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Development and evaluation of a polymerase chain reaction assay to detect *Bovine herpesvirus 1*

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Abstract

A polymerase chain reaction (PCR) assay to detect *Bovine herpesvirus 1* (BoHV-1) has been developed and evaluated. This assay was based on the amplification of a viral thymidine kinase (*tk*) gene fragment, for which a novel pair of primers and different PCR conditions were tested. Under optimal conditions, no amplification was observed from heterologous herpesviruses or other bovine viruses. However, a 202 bp band from BoHV-1 was obtained confirming the specificity of the assay. An analytical sensitivity of 0.15 $TCID_{50}$ per reaction led to the detection of BoHV-1 in nasal swab supernatants up to 11 days after experimental infection of cattle. There was a 100% match on the results until six days post-infection when PCR was compared with virus isolation and fluorescent antibody test (FAT). Starting the seven day post-infection and until the end of the experiment most of the positive samples were obtained by PCR. This simple PCR strategy do not use co-solvents in order to eliminate non-specific products or increase the efficiency of the amplification, provides a rapid method for diagnosis of BoHV-1 diseases in contrast with semi-nested or nested protocols with more time-consuming and a higher contamination rate, moreover, can be easily used in all diagnosis laboratories.

Additional key words: *Alphaherpesvirus*, molecular diagnosis, optimization, *tk* gene.

Resumen

Desarrollo y evaluación de un ensayo de reacción en cadena de la polimerasa para la detección de *Herpesvirus bovino 1*

Se ha desarrollado y evaluado un ensayo de reacción en cadena de la polimerasa (PCR) para la detección de *herpesvirus bovino 1* (BoHV-1). Este se basó en la amplificación de una porción del gen de la timidina quinasa (*tk*) viral, para lo cual se ensayaron un nuevo par de cebadores y diferentes condiciones de la PCR. Bajo condiciones óptimas, no se observó amplificación de herpesvirus heterologos u otros virus bovinos. Sin embargo, se obtuvo una banda de 202 pb lo que confirma la especificidad del ensayo. Una sensibilidad analítica de 0.15 $DICT_{50}$ por reacción permitió la detección de BoHV-1 en sobrenadantes de exudado nasal hasta 11 días después de la infección experimental de bovinos. Hubo un 100% de concordancia entre resultados hasta el día siete post-infección cuando la PCR fue comparada con el aislamiento viral y la prueba de anticuerpos fluorescentes (FAT). Desde el día ocho post-infección y hasta el final del experimento la mayoría de las muestras positivas se obtuvieron por PCR. Esta estrategia de PCR simple no usa co-solventes con el propósito de eliminar productos no específicos o incrementar la eficiencia de la amplificación, provee un método rápido para el diagnóstico de las enfermedades de BoHV-1 en contraste con el semi-nested o nested PCR con más consumo de tiempo y altas posibilidades de contaminación, además, puede ser fácilmente usada en todos los laboratorios de diagnostico.

Palabras clave adicionales: *Alfaherpesvirus*, diagnóstico molecular, optimización, gen *tk*.

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Abbreviations used: Acc. (accession), BoHV-1 (*Bovine herpesvirus* 1), DMEM (Dulbecco's modified essential medium), dNTP (deoxynucleoside triphosphate), ELISA (enzyme-linked immunosorbent assay), FAT (fluorescent antibody test), FCS (foetal calf serum), IBR (infectious bovine rhinotracheitis), IPV (infectious pustular vulvovaginitis), MDBK (Madin-Darby bovine kidney cells), OIE (Office International des Epizooties), PCR (polymerase chain reaction), SDS (sodium dodecyl sulfate), SK6 (swine kidney cells), $TCID_{50}$ (median tissue culture infective dose), tk (thymidine kinase).

Introduction

Bovine herpesvirus 1 (BoHV-1) infects the respiratory and genital tracts of domestic cattle and several ruminant species. Infection may result in different diseases such as infectious bovine rhinotracheitis (IBR), which has the most severe economic impact in cattle production (Bowland and Shewen, 2000), infectious pustular vulvovaginitis (IPV) in females, infectious pustular balanoposthitis (IPB) in males, conjunctivitis, abortion, enteritis and fatal systemic infection in newborn calves (Kendrick *et al.*, 1958; Abinanti and Plumer, 1961; McKercher and Wada, 1964; Gibbs and Rweyemamu, 1977).

