Phylogenetic study on the 5'-untranslated region of bovine viral diarrhoea virus isolates from Iran

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Summary
Bovine viral diarrhoea virus is a pathogen of bovids associated with reproduction system, causing in infected animals a range of ailments, from abortion to congenital defects. In this article, the nucleotide structure of the 5'-untranslated region (5-UTR) from 7 Iranian bovine diarrhoea virus (BVDV) isolates was characterized and subjected to comparative analysis against a panel of BVDV isolates from different sources. To this end, a 288 bp-long stretch of the internal ribosome entry site was amplified by RT-PCR. The PCR products subsequently cloned into PTZ57T vector and sequenced using T7 promoter primers. This resulted in detection of 3 new point mutations G→A and G→T in 2 isolates. When these findings were phylogenetically assessed, all the examined Iranian isolates were deemed to belong to the type1 of BVDV. Besides, 2 subtypes were identified among these isolates. In group A, a high level of similarity (99.2%) between Iranian isolates with a cytopathic Australian strain of BVDV-1c was detected; while in group B, the 4 Iranian isolates proved to be very similar to NADL-like BVDV-1a strains. We believe that the surprisingly high level of similarity between group A Iranian isolates and their corresponding Australian strain is likely to be an indication of a shared common ancestor. If correct, the most likely explanation of this observation is the introduction of such strains from Australia to Iran, possibly through exportation of infected live animals or animal productions (e.g. semen and meat) at some points in the past. Nevertheless, this hypothesis remains to be proved as further epidemiological work at genomic level is required to understand population of BVDV in Iran.

Studio filogenetico della struttura genomica di 5'-untranslated region di ceppi del virus della diarrea virale bovina in Iran

Parole chiave
5'-UTR,
Esportazione,
Filogenetica,
Genetica di popolazione,
Iran,
Sperma,
Virus della diarrea virale bovina (BVDV).

Riassunto
Il virus della diarrea virale bovina (BVDV) è un agente patogeno dei bovini associato alla riproduzione. Negli animali infetti si osservano aborto e difetti congeniti. Questo studio ha riguardato la caratterizzazione della struttura nucleotidica di 5'-untranslated region (5'-UTR) di 7 isolati iraniani di DVBV e l'analisi comparativa degli stessi con altri isolati di BVDV. A questo scopo, una porzione di 288 bp della parte interna del ribosoma è stata amplificata mediante RT-PCR. I prodotti ottenuti sono stati successivamente clonati in un vettore PTZ57T e quindi sequenziati usando T7 promoter primer. Questo processo ha portato all'identificazione di 3 nuovi punti di mutazione G→A and G→T in 2 ceppi. L'analisi filogenetica dei risultati ha mostrato l'appartenenza di tutti i ceppi di BVDV iraniani al tipo 1. Tra questi, sono stati identificati 2 sottotipi, gruppo A e gruppo B. Gli isolati iraniani appartenenti al gruppo A hanno mostrato un'alta similarità (99,2%) con il ceppo australiano citopatico di BVDV-1c. Nel gruppo B, 4 isolati iraniani sono risultati molto simili ai ceppi NADL e BVDV-1a. L'alta similarità degli isolati iraniani appartenenti al gruppo A con i ceppi australiani è, probabilmente, indicativa dell'origine comune dei due ceppi. In questo caso, è plausibile ipotizzare che ceppi australiani del virus siano stati introdotti in Iran con l'importazione di animali o prodotti come carne e sperma. Quest'ipotesi, ancora da confermare, richiede ulteriori studi epidemiologici e genetici allo scopo di acquisire una comprensione esaustiva della popolazione genetica del BVDV in Iran.

