D5.1: Serotype specific qRT-PCR for novel BTV serotypes (Partner 1, 2, 4, 5, 12, 16).

Partner 1 (UoN)

Nottingham (Partner 1) has been collaborating with colleagues at the Elizabeth Macarthur Agriculture Institute, in Menangle New South Wales, Australia to maintain and adapt the qRT-PCR assays previously developed by partner 1, to ensure that they are fully effective for the detection and identification of Australian (far eastern topotype) strains of different BTV serotypes.

A total of 12 BTV serotypes have previously been detected in Northern Australia. The original RT-PCR type-specific primers and probes developed by partner 1 were used in a "proof of concept" study using the MagPix platform, generating an assay that could detect 11 serotypes concurrently.

However, some of the original Taqman assays that were developed for western / African / European reference strains, failed to adequately detect some of the Australian BTV variants, particularly some of the more recent strains in current circulation. These assays have therefore been developed further by our Australian colleagues, involving modification of some primer/probe sequences, which in some cases required the design of completely new assays, targeting different sequences.

There are now 12 individual assays that detect the Australian variants each of the serotypes. The "Hofmann" pan-reactive assay targeting genome segment 10 is used as a benchmark with the aim to have comparable level of reactivity in both pan and type-specific assays. It is important to note that while the initial validation involves cell culture isolates, all subsequent validations have used blood from naturally infected cattle or sometimes also sheep.

The 12 individual assays have now been converted into 3 panels of 4 multiplex assays – which all work well on field samples. An inter-lab comparison of the assays has just been successfully completed with 4 other labs in Australia (validated with >2000 field samples). The plan is that these multiplex assays will be used routinely for BTV surveillance in sentinel herds throughout Australia. The results generated are very specific compared to VNT, generating results within a few hours, with indications of virus load that guide which samples to pursue for virus isolation or sequencing. These techniques have shown examples of the co-circulation of multiple individual serotypes and animals infected with at least 3 serotypes.

Partner 2 (ANSES-INRA)

An evaluation of three BTV-27-VP2 real-time RT-PCR assays BTV-27 has been performed from BTV-27 infected blood samples and collected from goats in 2014, 2015, 2016 and 2018.

BTV-27-VP2 real time RT-PCR assays

- FLI : in house (MIX 1)
- ANSES (Zientara et al, 2014)
- The Pirbright Institute (Maan et al, 2016)

Samples

Seventy one blood samples

- 43 identified as BTV-27 v01
- 1 identified as BTV-27 V02
- 14 identified as BTV-27 V03
- 13 BTV-27 (variant not determinated)

Results (see table)

The three BTV-27-VP2 real-time RT-PCR assays are able to detect BTV-27 v01 and v03. The assay described in Maan *et al* fails to detect BTV-27 v02 (only one sample). The FLI and ANSES assays detect the BTV-27 v02. The most sensitive assay is the FLI rtRT-PCR (68 out of 71 blood were detected positive).

The majority of blood that were non detected by one or other of the assays have been collected in 2015 or after. If blood samples with low viral load are excluded, it may be assumed that mismatching occured between viral genome and probes and/or primers.

Two samples (1529 and 1530) are not detected using the three assays while the CT value were respectively of 33 and 31 with Pan BTV rtRT-PCR. These samples were collected in 2016 in an herd where the BTV-27 was circulating. These results can suggest the potential presence of another variant or that the sequences targeted by the probes present to much mutations, inducing a lack of sensitivity.