One of the most important characteristics of BoHV-1 is the establishment of latent infection in neurons of sensory ganglia, such as trigeminal or sacral ganglia, and also in the tonsils (Winkler *et al.*, 2000; Jones, 2003). The latent infection can be reactivated under stress conditions or artificially by high doses of corticosteroids. Reactivation occurs usually subclinically making disease control very difficult, since animals without clinical signs can release virus to the environment and thus infect susceptible animals (Straub, 2001). Furthermore, BoHV-1 should be discarded in the diagnosis scheme of vesicular diseases for the similarity of clinical signs (OIE, 2004).

The laboratory diagnosis of BoHV-1 diseases is frequently based on classical methods such as fluorescent antibody test (FAT), virus isolation with subsequent identification by virus neutralization and specific antibodies detection in paired or single serum samples. Immunochemical techniques are faster than virus isolation but these are characterized by lower sensitivity and specificity; furthermore, good quality samples are very important to obtain satisfactory results. Virus isolation, in turn, is laborious, expensive and has a long turnaround time. The seroconversion tests take a minimum of 14 to 21 days and some persistently infected animals with low antibody titer may not be detected.

Faster and accurate BoHV-1 detection methods are necessary to prevent the virus spread to other animals, thus as to reduce the inherent risk from a disease not diagnosed or to prevent the antibiotics used in the treatment of diseases with a wrong presumptive diagnosis.

The polymerase chain reaction (PCR) assay has been used in veterinary virology by its high sensitivity, specificity and speed. Moreover, its use allows reducing handling and extensive amplification of the infectious agent (Belak and Ballagi-Pordany, 1993). Specifically in BoHV-1 detection, PCR has been a very useful tool mainly for detecting the virus in semen (Smits *et al.*, 2000; Deka *et al.*, 2005) and also in other clinical samples (Moore *et al.*, 2000; Takiuchi *et al.*, 2005). However, the existing PCR assays have some limitations. The assays developed by Rocha *et al*. (1998a,b) and Takiuchi *et al*. (2005) were based on a nested and semi-nested PCR strategy, respectively, these imply more time-consuming and a higher risk of cross-contamination. On the other hand, Kibenge *et al*. (1994) and Yason *et al*. (1995) described a PCR method, which has not proved to be suitable for BoHV-1 detection in clinical samples. In order to improve the amplification of GC rich fragment, as can be found in BoHV-1 genome, and subsequently to eliminate most non-specific products, the effect of the addition of several co-solvents such as formamide, dimethyl sulfoxide or glycerol has been evaluated (Cândido *et al*., 2000; Moore *et al*., 2000), but this is not always required.

Different genes of BoHV-1 genome such as *gB*, *gC*, *gD*, *gE* and *tk* gene have been used as targets in the PCR reactions (Wiedman *et al.*, 1993; Kibenge *et al.*, 1994; Santurde *et al.,* 1996; Fuchs *et al.*, 1999; Schynts *et al.*, 1999). The *tk* gene selection for BoHV-1 diagnosis has been effective in a large number of routine diagnosis submissions due to its importance for the virus pathogenicity of all bovine herpesviruses, therefore, the *tk* gene sequence is present in all pathogenic strains (Mittal and Field, 1989), and a large number of *tk* gene sequences has been published in free access databases. This paper describes the development and evaluation of a PCR assay in the region of BoHV-1 *tk* gene, which solves the limitations described above and can be used in all diagnosis laboratories.

Material and methods

Viruses and cells

BoHV-1 strain as well as other viruses used during the present study is listed in Table 1. Madin-Darby bovine kidney cells (MDBK; ATCC CCL 22) were used for bovine viral strain amplification and swine kidney cells (SK6; Kasza *et al.*, 1972) were used for Pseudorabies virus amplification. The cells were grown in Dulbecco's modified essential medium (DMEM, Gibco) supplemented with 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 7% foetal calf serum (FCS, Gibco). In the maintenance medium, FCS was reduced to 2%.

