Available online at www.sciencedirect.com
ScienceDirect
Journal of Virological Methods 144 (2007) 103–108
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Validation of a real-time PCR assay for the detection of bovine herpesvirus 1 in bovine semen

Jianning Wang [a,*], Joseph O'Keefe [a], Della Orr [a], Leo Loth [a], Malcolm Banks [b], Philip Wakeley [b], Donna West [b], Roderick Card [b], Georgina Ibata [b], Kees Van Maanen [c], Peter Thoren [d], Mats Isaksson [d], Pierre Kerkhofs [e]

[a] *Investigation and Diagnostic Centre-Wallaceville, Biosecurity New Zealand, Ministry of Agriculture and Forestry, Upper Hutt, New Zealand*
[b] *Veterinary Laboratories Agency, Weybridge, United Kingdom*
[c] *Animal Health Service, Deventer, The Netherlands*
[d] *National Veterinary Institute, Uppsala, Sweden*
[e] *Veterinary and Agrochemical Research Centre, Brussels, Belgium*

Received 17 February 2007; received in revised form 16 April 2007; accepted 25 April 2007
Available online 11 June 2007

Abstract

A real-time polymerase chain reaction (PCR) assay was developed for detection of the presence of bovine herpesvirus type 1 (BoHV-1) in extended bovine semen. The assay detects a region encoding a highly conserved glycoprotein B gene. The real-time PCR assay was validated for specificity, sensitivity and repeatability using spiked semen and semen from naturally infected animals. The real-time PCR was very rapid, highly repeatable and more sensitive (lower detection limits) than conventional virus isolation method for the detection of BoHV-1 in extended semen. The specificity of the assay is as expected. The assay had an analytical sensitivity of 0.38 $TCID_{50}$ virus spiked into negative semen. The second real-time PCR system for the detection of the bovine growth hormone (bGH) gene was applied as an internal control for the DNA extraction and PCR. The bGH PCR can be performed separately to BoHV-1 PCR, or in a duplex format. The real-time PCR assay is intended for use in international trade. The complete validation dossier based on this study and an international inter-laboratory ring trial has been accredited by the Office International des Epizooties (OIE) and has been recommended to be adopted as a prescribed test for international trade.

Keywords: Bovine herpesvirus 1; Real-time PCR; Semen; Validation

1. Introduction

Bovine herpesvirus 1 (BoHV-1) is an economically important pathogen in cattle causing different syndromes such as infectious bovine rhinotracheitis (IBR), and infectious pustular vulvovaginitis (IPV) in cows and balanoposthitis (IBP) in bulls (Gibbs and Rweyemamu, 1977). BoHV-1 infection can also cause reproduction problems such as abortion and reduced fertility (Wyler et al., 1989; Miller, 1991). BoHV-1 is distributed worldwide. This virus may be transmitted via respiratory and venereal routes, and by artificial insemination with virus-contaminated semen, whereby, samples of a single ejaculate may be inseminated into many females (Afshar and Eaglesome, 1990; Deas and Johnston, 1973; Kahrs et al., 1980; Kupferschmied et al., 1986; Straub, 1991). After acute infection, the virus can enter neural cells and establish a latent infection in sensory ganglia (Pastoret et al., 1984; Ackermann and Wyler, 1984). The latent virus can be reactivated both by stressful conditions and/or administration of glucocorticoids (Thiry et al., 1984; Wyler et al., 1989). BoHV-1 infected bulls may shed virus intermittently in their semen long after the primary infection (Dennett et al., 1976; Office International des Epizooties (OIE), 2004). Therefore, cattle infected with BoHV-1 are regarded as lifelong carriers and potential shedders of the virus.

BoHV-1 is of significance to international bovine semen trade. To guarantee the safety of semen for artificial insemination, rapid, sensitive and reliable tests for virus detection are crucial. The routine method for the detection of BoHV-1 in bovine semen is virus isolation using cell cultures of bovine origin, which is also the prescribed test for international trade by

* Corresponding author. Tel.: +64 4 8945644; fax: +64 4 8944973.
E-mail address: Jianning.Wang@maf.govt.nz (J. Wang).

0166-0934/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2007.04.002

the Office International des Epizooties (OIE, 2004). However, this method is time and labour consuming and lacks sensitivity (Weiblen et al., 1992). Moreover, virus isolation in cell culture is often impeded by the cytotoxicity or some virus neutralizing activity of the semen (Ackermann et al., 1990; Phillpott, 1993; Straub, 1991; Wyler et al., 1989). Therefore, new methods are needed to overcome the drawbacks of virus isolation.

