Investigation of bluetongue virus in Kurdish sheep in Kurdistan province of Iran

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Bluetongue (BT) is a noncontagious disease caused by an Orbivirus of the Reoviridae family. The virus is transmitted by arthropods of the genus Culicoides and its distribution worldwide is restricted to the regions that contain competent vectors. This study was conducted to determine the seroprevalence and molecular serotypes of bluetongue virus (BTV) of Kurdish sheep in Kurdistan province during 2007-2008. The results showed widespread presence of the anti-BTV antibodies in sheep population because 70 samples (51.85%) were found positive by C-ELISA. The conserved S7 segment of the virus was detected in one collected blood sample by RT-PCR and nested-PCR. Analysis of PCR product of this sample demonstrated a new sequence with high similarity to the strains reported from USA, Africa, and Europe. This sequence is categorized with BTV4 from Turkey.

Key words: Bluetongue, S7 segment, C-ELISA, PCR, Iran, seroprevalence.

INTRODUCTION

Bluetongue (BT) is an insect-borne viral disease to which all species of ruminants are susceptible. It occurs mostly during periods of high temperature and rainfall, and usually disappears with the first frost or severe cold weather (OIE, 2006). Hematophagous Culicoides insects are biological vectors that transmit BTV from infected to susceptible ruminants, thus the global distribution of BTV coincides with the distribution of competent Culicoides insect vectors and appropriate climatic conditions (Jafari-Shoorijeh et al., 2010; Lundervold et al., 2003). However, the distribution of specific insect vectors and different BTV serotypes differs remarkably throughout the world, so specific vectors exist with specific constellations of BTV serotypes and topotypes in relatively distinct global ecosystems (Balasuriya et al., 2008). There are 24 distinct BTV serotypes currently recognized, and recently described Toggenburg orbivirus (TOV) is proposed to be a 25th serotype (Hofmann et al., 2008) and, complete genome characterization of a novel 26th BTV serotype from Kuwait (Maan et al., 2011). Clinical signs in sheep may include fever, depression, excessive salivation, nasal discharge (serous, muco-purulent or bloody), facial edema, hyperemia and ulceration of the oral mucosa, coronitis, lameness, muscle weakness and death (Maclachlan, 2011). The mortality rate and the severity of the clinical signs seem to vary with the breed and age of the animal infected (with the older age groups being more susceptible), the type and strain of the virus and certain rather ill-defined interactions with the environment. For example, animals that stressed or subjected to strong solar radiation are reputed to develop more severe clinical signs (Maclachlan, 2011). Other domesticated species, namely cattle and goats, usually suffer from in apparent infection, but these species, particularly cattle, serve as important reservoirs of the virus (MacLachlan et al., 1994).

Various techniques have been used to detect antibodies against BTV. These include agar gel immunodiffusion (AGID), haemagglutination inhibition
(HI), complement fixation (CF), and ELISA, which are serogroup specific and serum neutralization, which is serotype specific (OIE, 2008). Assays based on reverse transcription polymerase chain reactions (RT-PCR), can be used to detect BTV RNA in clinical samples (for example, blood or spleen) without virus isolation, and do not require standardized serological reagents (Aradaib et al., 2005). The majority of published primer sets target BTV genome segment 5 (Seg-5 coding for NS1), or genome segment 7 (Seg-7 coding for VP7) (Anthony et al., 2007; Aradaib et al., 2005). These genome segments are relatively conserved across the BTV virus-species, and sufficiently divergent between distinct *Orbiviruses* to remain BTV specific. Indeed, VP7 is the major BTV serogroup specific antigen (Gumm and Newman, 1982).

BTV has been reported in several Middle Eastern countries (Egypt, Jordan, Syria, Turkey, Cyprus, and Iraq) since 1951 (OIE, 2009). In 2008, Egypt reported the absence of BT, and Egypt is the only country in the region to have prohibited BTV vaccination (OIE, 2009). Iran reported outbreaks of BT in 2008, and Saudi Arabia reported infection without clinical signs, although the serotype(s) were not identified (OIE, 2009). Kurdistan province, with an area of 28203 square kilometers, is one of the western provinces of Iran, having more than 230 km of shared border with Iraq. Kurdistan lies between 45° 33′ 11″ and 51° 13′ 7″ eastern longitude and between 34° 24′ 16″ and 37° 52′ 12″ northern latitude; this is a reason to determine the situation of BTV in this part of country during 2007-2008.

