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Research paper

Analysis of bluetongue serotype 3 spread in Tunisia and discovery of a novel strain related to the bluetongue virus isolated from a commercial sheep pox vaccine

Alessio Lorusso[a,b,*,1], Soufien Sghaier[c,1], Marco Di Domenico[a,b], Mohamed Elias Barbria[d], Guendalina Zaccaria[a,b], Aida Megdich[c], Ottavio Portanti[a,b], Imed Ben Seliman[e], Massimo Spedicato[a,b], Federica Pizzurro[a,b], Irene Carmine[a,b], Liana Teodori[a,b], Mejdi Mahjoub[f], Iolanda Mangone[a,b], Alessandra Leone[a,b], Salah Hammami[g], Maurilia Marcacci[a,b], Giovanni Savini[a,b]

[a] OIE Reference Laboratory for Bluetongue, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM), Teramo, Italy
[b] National Reference Center for Whole Genome Sequencing of microbial pathogens: database and bioinformatic analysis, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise, Campo Boario, 64100 Teramo, Italy
[c] Univérsité de Tunis El Manar, Institut de la Recherche Vétérinaire de Tunisie (IRVT), Laboratoire de virologie, 20 Rue Djebel Lakhdar, 1006 Tunis, Tunisia
[d] CRDA-Commissariats Régionaux au Developpement Agricole, Menzel Bouzelfa, Tunisia
[e] CRDA, Medenine, Tunisia
[f] CRDA, Gafsa, Tunisia
[g] Ecole Nationale de Médecine Vétérinaire de Sidi Thabet, Tunisia

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ABSTRACT

Bluetongue (BT), is one of the OIE-listed major diseases of ruminants. Following the official report of BT virus serotype 3 (BTV-3) in a sheep in Cap Bon (Tunisia), blood and serum samples of ruminants were collected from some areas of Tunisia to further investigate the presence of this virus in the country. A quantitative real time RT-PCR has been first developed for the detection and quantitation of BTV-3 RNA from field specimens. Out of 62 collected blood samples, 23 were shown to be positive for BTV-3 RNA. Isolation on cell cultures was also possible from six samples. Genome sequencing revealed the circulation of two unrelated western strains of BTV-3, one circulating in Cap Bon and neighboring areas, and the other circulating nearby the border with Libya. The presence of a putative novel BTV serotype (BTV-Y TUN2017) in sheep introduced from Libya to Tunisia, genomically related to the BTV strain contaminating a commercially-available sheep pox vaccine and to BTV-26, has been also demonstrated. This finding highlights the pressing need for a prompt production and release of a novel inactivated BTV-3 vaccine to be used in case of emergence or proactively in the areas of Southern Europe at major risk of BTV introduction. The assessment of a novel vaccine will certainly exalt the role and importance of surveillance activities and collaboration with Northern African countries.

1. Introduction

Bluetongue virus (BTV), the type species of the genus *Orbivirus* within the family *Reoviridae*, causes the bluetongue (BT), one of the OIE-listed major diseases of ruminants. BT has severe economic repercussions for the livestock industry (Velthuis et al., 2010) due to direct losses caused by the infection but also due to indirect losses because of restrictions on animal trade (Dal Pozzo et al., 2009; Méroc et al., 2009; Nusinovici et al., 2013; Zientara and Ponsart, 2014; Tago et al., 2014). BTV is transmitted by various species of *Culicoides* midges; thus, the global distribution of BTV coincides with that of competent vectors (Maclachlan, 2010; Verwoerd and Erasmus, 2004). The BTV genome consists of ten segments (Seg 1 to Seg 10) of linear dsRNA and codes for 7 viral structural (VP1-VP7) and 5 non-structural (NS1, NS2, NS3/NS3a, NS4 and S10-ORF2) proteins (Schwartz-Cornil et al., 2008; Ratinier et al., 2011; Stewart et al., 2015). Up to 2008, 24 serotypes of BTV were officially recognized (Maan et al., 2008). However, in the last years novel and generally asymptomatic BTV serotypes have been

* Corresponding author at: OIE Reference Laboratory for Bluetongue, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM), Teramo, Italy.
E-mail address: a.lorusso@izs.it (A. Lorusso).
[1] These authors equally contributed to this manuscript.

