

# Re-emergence of BTV serotype 4 in North Macedonia, July 2020

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## Abstract

Bluetongue virus serotype 4 (BTV-4) was confirmed in sheep in North Macedonia in July 2020. The full genome of this BTV-4 strain (MKD2020/06) was shown to be most closely related (99.74% nt identity) to the Greek GRE2014/08 and the Hungarian HUN1014 strains, indicating the re-emergence of this BTV serotype in the Balkan region since it was last reported in 2017.

## KEYWORDS

Bluetongue virus, Macedonia, outbreak

Bluetongue (BT) is a haemorrhagic disease of ruminants which is caused by the BT virus (BTV), the type species of the genus *Orbivirus* within the *Reoviridae* (Maclachlan et al., 2009). BTV is typically transmitted by haematophagous *Culicoides* species which can cause the disease to spread vast distances (Carpenter et al., 2009). There are 24 classical serotypes of BTV (BTV-1 to BTV-24) which are notifiable to the World Organization for Animal Health (OIE) (Kundlacz et al., 2019). In June 2014, a new BTV-4 strain was detected in southern Greece and BT spread throughout the Balkan Peninsula in what was then a BTV-4 naïve ruminant population. The highest morbidity (8.5%) and case fatality rates (37%) were observed in sheep, followed by goats and cattle (Kyriakis et al., 2015) although the severity of illness was not consistent throughout the Balkan countries (Katsoulos et al., 2016). The BTV-4 outbreak extended beyond the Balkan countries to Austria in 2016 and France in 2017 (OIE). The last recorded outbreak of BTV-4 from the Balkan countries was reported to the OIE in 2017 with an outbreak occurring in the Zlatiborski region of Serbia.

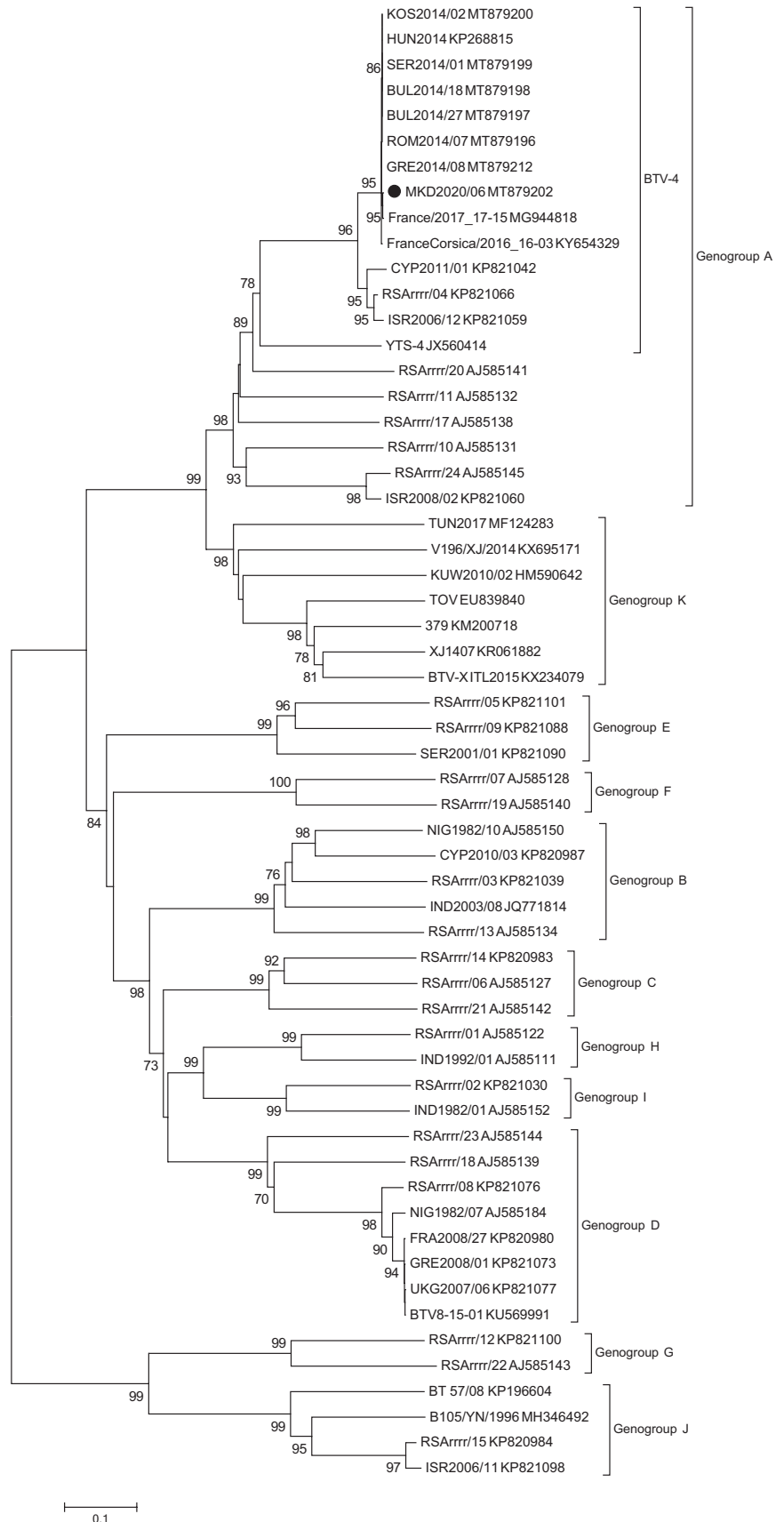
In July 2020, Macedonian veterinarian authorities notified the OIE of BT in sheep in the Makedonski Brod and Kicevo

