

Country-wide distribution of bluetongue virus with expanding host spectrum and evidence of vector competence in Hungary

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Summary. – Following the introduction of bluetongue virus type 4 (BTV-4) in 2014, country-wide monitoring of bluetongue (BT) disease was performed to see whether the virus has become enzootic in Hungary. To analyse the epizootiology of BT, over 110,000 samples collected from domestic and wild ruminants were screened for the presence of BTV RNA and virus-specific antibodies using real-time RT-PCR assay and commercial ELISA kit, respectively. During laboratory analysis, specimens collected from 333 (0.8%) cattle, 79 (2.2%) sheep, 4 (0.9%) goats, and 1 (2.3%) mouflon were found to be positive by viral RNA-detection assay. In addition, antibody to BTV was detected in 5.5% (3158/57,250) of cattle, 10.1% (517/5120) of sheep, 40% (116/290) of goat, and 5.6% (16/284) of buffalo origin samples. The majority of positive samples originated from south-western counties; however, 18 out of 19 counties reported cases or antibody prevalence in the examined animals. Genome sequencing of a representative BTV-4 strain from 2015 was also performed. When comparing this strain with the isolate BTV4-HUN2014 detected only a year earlier in Hungary, mutations at 14 sites were identified within the amplified and sequenced genome. Our findings reinforce the need for continued surveillance of BT disease in Hungary.

Keywords: reoviridae; orbivirus; cattle; sheep; goat; biting midge

Introduction

Bluetongue virus (BTV) is the causative agent of a vector-borne disease of ruminants principally transmitted by biting midges (*Culicoides spp.*). Bluetongue is a hemorrhagic disease with high mortality rates in sheep, while cattle and other domestic or wild ruminant species may act as reservoirs without clinical signs (Saegerman *et al.*, 2008; Casaubon *et al.*, 2013). The BTV genome comprises 10 double-stranded RNA segments that encode different viral and non-structural proteins (VP1-7; NS1-3). Based on VP2, the major capsid protein that is responsible for antigenic variability, 28 distinct

BTV serotypes and 3 putative serotypes have been reported so far (Maan *et al.*, 2011; Zientara *et al.*, 2014; Sun *et al.*, 2016; Savini *et al.*, 2017).

Previously, BTV infections were restricted to regions between latitudes of 40–50°N and 35°S, but since 2006–2007 BTV has spread to northern parts of the world mainly due to climate change and extended vector competence (Vangeel *et al.*, 2012). Appearance of the virus in Europe is associated with at least two unrelated incursion routes, such as the eastern and the western epidemiological system. Over the past two decades outbreaks caused by serotypes 1, 2, 4, 6, 8, 9, 11, 14, and 16 have occurred in several European countries (Breard *et al.*, 2007; Orłowska *et al.*, 2016). Since the first incursion to Europe (Greek Islands; late 1970s), serotype 4 BTV (BTV-4) strains have emerged in Bulgaria and Turkey. In addition, during the 21st century, outbreaks were also recorded in Italy, France, Spain, Portugal, the United Kingdom, Denmark, Norway, and Sweden (Breard *et al.*, 2007; Saegerman *et al.*, 2008; Durand *et al.*, 2010; Lorusso *et al.*,

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Abbreviations: BT = bluetongue; BTV = bluetongue virus; BTV-4 = BTV serotype 4; Ct = cycle threshold

2013). Phylogeographic analyses have identified two discrete strains of BTV-4 in Europe, a finding that is consistent with their geographical origin (Katsoulos *et al.*, 2016).

In 2007 serological testing has identified a BT-seropositive cattle in Hungary; this animal was imported from another European country (Malik *et al.*, 2008). Thereafter, country-wide monitoring was initiated to identify BT cases. As BTV has not been enzootic in Hungary, the majority of the ruminant population was immunologically naïve to the virus, enabling rapid spread of BTV under favorable ecological and epidemiological conditions. During 2014 increasing number of BTV cases was recorded, especially in the south-eastern counties of Hungary, which was linked to an ongoing outbreak reported in Greece and Bulgaria. Sequence analyses classified the Hungarian BTV strain to serotype 4 (Hornyák *et al.*, 2015). In September 2015, a new BTV outbreak was reported, therefore the aim of our study was to analyse the epizootiology of BT in Hungary during 2015. In this period the surveillance was extended to examine the possible role of wild ruminants in the maintenance of BTV and the role of biting midges for vector competence.

