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CONVENTIONAL VS REAL-TIME PCR FOR DETECTION OF BOVINE HERPES VIRUS TYPE 1 IN ABORTED BOVINE, BUFFALO AND CAMEL FOETUSES

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Summary

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In the present study, conventional and real-time PCR assays were developed in order to detect bovine herpes virus type 1 in clinical samples. A total of 16 (11.18%), 10 (11.11%) and 8 (10.12%) aborted bovine, buffalo and camel foetuses were respectively positive for the specific amplicon of 161 bp fragment for BHV-1 DNA in conventional PCR. After using real-time PCR, the glycoprotein B gene of BHV-1 was seen in 21 (14.68%), 14 (15.55%) and 10 (12.65%) of aborted bovine, buffalo and camel foetuses, respectively. There was no significant difference between the abilities of conventional and real-time PCR assays for detection of BHV-1 in abomasal contents of aborted foetuses. Statistical analysis showed significant differences ($P<0.05$) between buffalo and camel aborted foetuses with regard to the presence of the virus. The Ct values obtained from real-time PCR for presence of BHV-1 showed significant differences ($P<0.05$) between buffalo and camel aborted foetuses. Our results showed that the real-time PCR assay was 2 times more sensitive than the conventional PCR. The Gilan province had the highest BHV-1 prevalence and Kordestan: the lowest. Based on the results of this study, camels were the most resistant and buffaloes – the most sensitive to abortions caused by BHV-1. This is the first prevalence report of direct detection of BHV-1 in aborted bovine, buffalo and camel foetuses by evaluation of conventional and real-time PCR in Iran.

Key words: aborted foetuses, BHV-1, conventional PCR, real-time PCR

INTRODUCTION

Infectious bovine rhinotracheitis (IBR, respiratory disease) and infectious pustular vulvo-vaginitis (IPV, genital disease) are the most important diseases of domestic and wild cattle, goat, sheep and camelids around the world (Campos *et al*., 2009). They are caused by bovine herpes virus type 1 (BHV-1). BHV-1 is a member of the genus *Varicellovirus* in the subfamily Alphaherpesvirinae, which belongs to the Herpesviridae family, order Herpesvirales (Davison *et al*., 2009). The viral genome consists of double-stranded DNA that encodes for about 70 proteins, of which 33 structural and more than 15 nonstructural proteins have been identified. The viral glycoproteins, which are located in the envelope on the surface of the virion, play an important role in pathogenesis and immunity (OIE, 2010).

In addition to respiratory and genital disease, BHV-1 is one of the main causes of abortion in six to nine months of pregnancy (Muylkens *et al*., 2007) and sporadically, BHV-1 has been associated with encephalitis (Silva *et al*., 2007).

Therefore, in view of these economic losses associated with BHV-1, there is need for accurate and sensitive diagnostic methods for rapid identification and elimination of persistent carriers in the herds. There are various methods for diagnosis of BHV-1 such as cell culture, serological and molecular methods. Cell culture method is well established for detection of BHV-1, but is a difficult and lengthy process that requires experienced technicians and often takes weeks to achieve observable growth depending on the sample type, freedom from overgrowth by fungal and other viral contaminants.

Serological tests such as virus neutralisation (VN) (Saha *et al*., 2010) and enzyme linked immunosorbent assay (ELISA) (Graham *et al*., 1997) are frequently used for the detection of BHV-1 infection. Maternally-derived antibodies may be detected by serological tests for up to 7 months, but usually disappear in about 4–5 months (Graham *et al*., 1997). In addition, the serological tests usually give cross reactions with other pathogens. Therefore, in order to solve these problems, several conventional and real-time PCR assays which are simpler, faster, less hazardous and usually more sensitive than traditional methods have been developed (Moore *et al*., 2000; Grom *et al*., 2006; Guo *et al*., 2009).

