

Seroprevalence and S7 gene characterization of bluetongue virus in the west of Iran

Mohammad Khezri¹, Seyed Mahmoud Azimi²

1. Agricultural and Natural Resources Research Center of Kurdistan, Sanandaj, Kurdistan, Iran ;

2. Razi Vaccine & Serum Research Institute, Karaj, Alborz, Iran

Corresponding author: Mohammad Khezri, e-mail: khezri1836@yahoo.com

Received: 08-03-2012, Accepted: 06-04-2012, Published Online: 19-06-2012

doi: 10.5455/vetworld.2012.549-555

Abstract

Aim: The objective of this study was conducted to determine the seroprevalence and S7 gene characterization of *bluetongue virus* (BTV) of sheep in the West of Iran, during 2007-2008.

Materials and Methods: A total 372 sheep blood samples were collected from known seropositive regions in the West of Iran. Anti-BTV antibodies were detected in the serum samples by group specific, C-ELISA. Extractions of the dsRNA from whole blood samples were carried out. The One-step RT-PCR kit was used for the detection of S7 BTV gene in the blood samples. PCR products of the first amplification (RT-PCR) were used; template in the nested PCR. Products were separated by 1.2% Agarose gel electrophoresis. Nested PCR products of S7 segment from positive samples and the reference strain; BTV1 (RSA vvvv/01) were prepared for sequencing. All sequences were subjected to multiple sequence alignments and phylogenetic analysis.

Results: The results showed widespread presence of the anti-BTV antibodies in the province's sheep population, where 46.77% of the tested sera were positive on C-ELISA. Bluetongue viruses were diagnosed in some animals by RT-PCR and nested PCR, by targeting S7 segment. This genome segment was sequenced and analyzed in four samples as a conserved gene in BTV serogroup. This group was very similar to the West BTV strains from USA, Africa and Europe. This clustered was categorized with BTV4 from Turkey.

Conclusion: Increases in epidemic disease may constitute a serious problem for Iran's rural economy in future, and the situation is likely to worsen in the next few years as the proportion of unvaccinated livestock increases.

Keywords: Bluetongue, C-ELISA, PCR, Seroprevalence, S7 segment

To cite this article:

Khezri M, Azimi SM (2012) Seroprevalence and S7 gene characterization of bluetongue virus in the west of Iran, *Vet World*, 5(9): 549-555, doi: 10.5455/vetworld.2012.549-555

Introduction

Bluetongue (BT) is a non-contagious, arthropod borne viral hemorrhagic disease of ruminants, particularly of sheep and occasionally cattle and some species of deer [1]. It occurs mostly during periods of high temperature and rainfall, and usually disappears with the first frost or severe cold weather [2]. 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. Specifically, BTV exists in an extensive band that includes tropical, subtropical, and temperate regions of the world between latitudes of approximately 40° North and 35° South. Exceptions include regions of Asia and western North America, where BTV infection of ruminants

occurs as far as 50° North and most recently, northern Europe [3, 4].

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 [5]. There are 24 distinct BTV serotypes currently recognized, and the recently described Toggenburg Orbivirus (TOV) proposed to be a 25th serotype [6]. Due to its economic impact, BT is an OIE listed disease. Economic losses associated with BTV infection are through reduction in productivity and death; and indirectly through trade losses due to animal movement restrictions, and restrictions on the export of cattle semen [7]. In sheep, the clinical signs may include fever, excessive salivation, depression,

dyspnea and panting. Initially, animals have a clear nasal discharge; later, the discharge becomes mucopurulent and dries to a crust around the nostrils. The muzzle, lips and ears are hyperemic, and the lips and tongue may be very swollen. The tongue is occasionally cyanotic and protrudes from the mouth. The coronary bands on the hooves are often hyperemic and the hooves painful; lameness is common and animals may slough their hooves if they are driven. Pregnant ewes may abort their fetuses, or give birth to "dummy" lambs [2]. In sheep, the severity of disease varies with the breed of sheep, virus strain, and environmental stresses. The morbidity rate can be as high as 100% in this species. The mortality rate is usually 0-30%, but can be up to 70% in highly susceptible sheep [2].

