Transboundary and Emerging Diseases

ORIGINAL ARTICLE

A Rapid Field-Deployable Reverse Transcription-Insulated Isothermal Polymerase Chain Reaction Assay for Sensitive and Specific Detection of Bluetongue Virus

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Summary

Bluetongue is a non-contagious, haemorrhagic, *Culicoides*-borne disease of ruminants. The causative agent, bluetongue virus (BTV), is a member of the *Orbivirus* genus of the *Reoviridae* family. So far, 26 BTV serotypes have been identified worldwide. The global distribution of bluetongue has been expanding, and rapid detection of BTV, preferably in the field, is critical for timely implementation of animal movement restrictions and vector control measures. To date, many laboratory-based, molecular assays for detection of BTV have been developed. These methods require the samples to be shipped to a central laboratory with sophisticated instruments and highly skilled technicians to perform the assays, conduct analyses and interpret the results. Here, we report the development and evaluation of a rapid, portable, user-friendly, pan-BTV reverse transcription-insulated isothermal polymerase chain reaction (RT-iiPCR) assay that can potentially be used in low-resource field conditions. The total length of the assay was <60 min, and at the end of the assay, the results were automatically displayed as '+' or '−' without the need for data interpretation. The RT-iiPCR assay detected 36 BTV isolates and two *in vitro* transcribed RNA samples representing all 26 BTV serotypes. The assay did not cross-react with other animal viruses tested, including two closely related orbiviruses. The analytical sensitivity of the assay was as low as nine copies of *in vitro* transcribed double-stranded BTV RNA. Analysis of BTV-infected whole blood samples showed that the BTV RT-iiPCR assay was as sensitive as real-time RT-PCR. The assay can potentially be used for rapid screening of animals for BTV in routine diagnostics and for monitoring bluetongue outbreaks both in ruminants and in *Culicoides* vectors in the field and in the laboratory.

Introduction

Bluetongue (BT) is a non-contagious, haemorrhagic disease of ruminants transmitted by *Culicoides* midges. It affects both wild and domestic ruminants with the infection ranging from subclinical to fatal disease (Maclachlan et al., 2009). The most clinically susceptible hosts are sheep and North American white-tailed deer, while other domestic and wild ruminants generally serve as carriers for bluetongue virus (BTV). The disease is endemic in many tropical, subtropical and temperate regions, including Africa, Southern Asia, Northern Australia and the Americas. The causative agent, BTV, is a member of the *Orbivirus* genus of the *Reoviridae* family. BTV is a non-enveloped virus with a multilayered protein capsid which encloses the 10 double-stranded RNA segments of its genome (Roy et al., 2009).

BTV genome encodes seven structural proteins (VP1-7) and four non-structural proteins (NS1, NS2, NS3 and NS3a). To date, 26 BTV serotypes have been reported worldwide (Maan et al., 2011).

Bluetongue distribution is dependent upon availability of competent vectors, susceptible host animals, pathogenic serotypes and climatic conditions. Due to environmental restrictions on the distribution of competent vectors, it had been assumed that BT would only occur within certain latitudes (Tabachnick, 2004). However, recent European BT outbreaks (Wilson and Mellor, 2009) and emergence of exotic BTV serotypes in the United States of America (USA) (Johnson, 2011) suggest that there is a drastic change in BTV global distribution (Maclachlan and Guthrie, 2010). The emergence of BTV serotype 8 in Europe in 2008 caused unprecedented losses to the sheep and cattle industries due to its rapid spread and devastating morbidity and mortality. Five BTV serotypes (BTV-2, 10, 11, 13 and 17) are considered enzootic in the USA (DeHaven et al., 2004). However, since 1999, ten additional, previously exotic, BTV serotypes (1, 3, 5, 6, 9, 12, 14, 19, 22 and 24) have been detected primarily in the south-eastern regions of the USA (Johnson, 2011). The ability of multiple BTV serotypes to infect the same animal and the segmented nature of the BTV genome could result in reassortant viruses with unpredicted clinical outcome (Batten et al., 2008; Maan et al., 2012; Shaw et al., 2013; Celma et al., 2014). Despite widespread presence of BTV in the USA, Canada is considered free of BTV except in the Okanagan Valley, BC (Thomas et al., 1982; Dulac et al., 1988, 1989; Sellers and Maarouf, 1991; Shapiro et al., 1991; Clavijo et al., 2000). Based on previous and ongoing serological testing, BT incursions continue to occur in the Okanagan Valley. The majority of Canadian livestock is believed to be naïve to BTV, and therefore, BTV serotypes that are non-pathogenic to the USA livestock could cause devastating losses to the Canadian livestock and wildlife.