Therefore, the purpose of this study were to determine the actual prevalence rate of BHV-1 in aborted bovine, buffalo and camel foetuses in Iran and evaluate the sensitivity of conventional and real-time PCR assays for detection of BHV-1 in abomasal contents of aborted foetuses.

MATERIALS AND METHODS

Samples

From April 2010 to April 2011, a total of 143 bovine, 90 buffalo and 79 camel aborted foetuses were randomly collected from 100 commercial herds from different parts of Iran. All samples from abomasal contents of aborted foetuses were collected in sterile conditions and sent under refrigeration to the Biotechnology Research Center of the Islamic Azad University of Shahrekord Branch as soon as possible. Samples were stored at –20 ^{o}C until processing.

DNA extraction

In this present study the genomic DNA was isolated from abomasal contents of aborted foetuses using a genomic DNA purification kit (Invitrogen, Paisley, U.K) according to the manufacturer's instructions. The total DNA was measured at 260 nm optical density according to the method described by Sambrook & Russell (2001).

Conventional PCR

To detect BHV-1 DNA, the primer pair (F 5'-CTAACATGGAGCGCCGCTT-3' and R 5'- CGGGGCGATGCCGTC-3') was used, which gives a product of 161 bp. All oligonucleotide primers were obtained from a commercial source (Cinna Gen, Iran). The PCR reaction was performed in a total volume of 25 µL containing 2 µL of DNA sample, 0.5 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTP mix, 0.8 µm each primers and 0.5 U/reaction of Taq DNA polymerase (Promega, Madison, WI). Reactions were initiated at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 ^{o}C,

1 min at 61 °C and 1 min at 72 °C and a final extension time of 5 min at 72 °C. Some products were sequenced to confirm their identity (data not shown) in a DNA thermal cycler (ASTEC, Fukuoka, Japan). A negative control (sterile water), and a positive control DNA from BHV-1 were included in each amplification run.

Amplified samples were analysed by electrophoresis (120 V/208 mA) in 1.5% agarose gel. The gel was stained with 0.1% ethidium bromide (0.4 µg/mL) and viewed on UV transilluminator.

Real-Time PCR

The primers and probe selected from the sequences of the glycoprotein B gene of BHV-1 (Abril *et al*., 2004) were used for real-time PCR amplification and detection. The primers and probe were checked for their specificity using the database similarity search programme nucleotide-nucleotide BLAST (www.ncbi.nlm.nih.gov/blast) (Table 1) and the sequences were 100% homologous to those of BHV-1 strains deposited in the GenBank. All oligonucleotides were synthesised by Sigma Genesys (Australia). 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 optimise the PCR protocol, including concentration of reagents and PCR cycling parameters. The optimised 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 2× 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 final concentration of magnesium was 3 mM in each reaction. The real-time PCR was carried out in a Rotor Gene 3000 real time detection system (Corbett Research, Australia). The PCR cycling parameters were, 2 min at 50 °C (activation of UDG), 2 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 45 s of 60 °C. From each amplification plot, a threshold cycle (Ct) value was calculated representing the PCR cycle number at which the reporter dye fluorescence was detectable above an arbitrary threshold. The threshold was set at a level that was significantly higher than that of background. Samples were considered to be positive when the Ct value was less than or equal to 45 (negative samples have no Ct value).

Sensitivity of conventional and real-time PCR assays

At first the concentration of viral pathogens were measured using the tissue culture infectious dose ($TCID_{50}$) method (Meshkat *et al*., 2009). Suspensions of purified BHV-1 were prepared and DNA was extracted by a genomic DNA purification kit (Invitrogen, Paisley, U.K.) according to the instruction manual. Two

Table 1. Primers and probes used of real-time PCR for detection of BHV-1 and bGH gene

Oligonucleotide	Sequences (5'-3')	Genome position
gB-F (forward)	TGT GGA CCT AAA CCT CAC GGT	57499–57519
gB-R (reverse)	GTA GTC GAG CAG ACC CGT GTC	57595–57575
Probe (BHV-1)	FAM-AGGACCGCGAGTTCTTGCCGC-TAMRA	57525–57545

hundred μL of the solution was used, and the extracted DNA was dissolved in 100 μL of distilled water. Three μL of the DNA solution were used in the conventional and real-time PCR assays.

