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Isolation of BHV-1 from bovine semen and application of real time PCR for diagnosis of IBR/IPV from clinical samples

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ABSTRACT

Five hundred and twenty three semen samples were collected from fifteen different semen collection centres located across four states of South India. These samples were screened for infectious bovine rhinotracheitis virus by virus isolation studies at the Institute of Animal Health and Veterinary Biologicals, Bangalore, India. Bovine turbinate (BT) and Madin Darby Bovine Kidney (MDBK) cell lines were used for virus isolation. Out of the total samples, four semen samples showed cytopathic changes in cells and were further confirmed by regular PCR targeting amplification of the gC region of bovine herpesvirus-1. Real time PCR was applied for these virus isolates using primers and probes amplifying the gB region of bovine herpesvirus-1. Further, this real time PCR protocol was adapted for diagnosis of IBR/IPV directly on the clinical samples.

Key words: bovine herpesvirus-1, virus isolation, semen, real time PCR

Introduction

Bovine herpes virus type-1 (BHV-1), a member of the *Alphaherpesvirinae*, infects the respiratory and genital tracts of cattle, causing diverse ailments viz: infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), infectious balanoposthitis (GIBBS and RWEYEMAMU, 1977), keratoconjuctivitis (MEHROTRA et al., 1977) and neurological disorders (CARON et al., 2002). In BHV-1 genital tract infections of bulls, the virus replicates in the mucosae of the prepuce, penis and possibly in the distant part of the urethra. Semen is likely to be contaminated during ejaculation by the virus that sheds

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from the infected mucosae. Insemination of cows with such contaminated semen reduces the conception rate and may cause endometritis, abortion and infertility (ELUZHARY et al., 1980; KUPFERSCHMIED et al., 1986). During the primary infection the virus is transported along the axons and becomes latent in the sacral ganglion where it persists for the life of the animal (KERMAN and WYLES, 1984), and is reactivated under stress conditions, making the animal a carrier for life and a potential shedder of the virus.

To prevent transmission of BHV-1 through semen, only BHV-1 free semen should be used (ACKERMANN and ENGELS, 2006). Keeping in mind the emerging nature of the disease and its economic impact, the present study was designed to isolate the virus from semen samples and to apply/adapt a sensitive and rapid diagnostic test for early diagnosis of BHV-1infections from clinical samples including semen.

Materials and methods

Semen samples. Fresh semen samples were collected from 523 bulls, stationed at fifteen different semen collection centres, located across four states of India viz, Tamil Nadu, Kerala, Karnataka and Andhra Pradesh. Semen was collected in viral transport media made from Dulbeccos modified eagle media (DMEM) with 2% foetal calf serum and antibiotics. Samples were transported to the laboratory in cold chain condition.

Swabs from clinically ill animals. Nasal swabs, conjunctival and vaginal swabs from IBR ailing animals were collected in VTM from the Tumukur, Hasan and Mandya districts of Karnataka state, India. A total of 22 swabs were collected and subjected for PCR testing.

Cell lines. Bovine Turbinate (BT) and Madin Darby Bovine Kidney (MDBK) cells obtained from the National Centre for Cell Sciences, Pune, India, grown in DMEM growth media, supplemented with 5% FCS, were used in the entire screening work.

Virus isolation. 200 µL of semen sample in VTM was diluted with two mL of certified foetal calf serum (Gibco, Cat log no.160000-044, Lot no. 1127731, USA) mixed vigorously and incubated for 30 min at room temperature. One mL of semen-serum mixture was inoculated separately in each of BT and MDBK cells in three tubes. Each sample was given three blind passages. In each passage the cells were observed for changes for seven days. Tubes that did not show a cytopathic effect (CPE) by the third passage were discarded and treated as negative. Samples that produced CPE by the third passage were further passaged for virus propagation (ANONYM., 2008).

DNA isolation

a) DNA from semen samples was isolated using chelating resin along with Proteinase -K and DL-Dithiothretiol as described in ANONYM. (2008).

b) The DNA from cell culture supernatants and swabs, collected from clinically ill animals, was isolated as per the procedures of LOPASEV et al. (1991).