Sensitive, specific and early detection of BTV in infected animals, preferably in the field, is highly desirable in an outbreak situation. Early identification of BTV-infected animals allows rapid enforcement of animal movement restrictions and vector control measures, particularly during the vector season. Detection of BTV in *Culicoides* midges in a disease-free zone indicates introduction of the virus into the area (Meiswinkel et al., 2007; Dijkstra et al., 2008). Detection of BTV, especially in the heads of field-collected, non-blood-engorged nulliparous, parous or gravid *Culicoides* midges can be used to identify potential vector species during an outbreak (Elbers et al., 2013a,b; Veronesi et al., 2013). Specific detection of BTV currently relies on laboratory-based molecular assays (Wilson and Chase, 1993; Billinis et al., 2001; Jimenez-Clavero et al., 2006; Orru et al., 2006; Polci et al., 2007; Shaw et al., 2007; Toussaint et al., 2007; Hoffmann et al., 2009; Wilson et al., 2009; Leblanc et al., 2010; Yin et al., 2010). They require the samples to be shipped to a central laboratory equipped with high-cost, sophisticated instruments (thermal cyclers) and highly skilled technicians who can perform these assays and interpret the results. The current laboratory assays require at least 2–4 h assay time from start to completion plus the time required for the sample to reach the laboratory. Here, we report the development and laboratory evaluation of a field-deployable, highly sensitive and highly specific, yet user-friendly and cost-effective pan-BTV TaqMan probe-based reverse transcription-insulated isothermal polymerase chain reaction (RT-iiPCR) assay that can be performed within 60 min.

The BTV RT-iiPCR assay was designed based on Rayleigh–Bénard convective PCR method (Krishnan et al., 2002; Chou et al., 2011; Chang et al., 2012). In Rayleigh–Bénard convective PCR, a temperature gradient is created by heating the PCR vessel from the bottom at a fixed temperature. As a result, in theory, the denaturation, annealing and extension steps of PCR take place at the bottom, top and middle zones of the vessel, respectively. Unlike in a conventional PCR, denaturation, annealing and extension events occur simultaneously in different temperature zones in the same tube, generating detectable amount of amplicons within 30 min. This allows the ability to conduct these assays in a relatively simple, portable and an inexpensive platform, the POCKIT™ nucleic acid analyzer (GeneReach USA, Lexington, MA, USA). The POCKIT™ is composed of an insulated isothermal device, optical module and a microcontroller unit connected to an LCD display. The optical module detects the fluorescent signals generated by hydrolysis of the TaqMan probe during RT-iiPCR. To enable detection of RNA targets, the instrument accommodates a reverse transcription step prior to PCR amplification. The POCKIT™ nucleic acid analyzer is a component of the POCKIT™ Xpress mobile laboratory (GeneReach USA), which also includes a mini-centrifuge (Cubee™, GeneReach USA) and two micropipettes, all the equipment necessary to run an RT-iiPCR assay. This technology has been proven to be successful in amplification of both DNA and RNA viruses, and as sensitive as laboratory-based nested (Tsai et al., 2012) and real-time PCR assays (Balasuriya et al., 2014; Tsai et al., 2014; Wilkes et al., 2014; Lung et al., 2015).

Materials and Methods

Viruses

The specificity of the BTV RT-iiPCR was evaluated using laboratory-extracted viral RNA. A total of 36 BTV reference strains and field isolates encompassing BTV serotypes 1-24 were propagated in BHK-21 cells. The

serotypes of all BTV strains used in this study were confirmed by sequencing the VP2 gene, the major determinant of serotype identity. BTV serotypes 25 and 26 were recently isolated from goats in Switzerland and Kuwait, respectively (Hofmann et al., 2008; Maan et al., 2011). Viral RNA from these two isolates was not available for this study, and therefore, RNA i*n vitro* transcribed from plasmids containing synthesized and cloned BTV-25 and BTV-26 NS1 sequences was used. Epizootic haemorrhagic disease virus (EHDV) reference strains 1, 2, 4, 5, 6, 7 and 8, as well as a recent EHDV-6 US field isolate, were propagated in BHK-21 cells, and two African horse sickness virus (AHSV) strains belonging to serotypes 5 and 6 were propagated in Vero cells. Bovine diarrhoea virus (BVDV) type 1 and type 2, and border disease virus (BDV) were propagated in MDBK cells. Classical swine fever virus (CSFV) Alfort/187 RNA was kindly provided by Dr. Irene Greiser-Wilke (EU Reference Laboratory for CSF, Hanover, Germany). All the viruses and synthetic constructs used in this study are listed in Table 1.

Animal inoculation

Two six–month-old, weaned Holstein calves (#471 and #472) were inoculated with a cell culture passaged Canadian BTV serotype 11 isolate at 10^7 $TCID_{50}$/ml (Clavijo et al., 2000). On day '0', each calf was given 3 ml of BTV-11 subcutaneously and 1 ml intradermally (0.1 ml per site for 10 sites). On day 3, each animal was given 3 ml of the same virus intravenously. The animals were monitored visually for any clinical signs, and blood samples were collected daily for the first 7 days and every other day thereafter. BTV RNA in peripheral blood circulation was monitored using Virotype® BTV Plus pan-BTV real-time RT-PCR assay (from Labor Diagnostik GmbH Leipzig, Leipzig, Germany, distributed in North America by Qiagen). BTV RNA was detected in both calves at 7 day post-infection (dpi). One of the animals developed transient corneal oedema in the right eye at 21 dpi. The condition improved without any treatment and completely recovered by 28 dpi. No fever, loss of appetite or lameness was observed in any of the animals. The calves were euthanized at 31 dpi.

