Concurrent testing of breeding bulls for bovine herpesvirus 1 infection (BHV-1) in India

Chintu Ravishankar, Sukdeb Nandi, Vishal Chander & Tapas Kumar Mohapatra

Virus Laboratory, Centre for Animal Disease Research and Diagnosis, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, PIN 243 122 India
chinturavi@rediffmail.com

Keywords
Bovine herpesvirus 1 (BHV-1),
Breeding bulls,
ELISA,
Real time PCR,
Virus isolation,
Virus neutralisation test (VNT).

Summary
In this study, sera from 65 breeding and 19 training bulls from Uttar Pradesh State in north India were tested for bovine herpesvirus 1 (BHV-1) antibodies by enzyme linked immunosorbent assay (ELISA) and virus neutralization test (VNT). The VNT test could detect 56 (86.15%) and 9 (47.37%) of the samples from breeding and training bulls as positive for BHV-1 antibodies whereas in ELISA 63 (96.92%) and 10 (52.63%) were found positive, respectively. Semen samples from the breeding bulls were simultaneously tested by the Taqman based real time PCR (qPCR). Of the 65 samples screened, only 40 (61.54%) were found to contain BHV-1 DNA indicating that all the seropositive bulls are not shedding the virus in semen. When the RT-PCR positive samples were subjected to virus isolation on Madin-Darby bovine kidney (MDBK) cells, no virus isolates could be obtained. The advantages of concomitant testing of serum and semen of breeding bulls and measures for control of BHV-1 infections in bull farms are discussed.

Infezione da Herpesvirus bovino-1 (BHV-1) in tori da riproduzione in India

Parole chiave
ELISA,
Herpesvirus bovino 1 (BHV-1),
Isolamento virale,
Real time PCR,
Test di neutralizzazione virale (VNT),
Toro da riproduzione.

Riassunto
L'articolo riporta i risultati ottenuti analizzando i sieri prelevati da 65 tori da riproduzione e 19 tori in prova nello stato indiano dell'Uttar Pradesh per il rilevamento di anticorpi nei confronti dell'Herpesvirus bovino-1 (BHV-1). Il saggio di sieroneutralizzazione (VNT) ha evidenziato la positività per anticorpi anti BHV-1 in 56 (86,15%) tori da riproduzione e 9 (47,37%) in prova. L'ELISA ha permesso di riscontrare la positività in 63 (96,92%) esemplari da riproduzione e in 10 (52,63%) dei soggetti in prova. Contemporaneamente, con TaqMan real time PCR (qPCR), sono stati analizzati campioni di seme prelevati dai tori da riproduzione. Solo 40 (61,54%) dei 65 campioni hanno evidenziato la presenza di BHV-1, mentre i restanti 25 sono risultati negativi. L'isolamento del virus dai campioni risultati positivi con qPCR, tramite cellule Madin-Darby bovine kidney (MDBK), non ha avuto esito favorevole. L'articolo descrive i vantaggi dell'analisi contemporanea di siero e seme e propone misure atte al controllo delle infezioni da BHV-1 in allevamenti di tori da riproduzione.

Veterinaria Italiana 2013, **49** (2), 145-150. doi: 10.12834/VetIt.2013.492.145.150

Introduction

Bovine herpesvirus 1 (BHV-1) is the etiologic agent of a variety of disease conditions in bovines, like infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), infectious pustular balanoposthitis (IPB), conjunctivitis, abortion, enteritis, and a generalized disease of newborn calves. Bovine herpesvirus 1 belongs to the genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, family *Herpesviridae* (14). The virus can be differentiated into subtypes 1.1, 1.2a and 1.2b on the basis of restriction enzyme profiling of genomic DNA (18). Subtype 1.1 is mainly responsible for the respiratory form of the disease whereas 1.2a and 1.2b are associated with the reproductive system. However, BHV-1 subtype 1.1 may also be associated with the reproductive form of the disease and *vice versa*. Bovine herpesvirus 1.2 subtypes may be less virulent than subtype 1.1. Bovine herpesvirus subtype 1.2a is abortigenic whereas 1.2b is not (9). The virus is distributed worldwide with the exception of few BHV-1 free countries. It has been eradicated from some European countries and parts of Germany. Control programs are being implemented in several other countries such as Germany and Italy (36). Though an infection with BHV-1 does not cause high mortality among the affected animals, the economic loss due to reduced production, impaired work ability, abortion and so on, is enormous.