Introduction

Bovine viral diarrhoea virus (BVDV) is a pathogen of cattle associated with reproductive defects (Baker 1990). This virus is a *Pestivirus* carrying a positive-stranded RNA and it belongs to family *Faviviridae* (Poole *et al.* 1995). To these days, 3 types of BVDV have been identified, namely: BVDV-1, BVDV-2 and HoBi-like BVDV-3. Further, 2 biotypes of BVDV including cytopathic (cp) and non-cytopathic (ncp) types have been characterized, their co-existence has also been frequently reported in *in vitro* observations (Moennig and Plagemann 1992), HoBi-like viruses BVDV-3 have been initially described in 2004 in Europe, Brazil and Australia (Peletto *et al.* 2012, Schirrmeier *et al.* 2004, Stalder *et al.* 2005, Xia *et al.* 2011, Xia *et al.* 2013). The BVDV genome is about 12 kb long with 2 untranslated regions (UTRs) at the 5' and 3' ends and a single open reading frame (Meyers and Thiel 1996). The 5'UTR fragment is known to be the most conserved region of the virus genome (De Moerlooze *et al.* 1993). It has a highly structured internal ribosome entry site (IRES) (Poole *et al.* 1995, Chon *et al.* 1998, Le *et al.* 1998), which is involved in the regulation of replication and gene expression (Becher *et al.* 2000, Yu *et al.* 2000). A number of virulence markers have been characterized in 5'UTR region of BVDV genome (Topliff and Kelling 1998). There are a number of convincing evidences to implicate the 5'UTR in tropism and pathogenesis of both picornaviruses and hepaciviruses (Funkhouser *et al.* 1999, Lerat *et al.* 2000, Nakajima *et al.* 1996). The 5'UTR is a frequently-used locus in taxonomical and epidemiological studies (Baule *et al.* 1997, Harasawa and Giangaspero 1998, Hofmann *et al.* 1994, Pringle 1999, Sakoda *et al.* 1999). This article describes the genotyping and phylogenetic analysis of BVDV isolates from Iran, with a focus on genomic structure of the virus 5'UTR region.

Materials and methods

Virus isolation from clinical specimens

Blood specimens from 7 Holstein-Friesian calves bearing symptoms to arouse clinical suspicion of BVDV infection (high fever of 41-42 °C along with dysentery) were collected from Tehran province. All specimens were processed and cultured in MDBK cell line (Razi institute, Iran) as described elsewhere (Becher *et al.* 2000). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, San Francisco, California, USA) supplemented with 10% FCS serum (SIGMA-ALDRICH, Munich, Germany), 100 µg/m streptomycin and 100 U/ml penicillin, 5% CO_2 at 37 °C for 72 hours.

RNA extraction

Viral RNA was extracted from transfected and non-infected cells using an RNX reagent (Cinnagen, Iran). In brief, cells were centrifuged at 85,000×g at 4 °C for 2 hours. They were then re-suspended in 200 ml of PBS and 400 ml of RNX reagent was subsequently added. This cocktail was mixed throughly and incubated at room temperature for 5 minutes. Extraction was done with 0.2 ml chloroform/isoamylalcohol (24:1). The total RNA, in the aqueous solution, was precipitated by adding an equal volume of isopropanol. This mixture was subsequently centrifuged at 10,000×g for 20 minutes. The RNA pellet was washed by 75% ethanol and re-dissolved in 20 ml of RNase-free water.

RT-PCR amplification of 5'UTR fragment

A conventional RT-PCR, using universal pestiviruses BVF (5'-cat gcc cat agt agg act agc-3') and BVR (5'- tca act cca tgt gcc atg tac-3') primers, was conducted to amplify a 288 bp-long fragment of internal ribosome entry site of 5'UTR locus of BVDV. At the second step, 2 pairs of primers the

Table I. *Accession numbers, types and origins of 5'UTR sequences of BVDVs used in alignment.*

Accession no.	Type/Origin
AJ133739	NADL Non-cytopathic
AY763004	VR991 Australia
AJ312930	Ireland
AJ312931	Ireland
U65031	Sweden -Ovine BVDV1
AB078952	Japan
D50826	Japan
Z79781	Germany
Z79780	Germany
L32878	Canada - strain C1
AB003621	BVDV-2 Japan
AB003622	BVDV-2 Japan
AB003620	BVDV-2 Japan
U18059	BVDV-2 USA
U97427	M139B/91-South Africa
AB014339	BVDV-1 Italy
U97426	Southern Africa
U97428	M140B Southern Africa
U97429	M1515 Southern Africa
JN703311.1	BVDV-3 Italy 280/11-A
KC544256.1	BVDV-3 Brazil LPV-WR/BR11
DQ897641.1	BVDV-3 Thailand khonkaen
HQ403056.1	BVDV-3 Australia

