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Investigation of Bluetongue Virus in Ilam province, Iran

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Abstract: Bluetongue (BT) is a noncontagious disease caused by an Orbivirus of the family *Reoviridae*. Bluetongue virus (BTV) is transmitted by arthropods of the genus *Culicoides* and its distribution worldwide is restricted to regions that contain competent vectors. This study was to describe the distribution and seroprevalence of BTV infection of sheep in Ilam province of Iran by competitive ELISA, and BTV was diagnosed by RT-PCR and nested PCR, by targeting S7 segment. BTV infection was widespread in the province as 104 of 237 (43.88%) of the sheep sera evaluated contained antibodies to BTV. This genome segment was sequenced and analyzed in three samples as a conserved gene in BTV serogroup. The phylogenetic evaluation showed that I9, I84, I90 viruses were very similar to west BTV strains from US, Africa and Europe. These viruses were categorized with BTV4 from Turkey.
Keywords: Bluetongue, S7 gene, cELISA, PCR, Iran, Seroprevalence

INTRODUCTION

BT is an insect-borne viral disease of ruminants. Among domestic animals, clinical disease occurs most often in sheep, and can result in significant morbidity. Affected sheep may have erosions and ulcerations on the mucous membranes, dyspnea, lameness from muscle necrosis and inflammation of the coronary band. Some sheep may slough their hooves, and surviving animals can lose part or all of their wool. Some strains of the virus can result in mortality rates as high as 70% in highly susceptible sheep [1]. Transmission takes place via bite of certain species of *Culicoides* midges, which are biological vectors. It is not transmitted by direct or indirect contact between animals in the absence of the insects [2]. BTV is widely distributed around the world, particularly in warmer climates [3], although the disease is primarily associated with its introduction or at least introduction of a novel strain to a new geographical area [4]. BTV is a non-enveloped virus with a genome of approximately 19200 base pairs composed of ten linear segments of double-stranded RNA (dsRNA), containing 57% AU and 43% GC, with conserved 5_ and 3_ terminal sequences (GUUAAA at 5_, and ACUUAC at 3_ ends of the positive strand [5]. The 10 dsRNA segments are packaged within a triple layered icosahedral protein capsid (approximately 90 nm in diameter) [6,7]. The sub core consists of the 12 decamers of the VP3 protein (100 kDa), one centered on each of the fivefold axes of the icosahedral particle structure [8]. The 120 molecules of VP3 can be considered as a 'pseudo T = 2' icosaedral lattice, which houses the viral genome segments and three minor proteins involved in transcription and replication, namely the RNA-dependent RNA polymerase (VP1, 149 kDa), the RNA capping enzyme (VP4, 76 kDa) and the dsRNA helicase (VP6, 36 kDa) [9]. VP7 forms the outer layer of the transcriptionally active virus 'core' [10]. Non-structural proteins (NS1, NS2, NS3 and NS3A) probably participate in the control of BTV replication, maturation and export from the infected cell. Unlike most single stranded RNA (ssRNA) viruses, the Orbiviruses are genetically and antigenically stable throughout infection; point mutations do not appear to arise in vivo, at least at the high frequency noted with many non-segmented ssRNA viruses [11]. Due to its economic impact, BT is an Office International des Epizooties (OIE)-listed disease. Economic losses associated with BTV infection are caused directly through reductions in productivity and death and more importantly indirectly through trade losses due to animal movement restrictions, restrictions on the export of cattle semen [12]. Although cELISA can be used to detect BTV specific antibodies in serum samples [13], these antibodies are not generated until 7–10 days post-infection. The methods used to detect and serotype virus in blood can take several weeks, involving virus isolation (e.g. in embryonated chicken eggs), adaptation to cell culture and virus or serum neutralization tests (VNT or SNT). These assays also depend on the availability of highly characterized antigens and antibodies for the cELISA (to identify the virus species/serogroup), and reference antisera or reference strains for all 24 BTV serotypes for the neutralization assays (to identify virus serotype). Assays based on reverse transcription polymerase chain reactions (RT-PCR), can be used to detect BTV RNA in clinical samples (e.g. blood or spleen) without virus isolation and do not require standardized serological

reagents. However, many of the published RT-PCR based methods have not been fully evaluated for the detection of different BTV serotypes or topotype and some published methods will only detect certain BTV strains [14-16]. 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) [14,15]. Although these genome segments are relatively conserved across the BTV virus-species, they are sufficiently divergent between distinct Orbiviruses to remain BTV specific. Indeed, VP7 is the major BTV serogroup specific antigen [17,18]. NS1 is also highly conserved and Seg-5 is recommended as an RT-PCR target by the OIE [1].