Bovine herpesvirus 1 replicates to high titres in mucous membranes of the upper respiratory tract and, after genital infection, replication occurs in the mucous membranes of the vagina or prepuce. After initial replication in respiratory, prepuce or vaginal mucosal epithelium, the virus can enter neural cells and remain latent in the trigeminal (respiratory form) or sacral ganglia (reproductive form) probably for the entire life of the host (10, 19). Natural stress such as transportation, pregnancy, inclement weather and artificial stress, such as application of corticosteroids, can induce reactivation of the latent infection and potentially shed the virus intermittently in semen, respiratory or vaginal secretion (6). The virus can be transmitted through infected semen by natural mating and artificial insemination to susceptible cows (8, 23). The achievement and maintenance of BHV-1-free status is the best way to prevent the diseases caused by BHV-1. The World Organisation for Animal Health (*Office International des Épizooties*: OIE) prescribes that, for international trade, sera of animals should be tested for freedom from BHV-1 antibodies by enzyme-linked immunosorbent assay (ELISA) or virus neutralisation test (VNT) and semen for absence of BHV-1 by virus isolation or by real time PCR (qPCR).

In India, IBR was first reported in 1976 from Uttar Pradesh State in north India (17). At present the disease is endemic in the country and the seroprevalence of BHV-1 infections has been reported from cattle and buffalos from different parts of the country (15, 20, 21, 22, 25, 28). The Government of India has decided to screen breeding bulls for the infection before using semen for artificial insemination purposes. However, reports on simultaneous screening of serum and semen of breeding bulls for BHV-1 status are scarce. This article reports the evaluation of serum and semen of breeding bulls from Uttar Pradesh State for the presence of BHV-1 antibodies using VNT and ELISA, virus shedding using a Taqman probe based qPCR, and virus isolation. It also communicates the results obtained by testing training bulls for seropositivity.

Materials and methods

The samples of the study were from Uttar Pradesh state, North India, which is situated between 23°52´N and 31°28′N latitude and 77°3´E and 84°39´E longitude. The climate of the state is of tropical monsoon type, but variations exist because of differences in altitudes. The average temperature varies in the plains from 3°C to 4°C in January to 43°C to 45°C in May and June. Blood samples from 65 breeding bulls were collected in sterile vacutainers, allowed to clot and transported under chilling conditions to the Virus Laboratory, Centre for Animal Disease Research and Diagnosis (CADRAD), Indian Veterinary Research Institute, Izatnagar, for testing. Serum was separated from the clotted blood by centrifugation; heat inactivated at 56°C for 30 min and stored at –20°C until testing was done. Semen samples were also collected from the animals on the same day and transported to the laboratory under the conditions mentioned above. All the animals were apparently healthy and were not vaccinated against BHV-1 as this vaccination is not practiced in India.

The virus neutralisation test was used to determine the titre of BHV-1 antibodies and was performed following the standard protocol of the OIE (36) with some modifications. Two fold serial dilutions of all the test serum samples, including control positive and control negative serum samples, were made up to 1:32 in Dulbecco modified Eagle's medium (DMEM) containing 2% foetal bovine serum (FBS) and 50 µg/ml gentamicin. A total of 50 µl of each dilution was put in duplicate wells in a 96 well tissue culture microtitre plate. Undiluted serum samples were put in one well to test the toxicity of the serum to the Madin-Darby bovine kidney (MDBK) cells. A volume of 50 µl of BHV-1 containing 100 $TCID_{50}$ was added to each well excluding the cell control wells, and the plate was incubated at 37°C for 24 h in a CO_2 incubator. A volume of 100 µl of MDBK cell suspension containing 3×10^4 cells was added to all

