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Molecular typing of Bluetongue virus using the nCounter® analysis system platform

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ABSTRACT

Bluetongue virus (BTV) is a segmented double-stranded RNA virus, existing in multiple serotypes, belonging to the genus *Orbivirus* of the family *Reoviridae*. BTV causes Bluetongue (BT), a major OIE-listed disease of ruminants. Identification of BTV serotype is accomplished using multiple typing assays and tends to be executed based on the known epidemiological situation within a given country. Samples containing multiple serotypes, particularly those containing novel introductions, may therefore be missed. The aim of this work was to optimize the nCounter® Analysis System Microarray platform (NanoString technologies), that would simultaneously identify all BTV serotypes and co-infections in analyzed samples. Probes were designed according to all Seg-2 sequences, coding for VP2 proteins which determine serotype specificity, available on line. A specific BTV CodeSet of probes was optimized. Experiments were performed with 30 BTV isolates and with 46 field samples previously shown to be infected with BTV by classical molecular assays. All BTV isolates were correctly identified and the expected BTV serotype was recognized in 35 field samples with C_T values between 22.0–33.0. In turn, it was unable to identify 11 samples with C_T values between 29.0–38.0. Although specificity of the assay needs to be further investigated against a larger panel of BTVs collected worldwide, RNA loads, which are normally detected in blood samples during the acute phase of infection, are within the range of C_T values detectable by the BTV CodeSet. We propose the NanoString RNA microarray as a first-line molecular diagnostic tool for identification and typing of BTV. Once identification of the index cases is performed, diagnosis of the following samples may be performed by specific, more sensitive and cheaper PCR-based tools.

1. Introduction

Microarrays, real time PCR and next generation sequencing (NGS) are the three major existing tools in diagnostic medicine (Hamza and Billy, 2019). Whereas, in general, traditional microarrays and real time PCR based-assays show some limitations including low dynamic range of detection or limited number of targets per single run, respectively, NGS is gathering momentum as for its capability to test for the plethora of rare pathogens that might be expected to cause a given pathology and to identify new or unexpected pathogens. However, the laborious library construction steps and the medium-high costs for single run remain the major hurdle for the widespread use of NGS in molecular diagnostics. More recently, NanoString technology has developed an alternative to the standard 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 (DNA or RNA) 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 (Geiss et al., 2008), thus making NanoString adequate for even degraded clinical samples, in which purified nucleic acids are not easily amplified by PCR (Tsang

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et al., 2017). Although this technology is actually mainly used for gene expression experiments to gain insights into the signaling pathways implicated in cancer activity and therapeutic response (Cesano, 2015; Alì et al., 2018), it is also used as diagnostic tool for a multiplex approach to profile both pathogens and host-specific responses, simultaneously (Barczak et al., 2012; Xu et al., 2016; Speranza et al., 2017) ushering in new capability in infectious disease research at the basic, translational, and diagnostic levels.

Bluetongue (BT) is an OIE-listed infectious disease of domestic and wild ruminants caused by a segmented double-stranded RNA (dsRNA) virus (Bluetongue virus, BTV), belonging to the genus *Orbivirus* of the family *Reoviridae*. BT has severe economic repercussions for the livestock industry due to direct losses caused by the infection but also due to indirect losses because of restrictions on animal trade. BTV exists in multiple serotypes (1–24) which are generally transmitted by biting midges of the *Culicoides* genus. Any classical BTV serotype has potential to cause BT. Thus, identification of the occurring serotype is important for the assessment of preventive measures. Generally, two major geographic groups of BTVs are described and designated as eastern (e) or western (w) topotypes even within the same serotype (Maan et al., 2008). In addition, novel and generally asymptomatic BTV serotypes have been recently described in the field (Hofmann et al., 2008; Maan et al., 2011; Zientara et al., 2014; Schulz et al., 2016; Sun et al., 2016; Bumbarov et al., 2016; Savini et al., 2017; Marcacci et al., 2018; Lorusso et al., 2018). They are called atypical BTV serotypes also for some unique biological characteristics, demonstrated for few of them, including the apparent inability to be transmitted by the competent vector or to replicate in cell cultures derived from it and the capability to be transmitted by direct contact (Batten et al., 2013, 2014; Bréard et al., 2018).