Table 1. Virus strains

Abbreviation	Virus designation	Strain
BoHV-1	Bovine herpesvirus 1	E8[a]
BoHV-2	Bovine herpesvirus 2	Phylaxia[b]
BAdV- 8	Bovine adenovirus 8	Misk/67[b]
BAdV-9	Bovine adenovirus 9	Sofía[b]
PRV	Pseudorabies virus	V208[a]

[a] Virus Collection National Center of Animals and Plants Health, Havana, Cuba. [b] Virus Collection Veterinary Medicine Institute, Phylaxia, Hungary

The cells were infected and incubated at 37°C until a 90-100% cytopathic effect was observed. The supernatants were harvested after three freezing/thawing cycles (-80°C/room temperature) followed by centrifugation at 1500 rpm for 20 min at 4°C. The viral titer was determined by the method of dilution endpoint and the data were processed according to Reed and Muench (1938).

Collection of biological samples

Four male Holstein calves, between 1 to 4 months old and BoHV-1 seronegative, were used. Three calves (1, 2 and 3) were inoculated by intranasal route with 2 mL of E8 infected cells supernatant containing 10^5 $TCID_{50}$/50µL (1mL in each nostril) and another non-infected calf was taken as source of negative control (C). The animals were separated, then fed and treated similarly. Clinical examination was based on the daily assessment of the following aspects: temperature, presence or absence of secretions, nasal mucosa aspect and appetite lost. Nasal swabs were taken before inoculation and every day after inoculation, during 13 days. These samples were processed according to OIE (2004) and evaluated by virus isolation in MDBK cells, FAT and PCR. BoHV-1 fluorescent conjugate, obtained according to Delgado *et al.* (1992), was used for FAT.

DNA extraction

DNA extractions were conducted as previously described by Sambrook *et al.* (1989), with some modifications. Briefly: SDS and proteinase K at final concentration of 1% and 0.2 mg mL^{-1}, respectively, were added to 200 µL of infected and non-infected cell supernatants or 200 µL of nasal swab supernatants. After 2 h at 56°C, suspensions were mixed with 200 µL of buffer-saturated phenol and the phases were separated by centrifugation at 12000 x g for 5 min. The water phase was collected and the phenol extraction was repeated twice. Then, the water phase was mixed with 200 µL chloroform-isoamylalcohol (1:24 v/v) and the upper phase was collected after centrifugation at 12000 x g for 5 min. DNA was precipitated by incubation at -80°C for 2 h with 400 µL absolute ethanol and sodium acetate pH 5.2 at final concentration of 0.1 M. DNA pellet was obtained by centrifugation at 13000 x g for 15 min, washed with 400 µL of 70% ethanol and pelleted again by centrifugation. DNA pellet was dried at room temperature, resuspended in 20 µL TE and stored at -20°C. DNA yield and purity were determined by spectrophotometry (*Amersham Pharmacia Biotech* Ultrospec® 2100 pro).

Target selection and primer design

BoHV-1 primers 514S21, 5'-GCGGGCCTGGTTGCGTACTAC-3' and 695A21, 5'-AGCAGATCTTCCGCGTTGATC-3' were designed from published DNA sequences in *GenBank* database with the computer software program *Oligo* (version 6.31). These correspond to a region of 202 bp within 514 to 716 nt of BoHV-1 *tk* gene and do not hybridise to the *tk* nucleotide sequences of heterologous herpesviruses (Table 2).

PCR assay optimization

The volumes and concentrations of PCR reaction were those recommended by the Technical Bulletin N° 254, PCR Core System Promega®, where the following guidelines are established: 1 X Taq DNA polymerase reaction buffer, 0.5-3 mM $MgCl_2$ solution, 0.2 mM each dNTP, 0.1-1.0 µM primers, 1.25 U Taq DNA polymerase, 2 µL of DNA and water at a volume of 50 µL. As a negative control, DNA was replaced for the same amount of water. Amplifications were performed in a MJ-Research™ termocycler. The cycling conditions consisted of a first incubation step of 5 min at 94ºC followed by 35 cycles (30s-94ºC, 30s-65ºC and 30s-72ºC) and a final incubation at 72ºC-7 min. Optimization was carried out by methodical variation of each test parameters and always was performed at least three replicas by test.