Veterinaria Italiana 2013, **49** (2), 145-150. doi: 10.12834/VetIt.2013.492.145.150

the wells. The plate was further incubated at 37°C for 48 h in a CO_2 incubator and read for presence or absence of cytopathic effect (CPE). The end point titre was determined on the basis of the highest dilution of the test serum neutralising 100 $TCID_{50}$ of virus in 50% of cell culture wells. Any neutralisation by undiluted serum was considered as positive.

Serum samples were also tested for antibodies to BHV-1 by using an indirect ELISA (i-ELISA) kit (Svanovir® IBR-Ab, Svanova Biotech Ab, Uppsala, Sweden) following the protocol supplied by the manufacturer. After the test, the plate was read at 450 nm in ELISA reader. The percentage positivity (PP) was calculated using the formula PP = (OD of test sample/OD of positive control) × 100. Serum samples with a percentage positivity <25 were considered negative and those with a percentage ≥25 were positive. The relative sensitivity and specificity of VNT with respect to ELISA were calculated following the method of MacDiarmid and Hellstrom (13).

A Taqman probe based qPCR was used to detect BHV-1 genomic DNA in the semen samples. The assay detects a 97 bp portion of the highly conserved glycoprotein B gene of BHV-1. The extraction of DNA from the samples and qPCR were carried out as described previously (36). For extraction of DNA from the semen samples, 10 µl of the sample was added to 100 µl of Chelex 100 (Sigma-Aldrich, St. Louis, MO, USA) (10%, w/v, in sterile distilled water), 11.5 µl of 10 mg/ml proteinase K (Sigma), 7.5 µl of 1M DTT (Sigma) and 90 µl sterile distilled water. The sample mixture was mixed gently and incubated at 56°C for 30 min. Following brief vortexing, the sample tube was placed in a boiling water bath for 8 min. The vortexing was repeated and the samples were centrifuged at 10,000 × *g* for 3 min. The supernatant containing DNA was used directly or stored at -20°C for future use. For use as positive control, DNA from BHV-1 isolate was extracted using SDS-Proteinase K method protocol as described by Sambrook and Russell (27).

A 25 µl qPCR reaction mixture consisted of 12.5 µl of 2X Platinum Quantitative PCR SuperMix-UDG master mixture (Invitrogen, California, USA), 1 µl of each primer (4.5 mM), Taqman probe (3 mM), 4 µl of nuclease free water, 0.5 µl of 1:10 diluted ROX dye and 5 µl of template DNA. Positive control and no template control (NTC) were also included with each run. The cycling was carried out in a Stratagene Mx3005P RT-PCR machine and the cycling parameters were 50°C for 2 min, 95°C for 2 min followed by 45 cycles of 95°C for 15 sec and 60°C for 45 sec. Any sample with a cycle threshold (Ct) value equal or less than 45 was regarded as positive.

Virus isolation from semen was carried out as per the procedure described by the OIE (36) with slight modifications. Briefly, 200 µl of each fresh semen sample was diluted in 2 ml FBS and gentamicin was added to it at 500 µg/ml. The suspension was mixed vigorously and left at room temperature for 30 min. The mixture was inoculated into confluent MDBK cell monolayer in a six-well tissue culture plate and the plates were incubated for 1 h at 37°C. The mixture was removed and the monolayer washed twice with 5 ml maintenance medium (DMEM with 2% FBS and 50 µg/ml gentamicin) and 5 ml maintenance medium was added to each well. Known positive control and cell control (without sample) wells were also kept. The plates were observed daily for the appearance of a CPE. If no CPE was present after 7 days, the cultures were frozen and thawed three times, clarified by centrifugation, and the supernatant used to inoculate fresh MDBK monolayers. The samples were considered to be negative, if there was no evidence of a CPE after three passages.