Different PCR assays have been developed for the detection of BoHV-1 in fresh and extended semen (Masri et al., 1996; Rocha et al., 1998; Smits et al., 2000; Van Engelenburg et al., 1993, 1995; Vilcek et al., 1994; Wagter et al., 1996; Wiedmann et al., 1993). PCR has been reported to be more sensitive and much more rapid than virus isolation. Compared to the conventional PCR assays, real-time PCR has additional advantages in that it is more rapid, accurate and convenient, has a quantitative result and high sample throughput. Also, as amplification and detection in real-time PCR is performed in a closed-tube format, no post-amplification manipulation is needed. This significantly minimizes the risk of cross-contamination.

Thus far, there is no published information available about application of real-time PCR for the detection of BoHV-1 in bovine semen. We hereby describe an international collaborative study to develop and validate a real-time PCR assay that can be utilized for the detection of BoHV-1 in bovine semen and provide a sensitive alternative to virus isolation, applicable in international trade of bull semen.

2. Materials and methods

2.1. Viruses

A variety of BoHV-1 isolates/strains from different geographic locations were used in this study, including 35 BoHV-1.2 and 19 BoHV-1.1 strains (Table 1). Herpesviruses from several other species, including cervine, caprine, rangiferine and ovine, as well as bovine herpesvirus 2 were applied in this study. In addition, bovine viral diarrhoea virus (BVDV), bovine parainfluenza virus 3 (PI-3) and bovine respiratory syncytial disease virus (BRSV) were tested to evaluate the specificity of the assay. Several human herpesviruses, including human herpesvirus 1 (herpes simplex virus 1), 2 (herpes simplex virus 2), 3 (Varicella-Zoster virus), 4 (Epstein-Barr Virus) and 5 (human cytomegalovirus), were also included for assessment of the specificity.

Table 1
Herpesvirus isolates/strains used in this study

Virus type	Number of isolates/strains	Origin
Bovine herpesvirus 1.1	19	UK, Canada
Bovine herpesvirus 1.2	35	UK, New Zealand, Australia
Bovine herpesvirus 2	10	UK, New Zealand
Ovine herpesvirus 2	3	New Zealand
Caprine herpesvirus	4	UK, New Zealand
Cervine herpesvirus	3	UK, New Zealand
Rangiferine herpesvirus	1	UK

2.2. Virus isolation from semen

The virus isolation from extended semen was conducted according to the OIE guidelines (OIE, 2004) using primary bovine embryonic lung cells.

2.3. Semen samples

BoHV-1 contaminated extended semen samples were obtained from 10 naturally infected bulls from New Zealand, Australia and UK, and five experimentally infected bulls from The Netherlands (Smits et al., 2000). Negative semen samples used in this study were obtained from 45 uninfected bulls from Swiss Genetics, Bern, Switzerland, which is known to be free of BoHV-1.

2.4. Isolation of DNA

The method for extraction of DNA from semen was essentially according to Wiedmann et al. (1993) with some modifications. In brief, 10 μl of semen sample was added to 100 μl of Chelex 100 (Sigma) (10%, w/v, in sterile, distilled water), 11.5 μl of 10 mg/ml, proteinase K (Sigma), 7.5 μl of 1 M DTT (Sigma) and 90 μl sterile distilled water. The sample mixture was mixed gently and incubated at 56 °C for 30 min. Following brief vigourous vortexing (10 s), 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.

DNA isolation from cultured viruses was also carried out using above mentioned method.

2.5. Primers, probes and real-time PCR

2.5.1. PCR for the detection of BoHV-1

The primers and probe selected from the sequences of the glycoprotein B gene of BoHV-1 (Abril et al., 2004) were used for PCR amplification and detection. The glocoprotein B gene is a highly conserved gene of herpesviruses. The primers and probe were checked for their specificity using the database similarity search program nucleotide–nucleotide BLAST (www.ncbi.nlm.nih.gov/blast) and the sequences are 100% homology to those of BoHV-1 strains deposited in the GenBank. All oligonucleotides were synthesized by Sigma Genesys (Australia). The sequences of primers and probes are shown in Table 2.

The real-time PCR was carried out using the Platinum Quantitative PCR SuperMix-UDG Kit (Invitrogen, Life Technologies). A number of experiments were performed to optimize the PCR protocol, including concentration of reagents and PCR cycling parameters. The optimized PCR assay was established using a total volume of 25 μl. Briefly, for a single tube 4 μl of nuclease free water, 12.5 μl 2x Platinum Quantitative PCR SuperMix-UDG master mixture, 1 μl of each primer (final concentration at 180 nM) and probe (final concentration at 120 nM) were pooled as a master mixture. Finally, 5 μl DNA template was added. The