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Paper

Development and validation of a real time RT-qPCR assay for detection of the emerging Bluetongue virus serotype 5 from field samples

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Abstract

Bluetongue (BT) is a WOAH-notifiable economically important disease of ruminants caused by Bluetongue virus (BTV), transmitted by *Culicoides* spp. biting midges. Over the past two years, Italy has experienced a marked re-emergence of BT, with thousands of outbreaks reported due to the simultaneous circulation of several BTV strains belonging to serotypes 3, 4, and 8. Moreover, in September 2025, BTV-5 was detected in Sardinia, marking its first occurrence in Europe. Following the first identification by Whole Genome Sequencing, the development of a reliable real-time RT-qPCR-based assay capable of typing the novel BTV-5 ITA 2025 strain was essential, as currently available molecular typing methods targeting BTV segment 2, which encodes the outer capsid protein VP2, are unable to detect this newly emerging strain. Therefore, in this study we developed, optimised, and validated a real-time RT-PCR assay for the detection and typing of BTV-5 ITA 2025 in field samples. The assay is characterised by high sensitivity and specificity, as well as good reproducibility, and can be effectively applied for BTV-5 ITA 2025 diagnosis in the current epidemiological context, supporting surveillance and control strategies.

Keywords

Bluetongue, BTV-5, real time RT-qPCR, typing, validation

Introduction

Bluetongue (BT) is a WOAH-listed disease affecting domestic and wild ruminants that continues to cause considerable economic losses in the livestock industry, caused by the bluetongue virus (BTV), an arbovirus transmitted by *Culicoides* spp. biting midges. BTV is a member of the genus *Orbivirus*, family *Sedoreoviridae* (https://ictv.global/report/chapter/sedoreoviridae/sedoreoviridae/orbivirus) and has a triple-layered capsid, encasing a double-stranded RNA genome divided into ten segments (Seg-1 to Seg-10) that encode for seven structural proteins (VP1 to VP7) and five non-structural proteins (NS1 to NS5) (Schwartz-Cornil et al., 2008; Ratinier et al., 2011; Roy, 2017). Based on the highly variable, outermost capsid protein VP2, to date up to 36 BTV serotypes have been described, including 24 traditional BTV serotypes, associated with clinical BT and subjected to official control (Jimenez-Clavero, 2012).

During 2024-2025 seasons, in Italy the BT epidemiological dynamics were characterised by an intense co-circulation of multiple genomic constellations of BTV strains belonging to serotypes BTV-3, BTV-4, and BTV-8, several reassortment events among viruses of different lineages and the introduction of novel strains (Plebani et al., 2025). This scenario was strongly influenced by multiple factors, including Italy's geographic position, where climatic conditions create ideal ecological niches for the persistence and spread of different species of competent *Culicoides*. In addition, wind-borne incursions of these midges from North Africa across the central Mediterranean toward southern Europe, as well as intense animal movements across European borders, have contributed to this

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epidemiological framework (Calistri et al., 2004; Hammami, 2004).

The molecular diagnostic workflow for BTV includes a real time RT-PCR (RT-qPCR) assay targeting a conserved sequence of the Seg-10, in order to detect all known strains of all BTV serotypes, followed by a panel of typing RT-qPCR assays, detecting the Seg-2 and able to identify the serotype of the BTV strain responsible of infection (https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.01.03_BLUETONGUE.pdf). Typing assays are generally performed according to the prevailing epidemiological situation of a given country. In recent years, the increasingly complex epidemiological landscape observed in Italy and other European countries, characterised by the concurrent circulation of multiple viral serotypes within the same geographical areas, has posed significant challenges to diagnostic strategies. Moreover, the emergence of novel, untypable strains can lead to gaps in typing capacity due to the unavailability of specific diagnostic reagents, which require time to be developed and validated. Such delays may consequently impair the prompt implementation of appropriate preventive and control measures needed when a new BTV strain is introduced into a previously free territory.