Year	N°	Variant	CT BTV-27 FLI (mix 1)	CT BTV-27 Maan	CT BTV-27 ANSES	Pan rt-RT-PCR
2014	379	1	27,12	25,89	27,56	28,59
2014	2917	2	27,02	und	26,62	31,24
2014	4120	3	31,72	32,09	31,13	26,92
2014	4123	3	32,88	33,15	32,67	31,15
2014	4125	3	33,56	33,94	33,14	31,02
2014	4127	3	32,2	33,82	33,16	30,11
2014	4128	3	31,57	31,11	30,66	28,51
2014	4129	3	32,06	33,65	33,37	31,76
2014	4130	3	29,87	30,84	30,39	29,77
2014	4135	3	32,06	33,78	32,26	31,16
2014	4136	3	32,11	32,57	31,79	29,23
2014	4138	3	33,32	32,6	32,95	31,58
2014	4140	3	33,29	33,97	33,41	30,2
2014	4141	3	33,17	34,07	32,79	31,04
2014	4142	3	33,22	34,22	33,87	31,52
2014	4144	1	30,65	31,44	32,11	31,55
2014	4145	UN	33,42	33,1	34,19	33,44
2014	4147	1	33,55	32,35	34,19	31,25
2014	4148	1	32,69	29,18	32,55	31,24
2014	4149	UN	35,83	33,97	35,19	32,89
2014	4150	1	34,3	32,43	33,45	31,01
2014	4152	1	35,46	34,99	34,54	33,21
2014	4153	UN	35,73	34,16	35,82	33,84
2014	4154	1	33,68	33,27	35,57	32,4
2014	4155	1	32,17	31,58	33,38	30,41
2014	4156	1	33,87	33,73	34,58	31,86
2014	4161	1	27,55	28,54	28,44	27,85
2014	4167	1	32,52	31,02	32,55	30,91
2014	4169	1	32,42	32,92	32,68	31,34
2014	4771	UN	34,92	35,54	37,18	35,54
2014	4775	UN	34,22	32,99	34,3	36,24
2014	4778	3	31,24	31,58	33,12	32,88
2014	5292	1	32,65	39,79	32,6	31,37
2014	5302	1	33,67	33,2	34,28	31,92
2014	6102	UN	35,09	35,91	35,45	32,83
2014	6112	1	27,85	27	28,19	28,2
2014	6116	1	35,78	34,32	36,03	35,38
2014	6122	1	28,12	28,01	28,03	27,5
2014	8532	1	31,27	29,41	29,08	28,54
2014	8707	1	34,82	35,8	35,76	35,09
2014	8713	1	35,48	35,8	36,55	36,56
2014	8721	1	30,13	29,5	30,28	31,14
2015	10906	1	32,08	30,79	32,75	31,76
2015	10908	1	38,9	37,75	36,76	35,99

Year	N°	Variant	CT BTV-27 FLI (mix 1)	CT BTV-27 Maan	CT BTV-27 ANSES	Pan rt-RT-PCR
2015	10909	1	31,85	30,11	31,24	27,29
2015	10945	UN	35,04	und	33,1	36,12
2015	11463	1	32,26	31,02	30,49	35,2
2015	11467	UN	34,72	34,53	35,1	37,13
2015	11474	1	34,76	36,6	35,12	36,95
2015	11547	UN	33,94	34,99	35,47	36,31
2016	1525	1	35,51	und	36,37	34,32
2016	1528	1	34,52	36,87	34,52	34,26
2016	1529	UN	und	und	und	33,25
2016	1530	UN	und	und	und	31,01
2016	1531	1	33,78	36,59	34,86	33,31
2016	1533	1	29,41	31,72	32,03	30,76
2016	1534	1	37,27	38,36	38,01	34,43
2016	1536	1	34,83	36,26	34,49	33,49
2016	1537	1	34,62	37,31	38,29	33,41
2016	1539	1	30,25	32,74	30,97	31,57
2016	11355	1	30,04	33,7	und	31,12
2016	11357	1	26,99	30,7	36	27,2
2016	11358	1	27,74	32	und	27,57
2016	11359	1	31,84	und	und	34,41
2016	11360	UN	35,91	und	und	37,07
2016	11362	1	33,47	38,4	und	34,28
2016	11365	UN	36,78	und	und	35,74
2016	11368	1	und	und	und	32,32
2016	11369	1	26,52	und	und	28,16
2018	1421	1	35,17	und	37,35	35,35
2018	1422	1	32,7	und	38,16	33,49

UN : Unidentified
und : undetected

Partner 4 (IZSAM)

Development of a specific quantitative real time RT-PCR for detection and quantitation of western BTV-3 and an atypical novel BTV serotype detected in Sardinia (BTV-X ITL2015)

A specific real time for the newly emerged western BTV-3 strain was designed. 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 (CAGTCGGTAATTGATGATGGGTGGGACC) 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. 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-6VP2/VP5 TOV), 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 were also tested. RNAs purified from BTV-negative spleen homogenates (cattle, sheep, red deer and roe deer), blood (goat, sheep and cattle), midge homogenates (*Culicoides* spp.) and sterile water were also added to the analysis as negative controls and notemplate controls, respectively. Sensitivity (limit of detection) was evaluated by means of a pUC57 plasmid vector containing the BTV-3w Seg-2 insert (GenScript). The plasmid was linearized by digestion with BamHI for runoff transcription in vitro with the mMessage mMachine T7 Ultra Kit (Ambion) following manufacturer's instructions. Residual template DNA was removed by DNase treatment (1 µl TURBO DNase for 15 min at 37 °C) and the final purified cRNA product was quantified by Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Twenty-four replicates

