MOLECULAR EPIDEMIOLOGY FOR BTV8 TOPOTYPES IN NORTH-WESTERN ITALY*

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SUMMARY

Bluetongue (BT) is an infectious non contagious vector-borne disease of ruminants transmitted by *Culicoides* biting midges. It is caused by Bluetongue virus (BTV), a double-stranded RNA virus member of the *Orbivirus* genus within *Reoviridae* family.

The replication of the virus is characterized by a high mutation rates during sequential passage of BTV between hosts (insect and ruminant). This peculiarity contributes to the diversification and independent evolution of BTV by way of variations in genomic segments linked in this way with the geographic origin of the virus strains (topotypes).

In order to identify specific topotypes involved in the 2008 BTV-8 outbreaks occurring in North-Western Italy (Piedmont region), phylogenetic analyses based on nucleotide sequences of genomic segments were carried out.

Keywords: Molecular epidemiology, Bluetongue, Serotype 8, Topotypes.

RÉSUMÉ

La fièvre catarrhale ovine (maladie de la langue bleue, en anglais Bluetongue – BT) est une maladie virale des ruminants dont la transmission se fait par l'intermédiaire de moucherons piqueurs du genre *Culicoïdes*. Le virus responsable de la maladie (BTV) est un *Orbivirus* à ARN segmenté de la famille des *Reoviridae.*

La réplication du virus est caractérisée par plusieurs niveaux de mutation pendant la transmission du virus entre ses hôtes (moucherons et ruminants). Ces mutations contribuent à la diversification et à l'évolution indépendante de segments d'ARN, en lien avec l'origine géographique du type du virus (topotype).

Ce travail d'épidémiologie moléculaire se propose d'établir l'origine des topotypes impliqués dans les foyers de BTV-8 dans la région du Piémont, en Italie du Nord-Ouest.

Mots-clés : épidémiologie moléculaire, fièvre catarrhale ovine, sérotype 8, topotypes.

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I - INTRODUCTION

Bluetongue (BT) is an infectious non contagious viral vector-borne disease of domestic and wild species of ruminants. It is caused by the Bluetongue virus (BTV), a RNA virus member of the *Orbivirus* genus within *Reoviridae* family.

Nowadays 24 serotypes (BTV-1 to 24) are officially recognised [Schwartz-Cornil *et al.*, 2008]; all of them are capable of causing severe clinical disease at least in sheep and potentially 25th and 26th serotypes have also been proposed [Chaignat *et al.*, 2009; Maan *et al.*, 2011].

The virus is mainly transmitted among vertebrate hosts by biting midges belonging to the *Culicoides* species and it has been reported from 35°S to 53°N. of latitude.

The BTV genome is composed of ten linear segments (Seg-1 to Seg-10) of double-stranded RNA (dsRNA), which encode ten viral proteins (VP1 to VP10).

The BTV shows considerable genetic and antigenic variability [Dal Pozzo *et al.,* 2009], and its replication is characterized by high mutation rates during sequential passages of BTV between hosts (insecst and ruminants).

This contributes to the diversification and independent evolution of BTV genomic segments. Sequencing studies indicate that BTV strains in different geographic regions have evolved separately over long periods of time, acquiring multiple point mutations and developing characteristic regional variants, even related to the geographic origin of virus strains (topotypes). Seg-2 showed high levels of sequence variation related to each virus topotype. These studies show significant variations between strains of the same serotype from different geographical areas (different Seg-2 topotypes). In most cases, different isolates of the same serotype could be divided into an “eastern topotype” (from Australia, Indonesia and India) and a “western topotype” (from Africa and North or South America) [Mertens *et al.*, 2007].

Since 1998 several BTV strains belonging to five serotypes (i.e. BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16) have been circulating across large parts of the South-European Mediterranean Basin [Mellor *et al.*, 2002; Saegerman *et al.*, 2008]. In August 2006, BTV-8, a serotype previously reported only in the sub-Saharan region, Asia and South America, unexpectedly broke up in northern Europe and outbreaks were reported for the first time in several animal holdings in the Netherlands. This was the onset of a rapidly spreading BT-epidemic which involved Belgium, Germany, France and then many other countries [Mintiens *et al.*, 2008].