Materials and Methods

Samples. Between January and December 2015 a total of 47,273 and 63,146 samples were processed at the National Reference Laboratory of Veterinary Diagnosis Directorate of National Food Chain Safety Office for viral nucleic acid and virus-specific antibody detection, respectively. Anticoagulant-treated and non-treated blood samples collected from all over the country were collected from cattle, sheep, goat, buffalo and several wild ruminant species (e.g. roe deer, fallow deer, red deer, mouflon). Biting midges were collected by light traps in the proximity of the stalls. Midges were transported in 70% ethanol to the National Reference Laboratory to identify them and carry out PCR tests.

Real-time RT-PCR. BTV genomic RNA was identified by real-time RT-PCR method. In brief, BTV RNA was isolated with MagNa Pure LC Total Nucleic Acid Isolation kit (Roche, Germany). After RNA extraction, specific primers to Segment-10 (NS3) were used in a quantitative one-step RT-PCR (qRT-PCR) assay (Hofmann *et al.*, 2008). The reaction mixture was prepared in a final volume of 15 µl that contained 2 µl RNA, 3 µl 5x Qiagen OneStep RT-PCR buffer, 0.8 µl MgCl₂, 0.6 µl dNTP, 1 µl forward primer (5'-TGGAYAAAGCGATGTCAAA-3'), 1 µl reverse primer (5'-ACATCATCACGAAACGCTTC-3'), 0.6 µl probe (5'-FAM-ARGCTGCATTTCGCATCGTACGC-3' BHQ1), 0.6 µl Qiagen enzyme mix and 5.4 µl distilled water. The thermal profile of qRT-PCR consisted of 55°C for 30 min (reverse transcription), 95°C for 15 min (denaturation), followed by 45 cycles of 95°C for 20 s, 50°C for 30 s and 72°C for 30 s. Fluorescence was measured at the end of 50°C annealing step. Cycle threshold (Ct) values were determined from the point at which the sample fluorescence signal

crossed a threshold value. Samples with Ct values greater than 40 were considered as negative.

Antibody detection. BTV specific monoclonal antibody detection was carried out with INgezim BTV Compac 2.0 (Ingenasa, Madrid, Spain) ELISA test, which contains recombinant BTV VP7 protein as target antigen. The test was performed according to the manufacturer's instructions. The optical density (OD) of wells was measured at 450 nm. Samples were considered positive if their OD value was equal to or lower than the positive cut off and negative if their OD value was equal to or higher than the negative cut off. The positive results were confirmed by INgezim BTV DR immunoenzymatic assay (Ingenasa, Madrid, Spain).

Virus genome analysis. Two Hungarian BTV strains isolated from cattle during 2015 were selected for sequencing. Direct sequencing was carried out using published oligonucleotide primers (Eschbaumer *et al.*, 2011). The obtained partial sequence of the VP2 coding gene (540 bp) was used for phylogenetic analysis. BLAST analysis was carried out to compare nucleotide sequence of Hungarian BTV strains to other BTV strains with available sequence data. Multiple sequence alignments were generated by the Translator X online platform (<http://translatorx.co.uk/>), then phylogenetic analysis was performed with the MEGA 6 software (<https://www.megasoftware.net/>) to determine genetic relatedness among BTV strains. The best-fit substitution model with the lowest Bayesian information criterion value for each data set was used to construct maximum-likelihood tree. The tree topologies were validated by bootstrap analysis (1000 replicates). In order to identify possible changes within the genome, one BTV-4 isolate from 2015 was selected for whole genome sequencing. In brief, random-primed RT-PCR assay was used for the amplification of the whole genome that was followed by next generation sequencing using the Ion Torrent platform as described in detail elsewhere (Bányai *et al.*, 2014).

Results

Among the 47,273 samples tested by qRT-PCR, 424 (0.9%) gave positive results. The Ct values ranged from 15.98 to 39.98 (mean Ct value, 34.3; median Ct value, 34.9). Most samples were collected from cattle (42,841), of which 333 (0.8%) were positive for BTV. The majority of positive animals (n = 254; 76.3%) originated from the south-western regions (i.e. Baranya, Somogy, Tolna counties). Concerning ovine specimens, out of 3608 samples 79 (2.2%) were found to be positive, however, all of these were sampled from two counties, Baranya (n = 21) and Somogy (n = 58). All four BTV positive goat samples (0.9%) were collected from Baranya county. None of the 43 buffalo blood samples tested for BTV RNA gave positive results. Three positive cases (2.5%) were detected out of 119 samples from other ruminants, which were also involved in the study. One of these positives was collected from a mouflon (2.3%) (Table 1). The large number of positive samples in the south-western

