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S7 gene Characterization of bluetongue viruses in Iran

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ABSTRACT

Bluetongue is an infectious disease that primarily affects sheep. But due to serious socioeconomic consequence of it outbreaks on the international trade it has been included in the OIE notifiable diseases (list A). During 2007-8, total number of 130 blood samples gathered from suspected sheep to bluetongue disease in seropositive region including Khuzestan, Kurdistan, Fars, Ilam and Qum provinces. Blue tongue viruses were diagnosed in some animals by RT-PCR and nested PCR, by targeting S7 segment. This genome segment was sequenced and analyzed in 11 samples as a conserved gene in BTV serogroup. The phylogenetic evaluation showed that there were two distinct clusters. One cluster was significantly near to East BTV strains from China and India and also was classified with BTV9/16 from Turkey. The second group was very similar to West BTV strains from US, Africa and Europe. This cluster was categorized with BTV4 from Turkey.

Keywords: Characterization, Bluetongue, S7 gene, PCR

INTRODUCTION

Bluetongue virus (BTV) is an economically important insect transmitted disease of wild and domestic ruminants. The disease has a global distribution of 35 ºS and 40 ºN; although in parts of North America and China it has been reported as far as 50 ºN. BTVs are transmitted between their vertebrate hosts by the bites of certain 'vector-competent' species of biting midge (*Culicoides* sp.). This virus is the type spices of Orbivirus within the family *Reoviridae*. Twenty-four immunologically distinct serotypes of the virus have been determined worldwide (Mellor, 1990).

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BTV is a small (90 nm) icosahedral virus. Its genomes compose of ten segments of linear dsRNA which code for ten distinct viral proteins. The virus particle is arranged as three concentric capsid shells surrounding the viral genome. The outer layer (outer capsid) is composed of two structural proteins, VP2 and VP5, which are principally involved in virus attachment and penetration of the host cell during initiation of infection. These are the most variable of the viral proteins and the specificity of their interactions with neutralizing antibodies (particularly VP2) that determines virus serotype (Roy *et al* 1990). The inner layers forming the core of the BTV capsid contains VP1, VP3, VP4, VP6 and VP7 that they are encoded by segments 1, 3, 4, 9 and 7 respectively. The core proteins and non structural proteins NS1, NS2, NS3/3A (encoded by

seg. 5, 8 and 10 respectively) are thought to be relatively conserved, and are antigenically cross-reactive between different strains and serotypes of BTV. However, cross-hybridization and sequencing studies have shown that these genome segments can vary in manner that reflects the geographical origin of the virus strains (Pritchard *et al* 2004). Efficiency of PCR in diagnosis of BTV in several parts of the world, verified this technique as a rapid, reliable and sensitive diagnosis methods. The highly specific and sensitive nature of RT-PCR based assay makes it ideal for rapid detection of BTV genome segments in clinical samples without requirement to virus isolation (Anthony *et al* 2007). BTV infection previously has been determined in several parts of Middle East (Shoorijeh *et al* 2008). The history of BTV detection in this part of the world is more than 50 years. Serological evidence indicates that the virus has been present in Iran, at least since 1974 (Afshar & Keyvanfar, 1974).To our knowledge, except Turkey and Israel, there has never been detection based on molecular technique and genotypic analysis of BTV genes in Middle East. The purpose of this study was to use a valid RT-PCR assay to detect any BTV isolates in clinical samples, especially blood specimen. Then we tried to evaluate the genotypic variation of this gene among PCR positive samples and compared them with BTV strains that were isolated in other parts of the world.

MATERIALS AND METHODS

Clinical samples. During 2007-8, total number of 130 EDTA blood samples gathered from the affected sheep belong to seropositive regions in five provinces, including; Khuzestan, Kurdistan, Fars, Ilam and Qum.

Extraction of viral RNA. The dsRNA extractions were carried out by using the viral RNA Mini kit (QIAamp® viral RNA Mini Kit, cat. no. 52906) from whole blood samples. The extracted RNA was denatured by incubation them for 5 min in 95ºC, and cooling to 0 ºC.

Oligonucleotide primers. Two pairs of primers: (SZ1: 5'-GTAAAAATCTATAGAGATG-3'; SZ_2: 5'-GTAAGTGTAATCTAAGAGA-3') and (SA_1: 5'-GTTAAAAAATCGTTCAAGATG-3'; SA2: 5'-GTAAGTTTAAATCGCAAGACG-3') which amplify full length of BTV serogroup S7 gene (1156 bp), were used (Anthony *et al* 2007). For nested PCR, internal primers (IntS7F: 5'-ACAACTGATGCTGCGAATGA-3'; IntS7R: 5'-AACCCACACCCGTGCTAAGTGG-3' was applied (Anthony *et al* 2004). The second primer set amplified internal part of S7 segment in length of 770 bp. All Oligonucleotide primers were synthesized commercially (Cinnagen Co., Iran).