A study conducted on the sequence analysis of the full genome of BTV-8 from the Netherlands 2006 outbreaks and in comparison with other European strains, demonstrated that even for BTV-8, Seg-2 is the most variable among all genome segments and also showed variations that correlate with the geographic origin of the virus strain [Maan *et al.*, 2008].

At the beginning of 2008, BTV-8 was first detected in cattle farms located in the North-Eastern region of Italy while in late Autumn the infection was reported in the North-Western area Piedmont region)n (figure 1) [Giovannini *et al.*, 2008].

There is no certainty regarding the way BTV-8 entered Piedmont, even ithough the intense livestock import activity between France and the region may suggest a French origin. Around 20.000 bovines are every month importedfrom France into Piedmont for fattening.

In order to identify specific topotypes involved in the BTV-8 outbreaks occurring in the Piedmont region, phylogenetic analyses based on nucleotide sequences of genome segments werecarried out, and then compared to other published sequences of BTV-8 strains evaluated in an attempt to clarify the origin of the virus.

Figure 1

Map showing Italy with the location of the Piedmont region (dark grey)

II - MATERIALS AND METHODS

The characterization of BTV-8 was directly performed on viral genome positive blood (EDTA) samples collected from Piedmont herds and detected by mean of specific real-time RT-PCR [Polci *et al.*, 2007], Samples were collected from infected cattle during 2008 and 2009 outbreaks. The areas exposed to virus circulation were extended from the North-Western, to the North-Eastern and Southern parts of the region bordering with France (Provence-Alpes-Côte d'Azur and Savoie regions).

Serotyping of positive blood samples was previously confirmed at the OIE Reference Laboratory for BT in Teramo (I.Z.S. Abruzzo e Molise) using the serum neutralization assay [OIE, 2008].

Sequence analyses on viral genomes were carried out at the Istituto Zooprofilattico Sperimentale of Piemonte, Liguria and Valle d'Aosta.

Fifteen BTV-8 RNA genomes were directly extracted from whole blood (EDTA) samples of infected animals using Highly Pure Viral RNA kit (Roche) according to the manufacturers' instructions and stored below 80°C.

RNA samples were analysed for different genome segments by one-step RT-PCR amplifications method. The RT-PCR protocol consisted of SuperScript III OneStep RT-PCR System with Platinum *Taq* High Fidelity (Invitrogen) that includes all reagents required for reverse-transcription and PCR-amplification steps. The primers-template mix was heated to 95°C for 5 minutes, then RT-PCR mix was added to a final volume of 25 µl. Samples were first subjected to reverse transcription at 60°C for 30 min, then PCR amplifications were conducted using the following thermal profile: initial denaturation at 95°C for 2 min; 94°C for 30 s, annealing at primers specific T_m for 30 s and extension at 68°C for 2 min, for 40 cycles. There was also applied a final extension step of 68°C for 5 min. The primers set for the complete amplification and the sequencing protocol of Seg-2 of the virus genome were previously described by Mertens *et al.* [2007]. Briefly cDNA products were analysed by 2% agarose gel electrophoresis and visualized under UV light after staining with GelRed™ (Biotium), then recovered from the agarose gel using the Purelink PCR Purification kit (Invitrogene) according to the manufacturer's instructions. The purified cDNA was sequenced in both directions using the 'Big Dye 3.1 kit (ABI) and reactions were run on a

BABI PRISM 310 Genetic Analyzer Automated Capillary Sequencer.

The sequences obtained were then analysed using BioEdit Sequence Alignment Editor (version 7.0.5.2) and aligned with ClustalX (1.83.1) software. A neighbour-joining tree was drawn using the nucleotide sequences and compiled from multiple alignments of the VP2 sequences with Phylo Win 2.0 software [Galtier *et al.*, 1996], after comparison with some available GenBank® deposed sequences (http://blast.ncbi.nlm.nih.gov/), in order to evaluate the degree of similarity between local strains. The BTV-8 comparison was carried out considering various major BTV-8 geographical origins: South Africa; Kenya; Niger; Netherlands and France.