Table 2. Published DNA sequences used for primers design

Abbreviation	Virus designation	Acc. No.
BoHV-1	Bovine herpesvirus 1	D00438, AJ004801, Z78205
BoHV-2	Bovine herpesvirus 2	D00537
BoHV-4	Bovine herpesvirus 4	S49773, AB035517
BoHV-5	Bovine herpesvirus 5	AF113752
	Bovine herpesvirus 5 (strain SV507/99)	AY261359
EHV-1	Equine herpesvirus 1	X13209
PRV	Pseudorabies virus (strain LA)	AY173125S1
----	Herpesvirus Alcelaphine 1	NC 002531

PCR products were analyzed by electrophoresis in 2% agarose gels containing 0.8 μg mL^{-1} ethidium bromide solution.

Analytical specificity and sensitivity

Analytical specificity was determined by the evaluation of heterologous herpesviruses and other DNA viruses, cattle pathogens (Table 1).

Serial ten-fold dilutions in nasal swab supernatant from a healthy donor bovine of a nasal swab supernatant ($10^{3.88}$ $TCID_{50}$/50μL) were conducted. DNA was isolated from each dilution and was used as template for PCR.

Results

PCR assay optimization

The PCR optimization revealed that a 65°C annealing temperature allowed the best balance between sensitivity and specificity of primer alignments (Fig. 1A). The optimal primers and magnesium chloride concentrations were 0.6 μM and 1mM, respectively (Fig. 1B and C). However, an easily detectable reduction of the band intensity or not product amplification was observed at other concentrations. With the evaluation of 30 cycles, low intensity bands were obtained, while differences were not observed between 35 and 40 cycles (data not shown). Thirty-five were selected as the optimal number of reaction cycles. These results were consistently observed in all replicas.

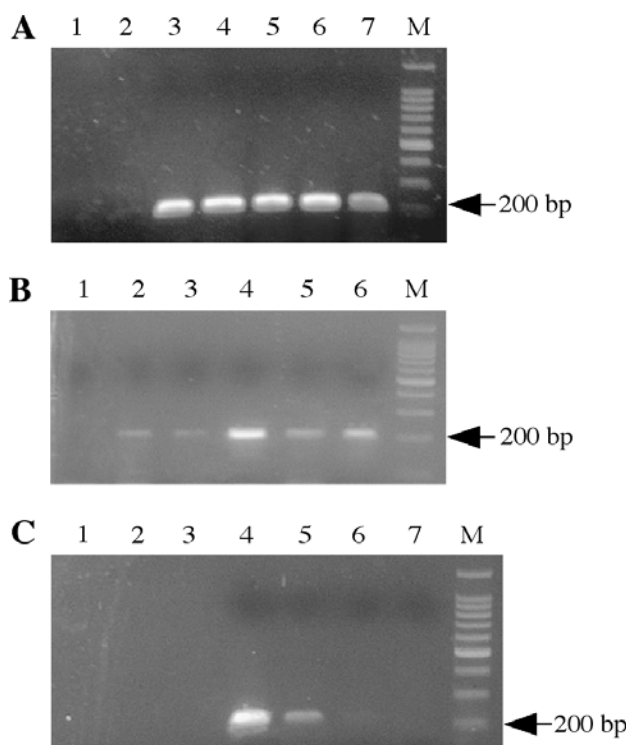

Figure 1. (A) Determination of the optimal temperature of primers annealing, DNA from BoHV-1 (E8 strain) infected cell supernatant. Lane 1: Negative control (water free of nucleases); 2: Negative control (DNA from non-infected MDBK cell supernatant); 3: 57ºC; 4: 60ºC; 5: 62ºC; 6: 65ºC; 7: 68ºC. (B) Determination of the final optimal concentration of primers, DNA from BoHV-1 (E8 strain) infected cell supernatant. Lane 1: Negative control (water free of nucleases); 2: 0.3 μM; 3:0.5 μM; 4: 0.6 μM; 5: 0.7 μM; 6: 0.9 μM. (C) Determination of the final optimal temperature of magnesium chloride, DNA from BoHV-1 (E8 strain) infected cell supernatant. Lane 1: Negative control (water free of nucleases); 2: Negative control (DNA from non-infected MDBK cell supernatant); 3: 0.5 mM; 4: 1 mM; 5: 1.5 mM; 6: 2 mM; 7: 2.5 mM; M: 100 bp DNA ladder (Promega).