**MATERIALS AND METHODS**

**Blood samples**

A total of 135 sheep blood samples were collected from various regions in Kurdistan. The whole blood and sera samples were used in the viral RNA extraction and serological assays respectively.

**Extraction of viral RNA**

Extractions of the dsRNA from whole blood samples were carried out using the viral RNA Mini kit (QiAmp®viral RNA Mini Kit, USA) according to manufacturer instruction.

**Oligonucleotide primers**

Two pairs of primers; (SZ1: 5'-GTTAAAAATCTATAGAGATG-3', SZ2: 5'-GTAAGTGAATCTAAAGAGA-3'), and (SA1: 5'-TTAAAAATCCTGTCAGATG-3'; SA2: 5'-GTAAGTTAATGCGCAAGAGC-3') which amplify full length of BTV serogroup S7 gene (1156bp), were used (Anthony et al., 2007) for nested PCR, internal primers (IntS7F: 5'-ACAACCTGATGCTGCGAATGTA-3'; intS7R: 5'-AACCCACACCGTGGTGTAGTG-3') were applied. The second primer set amplified internal part of S7 segment in length of 770bp. All Oligonucleotide primers synthesized commercially (Cinnagen Co., Iran).

**One step RT-PCR**

The One-step RT-PCR kit (QIAGEN® One-Step RT-PCR Kit, USA) used for detection of S7 BTV gene in the blood samples. The master mix was made as follows: 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 and 28 μl of RNase free water. Then 6 μl of denatured RNA added to the prepared master mix. In the RT-PCR, the extracted RNAs were initially reverse-transcribed at 45°C for 30 min, followed by a step at 95°C for 15 min. Forty amplification cycles were performed at 95°C for 1 min, 45°C for 1 min and 72°C for 2 min. The PCR cycles terminated by final extension step at 72°C for 10 min.

**Nested PCR**

PCR products of first amplification (RT-PCR) were used as template in the nested PCR. The mixture of master mix contained 5 μl of 10x PCR buffer, 1 μl dNTPs (10 mM), 1 μl MgCl₂ (50 mM), 1 μl (20 pmol) of IntS7F and IntS7R primers, 0.5 μl Taq polymerase (2.5 U), 35 μl RNase free water and 5 μl of template that were added to the reaction at the end. The thermal cycler (Master cycler personal, Eppendorf) was set to amplify the nested fragment as follows: first step was 95°C for 1 min, then 30 cycles were performed at 95°C for 1 min, 59°C for 1 min and 72°C for 1 min. The reaction was stopped by extension at 72°C 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 analyzed using Gel Documentation System (Bio Doc-It Imaging system, UK).

**PCR product sequencing**

Nested PCR products of S7 segment from positive samples and the reference strain; BTV1 (RSA vvvv/01) were prepared for sequencing. The BTV1 strain was received from Institution of Animal Health, Pirbright, UK and used as positive control. The amplified products were purified from agarose gel by high pure PCR product purification Kit, (Roche, USA) and sent for sequencing to MWG DNA Sequencing Services (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 Cluster W (Della-Porta et al. 1985). 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**

70 of 135 serum samples were positive for BTV-specific antibodies by C-ELISA (ID- Vet, Montpellier, France) test. Prevalence of BTV antibody was detected in Kurdistan (Table 1). The prevalence of BTV antibodies in sheep in
Table 1. Seroprevalence of bluetongue antibodies in Kurdish sheep from various regions of Kurdistan Province, Iran.

<table>
<thead>
<tr>
<th>Region</th>
<th>No. of serum sample</th>
<th>Positive</th>
<th>Seropositive rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center (Sanandaj)</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Northern (Divandareh, Saquez)</td>
<td>48</td>
<td>29</td>
<td>60.42</td>
</tr>
<tr>
<td>Southern (Kamyaran)</td>
<td>22</td>
<td>6</td>
<td>27.3</td>
</tr>
<tr>
<td>Western (Marivan)</td>
<td>22</td>
<td>9</td>
<td>40.9</td>
</tr>
<tr>
<td>Eastern (Bijar)</td>
<td>31</td>
<td>26</td>
<td>83.87</td>
</tr>
<tr>
<td>Total</td>
<td>135</td>
<td>70</td>
<td>51.85</td>
</tr>
</tbody>
</table>

Table 2. The result of sequence identity analysis between sample in Kurdistan (KO215) and other BTV strains from Genbank.

