

## SEQUENCING AND PHYLOGENETIC ANALYSIS OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS *gB* AND *gD* GENES IN IRAN

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### Summary

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Bovine herpesvirus 1 (BHV-1) belongs to the *Varicellovirus* genus, *Herpesviridae* family and causes different respiratory, reproductive and nervous system disorders in cows, including infectious bovine rhinotracheitis (IBR). For determination of the genetic relationship of *gB* and *gD* – two of main BHV-1 glycoprotein genes in Iran with those in other countries, DNA fragments of 671 bp corresponding to *gB* and 1002 bp corresponding to *gD* from four IBR viral strains have been isolated from bovine respiratory disorders in Iran, then were amplified in PCR system, sequenced for determining nucleotide sequence and compared with identified nucleotide sequences of these genes in other countries. The results indicated 6.6% to 14.5% variability in *gB* gene and 1.1% to 13.3% variability in *gD* gene in four sequenced samples. A comparison made on *gB* and *gD* genes in Iran with other countries showed 6.4% to 59% variability in *gB* gene and 8.7% to 29.2% variability in *gD* gene.

**Key words:** bovine herpesvirus 1, *gB* and *gD* genes, Iran, phylogenetic relationship

### INTRODUCTION

Bovine herpesvirus type 1 (BHV-1), the cause of infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), is a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae* and is associated with a number of different clinical syndromes in cattle (Roizman *et al.*, 1992; Tikoo *et al.*, 1995; D'Arce *et al.*, 2002; Meurens *et al.*, 2004; Toussaint *et al.*, 2005; Muylkenes *et al.*, 2006).

All herpesviruses encode a large number of proteins involved in nucleic acid metabolism, DNA synthesis and protein processing (Roizman & Pellett, 2002; Keil *et al.*, 2005). At least 33 of the BHV-1 encoded proteins are structural proteins (Misra *et al.*, 1981). Of these, 13 are probably associated with the envelope and 10

have the potential to encode glycoproteins (Liang *et al.*, 1996; Schwyzer & Ackermann, 1996). There are 8 known glycoproteins: *gB*, *gC*, *gD*, *gE*, *gH*, *gL*, *gK* and *gL*; the major envelope glycoproteins being *gB*, *gC* and *gD*. The *gC*, *gD*, *gE*, *gG*, *gL*, *UL49h* and thymidine kinase genes are involved in viral virulence and are useful targets for diagnosis, prevention or antiviral treatment (Smith *et al.*, 1994; van Engelenburg *et al.*, 1994; Young & Smith, 1995; Schwyzer & Ackermann, 1996; van Oirschot *et al.*, 1996; Liang *et al.*, 1997; Kaashoek *et al.*, 1998).

In the present study, partial sequences of the BHV-1 *gB* and *gD* genes obtained from four IBR viral strains isolated in

different regions of Iran were compared with other BHV-1 *gB* and *gD* gene sequences of strains isolated in other countries and registered in GenBank.

## MATERIALS AND METHODS

### Viruses

Four IBR viral strains isolated in different regions of Iran were propagated in Madin-Darby Bovine Kidney (MDBK) cells grown in Eagle's minimum essential medium (MEM; Invitrogen) supplemented with 10% fetal calf serum (FCS; Invitrogen).

### Polymerase chain reaction

Viral DNA were purified from IBR-infected MDBK cells using DNA isolation kit for cell and tissues (Roche Applied Science).

Primers for PCR amplification of the genes coding for *gB* and *gD* glycoproteins were designed from the published sequence of BHV-1 *gB* and *gD* genes in GenBank (Table 1).

The amplification reactions were performed in 50 µL reaction mixtures containing 0.1 mM of each deoxynucleotide, 15 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH=9), 2 mM MgCl<sub>2</sub>, 10% dimethyl sulfoxide (DMSO, Sigma), 1.5 U of Taq DNA polymerase (Sigma) and 40 ng of template DNA. The PCR reaction was carried out in a PCR programmed thermocycler (Eppendorf, Mastercycler 5330, Eppendorf-Nethel-Hinz

GmbH, Germany) using the thermal profiles: initial cycle 94 °C for 9 min, followed by further 35 cycles: denaturation at 95 °C for 60 s; annealing at 58–61 °C for 60 s, depending on the melting temperature of the primers used and considering previous amplification results, and extension by polymerase at 72 °C for 60 s. The final cycle was run at 72 °C for 7 min (Ros & Belak, 1999). PCR products were purified with High pure PCR product purification kit (Roche Applied Science) according to manufacturer's recommendations. Single DNA strands were sequenced with ABI 3730 XL device and Sanger sequencing method (Macrogen, Korea) (Sambrook & Russell, 2001).