Statistical analysis

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA), ANOVA test analysis were performed and differences were considered significant at $P<0.05$.

RESULTS

In this study, a total of 312 aborted bovine, buffalo and camel foetuses were tested for presence of BHV-1 by both conventional and real-time PCR assays.

Quality of DNA extracted after 1.5% agarose gel electrophoresis from abomasal contents of aborted bovine, buffalo and camel foetuses was evaluated as suitable for PCR assay.

Agarose gel electrophoresis of the amplification products showed the presence of bands with the size of 161 bp fragment for BHV-1 (Fig. 1). Results showed that 34 out of 312 samples (10.89%) were positive for presence of BHV-1 in abomasal contents of aborted foetuses. After real-time PCR, the glycoprotein B gene of BHV-1 was determined in 45 out of 312 specimens (14.42%) diagnosed as positive for BHV-1 (Table 2).

Sensitivity between the conventional and real-time PCR assays to detect BHV-1 DNA was compared. The conventional PCR assay detected 68 microorganisms/PCR-tube, whereas the real-time PCR assay with the specific primers to glycoprotein B gene of BHV-1, detected 34 microorganisms/PCR-tube (data not shown), indicating that the real-time PCR assay was twice more sensitive than the conventional PCR. There were no significant differences between the abilities of conventional and real-time PCR assays to detect BHV-1 in abomasal contents of aborted foetuses.

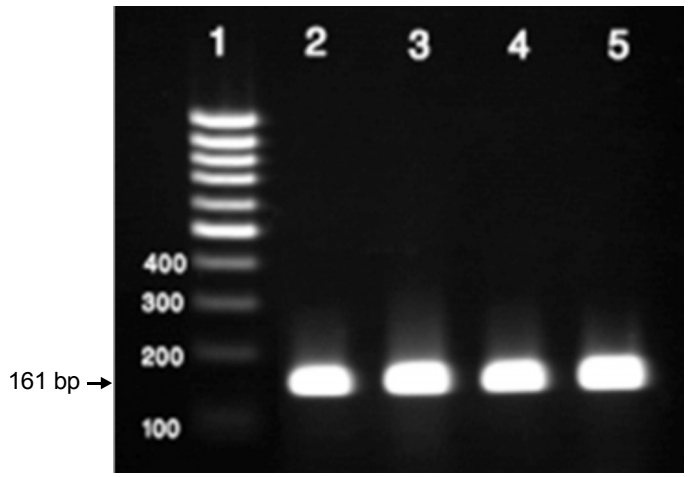

Fig. 1. Conventional PCR for detection of BHV-1 in aborted foetuses.
Lane 1: 100 bp ladder; lanes 2–4: positive samples for aborted bovine, buffalo and camel foetuses respectively; lane 5: positive control.

Table 2. Distribution of BHV-1 in abomasal contents of aborted bovine, buffalo and camel foetus samples in each province in Iran by evaluation of conventional and real-time PCR

Province	Conventional PCR number positive/total number (%)			Real-time PCR number positive/total number (%)		
	Bovine	Buffalo	Camel	Bovine	Buffalo	Camel
Tehran	3/40 (7.5)	2/14 (14.28)	1/8 (12.5)	4/40 (10)	3/14 (21.42)	1/8 (12.5)
Isfahan	3/20 (13.63)	1/14 (7.14)	3/28 (10.71)	4/20 (18.18)	2/14 (14.28)	4/28 (14.28)
Khorasan	1/25 (4)	1/16 (6.25)	2/20 (10)	2/25 (8)	2/16 (12.5)	2/20 (10)
Khozestan	4/19 (21.05)^{C}	4/25 (16)	1/12 (8.33)^{C}	5/19 (26.31)^{D}	5/25 (20)	2/12 (16.66)^{D}
Gilan	4/16 (25)	2/8 (25)	1/5 (20)	5/16 (31.25)	2/8 (25)	1/5 (20)
Kordestan	1/21 (4.76)	0/13 (0)	0/6 (0)	1/21 (9.52)	0/13 (0)	0/6 (0)
Total	16/143 (11.18)^{A}	10/90 (11.11)	8/79 (10.12)^{A}	21/143 (14.68)^{B}	14/90 (15.55)	10/79 (12.65)^{B}