Results

Fifty-six (86.15%) and 9 (47.37%) samples out of the 65 bull and 19 training bull sera tested by VNT were found to have BHV-1 antibodies. The VNT titre of the positive samples ranged from 1:2 to 1:16. In the ELISA test, 63 (96.92%) out of the 65 samples tested were found to be positive for BHV-1 antibodies. Percentage positivity values ranged from 40.83 to 95.96%. Ten (52.63%) of the sera of the 19 training bulls tested were found to be positive and the PP values ranged from 55.70 to 95.19%. The samples that were positive in ELISA were positive also in VNT. The two ELISA negative sera were negative also in VNT. When the relative sensitivity of ELISA with respect to VNT for detection of BHV-1 antibodies was calculated, it was found to be 100%. The relative specificity of detection of ELISA was calculated to be 100% for breeding bulls and 90% for training bulls (Table I). A total of 40 (61.64%) of the 65 semen samples tested were found to be positive for BHV-1 as evidenced by Ct values ranging from 33.74 to 38.58. All the qPCR positive semen samples were tested as positive in ELISA; however, five samples were detected as negative in the VNT. Virus isolation trials could not recover any BHV-1 isolate from the qPCR positive semen samples.

Discussion

Bovine herpesvirus 1 causes conditions in bovines that are important from the economic point of view. Some of the characteristics of the virus which make it an important pathogen are:

- its capacity to spread quickly in a herd with a basic reproduction number (R0) of more than 3,

Table I. *Results of virus neutralisation test and enzyme-linked immunosorbent assay for bovine herpesvirus 1 antibodies performed on sera of breeding and training bulls.*

Breeding bulls					Training bulls				
Test		VNT			Test		VNT		
		Positive	Negative	Total			Positive	Negative	Total
ELISA	Positive	56	7	63	ELISA	Positive	9	1	10
	Negative	0	2	2		Negative	0	9	9
	Total	56	9	65		Total	9	10	19

VNT = Virus neutralisation test; ELISA = Enzyme-linked immunosorbent assay.

- its capacity to remain latent in the nervous system,
- to be reactivate intermittently,
- to shed in semen, and
- be transmitted by natural mating and artificial insemination (34).

Artificial insemination with BHV-1 contaminated semen can result in reduction of conception rates, shortened oestrus cycles and endometritis that leads to huge economic losses as well as reduction in production (31). Abortion caused by BHV-1 infection is observed at 4 - 8 months of gestation (19).

In the present study, it was observed that a very high percentage of breeding bulls (96.92%) were positive for BHV-1 antibodies indicating that they have been exposed to BHV-1. In breeding bulls, 95% seropositivity from Tamil Nadu, 41% from Karnataka, and 32.34% from Punjab have been reported from India (25, 29). Another important finding is that 52.63% of the training bulls were also found to be seropositive and this should be taken seriously as semen will be collected from these bulls in the future. Among the serological tests conducted, VNT could detect a lesser percentage of animals in both groups as positive.

The greater sensitivity of ELISA is due to the fact that ELISA detects both neutralising and non-neutralising antibodies whereas VNT detects only the former (20).

There are many reports of the use of conventional PCR assays for the detection of BHV-1 DNA in bovine semen samples (5, 11, 16, 30, 33, 35, 37). However, qPCR has the advantage to be more sensitive and specific than conventional PCR; besides, results can be visualised in real time as and when the reaction is progressing without the need for post PCR processing. Real time PCRs for the detection of BHV-1 in samples have been reported (1, 7). In the present study, the Taqman based qPCR could detect only 40 (61.54%) out of the 65 semen samples as positive. However, the percentage of seropositive animals was much higher (96.92%). This indicates that not all the seropositive bulls shed virus in the semen at that time. The percentage of positivity is similar to that obtained by Deka *et al.* (5) in India wherein 14 (58.33%) of 24 semen samples were tested positive by conventional PCR. In another study conducted in India, Rana *et al.* (24) used qPCR to detect BHV-1 in frozen semen batches and reported that 86 (19.5%) of the 439 bull samples tested were positive. This positivity percentage is lower compared to the one obtained in this study and it may be due to a lower number of animals shedding the virus or to the fact that frozen (extended) semen in which virus load becomes diluted was used for testing.