### Sequence analysis

The nucleotide sequences were edited using Edit View v.1.0.1 (Applied Bioscience, Australia). The 14 sequences registered in GenBank (accession numbers AY758382.1; EF175730.1; M21474.1; AF078724.1; DQ006857.1; AF078725.1; EF624475.1; DQ006854.1; AY755875.1; AJ004701.1 for *gB* and AF078730.1; M59846.1; AY690484.1; AF078731.1 for *gD* gene) were aligned separately using the Clustal W v1.81 in order to obtain a consensus sequence. Subsequently, the sequences were analysed using the BioEdit package v.7.0.4.1 to compare the nucleotide sequences.

The four nucleotide sequences of the Iranian IBR *gB* and *gD* genes were com-

**Table 1.** Primers used for PCR analysis of IBR virus *gB* and *gD* genes

Primer	Primer sequence	Size of product (bp)	Accession number
IBR- <i>gB</i> -F	5'-GTACACG TTCAAGGCCTACA-3'	671	DQ006853.1
IBR- <i>gB</i> -R	5'-TCGTCTCGCAGCATTTC-3'		
IBR- <i>gD</i> -F	5'-GCGGTGAGCTTGCCTACA-3'	1002	AY690484.1
IBR- <i>gD</i> -R	5'-GTGCGTGATGGCTTCGAG-3'		

**Table 2.** Sequence identity matrix of partial *gB* gene of Iranian IBR virus isolates in comparison with 10 known reference sequences

Seq <i>gB</i> IBR	Iran 2	Ref 1	Ref 2	Ref 3	Ref 4	Ref 5	Iran 4	Ref 6	Iran 3	Ref 7	Ref 8	Ref 9	Iran 1	Ref 10
Iran 2		0.933	0.936	0.936	0.936	0.936	0.872	0.749	0.934	0.926	0.926	0.916	0.878	0.41
Ref 1	0.933		0.996	0.996	0.996	0.996	0.917	0.803	0.902	0.983	0.983	0.972	0.916	0.434
Ref 2	0.936	0.996		1.000	1.000	1.000	0.921	0.800	0.905	0.987	0.987	0.976	0.919	0.438
Ref 3	0.936	0.996	1.000		1.000	1.000	0.921	0.800	0.905	0.987	0.987	0.976	0.919	0.438
Ref 4	0.936	0.996	1.000	1.000		1.000	0.921	0.800	0.905	0.987	0.987	0.976	0.919	0.438
Ref 5	0.936	0.996	1.000	1.000	1.000		0.921	0.800	0.905	0.987	0.987	0.976	0.919	0.438
Iran 4	0.872	0.917	0.921	0.921	0.921	0.921		0.769	0.924	0.917	0.917	0.907	0.864	0.421
Ref 6	0.749	0.803	0.800	0.800	0.800	0.769	0.769		0.723	0.797	0.797	0.786	0.757	0.436
Iran 3	0.934	0.902	0.905	0.905	0.905	0.924	0.924	0.723		0.902	0.902	0.892	0.855	0.399
Ref 7	0.926	0.983	0.987	0.987	0.987	0.917	0.917	0.797	0.902		1.000	0.989	0.917	0.438
Ref 8	0.926	0.983	0.987	0.987	0.987	0.917	0.917	0.797	0.902	1.000		0.989	0.917	0.438
Ref 9	0.916	0.972	0.976	0.976	0.976	0.907	0.907	0.786	0.892	0.989	0.989		0.907	0.434
Iran 1	0.877	0.916	0.919	0.919	0.919	0.864	0.864	0.757	0.855	0.917	0.917	0.907		0.430
Ref 10	0.41	0.434	0.438	0.438	0.438	0.421	0.421	0.436	0.399	0.438	0.438	0.434	0.430	