The emergence of a novel BTV-5 strain was first documented in Italy in the Sulcis-Iglesiente province of Sardinia between late August and early September 2025. Notably, this occurred on the same island where, two months earlier, the first Italian outbreak of Lumpy Skin Disease had been reported (Marcacci et al., 2025). Spleen samples collected from two sheep displaying clinical signs consistent with BT were positive using a RT-qPCR targeting Seg-10, but negative to the subsequent serotype panel of RT-qPCR assays, specific for Seg-2 of European BTV serotypes, suggesting that the serotype responsible for the infection differed from those circulating at that time. The Center for Exotic Diseases (CESME) at IZSAM confirmed these results and to obtain further information on this strain, a spleen sample was subjected to whole genome sequencing (WGS). Analysis of the consensus sequences obtained showed the highest nucleotide identity with a strain identified in Nigeria in 1982, belonging to serotype 5, allowing the Sardinian strain to be typed as BTV-5 (GenBank accession numbers PX460302-PX460311) (Marcacci et al., 2026).

To date, 144 outbreaks involving BTV-5 were recorded in Sardinia, mainly in the southern part of the island (https://www.izs.it/BENV_NEW/datiemappe.html, accessed on February 12, 2026), but the real spread of BTV-5 could be underestimated, primarily due to undetected subclinical infections, particularly in cattle and goats.

In order to provide a suitable molecular diagnostic tool to detect the new BTV-5 ITA 2025 strain, applicable also to surveillance activities aimed at determining its actual distribution, in this paper we describe the development and validation of a RT-qPCR assay for the reliable detection of the emerging BTV-5 from field samples.

Materials and methods

Virus isolation, reference strains, field samples collection and spike-in samples

Strain BTV-5 ITA 2025 (IZSAM internal ID 2025TE23238; BTV5 2025.TE.23238.2.6) was isolated from a blood sample collected from a sheep during the 2025 BTV outbreak in Sardinia, Italy.

Virus isolation was attempted from a BTV RT-qPCR-positive EDTA blood sample by performing a first blind passage on the *Culicoides sonorensis* cell line (KC cells; RRID: CVCL_RW99), followed by two passages on African green monkey kidney cells (Vero cells; RRID: CVCL_0059).

Pre-seeded KC cell monolayers (24 h at 28°C) were infected with 0.2 mL of a lysed blood sample and incubated for 2 h at 28°C. The monolayers were then washed with serum-free medium and incubated for 10 days at 28°C in Schneider's insect medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 3% foetal calf serum. The cells were subsequently scraped, and 0.2 mL of the supernatant obtained after centrifugation was inoculated onto a pre-seeded Vero cell monolayer (24 h at 37°C with 5% CO_2) and incubated for 5 days (as first passage) at 37°C with 5% CO_2 in Minimal Essential Medium (MEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 3% foetal calf serum.

A second passage on Vero cells was performed, and a cytopathic effect (CPE) was observed after 3 days (as second passage). Viral isolation was confirmed by BTV RT-qPCR.

Endpoint virus titration of the isolate was performed on Vero cells using 10-fold serial dilutions starting from 10^{-1}. The viral titre was 7.0 $\log_{10}$ $TCID_{50}$/mL. The endpoint titre was calculated according to the method of Reed and Muench

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(1938).

BTV serotype 1 to BTV-24 and African horse sickness virus (AHSV) serotypes 1, 2, 3, 4, 6, 7, 8, and 9 were provided by Onderstepoort Veterinary Institute (OVI; Pretoria, Republic of South Africa), whereas BTV-26, AHSV-5, and epizootic haemorrhagic disease virus (EHDV) serotypes 1, 2, 4, 5, 6, 7, and 8 were supplied from Pirbright Institute, UK. BTV-25 was provided by Central Veterinary Institute of Wageningen (CVI, Lelystad, The Netherlands). The BTV-27 strain was provided from ANSES Institute (Maisons-Alfort Cedex, France). BTV-X-IT 2021 and EHDV-8/17 TUN 2021 were available at IZSAM.

The viruses were propagated and titrated in TC25 baby hamster kidney- clone BSR (BSR, CCLV-RIE 582) or in African green monkey kidney (Vero, BS-C-1 clone 86) cell cultures.