Details of BTV-8 isolates retrieved from GenBank® are given in table 1.

Table 1

BTV-8 sequences downloaded from GenBank® and employed for the comparison with Piedmont sequences

Serotype	Country of origin	Accession number
BTV-8	South Africa	AJ585129
BTV-8	Kenya	AJ585183
BTV-8	Niger	AJ585184
BTV-8	Netherland (2006)	AM498052
BTV-8	Netherland (2007: NET2007)	GQ506452
BTV-8	France (98% homology)	FJ183375

III - RESULTS

The results of our analysis demonstrate that the BTV-8 Seg-2 sequences from diagnostic RT-PCR positive samples of Piedmont infected herds showed high levels of similarity with the French BTV-8 group, compared through GenBank® sequence (accession number FJ183375), suggesting a phylogenetic relationship between the Piedmont field strains and the French virus closer than with other topotypes.

The neighbour-joining tree showing relationships between nucleotide sequences of Seg-2 from BTV-8 sequences analysed in this work is displayed in figure 2.

In the regional analysis, four large clusters were identified (Group 1 to Group 4 - figure 3):

- Group 1 (triangle-shaped dots): sequence number 1, 2, 10, 11 and 15. Highest similarity level with French topotype. BTV-8 topotypes that have circulated in North-Western and North-Eastern areas of the region.
- Group 2 (square-shaped dots): sequence number 8, 9, 12, 13 and 14. BTV-8 topotype that have circulated both in Northern and in Southern areas of the region.
- Group 3 (diamond-shaped dots): sequence number 3 and 4. BTV-8 topotype that have circulated in the North-Eastern area of the region.
- Group 4 (circle-shaped dots): sequence number 5, 6 and 7. BTV-8 topotypes that have circulated in Northern area of the region.

Figure 4 shows in detail the Northern area of Piedmont region involved in BTV-8 circulation and the patterns of topotype extension. Arrows indicate the ways by which topotypes have evolved in time of official infection confirmation by the OIE Reference Laboratory.

However phylogenetic analysis demonstrated that all the Piedmont BTV-8 sequences are grouped together and few changes between clusters were evident.

Figure 2

Dendrogram elaborated by Phylo Win 2.0 with "Neighbour Joining" analysis. Numbers 1 to 15 represent BTV-8 RNA genomes extracted from blood samples in Piedmont infected cattle

