rRT-PCR ASSAYs FOR TYPING BLUETONGUE VIRUS: notifiable serotypes (1 to 24) (Maan *et al.* 2016)

Date: 20/12/2024	Document code: GL-LCV-15	*Rev. 01*

1. PURPOSE

The purpose of this methods is to rapidly provide Bluetongue virus (BTV) serotype identification by using twenty-eight real time reverse transcription polymerase chain reaction assays (rRT-PCR) assays targeted to the segment-2 (VP2) of the virus genome.

2. SCOPE

The method is applied to specifically detect BTV RNA in clinical samples from ruminants, such as EDTA-blood or tissue homogenates, and inoculated cell culture. These methods should be applied on samples or viral suspension previously confirmed as positive to BTV genome by a rRT-PCR serogroup specific (first step of the BT diagnosis).

The rRT-PCRs described in this procedure are used to determine the notifiable serotypes (1 to 24). RT-PCRs for atypical strains are not included in the scope.

3. MATERIALS AND EQUPMENTS

Material

Disposable hand gloves

Microcentrifuge tubes (1,5 - 2 ml), PCR clean, RNase free

Centrifuge tubes (12 ml)

96-well PCR plate

Pippete filter tips, sterile (10µl, 100-200µl and 1000µl)

PCR water

Optic sealing film for real time PCR

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Reagents (see description in point 4. Reagents)

rRT-PCR Kit

Primers

Probes

Equipment

Adjustable micropipettes (full range)

Laminar flow cabinet

Ice or Labtop Cooler

Real time Thermal cycler

Refrigerator (-20ºC)

Micro-centrifuge (up to 15000 rpm)

Vortex mixer

4. REAGENTS

Amplification kit

AGPATH-ID ONE-STEP RT-PCR KIT (for fast rRT-PCR)	
Components:	RT-PCR Buffer RT-PCR Enzyme Mix Nuclease-free water

rRT-PCR AgPath-ID™ One-Step RT-PCR (Thermo Fisher Scientific) Kit manual

NOTE: several one step real-time RT-PCR kits are commercially available, which can be used depending upon local/case-specific requirements and equipment available.

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Primers Forward F) / Reverse (R) and Probes (P) (Maan *et al.* 2016)

BTV serotype	Oligo name	Oligo sequence (5´- 3´)
BTV-1 Eastern(e) & Western(w) strains	BTV-1/2604-2627P(w)	CCGATCACACATCCGAACAAATGC
	BTV-1/1632-1662P(e)	CAACGACRGAAYGATGAYCCRATGGTGAAAC
	BTV-1/2575-2597F(w)	GTATTTCTGAYGGTATTGTYTGG
	BTV-1/1599-1623F(e)	GCYAAATTRCGAATCAARCATRGYG
	BTV-1/2653-2633R(w)	TCATCAGATACCTCGATCGCT
	BTV-1/1711-1689R(e)	GTTARCCTCTGCAAYACAATAGG
BTV-2 Eastern(e) & Western(w) strains	BTV-2/1455-1428P(w)	CATTCCATCCACCATCTATAATTTCCCC
	BTV-2/116-139P(e)	CCAAGATGGCCGACATGACGTATC
	BTV-2/1401-1421F(w)	GATGAYRYAARTAYTCTGAG
	BTV-2/60-81F(e)	GAGCATTTGTTGAAARGTTATG
	BTV-2/1528-1503R(w)	GYATCYYTTTCGAARTCRATTGTRAG
	BTV-2/170-148R(e)	GATATCRAAYGCGTACATYTCTG
BTV-3 Western(w) strains	BTV-3/S2/656-688Pw	CYCCRCAGTTTCAYACAATACAGAGGAACCATC
	BTV-3/S2/619-640Fw	GARCGGTTRTCRACGGAWGARG
	BTV-3/S2/718-694Rw	TATCRTAAGCGTTATCTCCTARCYG
BTV-4 Western(w) strains	BTV-4/S2/2502-2529P2	TACCTGTTGTGACRTCCAAGTTGGACAC
	BTV-4/S2/2470-2488F2	GAACACGAAGATATCGCAG
	BTV-4/S2/2557-2532R2	GCATARAGAAGCTARATGTATCTTCA
BTV-5 Western(w) strains	BTV-5/S2/36-61Pw	CCGATWTTKCGRTCGAGCCAAGTTCC
	BTV-5/S2/08-26Fw	GCTTCTCAGGATGGATGAG
	BTV-5/S2/101-79Rw	CARRTCRAYCTTAAYRTCRTAYC
BTV-6 Western(w) strains	BTV-6/2086-2061P	CACCTTGAYTCATCCACACTACGAAC
	BTV-6/2001-2023F	GTCGATGTYACACAGTTGATCGT
	BTV-6/2112-2090R	AGCACGTCTAATCGTTTCTATG
BTV-7 Western(w) strains	BTV-7/S2/1635-1660Pw	CCACAATCTAGACCCGGCAATATCGC
	BTV-7/S2/1608-1631Fw	AGTATGTGAGACGTCAATCTCAGA
	BTV-7/S2/1704-1682Rw	GTCTAATAGGTCCGCAGCTTTAG
BTV-8 Western(w) strains	BTV-8/132-106P(w)	CGGGCTCATCACCTTCCTCTTCAACAC
	BTV-8/72-93F(w)	GATGGRTATGATTACATCATTG
	BTV-8/159-138R(w)	GAATTYCTGTYACATCGTGTCG