Table 1. County-based detection of BTV RNA and antibody in Hungary, 2015

	Number of positive samples											
	Cattle		Sheep		Goat		Buffalo		Biting midge	Other animal		
	Ab	RNA	Ab	RNA	Ab	RNA	Ab	RNA	RNA	Ab	RNA	
Baranya	454	48	132	21	62	4	0	0	0	0	0	0
Borsod-Abaúj-Zemplén	83	6	1	0	0	0	0	0	1	0	0	0
Bács-Kiskun	464	7	2	0	1	0	4	0	0	0	0	0
Békés	150	0	0	0	0	0	0	0	0	0	0	0
Csongrád	113	7	0	0	0	0	0	0	1	0	0	0
Fejér	226	8	0	0	0	0	0	0	0	0	0	0
Győr-Moson-Sopron	13	1	2	0	0	0	0	0	0	0	0	0
Hajdú-Bihar	68	4	1	0	0	0	1	0	0	0	0	0
Heves	26	0	1	0	0	0	0	0	0	0	0	0
Jász-Nagykun-Szolnok	36	1	4	0	0	0	0	0	1	0	0	0
Komárom-Esztergom	0	0	0	0	0	0	0	0	0	0	0	0
Nógrád	220	34	0	0	0	0	0	0	0	0	0	0
Pest	284	7	5	0	0	0	0	0	1	0	3*	0
Somogy	416	137	352	58	53	0	6	0	0	0	0	0
Szabolcs-Szatmár-Bereg	122	3	0	0	0	0	0	0	0	0	0	0
Tolna	405	69	17	0	0	0	0	0	0	0	0	0
Vas	10	0	0	0	0	0	0	0	0	0	0	0
Veszprém	46	1	0	0	0	0	5	0	0	0	0	0
Zala	22	0	0	0	0	0	0	0	1	0	0	0
Total	3158	333	517	79	116	4	16	0	5	0	3	0

*One mouflon and two unidentified species.

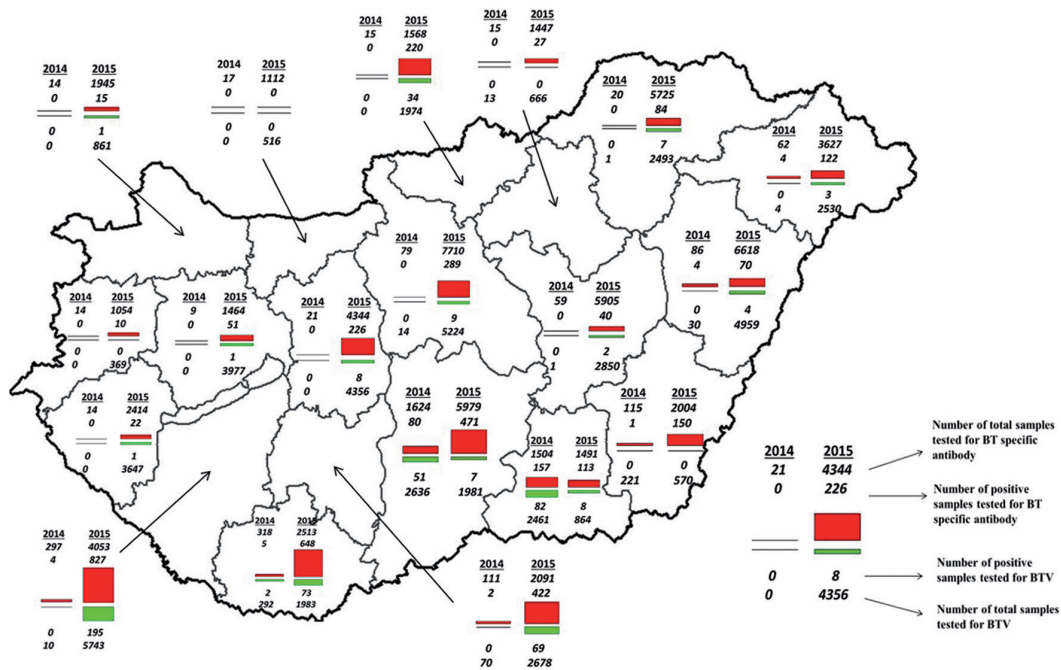


Fig. 1

Geographic distribution of BTV in 2014 and 2015, Hungary

The green and red rectangles represent the number of qPCR- and antibody ELISA-positive animals, respectively. The number of animals tested by both methods is also shown in each county.