in two different runs of five two-fold cRNA serial dilutions were analyzed by Probit analysis (Finney, 1971). Furthermore, three replicates of five ten-fold cRNA dilutions were examined to create the

standard curve. Efficiency (E) of the RT-qPCRBTV$_3$ was calculated according to the formula E=(10–1/slope−1)×100. To assess the intra- and inter-assay coefficient of repeatability, four replicates of three different cRNA dilutions within the operating range (10^5, 10^4, 10^3 copies) were analyzed repeating the assay in three different runs. Repeatability was estimated calculating the coefficient of variation (Cv=µ / σ)×100. Runs were performed on the 7900HT Fast Real-Time PCR System (Applied Biosystems). Neither BTV isolates nor BTV-infected organs, except reference BTV-3w regularly used for SN at IZSAM, was detected by this new specific assay. Moreover, the no-template controls, BTV negative specimens and RNA originating from EHDV and AHSV isolates did not produce any detectable fluorescence signal endorsing the specificity of the test. The LOD with 95% probability point, established by Probit analysis, was 65 copies with 51 and 100 as lower and upper confidence limits, respectively. The efficiency was 97%. The newly developed real-time PCR has been also used with 62 field samples recently collected in Tunisia for BT surveillance. Out of 62 blood samples, 31 were shown to be positive by RTqPCR$_{NS3}$. These samples were further serotyped by BTV European typing kit. Only two samples were identified as BTV-1 while the remaining 29 samples were negative. Twenty-three out of 29 were identified and characterized as BTV-3 by RT-qPCR$_{BTV-3}$.

The same strategy and validation protocol have been adopted for the design of a specific diagnostic molecular test for the identification and quantitation of BTV-X ITL2015, a novel atypical BTV strain identified in Sardinia (Italy during BT surveillance activities. Primers used are the following: Forward CTGGGTTGGTACAAGTAGAGGTAAGA, Reverse

ACTTATTTTTAATTACGTCAAGCCGTG, Probe FAM-AAGCGCCCAAACGATATCGCAAGTG-TAMRA. They were designed to amplify a fragment of the VP2 encoding gene segment of BTV-X ITL2015 (KX234079). The 25 µl reaction volume for each sample contained about 5 µl of total purified RNA, 12.5 µl of 2× Reaction Mix, 0.5 µl of SuperScript™ III RT/Platinum® Taq High Fidelity Enzyme Mix, 400 nM of primers forward and reverse, 250 nM of probe, 0.5 µl of ROX Reference dye and 5.8 µl of ultrapure DNase–RNase-free distilled water. The thermal profile consisted of a single cycle of reverse transcription for 15 min at 50 °C and 2 min at 95 °C 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 for 30 s at 60 °C. The increase of fluorescent signal was registered during the annealing step of the reaction. The same set of reference isolates or purified RNAs has been used for the validation of this novel test. All tested BTV reference isolates and BTV-infected organs did not produce any amplification. Moreover, no-template controls and BTV-uninfected specimens did not produce any detectable fluorescence signal endorsing the high specificity of the test. The LOD with 95% probability, established by Probit analysis, was 53 copies. Intra-assay repeatability showed CV % values as follows: 0.9%, 0.3%, 0.6% for 10^5, 10^4 and 10^3 copies respectively, and 0.8%, 0.3%, 0.6% for 10^5, 10^4 and 10^3 copies, respectively, for inter-assays repeatability. Moreover, the Efficiency calculated on cRNA was 101%. This novel test has been also used with field samples originating from the two farms (F1 and F2) where the virus was firstly discovered. All samples from farms F1 and F2 which tested positive with RT-qPCR$_{NS3}$ were also tested by RT-qPCR$_{BTV\text{-}X\ ITL2015}$. All samples resulted positive (CT range 30–40).

Development of an RNA microarray panel (Nanostring technologies) for BTV genotyping

As for the existence of multiple BTV serotypes, multiple typing assays are required to obtain the identification of circulating strains in order to assess adequate countermeasures. Currently, at IZSAM, the diagnostic workflow for BTV consists of a real time RT-qPCR able to detect the Seg-10 of the viral genome (RT-qPCR$_{NS3}$, VetMAX™ BTV NS3 All Genotypes Kit, Life technologies) and of a genotyping kit (RT-qPCR$_{LSI}$, LSI VetMAX European BTV Typing Real time PCR). This last has the fundamental limitation of identifying only those serotypes which had circulated or are currently circulating in Europe, including BTV-1, -2, -4, -6, -8, -9, -11 and -16. In addition, genotyping is often performed by using only one or two probes included in the LSI kit according to the epidemiological scenario of a given area. Therefore, samples potentially infected with multiple serotypes may not be correctly diagnosed. The complete identification process, even for one single serotype, could be therefore time consuming and expensive.