To assess diagnostic specificity, a total of 125 BTV-5 ITA 2025–negative samples were analysed, including 41 ovine, caprine, and bovine EDTA blood specimens; 41 spleen homogenates from ovine, caprine, bovine, and wild ungulates; and 43 *Culicoides* spp. pools. All samples were collected at the beginning of the 2025 BTV season, in June and July, when BTV-5 was not yet officially identified in Italy. Diagnostic sensitivity assessment was performed by analysing 57 naturally infected BTV-5 ITA 2025, including 48 ovine EDTA blood and 9 spleen samples and 25 BTV–negative *Culicoides* spp. pools spiked with the titrated BTV-5 ITA 2025 strain (Table 1).

Negative samples							**BTV-5 ITA 2025 positive samples**
Animal species / matrix	**BTV negative**	**BTV-3**	**BTV-4**	**BTV-8**	**BTV-4+BTV-8**	**BTV-25**	
Bovine blood	20	2	4	8	-	-	-
Ovine blood	1	-	-	-	-	-	48
Caprine blood	-	-	-	-	-	6	-
Bovine spleen	10	-	-	-	-	-	-
Ovine spleen	5	-	-	14	1	-	9
Caprine spleen	1	-	-	-	2	-	-
Roe deer spleen	5	-		-	-	-	-
Red deer spleen	-	-	-	1	1	-	-
Chamois spleen	-	-	-	1	-	-	-
Culicoides spp. pool	23	-	-	20	-	-	25 (spiked)
Total	65	2	4	44	4	6	83

Table I. BTV-5 ITA 2025 negative and positive samples used to determine the diagnostic specificity and sensitivity.

Primers and probe

Primers and probe design for the specific detection of BTV-5 ITA 2025 was carried out by aligning Seg-2 nucleotide (nt) sequences of the BTV-5 ITA 2025 strain (GenBank accession no. PX460303), and the BTV-5 Nigeria 1982 reference strain (AJ585182.1) using Geneious Prime® software version 2023.2.1 (Biomatters Ltd, Auckland, New Zealand). A conserved 109-bp target region (nt positions 456–564) was identified and selected for assay development, and the following primers and TaqMan probe were designed: BTV-5_ITA2025_Fw: 5'-GCCATTTCATACAGATATATATCGATAG-3', BTV-5_ITA2025_Rv: 5'-GCTCCACGTTATACATTCCACA-3' and BTV-5_ITA2025_Probe: 5'-FAM-TGAATCCAAAGGATGTGACCATACACAG-BHQ1-3'.

Primers and probe sequences were evaluated using Primer Express™ software version 3.0.1 (Thermo Fisher Scientific, Waltham, MA) to assess melting temperatures (Tm) and to exclude the presence of primer–dimers and potential secondary structures. All oligonucleotides were synthesised and purified by a commercial company (Eurofins Genomics, https://eurofinsgenomics.eu). In addition, VetMax Xeno Internal Positive Control-VIC Assay (Applied Biosystems, St. Austin, TX, USA) was included to detect exogenous internal positive control (IPC) consisting of VetMax Xeno Internal Positive Control RNA (Applied Biosystems, St. Austin, TX, USA).

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Nucleic acid purification

Total RNA was extracted from 200 µL of blood, 10% (w/v) spleen homogenates, cell culture supernatants, and spiked-in *Culicoides* spp. pools, using the MagMAX CORE Nucleic Acid Purification Kit (Applied Biosystems, St. Austin, TX, USA) on a KingFisher Flex Purification System (Thermo Fisher Scientific, MA, USA), according to the manufacturer's instructions. Two microliters of VetMax Xeno Internal Positive Control (IPC) RNA were added to each sample prior to nucleic acid extraction.

RT-qPCR

Primer and probe concentrations were optimised for a duplex RT-qPCR assay with the VetMax Xeno Internal Positive Control-VIC Assay. Amplification was performed using TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, St. Austin, TX, USA). Master mix composition and RT-qPCR conditions are shown in Table 2. After RNA denaturation at 95 °C for 5 min, 5 µL of RNA were added to 20 µL of reaction mix. RT-qPCR was run on a QuantStudio 7 Flex system (Applied Biosystems, St. Austin, TX, USA) using the following thermal profile: 50 °C for 15 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 55 °C for 30 s, with fluorescence acquired during the annealing/extension step. Samples with Ct values <40 were considered positive for BTV-5 ITA 2025, whereas a Ct <40 for the IPC confirmed the absence of PCR inhibition. Undetectable samples were reported as 'No Ct'. Quantification of the viral loads was determined based on Ct values.