Table 1. Viruses and synthetic constructs used in this study

Targets (Origin)	Non-targets (Origin)
Bluetongue virus	**Epizootic haemorrhagic disease virus**
BTV-1 (SA)[a], BTV-1 (US FL10)	EHDV-1 (USA NJ)[a], EHDV-1 (NG)
BTV-2 (USA)[a]	EHDV-2 (CA Alb)[a]
BTV-3 (SA)[a], BTV-3 (USA FL01), BTV-3 (USA AR08),	EHDV-4 (NG)[a]
BTV-3 (USA SD12)	EHDV-5 (AU)[a]
BTV-4 (SA)[a]	EHDV-6 (AU)[a], EHDV-6 (USA SD13)
BTV-5 (SA [a], BTV-5 (USA FL03)	EHDV-7 (AU)[a]
BTV-6 (SA)[a], BTV-6 (USA FL06)	EHDV-8 (AU)[a]
BTV-7 (SA)[a]	**African horse sickness virus**
BTV-8 (SA)[a]	AHSV-5 (SA)[a]
BTV-9 (SA)[a], BTV-9 (USA FL08)	AHSV-6 (SA)[a]
BTV-10 (USA)[a]	**Bovine viral diarrhoea virus**
BTV-11 (CA)	BVDV type 1a Singer (USA)
BTV-12 (BR W01), BTV-12 (USA LA12)	BVDV type 2 Ames 125 (USA)
BTV-13 (USA)[a]	**Border disease virus**
BTV-14 (SA)[a], BTV-14 (USA OK09)	BDV Coos Bay 5 ncp (?)
BTV-15 (SA)[a]	**Classical swine fever virus**
BTV-16 (SA)[a]	CSFV Alfort/187 (FR)
BTV-17 (USA)[a]	
BTV-18 (SA)[a]	
BTV-19 (SA)[a], BTV-19 (USA FL03)	
BTV-20 (SA)[a]	
BTV-21 (SA)[a]	
BTV-22 (SA)[a], BTV-22 (USA FL02)	
BTV-23 (SA)[a]	
BTV-24 (SA)[a], BTV-24 (USA FL07)	
BTV-25 NS1 gene (Synthetic)	
BTV-26 NS1 gene (Synthetic)	

[a]Reference Strain, AU = Australia, BR = Brazil, CA = Canada, FR = France, NG = Nigeria, USA = United States of America, SA = South Aftrica, ? = unknown, Alb = Alberta, NJ = New Jersey, AR = Arkansas, FL = Florida, LA = Louisiana, SD = South Dakota, OK = Oklahoma, ncp = non-cytopathic.

Four Rideau cross sheep (#2296, #2297, #2305 and #2311), approximately 6 months of age, were acclimated for 16 days in a biosafety level 3 (BSL3) animal cubicle prior to inoculating with BTV serotype 7. A South African isolate of BTV-7 that was propagated in BHK-21 cells and titered at 10^7 $TCID_{50}$/ml was used as the inoculum. Each animal initially received 4 ml subcutaneously split over two injection sites and 1 ml intradermally split over four injection sites. Three days later each animal received an additional 2.2 ml of inoculum given intravenously. Whole blood samples were collected from sheep #2296 and # 2297 on 0, 4, 6, 8 and 10 dpi, and from sheep #2305 and #2311 on 0, 5, 7 and 9 dpi. The animals were sampled on different days to minimize the stress on animals and to reduce the work load to animal care staff. All four sheep showed slight increase in rectal temperature and mild facial hyperaemia at 6 and 7 dpi. Sheep #2296 and #2297 were euthanized on 40 dpi, while sheep # 2305 and # 2311 were euthanized on 41 dpi.

All blood samples were stored at 4°C until use. All animal experiments were conducted in accordance with guidelines of the Canadian Council on Animal Care. The design of the experiment was approved by the Institutional Animal Care Committees, and all efforts were made to minimize animal discomfort. The virus stocks used in this study were free from bacterial and other viral contaminants.

Nucleic acid extraction

Total RNA from virus-infected cell culture supernatant was extracted using TriPure® reagent (Roche, Indianapolis, IN, USA), Trizol® LS reagent (Life Technologies, Grand Island, NY, USA) or Qiagen viral RNA mini kit (Qiagen, Valencia, CA, USA) as per manufacturer's instructions.

Total nucleic acid (DNA and RNA) was extracted from blood samples using PetNAD™ Nucleic acid Co-prep kit (GeneReach USA) according to the manufacturer's instructions. Briefly, 200 μl of whole blood was mixed with 600 μl of PB1 and vortexed for 1 min. Six hundred μl of PB2 (supplemented with ethanol) was added and vortexed for 10 s. Six hundred μl of the supernatant was transferred to a spin column and spun at top speed for 1 min in a Cubee™ single speed mini-centrifuge with 8-sample capacity (GeneReach USA). The column was washed once with PB3, once with PB4, and the bound nucleic acid was eluted in 50 μl of PB5. The eluted RNA was immediately used or stored at −80°C until use. The total extraction time for eight blood samples was <15 min.