optimized 50 ml RT-PCR mixture contained 10 ml of 5X reaction buffer, 4 ml of dNTPs (2.5 mM each), 1 ml of AMV enzyme (Titan one tube RT-PCR system kit, Roche Diagnostic, Mannheim, Germany), 1 ml (10 rmol) of each primer, 4 ml of RNA template, 2.5 ml of DTT, 3 ml of $MgCl_2$ (25mM), and 23.5 ml of PCR water. RT-PCR conditions comprised a single cycle of 42 °C for 30 minutes, followed by 94 °C for 3 minutes, plus 30 cycles of 94 °C for 30 seconds, 52 °C for 30 seconds, and 72 °C for 40 seconds, followed by a final extention step of 72 °C for 5 minutes. For negative control, DNA templates obtained from non-infected cell culture were used accordingly. In order to identify type 1 and type 2 of BVDV, a second PCR was conducted using BVDV1f (5'-ggtagc aac agt ggt gag-3') and BVDV1r (5'-gta gca ata cag tgg gcc-3'), specific for type 1 along with BVDV2f (5'-act agc ggt agc agt gag-3') and BVDV2r specific for type 2. In this PCR protocol, a typical BVDV type1 is expected to produce a 288 bp-long amplicon; while amplification of a BVDV type 2 produces a 225 bp-long product.

Cloning and sequencing of PCR products

For ligation, the reaction contained 0.165µg of plasmid pTZ57T, 100 Ng of purified PCR fragment, 3 µl 10X ligation buffer, 1 µl PEG 4000, 5 units of T4 DNA ligase and deionized water to make up the final volume of 30 µl. The ligation reactions were incubated at 22 °C for 16 hours and transformed into XL1-blue cells. The white colony selected by LcZ genetically marker according to the method of Sambrook. The recombinant plasmid was purified by purification kit (Roche Diagnostic, Mannheim, Germany). For sequencing of the PCR products, the dideoxy method was employed, the sequencing itself was conducted by the collaborating laboratory, MWG (Ebersberg, Germany) using T7 promoter primer in both the forward and reverse directions. Compilation and analysis of obtained sequences was performed using the MegAlign program (DNASTAR Inc., Madison, USA).