BTV genome constellation is composed by 10 segments (Seg-1 to Seg-10) of dsRNA coding for 7 structural proteins (VP1-VP7) and 5 non-structural proteins (NS1, NS2, NS3/NS3a, NS4 and S10-ORF). Seg-2 and Seg-6 encode for the outer capsid proteins of the virion (VP2 and VP5, respectively) which determine (mostly VP2) serotype specificity (Huismans and Erasmus, 1981; Shaw et al., 2013). VP2 is the outermost, least conserved viral protein and represents the primary target antigen for binding of neutralizing antibodies.

In general, the molecular diagnostic workflow for BTV consists of a real time RT-PCR assay detecting a portion of the Seg-10 (coding for NS3/3a) of all known BTV serotypes (qPCR$_{NS3}$), and typing assays which identify the Seg-2 of the occurring strain/s. Several factors may hamper a fast and accurate diagnosis of BTV. Multiple BTV serotypes may co-circulate in the same region (Lorusso et al., 2013, 2018) and more importantly typing assays tend to be executed based on the known epidemiological situation within a given country. Samples containing multiple serotypes (particularly those containing novel introductions or atypical BTV strains) may therefore be missed. The complete identification of even a single BTV strain can therefore be expensive and time consuming. In this manuscript we optimized the NanoString platform for the identification of BTV serotypes from supernatant of cell cultures and from different field specimens including internal organs and whole blood of infected animals.

2. Materials and methods

2.1. Samples, RNA extraction and RT-qPCR test

Two studies were performed using BTV isolates and BTV-infected field samples, respectively. In the first study, reference BTV isolates consisting of serotypes 1–24, chimeric BTV-6 harboring BTV-25 and BTV-X ITL2015 surface proteins (van Rijn et al., 2016; Savini et al., 2017), BTV-26 and BTV-27 (all three variants) available at IZSAM, were selected to test the performance of the nCounter® NanoString assay to determine the serotype. Remaining atypical BTV serotypes were not available for the experiments. To assess the linear dynamic range and the limit of detection of the method, two BTV isolates (BTV-3 and BTV-4), at a concentration of 10^6 TCID$_{50}$/ml, were serially 10-fold diluted into sterile cell-growth medium and tested. Moreover, all BTV-3 and BTV-4 dilutions were used to prepare spike-in samples into ovine blood. Additional five samples were also prepared to obtain a final concentration of BTV-3 10^6 TCID$_{50}$/ml and BTV-4 10^6 TCID$_{50}$/ml (sample 1), BTV-3 10^2 TCID$_{50}$/ml and BTV-4 10^2 TCID$_{50}$/ml (sample 2), BTV-3 10^6 TCID$_{50}$/ml and BTV-4 10^2 TCID$_{50}$/ml (sample 3), BTV-3 10^2 TCID$_{50}$/ml and BTV-4 10^6 TCID$_{50}$/ml (sample 4), BTV-3 10^3 TCID$_{50}$/ml and BTV-4 10^3 TCID$_{50}$/ml (sample 5) in ovine blood. For the second study 46 field samples, including internal organs (spleen, lymph nodes, and brain) and whole blood tested positive for BTV-1w, -2w, -4w, -8w, -9e, and -16e, were employed. These samples were collected during BTV outbreaks which occurred in Italy between 2000 and 2015 and stored at -80°. Samples were classified according to serotype, year, origin, isolation source and host. Nine field specimens tested negative for BTV, including three pools of *C. imicola*, the main vector of Bluetongue in the Mediterranean basin, were also used for the study. Total RNA was extracted from isolates, spike-in blood samples and field specimens by using the High Pure Viral Nucleic Acid Kit following manufacturer's guidelines (Roche, Basel CH). All samples were tested by qPCR$_{NS3}$ (VetMAX™ BTV NS3 All Genotypes Kit, Life technologies) and typed by using the LSI VetMAX European BTV Typing Real time PCR Kit (qPCR$_{ET}$). This commercially available typing assay is able to identify BTV serotypes (BTV 1-2-4-6-8-9-11 and 16) which have been circulating, in previous years, in Europe and in the Mediterranean basin.