<table>
<thead>
<tr>
<th>Sample of Kurdistan</th>
<th>BTV strains from Genbank East topotype</th>
<th>West topotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>KO215</td>
<td>BTV7-USA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BTV3-CHI</td>
<td>BTV7-USA</td>
</tr>
<tr>
<td></td>
<td>BTV16-CHI</td>
<td>BTV1-S.Afr</td>
</tr>
<tr>
<td></td>
<td>BTV?-IDN</td>
<td>BTV1-PORT</td>
</tr>
<tr>
<td></td>
<td>BTV9-IND*</td>
<td>BTV4/CHI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BTV4/GRE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BTV8-NET*</td>
</tr>
<tr>
<td></td>
<td>67-81%</td>
<td>82-95%</td>
</tr>
</tbody>
</table>

*Accession numbers: BTV3 CHI - AF172827.1 BTV16 CHI - AF172831.1 BTV9 IND - DQ399383.1, BTV7 IND - AM261981.1 BTV2 USA - AF188669.1 BTV2 USA - AF188670.1 BTV1 S.Afr - AY776331.1 BTV1 POR - EU498675.1 BTV1 CHI - AY839949.1, BTV4 GRE - AY841352.1 BTV4 GRE - AY841351.1 BTV8 NET - AM498057.2.

Kurdistan Province was 51.85%. The highest infection was recorded in the Eastern district (83.87%). The minimum prevalence was determined in the Center District (zero percent). The relationship between prevalence the disease and cites was significant (p<0.05). In this study, one animal (KO215) was detected positive by PCR. PCR products of seg-S7 (770bp) from this sample and BTV1 (RSA vvvv/01) were sequenced and evaluated. The sequence data of KO215 compared with registered BTV strains in GenBank. After blasting, the viruses that showed maximum identity with KO215 (Max Iden. >92%, E value=0), were chosen. The result of sequence identity evaluation between detected sample and BTV strains from Gene bank shown in Table 2. This sequence co-clustered with American (BTV7-502172-USA, BTV7-609558-USA), African (BTV1-S. Africa, BTV1-RSA vvvv/01) and some European (BTV4-Corsica, BTV4-Greece, BTV8-Netherlands, BTV4-Portugal) strains. They had 71 to 77% identity with BTV9/16-Turkey but 82 to 87% with BTV4-Turkey. The similarity of KO215 sequence with BTV1 (RSA vvvv/01) was determined 96-99%. The S7 sequence identity between this sample and Epizootic hemorrhagic disease virus (EHDV), as out-group of BTV spices, was determined 56 to 57% and 53 to 55%, respectively (Figure 1).

DISCUSSION

Although BTV infection of ruminants is often subclinical or in apparent, infection also can lead to severe disease with mortality in susceptible animals (Maclachlan, 2011; Maclachlan et al., 2009). Insect vector transmission, prevalence of multiple serotypes, broad host range and non-availability of a suitable vaccine are playing a major role in establishing and causing regular outbreaks of BT in the Iran. Since there is no control over the prevalence of BTV serotypes and vector control is a difficult task, the best alternative is to develop a suitable vaccine to control the disease. In Iran where BTV is known to be present, seroprevalence of 34.3 to 74.4% in sheep, 2.13% in cattle, 100% in camel have been reported (Hasanpour et al., 2008; Jafari-Shoorijeh et al., 2010; Mahdavi et al., 2006; Mozaffari et al., 2012). The overall prevalence of the BTV antibodies in Kurdish sheep in this state was found to be 51.85%. However, this suggests that BTV is widespread and endemic in country. The highest prevalence of disease was in Eastern (Bijar) and Northern (Divandareh, Saquez) districts. This may be attributed to the presence of many insects in these areas. Climatic factors play an important role in the occurrence of BTV infection in animals and influence the size of vector populations and periods of their seasonal activity (Ward and Thurmond, 1995). Considering that no vaccination against BT is practiced in the Iran, the results of this serological survey would clearly indicate that BT infection occurs in sheep of the Iran as evidenced by the demonstration of specific antibodies in the sera of sheep (Mellor and Widmann, 2002).