A total of 20 *Culicoides sonorensis* specimens collected as a part of an ongoing field survey in Western Canada were used in this study. Individual midges were decapitated using a hypodermic needle (26 G × 3/8 inch; Becton Dickinson & Co., Rutherford, NJ, USA) under a stereomicroscope and the heads were pooled into two groups (10 each). Each pool was hand-homogenized in 200 μl of sterile water using a Kimble-Chase Kontes™ Pellet Pestle™ (Thermo Fisher Scientific, Ottawa, ON, Canada). The homogenate was spun down in Cubee™ mini-centrifuge for 2 min, and total nucleic acids from the supernatant were extracted using PetNAD™ Nucleic acid Co-prep kit according to the manufacturer's instructions. The eluted nucleic acid was stored at −80°C until use. Prior to use in the BTV RT-iiPCR assay, 5 μl of *C. sonorensis* total RNA was spiked with BTV-11 RNA equivalent to 100 $TCID_{50}$.

In vitro transcribed RNA preparation

Double-stranded BTV-24 *NS1* RNA was synthesized and used to determine sensitivity of the RT-iiPCR assay. Briefly, a plasmid containing a partial sequence of the *NS1* gene of the South African BTV-24 reference strain NVSL053191 (GenBank Accession # FJ713355.1) was used to generate positive and negative strand RNA by *in vitro* transcription using the MAXIscript® T7 kit and MEGAscript® SP6 Kit (Life Technologies), respectively. After residual DNA was removed using the Ambion® Turbo DNA-free™ kit (Life Technologies), the two RNA products were annealed to form double-stranded RNA. Following removal of residual single-strand RNA by RNase A treatment, double-stranded RNA was purified by phenol–chloroform extraction (Sambrook and Russell, 2006). Integrity of the RNA preparation was confirmed by non-denaturing agarose (1%) gel electrophoresis analysis in TAE buffer (40 mM Tris–acetate, pH 8.0 and 1 mM EDTA). Concentration of RNA was determined in a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Houston, TX, USA). The copy number of each RNA sample was calculated based on the concentration measured and molecular weight of the RNA molecule. Single-use aliquots were stored at −80°C. Serial dilutions of double-stranded RNA were made in 40 ng/μl yeast tRNA.

To generate *in vitro* transcribed BTV-25 and 26 RNA, 145 nucleotides from the 5′ end of the *NS1* gene of BTV isolate TOV (Serotype 25, GenBank Accession # EU839841) and BTV isolate KUW201010/02 (Serotype 26, GenBank Accession # JN255158.1) were synthesized and cloned into pIDT blue vector downstream of the T7 promoter (Integrated DNA Technologies, Coralville, IA, USA). The two BTV pIDT constructs were used to generate single-strand RNA using MEGAscript® *in vitro* transcription kit. *In vitro* transcribed single-strand RNA was quantified using a Qubit® RNA assay kit (Life Technologies), and single-use aliquots were stored at −80°C until use.

BTV RT-iiPCR primer and probe design

The highly conserved and highly expressed BTV *NS1* gene was selected as the target for primer/probe design. To determine the most appropriate *NS1* gene region for RT-ii-PCR primer/probe design, 61 GenBank sequences representing all known 26 BTV serotypes were aligned. Regions that have potential to form major secondary structures, as predicted by MFold (http://mfold.rna.albany.edu/?q=mfold), were excluded. Primers and probes were designed according to the RT-iiPCR design guidelines (http://www.iipcr.com/eweb/uploadfile/20130522114104277.pdf) using Primer Express 3® Software (Life Technologies). The primers with more than five nucleotide repeats, four or more G/C repeats, 3′ hair pins or potential 3′ dimers were excluded. The GC content of primers was maintained between 40% and 60%, and melting temperatures were kept between 56 and 60°C (predicted by Primer Express 3®). The maximum size of the amplicon was restricted to 100 base pairs. The lengths of the probes were kept below 30 nucleotides, and the GC content of each probe was maintained between 30% and 80%. Minor groove binder (MGB) probes have higher melting temperatures (Tms) and increased specificity and therefore recommended for iiPCR assays. Only the MGB probes that had melting temperatures 10°C above those of the primers were selected. A total of three primer/probe sets were designed and evaluated, and the primer/probe set with the highest sensitivity and specificity (Table 2) was selected for validation. The final probe was 100% conserved across all BTV serotypes. It was labelled at the 5′ end with FAM and at the 3′ end with major grove binder (MGB) and NFQ1, a non-fluorescent quencher. The forward primer sequence had two mismatches with the BTV-24 reference sequence (GenBank: JN671910.1), but it was 100% conserved across all other BTV serotypes. The reverse primer sequence was the least conserved across BTV serotypes, with two and four mismatches with the BTV-25 (GenBank: EU839841) and 26 (GenBank: JN255158.1) reference sequences, respectively. The primer (0.625 μM each) and probe (0.0625 μM) concentrations were optimized using *in vitro* transcribed BTV-24 double-stranded RNA.