Legend: Ref 1=AF078724.1-Sweden; Ref 2=M21474.1-Philadelphia; Ref 3=DQ006857.1-Brazil; Ref 4=AY745875.1-Brazil; Ref 5=AJ004701.1-Switzerland; Ref 6=EF624475.1-Belgium; Ref 7=DQ006854.1-Brazil; Ref 8=AF078725.1-Sweden; Ref 9=AY758382.1-Brazil; Ref 10=EF175730.1-India.

pared with the corresponding sequences from other regions of the world. Unrooted dendrogrammes were constructed using the Njplot software. Statistical support for dendrogrammes was obtained by bootstrapping using 1000 replicates.

RESULTS

The nucleotide sequences of the 630 bp fragments of the IBR *gB* gene and the 785 bp fragments of the IBR *gD* gene from Iranian isolates were compared with the sequences of the *gB* and *gD* genes from the known reference sequences obtained from the GenBank nucleotide sequence database (10 sequences corresponding to *gB* and 4 sequences corresponding to *gD*). The nucleotide sequences had a variability of 6.4–59% for *gB* gene (Table 2) and 8.7–29.2% for *gD* gene (Table 3) and variations consisted only in nucleotide substitution. Frameshift, deletion, insertion and nonsense mutations were not observed.

A comparison made on the sequences of *gB* and *gD* between Iran and other countries showed 6.4–59% variability in *gB* and 8.7–29.2% variability in *gD*. The

greatest sequence similarity exists between Iranian *gB* sequence and M21474.1-Philadelphia, DQ006857.1-Brazil and AY745875.1-Brazil with a sequence similarity of 93.6% and the least relationship – between Iranian *gB* sequence and EF175730.1-India with a similarity of 41%. In case of *gD* gene, the greatest sequence similarity is also determined between the sequences of this gene in Iran with AY690484.1-Egypt with a similarity of 91.3% and the least – with AF078730.1-Sweden with a similarity of 70.8%.

Classification of Iranian IBR virus isolates using sequence alignment analysis and construction of the phylogenetic tree of *gB* and *gD* genes revealed that they fell into two closely related clusters that had 6.6–14.5% variability for *gB* gene and 1.1–13.3% variability for the *gD* gene (Fig. 1 and 2).

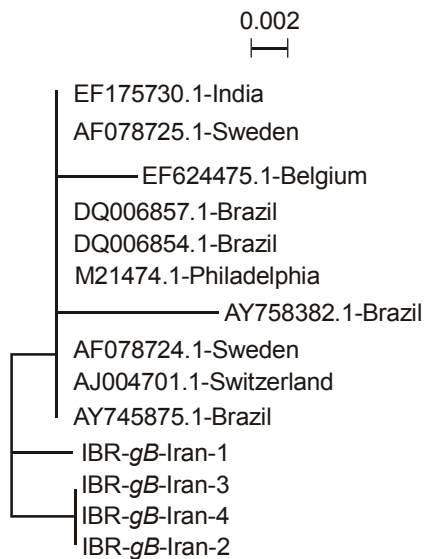
DISCUSSION

Among BHV-1 structure genes, those coding for glycoproteins *gB*, *gC* and *gD* are considered as major and relatively protected genes. Today, most molecular

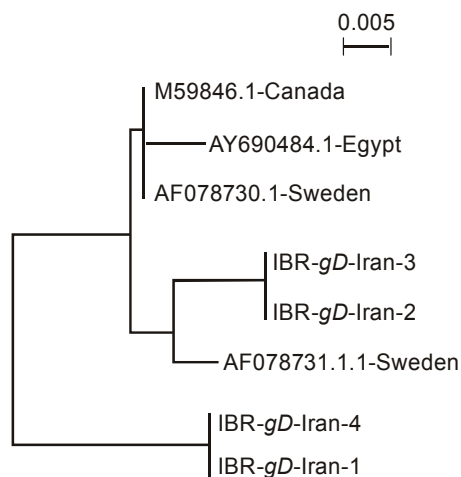
**Table 3.** Sequence identity matrix of partial *gD* gene of Iranian IBR virus isolates in comparison with 4 known reference sequences