METHODOLOGY

Ilam is located in the southwestern part of the Iran, bordering Khuzestan, Lurestan, and Kermanshah provinces and Iraq in the west with 425 kilometers of common border. Ilam province covers an area of 19,086 square kilometers and is among the warmer regions of Iran. However, the mountainous areas of north and north eastern Ilam are relatively cold. Average annual rainfall in the province is 578 mm. Absolute maximum temperatures is 38 °C in August and the minimum temperature is 0.4 °C in February. The areas selected for 237 EDTA sheep blood sample collection were seropositive regions in Ilam province, West Iran.

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'-TTAAAAAATCGTTCAAGATG-3'; SA2: 5'-GTAAGTTTAAATCGCAAGACG-3') which amplify full length of BTV serogroup S7 gene (1156 bp), were used [19]. For nested PCR, internal primers (IntS7F: 5'-ACAACTGATGCTGCGAATGA-3'; IntS7R: 5'-AACCCACACCCGTGCTAAGTGG-3') was applied [20]. 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 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, 28 µl of RNase free water. Then 6 µl of denatured RNA added to master mix. The RNA initially reverse transcribed at 45 ºC for 30 min in RT-PCR. Then, a step at 95 ºC for 15 min, to simultaneously activation of DNA polymerase and inactivation of reverse transcriptase. Forty amplification cycles were performed at 95 ºC for 1 min, 45 ºC for 1 min and 72 ºC for 2 min. The final extension step was 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 (Master cycler personal, Eppendorf) was set to amplify the nested fragment as fallow: first step was 95 ºC for 1 min, then 30 cycles were performed 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 (1µg/ml). The gels were analyzed using Gel Documentation System (Bio Doc-It Imaging system).

PCR product sequencing

Nested PCR products of S7 segment 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.

Competitive enzyme-linked immunosorbent assay

Sera that recorded complete or incomplete dot formation by dot ELISA were cross-checked, using a monoclonal antibody-based c-ELISA kit (ID-Vet. Innovative diagnostics 34070 Montpellier, France). The test was carried out as described in the protocol supplied by the manufacturers. Samples with PIs equal to or greater than 40% were considered to be negative, and those with PIs of less than 40% were taken as positive.

Computer analysis of the sequences

All sequences were subjected to multiple sequence alignments and phylogenetic analysis using the Cluster W [21]. 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 AND DISCUSSION

One hundred and four of 237 serum samples were positive for BTV-specific antibodies by c-ELISA (ID-Vet, Montpellier, 34070, France) test. A prevalence of BTV antibody was detected in 7 of districts of Ilam Province (Table1).

Table1: Seroprevalence of bluetongue antibodies in sheep from various regions of Ilam province, Iran

Regions	No of serum samples	No of positive	Seropositive Rate%
DarehShahr	52	22	42.30
Mehran	64	22	34.37
Ilam	21	18	85.71
Dehloran	43	16	37.20
Abdanan	17	7	41.17
Shirvan	21	10	47.61
Aivan	19	9	47.36
Total	237	104	43.88

The prevalence of BTV antibodies in sheep in Ilam province was 43.88%. The highest recorded in sheep from Ilam city, where 18 of 21 sheep (85.71%) were positive. The lowest prevalence was recorded among sheep from the Mehran city, where 22 of the 64 samples (34.37%) were positive. The prevalence values for the seven affected districts ranged from 34.37% to 85.71%.