POCKIT™ RT-iiPCR assay

The RT-iiPCR assay was performed on the POCKIT™ instrument as described previously (Balasuriya et al., 2014; Wilkes et al., 2014; Lung et al., 2015). Briefly, 50 μl of Premix Buffer B (GeneReach USA) was added to individual Premix tubes containing custom lyophilized reagents [primers, probe, dNTPs, reverse transcriptase and *Taq* polymerase (all from GeneReach USA)], and the tubes were briefly spun in a Cubee™ mini-centrifuge. Five μl of heat-denatured (98°C for 5 min) unquantified nucleic acid (50 × diluted RNA extracted from virus-infected cell culture supernatant, undiluted total nucleic acid extracted from blood or *Culicoides* midges) was added to each reconstituted BTV Premix, and 50 μl of the mixture was transferred into pre-labelled R-tubes (GeneReach USA). The tubes were spun for 10 sec, placed in the POCKIT™ analyzer, and the 'Run' button was pressed. The POCKIT™ instrument measured the fluorescence intensity of each sample once prior to and once after the PCR amplification, and automatically calculated the ratio between initial and final fluorescence intensities or signal to noise ratio (R1). If the R1 value was above the manufacturer-established threshold (1.3), a '+' result was displayed, while a '-' result was displayed if the R1 value was below 1.2. Rarely a '?' result was displayed if the R1 value is equal or between 1.2 and 1.3 ($1.2 \leq R1 \leq 1.3$), indicating an unreliable result and this required retesting of the sample.

Real-time RT-PCR

Commercially available Virotype® BTV Plus pan-BTV real-time RT-PCR kit was used according to the manufacturer's instructions. According to the manufacturer, this assay detects BTV serotypes 1-24 with a limit of detection (LOD) of 10 copies of *in vitro* transcribed BTV RNA per reaction. Briefly, 5 μl of heat-denatured nucleic acid (50 × diluted RNA extracted from virus-infected cell culture supernatant or total nucleic acid extracted from blood) was added to 20 μl of master mix for performing the real-time RT-PCR in an Mx3005P qPCR system (Stratagene, La Jolla, CA, USA). The reaction conditions were 50°C for 20 min, 95°C

Table 2. BTV RT-iiPCR primers and probes

Primer/Probe	Sequence (5′–3′)	Location[a]	Length	Temperature[b] (°C)	GC content (%)
Forward	CTCTAGTTGGCAACCACCAAAC	13–34	22	57.4	50
Reverse	GGCATTTGCATAATCTCCACTGAT	85–62	24	60.2	42
Probe (5′FAM-3′MGBNFQ1)	ATGGAGCGCTTTTTGAG	35–51	17	71	47.10

[a]Based on the BTV-24 reference strain NVSL053191 (GenBank Accession # FJ713355.1).
[b]Calculated using Primer Express 3® software (Life Technologies).

for 10 min, 42 cycles of 95°C for 30 s, 57°C for 45 s and 68°C for 45 s. As recommended by the manufacturer, C_t-values that were ≤35 for the FAM signal were considered positive.

The BTV RT-iiPCR assay when performed in the POCKIT™ analyzer is a qualitative end-point detection assay that cannot be used to quantify BTV. Therefore, to determine whether BTV RT-iiPCR reagents can be used in a real-time RT-PCR assay when quantification is needed, a dilution series of BTV-11 RNA was run in a Mx3005P qPCR system™ using BTV RT-iiPCR reagents. Virotype® BTV Plus pan-BTV real-time PCR kit uses hot-start *Taq* polymerase that requires activation at 95°C for 10 min. The *Taq* polymerase used in the BTV RT-iiPCR assay does not require hot start, and therefore, this step was removed when RT-iiPCR reagents were run on the Mx3005P qPCR system.

Statistical analysis

Probit analysis, a nonlinear regression model, was performed using commercial software SPSS 14.0 (SPSS Inc., Chicago, IL, USA) to determine LOD with 95% confidence. The experimental input variables were the different concentrations (copy number) of RNA tested, and the proportion of positive results at each concentration.

Results

Specificity of the BTV RT-iiPCR assay

The BTV RT-iiPCR assay was designed to amplify a 72-base pair amplicon from the highly conserved 5' region of the *NS1* gene of all 26 BTV serotypes. The assay consists of a forward primer, a highly conserved TaqMan probe and a reverse primer (Table 2). The specificity of the assay was determined using RNA extracted from cell culture grown 36 BTV RNA isolates representing serotypes 1 to 24, and two *in vitro* transcribed BTV NS1 RNA samples representing serotypes 25 and 26. The BTV RT-iiPCR assay detected all BTV RNA samples tested. In contrast, the assay did not detect EHDV, AHSV, BDV, BVDV and CSFV RNA.

Analytical sensitivity of BTV RT-iiPCR assay

A serial dilution of *in vitro* transcribed BTV-24 *NS1* double-stranded RNA was used to determine the LOD of the BTV RT-iiPCR assay. The percentage of positive results were 100% (14/14), 100% (20/20), 100% (20/20), 75% (15/20) and 0% (0/24) for 100, 50, 20, 5 and 0 copies of double-stranded RNA, respectively. Calculation by probit regression analysis determined that the ≥95% detection rate of the reaction were nine copies of double-stranded RNA per reaction.