Analytical specificity, analytical sensitivity and limit of detection

A panel comprising 29 BTV serotypes, including the strains belonging to the atypical serotypes BTV-25, BTV-26, BTV-27, and BTV-X-IT 2021, eight EHDV serotypes including EHDV-8/17 TUN 2021 strain, and nine AHSV serotypes was tested to evaluate the analytical specificity of the BTV-5 ITA 2025 RT-qPCR. The analytical sensitivity was determined using tenfold serial dilutions (10^{-1} to 10^{-10}), starting from 10^6 $TCID_{50/mL}$ of a titrated BTV-5 ITA 2025 cell culture supernatant. Each dilution was subjected to nucleic acid extraction and tested in triplicate by BTV-5 ITA 2025 RT-qPCR assay. Linear regression analysis was performed across the cycle threshold (Ct) values obtained for each replicate at each serial dilution. The slope derived from linear regression was used to calculate the efficiency according to the formula: $E\% = (10^{-1/slope}-1) \times 100$, the coefficient of determination (R^2) and linear dynamic range analysis. To evaluate the limit of detection (LoD), defined as the lowest $TCID_{50/mL}$ detected in 95% of replicates, twenty replicates of three dilutions, including the dilution corresponding to the analytical sensitivity, were subjected to extraction and analysed using the BTV-5 ITA2025 RT-qPCR.

Reagents	Concentration	Volume/reaction
BTV-5_ITA2025_Fw	800 nM	1 µL
BTV-5_ITA2025_Rev	800 nM	1 µL
BTV-5_ITA2025_Probe	400 nM	0.5 µL
VetMax Xeno Internal Positive Control-VIC Assay	-	1 µL
TaqMan Fast Virus 1-Step Master Mix	4x	6.25 µL
Nuclease free water	-	10.25 µL

Table II. Table 2. BTV-5 ITA 2025 typing RT-qPCR master mix composition

Diagnostic specificity and diagnostic sensitivity

To assess the presence of BTV, all the samples included in this study were first screened using a RT-qPCR assay simultaneously detecting both BTV and EHDV RNA (pan-BTV/pan-EHDV; Portanti et al., 2025). Furthermore, samples testing positive for BTV RNA were then serotyped using: i) the VetMAX European BTV Typing Kit (Applied Biosystems, St. Austin, TX, USA), capable of detecting serotypes 1, 2, 4, 6, 8, 9, 11, and 16 circulating or previously circulating in the Mediterranean basin; ii) an in-house RT-qPCR assay targeting Seg-2 of BTV-3 (Lorusso et al., 2018);

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iii) the ID Gene™ Bluetongue Genotype 12 Duplex (Innovative Diagnostics, Grabels, France), a RT-qPCR kit for the qualitative detection of BTV-12 RNA and iv) atypical serotypes, including BTV-25 (Hofmann et al., 2010), BTV-26 (Maan et al., 2011), BTV-27 (Zientara et al., 2014), and BTV-X -IT2021 (Spedicato et al., 2023). Specimens that tested positive for BTV by pan-BTV/pan-EHDV, but negative for all the tested serotypes were considered putatively positive for BTV-5 ITA 2025, based on WGS results obtained from representative Sardinian spleen samples. While not all untypable samples were confirmed by WGS, epidemiological and molecular evidence strongly supports their classification as BTV-5, although this assumption may introduce a minor overestimation of diagnostic sensitivity.

Diagnostic specificity (dSp) was determined by assessing a panel of 125 BTV-5 ITA 2025–negative samples including 41 ovine, caprine, and bovine EDTA blood specimens; 41 spleen homogenates from ovine, caprine, bovine, and wild ungulates; and 43 *Culicoides* spp. pools, all collected during a period when no BTV-5 circulation had been reported in Italy. Among these samples, 65 were BTV-negative, whereas 60 specimens tested positive for BTV-3, BTV-4, BTV-8, or BTV-25, or were co-infected with BTV-4 and BTV-8. In addition, 57 naturally infected BTV-5 ITA2025 samples, including 48 ovine EDTA blood and nine spleen samples and twenty-five BTV–negative *Culicoides* spp. pools spiked with the titrated BTV-5 ITA 2025 strain were analysed to determine diagnostic sensitivity (dSe) (Table 1).