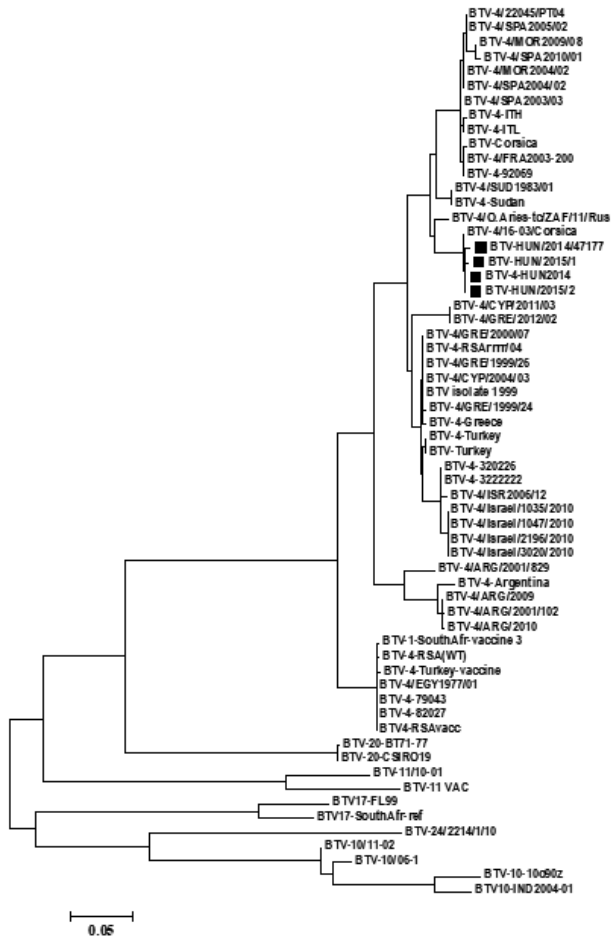


Fig. 2

Phylogenetic analysis of BTV strains using nucleotide sequences of Segment-2

Hungarian BTV strains are highlighted with black squares. The scale bar is proportional to the genetic distance.

counties was related to outbreaks first reported in Tolna in September 2015 (http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/WI). Additionally, viral RNA was detected in five biting midges (*Culicoides* sp.), each collected from a different county (Table 1).

During 2015, out of 63,146 samples tested for the presence of BTV-specific antibodies, 3807 (6%) proved to be positive. In cattle, out of 57,250 samples, 3158 were seropositive (5.5%). At least 10 seropositive cattle were detected in each county, except for Komárom-Esztergom, where no animals positive for BTV antibody were identified at all. Higher seropositivity rate was observed in sheep (517 positive out of 5120 samples; 10.1%) and goat (116 positive out of 290 samples; 40%), and positive samples originated mainly from Baranya (sheep, n = 132; goat, n = 62) and Somogy (sheep, n = 352; goat, n = 53) counties. The prevalence of BTV-specific antibodies in buffalo was 5.6% (16 positive out of 284 samples) (Table 1).

In 2015, at least one BTV-4 outbreak was reported in almost every county, indicating the virus circulation among ruminants. In comparison with the data observed in 2014, fewer BTV-positive animals were identified in Bács-Kiskun county and Csongrád county. In addition, a relatively high number of BTV-positive animals was reported in Nógrád county. Moreover, simultaneously with the dissemination of BTV, the seroprevalence among livestock showed marked increase (Fig. 1).

Comparison of the nucleotide sequences of Segment-2 of Hungarian BTV strains detected in 2015 and the reference BTV strains with sequence data available in the GenBank showed moderate-to-high sequence similarities and 100% nt sequence identity with two earlier Hungarian isolates, BTV4-HUN2014 and BTV-HUN/47177 (Fig. 2). Shotgun whole genome sequencing of a selected BTV-4 isolate determined