Although BTV is an Orbivirus, it was also shown to be transmitted directly from vertebrate to vertebrate, in semen and transplacentally. However, these forms of transmission happen occasionally [8]. 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 validated for the detection of different BTV serotypes or "topotype" and some published methods will only detect certain BTV strains [9]. 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) [9, 10]. These genome segments are relatively conserved across the BTV species and sufficiently divergent between distinct Orbiviruses to remain BTV specific [11]. In most cases the serotypes involved are not identified but BTV serotypes 1, 2, 4, 5, 6, 8, 10, 15, 16, 24, [12,13] and a putative new serotype, KUW 2010/02 [14] have been recognized in Kuwait [14].

The purpose of this study was to use a valid RT-PCR method to detect any BTV isolates in blood samples. Then we 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

A total 372 sheep blood samples were collected from known seropositive regions in the West of Iran, where 135 were from Kurdistan and 237 originated from Ilam. Bleeding was from the Jugular vein and vacutainer tubes with and without EDTA were used for blood and sera collection, respectively. The samples

without EDTA were stored at 4°C and serum separation was achieved by centrifuging, and then stored at -20°C until required. The samples with EDTA were stored at 4°C.

C-ELISA: Anti-BTV antibodies were detected in the serum samples by group specific, C-ELISA, using ID-Vet kit (Monpellier-France). The test is based on the competition between test sera and an anti-VP7 MAB for a VP7 antigen previously bound to the solid phase of an ELISA plate.

Extraction of viral RNA: Extractions of the dsRNA from whole blood samples were carried out using the viral RNA Mini kit (QIAamp®viral RNA Mini Kit, USA) according to manufacturer's instruction. The extracted RNA was denatured by incubation them for 5 min in 95 °C, and cooling to 0 °C.

Oligonucleotide primers: Two pairs of primers; (SZ1: 5'-GTAAAAATCTATAGAGATG-3', SZ2: 5'-GTAAGTGTAATCTAAGAGA-3'), and (SA1: 5'-TTAAAAAATCGTTCAAGATG-3'; SA2: 5'-GTAAGTTTAAATCGCAAGACG-3') which amplify the S7 gene (1156bp) of BTV were used for nested PCR [10]. Internal primers (IntS7F: 5'-ACAACGTGATGCTGCGAATGA-3'; IntS7R: 5'-AACCCACACCCGTGCTAAGTGG-3') were applied, which amplified the internal 770bp region of the S7 gene segment. All Oligonucleotide were synthesized commercially (Cinnagen Co., Iran).

One-step RT-PCR: The One-step RT-PCR kit (QIAGEN ® One-Step RT-PCR Kit, USA) was used for the detection of S7 BTV gene in the blood samples. The master mix was made as follows (44µl): 10µl of 5 x Qiagen RT-PCR buffer, 2µl dNTPs mixture (0.2mM each), 0.5µl (20pmol) of each of the four primers (SZ1, SZ2, SA1, SA2), 2µl Qiagen Enzyme Mix and 28µl of RNase free water. Then 6µl of denatured RNA added to the prepared master mix. In the RT-PCR, extracted RNA initially reverse-transcribed at 45°C for 30 min., followed by a step at 95°C for 15min. Forty amplification cycles were performed at 95°C for 1 min, 45°C for 1min and 72°C for 2min. The PCR cycles were terminated by final extension step at 72°C for 10 min.