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BTV-9 Eastern(e) & Western(w) strains	BTV-9/1703-1727P(w)	CTTATATGACACTCGCCCTGCCATC
	BTV-9/1735-1762P(e)	CAACCCTATCAATGAGACAACGCCAGAC
	BTV-9/1673-1694F(w)	GGTTATGCTTCAATTACGAACG
	BTV-9/1706-1724F(e)	GTATGATACCAGGCCAGCG
	BTV-9/1779-1756R(w)	GGGTCTTATGTAGGGATGTCTGTG
	BTV-9/1803-1783R(e)	GTTCATTTTGAGGATCATCCA
BTV-10 Western(w) strains	BTV-10/S2/1519-1552P(w)	YCTTGGYNCGCGYTCTGAATTAGTATTYCCRCCY
	BTV-10/S2/1470-1488Fw	TATTRACWACWGAACCAAACCT
	BTV-10/S2/1577-1557Rw	GYGARTTRATCCRTTTGTCAT
BTV-11 Western(w) strains	BTV-11/S2/1540-1573Pw	YGTGCTCCCAAGTTATTTCGATCAATGGATCTAC
	BTV-11/S2/1510-1530Fw	GGATGCGYAYYTGAATATTAG
	BTV-11/S2/1617-1596Rw	ATCTCTCCATGAGTTATTCGCA
BTV-12 Western(w) strains	BTV-12/S2/1101-1077Pw	CTCCACCATATGCGCCAACGATAGC
	BTV-12/S2/999-1019Fw	ATACAATTCAGGCTATCCRGA
	BTV-12/S2/1136-1116Rw	CAATGATYGTTCCTCGTAAGC
BTV-13 Western(w) strains	BTV-13/S2/1206-1175Pw	CTTATATCCCTCACGTACGCTCCAYTCATACC
	BTV-13/S2/1147-1169Fw	GGTGACGTYTATTATAAATTGCG
	BTV-13/S2/1225-1207Rw	GGCGATCCARATCYCGWGG
BTV-14 Western(w) strains	BTV-14/S2/2663-2683Pw	CCGGCTTCGCGCGAGRTTYCC
	BTV-14/S2/2616-2636Fw	GCCATTGARTTTTCTGAYGAYAG
	BTV-14/S2/2758-2734Rw	TCWGTATAYGCCTTAACYGCTCT
BTV-15 Western(w) strains	BTV-15/S2/130-105Pw	CCCTCCCGATAAAGCGACCATATTCC
	BTV-15/S2/29-47Fw	CCTGTGAGCGTGATCGAAC
	BTV-15/S2/177-156Rw	CTTACACCTATGTTTCGCACTC
BTV-16 Eastern(e) & Western(w) strains	BTV-16/1291-1264P(W)	CCTTCGTTGCTGGCTCTCCCTCTAGATC
	BTV-16/1291-1264P(e)	CCTTCGTTGCRGGCTCTCCTTCTAAGTC
	BTV-16/1221-1243F(W)	GCGAGAGCAAGAGAAGTATATCG
	BTV-16/1193-1213F(e)	GACCTGAATATAAACCGCGAG
	BTV-16/1337-1319R(W)	GATGTTCGATACGTCTGGG
	BTV-16/1320-1297R(e)	ATTAATCAATTCGTACTCCCAGTG