Polymerase chain reaction. The DNA isolated from cell culture supernatants and clinical samples collected from ailing animals were subjected to routine PCR in 20 µL of a reaction mixture containing a final concentration of 10 mM Tris (pH 9.0), 50 mM KCl, 1.9 mM $MgCl_2$, 0.2 mM deoxy nucleoside triphosphate, 0.1 µL primer P1 (5'CTGCTGTTCGTAGCCCACAACG 3'), 0.1 µm primer P2 (5' TGTGACTTGGTGCCCATGTCGC 3') and 1U of Taq Polymerase per reaction. All the reagents and primers were obtained from Bangalore Genie Ltd., Bangalore, India. The primer sequence is based on the sequence of BHV-1 glycoprotein C (gC) gene (AFSHAR and EAGLESOME, 1990). The PCR mixture was amplified by 38 repeated cycles: first 15 cycles at 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1min and rest 23 cycles at 95 °C for 1min, 60 °C for 1 min and 72 °C for 1 min 4 seconds auto segment extension (VAN ENGELENBURG et al., 1993). Negative control reactions (reaction without template and reaction with BHV negative semen) were added to each set of PCR. The PCR products were analysed by agarose gel electrophoresis (2% agarose) using 5 µL of the each PCR product mixed with 10 µL of ethidium bromide dye.

Real time PCR. In the present study we used a pair of sequence-specific primers for amplification of target DNA and a 5'-nuclease oligoprobe (Taqman) for the detection of amplified products. The oligoprobe was a single, sequence-specific oligonucleotide, labelled with two different flurophores, the reporter/donor, 5-carboxyfluorescein (FAM) at the 5' end, and the acceptor/quencher 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. This real-time PCR assay was designed to detect the viral DNA of all BHV-1 strains, including subtype 1 and 2, from bovine semen as well as from clinical samples. Details of the primers and probes are as follows.

i) Primers and probe sequences. The primers and probe were selected as per LOVATO et al. (2003) and ABRIL et al. (2004) and sequence were as follows

Primer gB-F: 5'-TGT GGA CCT AAA CCT CAC GGT-3'

Primer gB-R: 5'-GTA GTC GAG CAG ACC CGT GTC-3'

Taq Man probe: 5'- FAM AGG ACC GCG AGT TCT TGC CGC TAMRA-3'.

ii) Preparation of reaction mixtures. The PCR reaction mixtures were prepared in a clean laboratory room. All the reagents, except the test samples, were mixed before distribution to each individual reaction tube. Non templates control (NTC, reagents only) and negative controls were included in each test. The PCR amplifications were carried out in a volume of 25 µL which contained:

2× PCR Super mix	12.5 µL
ROX reference dye	0.5 µL
Forward primer (gB-F, 4.5 µM)	1 µL
Reverse primer (gB-R, 4.5 µM)	1 µL
Probe (3 µM)	1 µL
Nuclease free water	4 µL

5 µL of the DNA isolated from cell culture supernatants/clinical swabs/semen samples was added to the PCR reagent mixture to make final volume of 25 µL.

The primers, probes and PCR mix were obtained from M/S Applied Bio systems, United Kingdom.

iii) Parameters of real-time (Taq Man) polymerase chain reaction. The PCR tubes were placed in the real-time PCR detection system (7500, Applied bio systems) with the following programme parameters:

One cycle:	Hold 50 °C	2 minutes
One cycle:	Hold 95 °C	10 minutes
45 cycles:	Hold 95 °C	15 seconds
	Hold 60 °C	45 seconds

The results were analysed using SDS 3.1 software provided with a 7500 real time PCR system. Though real time PCR does not require running of gel for PCR products, as a means of authenticity, the real time PCR products were analysed by agarose gel electrophoresis (2% agarose) using 5 µL of each PCR product mixed with 10 µL of ethidium bromide dye.

Results

During our present study of virus isolation, the semen samples produced extensive degeneration and detachment of cells within 24 hours in the first passage and a few of the samples had similar effects even during the second passage. Of the 523 semen samples tested, four samples developed CPE in the cell lines used. CPE was noticed in BT cells by the third passage in all the four virus isolates. However, the same samples developed CPE-like changes by the fourth and fifth passages in the MDBK cell line. The CPE comprised rounding of cells 48-72 hr post infection (PI) with the characteristic " bunch of grapes" like aggregation developed by 72 hr, finally leading to complete destruction of the cell sheet by 96-120 hrs.

The DNA isolated from cell cultures showing CPE when subjected to routine PCR, using primers amplifying the gC region of BHV-1, yielded a specific PCR product of 173 bp as indicated in Fig. 1. In this PCR all the four tissue culture fluids collected from four BHV-1 isolates showing CPE yielded the desired amplicon. Further, out of 22 clinical swabs collected and tested, 14 were found positive with an amplicon size of 173 bp.