Seq <i>gD</i> IBR	Iran 1	Iran 4	Ref 1	Ref 2	Iran 2	Iran 3	Ref 3	Ref 4
Iran 1		0.975	0.723	0.716	0.885	0.875	0.878	0.868
Iran 4	0.975		0.741	0.733	0.876	0.867	0.898	0.882
Ref 1	0.723	0.741		0.988	0.715	0.708	0.733	0.717
Ref 2	0.716	0.733	0.988		0.717	0.709	0.726	0.71
Iran 2	0.885	0.876	0.715	0.717		0.989	0.913	0.895
Iran 3	0.875	0.867	0.708	0.709	0.989		0.903	0.886
Ref 3	0.878	0.898	0.733	0.726	0.913	0.903		0.946
Ref 4	0.868	0.882	0.717	0.710	0.895	0.886	0.946	

Legend: Ref 1= AF078730.1-Sweden; Ref 2= AF078731.1-Sweden; Ref 3= AY690484.1-Egypt; Ref 4= M59846.1-Canada.



**Fig. 1.** Dendrogramme based on sequence alignment analysis of 4 Iranian isolates and 10 of the reference isolates from other regions of the world for *gB* gene of IBR virus.



**Fig. 2.** Dendrogramme based on sequence alignment analysis of 4 Iranian isolates and 4 of the reference isolates from other regions of the world for *gD* gene of IBR virus.

biological diagnostic methods are based on detection of these genes (Yan *et al.*, 2008). The *gB* gene plays a main role in

BHV-1 penetration into a host cell and it is the major viral antigen inducing a protective immune response in naturally infected hosts. The *gB* antibodies are extensively used in most diagnostic serum tests for BHV-1 infections with despite the potential cross reaction with other herpesviruses (Gao *et al.*, 1994; Kramps *et al.*, 1994; McGeoch & Cook, 1994; Ros & Belak, 1999; 2002).

On the other hand, *gB*, *gC* and *gD* genes play main roles in genetic studies for determination of the phylogenic relationship among different herpesviruses and today, nucleotide sequences of these genes have been identified in many herpes viruses.

In the study of Ros & Belak (1999), conducted to determine the genetic relationship among different alpha herpesviruses, a relationship of 87.2% to 99.6% was observed between nucleotide sequence of *gB* in five alpha herpesviruses: BHV-1, BHV-5, caprine herpesvirus1 (CapHV-1), cervine herpesvirus 1 (CerHV-1) and rangiferine herpesvirus 1 (RanHV-1) while the phylogenetic relationship of *gD* gene in these viruses appeared to be equivalent to 71.3% to 98.9%.

In another study (Ros & Belak, 2002), aimed at detection of genetic relationship of the *gB* gene in ruminant alpha herpesviruses, it was reported that the greatest relationship of *gB* gene exists in BHV-1 with BHV-5 and buffalo herpesvirus 1 (BuHV-1) with a sequence similarity of 91.9% and the least relationship is between BHV-1 and CapHV-1 with a relationship of 77.3% .

The present study was conducted for the first time in Iran to detect *gB* and *gD* genes in IBR viral strains isolated from bovine respiratory infections to determine nucleotide sequences of these genes and to compare obtained sequences with those from other countries. In this research, the

fragments 671 bp (corresponding to *gB*) and 1002 bp (corresponding to *gD*) isolated from four IBR viral strains in Iran were amplified and sequenced in PCR system.

The presence of high genetic variability of IBR virus in Iran with other countries may be associated with the transmission of cattle as well as biological products. The main origin of the IBR virus is the region of Colorado in America and it was spread from America to other countries. As the first isolation of the IBR virus in Iran was performed from cows and biological materials mostly imported from European and American countries, it seems that the IBR virus encountered in Iran is of the same origin and has been involved in highly genetic variability during the time of its adaptation to Iranian bovine strains. For the same reason, most determined sequences of *gB* and *gD* genes of this virus in Iran have been arranged in a separate phylum in the phylogenetic tree. This view could be more definitely supported with evidence from determination of the complete nucleotide sequence of this virus in Iran and its comparison to that of other countries.

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