municipalities. Farmers notified veterinary authorities of suspected BT in sheep on their small holdings, which were located in the same region as the initial report of BT in 2014. On the 8th July, an official veterinarian visited 3 holdings and found that the sheep were exhibiting classical signs of BT such as facial oedema, fever, lameness and recumbency. Twelve EDTA blood samples were taken and were sent to the Faculty of Veterinary Medicine in Skopje who detected BTV-4 using RT-qPCR and partial sequence analysis of BTV segment 2. EDTA blood samples ( $n = 6$ ) from the three holdings were submitted to the OIE reference laboratory for BT at The Pirbright Institute, UK, for confirmatory diagnosis. Nucleic acid was extracted from EDTA blood samples using the LSI MagVet Universal isolation kit (ThermoFisher Scientific) on the KingFisher Flex (ThermoFisher), and BTV was detected using an RT-qPCR assay targeting BTV segment 10 (Hofmann et al., 2008). BTV serotype identification was performed using an assay targeting BTV segment 2 (Maan et al., 2016) for serotype BTV-1, BTV-4, BTV-8 and BTV-9. BTV was isolated on *Culicoides sonorensis* KC cells, following which full-genome sequencing was performed using the Nextera XT DNA Library Prep kit (Illumina) on the Illumina MiSeq

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**FIGURE 1** Phylogenetic relationship of BTV-4 (MKD2020/06) from an outbreak in Northern Macedonia in 2020 (identified by black circle) to publicly available BTV isolates based on segment 2 of the genome. A neighbour-joining tree (1,000 bootstrap replicates) was constructed using the MEGA7.0 software



platform. Sequences were assembled using the BWA-MEM tool (Li & Durbin, 2010) as previously described (Rajko-Nenow et al., 2020), and a neighbour-joining tree of BTV segment 2 was built using the

default settings in MEGA7.0 software (Kumar et al., 2016). The full-genome sequence of isolate MKD2020/06 has been deposited in GenBank under accession numbers MT879201–MT879210.

Segment	Length (bp)	GenBank accession no.	Strain ID	%nt identity
1	3,944	MG944817	France/2017	99.82
		KP268814	HUN2014	99.72
		KY654328	FranceCorsica/2016	99.70
		MT879211	GRE2014/08	99.77
2	2,926	MG944818	France/2017	99.76 <sup>a</sup>
		KP268815	HUN2014	99.66
		KY654329	FranceCorsica/2016	99.56
		MT879212	GRE2014/08	99.73
3	2,772	MG944819	France/2017	99.89 <sup>a</sup>
		KP268816	HUN2014	99.82
		KY654330	FranceCorsica/2016	99.78
		MT879213	GRE2014/08	99.86
4	1,981	MG944820	France/2017	99.85
		KP268817	HUN2014	99.90
		KY654331	FranceCorsica/2016	99.70
		MT879214	GRE2014/08	99.90
5	1,774	MG944821	France/2017	99.83
		KP268818	HUN2014	99.89
		KY654332	FranceCorsica/2016	99.89
		MT879215	GRE2014/08	99.89 <sup>a</sup>
6	1,637	MG944822	France/2017	99.57 <sup>a</sup>
		KP268819	HUN2014	99.39
		KY654333	FranceCorsica/2016	99.45
		MT879216	GRE2014/08	99.39
7	1,156	MG944823	France/2017	99.83
		KP268820	HUN2014	100
		KY654334	FranceCorsica/2016	99.91
		MT879217	GRE2014/08	99.65 <sup>a</sup>
8	1,125	MG944824	France/2017	99.73 <sup>a</sup>
		KP268821	HUN2014	99.73
		KY654335	FranceCorsica/2016	99.64
		MT879218	GRE2014/08	99.73
9	1,049	MG944825	France/2017	99.70 <sup>a</sup>
		KP268822	HUN2014	99.62
		KY654336	FranceCorsica/2016	99.52 <sup>a</sup>
		MT879219	GRE2014/08	99.62
10	822	MG944826	France/2017	99.88
		KP268823	HUN2014	99.64
		KY654337	FranceCorsica/2016	99.64
		MT879220	GRE2014/08	99.51