Nested PCR: PCR products of the first amplification (RT-PCR) were used; template in the nested PCR. The mixture of the master mix contained, 5µl of 10x PCR buffer, 1µl dNTPs (10mM), 1µl MgCl₂ (50mM), 1µl (20pmol) of each IntS7F&R primers, 0.5µl Taq polymerase (2.5U), 35µl RNase free water and 5µl of template that was added to the reaction at the end. The thermal cycler (Master cycler personal, Eppendorf)

Table-1. Seroprevalence of bluetongue antibodies in sheep in the West of Iran

Regions(province)	No.	Positive	Infection (%)
Kurdistan	135	70	51.85
Ilam	237	104	43.88
Total	372	174	46.77

* Accession numbers

BT3 CHI - AF172827.1 BT16 CHI - AF172831.1 BT9 IND - DQ399836.1
 BT7 IND - AM261981.1 BT7 USA - AF188669.1 BT7 USA - AF188670.1
 BT1 S.Afr - AY776331.1 BT1 POR - EU498675.1 BT1 COR - AY839949.1
 BT4 GRE - AY841352.1 BT4 GRE - AY841351.1 BT8 NET - AM498057.2

Table-2. The result of sequence identity analysis between samples in the West of Iran and other BTV strains from Gene bank

BTV strains from Gene bank	East toptotype	West toptotype
KO215,I9,I84,I90 (BT1RSA vvvv/01)	BT3-CHI BT16-CHI BT7-IDN BT9-IND* GRE/GRE	BT7-USA/ BT2-USA BT1-S.Afr/ BT1-POR BT4/COR/ BT8-NET*
	67-81. %	82-95. %

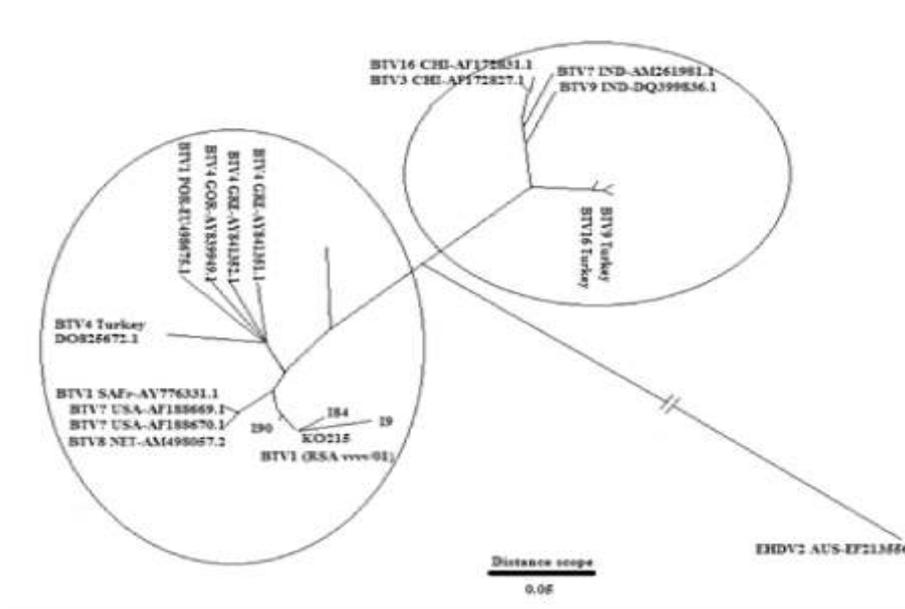


Figure-1. Phylogram of the S7 gene of Bluetongue virus in Iran. The phylogenetic tree generated by neighbor-joining analysis of 770bp of the S7 gene (1000 bootstrap replicates). The figure shows relationships between BTV in the West of Iran with some East and West BTV strains. The GenBank accession numbers 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.

was set to amplify the nested fragment as follows: first step was 95°C for 1min, then 30 cycles were performed at 95°C for 1 min, 59°C for 1 min and 72°C for 1min. The reaction stopped by extension at 72°C for 10 min.