NanoString technology has developed a DNA/RNA microarray assay based on a novel digital barcode technology for direct multiplexed measurement of analytes. The nCounter® Analysis System (NanoString technology, Seattle, USA) relies on digital detection and direct molecular barcoding of target molecules through the use of a color coded probe pair. The

probe pair consists of a Reporter Probe, which carries the signal on its 5' end, and a Capture Probe which carries a biotin on the 3' end. This system provides the ability to analyze up to 800 target genes per reaction through molecular fluorescent barcodes. The digital quantitation of nucleic acids is made by direct counting of target molecules without reverse transcription or amplification steps. This aspect makes NanoString adequate for degraded clinical samples, in which extracted nucleic acids are not amplified by polymerase chain reaction.

Therefore in this task we decided to set up a diagnostic panel for BTV serotype identification based on the Nanostring technology. The NanoString BTV CodeSet assay has been optimized to identify RNA of BTV serotypes from supernatant of infected cell cultures and from different biological specimens including internal organs, whole blood and *Culicoides* midges.

Two different studies were performed using nucleic acids purified from tissue culture adapted BTV isolates and field samples, respectively. In the first study, reference BTVs strains, including atypical BTV serotypes, were employed. To assess the linear dynamic range and the limit of detection (LOD) of the method, 10-fold dilution series were prepared with two BTV isolates (serotypes -3 and -4) with 1, 10, 10^2,10^3, 10^4 and 10^5 viral copies; BTV-3 and BTV-4 were used to prepare spike-in ovine blood samples with 1, 10, 10^2,10^3, 10^4 and 10^5 viral copies and five mixed samples as follows: sample 1, BTV-3 10^6 copies and BTV-4 10^6 copies; sample 2, BTV-3 10^2 copies and BTV-4 10^2 copies; sample 3, BTV-3 10^2 copies and BTV-4 10^6 copies; sample 4, BTV-1 10^2 copies and BTV-4 10^2 copies; and sample 5, BTV-1 10^3 copies BTV-4 10^3 copies. For the second study 55 field samples, including internal organs (spleen, lymph node, brain), whole blood and *Culicoides* midges positive for BTV-1, -2, -4, -9 and -16 were selected. Negative controls were also included. Total RNA was extracted from isolates, spike-in blood samples and field specimens by using High Pure Viral Nucleic Acid Kit following manufacturer's guidelines (Roche, Basel CH) and tested by RT-qPCR$_{NS3}$. Positive samples were then genotyped by RT-qPCR$_{LSI}$.

The BTV CodeSet consisted of 64 pairs of reporter and capture probes. Multiple VP2 sequences were selected for the different topotypes within the single serotypes. Sequence of Seg-5 and Seg-10 were also selected as BTV housekeeping gene. The CodeSet includes 6 couples of probes designed onto mRNA sequences from *Bos Taurus* and rRNA or mithocondrial DNA sequence from different *Culicoides* midges. All the probes were designed and synthesized at NanoString Technologies (Seattle, WA).

The Nanostring protocol included three basic steps including hybridization, purification/immobilization and data collection. Briefly, 100 ng of purified RNA were denaturated at 95°C for 5 min and then hybridized with the probe set at 65 °C overnight. Probeset-target RNA complexes were purified and immobilized on nCounter® Cartridges using an nCounter® Prep Station and data Collection is carried out in the nCounter® Digital Analyzer. At the highest standard data resolution, 555 (FOV) are collected per flow cell using a microscope objective and a CCD camera yielding data of all the target molecule counts.

All reference BTV serotypes were correctly identified by the nCounter® Analysis System. All probes were able to detect the respective VP2 target allowing geno-/sero-types identification. These results were compared with those obtained by RT-qPCR$_{NS3}$. C_T values of isolates ranged between 15.0 and 21.0. For all isolates, counts obtained for housekeeping gene NS1 were higher than those registered for NS3 target. The serial 10-fold dilutions of BTV-3 and BTV-4 isolates were correctly identified from the BTV CodeSet up to 10 copies and counts were linear across the dilutions that have been made. Only isolates containing 1 viral copy were not detected. Also the serial 10-fold dilutions of BTV-3 and BTV-4 spike-in showed linear counts across the dilutions, but they were detected counts up to 10^2 copies. Isolates and spike-in samples were also tested by RT-qPCR$_{NS3\ and}$ RT-qPCR$_{LSI}$. Undeniably, for this set of samples, both PCR –based tests revealed that the LOD of Nanostring counts corresponds to C_T value of 28.