One step RT-PCR. The One step RT-PCR kit (QIAGEN® OneStep RT-PCR Kit cat. no. 210210) was used for detection of S7 BTV gene in blood samples. The master mix was made as fallows: 10 µl of 5x Qiagen RT-PCR buffer, 2 µl dNTPs mixture (0.2 mM each), 0.5 µl (20 pmol) of each of four primers (SZ1, SZ2, SA1, SA2), 2 µl Qiagen Enzyme Mix, 28 µl of RNase free water. Then 6 µl of denatured RNA added to master mix. In RT-PCR the RNA initially reverse-transcribed at 45 ºC for 30 min. Then fallowed by a step at 95 ºC for 15 min, to simultaneously activation of DNA polymerase and inactivation of reverse transcriptase. Forty amplification cycles were preformed at 95 ºC for 1 min, 45 ºC for 1 min and 72 ºC for 2 min. The PCR cycles terminated by final extension step at 72 ºC for 10 min.

Nested PCR. PCR products of first amplification (RT-PCR) were used as template in nested PCR. The mixture of master mix contained, 10x PCR buffer 5 µl, dNTPs 1 µl (10 mM), Mgcl2 1 µl (50mM), each of primers (IntS7F and IntS7R) 1 µl (20pmol), Taq polymerase (2.5U) 0.5 µl and RNase free water 35 µl, at the end 5 µl of template was added to the reaction. The thermal cycler (Mastercycler personal, Eppendorf) was set to

amplify the nested fragment as fallow: first step was 95 ºC for 1 min, then 30 cycles were preformed at 95 ºC for 1 min, 59 ºC for 1 min and 72 ºC for 1 min. The reaction was stopped by extension at 72 ºC for 10 min.

Analysis of PCR products. All PCR products were separated by 1.2% agarose gel electrophoresis, and stained for 20 min in ethidium bromide (1 µg/ml). The gels were analysed using Gel Documentation System (Bio Doc-It Imaging system).

PCR product sequencing. Nested PCR products of S7 seg. from positive samples and reference strain BTV1 (RSAvvvv/01, which was received from Institution of Animal Health, Pirbright, UK and used as positive control) were sequenced. At first, the amplification products were purified from agarose gel (High pure PCR product purification Kit, Roche) and then sent for sequencing to MWG (Germany). Both strands of each sample were sequenced by forward and reverse primers.

Computer analysis of the sequences. All sequences were subjected to multiple sequence alignments and Phylogenetic analysis using the Clustal W (Thompson *et al* 1994). Sequence identity matrix was calculated by BioEdit program (BioEdit Sequence Alignment Editor Copywrite® 1997-2007 Tom Hall). The resulting dendrogram was viewed and edited by Tree View (1.6.6) software.

RESULTS

In this study among the suspected sheep to bluetongue disease, eleven animals detected positive. PCR products of seg-S7 (770bp) from these samples and BTV1 (RSA vvvv/01) were sequenced and evaluated (Table 1). The sequence data of samples were compared with each other and BTV strains that were registered in GenBank. After blasting, the viruses that showed maximum identity with the samples (Max Iden. >92%, E value=0), were chosen.

Table 1. The origin of samples that were used for seg-7 sequence analysis.

Origin(Province)	Number of Samples	Name
Qum	3	GO75,GO76,GO128
Ilam	3	E9,E84,E90
Kurdistan	1	KO215
Fars	2	SH30,SH31
Khuzestan	2	KH6,KH7

BTV strains that used for nucleotide sequence comparison were listed in table 2.The alignment of sequences showed that they can be segregated into two distinct clusters (referred as Group I and II); in each group more than 84% overall nucleotide similarity was determined. However between these two groups 71-78% homogeneity of S7 gene can be seen. The result of sequence identity evaluation between detected viruses and BTV strains from GeneBank is shown in Table 2. Group I consisted of four samples which they had genetically closely related to each other (more than 95%).

Table 2. The result of sequence identity analysis between Group I & II and other BTV strains from GeneBank.