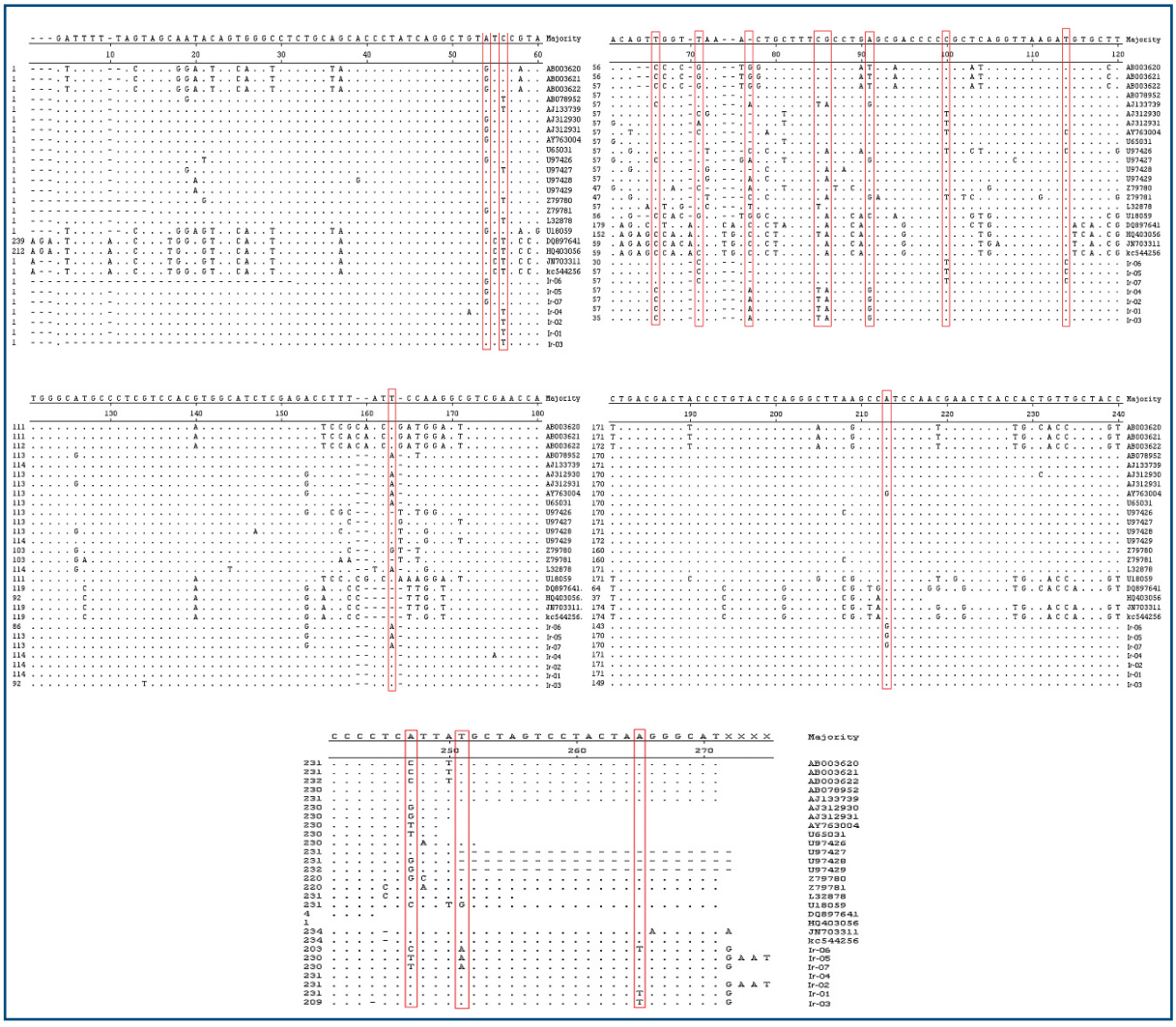

Figure 1. *Multiple alignment of different types of BVDV and Iranian isolates based on 5'UTR sequences.*

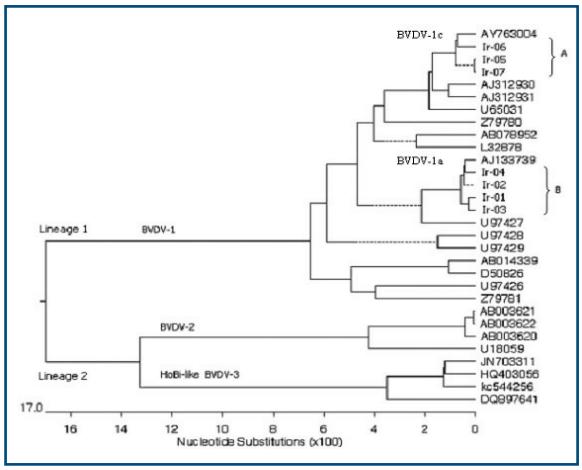

Figure 2. *Phylogenetic tree designed by MegAlign software based on 5'UTR sequences of BVDV.*

Phylogenetic analysis

For multiple alignment of the obtained sequences Clustal V method was used. This was conducted by MegAlign program. Similarly, calculation of distance matrices as well as generation of the phylogenetic tree was performed by the same software, where the neighborhood joining method was endorsed for the analysis of both the distance and alignment data.