The primers and probes used in the Real time PCR assay in this study selectively amplified a 97 basepair sequence of the glycoprotein B (gB) gene. The DNA isolated from cell cultures showing CPE when subjected to Real time PCR yielded a highly acceptable cycle threshold (CT) value range of 13 to 14 (Fig. 2).

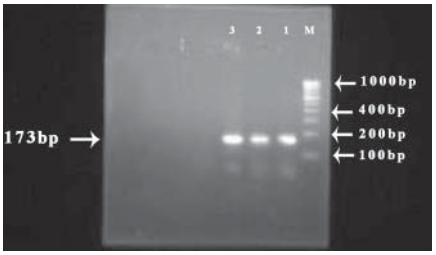

Fig. 1. Gel showing amplification of gC region of BHV-1 at 173bp. (M = Marker)

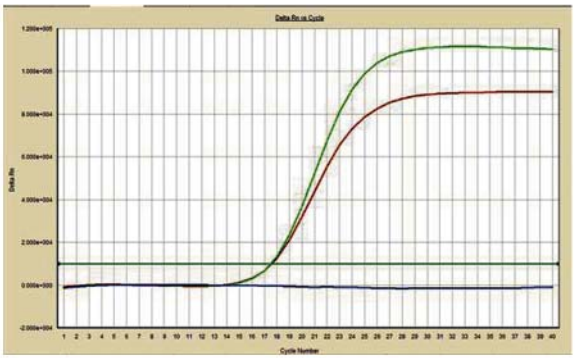

Fig. 2. Real time PCR amplification plot showing amplification of gB region of cell culture adopted BHV-1. [Green and Red curve → Tissue culture adapted virus; Blue curve→ Non template control (NTC)].

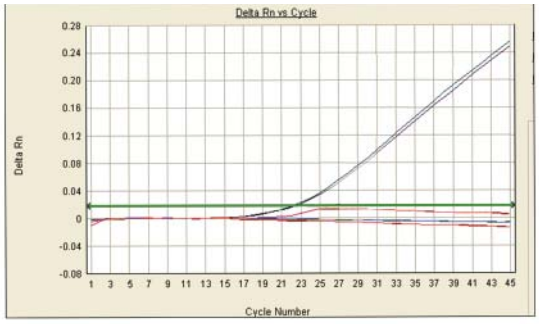

Fig. 3. Real time PCR amplification plot showing atypical amplification curve of a few of the neat swabs. [Blue line → Non template control; Red line → Negative controls; Blue & purple curves → Swab samples].

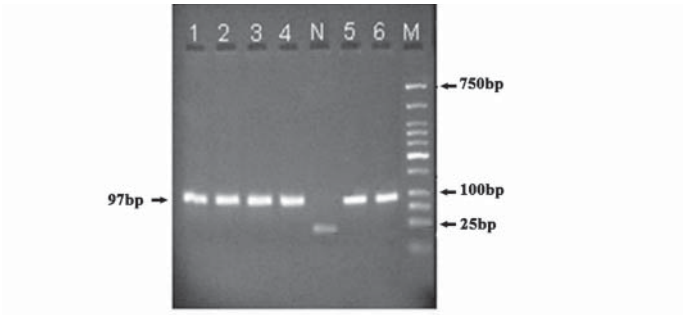

Fig. 4. Gel showing gB gene specific amplicon of BHV-1 at 97bp (real time PCR product). (N = Negative control, M = Marker)

The four DNA samples isolated from four semen samples from which the viruses were isolated yielded CT values ranging from 34 to 39, which was possibly suggestive of a low viral DNA concentration in the semen samples. In spite of repeated attempts, the neat semen samples (without DNA isolation) failed to produce any amplification in the real time PCR assay.

The 14 swab samples, which were found positive by regular PCR (amplifying gC region of the virus), were subjected for Real time PCR assay. The neat swabs (without DNA isolation) boiled in a water bath for 10 min and were subjected to Real-time assay. All the samples gave indications of PCR amplification with a CT value ranging from 19 to 35 for different samples, however, the amplification plot of two vaginal and two nasal swabs did not yield a typical curve as we obtained in the cell culture adopted virus or the rest of the ten clinical samples. These four samples resulted in amplification plots which were relatively a straight line (Fig. 3).

When the DNA isolated from these fourteen swabs were subjected to real time PCR, all the samples had better CT values (ranging between 17 to 28) compared to their respective neat (without DNA isolation) swab samples and further, all the DNA samples recorded typical, sigmoid amplification plots as we noticed in the cell culture adopted virus.