Analytical specificity and sensitivity

DNA amplification was not produced when heterologous herpesviruses and other bovine viruses were evaluated. A fragment to the correct molecular weight was only obtained for E8 strain (Fig. 2A).

Serial ten-fold dilutions were performed to establish the PCR lowest detection limit. The value was obtained bearing in mind the volume of sample (200 μL) and the fraction of nucleic acid extract used in each reaction (20%). Clear bands in the electrophore-

sis gels were observed until 0.15 $TCID_{50}$ per reaction (Fig. 2B).

BoHV-1 detection in clinical samples

Fever between 39.5 to 40.3ºC since two to five days post-inoculation, tearing, nasal and ocular serous secretions were observed according to the animal. Subsequently, the animal 2 was recovered from the disease while animals 1 and 3 were with tearing and serous secretion which became mucopurulent. Hyperaemia in the mucous membrane or inappetence was not present in the animals. The control did not present clinical signs during the 13 days.

Fifty-six nasal swab samples were analyzed by virus isolation and PCR. Only 47 samples were analyzed by FAT because 9 samples had not a good quality for this purpose (Table 3). Virus isolation positive samples were found PCR positive. However, from five PCR-positive samples, virus was not obtained after three passages in cell cultures. Two of these samples were positive by FAT indicating that viral antigens were present in the absence of infective virions.

In general, it was noted that until six days post-inoculation, there was a 100% match on the results obtained by the three tests analyzed with the highest number of fluorescent cells by fluorescent antibody test and the highest intensity of the electrophoresis bands. During this period, the largest viral excretion and the appearance of the clinical signs were also observed. Starting the seven day post-inoculation and until the end of the experiment, differences in results between virus isolation, FAT and PCR were observed. At this time, most of the positive samples were obtained by PCR and the number of infective viral particles in the nasal swab supernatants were significantly reduced (Table 3).

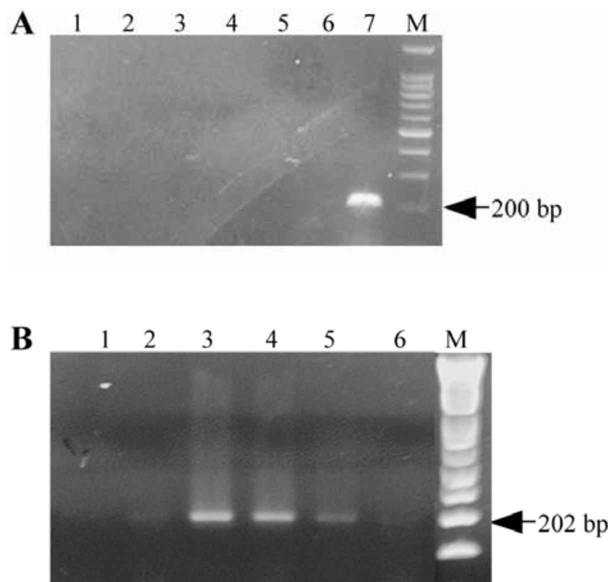

Figure 2. (**A**) PCR analytical specificity. Lane 1: Negative control (water free of nucleases); 2: Negative control (DNA from non-infected MDBK cell supernatant); 3: Sofía strain DNA; 4: Misk/67 strain DNA; 5: Phylaxia strain DNA; 6: V208 strain DNA; 7: E8 strain DNA; M: 100 bp DNA ladder (Promega). (**B**) PCR analytical sensitivity from DNA of nasal swab supernatants. Lane 1: Negative control (water free of nucleases); 2: Negative control (DNA from negative nasal swab supernatant); 3, 4, 5 and 6: nasal swab supernatant with a titer of $10^{3.88}$ $TCID_{50}$/50mL, serial ten-fold dilutions; 3: 10^{-1}; 4: 10^{-2}; 5: 10^{-3}; 6: 10^{-4}; M: 1Kb plus DNA ladder (Invitrogen).