Results

Amplification of BVDV-1- specific locus of 5′ UTR using BVF1 and BVR1 primers was successful for all of the blood specimens. No amplicon was observed in non-infected cell cultures. Multiple alignment of sequences from 7 Iranian studied isolates along with 23 more strains (Table I) from around the world resulted in detection of 13 Specific Nucleotide Patterns (SNP) in 5' UTR region of BVDV isolates subtype A_{10}, T_{17}, $CCC_{55\text{-}59}$, A_{62}, A_{70}, Y_{74}, R_{75}, T_{80}, G_{95}, W_{115}, A_{117}. Nine specific nucleotide patterns in all sequences of BVDV-2 at nucleotide positions T_{36}, A_{58}, G_{71}, T_{76}, A_{94}, A_{169}, R_{205}, T_{219} and T_{250} were identified (Figure 1). Surprisingly, at nucleotide position 213 a unique SNP (G_{213}) was characterized in Iranian isolates belonged to group A and an Australian-VR991 isolate (Figure 2). Similarly, an identical SNP was identified at each of the nucleotide positions 54, 71, 100 and 114, mutually shared by group A Iranian isolates and the Australian-VR991 isolate (Figure 2).

Four Iranian isolates (Ir01, Ir02, Ir03 and Ir04) hold 5 specific SNPs including T_{95}, C_{105}, A_{113}, $TA_{121\text{-}122}$, G_{127} (Figure 1). The other Iranian isolates of Ir05, Ir06 and Ir07, represented 9 SNPs at positions G_{93}, C_{109}, T_{136}, C_{150}, G_{189}, A_{199}, G_{249}, $T\backslash C_{283}$, A_{287} (Figure 1), all these SNPs also existed in a cytopathic Australian and in 2 Irish isolates. Six of the 9 SNPs, C_{109}, T_{136}, C_{150}, G_{189}, A_{199}, G_{249} were identically shared by both Iranian subtype A and Australian isolates. A comparative SNP analysis of Iranian isolates with their matching isolates in GenBank, resulted in detection of 3 novel polymorphism in 2 of the Iranian isolates. The isolate carried 2 novel points mutation (G→A) at nucleotide positions 52 and 175 (Figure 1). Moreover, in isolate Ir03, another G→T at nucleotide position 134 (Figure 1).

Phylogenetic analysis

The generated phylogram based on nucleotide structure of the 5' untranslated region of BVDVs classified all the isolates into 2 lineages (Figure 2): BVDV 1 isolates stood alone in a single lineage; while both BVDV-2 and HoBi-like BVDV-3 isolates were co-located in another lineage. The largest observed genomic divergence level was as high as 17% detected among the BVDV-1, BVDV-2 and BVDV-3 HoBi like isolates. The study panel included 30 isolates, 7 Iranian and 23 from around the world. All the Iranians isolates were classified into lineage 1 under 2 groups: group A holding 3 Iranian isolates (Ir-06, Ir-05 and Ir-07) and a single Australian BVDV-2 isolate (AY763004), the remaining Iranian isolates (IR-01, Ir-02, Ir-03 and Ir-04) along with a Japanese NADL-non cytopathic isolate (AJ133739) were gathered in group B. No lineage 2 Iranian isolate was identified.

Discussion

This work pioneers in population genetic study of BVDV in Iran, BVDV infected bulls have the potential to transmit the infection through breeding and also shed the virus through the semen. The very high genomic similarity observed between Iranian and Australian BVDV isolates might be an indication of epidemiological link between the 2 sets of isolates. Australia has been one of the major cattle and also bovine semen exporting countries to Iran over the last 30 years. We assume this cattle-farming activity might have played a role in introduction of BVDV to the Iranian cattle herd, but this needs to be investigated through more epidemiological studies. Besides, a high level of genomic similarity was detected between 2 Iranian isolates (Ir-03 and Ir-04) and a Japanese NADL strain as all the 3 carried 3 new points of mutations in their genomes.

BVDV has the capability to cross placenta and infect fetus. Persistent infected (PI) calves with BVDV are known to shed virus and infect susceptible animals (Pringle 1999). In those PI calves that mature, at some stages, a non cytopathotic infection, might spontaneously turn to a cytopathic infection

resulting in the onset of fatal mucosal disease (Brownlie *et al.* 1984, Meyers *et al.* 1991, Meyers and Thiel 1996).

The findings of the present work can help veterinary authorities to consider BVD as an emerging trans-boundary transmissible disease in the Iranian environment with practical measures required to control it.

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