2.2. Design of NanoString nCounter® probes

The BTV CodeSet consisted of 64 pairs of reporter and capture probes which were designed according to the complete Seg-2 sequences (coding for VP2) of all BTV strains available on line (Supplemental Material 1). Thus, 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). Specific probes for some atypical BTV serotypes, including SP-vaccine derived BTV, BTV-Z ITA2018 and BTV-Y TUN2017, were not designed at the time this CodeSet was planned. For 17 serotypes, multiple probes (up to 7) were designed. At the time the experiment was planned, probes for Seg-5 and Seg-10 were also designed for classical serotypes and included as BTV internal control genes. The CodeSet included also 2 couples of probes designed for beta actin mRNA sequence and for the internal transcribed spacer 2 (ITS2) RNA gene of *Culicoides*. All probes were finalized and synthesized at NanoString Technologies (Seattle, WA-USA). In this regard, for an identified target sequence of 100bp, two probes, flanked to one another and complementary to the sequence, were designed: the reporter probe, 5′ labeled with a 6-fluorophore sequence whose combination is unique for each target, and the 3′ biotinylated capture probe.

2.3. Fluorescent barcode probe assay

The nCounter can process 12 samples per run. The NanoString protocol included three basic steps of hybridization, purification/immobilization and data collection. 100 ng of purified RNA were denatured 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 (3 h and 20 min total time). Data collection was carried out in the nCounter® Digital Analyzer; at the highest standard data resolution, 555 Fields of View (FOV) were collected (6 h total time) per flow cell using a microscope objective and a CCD camera yielding data of all the target molecule counts.

2.4. Data normalization

Raw data were collected and exported into an Excel worksheet using the nSolver™ Analysis Software version 2.0 from NanoString Technologies (NanoString Technologies, WA, USA). Resulting NanoString data consists in a direct count of barcoded probes hybridized with the target sequence in a 1:1 ratio. Therefore, counts number obtained is proportional to the number of targets present in a given sample. Data were normalized according to NanoString's guidelines. Six spike-in positive controls of the CodeSet kit (128, 32, 8, 2, 0.5, 0.125 fM) were used to eliminate systematic differences between individual hybridization experiments: the geometric mean of the counts from the six positive control probes was calculated for every individual sample, the mean of these values was then used to calculate the normalization factor of every single samples. A target was considered not detected if the mean count was the same or below the geometric mean of the negative control counts.

3. Results

In the first study, all isolates available at IZSAM were identified by the *ad hoc* designed BTV CodeSet. These samples were also tested by qPCR$_{NS3}$ and results are showed in Table 1. Overall, C_T values of isolates ranged from 15.0 and 29.0. The NanoString counts obtained for the Seg-5 were higher than those registered for Seg-10 in all tested isolates (data not shown). As expected, counts for Seg-5 and Seg-10 were not evidenced for the atypical BTV serotypes. The serial 10-fold dilutions of BTV-3 and BTV-4 isolates were identified up to 10 TCID$_{50}$/ml. (Fig. 1a). Counts were also linear across the dilutions (Fig. 1a). Likewise, the serial 10-fold dilutions of BTV-3 and BTV-4 spiked into ovine blood showed linear counts across the dilutions, but in this case, samples containing 10 TCID$_{50}$/ml were not detected by the BTV CodeSet (Fig. 1b). BTV-3 and BTV-4 dilutions and spiked-in samples were also tested by qPCR$_{NS3}$. Apparently, in this case the qPCR$_{NS3}$ revealed that the limit of detection of NanoString counts corresponded to a C_T value of 28.0. Five BTV-3 and BTV-4 spike-in mixed samples (from sample 1 to sample 5) were also identified. The probes detected both strains, even the high dilutions (Fig. 1c). In the second study, 46 field samples including whole blood and tissues tested previously positive for BTV-1, -2, -4, -8, -9, and -16, were analyzed (Table 2). Thirty-five samples with C_T ranging between 22.0 and 33.0 (by qPCR$_{NS3}$) were correctly identified. Mixed infections were also evidenced. Within these, two samples, one positive for serotypes -1 and -4 (C_T 31.5 by qPCR$_{NS3}$) and the other for BTV-4 and -16 (C_T 24.0 by qPCR$_{NS3}$) were correctly detected by the BTV CodeSet for the presence of both serotypes. In turn, one sample from 2004 given positive for BTV-16 resulted positive also for BTV-4; three samples from 2002 given positive for serotypes -2 and -9 (C_T values 27.1, 28.9 and 28.9) were identified by the BTV CodeSet only as BTV-9; one tissue sample given positive from 2015 for BTV-4 (C_T 27.0 by qPCR$_{NS3}$) was identified by the BTV CodeSet as BTV-8. Further screening using novel nucleic acid purified from the stocked original material confirmed the NanoString results of these conflicting samples, thus suggesting that mislabeling or typing errors had occurred at some point. Eleven samples with C_T between 29.0–38.0 were not identified by the CodeSet. Nine BTV-negative field specimens, including pools of midges, tested also negative by the NanoString CodeSet.