In addition to dSp and dSe, accuracy and Cohen's kappa index were estimated. Confidence intervals were calculated using binomial distribution.

Repeatability

Repeatability of BTV-5 ITA 2025 RT-qPCR was assessed by calculating the intra- and inter-assay coefficients of variation (CVs). A panel of 12 positive samples with different viral loads (Ct range: 22-35) comprising 3 ovine blood samples, 3 ovine spleen specimens, and 6 *Culicoides* spp. pools spiked with titrated BTV-5 ITA 2025 strain was tested in ten replicates across two independent RT-qPCR runs. CVs were calculated for each sample and were defined as follows: intra-assay CV = (average of each session Ct standard deviation (SD)/average of each session Ct average) x 100, whereas inter-assay CV = (SD of the Ct averages for each session/average of the averages for each session) x 100.

Results

Analytical specificity, analytical sensitivity and limit of detection

The analytical specificity of the BTV-5 ITA 2025 RT-qPCR was investigated on BTV, EHDV, and AHSV serotypes. The results showed that specific amplification curves were produced only for BTV-5 ITA 2025, while no amplification was observed for the other viruses investigated.

Regarding the analytical sensitivity, the assay was able to detect RNA up to a dilution corresponding to 10^2 $TCID_{50/mL}$. The generated standard curve showed a strong linear correlation over the range from 10^6 $TCID_{50/mL}$ to 10^2 $TCID_{50/mL}$. Linear regression analysis revealed a slope = -3.36, coefficient of determination (R^2)of 0.993 and an efficiency of amplification (E%) of 98.4% (Figure 1). The LOD (95%) of the assay was 10^2 $TCID_{50/mL}$ (Table 3).

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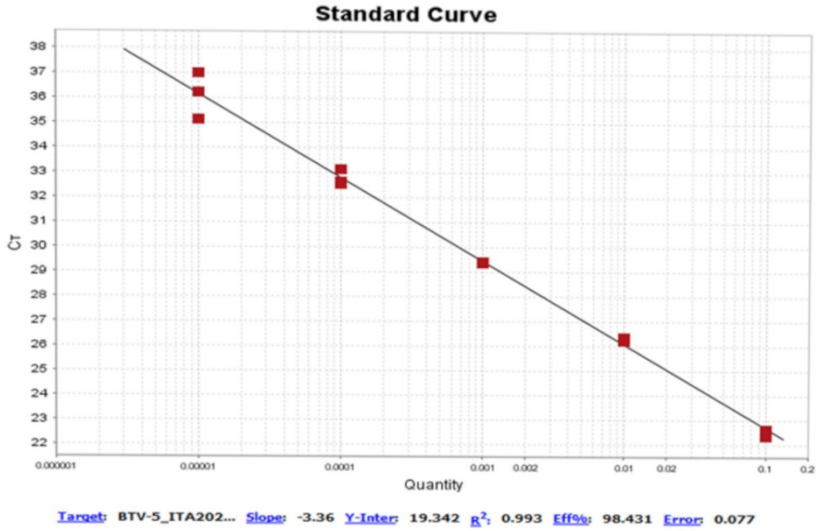

Figure 1. BTV-5 ITA 2025 RT-qPCR standard curve.