Discussion

In Cuba, as in many regions of the world, infections caused by BoHV-1 are endemic, suggesting that BoHV-1 should be frequently discarded in diagnosis laboratories. Development and evaluation of a PCR to detect BoHV-1 in clinical samples are considered a contribution to the virological veterinary diagnosis of the country, which is still deficient in terms of international standards for speed, quality and costs.

The PCR assay developed was specific and sensitive. The specific detection of BoHV-1 showed that the primers design is correct and the *tk* gene region selected has diagnosis quality. This assay was as sensitive as others previously described (Vilcek *et al*., 1994; Masri *et al*., 1996; Moore *et al*., 2000) and more sensitive than the PCR described by Yason *et al*. (1995). The protocol described in this paper is simple, in contrast with semi-nested or nested PCR protocols used in order to achieve greater sensitivity (Rocha *et al*., 1998a,b; Takiuchi *et al*., 2005). Only a real-time PCR for BoHV-1 detection has been developed (Abril *et al*., 2004). This assay has several advantages over conventional or nested PCR methods, including a much lower risk of contamination, rapidity, a higher sensitivity and the possibility to obtain quantitative measurements (OIE, 2004), but the necessary equipment required to perform the real-time PCR assays is not accessible for all laboratories, and at present such assays are too expensive for routine diagnosis.

The clinical signs observed after experimental infection of cattle were consistent with a mild infection,

Table 3. Identification of BoHV-1 positive samples by virus isolation, fluorescent antibody test (FAT) and PCR

	Days														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	Total
Virus isolation	0	3	3	3	3	3	3	2	2	2	1	1	0	0	26
FAT	0	3	3	3	3	3	2[a]	3	3	2	1	0[a]	0	0[a]	26
PCR	0	3	3	3	3	3	3	3	3	3	2	2	0	0	31
log titer[b]	0	3.6	4.18	3.45	4.52	5.22	4	0.93	1.1	1.72	2.57	2.4	0	0	
Temperature[c]	39.3	39.1	39.5	39.9	40	38.8	38.9	38.7	38.2	38.6	38.3	38.4	38	37.5	

[a] Slide preparations with poor quality. [b] log titer average from virus isolation positive samples. [c] Temperature average from the three infected animals.

according to Young (1993), who classifies BoHV-1 infections in subclinical, mild or severe. Taking into account that nasal swabs are the normal form of sample for the diagnosis of IBR, the observations made during the experimental inoculation are useful to guide the sampling period to animals showing clinical signs of high respiratory infection (fever, tearing, serous ocular or nasal secretion etc). In this period an excellent correlation between virus isolation, FAT and PCR was obtained. It is the moment of high viral excretion and the samples taken at this time enhance the diagnosis effectiveness. However, for animals that recovered quickly, the PCR could be a recommended tool for diagnostic confirmation according to the results obtained by test comparison.

Virus isolation, gold standard for the diagnosis of many viruses, requires intact virions able to multiply in susceptible cells. Currently, it has been demonstrated that the sensitivity and specificity of PCR are generally higher than virus isolation or capture ELISA procedures (OIE, 2004). FAT, which provides an alternative for rapid diagnosis, is also described with a low sensitivity regarding PCR or virus isolation due to the principle of the technique and the poor detection in low quality samples (Keuser *et al.*, 2004). The highest PCR sensitivity described is based on the amplification of million copies of the target (Belak and Ballagi-Pordany, 1993); furthermore, adding the ability to detect viable and non-viable viral particles (Williams and Kwok, 1992).

To avoid cross-contamination during the execution of the PCR, special precautions recommended by the OIE (2004) were taken into consideration. Moreover, the PCR results were guaranteed by the inclusion of negative controls during extraction and amplification procedures (non-infected MDBK cell supernatant, negative nasal swab supernatant and water nucleases free).

Further studies will be required to evaluate 514S21 and 695A21 primers for the BoHV-1 detection in semen and other biological fluids taking into account that BoHV-1 is frequently transmitted by artificial insemination and it is a known contaminant of foetal calf serum (Kupferschmied *et al.*, 1986; Erickson *et al.*, 1991).

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