BTV strains from Genebank		**East topotype**	**West topotype**
		BTV3-CHI BTV16-CHI BTV?- IND BTV 9- IND*	**BTV?-USA/ BTV?-USA BTV1-S.Afr/ BTV1-POR BTV4/COR/ GRE/GRE BTV8-NET***
Group I	**SH30,SH31 KH6,KH7**	**84-92.%**	**72-78.%**
Group II	**GO75,GO76 ,GO128 E9,E84,E90 KO215 BTV1 (RSA vvvv/01)**	**67-81.%**	**82-95.%**

**** Accession numbers***

BTV3 CHI - AF172827.1 BTV16 CHI - AF172831.1 BTV9 IND - DQ399836.1
BTV? IND - AM261981.1 BTV? USA - AF188669.1 BTV? USA - AF188670.1
BTV1 S.Afr - AY776331.1 BTV1 POR - EU498675.1 BTV1 COR – AY839949.1
BTV4 GRE – AY841352.1 BTV4 GRE – AY841351.1 BTV8 NET – AM498057.2

The viruses in Group I were co-clustered with Asian strains from China (BTV3-China, BTV16-China) and India (BTV?-India, BTV9-India). Also they had 99-95% sequence similarity with BTV9 and BTV16

from Turkey, but they showed 72-74% identity with BTV4 Turkish strain. The homogeneity of S7 between this group and BTV1 (RSAvvvv/01) was determined 75-78%. Group II, consisted of seven samples beside of BTV1 (RSA vvvv/01). The members of this cluster showed 84-99% similarity with each other. This group co-clustered with American (BTV?-502172-USA, BTV?-600558-USA), African (BTV1-S. Africa, BTV1-RSAvvvv/01) and some European (BTV4-Corsica, BTV4-Greece, BTV8-Netherlands, BTV1-Portugal) strains. They had 71-77% identity with BTV9/16-Turkey but 82-87% with BTV4-Turkey.

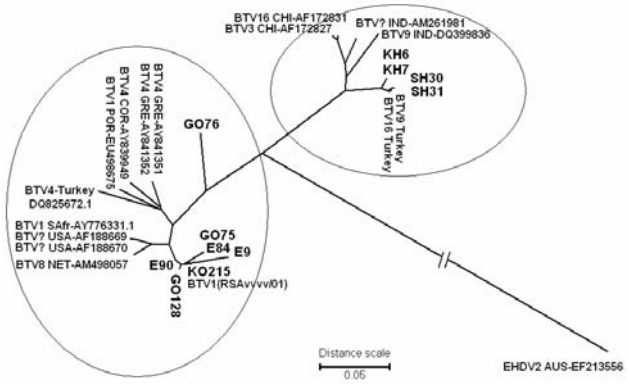

Figure 1. Phylogram of the S7 gene of Bluetongue virus in Iran. The phylogenetic tree generated by neighbor-joining analysis of 770bp of the S7 gene (1000 bootstrap replicates). The figure shows relationships between BTV in some parts of Iran with some East and West BTV strains. The GenBank accession numbers of BTV strains are mentioned in the figure. The accession numbers of BTV9/16-Turkey for S7 segment not available and they are used by permission of Dr P.P.C Mertens from IAH, Pirbright Laboratory.

The similarity of this group with BTV1 (RSA vvvv/01) was determined 96-99%. The S7 sequence identity between Groups I & II and EHDV2, as outgroup of BTV spices, were determined 56-57% and 53-55% respectively. The result of S7 phylogenetic analysis of positive samples which were detected during this investigation was depicted in Figure 1.

DISCUSSION

Molecular technique has a valuable and exclusive application comparing to the other diagnostic methods. For example, it provids the opportunity to find the origin of BTV in outbreaks and study about genetic variation of this virus (White *et al* 2006). It has been suggested that the strains of BTV classified as "topotype", in which the sequence of a conserved gene is used to assign a virus isolate to a geographical region, regardless of its serotype (Bonneau *et al* 1999). Previous studies showed that East and West topotype was a specific character for majority of genome segments, specially conserved genes, of BTV (Gould & Pritchard 1991, Mann *et al* 2008). The ability to differentiate isolates, on the basis of genome sequence, between geographical origins dose not confined to BTV and has been shown for other Orbiviruses, like EHDV (Gould & Pritchard 1991). Wilson *et al* (2000) compared genetic diversity of S7 segment among isolates from US, Caribbean Basin and Central America (west group of BTV) and found several distinct clads. In this study, the source of BTV2, BTV3 and BTV17 was presented. BTV2-OnaB showed 99.8% identity with BTV2-S.Africa, but only 79.4% with BTV11-US. BTV3 strains which circulated in Caribbean Basin and Central America had nearly complete identical S7 gene with BTV3-S.Africa. These data confirmed that the viruses in the related regions can be originated from South Africa. But BTV17 in Caribbean Basin and Central America probably came from US. Because they are phylogentically near to BTV17-US. During the last outbreak of bluetongue in Portugal (2004-2006), molecular investigation was performed to find the origin of detected viruses. Barros *et al* (2007) found BTV4 and BTV2 according S7, L2 and S10 genes, phylogentically related to Corsican/Italian BTV4 (99.3%) and South African BTV2 (99.9%), respectively. The authors concluded that these two Portuguese strains came from far separate origin because the low nucleotide identity (less than 75%).