<sup>a</sup>Query cover < 100%.

All samples ( $n = 6$ ) were positive using the segment-10 RT-qPCR assay ( $C_T$  range 18.75–23.10) and using the BTV-4 serotype-specific RT-qPCR assay confirming the clinical diagnosis and the re-emergence of this serotype in the Balkan region since it was last reported in 2018. No amplification was observed for the

**TABLE 1** The per cent of nucleotide sequence (%nt) identity for individual segments of BTV-MKD2020/06 with the closest matching BTV sequences in GenBank

segment-2-targeted RT-qPCR assays for BTV-1, BTV-8 or BTV-9. A phylogenetic analysis of segment 2 showed the Macedonian 2020 isolate (MKD2020/06) was most closely related to BTV-4 field strains isolated during the previous Balkan BTV-4 epizootic (Figure 1). Pairwise alignments of full genomes were performed

on EMBOSS Needle ([https://www.ebi.ac.uk/Tools/psa/emboss\\_needle/](https://www.ebi.ac.uk/Tools/psa/emboss_needle/)) using default settings. Across all 10 segments, MKD2020/06 shared 99.74% nucleotide (nt) identity with BTV-4 field strains from Hungary (HUN2014) and Greece (GRE2014/08) in 2014. A BLAST analysis of BTV segment 2, the determinant of BTV serotype, against the available BTV-4 segment-2 sequences revealed that MKD2020/06 was most closely related to: France/2017 (BTV-4/17-15) (99.76%), HUN2014 (99.66%), GRE2014/08 (99.73%) and FranceCorsica/2016 (BTV-4/16-03) (99.56%). The sequences of the remaining segments were also subjected to a BLAST analysis to find the closest relative(s) (Table 1). Based on the %nt identity and the identification of the closest relative in individual segments, MKD2020/06 is a re-emergent BTV-4 strain rather than a novel reassortant virus.

Small ruminant production has historically been an important part of the Macedonian economy. Approximately 44% of the population live in rural regions with farming (typically in smallholdings) providing a dominant part of the agriculture sector (Food & Agriculture Organization of the United Nations, 2020). Indeed, in 2019 there were approximately 685,000 sheep and 87,500 goats (Republic of North Macedonia State Statistical Office, 2020). Transboundary diseases such as BT present a significant concern as they not only have a direct impact on the health and welfare of livestock but also endanger the sustainability of farming and ultimately food security. While the Macedonian BTV-4 strain has not yet been associated with mortalities in sheep, concomitant infections can exacerbate BT in the field (Kyriakis et al., 2015); thus, this BTV-4 strain may yet have an impact on the health status of sheep in affected flocks. The re-emergence of BTV-4 should be of concern to the Balkan and neighbouring countries as the possibility exists that it may take the course of the previous epizootic in 2014 whereby six countries were affected within 4 months of the outbreak with significant animal health impacts and trade restrictions.

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#### ETHICAL APPROVAL

This investigation was performed at the Pirbright Institute in their capacity as an OIE Reference laboratory for BT and as such, no ethical approval was required for the work carried out.

#### CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study have been deposited in GenBank under accession numbers MT879201–MT879210 (MKD2020/06 segment 1–10), MT879211–MT879220 (GRE2014/08 segment 1–10), MT879196 (ROM2014/07 Segment 2), MT879197

(BUL2014/27 Segment 2), MT879198 (BUL2014/18 Segment 2), MT879199 (SER2014/01 Segment 2) and MT879200 (KOS2014/02 Segment 2).

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