. These titres indicate that some of the bulls were already infected and became the source of infection to others. The titres of the second samples collected 21 days after the appearance of clinical signs varied from 1:2 to 1:64. The two- to fourfold increase in virus neutralising titre of the second serum samples indicated an outbreak of IPB (11, 13). Results of the cELISA were in line with those obtained in the VNT. Four bulls (1 Jersey, 3 crossbreeds) did not

have detectable antibody in the VNT at the time of infection (day 0 of appearance of clinical symptoms), but two of them (crossbreeds) tested positive in the cELISA.

The results of testing serum samples from 43 in-contact bulls, which did not show clinical symptoms, are shown in Table II. Twenty-one days after the start of the outbreak, 13 of the bulls had no detectable antibody levels in the VNT but the other 30 had antibody titres of more than 1:4, indicating recent introduction of infection.

Primers based on the gC region of the BoHV1 genome have been found more sensitive than the gE region in detection of the virus in PCR assay (12), therefore primers for amplification of the gC region were used. In the PCR analysis, four DNA samples from semen specimens tested positive after specific amplification of the gC region of the BoHV1 genome. It was not possible to relate the PCR results to particular clinically affected or in-contact bulls.

Comparison of the virus neutralisation test and competitive enzyme-linked immunosorbent assay

When serum samples were tested in both serological tests the following points were observed. In the first of paired serum samples from ten IPB-infected bulls, antibodies to BoHV1 were not detected by VNT in two bulls but the animals tested positive in the cELISA. All the second serum samples collected from the infected bulls tested positive in both the VNT and the cELISA.

Among the 43 serum samples from in-contact bulls, five tested positive in the VNT but were negative in cELISA, five others were negative in VNT but positive in the cELISA, and a further eight were negative in both tests. Comparison of serological results obtained from the in-contact bulls showed that sensitivity was greater than specificity (Table III). The overall agreement in detection of BoHV1 antibodies in the VNT and the cELISA was 76.74%.

Discussion

Male sex, older age and large herd size are risk factors for higher seropositivity of BoHV1 (14). Bulls kept in large numbers at frozen semen banks must therefore be examined thoroughly and periodically for signs of clinical disease and samples must be tested in the laboratory. Among the infected bulls in the present study, 70% recovered in about 11 days and all recovered within two weeks. This is in agreement with the observations of Miller, who reported that recovery usually occurs in one to two weeks (15). In the present outbreak, antibiotic was administered to prevent secondary bacterial infection and thus the disease course did not extend beyond the period reported above.

Many workers have reported isolation of BoHV1 from clinical samples consisting of nasal swabs, preputial scrapings and semen (16, 17, 18). BoHV1 has been isolated in an outbreak of abortion by adapting the virus first on primary cow calf kidney cells and later on MDBK cells (7). The virus has also been isolated from uterine swabs from repeat breeder cows, but virus isolation failed when a nasal swab from a case of clinical rhinitis was used (19). Failure of virus isolation in the present study may be due to the difficulty of detecting virus more than ten days post-infection, as maximal virus replication and shedding occur between three and six days after infection (20). There is frequently a time lapse in attending natural outbreaks and viral shedding may have declined to an undetectable level.

The outbreak of BoHV1 was not noticed in the frozen semen bank before the investigation. The semen bank in the present study is located at a riverside and is surrounded by a dense human population and stray animals. Strict quarantine measures are routinely followed before the introduction of new animal/breeding bull into the herd and it is supposed that the disease might have been contracted from infected stray animals or be due to unknown factors. To avoid this problem, frozen semen banks should be located in isolated places where appropriate biosecurity measures can be adopted.

Economic losses can result from poor-quality infected semen, decreased reproductive efficiency and reduced draught power. Bulls with lesions on penile and preputial mucous membranes can transmit semen contaminated with BoHV1 during natural breeding or through AI. The process of AI is also at risk from semen collected from apparently healthy seropositive bulls with latent infections of BoHV1 (21). Vaccination against BoHV1 in breeding bulls is restricted by the authorities in India, although vaccination with a gE-deleted marker vaccine is advocated (11) for prevention and control of the disease. For religious and ethical reasons, seropositive bulls in India are not culled to maintain the BoHV1-free status of a herd, although culling is used elsewhere in the world. After the present outbreak, the bulls at the semen bank were not used for semen collection for a period of two months.