Table 3. Analytical sensitivity of BTV RT-iiPCR assay using BTV-11 RNA

$TCID_{50}$	POCKIT™ result	Virotype® BTV plus real-time RT-PCR. C_t-value (Result)
No template	–	No C_t (–)
10^6	+	17.67 (+)
10^5	+	23.58 (+)
10^4	+	24.56 (+)
10^3	+	26.78 (+)
10^2	+	30.73 (+)
10^1	+	32.78 (+)
10^0	–	39.26 (–)

A C_t-value of <35 was used as a cut-off for positivity in real-time RT-PCR. (–), negative; (+), positive; No C_t, no C_t-value.

The analytical sensitivity of the RT-iiPCR assay was also compared to a commercially available Virotype® BTV Plus real-time RT-PCR kit using a serial dilution of BTV RNA extracted from a virus stock with a known titre (10^7 $TCID_{50}$/ml – the same virus stock used to inoculated calves). Both assays were able to detect as low as 10 $TCID_{50}$ of the tissue culture adapted BTV-11 virus strain (Table 3). Heat denaturation of viral RNA in the field can be challenging, and removal of this step can simplify the workflow. Therefore, we evaluated the sensitivity of the assay using non-denatured viral RNA, and it resulted in a 5- to 10-fold higher detection limit (data not shown).

Inter-assay, intra-assay and inter-instrumental variability

To evaluate the precision and reproducibility of the BTV RT-iiPCR assay, inter-assay and intra-assay variabilities were assessed using a dilution series of BTV-11 RNA. For inter-assay variability, the BTV RT-iiPCR assay was run three separate times using the same POCKIT™ instrument. To assess intra-assay variation, two BTV-11 RNA dilutions (10^{-4} and 10^{-5}) were tested in the same instrument in quadruplets. The performance results for the inter-assay and intra-assay evaluations are summarized in Table 4A and B, respectively. A percentage coefficient of variation (% CV – the ratio of the standard deviation to the mean) less than or equal to 15% was considered acceptable (Burd, 2010). In the inter-assay comparison, dilutions up to 10^{-5} (equivalent to 10 $TCID_{50}$) showed % CV values ≤2%, indicating excellent inter-assay reproducibility. At 10^{-6} dilution, only one of the three replicates was positive. This resulted in a higher % CV value for the 10^{-6} dilution. The % CV for intra-assay variability ranged from 0.8 to 4, indicating that the BTV RT-iiPCR assay is highly reproducible. To assess potential inter-instrumental variability, a serial dilution of BTV-11 RNA was tested using three different POCKIT™ instruments. As the results indicate in Table 4C, performances of all three POCKIT™ instruments were compara-

Table 4. Precision and reproducibility of the BTV RT-iiPCR assay (A) inter-assay variability, (B) intra-assay variability and (C) inter-instrumental variability. % CV = (standard deviation/mean) × 100

(A)

BTV-11 RNA dilution	Runs 1-3		
	Positive	Average R1 ± SD	% CV
No template	0/3	1.01 ± 0.018	1.2
10^{-1}	3/3	4.91 ± 0.056	0.9
10^{-2}	3/3	4.93 ± 0.055	1.1
10^{-3}	3/3	4.91 ± 0.056	1.1
10^{-4}	3/3	4.88 ± 0.098	2.0
10^{-5}	3/3	4.92 ± 0.024	0.5
10^{-6}	1/3	1.59 ± 0.992	62.4

(B)

BTV-11 RNA dilution	Positive	Average R1 ± SD (Repeat 1-4)	%CV
10^{-4}	4/4	4.92 ± 0.041	0.8
10^{-5}	4/4	4.79 ± 0.204	4

(C)

BTV-11 RNA dilution	POCKIT™ Instruments 1-3		
	Positive	Average R1 ± SD	%CV
No template	0/3	1.02 ± 0.004	0.4
10^{-1}	3/3	4.86 ± 0.029	0.6
10^{-2}	3/3	4.95 ± 0.051	1.0
10^{-3}	3/3	4.76 ± 0.082	1.7
10^{-4}	3/3	4.84 ± 0.050	1.0
10^{-5}	3/3	4.68 ± 0.287	6.1
10^{-6}	0/3	0.99 ± 0.007	0.7

ble. The % CV ranged from 0.4 to 6.1, increasing gradually as the viral RNA concentration decreased.

BTV RT-iiPCR assay performance with clinical and *Culicoides* samples

Whole blood samples collected from experimentally infected sheep and cattle were tested with the BTV RT-iiPCR assay, and the results were compared with that of the Virotype® BTV Plus pan-BTV real-time RT-PCR assay. Similar to the real-time RT-PCR assay, the RT-iiPCR assay was able to detect BTV RNA in sheep blood starting at 4 dpi and in cattle blood starting at 7 dpi (Table 5A and B, respectively).