BTV-5 ITA 2025 RT- qPCR (Ct)			
Replicates	**10^{3} $TCID_{50/mL}$ (dilution 10^{-4})**	**10^{2} $TCID_{50/mL}$ * (dilution 10^{-5})**	**10^{1} $TCID_{50/mL}$ (dilution 10^{-6})**
1	32.6	36.5	no Ct
2	33.3	37.1	no Ct
3	33.5	37.3	no Ct
4	33.3	36.8	no Ct
5	33.3	37.2	36.1
6	32.2	35.7	no Ct
7	32.6	38.4	33.3
8	32.8	37.6	no Ct
9	32.9	37.5	no Ct
10	33.4	36.9	36.3
11	33.7	37.5	no Ct
12	34.2	37.1	no Ct
13	32.7	37.3	37.6
14	32.8	36.0	no Ct
15	33.0	37.2	no Ct
16	33.3	36.8	no Ct
17	33.0	36.4	no Ct
18	32.4	35.5	no Ct
19	33.0	no Ct	no Ct
20	33.4	37.2	no Ct

Table III. Limit of detection of BTV-5 ITA 2025 RT-qPCR. *LOD (95%) was 102 TCID50/mL

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Diagnostic specificity and diagnostic sensitivity

All the 57 positive ovine blood and spleen samples and twenty-five BTV–negative *Culicoides* spp. pools that were spiked, tested positive for BTV-5 ITA 2025 using the developed RT-qPCR assay, whereas no fluorescence signal was detected in the 125 BTV-5 ITA 2025 negative samples. The assay demonstrated 100% diagnostic sensitivity (95% confidence interval (CI): 93.8%-100.0%) and 100% diagnostic specificity (95% CI: 97.7%-100.0%), achieving an overall accuracy of 100% (95% CI: 98.6%–100%) and a Cohen's kappa index of 1 (Table 4).

BTV-5 ITA 2025 RT- qPCR	
Target	Seg-2
Slope	-3.36
R ^{2}	0.993
Efficiency %	98.4
LOD($TCID_{50/mL}$)	$10^{\wedge 2}$
Diagnostic Specificity (Sp)%	100
CI* at 95% of Sp	97.7%-100.0% (n = 125)
Diagnostic Sensitivity (Se)%	100
CI* at 95% of Se	93.8-100% (n = 82)
BTV-5 ITA 2025 Repeatability(Intra-assay variation) (CV**)%	0.26-1.66
BTV-5 ITA 2025 Reproducibility(Inter-assay variation) (CV**)%	0.82-1.73
IPC Repeatability(Intra-assay variation) (CV**)%	0.16-1.34
IPC Reproducibility(Inter-assay variation) (CV**)%	0.46-3.65

Table IV. Validation data of BTV-5 ITA 2025 RT-qPCR. * CI: confidence interval; **CV: coefficient of variation

Repeatability

Using the BTV-5 ITA 2025 typing RT-qPCR, consistent results were obtained for the tested panel across all replicates. For the BTV-5 ITA 2025 target, the intra-assay CV ranged from 0.26% to 1.66%, while the inter-assay CV ranged from 0.82% to 1.73%. For the IPC target, the intra-assay CV ranged from 0.16% to 1.34%, while the inter-assay CV ranged from 0.46% to 3.65%.

Discussion

Climate change and animal movements are widely recognised as key drivers of the global BTV distribution and epidemiology (Alkhamis et al., 2020). In addition, in southern Europe, the geographical proximity to North Africa has facilitated the cross-continental spread of infected *Culicoides* biting midges, occasionally transported over long distances by wind currents and sandstorms. This phenomenon has led to the introduction of novel BTV serotypes, which may rapidly spread among immunologically naïve animal populations (Lorusso et al., 2013; Lorusso et al., 2014; Lorusso et al., 2017; Cappai et al., 2019; Sghaier et al., 2022; Lorusso et al., 2023; Martinez et al., 2025; Marcacci et al., 2026).

At the end of August 2025, the emergence of BTV-5, which had never previously been detected in Italy, posed a significant diagnostic challenge, highlighting the urgent need for a molecular tool capable of rapidly and specifically

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detecting this new strain to enable prompt measures to prevent its spread.