Table 1
Study 1, qPCR$_{NS3}$ and NanoString results of BTV isolates. For those serotypes having multiple probes, the sum of the counts of all probes is provided. *Chimeric viruses obtained by reverse genetics.

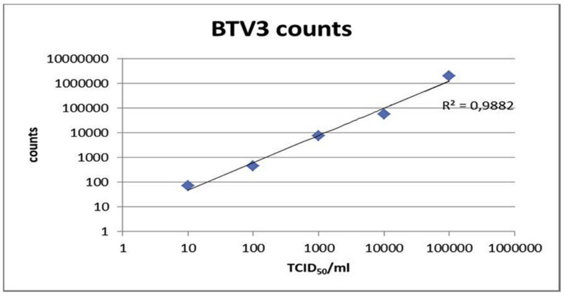
isolate	C_T qPCR$_{NS3}$	nCounter BTV CodeSet serotype identified	counts
BTV-1	16	BTV-1	312854.42
BTV-2	18	BTV-2	35024.02
BTV-3	20	BTV-3	18633.41
BTV-4	17	BTV-4	140295.25
BTV-5	19	BTV-5	31200.96
BTV-6	18	BTV-6	41899.13
BTV-7	19	BTV-7	38925.30
BTV-8	18	BTV-8	50141.11
BTV-9	15	BTV-9	453429.48
BTV-10	16	BTV-10	425136.36
BTV-11	16	BTV-11	394701.64
BTV-12	16	BTV-12	538677.06
BTV-13	18	BTV-13	73020.65
BTV-14	17	BTV-14	258124.97
BTV-15	17	BTV-15	185447.61
BTV-16	17	BTV-16	99630.47
BTV-17	18	BTV-17	48346.14
BTV-18	17	BTV-18	240196.75
BTV-19	18	BTV-19	60323.97
BTV-20	17	BTV-20	187224.24
BTV-21	18	BTV-21	54862.35
BTV-22	16	BTV-22	490027.33
BTV-23	19	BTV-23	26878.19
BTV-24	17	BTV-24	13085.21
*BTV-6$^{VP2\ TOV}$	27	BTV-25	86.21
BTV-26	21	BTV-26	2703.46
BTV-27	27	BTV-27 v01	83.49
BTV-27	28	BTV-27 v02	57.51
BTV-27	26	BTV-27 v03	88.75
*BTV-6$^{VP2\ XITL2015}$	29	BTV-X ITL2015	43.08