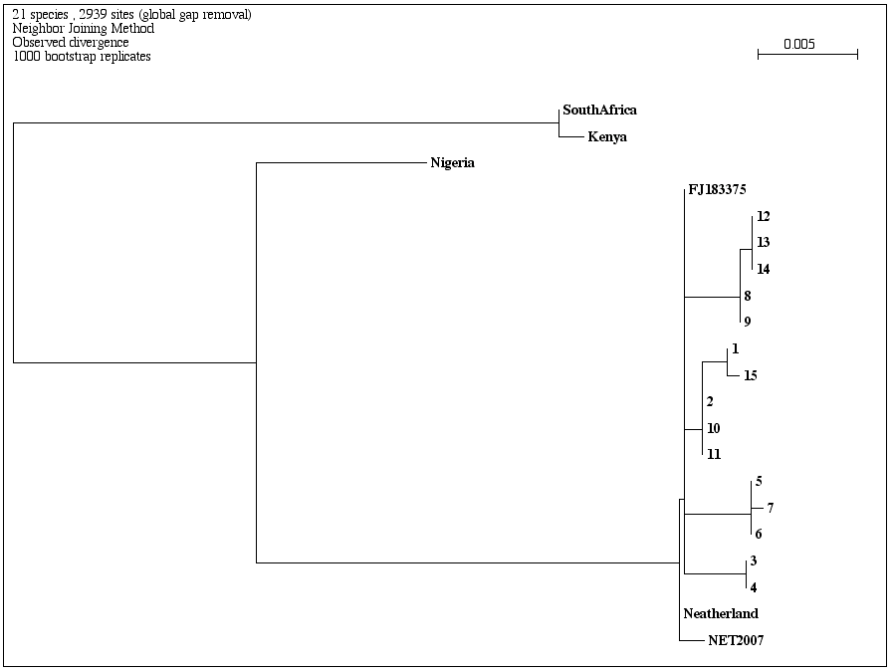


Figure 3

Map showing clusters of BTV-8 topotypes in Piedmont region

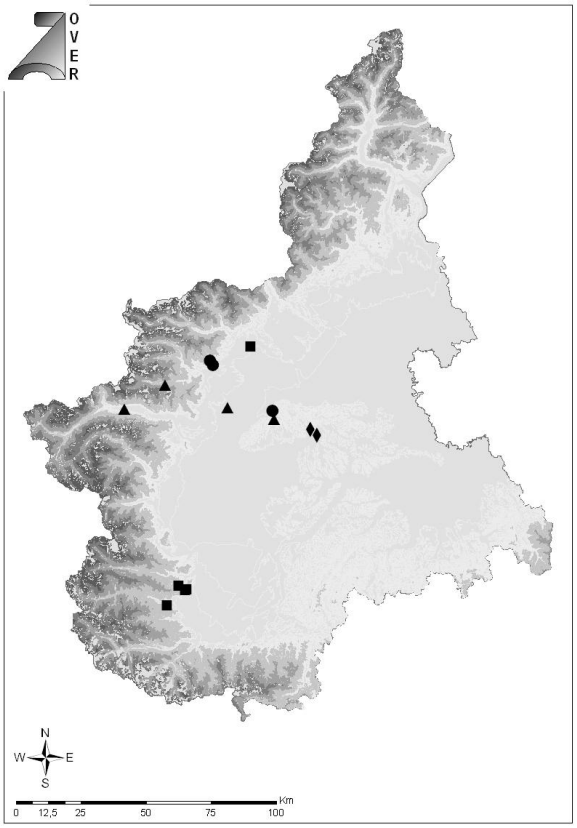
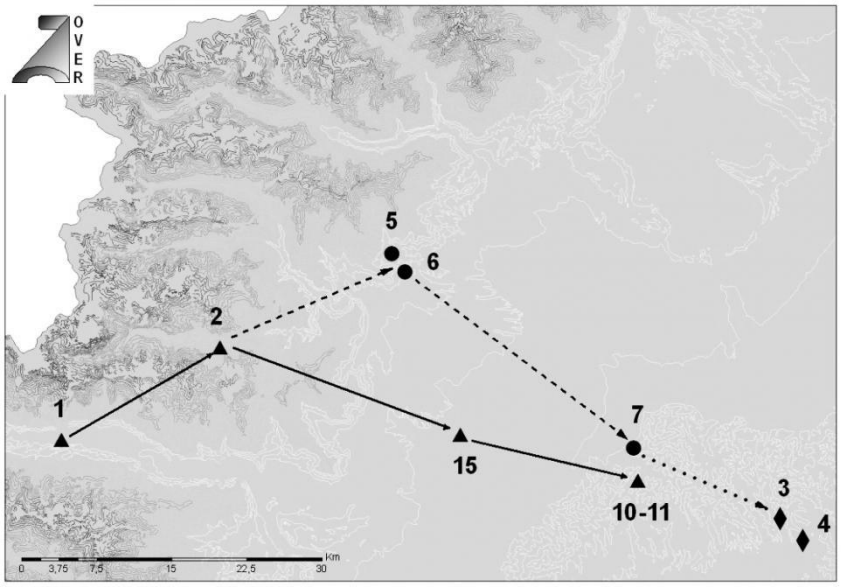


Figure 4

Pathways of BTV-8 topotypes (Groups 1, 3 and 4) in the North of the region, detail. Groups 3 (circle-shaped dots) and 4 (diamond-shaped dots) derive from outbreak identified by number 2 (part of Group 1: triangle-shaped dots)



IV - DISCUSSION AND CONCLUSION

This study provided epidemiological information that could help to identify the origins, movements and spreading of BTV-8 individual strains occurred in the Piedmont region by the Seg-2 phylogenetic approach.