The five mixed spike-in samples were also correctly identified: probes detected both strains, BTV-3 and BTV-4, even in those showing higher dilutions. In the second study, 55 field samples including whole blood, tissue and midges tested positive and negative for BTV-1, -2, -4, -9, -16 with C_T values ranging from 22.0 to 50.0 were also analyzed. In this case, thirty-five field samples with C_T ranging from 22.0 to 35.0 were correctly identified. Out of these, in two different samples, one positive for BTV serotypes -1 and -4 (C_T 31.5 for both serotypes) and one positive for BTV-4 and -16 (C_T 24.0 for both serotypes), respectively, the two BTV combinations were evidenced. Instead, in three samples contemporarily positive for BTV-2 (C_T 33.5) and BTV-9 (C_T 29.0), only BTV-9 was correctly identified. In one archival sample supposedly to be positive for BTV-16 by RT-qPCR, BTV-4 was also detected by Nanostring. Nineteen samples showing C_T values ranging from 29.0 to 50.0 were not identified by the BTV probe set. One single discrepancy was evidenced in one tissue sample identified as BTV-4 by the LSI kit (CT 27.0) but as BTV-8 by the Nanostring assay. The sample was lately identified as BTV-8 also by the LSI kit. A sample switch occurred during preparation of RNA.

In conclusion, we tested this rapid and specific multiplexed platform for detection and typing of BTV also directly from clinical samples. Specificity of the assay needs to be further investigated analyzing a large number of BTV positive samples collected worldwide and additional experiments are currently ongoing to increase the analytical sensitivity.

Partner 5 (FLI)

Develop a Low density TaqMan-RT-qPCR array for quick and easy serotyping of BTV.

Bluetype – a low density TaqMan-RT-qPCR array

Bluetongue virus

Bluetongue virus (BTV) is an arthropod-borne Orbivirus in the family Reoviridae and a major pathogen of ruminants causing Bluetongue disease. It consists of 10 genomic dsRNA segments [1].

Primers and probes.

The PAN IVI NS3 assay is used as positive control in Bluetype array [2]. For serotyping, TaqMan assays targeting the segment 2 were selected. For all 24 classical serotypes, published serotype specific assays considering western and eastern topotypes were used. Their analytical and diagnostic sensitivity and specificity was tested in this previous work of the Pirbright Institute [3]. The array was expanded with serotype specific assays for serotypes 1, 6 and 8 published by the FLI [4] and FLI-in-house assays for serotypes 4, 5, 14 and 15, as well as new designed assays for serotypes 23 and 24. Two new Pan Assays, one detecting only atypical BTV strains, the other one detecting only classical BTV strains were developed but need further validation until usage. The Beta Actin assay acts as extraction control. The BTV serotype specific and pan assay probes, as well as the Beta Actin probe were labeled with 6-carboxyfluorescein (FAM) at the 5' end, and a black hole quencher 1 was attached to the 3' end. The used EGFP Mix 1 (limit 5) contains a HEX labelled probe [5]. Oligos were synthesized by Metabion GmbH (Martinsried, Germany) and stored at − 20 °C. All primers and probes used in the Bluetype Array are shown in Table 1.

RNA Panel 1-24.

The RNA of the serotypes 1-24 was obtained from the Orbivirus reference collection at the Pirbright Institute and diluted in 10 fold dilution series in RSB_{50}. For serotypes 1, 2, 4 and 9 only the western topotype RNA was available, for serotype 16 only RNA of an eastern topotype.

RNA Panel 25-28.

For serotype 25 a full-length in vitro transcript of segment 2 of TOV from Switzerland, for BTV was used. For serotype 26 an isolate of the Orbivirus reference collection at the Pirbright Institute grown on Vero cells (V/168), for BTV 27 x, y, z isolates grown on BHK-BSR cells (V8,V14,V3) and for BTV 28, an Israeli field virus isolate of 2015 grown on BHK-BSR cells was used. (V27) The viral RNA of all virus isolates grown on cell culture was extracted manually using the Qiagen Viral RNA kit (Qiagen, Hilden, Germany) and diluted in 10 fold dilution series in RSB_{50}.

Individual RT-qPCRs and plate arrangement.