Culicoides sonorensis is the primary vector of BTV in North America. Based on membrane feeding experiments, it is estimated that the head of a *C. sonorensis* at 7 days post-feeding has a BTV viral load of 100–1000 $TCID_{50}$ (Veronesi et al., 2013). To evaluate the possible use of this assay to detect BTV in field-collected midges, total nucleic

Table 5. BTV RT-iiPCR assay performance with clinical and *Culicoides* samples. Clinical sensitivity and specificity of the RT-iiPCR assay using whole blood samples from BTV-infected sheep (A) and calves (B). The samples were tested individually, and RT-iiPCR R1 value and real-time RT-PCR C_t-value for each blood sample are depicted in the table. Positive control = BTV-11 viral RNA. A C_t-value of <35 was used as a cut-off for positivity in real-time RT-PCR. (C) Detection of BTV RNA in *C. sonorensis* samples. Positive control = BTV-11 RNA without *C. sonorensis* nucleic acids. No C_t = No C_t-value. ND = Not done

(A)

Sheep (4)	RT-iiPCR Assay	Virotype® BTV plus real-time RT-PCR assay
#2296/#2297	R1 value (Result)	C_t-value (Result)
dpi 0	1.05/0.96 (−)	No C_t/No C_t (−)
dpi 4	4.89/4.90 (+)	35.91/29.40 (−/+)
dpi 6	4.90/4.89 (+)	28.84/25.17 (+)
dpi 8	4.81/4.86 (+)	29.05/26.92 (+)
dpi 10	4.94/4.88 (+)	31.75/28.57 (+)
#2305/#2311		
dpi 0	0.93/0.94 (−)	No C_t /No C_t (−)
dpi 5	4.90/4.86 (+)	27.32/27.44 (+)
dpi 7	4.85/4.80 (+)	27.18/28.13 (+)
dpi 9	4.96/4.93 (+)	28.98/29.80 (+)
Positive control	4.74 (+)	ND
No template control	0.94 (−)	ND

(B)

Cattle (2)	RT-iiPCR assay	Virotype® BTV plus real-time RT-PCR assay
#471/#472	R1 value (Result)	C_t-value (Result)
dpi 0	1.01/0.94 (−)	No C_t /No C_t (−)
dpi 7	4.97/4.94 (+)	30.30/30.55 (+)
dpi 10	4.98/4.98 (+)	26.61/26.61 (+)
dpi 15	4.99/4.98 (+)	24.89/27.00 (+)
dpi 17	4.92/4.94 (+)	27.13/29.22 (+)
dpi 19	4.96/4.96 (+)	29.71/30.15 (+)
dpi 25	4.85/4.96 (+)	29.79/31.51 (+)
dpi 31	4.99/4.94 (+)	30.26/34.87 (+)

(C)

C. sonorensis samples	RT-iiPCR assay R1 (Result)
Head homogenate pool # 1	0.99 (−)
Head homogenate pool # 2	0.98 (−)
# 1 Spiked with 100 $TCID_{50}$ BTV-11	4.50 (+)
# 2 Spiked with 100 $TCID_{50}$ BTV-11	4.83 (+)
Positive control	4.81 (+)
No template control	0.99 (−)

Table 6. Comparison of BTV RT-iiPCR and real-time RT-PCR reagents on a Stratagene Mx3005P real-time PCR instrument. A C_t-value of <35 was used as a cut-off for positivity in real-time RT-PCR. Positive control = BTV-11 viral RNA. No C_t = No C_t-value

Dilution	RT-iiPCR reagents C_t-value (Result)	Virotype® BTV Plus real-time RT-PCR reagents C_t-value (Result)
10^{-1}	26.56 (+)	24.35 (+)
10^{-2}	27.72 (+)	28.12 (+)
10^{-3}	29.91 (+)	30.39 (+)
10^{-4}	33.91 (+)	34.72 (+)
10^{-5}	36.51 (−)	No C_t (−)
Positive control	26.18 (+)	26.34 (+)
No template control	No C_t (−)	No C_t (−)

acid extracted from two pools of *C. sonorensis head* homogenates spiked with BTV-11 RNA equivalent to 100 $TCID_{50}$ *was* tested in the BTV RT-iiPCR assay. BTV RNA present in *Culicoides* nucleic acid samples was readily detected in the assay (Table 5C).

Use of BTV RT-iiPCR reagent in real-time PCR instrument

To determine whether BTV RT-iiPCR reagents can be used in a real-time RT-PCR assay, a dilution series of BTV-11 RNA was run in a Mx3005P qPCR system™ using BTV RT-iiPCR reagents (Table 6). The C_t -values were comparable to those obtained using Virotype® BTV Plus pan-BTV real-time RT-PCR reagents. Therefore, if quantification of BTV RNA is required, the same reagents can be used to perform a quantitative real-time PCR assay on a real-time PCR instrument. The ability to use the same reagents on two different detection platforms (in the field on POKIT™ analyzer for detection and in the laboratory on a real-time PCR machine for quantification) could potentially reduce the cost and complexity of the diagnostic workflow. These results also suggested that the performance of the POCKIT™ was comparable to the performance of the Mx3005P qPCR system™ when using the same RT-iiPCR regents.

Discussion

Bluetongue is an economically important viral disease in domestic and wild ruminants. To date, a number of laboratory-based conventional and real-time PCR assays for detection of BTV have been developed. Rapid detection of BTV, preferably in the field, is highly desirable in BTV outbreak situations especially during the vector season. This study describes the development of a highly sensitive and specific RT-iiPCR assay that can be performed in low-resource settings (in the field and in a less well-equipped laboratory).