Topotyping provides a powerful tool for epidemiological detective work. It helps to identify probable geographic origin of a virus incursion and help to understand the mechanisms and routes by which they have spread, tracing an outbreak to a source.

The sequence data allowed the creation of a BTV-8 regional sequence database, which will support more rapid molecular methods for BTV-8 topotypes identification in case of new outbreaks.

BIBLIOGRAPHIE

Chaignat V., Worwa G., Scherrer N., Hilbe M., Ehrensperger F., Batten C., Cortyen M., Hofmann M., Thuer B. - Toggenburg Orbivirus, a new bluetongue virus: initial detection, first observations in field and experimental infection of goats and sheep. *Vet. Microbiol.,* 2009, **138**, 11-19.

Dal Pozzo F., Saegerman C., Thiry E. - Bovine infection with bluetongue virus with special emphasis on European serotype 8. *Vet. J.,* 2009, **182**(2), 142-51. *Epub*, 2009 May 27.

Felsenstein J. - PHYLIP -- Phylogeny Inference Package (Version 3.2). *Cladistics, 1989,* **5,** 164-166.

Galtier N., Gouy M., Gautier C. - « SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny » *Comput. Appl. Biosci.,* 1996, **12**(6), 543-548.

Giovannini A., Calistri P., Conte A., Goffredo M., Paladini C., Lelli R., Caporale V. - Bluetongue virus serotype 8 in Europe in

2007–2008 and the risk of virus introduction and spread in Italy. Symposium on ‘‘Bluetongue in Europe back to the future’’. Brescia (Italy), 7 June 2008.

Hall T.A. - BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series,* 1998, **41**, 95–98.

Maan S., Maan N.S., Ross-Smith N., Batten C.A., Shaw A.E. - Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to the other European strains. *Virol*., 2008, **377**, 308-318.

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. - Novel bluetongue virus serotype from Kuwait. *Emerg. Infect. Dis*., 2011, **17**, 886-9.

Mellor P.S., Wittmann E.J. - Bluetongue virus in the Mediterranean basin 1998-2001. *Vet. J.,* 2002, **164**, 20-37.

Mertens P.P.C., Maan N.S., Prasad G., Samuel A.R., Shaw A.E., Potgieter A.C., Anthony S. J., Maan S. - Design of primers and use of RTPCR assays for typing European bluetongue virus isolates: differentiation of field and vaccine strains. *J. Gen. Virol.,* 2007, **88**, 2811-2823.

Mintiens K., Méroc E., Staubach C., Gerbier G., Elbers A.R.W., Hendrickx G., De Clercq K. - Possible routes of introduction of bluetongue virus serotype 8 into the epicentre of the 2006 epidemic in north-western Europe. *Prev. Vet. Med*., 2008, **87**, 131-44.

Office International Des Epizooties (OIE). - Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, OIE Standards Commission (Eds), 6th eds., 2008, 1344.

Polci A., Cammà C., Serini S., Di Gialleonardo L., Monaco F., Savini G. - Real time polymerase chian reaction to detect bluetongue virus in blood samples. *Vet. It*., 2007, **43**, 65-76.

Saegerman C., Berkvens D., Mellor P. S. - Bluetongue epidemiology in the European Union. *Emerg. Infect. Dis*., 2008, **14**, 539–544.

Schwartz-Cornil I., Mertens P.P., Contreras V., Hemati B., Pascale F., Bréard E., Mellor P.S., MacLachlan N.J., Zientara S. - Bluetongue virus: virology, pathogenesis and immunity. *Vet. Res*., 2008 Sep-Oct, **39**(5), 46. *Epub*, 2008 May 22.