All RT-qPCRs were run on the Bio-Rad CFX 96 real-time PCR cycler (Bio-Rad, Munich, Germany) with the AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems™). The temperature profile for Bluetype and all RT-qPCR amplifications was 10 min at 45 °C (reverse transcription) and 10 min at 95 °C (inactivation of the reverse transcriptase/activation Taq polymerase), followed by 42 cycles of 15 sec at 95 °C (denaturation), 20 sec at 56 °C (annealing) and 30 sec at 72 °C (elongation). Fluorescence values (FAM, HEX) were collected during the annealing step. The compatibility of each single RT-qPCR assay selected for Bluetype was tested by comparing their performances with and without internal control system to the PAN NS3 IVI results by using the10-fold dilution series of RNA. When more Assays were selected for one serotype, the compatibility in a single reaction was tested with and without internal control in comparison to the PAN IVI NS3 as well. For the assays for serotype 1, 2, 4, 9 targeting the eastern topotype and for serotype 16 western topotype no Reference RNA was available. For those seroptype specific assays an in vitro transcript of the targeted sequences in a row was synthesized. This transcript serves as universal positive control for each serotype. Using a heterologous internal control system has the advantage of assuring the integrity of the reagents and excluding inhibition phenomena [5]. The composition of a single reaction with one (or two) specific primer-probe-mixtures was as followed: 2.25 µl (1.25 µl) of RNase-free water, 6.25 µl of 2x RT-PCR buffer, 0.5 µl of RT-PCR Enzyme Mix and 1 µl (2 µl) of BTV specific primer-probe-mix. The composition of a single reaction including the internal control system with one (or two) specific primer-probe-mixtures as used in the BlueType Array was: 1.0 µl (0 µl) of RNase-free water, 6.25 µl of 2x RT-PCR buffer, 0.5 µl of RT-PCR Enzyme Mix, 1.0 µl (2 µl) of BTV specific primer-probe-mix, 1 µl of primer-probe-mix for the internal control and 0.25 µl of internal control template. Finally, in both compositions 2.5 µl of RNA template was added. The Bluetype array in its current layout consists of 32 wells including Beta Actin, PAN IVI NS3 and serotype specific RT-qPCR assays for all 28 BTV serotypes. Running three samples in parallel is possible. The serotype specific RT-qPCRS assays can be exchanged or reduced at discretion. Additional or in replacement, two new developed assays can be included in the plate arrangement of Bluetype for fast distinction between notifiable (classical BTV serotype 1-24) and not notifiable BTV serotype (all atypical BTV serotypes). The setup of one Bluetype array starts with prefilling the 96 well plate with 1.0 µl (2 µl) of each serotype specific assay, 1.0 µl of EGFP Mix 1 (limit 5) and 1.0 µl (0 µl) of RNase-free water. A storage stock of array plates can be freezed at − 20 °C without loss of sensitivity for 9 months. Into each well of the thawed array plate 2.5 µl of the template RNA mixed with internal control RNA in ratio 1/10 was filled. The plate was heat denaturated for 3-5 min followed by the immediate cooling step. Afterwards 7.5 µl of Mastermix consisting of 6.25 µl 2x RT-PCR buffer, 0.5 µl of RT-PCR Enzyme Mix and 0.75 µl of RNase-free water is added to each well prior to starting the amplification.

Analysis of a no template control (NTC) and a positive control was required after a new production of batch of Bluetype arrays before use. The usage of RNase free water as NTC may identify cross-contamination of the master mix or pre-prepared array plates. A RNA

construct was synthesized containing all target primer sequences and can therefore be used as universal positive control. Furthermore, the synthesized positive control allowed the evaluation of the assays targeting the missing topotype RNA of the serotypes 1, 2, 4, 9 and 16 by testing the assays in combination with the IC Mix.

Results

RT-qPCRs.

The concentration of all primers used in the Assays is 100 pmol/ µl. Performances of all serotype specific TaqMan assays were tested with 10 fold dilution series, with and without internal control system and compared to PAN NS3 IVI results. For serotypes with two selected serotype specific assays, the assays were combines and tested with and without IC system. For serotypes with no available RNA, the in vitro transcript of the target sequences of the primer and probes is used. The duplicate testing's of 4 dilution steps with Pan Ct-values ranging between 20-35 showed, that the serotypes 1-22 did not deviate more than 2.5 Ct-values on average from the Pan BTV result. For BTV 23 and BTV 24 a deviation of 5.7 and 3.23 Ct-values on average from the Pan BTV result were observed, therefore newly designed primer-probe were added to Bluetype array. The amplification plots for BTV 2 were showing no plateau phase, whereas the amplification plots for BTV 1, 5, 6, 9, 14 and 18 were flattening in higher dilutions steps. Newly developed assays on segment 1 for only targeting atypical serotypes and another one targeting only the classical serotyoes will be added to Bluetype array as soon as possible, when the sensitivity and specificity testing's are completed. All new designed primer and probes were selected by in silico analyses of published sequence data using Geneious.