However, the incidence rates 54.1, 21.4, 34.7, and 6.1% were reported from Saudi Arabia (Yousef et al., 2012), Kazakhstan (Lundervold et al., 2003), South Eastern Turkey (Gür, 2008), and Iraq (Hafez et al., 1978), respectively. Iran is immediately adjacent to the BT zone where the situation (existence of disease) is unstable (Afghanistan, Iraq, Pakistan and Turkey) (Mellor and Widmann, 2002). The agricultural economy in that area is based on pasture in extensive semi-arid rangeland; therefore, domestic ruminants come into contact when grazing (Mahdavi et al., 2006; Mellor and Widmann, 2002). Considering the seasonal movements of different animals, it is suggested that a risk-based approach
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 Kurdistan province 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.

should be adopted (Akhtar et al., 1997). Iran's strategic location in the southeast of Europe makes it an important potential source of BTV strains and serotypes that might incur into adjacent areas (Purse et al., 2005).

Although >1000 species of Culicoides are known worldwide, relatively few of these species have been incriminated as vectors of BTV (Meiswinkel et al., 2004). Species of vector insects that transmit BTV differ amongst regions, and are especially poorly characterized in the portions of Asia that are devoid of Culicoides imicola, the traditional African-Asian vector of BTV (Jafari-Shoorijeh et al., 2010; Maclachlan, 2010, 2011). None of the midges (Culicoides spp.) identified as vectors of BTV are known to exist in Iran, however other Culicoides spp. do exist there.

In this study genome segment 7 was chosen as a target gene for an RT-PCR assay because it codes for the major BTV species specific and immuno-dominant antigen. Also for confirmation of PCR positive sample, second amplification applied by nested PCR primer. This gene repeatedly used for topotypes of BTV isolate in the literature. It was previously reported that Chinese and Australian strains of BTV-15 could show up to 30% genetic divergence in Seg-7, from isolates of other BTV serotypes (Bonneau et al., 2000). Molecular technique has a valuable and exclusive application comparing to the other diagnostic methods. For example, it provides the opportunity to find the origin of BTV in outbreaks and study about genetic variation of this virus (White et al., 2006). It has 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 the majority of genome segments, specially conserved genes, of BTV (Gould and Pritchard, 1991). The ability to differentiate isolates, based on genome sequence, between geographical origins dose not confine to BTV and has been shown for other Orbiviruses, like Epizootic haemorrhagic disease virus (EHDV) (Bonneau et al., 1999). 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. During the last outbreak of BT in Portugal (2004-2006), molecular investigation performed to find the origin of
detected viruses. Barros et al. (2007) found BTV4 and BTV2 according S7, L2 and S10 genes, phylogenetically 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 of the low nucleotide identity (less than 75%). In this 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 attempted to investigate the genetic variation of detected BTV in our province by sequencing of seg-7.

According to 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 circulated in these two macro-environments. In addition, this area can play an important role for transferring BTV strains between these two ecosystems. Therefore, it can anticipate that both east and west BTV strains find in this part of the world. The reason not clearly defined, but extensive animal transportation in this part of the country, and resentment ability of BTV may be explained it.

Comparison of S7 gene of detected virus with Turkish strains (BTV4, BTV9, and BTV16) showed that this virus 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. It can refer to the presence of common long border between Iran and Turkey that facilitate the transportation of vertebrate and invertebrate host. Furthermore, similar ecosystem and vectors can support this idea. 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 (Bread et al., 2007).

As per our Knowledge, this is the first study to evaluate the prevalence of antibodies to BTV in Kurdish sheep in Kurdistan province of Iran. The results showed that a high incidence rate of BT antibodies has been detected in sheep in Iran that indicate serological evidence of exposure to infection was widely distributed in Iran. There are no restrictions on the movement of animals from one region to another within the country. Thus, outbreaks may also occur due to transportation of animals. Consequently, a well-defined control strategy for preventing and controlling the BTV may be based not only on vaccination plans and vector eradication but also restriction on the movement of animals from one region to another within the country.

The results support the conclusion that BTV was widespread in areas of Iran, suggest that it may be endemic, and need for further research. If BTV is endemic, it may cause only sporadic deaths, which could easily be attributed to their causes.

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