The Real time PCR products, when subjected to gel electrophoresis, yielded a specific amplicon of 97 bp (Fig. 4).

Discussion

The greatest problem of herpesviral infections is the carrier status they induce in the animals they prevail in, consequent to which the presence of antibodies in an animal may not indicate an active infection. Considering this major drawback in serum based tests,

detection of virus or its antigen becomes mandatory to designate any animal as positive for BHV-1 (ANONYM., 2008).

The semen samples produced extensive destruction of cells in the first passage of virus isolation, which is attributed to lytic enzymes present in the seminal fluids and this finding was in total agreement with ANONYM. (2008). The finding of CPE of BHV-1 with the "bunch of grapes" appearance of the virus infected cells was in correlation with previous BHV-1 isolation studies (MURPHY et al., 1999**)**.

The results of PCR targeting the gC region of BHV-1 was in concurrence with several previous studies (AFSHAR and EAGLESOME, 1990 ; VAN ENGELENBURG et al., 1993). The PCR was specific since the coding region of BHV-1, gC codes for nucleotides sequence which is proved to be present in all BHV-1 strains (VAN ENGELENBURG et al., 1993).

Real time PCR differs from standard PCR in that the amplified PCR products are detected directly during the amplification cycle using a hybridisation probe, which enhances assay specificity. Real-time PCR assays have several advantages over conventional PCR methods. Real-time PCR assays, using only one pair of primers, are able to provide sensitivity close or equal to nested PCR methods, with a much lower risk of contamination. The amplification and detection of the target is conducted simultaneously. There is no post amplification PCR product handling, which significantly reduces the risk of contamination (ANONYM., 2008).

The CT value obtained for positive samples was in agreement with ANONYM (2008) who prescribed a CT value of ≤45 to indicate any sample positive for BHV-1. Further, the results were in concurrence with the findings of LOVATO et al. (2003) and ABRIL et al. (2004) who used similar protocols for BHV-1 detection.

The four DNA samples isolated from four semen samples from which the viruses were isolated yielded CT values ranging from 34 to 39, which was possibly suggestive of low viral DNA concentrations in the semen samples.

The amplification plot of two vaginal and two nasal swabs did not yield a typical curve like the one we obtained in the cell culture adopted virus or the rest of the ten clinical samples. These four samples resulted in amplification plots which were relatively a straight line. We could not come to any concrete conclusions regarding this atypical line and it was possibly due to the PCR inhibitors present in the thick mucus rich swabs.

Though real time PCR gives indications of amplification when directly applied to the clinical samples, the present study recommends Real time PCR only after DNA isolation from the clinical sample and not directly on the clinical samples.

The real time PCR products, when subjected for gel electrophoresis yielded a specific amplicon of 97 bp which was in agreement with (ABRIL et al., 2004). This further augmented the specificity of gB based real-time assays for BHV-1 diagnosis.

The set of real time primers and probes used in this study was recommended by OIE for detection of BHV-1 in bovine semen, the present study records and recommends that the same can also be used for diagnosis of BHV-1 in clinically suspected samples like swabs and tissue culture fluids.

The serum based screening of IBR in cattle is proving to be a futile exercise as several of the breeding bulls that were seropositive for IBR antibodies failed to yield the virus in their semen and also because the antibodies, in the majority of the bulls are due to the respiratory form of the disease. Since the genital form of IBR is an emerging threat, virus detection stands as the only alternative in confirming the genital form of the disease and Real time PCR, which is highly specific and cost effective, can be easily adopted for routine diagnosis of BHV-1 in semen as well as from clinically suspected swabs/tissue samples.

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CHANDRANAIK, B. M., CHETHANA, SHIVARAJ, S. KUMAR, C. RENUKAPRASAD: Izdvajanje goveđeg herpesvirusa 1 iz bičjeg sjemena i primjena lančane reakcije polimerazom u stvarnom vremenu za dijagnosticiranje zaraznog rinotraheitisa/pustularnoga vulvovaginitisa pretragom kliničkoga materijala. Vet. arhiv 80, 467-475, 2010.