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BTV-17 Western(w) strains	BTV-17/S2/2224-2254Pw BTV-17/S2/2178-2202Fw BTV-17/S2/2315-2295Rw	CCTCCCTCTGATGTTCCTTGTTCATGATAAC TGCTRAAAGAGATCAAATTTGTRCGG ACTTGATCGTATCGTCAAACA
BTV-18 Western(w) strains	BTV-18/S2/387-414Pw BTV-18/S2/357-381Fw BTV-18/S2/451-425Rw	CATGTACCATCACGGATAAGCCACGCCC GATTATCAACCACTTAAGGTCGACG GCTCTCTTTGCGTGTAACCTTACCGTG
BTV-19 Western(w) strains	BTV-19/S2/2378-2346Pw BTV-19/S2/2313-2336Fw BTV-19/S2/2410-2379Rw	CCAAACCTATTATARTACGCACCRAGCTCAACC AGTGTTGRTATCRCATAAATTACG GGAAAGTYAGATGCGAAATYARRGAAGTCAAT
BTV-20 Western(w) strains	BTV-20/S2/1876-1902Pw BTV-20/S2/1838-1856Fw BTV-20/S2/1928-1909Rw	CCGTAAAACCGCTTTGATGCTGATGGC GCAATATGTCCGCATGCTG GCTCCGGGCTTAATTTTTCG
BTV-21 Eastern(e) strains	BTV-21/S2/1613-1636P BTV-21/S2/1584-1603F BTV-21/S2/1686-1669R	CGCTCAACGTAAAGCAGATGACCC GCCAGATTAAAGATAACGCA GTAAATCGATAGGGTCCG
BTV-22 Western(w) strains	BTV-22/S2/1101-1077Pw BTV-22/S2/1013-1032Fw BTV-22/S2/1124-1148Rw	CTCCACCAGATACGCCACCGATAAC ATCTCAAGCGGTCAAACAGA CCATTTCACAYGCTATTATAGTTCC
BTV-23 Eastern(e) strains	BTV-23/S2/92-118P BTV-23/S2/60-81F BTV-23/S2/148-126R	CGAYGTAAGCACACGYATCGATGAACC GCGGARYTGTTAGATGGCTATG GGAATTTGWGYRACRTCATGACG
BTV-24 Western(w) strains	BTV-24/S2/1944-1973Pw BTV-24/S2/1901-1919Fw BTV-24/S2/2016-1994Rw	CATCAGACTTACAYGCACCCGAARATAAAY GAACTAYGAGAAGCTTAYR GCGAAAARTCYYTCATATCTA

Controls

- Extraction controls.

  -*Extraction Positive Control* (EPC) should contain the target virus (one for each specific serotype/topotype)

  -*Extraction Negative Control* (ENC): water or buffer is normally used

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- Amplification controls.

  -Amplification Positive Control (APC): PCR mix prepared with the same volume and concentration of reagents as the PCR mix being used to test specimens, but with the addition of target nucleic acid (one for each specific serotype/topotype)

  -Amplification Negative Control (ANC): PCR mix being used to test specimens, but with the addition of a volume of sterile nuclease free water or sterile nuclease free buffer instead of extracted template nucleic acid.

5. METHOD

General Aspects

- The procedure describes 28 assays which amplifies 28 RNA fragments of 78 to 148 bp of the BTV segment2 genome region.
- Probes are labelled at their 5´and 3´extremes with 6-carboxyfluorescein (6-FAM) and Black Hole Quencher-1 (BHQ-1) respectively.
- The rRT-PCR is carried out in a final volume of 20 µl.
- RNA sample is added to each reaction tube containing the primers mix and heat denatured.
- One step RT-PCR master mix including the probe is added to reaction tubes after denaturation.
- Commercial kits to extract viral nucleic acid are widely available. RNA extraction from blood and tissue samples can be performed following manufacturer´s instructions.