Construction of a 32-well PCR array.

The FAM labeled Beta Actin well in the Bluetype array is combined with the HEX labeled EGFP Mix 1 (limit 5), as well as the FAM labeled PAN NS3 IVI Mix in another well. All serotype specific assays are run in parallel in separately wells combined with the HEX labeled EGFP Mix 1. For serotypes 1 and 4 three assays were selected, split in two wells where one well consists of two assays and the remaining third one in a separately well. For all other serotypes one or two FAM labeled serotype specific assays were combined with the IC Mix. The current BlueType layout consists of 32 wells covering serotypes 1-28, Beta actin and the PAN NS3 IVI. Two additional wells can be used for the new typical and atypical specific assays or replace the atypical serotypes at discretion. The layout of 32 wells allows three samples run in parallel.

Diagnostic performance of BlueType.

Three diagnostic samples were tested with the Bluetype array for evaluating the diagnostic sensitivity and further testing's are planned. The array run was successful for positive samples, when all wells exhibited amplifications plots in HEX together with amplification

plots in FAM for Beta Actin and PAN IVI wells. The array run is valid for a negative sample, when in all wells amplifications plots in the HEX channels and Beta Actin in the FAM channel exhibited amplifications plots. A field blood sample taken of a cow shortly after BTV 4 vaccination was tested with the Bluetype array. The Beta actin, PAN NS3 IVI and the assay for BTV 4 showed amplifications plots in the FAM channel, as well as all EGFP HEX Plots. Thereby BTV 4 vaccine genome was detected in small amount in this field sample shown in figure 1. The next sample was a BTV positive caprine blood sample. The valid array run detected a positive amplification of BTV 25 well shown in figure 2. Another sample of the BTV ring trial 2018 was tested in the Bluetype array. The double serotype mixture of BTV-1 positive blood (MOR2007/01) and BTV-4 positive blood (MOR2009/07) diluted in negative ovine blood and the double infection was successfully identified shown in figure 3.

References:

1. Schwartz-Cornil, I., et al., *Bluetongue virus: virology, pathogenesis and immunity.* Vet Res, 2008. **39**(5): p. 46.
2. Hofmann, M., et al., *[Bluetongue disease reaches Switzerland].* Schweiz Arch Tierheilkd, 2008. **150**(2): p. 49-56.
3. Maan, S., et al., *Development and Evaluation of Real Time RT-PCR Assays for Detection and Typing of Bluetongue Virus.* PLoS One, 2016. **11**(9): p. e0163014.
4. Hoffmann, B., M. Eschbaumer, and M. Beer, *Real-time quantitative reverse transcription-PCR assays specifically detecting bluetongue virus serotypes 1, 6, and 8.* J Clin Microbiol, 2009. **47**(9): p. 2992-4.
5. Hoffmann, B., et al., *A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses.* J Virol Methods, 2006. **136**(1-2): p. 200-9.

Figure 1. Detection of the BTV 4 virus genome in a bovine blood sample.