Similar letters within rows indicate statistically significant difference at $P<0.05$.

The statistical analysis showed significant differences ($P<0.05$) between positive bovine and camel aborted foetuses.

This present study showed that the real-time PCR assay was more sensitive and accurate than conventional method for detection of BHV-1 in abomasal contents of aborted foetuses.

In this study 21 (16.68%), 14 (15.55%) and 10 (12.65%) samples were positive for presence of BHV-1 by real-time PCR assay out of 143, 90 and 79 aborted bovine, buffalo and camel foetuses respectively (Table 2), indicating the prevalence rate of BHV-1 in aborted foetuses in Iran. Our results showed that camels were the most resistant and buffaloes – the most sensitive to abortion caused by BHV-1. The Gilan province had the highest and Kordestan province – the lowest prevalence rate of BHV-1 in Iran.

DISCUSSION

Previously used diagnostic assays such as cell culture, VN test and ELISA usually pose the problem of cross reaction with other pathogens. This present study was conducted to detect BHV-1 in abomasal contents of aborted bovine, buffalo and camel foetuses by conventional and real-time PCR assays. They were designed based on the glycoprotein B gene that is a highly conserved gene of herpesviruses, which was confirmed by the higher accuracy of these tests.

PCR has been developed for detection of BHV-1 in a wide variety of clinical samples such as artificially infected semen (Grom *et al*., 2006), naturally infected semen (Deka *et al*., 2005), blood (Fuchs *et al*., 1999), nasal swabs (Saha *et al*., 2010), vaginal swabs (Saha *et al*.,

2010) and faecal swabs (Mahmoud & Ahmed, 2009). In all these studies PCR was found to be accurate, sensitive and fast method for detection of BHV-1.

The real-time PCR assay used in this present study was more accurate, sensitive and faster than conventional PCR for the detection of BHV-1. In addition, the real-time PCR assay had some advantages compared to the conventional PCR: it was demonstrated as an important diagnostic tool yielding reliable and reproducible results, which does not require post-PCR analysis (gel electrophoresis, hybridisation), and the risk of cross contamination appeared more limited than in conventional PCR. This study showed that the conventional PCR method for detecting BHV-1 is more technically time-consuming and labour-intensive than real-time PCR assay. However, the real-time PCR is more expensive than the conventional. The real-time PCR assay that was used in this study could simplify the procedure by testing presumptive BHV-1 genome taken directly from abomasal contents of aborted foetus samples. Also, compared with virus isolation, the PCR could detect episomal DNA of the non-replicating virus in sensory ganglia (Van Engelenburg *et al*., 1993), such as the trigeminal ganglion, in the latent phase of infection. The risk of contamination is markedly reduced by real-time or quantitative PCR (Lovato *et al*., 2003; Abril *et al*., 2004).

Prevalence studies of BHV-1 from different parts of Iran including Ahvaz (31.48%; Hajikolaei & Shapouri, 2006), Kerman (30.39%; Sakhaee *et al*., 2009), Chaharmahal & Bakhtiari (46.68%; Hemmat Zade *et al*., 2002) and Urmia (48.9%; Mahmodian *et al*., 2002) indicated that IBR/IPV is one of the most important endemic viral diseases in Iran. The most recent previous study from Iran showed a total prevalence of BHV-1 of 27.68% in 2010 (Badiei *et al*., 2010) but our study showed that the prevalence of this virus was 22.39%. Investigations on dairy herds have established IBR prevalence to be 17.3-54.0 % on a global basis (Eiras *et al.*, 2009).