Analysis of PCR products: Products were separated by 1.2% Agarose gel electrophoresis, and stained for 20min in Ethidium bromide (1µg/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 the Institution for Animal Health, Pirbright, UK and used as positive control. The amplified products were purified from the Agarose gel (High pure PCR product purification Kit, Roche, USA), and then sent for sequencing at 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 Cluster W program [15]. The sequence identity matrix was calculated using Bioedit program (Bio Edit Sequence Alignment Editor Copywrite©1997-2007 Tom Hall). The resulting dendrogram was viewed and edited by Tree View (1.6.6) software.

Results

Mention the ELISA test under materials and methods, and briefly explain its principle and the nature of the antigen used to coat the plates. One hundred and seventy four serum samples were positive for BTV-specific antibodies by cELISA test. The total BTV prevalence in western Iran was 46.77% (Table 1), where prevalence in Kurdistan and Ilam were 51.85% and 43.88% respectively. BTV strains that

used for nucleotide sequence comparison were listed in Table 2. The alignment of sequences showed that each samples more than 84% overall nucleotide similarity was determined. However, between these samples 71-78%, homogeneity of S7 gene can be seen. The result of sequence identity evaluation between detected this group and BTV strains from GeneBank are shown in Table 2. This Group consisted of four samples beside of BTV1 (RSA vvvv/01). The members of this cluster showed 84-99% similarity with each other. The western Iranian BTV sequences co-clustered with those of American (BTV?-502172-USA, BTV?-600558- USA), African (BTV1-S. Africa, BTV1-RSAvvv/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 these sequences (KO215, I9, I84, I90) with BTV1 (RSA vvvv/01) was determined 96-99%. The identity of the BTV1 (RSA vvvv/01) S7 sequence and the newly generated ones with that of the out group, Epizootic hemorrhagic disease virus (EHDV), were 56-57% and 53-55% respectively (Figure-1).

Discussion

BTV exists throughout many parts of the world including, the America, 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 affected before was attributed mainly to climate change and was linked to the northern expansion of the major Old World vector *Culicoides imicola* (Kieffer), which is an Afro-Asiatic species of biting midge [16]. This study reports, for the first time, the prevalence of BT antibodies (46.77%) in sheep in the West of Iran. The various proportions of seropositive animals came from Northern 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 [17].

Higher seropositivity rates 76.44%, 54.1%, and 48.4%, detected in the East-Azerbaijan in the North of Iran [18], Saudi Arabia [19], and Pakistan [20], respectively. However, lower incidence rates 8.3%, 29.5%, and 34.7%, reported from India [21], and the West-Azerbaijan in the North-West of Iran [4], South-Eastern Turkey [13], respectively. 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 [3]. The vast majority of infections with bluetongue are clinically inapparent. In a percentage of infected sheep and occasionally other ruminants, more severe disease can occur [2]. Iran's strategic location in the South-East of Europe makes it an important potential source of BTV strains and serotypes that might infiltrate adjacent areas [16], thus more intensive epidemiological investigations are clearly warranted within the region [4]. 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 DNA 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). Oligonucleotide primers targeting the near terminal regions of the segment 7 genome fragments were designed for detecting many BTV strains for which sequences were available in GenBank, with the exception of the highly divergent serotypes 7, 15 and 19 sequences. 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 [22]. Molecular techniques have, compared to a valuable and exclusive application comparing to the other diagnostic methods. For example, it provides the opportunity to trace the origin of BTV in outbreak situations and study their genetic variation [23]. Previous studies have showed that East and West BTV topotype had specific characters in the majority of genome segments studied, especially the ones [24]. The ability to differentiate isolates from different geographical origins based on genomic sequences has been demonstrated for other Orbiviruses like Epizootic haemorrhagic disease virus (EHDV) [25]. Wilson *et al.*, (2000) compared the genetic diversity of S7 segment among isolates from the USA, Caribbean Basin and Central America (west group of BTV) and found several distinct clads [26].