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described in the field. These include BTV-25 (TOV strain) from Switzerland, BTV-26 from Kuwait, BTV-27 (variants 01, 02 and 03) viruses from Corsica (France), BTV-XJ1407 from China, a BTV strain isolated from a sheep pox vaccine (SP vaccine derived BTV) and BTV-X ITL2015 from Sardinia (Italy) (Hofmann et al., 2008; Maan et al., 2011; Zientara et al., 2014; Schulz et al., 2016; Sun et al., 2016; Bumbarov et al., 2016; Savini et al., 2017). As for the SP-vaccine derived BTV only partial (Seg 1, Seg 4, Seg 7, Seg 8, Seg 9 and Seg 10) genome sequences are publicly available (Bumbarov et al., 2016). A putative novel BTV serotype has been also described from an Alpaca in South Africa (Wright, 2014; Belbis et al., 2017). Generally, two major geographic groups of BTVs are described and designated as eastern (e) or western (w) topotypes even within the same serotype. The eastern topotype includes viruses from Australia and the Middle/Far East, the western viruses originating from Africa and the Americas (Maan et al., 2008).

Since 1998, Southern Europe has experienced multiple incursions of different serotypes and topotypes of BTV. Strains of BTV-1e, BTV-4w, BTV-9e and BTV-16e have all entered the eastern Mediterranean region. In addition, BTV-1w, BTV-2w and BTV-4w have entered Southern Europe because of wind-driven dissemination of infected midges from Northern African countries. Specifically, the virus had been likely introduced to Europe from Northern Africa *via* two major gateways: (i) from Morocco to Spain through the Straits of Gibraltar, (ii) from Tunisia to Italy through Sicily or Sardinia (Wilson and Mellor, 2008). In December 1999, BTV-2w was recorded in the northeastern part of Tunisia (Hammami, 2004) and during summer of 2000 BTV-2w spread to Algeria. In the same summer, BTV-2w appeared in Sardinia (Italy), Corsica (France) and Balearic Islands (Spain). BT outbreaks in Sardinia were immediately linked with BTV-2w circulation in Tunisia (Calistri et al., 2004). During the first epidemic, BTV-2w occurred also in Sicily and Calabria (mainland Italy) regions. Further outbreaks occurred again in 2001 and 2002 (Savini et al., 2003). The genome of the initial Tunisian isolates of BTV-2w was revealed to be almost identical to that of the strains from Corsica and Sardinia (Ben Fredj et al., 2003), demonstrating that BTV-2w had spread northward from Northern Africa to Italy and the western Mediterranean islands. In 2003, BTV-4w was first observed in Sardinia and in several western Mediterranean islands. Genome analysis has shown that this virus was distinct from BTV-4w that had been circulating in the eastern regions of the Mediterranean basin since 1999 (Breard et al., 2007). BTV-4w strain possibly entered Europe from Northern Africa, possibly from Tunisia or Algeria. The same strain was subsequently isolated from Morocco and Spain in 2004 where it persisted into 2005. In 2012, a novel reassortant strain of BTV-4w was identified in Sardinia together with BTV-1w (Lorusso et al., 2013). This reassortant BTV-4w has been shown to be closely related to BTV-4w isolated in Tunisia in 2007 and 2009 (Lorusso et al., 2013). BTV-1w, isolated for the first time in Algeria in 2006, (Cêtre-Sossah et al., 2011) had spread in different incursions all over the south Mediterranean basin and, surprisingly, northward reaching France and threatening the introduction into the UK. BTV-1w was remarkably detected in Sardinia together with the BTV-4w reassortant strain in 2012 (Lorusso et al., 2013) and reappeared alone in the following year (Lorusso et al., 2014a). In 2013, BTV-1w spread all over the Island of Sardinia invading Corsica (Sailleau et al., 2015), Sicily and mainland Italy. Phylogenetic analysis revealed that BTV-1w strains isolated in Italy in 2012 and 2013 have a direct Northern African origin. The Italian strains originated from a strain closely related to a BTV-1w isolated in Tunisia in 2011 (Lorusso et al., 2014b). In November 2016, a novel BTV-3w was identified in a symptomatic sheep located in the Municipality of Hannous (Governorate of Nabeul; Delegation of Beni Khalled), in the north-eastern part of Tunisia (36°37,059.04″N-10°42,039.52″E), in the middle area of Cap Bon (Sghaier et al., 2017). This outbreak was notified to the OIE and the genome sequence of BTV-3 TUN2016 was deposited into GenBank (KY432369-KY432378). Considering the epidemiology of BT in the Mediterranean basin (Calistri et al., 2004), it would not be surprising to observe the spread of this virus all over Tunisia and eventually the incursion of BTV-3w in Southern Europe. Therefore, in this study, blood and serum samples collected from ruminants from some regions of Tunisia were tested for the presence of BTV RNA and antibodies, respectively.