Studies showed that the prevalence of virus in European countries such as Portugal (47.2%; Soares *et al*., 2006), Italy (61%; Cavinari, 2006) and Belgium (84%; Boelaert *et al*., 2000) is considerably higher than that in Iran. In addition, a recent study has established herd prevalence of BHV-1 for Europe ranging from 10–80% (Borchers, 2006). It therefore appears that the prevalence of IBR in the countries of the European Economic Area (EEA) is highly variable, which may be a factor of control measures.

Studies on the prevalence of BHV-1 in camels are limited, but the detection of antibodies to IBR in camels has been reported in Tunisia (Burgemeister *et al*., 1975) and Egypt (Eisa, 1998). Nawal *et al.* (2003) were the first to report detection and isolation of BHV-1 from camels in Egypt. Similarly, there is paucity of prevalence studies of BHV-1 in buffaloes.

The 14.68% prevalence rate of BHV-1 cattle reported in the present study, was higher than that in Uzeyir (0.8% – Iscan & Duman, 2011) but lower than that in Spain (38.4% – Eiras *et al*., 2009), Ireland (74.9% – Bosco Cowley *et al*., 2011), Hungary (64.1% – Tekes *et al*., 1999) and Uruguay (37% – Guarino *et al*., 2008). The prevalence of BHV-1 in camels of this study (12.65%) was lower than a previous study in Sudan (76.9%) (Intisar *et al*., 2009).

Variations in prevalence rates of BHV-1 in aborted foetuses found in this study may be dependent on environmental condition, animal movement (especially purchasing of replacement animals) and

the chance of exposure of these animals to BHV-1 infection. To our knowledge, the close contact between infected cattle with camels and buffaloes especially in traditional condition in Iran is the most likely reason for the high rate of abortions in these species.

Other studies showed that in some countries, particularly in Iran where cattle are kept in close association with camels and buffaloes, infections and abortions due to BHV-1 could occur in latter species. On the other hand, the high presence of BHV-1 in abomasal content samples of unspecific hosts (buffaloes and camels) of this present study may show that these animals had been maintained in close association with infected cattle. In addition, a previous study (Boelaert *et al.,* 2005) showed that the herd size may also be a risk factor for IBR/IPV prevalence.

In rural conditions, small and large ruminants and in some area camels are pastured together. Moreover, in small holder family farms, it is common to harbour these species in the same location. Hence, this system of breeding may allow cross transmission of the agent among the species. This risk may be a factor in the efficiency of BHV-1 control activities. On the other hand, newly purchased and introduced cattle, sheep and goats if latently infected, can introduce the virus to uninfected buffalo and camel populations.

Prevention and control of BHV-1 infections are based on thorough farm management including hygienic measures, vaccination schedules and removal of infected animals. Natural mating should be avoided and only semen from BHV-1 negative bulls should be used.

To our knowledge, this study is the first report on the prevalence of BHV-1 in aborted bovine, buffalo and camel foetuses using both conventional and real-time PCR. This present study suggested that real-time PCR was more sensitive and accurate than conventional PCR and can be used as a safe, fast and reliable diagnostic method for detection of BHV-1 in aborted foetuses. Our study also established the current prevalence of BHV-1 in aborted bovine, buffalo and camel foetuses in Iran.

The used PCR assays were found to be specific and sensitive for the detection of BHV-1 in the abomasal contents of aborted foetuses, thus they have practical diagnostic value. Our results indicated that the camel was the most resistant and buffalo – the most sensitive species to BHV-1 abortions. In some areas (Isfahan, Khozestan, Gilan and Kordestan provinces) the prevalence of BHV-1 abortions in cattle was higher than in buffaloes.

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