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 BTV3S.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 phylogenetically near to BTV17-US. During the 2004-2006 BT outbreaks in Portugal, molecular investigations were performed to trace the origin of the outbreak strains. BTV genome segments S7, L2 and S10 for molecular analysis of BTV2 and BTV4 isolated, where it was shown that the homology at nucleotide level between the two isolates was less than 75%, but 99.9% and 99.3% with the South African BTV2 and Cortisone/Italian BTV4 respectively. It was concluded that the two Portuguese viruses had distant origins [27]. Mann et al., (2008) compared segment 7 of 41 BTV strains from all around of world. They found S7 gene indicated significant level of variation. They found 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 [28].

In this study the high percentage of homology 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 the West of Iran by sequencing of seg-7. This result was consistent with previous studies that reported the variability of S7 gene in BTV up to 30% [22,26,28]. 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 in transferring BTV strains between these two ecosystems. Therefore, it can be anticipated that both East and West BTV strains are found in this part of the world, possibly because of the extensive animal transportation in this part of the country, and reassortment 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. BT is a transboundary

disease and there are several reasons that could explain the observed similarities between the viruses from western Iran and Turkey, such as the long border through which vertebrate and invertebrate hosts are transported between the two countries. Moreover, the ecosystems in the two countries are similar and can support the same vectors. In the previous study BTV4-Turkey was grouped with other European (Greece, Spain, Italy, Bulgaria and Corsica) and African strains (Morocco), suggesting that the BTV4 strain which invaded Europe and the Eastern Mediterranean region since 1999 belonged to the western BTV lineage [29,30]. Co-clustering reference strain BTV1 (RSA/vvvv01) with this group, which originated from west (South Africa), supported the categorization of these viruses with west origin BTV strains. However, some other investigator made evidence that invasion of BTV1 lineage to Europe could be form bout east and west sources [30-32]. By assessment of the genetic diversity of BTV gene, lots of valuable information about epidemiology of this virus can be collected. In our study we concluded that probably there are west BTV strains in the West of Iran.

Conclusions

Increases in epidemic disease may constitute a serious problem for Iran's rural economy in future, and the situation is likely to worsen in the next few years as the proportion of unvaccinated livestock increases. Hence, there is a need to act now to strengthen veterinary services in rural areas.

Author's contribution

M Khezri prepared samples. SM Azimi implemented the study design and analyzed data. M Khezri drafted and revised the manuscript. All the authors read and approved final Manuscript.

Acknowledgments

We are very grateful to the staff of the Veterinary Division of Agricultural, and Natural Resources Research Center of Kurdistan, particularly Dr. Baharake Mohammadian, Babak Rokhzad, Homan Khanbabaie. This study was supported with a grant of Razi Vaccine and Serum Research Institute.

References

1. Balam, D., Daggupati, S., Maddireddy, H. (2011). Studies of the Antigenic relationships between Bluetongue virus serotypes 2, 9 & 15 isolated in Andhra Pradesh, India. *Vet. World* 4 (10): 444-448.
2. OIE. (2006). World Organization for Animal Health. Press release, Bluetongue detected for the first time