In the present study, neutralising antibody was undetectable in the first of paired serum samples from two bulls showing clinical signs of disease. These animals tested positive in the cELISA, thus the cELISA appears to be more sensitive than the VNT. However, screening of in-contact bulls identified five serum samples positive in the VNT (one serum sample titre >1:2, four samples titre >1:4) but negative in the cELISA; the opposite was found for a further five serum samples. The results indicate a lack of specificity in the cELISA compared with the VNT in detection of anti-BoHV1antibodies.

A neutralising antibody titre of 1:8 has been observed in experimentally infected bulls on day 8 after infection, remaining the same up to day 28 of the observation period (22). Higher antibody titres ranging from 1:12 to 1:48 in serum samples collected at days 20 to 40 after experimental infection have also been reported (16). These higher titres may be due to the higher dose of virus used to inoculate the animals; such variation in results from serum samples of experimentally or naturally infected animals at different days of infection has been reported (23, 24). A lower level of antibody in recent BoHV1 infection is also considered a factor for variation in serological results (12).

In the present study of a natural outbreak of BoHV1, the VNT-positive but cELISA-negative results may be attributed to artefacts or other factors. The findings also indicate that neither of the tests was completely reliable when evaluating serum samples from the in-contact bulls. However, the focus of the study was not to demonstrate superiority of any one serological test over another but to understand the overall agreement between the two tests; this was 76.74% and indicates that few seropositive bulls escaped detection. Regular testing of the semen of breeding bulls, irrespective of the serological outcome, is vital for controlling spread of the disease.

Specific PCR amplification of the gC region of the BoHV1 genome in DNA of semen samples confirmed that a few bulls were infected initially and acted as source of infection to other susceptible bulls. A nested PCR was used to confirm the first PCR (results not shown). The sensitivity of the PCR is higher than that of virus isolation, thus the presence of the virus was confirmed by this reaction (25, 26). Previously isolated BoHV1 virus is maintained in the laboratory and sequencing work is in progress. The sequencing data will be used to further characterise the subtype of BoHV1 and its phylogenetic relationship with other BoHV1 isolates elsewhere in the world. Molecular tests must be used to identify the source of an outbreak and to diagnose the disease with a high degree of confidence, which in turn helps in tackling the menace of IBR/IPV/IPB more effectively.

Continuous serosurveillance of bulls and the exclusion of BoHV1-positive bulls is a priority for maintaining the BoHV1-free status of bulls at a frozen semen bank. In addition, sufficient funds are required for construction of animal sheds for quarantine and isolation and to maintain the healthy animals in separate sheds. In India this is sometimes difficult, therefore the semen of seropositive bulls is screened, and semen free from BoHV1 is generally used for AI purposes. The disease has become endemic in India and BoHV1 infections are reported from many parts of the country throughout the year. There is no vaccination programme at present but such a programme should be implemented, particularly with a gE-deleted marker vaccine. Continuous monitoring with a gE-ELISA could be an

alternative method of screening breeding bulls in endemic situations. The present outbreak of IPB was confirmed by PCR detection of the BoHV1 viral genome in semen samples and by a fourfold rise in the antibody titre of infected bulls. Frozen semen banks cater for the needs of many veterinary hospitals and AI centres for insemination of large populations of cows and she-buffaloes, therefore the utmost care must be paid to the quality of the semen samples of the bulls. No semen should be used before certifying it negative for BoHV1 in TaqMan-based real-time PCR, an OIE recommended test for the international trade.

Conclusion

In a herd of 63 breeding bulls at a frozen semen bank, a total of 11 bulls developed IPB with clinical symptoms. Paired serum samples from ten clinically affected bulls showed increasing titres of BoHV1 antibodies from 1:2 to 1:64 in a VNT. Infection with BoHV1 was confirmed by a fourfold rise in the antibody titre and by PCR detection of the BoHV1 viral genome in semen samples.