Mann *et al* (2008) compared segment 7 of 41 BTV strains from all around of the world. They found S7 gene indicated significant level of variation. They found that the viruses can be segregated into six clads, three in western and the others in eastern topotype. The authors suggested that the source of the north European BTV8 (Netherlands 2006/04) came from west. Because genome segment 7, like the other genes, showed very close relationship with this category. For example BTV8 had 97% sequence identity with BTV1-Honduras and BTV1-S.Africa from western topotype.

In our study the high percentage of homology (Max Iden. >92%, E value=0) between the nucleotide sequence of S7 gene and published BTV strains in GenBank, confirmed the identity of detected agents as BTV. We have attempted to investigate the genetic variation of detected bluetongue virus in our country by sequencing of seg-7. We found noticeable nucleotide variation among the samples in this study (between 0-29 percent). This result was consistent with pervious studies that reported the variability of S7 gene in BTV up to 30% (Bonneau *et al* 2000, Mann *et al* 2008, Wilson *et al* 2000). According the epidemiology of BTV in the world, the situation of Middle East is unique. Because it is between east and west hemisphere, and may be invaded by BTV strains that are circulated in these two macro-environments. Also this area can play a important role for transferring BTV strains between these two ecosystems. Therefore, it can be anticipated that both east and west BTV strains find in this part of the world. In our study evaluation of recognized viruses divided them into two monophyletic groups. Group I had very homogeneity and closely related to BTV strains that were originated from eastern topotype (BTVs from India and China). However, Group II phylogentically categorized with western topotype (BTVs from US, South Africa and Europe). The homogeneity of the viruses in Group II is less than the previous group. For example GO76 show obvious different (10-16% distances) from the other members of this group. The reason not clearly defined, but extensive animal transportation in this part of the country, and reassortment ability of BTV may be explained it. Unfortunately, except Turkey, we couldn't find any BTV genome sequences in GenBank or articles from the other neighbor country of Iran. Comparison of S7 gene of detected viruses with Turkish strains (BTV4, BTV9, and BTV16) showed that Group I closely related with BTV9 and BTV16 but Group II is more near to BTV4. There are several reasons that can explain the similarity of BTV strains, as a trans-boundary virus, between these two countries. For example it can be referred to the presence of common long border between Iran and Turkey that facilitate the transportation of vertebrate and invertebrate host. Also similar ecosystem and/ vectors can support this hypothesis. It can be supposed that S7 gene of BTV9/16-Turkey and viruses in Group I probably have a same origin. The investigation showed that the first incursion of BTV9 (GRE1998/01) to Europe in 1998 was related to eastern strains from Indonesia and Australia. Also this strain closely related to BTV9 isolated in Greece, Bulgaria, Turkey, Bosnia, Kosovo and Serbia, it means that BTV9 in the eastern and central Mediterranean region came from this eastern source. Phylogenetic analysis confirmed, initial European strain of BTV16 (isolated in Greece during 1999) is from an eastern lineage and is very similar to strains of BTV16 from Turkey and strain which was originally derived from an outbreak in Pakistan (Mertens, 1998). In the previous study BTV4-Turkey was grouped with other European (Greece, Spain, Italy, Bulgaria and Corsica) and African strains (Morocco), this suggests that the BTV4 strain which invaded Europe and Eastern Mediterranean region since 1999 came from western BTV lineage. (Breard *et al* 2007, Mertens 1998). Co-clustering reference strain BTV1 (RSA/vvvv01) with Group II, which originated

from west (South Africa), supported the categorization of these viruses with west origin BTV strains. However some others investigator made evidence that invasion of BTV1 lineage to Europe could be from both east and west sources (Batten *et al* in press; Mertens, 1998, Monaco *et al* 2005). By assessment of the genetic diversity of BTV genes, lots of valuable information about epidemiology of this virus can be collected. In our study we concluded that probably there are both east and west BTV strains in Iran. Although beside of S7 segment, for better understanding about relationship of active BTV strains, it recommended to analyze nucleotide sequence of two other genome segments like S10 and L3.

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