- in Northern Europe. OIE; Aug. Available at:http://www.oie.int/eng/press/en_060823.htm.
3. Lundervold, M., Milner-Guilland, E.J., O'Callaghan, C.J., Hamblin, C. (2003). First evidence of bluetongue virus in Kazakhstan. *Vet. Microbiol.* 92:281-287.
 4. Jafari-Shoorijeh, S., Ramin, A.G., Maclachlan, N.J., Osburn, B.I., Tamadon, A., Behzadi, M.A., Mahdavi, M., Araskhani, A., Samani, D., Rezajou, N., Amin-Pour, A. (2010). High seroprevalence of bluetongue virus infection in sheep flocks in West Azerbaijan, Iran. *Comp. Immunol. Microbiol. Infect. Dis.* 33:243-247.
 5. Balasuriya, U.B., Nadler, S.A., Wilson, W.C., Pritchard, L.I., Smythe, A.B., Savini, G., Monaco, F., De Santis, P., Zhang, N., Tabachnick, W.J., Maclachlan, N.J. (2008). The NS3 proteins of global strains of bluetongue virus evolve into regional topotypes through negative (purifying) selection. *Vet. Microbiol.* 126:91-100.
 6. Hofmann, M.A., Renzullo, S., Mader, M., Chaignat, V., Worwa, G., Thuer, B. (2008). Genetic characterization of Toggenburg Orbivirus, a new bluetongue virus, from goats, Switzerland. *Emerg. Infect. Dis.* 14:1855-1861.
 7. Anonymous. (2007). Commission regulation (EC) No 1266/2007 of 26 October 2007 on implementing rules for Council Directive 2000/75/EC as regards the control, monitoring, surveillance and restrictions on movements of certain animals of susceptible species in Relation to bluetongue, Official Journal of the European Union L 283, 27.10. p.37-52.
 8. Maclachlan, N.J. (2011). Bluetongue: History, global epidemiology, and pathogenesis. *Prev. Vet. Med.* 102: 107-111.
 9. Aradaib, I.E., Mohamed, M.E., Abdalla, T.M., Sarr, J., Abdalla, M.A., Yousof, M.A., Hassan, Y.A., Karrar, A.R. (2005). Serogrouping of United States and some African serotypes of bluetongue virus using RT-PCR. *Vet. Microbiol.* 111:145-150.
 10. Anthony, S., Jones, H., Darpel, K.E., Elliott, H., Maan, S., Samuel, A., Mellor, P.S., Mertens, P.P. (2007). A duplex RT-PCR assay for detection of genome segment 7 (VP7 gene) from 24 BTV serotypes. *J. Virol. Methods* 141:188-197.
 11. Shaw, A.E., Monaghan, P., Alpar, H.O., Anthony, S., Darpel, K.E., Batten, C.A., Guercio, A., Alimena, G., Vitale, M., Bankowska, K., Carpenter, S., Jones, H., Oura, C.A.L., King, D.P., Elliott, H., Mellor, P.S., Mertens, P.P.C. (2007). Development and initial evaluation of a real-time RT-PCR assay to detect bluetongue virus genome segment 1. *J. Virol. Methods* 145:115-126.
 12. OIE. (2009). Final report of the 10th conference of the OIE Regional Commission for the Middle. (East Doha Qatar), 26–29 October 2011 Nov 4, http://www.rr-middleeast.oie.int/download/pdf/Final%20Report_Doha_%20Qatar.pdf.
 13. Ozmen, O., Kale, M., Haligur, M., Yavru, S. (2009). Pathological, serological, and virological findings in sheep infected simultaneously with Bluetongue, Peste-des-petits-ruminants, and Sheep pox viruses. *Trop. Anim. Health. Pro.* 41:951-958.
 14. Maan, S., Maan, N.S., Nomikou, K., Batten, C., Antony, F., Belaganahalli, M.N., Samy, A.M., Reda, A.A., Al-Rashid, S.A., El Batel, M., Oura, C.A., Mertens, P.P. (2011). Novel bluetongue virus serotype from Kuwait. *Emerg. Infect. Dis.* 17:886-889.
 15. Della-Porta, A.J., Parson, I.M., McPhee, D.A. (1985). Problems in the interpretation of diagnostics tests due to cross reactions between orbiviruses and broad serological responses in animals. In: Barber, T.L., Jochim, M. (Eds.), *Bluetongue and Related Orbiviruses*. Alan R. Liss, New York, pp. 445-453.
 16. Purse, B.V., Mellor, P.S., Rogers, D.J., Samuel, A.R., Mertens, P.P., Baylis, M. (2005). Climate change and the recent emergence of bluetongue in Europe. *Nat. Rev. Microbiol.* 3:171-181.
 17. Ward, M.P., Thurmond, M.C. (1995). Climatic factors associated with risk of seroconversion of cattle to bluetongue viruses in Queensland. *Preventive Vet. Medicine* 24:129-136.
 18. Hasanpour, A., Mosakhani, F., Mirzaii, H., Mostofi, S. (2011). Seroprevalence of Bluetongue Virus Infection in Sheep in East-Azarbagan Province in Iran. *Res. J. Biol. Sci.* 3(11):1265-1270.
 19. Yousef, M.R., Al-Eesa, A.A., Al-Blowi, M.H. (2012). High seroprevalence of bluetongue virus antibodies in Sheep, Goats, Cattle and Camel in different districts of Saudi Arabia. *Vet World* 5: 389-393.
 20. Akhtar, S., Djallem, N., Shad, G., Thiemo, O. (1997). Bluetongue virus seropositivity in sheep flocks in North West Frontier Province, Pakistan. *Prev. Vet. Med.* 29:293-8.
 21. Ravishankar, C., Krishnan, N., Air, G.M., Jayaprakasan, V. (2005). Seroprevalence of bluetongue virus antibodies in sheep and goats in Kerala State, India. *Rev. Sci. Tech. Off. Int. Epiz.* 24(3): 953-958.
 22. Bonneau, K.R., Zhang, N., Wilson, W.C., Zhu, J., Zhang, F., Li, Z., Zhang, K., Xiao, L., Xiang, W., Maclachlan, N.J. (2000). Phylogenetic analysis of the S7 gene does not segregate Chinese strains of bluetongue virus into a single topotype. *Arch. Virology* 145:1163-1171.
 23. White, D., Blair, C., Beaty, B. (2006). Molecular epidemiology of bluetongue virus in northern Colorado. *Virus Res.* 118(1-2):39-45.
 24. White, D., Blair, C., Beaty, B. (2006). Molecular epidemiology of bluetongue virus in northern Colorado. *Virus Res.* 118(1-2):39-45.
 25. Bonneau, K., Zhang, N., Zhu, J., Zhang, F., Li, Z., Zhang, K., Xiao, L., Xiang, W., Maclachlan, N. (1999). Sequence comparison of the L2 and S10 genes of bluetongue viruses from the United States and the People's Republic of China. *Virus Res.* 6(12):153-160.
 26. Wilson, W., Ma, H., Venter, E., Van Djik, A., Seal, B., Mecham, J. (2000). Phylogenetic relationships