In the present study no isolate could be recovered from any of the semen samples tested. There are contrasting reports on the sensitivity of virus isolation from semen employing cell cultures. Brunner *et al.* (3) and Drew *et al.* (8) could detect as little as $5TCID_{50}$ in 0.5 ml and $1TCID_{50}$ in 0.1 ml of artificially contaminated semen, respectively. Some reports, on the contrary, suggest that virus isolation in cell culture is not sensitive enough to detect BHV-1 that is still infectious for bovines (2, 12). However, many authors have reported the isolation of BHV-1 from semen samples with varying success rates (4, 5, 32, 33). The failure to get any isolates in the present study may be due to the very low concentration of virus in the samples which was further reduced by dilution (1:10) prior to inoculation, inactivation of virus during transport of semen samples to the laboratory, and to the inherent toxicity of bovine semen to cell cultures.

The establishment of a BHV-1 free herd/bull station can be effected by strict quarantine and periodic testing of animals for BHV-1 antibodies. Testing random serum samples (20%) collected one month apart are a safe surveillance system to monitor BHV-1 infection in bull farms (34). In a country like India, where BHV-1 is endemic, it is difficult, although not impossible, to maintain a BHV-1 free herd. However, it should also be borne in mind that seronegative animals have also been shown to excrete the virus in semen (5, 26). Bulls shed BHV-1 virus in the semen intermittently and following natural and artificially induced stress.

Real time PCR has several limitations such as need for sophisticated and expensive laboratory instruments, costly chemicals and reagents, and skilled manpower for carrying out of the test and interpretation of results. However, these drawbacks are negligible as compared to the huge economic losses brought about by the release of batches of infected semen into the susceptible population. Further, the adoption of programs to vaccinate the animals with a suitable potent and efficacious vaccine would definitely reduce the incidence of disease and associated economic losses.

Conclusions

In the present study breeding bulls were tested for the presence of BHV-1 infection by VNT, ELISA, qPCR and virus isolation. Both the serological tests could detect BHV-1, though ELISA was superior to VNT in this regard. The presence of the virus in semen samples could be detected by a highly sensitive qPCR. However, the virus could not be isolated from the positive samples. Since serological results do not always correlate well with the presence of virus in the semen, and since virus isolation from semen is a laborious process, the only prudent approach to check the spread of BHV-1 through semen would be to test each batch of semen by a sensitive test like qPCR and release batches of semen found negative.

Acknowledgments

The authors wish to thank the Director of the Indian Veterinary Research Institute for providing the facilities in which this study was conducted.