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Table I
Virus neutralisation titres of anti-bovine herpesvirus 1 antibodies and competitive enzyme-linked immunosorbent assay results in paired serum samples from ten bulls with infectious pustular balanoposthitis

Bull No.	VNT titres		cELISA	
	1st sample	2nd sample*	1st sample	2nd sample
Jersey bulls				
JY3272	Un	1:4	ND	Positive
JY3292	1:4	1:8	Positive	Positive
JY3443	NA	1:4	NA	Positive
Red Sindhi bulls				
RS63	1:4	1:4	Positive	Positive
RS99	1:2	1:32	Positive	Positive
Crossbreed bulls				
CB133	Un	1:16	ND	Positive
CB143	NA	1:2	NA	Positive
CB152	Un	1:4	Positive	Positive
CB166	1:32	1:64	Positive	Positive
CB191	Un	>1:2	Positive	Positive

cELISA: competitive enzyme-linked immunosorbent assay

* samples collected 21 days after first serum collection

NA: not available

ND: not done

Un: antibody undetectable in neat serum

VNT: virus neutralisation test

Table II

Virus neutralisation titres of anti-bovine herpesvirus 1 antibodies and competitive enzyme-linked immunosorbent assay results of 43 in-contact bulls in an outbreak of infectious pustular balanoposthitis

Bull No.	VNT titres	cELISA	Bull No.	VNT titres	cELISA
Jersey bulls			Crossbreed bulls continued…		
JY3411	>1:2	Negative*	CB155	>1:4	Positive
JY3517	>1:4	Positive	CB156	>1:4	Positive
JY3609	>1:4	Positive	CB158	>1:4	Positive
JY3637	>1:4	Positive	CB161	>1:4	Positive
Hariana bulls			CB164	>1:4	Negative*
H26	>1:4	Negative*	CB165	>1:4	Positive
H27	>1:4	Positive	CB168	Un	Positive
Red Sindhi bulls			CB169	Un	Positive
RS19	>1:4	Positive	CB170	>1:4	Positive
RS45	>1:4	Positive	CB171	Un	Negative
RS49	Un	Negative	CB173	Un	Negative
RS57	Un	Negative	CB175	>1:4	Positive
RS69	>1:4	Positive	CB176	Un	Negative
RS77	>1:4	Positive	CB178	>1:4	Negative*
RS83	Un	Positive	CB179	Un	Negative
RS93	>1:4	Positive	CB180	>1:4	Positive
RS101	>1:4	Positive	CB181	>1:4	Positive
RS103	Un	Negative	CB182	Un	Negative
Crossbreed bulls			CB185	Un	Positive
CB130	>1:4	Positive	CB186	Un	Positive
CB140	>1:4	Positive	CB187	>1:4	Positive
CB149	>1:4	Negative*	CB189	>1:4	Positive
CB154	>1:4	Positive	CB190	>1:4	Positive

cELISA: competitive enzyme-linked immunosorbent assay

VNT: virus neutralisation test

* serum positive in VNT but negative in cELISA

Un: antibody undetectable in neat serum

Table III

Comparison of virus neutralisation test and competitive enzyme-linked immunosorbent assay results of 43 in-contact bulls in an outbreak of infectious pustular balanoposthitis

		VNT		Sensitivity[a]	Specificity[b]	Overall agreement[c]
		Positive	Negative			
cELISA	Positive	25	5	83.33%	61.54%	76.74%
	Negative	5	8			

cELISA: competitive enzyme-linked immunosorbent assay

VNT: virus neutralisation test

a) %sensitivity = (VNT+cELISA positives / cELISA positives) x 100

b) %specificity = (VNT+cELISA negatives / cELISA negatives) x 100

c) %overall agreement = (VNT+cELISA positives) + (VNT+cELISA negatives) / (cELISA positives + cELISA negatives)

Fig. 1
Scheme for serum sampling of the cattle breeding bulls