4. Discussion

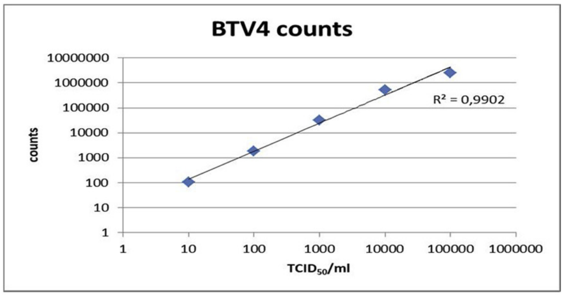
In this study we evaluated if the nCounter® NanoString assay could be used for the simultaneous diagnosis and typing of BTVs from a wide range of specimens including isolates, whole blood, and tissues samples. Thirty BTV isolates and forty-six field samples, previously quantified and typed by standard molecular assays, were selected for the analysis with this novel technology. BTV isolates were all recognized by the developed BTV CodeSet. Field specimens showed C_T values, by qPCR$_{NS3}$, ranging between 22.0–40.0 (negative). These values satisfactorily represent the broad range of samples, in terms of RNA load, which routinely arrive at IZSAM for diagnosis or confirmation. The BTV CodeSet identified the expected BTV serotype in 35 field samples with C_T values between 22.0–33.0, whereas it was unable to identify 11 samples with C_T values between 29.0–38.0. Negative samples were correctly identified. Two different targets of BTV genome (Seg-5 and Seg-10) were also included in the CodeSet as well as two housekeeping genes, the beta actin mRNA sequence of ruminants and the *ITS2* RNA gene of *Culicoides*. Counts for Seg-5 were evidenced in the 100% of classical BTV serotypes (either isolates or field samples), while Seg-10 counts have been evidenced only in a few samples. Considering the high sequence variability of Seg-10 (the fourth most variable gene of BTV genome) and the length of the NanoString probe set (50 + 50 bp) with respect to a standard real time probe, this result was not surprising. Nevertheless, Seg-5 and Seg-10 were included to have BTV detection in case of novel introductions by novel classical BTV strains which are not recognized by the established Seg-2 CodeSet. In this perspective, other targets (e.g. Seg-1 and Seg-3), coding for conserved structural proteins, are currently being considered as well as conserved genes for atypical BTV serotypes.

This study has certainly some flaws. Indeed, only few biological samples were analyzed, some atypical BTV serotypes were not included, and accurate comparison with traditional molecular typing assays was not performed. Moreover, all field samples originated from Italy, therefore the complete plethora of circulating serotypes was lacking. However, this assay was able to detect and type BTV RNA in field samples up to a viral load of 33.0 C_T by qPCR$_{NS3}$. On one hand, this clearly evidenced that this method had less sensitivity than qPCR$_{NS3}$ but on the other, the BTV CodeSet was able to simultaneously type the occurring strain, also in mixed infections. This aspect is terrifically a

TCID$_{50}$/ml	C$_T$ qPCR$_{NS3}$	BTV3 counts
1	31	undetectable
10	27	72.06
100	24	461.63
1000	21	7413.97
10000	17	57736.45
100000	15	2041131.33

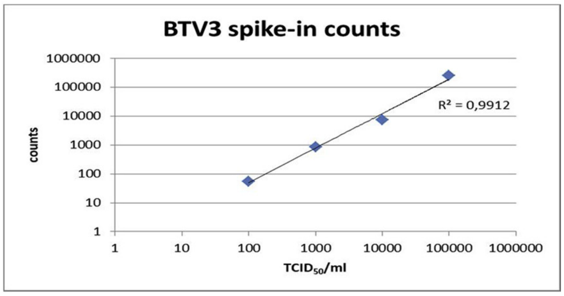


TCID$_{50}$/ml	C$_T$ qPCR$_{NS3}$	BTV4 counts
1	32	undetectable
10	28	104.25
100	24	1856.36
1000	19	31686.42
10000	15	528302.42
100000	13	2552463.12

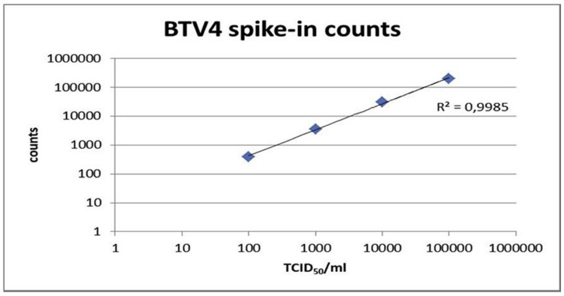


b

TCID$_{50}$/ml	C$_T$ qPCR$_{NS3}$	BTV3 spike-in counts
1	33	undetectable
10	30	undetectable
100	28	55.52
1000	24	857.14
10000	22	7543.95
100000	16	254350.29