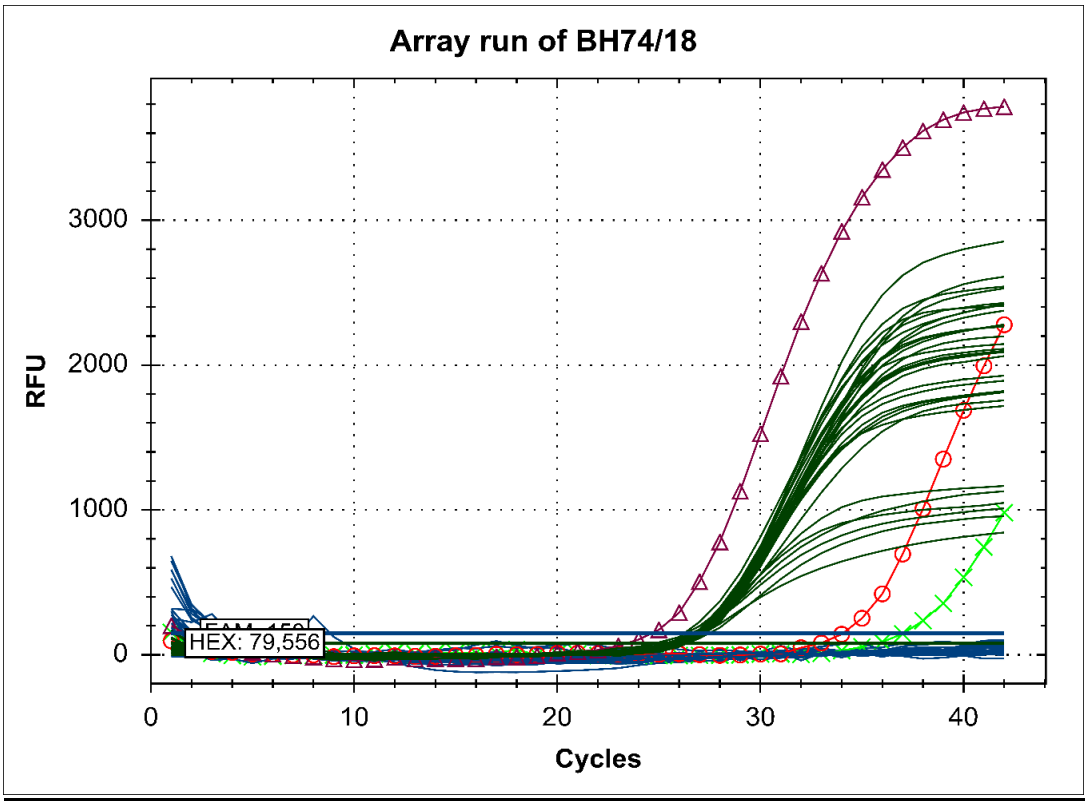

Figure 2. Detection of BTV 25 in a goat blood sample.

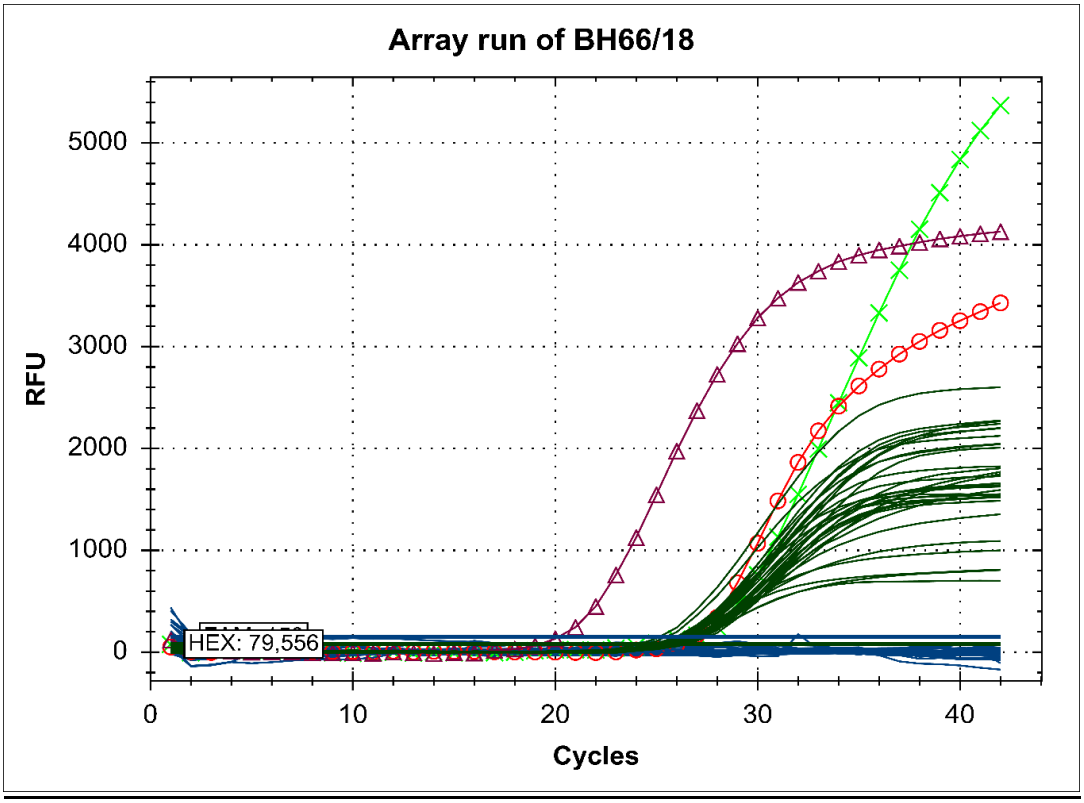

Figure 3. Detection of BTV-1 positive blood (MOR2007/01) and BTV-4 positive blood (MOR2009/07) diluted in negative ovine blood of the BTV Ring trial 2018.