SAŽETAK

Ukupno su bila prikupljena 523 uzorka sjemena bikova iz 15 različitih središta za prikupljanje sjemena smještenih na području četiriju država u Južnoj Indiji. Uzorci su bili pretraženi na prisutnost virusa zaraznoga rinotraheitisa goveda (ZRG) u Institutu za zdravlje životinja i veterinarske pripravke u Bangaloreu u Indiji. Za izdvajanje virusa rabljena je stanična linija podrijetlom od stanica goveđih turbinata (engl. bovine turbinate - BT) i stanična linija podrijetlom od goveđega bubrega (engl. Madin Darby Bovine Kidney –MDBK). Od svih pretraženih, četiri uzorka pokazivala su citopatski učinak u rabljenim staničnim kulturama što je bilo potvrđeno uobičajenom lančanom reakcijom polimerazom uz uporabu sekvence u području glikoproteina gC goveđega herpesvirusa 1. PCR u stvarnom vremenu primijenjen je za identifikaciju tih izolata uporabom početnica specifičnih za glikoprotein gB virusa ZRG. Protokol lančane reakcije polimerazom u stvarnom vremenu razvijen je za dijagnosticiranje ZRG izravno u kliničkim uzorcima.

Ključne riječi: goveđi herpesvirus 1, izdvajanje virusa, sjeme, lančana reakcija polimerazom u stvarnom vremenu

Book Review

Rinderpest and Peste des Petits Ruminants: Virus Plagues of Large and Small Ruminants. T. Barrett, P. Pastoret and W. P. Taylor, editors. Academic Press, Elsevier, 84 Theobald's Road, London WC1X 8RR, UK, ISBN-13: 978-0-12-088385-1, Pages: 341; Price: US$ 88.73

In this aptly titled book "Rinderpest (RP) and Peste des Petits Ruminants (PPR): Virus Plagues of Large and Small Ruminants", T. Barrett, a senior researcher and morbillivirus group leader (sadly saying, who had passed away in September, 2009) at the Institute for Animal Health, Pirbright Laboratory, U.K., his co-editors have presented a subtle and instructive brief touches on the key milestones of both deadly diseases, and incorporated a great deal of information regarding history, infection biology, disease diagnosis, control and eradication. This book is compiled of high quality scientific and historical research arose from the editors' vast self-experience in morbilliviruses and their well-built professional relations with other contributors around the globe. This monograph consists of 17 chapters, contributed by 22 well-known scientists having holding expertise opinions in various disciplines of infectious diseases.

Throughout the book, the contributors have tried to maintain the appropriate balance between the RP and PPR on one hand and compared the PPRV nicely with well known RPV, on the other. This hypothesis based balancing act, but an essential one is important to highlight the importance and comparative understanding of PPRV, the base on which an adage is inferred, "If rinderpest becomes a disease of the past, PPR is certainly a disease of the future". The text is skillfully started a counter history of the RP and PPR disease which is fully accomplished with monumental pictures dated back to eighteenth century. These photographs are fascinating, illustrative, and large enough for detail to be respected. Out of others, a condolence letter written by Emile Roux from *Institute Pasteur* to the widow of Joseph Hamoir (to whom he worked on rinderpest) and a group photo of Robert Koch on visiting the Imperial Veterinary Laboratory in 1897 to see the effect of immunization with the bile of succumbed animals are extinct to see otherwise.

The editors and contributors to this book reserve their greatest focus on more general mantra of the importance that covers other argumentative issues: relative position of each member in the genera; comparative molecular biology; infectious disease; pathophysiology of the infectious diseases; global epidemiological pattern; contribution of countries in the eradication of the disease under PARC (Pan African Rinderpest Campaign) and PACE (Pan African Programme for the Control of Epizootics) programmes; viral immune suppression and molecular diagnostic approaches being developed for PPRV and RP. Each of these claims could highlight the importance of diagnosis, managing and preventing infectious diseases for the clinicians. Due to the current sensitivity and emergence of PPR and the successful Global Rinderpest Eradication Programme (GREP), contributors believe to control PPR likewise, before it gets even worse. For this reason, the last seven chapters are dedicated to emphasize the old prophylactic measures, potency and probably of vaccine use, the history of vaccine improvement, recent advances in the vaccine development, implementation of the international control campaigns for the eradication of RP and PPR by the use of vaccine and a brief overview of the pathogenesis and eradication of measles virus. Finally, the editors are scrupulously honest in assessing the real cost and benefits of campaign for GREP and are owed to shortly confirm the RP free world, with comparatively limited resources.

Although the text provides best snapshots of the state of the art in term of data available at that time but few chapters emerge to have been printed around 2000, and after this a surge in PPRV research is observed. Therefore readers will require another source to search most recent developments elsewhere. Still to my understanding, this is the only book available describing the PPRV comprehensively, which is not only appropriate for academic and research but also for virologists, infectious disease specialists, vaccine researchers and clinicians. Moreover, this book is an understanding source of scientific information utilized by undergraduate and graduate students.

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