TCID$_{50}$/ml	C$_T$ qPCR$_{NS3}$	BTV4 spike-in counts
1	33	undetectable
10	30	undetectable
100	27	387.85
1000	24	3570.45
10000	21	30206.33
100000	17	195375.38



c

	C$_T$ qPCR$_{BTV3}$	C$_T$ qPCR$_{ET}$	BTV3 counts	BTV4 counts
BTV3 10^6_ BTV4 10^6	14	17	238780.435	341474.29
BTV3 10^2_ BTV4 10^2	25	27	46.84	79.63
BTV3 10^6_ BTV4 10^2	13	28	282316.475	25.185
BTV3 10^2_ BTV4 10^6	26	18	198.61	339073.415
BTV3 10^3_ BTV4 10^3	24	26	160.05	296.83

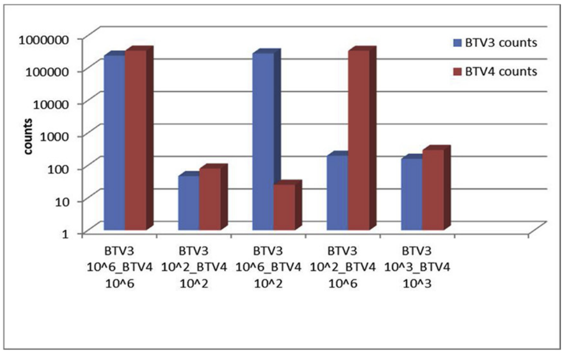


Fig. 1. Study 1, qPCR$_{NS3}$ and NanoString results of serial 10-fold dilutions of BTV-3 and BTV-4 isolates (a); BTV-3 and BTV-4 spiked into ovine blood (b); mixed BTV-3 and BTV-4 spiked into ovine blood (c). In this last experiment, BTV-3 was quantified by a specific BTV-3 real time PCR (qPCR$_{BTV-3}$, Lorusso et al., 2017, 2018) whereas BTV-4 by qPCR$_{ET}$. On the right, correlation between normalized counts quantified by nCounter® assay and viral concentration in TCDI$_{50}$/ml (50% Tissue Culture Infectious Dose).

Table 2

Study 2, qPCR_{NS3} and NanoString results of field samples. *NanoString results were lately confirmed by standard typing assays. Midges were included to test the housekeeping genes of the CodeSet.

No.	z	Year	Biological specimens	Species	Typing	C_T qPCR_{NS3}	NanoString
1		2004	spleen	sheep	BTV-16	27.8	BTV-16
2		2014	spleen	sheep	negative	negative	negative
3		2014	lung	sheep	BTV-1	35.1	not identified
4		2014	spleen	sheep	BTV-1	26.7	BTV-1
5		2014	spleen	sheep	negative	negative	negative
6		2015	midges	*C. imicola*	negative	negative	negative
7		2001	spleen	sheep	BTV-9	27.2	BTV-9
8		2002	spleen	sheep	BTV-2, -9	38.0	not identified
9		2004	spleen	sheep	BTV-16	26.8	BTV-16
10		2015	lymph node	sheep	negative	negative	negative
11		2014	midges	*C. imicola*	negative	negative	negative
12		2015	blood	cattle	BTV-4	31.9	BTV-4
13		2015	blood	sheep	BTV-4	31.2	BTV-4
14		2015	lymph node	sheep	BTV-4	31.7	not identified
15		2001	spleen	sheep	BTV-2	34.8	not identified
16		2015	blood	goat	BTV-4	27.6	BTV-4
17		2015	blood	cattle	BTV-4	31.7	BTV-4
18		2015	midges	*C.imicola*	negative	negative	negative
19		2015	blood	cattle	BTV-4	27.2	BTV-4
20		2012	blood	sheep	BTV-1, -4	31.5	BTV-1, -4
21		2015	blood	cattle	BTV-1, -4	32.6	not identified
22		2000	lymph node	sheep	BTV-2	30.4	BTV-2
23		2001	spleen	sheep	BTV-2	27.1	BTV-2
24		2002	spleen	sheep	BTV-2, -9	35.4	not identified
25		2002	spleen	sheep	BTV-2, -9	27.1	BTV-9*
26		2002	spleen	sheep	BTV-2, -9	28.9	BTV-9*
27		2002	spleen	lamb	BTV-2, -9	28.9	BTV-9*
28		2002	spleen	lamb	BTV-2, -9	32.6	not identified
29		2015	blood	cattle	BTV-4	31.0	BTV-4
30		2002	brain	sheep	negative	negative	negative
31		2007	blood	cattle	negative	negative	negative
32		2015	spleen	fallow deer	BTV-1	35.1	not identified
33		2015	spleen	sheep	BTV-4	27.2	BTV-8*
34		2007	blood	cattle	BTV-8	28.9	not identified
35		2007	blood	cattle	BTV-8	35.2	not identified
36		2007	blood	cattle	BTV-8	34.9	not identified
37		2004	spleen	sheep	BTV-16	27.2	BTV-16
38		2004	spleen	sheep	BTV-16	27.1	BTV-16
39		2004	spleen	sheep	BTV-16	27.6	BTV-16
40		2014	midges	*C. imicola*	negative	negative	negative
41		2014	spleen	sheep	BTV-1	28.9	BTV-1
42		2000	spleen	sheep	BTV-2	25.0	BTV-2
43		2001	spleen	sheep	BTV-2	29.0	BTV-2
44		2001	spleen	sheep	BTV-9	30.0	BTV-9
45		2001	spleen	sheep	BTV-9	28.0	BTV-9
46		2004	spleen	sheep	BTV-16, 4	24.0	BTV-16, -4
47		2004	spleen	sheep	BTV-16	26.0	BTV-16
48		2007	blood	cattle	BTV-8	30.0	BTV-8
49		2004	spleen	sheep	BTV-16	33.0	BTV-16, -4*
50		2007	blood	sheep	BTV-4	26.0	BTV-4
51		2012	blood	sheep	BTV-4	22.0	BTV-4
52		2015	blood	cattle	BTV-4	30.0	BTV-4
53		2015	blood	cattle	BTV-4	29.0	BTV-4
54		2015	blood	cattle	BTV-4	31.0	BTV-4
55		2015	blood	cattle	BTV-1	33.0	BTV-1