- of bluetongue viruses based on gene S7. *Virus Res.* 67(2):141-151.
27. Barros, S., Ramos, F., Luis, T., Vaz, A., Duarte, M., Henriques, M., Cruz, B., Fevereiro, M. (2007). Molecular epidemiology of bluetongue virus in Portugal during 2004–2006 outbreak. *Vet. Microbiol.* 124(1-2):25-34.
28. Maan, S., Maan, N., Ross-smith, N., Batten, C., Shaw, A., Anthony, S., Samuel, A., Darpel, K., Veronesi, E., Oura, C. (2008). Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. *Virology* 377 (2): 308-318.
29. Breard, E., Sailleau, C., Nomikou, K., Hamblin, C., Mertens, P., Mellor, P., El Harrak, M., Zientara, S. (2007). Molecular epidemiology of bluetongue virus serotype 4 isolated in the Mediterranean Basin between 1979 and 2004. *Virus Res.* 125(2):191-197.
30. Mertens, P.P.C. (1998). Bluetongue virus incursions into Europe since 1998. http://www.iah.bbsrc.ac.uk/press_release/BT_UK_2007/BT_PressRel_23jul07.htm.
31. Batten, C., Maan, S., Shaw, A., Maan, N., & Mertens, P. (2008). A European field strain of bluetongue virus derived from two parental vaccine strains by genome segment reassortment. *Virus Res.* 137 (1): 56-60.
32. Monaco, F., Cammà, C., Serini, S., Savini, G. (2006). Differentiation between field and vaccine strain of bluetongue virus serotype 16. *Vet. Microbiol.* 116(1-3): 45-52.

* * * * *