References

1. Abril C., Engels M., Liman A., Hilbe M., Albini S., Franchini M., Suter M. & Ackermann M. 2004. Both viral and host factors contribute to neurovirulence of bovine herpesviruses 1 and 5 in interferon receptor-deficient mice. *J Virol*, **78**, 3644-3653.
2. Bielanski A., Loewen K.G. & Hare W.C. 1988. Inactivation of bovine herpesvirus 1 (BHV-1) from *in vitro* infected bovine semen. *Theriogenology*, **30**, 649-657.
3. Brunner D., Engels M., Schwyzer M. & Wyler R. 1988. A comparison of three techniques for detecting bovine herpesvirus 1 (BHV-1) in naturally and experimentally contaminated semen. *Reprod Domest Anim*, **23**, 1-9.
4. Chandranaik B.M., Chethana, Shivaraj, Kumar S. & Renukaprasad C. 2010. Isolation of BHV-1 from bovine semen and application of real-time PCR for diagnosis of IBR/IPV from clinical samples. *Vet Arhiv*, **80**, 467-475.
5. Deka D., Ramneek, Maiti N.K. & Oberoi M.S. 2005. Detection of bovine herpesvirus-1 infection in breeding bull semen by virus isolation and polymerase chain reaction. *Rev Sci Tech Off int Epiz*, **24**, 1085-1094.
6. Dennet D.P., Barasa J.Q. & Johnson J.H. 1976. Infectious bovine rhinotracheitis virus: studies on the venereal carrier status range of cattle. *Res Vet Sci*, **20**, 77-83.
7. Diallo I.S., Corney B.G. & Rodwell B.J. 2011. Detection and differentiation of bovine herpesvirus 1 and 5 using a multiplex real-time polymerase chain reaction. *J Virol*, **175**, 46-52.
8. Drew T.W., Hewitt-Taylor C., Watson L. & Edwards S. 1987. Effect of storage conditions and culture technique on the isolation of infectious bovine rhinotracheitis virus from bovine semen. *Vet Rec*, **121**, 547-548.
9. Edwards S., White H. & Nixon P. 1990. A study of the predominant genotypes of bovid herpesvirus 1 found in U.K. *Vet Microbiol*, **22**, 213-223.
10. Engels M. & Ackermann M. 1996. Pathogenesis of ruminant herpesvirus infections. *Vet Microbiol*, **53**, 3-15.
11. Grom J., Hostnik P., Toplak I. & Barlic-Maganja D. 2006. Molecular detection of BHV 1 in artificially inoculated semen and in the semen of a latently infected bull treated with dexamethasone. *Vet J*, **171**, 539-544.
12. Kupferschmied H.U., Kihm U., Bachmann P., Muller K.H. & Ackermann M. 1986. Transmission of IBR/IPV virus in bovine semen: a case report. *Theriogenology*, **25**, 439-443.
13. MacDiarmid S.C. & Hellstrom J.S. 1987. An intradermal test for the diagnosis of brucellosis in extensively managed cattle herds. *Prev Vet Med*, **4**, 361-369.
14. MacLachlan N.J. & Dubovi E.J. 2011. Fenner's veterinary virology. Academic Press, Amsterdam, 507 pp.
15. Malmurugan S., Raja A., Saravanabava K. & Dorairajan N. 2004. Seroprevalence of infectious bovine rhinotracheitis in cattle and buffaloes using Avidin-Biotin ELISA. *Tamilnadu J Vet Anim Sci*, **33**, 146-149.
16. Masri S.A., Olson W., Nguyen P.T., Prins S. & Deregt D. 1996. Rapid detection of bovine herpesvirus 1 in the semen of infected bulls by a nested polymerase chain reaction assay. *Can J Vet Res*, **60**, 100-107.
17. Mehrotra M.L., Rajya B.S. & Kumar S. 1976. Infectious bovine rhinotracheitis (IBR) / keratoconjunctivitis in calves. *Indian J Vet Path*, **1**, 70-73.
18. Metzler A.E., Matile H., Gassmann U., Engels M. & Wyler R. 1985. European isolates of bovine herpesvirus 1: a comparison of restriction endonuclease sites, polypeptides, and reactivity with monoclonal antibodies. *Arch Virol*, **85**, 57-69.
19. Muylkens B., Thiry J., Kirten P., Schynts F. & Thiry E. 2007. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. *Vet Res*, **38**, 181-209.

20. Nandi S. & Kumar M. 2011. Comparison of ELISA and virus neutralization test in assaying serum antibodies to bovine herpesvirus 1. *Acta Virol*, **55**, 175-177.

21. Nandi S., Pandey A.B., Sharma K., Audarya S.D. & Chauhan R.S. 2007. Seroprevalence of infectious bovine rhinotracheitis in cattle of an organized farm by indirect ELISA. *The Indian Cow*, **7**, 50-53.