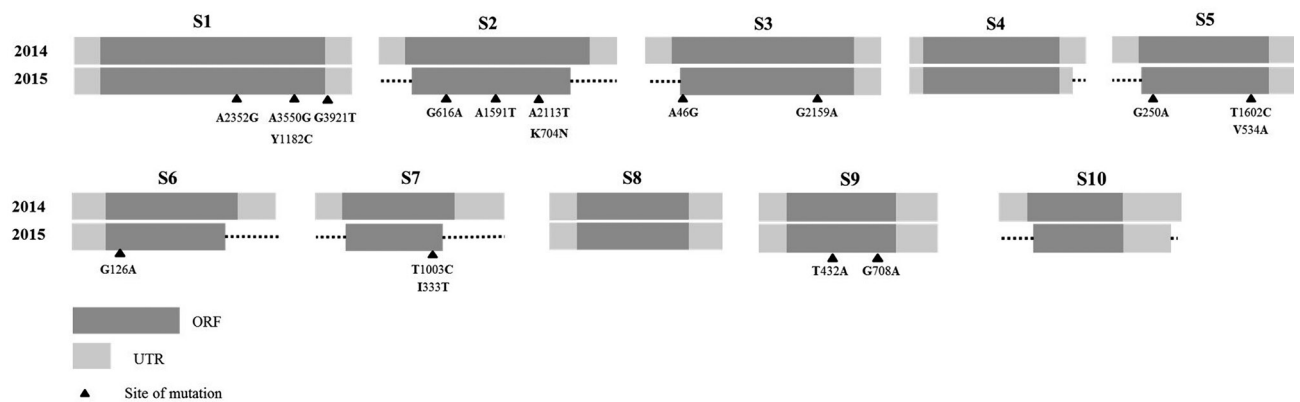


Fig. 3

Sequence variation between representative BTV strains isolated in 2014 and 2015 in Hungary

Black triangles represent the site of mutation in the open reading frame (ORF) and the untranslated region (UTR). Only mutations resulting in amino acid changes are indicated.

the full-length coding region of genome segments S1, S4, S5 and S8, whereas partial genome sequences were determined for the remaining segments (S2, 94.9%; S3, 99.1%; S6, 99.1%; S7, 95%; S9, 99.1%; S10, 99.3%). We identified 14 mutation sites in this strain when compared to the BTV-4 strain detected in the previous year in Hungary. Thirteen nucleotide mutations were located in the coding regions, out of which four point mutations resulted in amino acid changes (Fig. 3). Overall, 18,624 nucleotides were determined, which represents 97.1% of the whole genome.

Discussion

In 2014 a novel, multireassortant BTV-4 emerged in Hungary causing BT epizootics, which coincided with a large outbreak in the Balkan Peninsula (Hornýák *et al.*, 2015). Since the first occurrence of BT in the country, nucleic acid detection and serologic assays were carried out in order to monitor the spread of the disease and detect circulating virus strains. In September 2015, re-emergence of the disease was reported in Tolna and Somogy counties and the outbreak expanded to north, reaching Győr-Moson-Sopron county in November, the most distant county from Csongrád and Bács-Kiskun counties, where BT epizootics restricted in 2014. Genetic analysis of selected 2015 BTV isolates identified their high genetic relatedness to the BTV-4 strain reported from the 2014 outbreak in Hungary (Hornýák *et al.*, 2015). This finding suggested that BTV-4 could have overwintered in infected competent vectors or a ruminant host. In addition, sequencing and phylogenetic analysis showed high genetic relatedness among Hungarian BTV-4 strains from 2014 and 2015 and those, which emerged in Corsica in November 2016 (Sailleau *et al.*, 2017). All 10 genomic segments of the Hungarian BTV strain were closely related to the BTV-4 strain introduced to Corsica from Sardinia, a finding that is consistent with the theory that BTV-4 incursions to Europe are linked to outbreaks in North Africa (Sailleau *et al.*, 2017).

Wild ungulates may play an important role in the transmission and maintenance of BTV (Ruiz-Fons *et al.*, 2014; Niedbalski, 2015) and in this surveillance study we found a BTV-positive mouflon, providing evidence for the infection in wild mammals. Furthermore, we provided evidence for biting midges carrying the virus. As vaccination and movement restrictions can only be applied in farmed ruminants, it is essential to extend our knowledge on the relationship between wild ruminants and BTV in Hungary.

In conclusion, the detection of BTV genomic RNA in different geographic regions and the increased seroprevalence demonstrates that resurgence of the virus was likely associated with the overwintering of a multireassortant BTV-4 strain that emerged in 2014 in Hungary. Epidemiologic surveillance also provided evidence for vector competence

of the local populations of biting midges as well as the potential role of wildlife reservoirs in disease maintenance. These findings along with adequate implementations of control measures and continued epidemiologic monitoring of ruminant population may help understanding the geographic dispersal of BT disease and the success of vaccination campaigns in Hungary.

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