The BTV RT-iiPCR assay targets *NS1*, one of the highly conserved and most abundantly expressed BTV genes during infection (Owens et al., 2004). Despite a few sequence mismatches observed between sequences in the GenBank and the forward and reverse primers, BTV RT-iiPCR assay was able to detect all BTV serotypes tested. One hypothesis is that the primer annealing in an iiPCR reaction can occur within a temperature gradient which may allow the primers to hybridize with sequences with multiple mismatches and still initiate chain elongation. However, the effect of sequence mismatches between primers and viruses on the analytical sensitivity of the BTV RT-iiPCR assay for different serotypes, especially BTV-26, requires further investigation.

The BTV RT-iiPCR assay did not detect other animal viruses including two closely related orbiviruses (EHDV and AHSV). The assay can be completed within 60 min (from nucleic acid addition to detection) in a field-deployable POCKIT™ analyzer without the need for any data analysis or interpretation by the user. The assay was highly reproducible, and no significant difference in assay performance was observed between POCKIT™ analyzers. The analytical sensitivity of the assay was as low as nine copies of heat-denatured double-stranded RNA per reaction or 10 $TCID_{50}$ of BTV, and these results were comparable to real-time RT-PCR assay. Heat denaturation of viral RNA is not essential but increased the sensitivity of the assay by 5- to 10-fold.

Diagnostic sensitivity and specificity of the BTV RT-iiPCR assay were estimated using blood samples from experimentally infected cattle and sheep. The onset and duration of BT viremia depend on the ruminant species, virus strain, blood fraction examined and virus detection system. In general, sheep develop viremia earlier (~4 day post-infection) than cattle (~5–7 days post-infection) (Katz et al., 1993; Perez de Diego et al., 2013). The BTV RT-iiPCR assay was able to accurately identify BTV-infected animals, and the results were comparable to those obtained using the Virotype® BTV Plus real-time PCR assay. Washed peripheral blood cells are the most commonly used clinical sample used in molecular detection of BTV infection in ruminants. However, to expedite detection, whole blood instead of washed blood cells was used in this experiment.

Isolation of nucleic acids from clinical samples in field situations could be challenging. The use of column-based, rapid total nucleic acid extractions systems minimizes these challenges and enables isolation of high-quality RNA from clinical samples. The extracted RNA should be immediately used in the RT-iiPCR assay to prevent potential degradation or contamination. Potential RNase contamination

during sample processing can be further reduced by performing the assay in a cleaner environment such as a commercially available portable clean room system. Assessment of the quality and the quantity of extracted nucleic acid is almost impossible in the field. One of the ways to overcome this is to introduce an internal control to the existing assay. The POCKIT™ analyzer is equipped with dual channel fluorescence detection, and therefore, it is possible to incorporate an internal control to the BTV RT-iiPCR assay. RNA or total nucleic acid extraction is not required for detection of viruses in some clinical samples. We have successfully demonstrated that sample types such as serum (Lung et al., 2015) and vesicular fluid (A. Ambagala, O. Lung, M. Fisher, T. Furukawa-Stoffer, C. Nfon, unpublished data) can be directly used in RT-iiPCR assays without being processed. The POCKIT™ analyzer can process only eight samples per run and therefore not suitable for high-throughput applications. However, an RT-iiPCR analyzer that can process up to 32 samples is available for high-throughput applications in the laboratory.

To our knowledge, this is the first report of development of a portable assay that can detect all 26 BTV serotypes. In 2004, Wilson and others described a real-time PCR assay that can be performed on a field-deployable pathogen detection system; however, it was able to detect only eight of the 19 BTV serotypes tested (Wilson et al., 2004). Recently, an accelerated reverse-transcription loop-mediated isothermal amplification (RT-LAMP) has been developed for pen-side detection of BTV-8 (Mulholland et al., 2014). The BTV-8 RT-LAMP assay was specific to BTV-8 and did not amplify other BTV serotypes or EHDV. The assay consisted of six primers, and using heat-denatured viral RNA, it was able to detect 5×10^2 copies of BTV-8 RNA within 60 min. The amplicons were analysed with a hand-held UV light after addition of a fluorescent intercalating dye. LAMP is a rapid, cost-effective method that does not require expensive precision instrumentation and therefore has the potential to be used in the field as a 'pen-side' assay. However, sensitivity of LAMP assays is considerably lower when compared to real-time PCR assays (Jaroenram et al., 2009; Hayasaka et al., 2013).

In conclusion, this study describes development and initial laboratory validation of a highly sensitive and specific field-deployable pan-BTV RT-iiPCR assay. The assay uses lyophilized reagents that can be stored and shipped at ambient temperatures. It can be used in the field and in less-equipped laboratories for on-site testing of ruminants during routine diagnostics. It also has the potential to be used in entomological surveys to detect BTV in field-collected *Culicoides* midges as an 'early warning system' and to identify potential BTV vectors in an outbreak. Considering the overall speed, ease of performance, versatility, cost and portability, the BTV RT-iiPCR assay described here warrants further evaluation on additional BTV strains, clinical samples and a comprehensive field validation.

During the review process of this manuscript, a novel BTV serotype (putative BTV-27) was reported from goats in Corsica, France (Jenckel et al., 2015).

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Conflict of Interest

Pei-Yu Alison Lee is an employee of GeneReach USA, and she helped to design BTV RT-iiPCR primers and conducted the experiments related to analytical sensitivity of the BTV RT-iiPCR assay. None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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