Considering the incursion pathways reported since 2000 for several strains of BTV (BTV-1, BTV-2, BTV-3, and BTV-4) and epizootic haemorrhagic disease virus serotype 8 (EHDV-8) (Lorusso et al., 2013; Lorusso et al., 2014; Lorusso et al., 2017; Cappai et al., 2019; Sghaier et al., 2022; Lorusso et al., 2023; Martinez et al., 2025), the emergence of the novel BTV-5 strain detected in Sardinia, Italy, during the summer of 2025 (Marcacci et al., 2026) was hypothesised to be linked to wind-borne dispersal of infected Culicoides vectors from North Africa. This scenario was further supported by the official notification by Tunisia of BTV-5 circulation through the WOAH WAHIS platform in January 2026 (https://wahis.woah.org/#/in-review/7169), which reported that the virus had been present in the country since at least August 2025. Notably, the Tunisian BTV-5 identification was confirmed by molecular tools available at IZSAM, including the RT-qPCR for BTV-5 ITA 2025 and WGS (data not shown).

Although BTV-5 ITA 2025 could be detected by the screening RT-qPCR targeting Seg-10, no serotype-specific RT-qPCR assays targeting Seg-2, which were required for strain typing, were available. Consequently, the first identification of Italian BTV-5 was performed on spleen samples using a SISPA-orbivirus protocol based on random-tagged primers and orbivirus-specific tagged primers, followed by Illumina sequencing (Marcacci et al., 2026). Based on the obtained Seg-2 sequence, a specific set of primers and TaqMan probe capable of amplifying the BTV-5 ITA 2025 RNA was designed, and a reliable RT-qPCR for the typing of this strain was developed, optimised, and validated. We propose this assay as a reliable molecular method for the detection of BTV-5 ITA 2025. The assay showed good performance for diagnostic sensitivity and specificity, as all 28 BTV serotypes, as well as other orbiviruses included in this study tested negative, with positive results exclusively obtained for BTV-5 ITA 2025. Furthermore, the inclusion of an internal positive control for nucleic acid extraction and amplification reduced the risk of false-negative results associated with the inhibition of enzymatic reactions or technical errors. Finally, testing of samples in ten replicates across different runs demonstrated good reproducibility, thereby increasing confidence in the assay performance.

Primers and probe set previously developed for the identification of serotype 5 strains are not suitable for the typing of the strain currently circulating in Italy (Kirkland et al., 2022), due to the substantial level of sequence variation that can occur in Seg-2 among BTV isolates of the same serotype originating from different geographic regions, with nucleotide (nt) divergence of up to 32% (Maan et al., 2012). Such variability is likely to result in mismatches within primer or probe binding sites. Therefore, careful attention should be given to the design and re-evaluation of primers and probes to ensure reliable detection of currently circulating strains. For example, there is only 85.7% Seg-2 nucleotide identity between the Italian strain and the isolate USA2003/FL 280559-7 (KX164060.1) detected in Florida, USA in 2003 in cattle blood, or 84.7% Seg-2 nt identity with the strain OV1688_FL_BTV5 OC1 (PQ625403.1) identified in 2022 in a spleen sample of a white-tailed deer in USA (Marcacci et al., 2026).

In conclusion, this assay provides a valuable tool for the rapid, sensitive, and specific identification of BTV-5 strains currently circulating in Italy and can be employed in disease surveillance programs and to support control measures, which, in turn, can help prevent economic losses in the livestock industry.

Ethical approval

No ethical authorisation was required. All included samples were collected for routine testing and surveillance programmes for BTV.

Conflict of interest

The authors declare no conflicts of interest. They declare that no competing interests exist. Mention of trade names or commercial products in this article is solely for providing specific information and does not imply recommendation or endorsement by the IZSAM.

Author Contributions

Andrea Palombieri: Methodology, Investigation, Formal analysis, Validation, Writing – original draft**;** Maurilia Marcacci: Methodology, Software, Formal analysis, Data curation, Validation; Gardenia Gatta: Investigation, Data curation; Alessandra Leone: Methodology, Investigation; Liana Teodori: Methodology, Investigation; Eugenia Ciarrocchi: Investigation, Data curation; Mariassunta Iannetta: Investigation, Data curation; Nicola D'Alterio: Project

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administration, Funding acquisition, Supervision; Massimo Spedicato: Methodology, Investigation, Data curation; Alessio Lorusso: Conceptualisation, Project administration, Funding acquisition, Writing – review & editing, Supervision; Ottavio Portanti: Conceptualisation, Methodology, Investigation, Formal Analysis, Writing –review & editing, Supervision, Validation, Data curation.

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