22. Pandita N. & Srivastava R. N. 1993. A study on seroepidemiology of bovine herpes virus in Haryana. *Indian J Virol*, **9**, 31-38.

23. Parsonson I.M. & Snowdon W.A. 1975. The effect of natural and artificial breeding using bulls infected with, or semen contaminated with, infectious bovine rhinotracheitis virus. *Aust Vet J*, **51**, 365-369.

24. Rana S.K., Kota S.N., Samayam P.N., Rajan S. & Srinivasan V.A. 2011. Use of real-time polymerase chain reaction to detect bovine herpesvirus 1 in frozen cattle and buffalo semen in India. *Vet Ital*, **47**, 313-322.

25. Renukaradhya G.J., Rajasekhar M. & Raghavan R. 1996. Prevalence of infectious bovine rhinotracheitis in southern India. *Rev Sci Tech Off int Epiz*, **15**, 1021-1028.

26. Rocha M.A., Barbosa E.F., Guimaraes S.E., Dias Neto E. & Gouveia A.M. 1998. A high sensitivity-nested PCR assay for BHV-1 detection in semen of naturally infected bulls. *Vet Microbiol*, **63**, 1-11.

27. Sambrook J. & Russell D.W. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

28. Singh B., Ramakhant K. & Tongaonakar S.S. 1983. Adaptation of infectious bovine rhinotracheitis virus in MDBK cell line and testing of buffalo sera for neutralizing antibodies. *Indian J Comp Microbiol Immunol Infect Dis*, **4**, 6-10.

29. Singh B.K., Sreenivasan M.A., Tongaonkar S.S., Ramakant K. & Roychoudhary P.N. 1986. Isolation of infectious bovine rhinotracheitis from semen and aborted materials of dairy cattle. *Indian J Ani Sci*, **56**, 823-826.

30. Van Engelenburg F.A., Maes R.K., van Oirschot J.T. & Rijsewijk F.A. 1993. Development of a rapid and sensitive polymerase chain reaction assay for detection of bovine herpesvirus type 1 in bovine semen. *J Clin Microbiol*, **31**, 3129-3135.

31. Van Oirschot J.T. 1995. Bovine herpesvirus 1 in semen of bulls and the risk of transmission: A brief review. *Vet Quart*, **17**, 29-33.

32. Van Oirschot J.T., Straver P.J., van Lieshout J.A.H., Quak J., Westenbrink F. & van Exsel A.C. 1993. A subclinical infection of bulls with bovine herpesvirus type 1 at an artificial insemination centre. *Vet Rec*, **132**, 32-35

33. Weiblen R., Kreutz L.C., Canaborro T.F., Schuch L.F. & Rebelatto M.C. 1992. Isolation of bovine herpesvirus 1 from preputial swabs and semen of bulls with balanoposthitis. *J Vet Diagn Invest*, **4**, 341-343.

34. Wentink G.H., Frankena K., Bosch J.C., Vandehoek J.E.D. & Van den Berg Th. 2000. Prevention of disease transmission by semen in cattle. *Livest Prod Sci*, **62**, 207-220.

35. Wiedmann M., Brandon R., Wagner P., Dubovi E.J. & Batt C.A. 1993. Detection of bovine herpesvirus-1 in bovine semen by a nested PCR assay. *J Virol Methods*, **44**, 129-139.

36. World Organization for Animal Health (Office International des Épizooties: OIE) 2010. Infectious bovine rhinotracheitis / infectious pustular vulvovaginitis. OIE, Paris, p. 1-17 (oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.13_IBR_IPV.pdf accessed on 9 July 2012).

37. Xia J.Q., Yason C.V. & Kibenge F.S. 1995. Comparison of dot blot hybridization, polymerase chain reaction, and virus isolation for detection of bovine herpesvirus-1 (BHV-1) in artificially infected bovine semen. *Can J Vet Res*, **59**, 102-109.