useful for the fast diagnosis of an *ex abrupto* BTV outbreak as it can provide the serotype identification in less than 30 h. Shallowly one could reasonably argue that genogroup identification and typing assays can be performed in less time. This is undeniably true when the epidemiological situation is known (e.g. countries with a surveillance program). Conversely, if the available typing assays are unsuccessful, the only valid strategy to make diagnosis would be testing the nucleic acids by specific typing assays for all existing BTVs, which certainly requires more than 30 h or alternatively, in absence of typing assays, by metagenomics as it was done previously in our laboratories for atypical BTV strains and the recently emerged BTV-3 (Savini et al., 2017; Sghaier et al., 2017; Marcacci et al., 2018; Lorusso et al., 2018). Considering that RNA loads, which are normally detected in ruminant blood samples during the acute phase of infection, are within the range of C_T values detectable by the BTV CodeSet (Lorusso et al., 2017; Spedicato et al., 2017; Cappai et al., 2019), this assay may be suitable for molecular diagnosis during a field outbreak. Therefore, we propose the NanoString RNA microarray as first-line molecular diagnostic tool for BTV. Once identification of the index cases is performed, diagnosis of following samples can be performed by specific and more sensitive existing molecular assays. This is particularly important considering the costs of the single NanoString BTV run(around 100 Euros) and the need for specialized personnel. However, more efforts are warranted for the diagnosis of atypical BTV serotypes which, conversely, show very low viral titers in the blood of infected hosts. In conclusion, NanoString technology offers a rapid, plastic and specific multiplexed detection and typing of hundreds of pathogens, directly from clinical samples without PCR amplification. This new platform is able to direct multiplexed measurement of targets and it represents a powerful tool for detection and typing at the same time of different pathogens. DNA and RNA are measured directly without amplification and therefore no gene-specific or 3′ biases are introduced (Geiss et al., 2008). In our settings, the nCounter® system provided a digital readout of the amount of target in a given sample; these counts were linear across a large dynamic range, they exhibited less background noise, and analysis of data was simple requiring only a normalization strategy. In the BTV context, specificity of the assay needs to be further investigated analyzing a large number of BTV positive samples, including infected midges, collected worldwide and additional experiments are currently ongoing to increase the analytical sensitivity. Importantly, in order to keep this diagnostic system updated, BTV genome sequences need to be shared within the scientific community once new strains are discovered and sequenced. Hence, new probes may be eventually implemented within the existing CodeSet.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.04.002.

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