2. Material and methods

2.1. Ethical statement

The study did not involve any animal experiments. Blood and serum samples were collected from animals by the Tunisian Veterinary Services with standard procedures avoiding suffering.

2.2. Development of a specific BTV-3w quantitative real time PCR, RT-qPCR$_{BTV-3}$

Seg 2 sequences of all publicly available BTV serotypes were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and aligned using the DNAStar software package (DNAStar Inc., Madison, WI, USA). Primers were designed to amplify a specific 104 bp fragment of Seg-2 sequence of BTV-3w (BTV-3 TUN2016, KY432370) and then verified by the Primer Express 3.0.1 software test tool (Applied Biosystems). Primer BTV-3w forward sequence was 5′-AAATTTAATGAAGATAGATATCGTGAGATGATC-3′ (position 1393–1425), and primer BTV-3w reverse sequence was 5′-TTACCTTCTTCCTCAAGGATYTTATACATT-3′ (position 1496–1467). Probe and primers were synthesized by Eurofins Genomics (Ebersberg, Germany). BTV-3w TaqMan probe (CAGTCGGTAATTGATGATGGGTG GGACC) was dual-labelled with 6-carboxyfluorescein (FAM) at the 5′ end and with tetramethylrhodamine (TAMRA) at the 3′ end (position 1426–1453). The 25 µl reaction volume contained 5 µl of total purified and previously heat denatured RNA, 12.5 µl of 2× Reaction Mix, 0.5 µl of SuperScript™ III RT/Platinum® Taq High Fidelity Enzyme Mix, 0.5 µl of ROX Reference dye (SuperScript One-Step RT-PCR System with Platinum Taq DNA Polymerase, Invitrogen), 1 µl of Armored RNA West Nile Virus (HNY1999) (Asuragen) as non-competitive exogenous internal amplification control (EIAC), a final concentration of 200 nM for each EIAC primer (NS5-2-F, GAAGAGACCTGCGGCTCATG; NS5-2-R, CGGTAGGGACCCAATTCACA), 160 nM for EIAC probe (NS5-2-P, CCAACGCCATTTGCTCCGCTG), 600 nM for both BTV-3w forward and reverse primers, 300 nM for BTV-3w probe and nuclease-free water up to final volume. The thermal profile consisted of a single cycle of reverse transcription at 50 °C for 15 min followed by a denaturation step at 95 °C for 2 min for reverse transcriptase inactivation and DNA polymerase activation. The amplification of cDNA was performed by 45 cycles including denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s.

2.3. RT-qPCR$_{BTV3}$: specificity, sensitivity and repeatability

Total RNA was purified from whole blood samples, tissue homogenates, tissue culture supernatants and insect homogenates using the High Pure nucleic acid extraction kit (Roche, Nutley, NJ) according to the manufacturer's instructions. To assess specificity, RNAs purified from reference isolates of each BTV serotype available at the IZSAM including BTV-26 from Kuwait, BTV-27s from Corsica and a chimeric BTV-6 expressing VP2 and VP5 of TOV (BTV-6$^{VP2/VP5\ TOV}$, van Rijn et al., 2016), were included in the analysis. RNAs purified from biological samples (whole blood and spleen homogenates of cattle and sheep) previously tested positive for BTV-1w, BTV-2w, BTV-4w, BTV-8w, BTV-9e and BTV-16e were also included. Moreover, RNAs from reference isolates of two additional viral species belonging to the *Orbivirus* genus available at IZSAM, namely Epizootic hemorrhagic disease virus (EHDV) and African horse sickness virus (AHSV), were also included in the analysis. BTV-X ITL2015-positive blood samples of goats (Savini et al., 2017) were also tested. RNAs purified from BTV-negative