BT virus is present in much of the American, Africa, southern Asia and northern Australia. While, the virus is occasionally present in some areas in the southern part of Europe, recent developments indicate that it may be extending its range northwards into areas of Europe that have never been affected before [2]. In an ideal world, it would be necessary to carry out ad hoc surveys to assess the test performance for each of these purposes. But it is not always a priority, especially during the management of an unexpected disease, although it is exactly in these particular cases that tests should be re-validated [1]. The objectives of the cELISA test used were both to confirm the BTV infected status of sheep in suspicious holdings in Ilam (diagnostic test) and to detect the incursion of BTV in West Iran (screening test). Thus, its use has been more and more often abandoned and replaced by ELISA tests which are rapid and easier to use, more sensitive and specific [22,23]. This study showed, for the first time, the prevalence of BT antibodies from Ilam province in the west of Iran. The seroprevalence of BT antibodies in sheep was 48.88%. The highest proportion of seropositive sheep came from Ilam city (85.71%), the second highest rate was from the Shirvan city (60.42%). 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 also influence the size of vector populations and periods of their seasonal activity [24]. Higher seropositivity rate 76.44% was detected in East-Azerbaijani province in the West-North of Iran [25]. However, the rate of BT antibodies in our study is close to some studies were reported of Pakistan, India, and Kurdistan province of Iran, 48.4, 45.7, and 51.85%, respectively [26-28]. Lower incidence rates 8.3%, 34.7% and 29.5% were reported of India, Northwestern Iran, and Southeastern Turkey, respectively [29-31]. Although BTV infection of sheep is clearly widespread in northwest Iran, the specific virus serotypes and vector insects that occur within the region remain uncharacterized, as they are in adjacent countries such as Kazakhstan [32]. Furthermore, BTV infection of sheep apparently is largely subclinical. Iran's strategic location to the southeast of Europe makes it an important potential source of BTV strains and serotypes that might incur into adjacent areas [2,33].

PCR products of seg-S7 (770bp) from these samples and BTV1 (RSA vvvv/01) was sequenced and evaluated. The sequence data of sample was compared with each other and BTV strains that were registered in GenBank. After blasting, the viruses that showed maximum identity with the sample (Max Iden. >92%, E value=0), was chosen. The result of sequence identity evaluation between detected virus and BTV strains from Genbank is shown in Table 2. This group (I9, I84, I90), consist of three 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. The similarity of this viruses (I9, I84, I90) with BTV1 (RSA vvvv/01) was determined 96-99%. The S7 sequence identity between this virus and EHDV2, as out group of BTV spices, were determined 56-57% and 53-55% respectively.

Table2. The result of sequence identity analysis between sample in Ilam (I9, I84, I90) and other BTV strains from GenBank

BTV strains from Gene bank	East topotype	West topotype
	BTV3-CHI BTV16-CHI BTV?-IDN BTV9-IND*	BTV?-USA/ BTV?-USA BTV1-S.Afr/ BTV1-POR BTV4/COR/ GRE/GRE BTV8-NET*
Sample of Ilam I9, I84, I90 (BTV1RSA 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

Overall, 24 serotypes have been reported around the world, generating only low levels of cross-protection and complicating vaccination strategies. Due to its economic impact, BT is an Office International des Epizooties OIE)-listed disease. Economic losses associated with BTV infection are caused directly through reductions in productivity and death and more importantly indirectly through trade losses due to animal movement restrictions, restrictions on the export of cattle semen [12]. BTV 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 VP7, and it is therefore considered likely to show variations that mimic the antigenic variation of different orbivirus strains and species as detected in serogroup-specific serological assays. Seg-7 is therefore also considered unlikely to show cross reactions between Orbivirus species that might be detected in more conserved genes. Since Seg-7 is highly conserved, differences between BTV isolates (even from different geographic origins) are in most cases likely to be relatively small, often representing changes in the third base position (and thus maintaining the conservation at the amino acid level). Indeed it was possible to design oligonucleotide primers, which target the near terminal regions of genome segment 7, to detect most of the BTV strains for which sequences are available (except for the available isolates of serotypes 7, 15 and 19). It was previously reported that Chinese and Australian strains of BTV-15 can show up to 30% genetic divergence in Seg-7, from isolates of other BTV serotypes [34]. 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 [35]. 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 [36]. Previous studies showed that East and West topotype was a specific character for majority of genome segments, specially conserved genes of BTV [37,38]. 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 epizootic hemorrhagic disease viruses (EHDV) [36]. During the last outbreak of BT in Portugal (2004-2006), molecular investigation was performed to find the origin of detected viruses, compared genetic diversity of S7 segment among isolates from US, Caribbean Basin and Central America (west group of BTV) and found several distinct clads [39]. The authors 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. concluded that these two Portuguese strains came from far separate origin because the low nucleotide identity (less than 75%) [40].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 have attempted to investigate the genetic variation of detected BTV in our province by sequencing of seg-7. 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 an 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. 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 these viruses are 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. 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 [10]. In this study we concluded that probably there are both east and west BTV strains in Ilam province. 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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