Protocol (For each of the 28 rRT-PCR assays only the corresponding primers and probe and positive controls will be varied)

a. Thaw all reagents and store in ice, except the enzyme mix.
b. Maintain an RNAse and DNAse free work environment.
c. Mix all individual reagents thoroughly and spin down.

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d. A test plate layout should be designed and loaded into the real time PCR machine software. Using the layout as a guide 2.5 µl of each primer working stock 8 µM (final concentration 1 µM) is added to each well. The plate is held on ice.

e. Add 2 µl of RNA sample, positive and/or negative controls of extraction (EPC, ENC) or amplification (AMP, ANC). to each reaction well containing the primers mix.

Table 1. Primers and sample. Volumes and concentrations

Reagent	µl/well	Working concentration	Final reaction mix concentration
Primer Forward	2,5	8 µM	1 µM
Primer Reverse	2,5	8 µM	1 µM
Sample (RNA)	2		
Total Volume	**7**		

Note: PCR plates can be replaced with tubes or strips as appropriate.

f. Heat denaturation at 95°C for 5 minutes, followed by rapid cooling on ice for further 5 minutes.

g. An appropriate volume of real time one-step complete RT-PCR mix for the number of samples to be tested is prepared following manufacturer´s instructions. Probe should be included in a final concentration of 0.25 µM.

Table 2. Complete RT-PCR mix. Volumes and concentrations

Reagent (AgPATH-ID One Step RT-PCR kit)	µl/well	Working concentration	Final reaction mix concentration
RT-PCR Buffer	10	2x	
RT-PCR Enzyme	0,8	25x	
Probe	0,1	50µM	0.25 µM
RNase free water	2,1		
Total Volume	**13**		

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h. 13 µl of complete RT-PCR mix is distributed in each well on the PCR plate containing denatured primer mix + sample RNA (7 µl). Total volume 20 µl

i. The plate is placed in a real time thermal cycler programmed with the following profile:

Step	Cycle step	Temperature	Time	Nº cycles
Reverse transcription		48°C	10 min	1x
"Hot start"		95°C	10 min	1x
PCR cycle	DNA denaturation	95°C	15 sec	40x
	Primer annealing/extension	60°C*	30 sec	

* Fluorescence data are acquired at the end of the 60°C step.

6. ASSAY VALIDATION

Test validation

Each PCR assay will be valid if after completion of thermocycling program:

- All specific positive controls (EPC, APC) should produce amplification curves. Positive control Ct values should be in the range of the previously assigned Ct value ±2. If not, the assay should be repeated.
- All negative controls (ENC, ANC) should not produce any amplification curve and the finding of PCR amplification curves in any negative control means that the assay should be repeated. If only ANC showed amplification, the new PCR test could be performed using the same extracted nucleic acid, otherwise the sample should be retested from the nucleic acid extraction.

Sample results interpretation

- The sample result is **POSITIVE** when a typical amplification curve is obtained and the Ct value (cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold) is lower or equal to the defined Ct threshold (35) within 40 PCR cycles (Ct≤35).

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- The sample result is **INCONCLUSIVE** when a typical amplification curve is obtained and the Ct value is higher to the defined Ct threshold (35) within 40 PCR cycles (Ct>35).
- The sample result is **NEGATIVE** when a horizontal amplification curve is obtained and does not cross the threshold line within 40 PCR cycles (No Ct).

If atypical amplification curves are obtained the sample result is not valid and the assay must be repeated.

7. REFERENCES

WOAH. Manual for Diagnostic Tests and Vaccines for Terrestrial Animals. English version in force at date. Chapter 3.1.3.: Bluetongue (Infection with Bluetongue virus).

Maan S, Maan NS, Belaganahalli MN, Potgieter AC, Kumar V, Batra K, Wright IM, Kirkland PD,Mertens PC Development and Evaluation of Real Time RT-PCR Assays for Detection and Typing of Bluetongue Virus. PLOS ONE | DOI:10.1371/journal.pone.0163014 September 23, 2016

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