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A multiplex real-time reverse transcription polymerase chain reaction assay for detection and differentiation of *Bluetongue virus* and *Epizootic hemorrhagic disease virus* serogroups

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Abstract. *Bluetongue virus* (BTV) causes disease in domestic and wild ruminants and results in significant economic loss. The closely related *Epizootic hemorrhagic disease virus* (EHDV) has been associated with bluetongue-like disease in cattle. Although U.S. EHDV strains have not been experimentally proven to cause disease in cattle, there is serologic evidence of infection in cattle. Therefore, rapid diagnosis and differentiation of BTV and EHDV is required. The genetic sequence information and bioinformatic analysis necessary to design a real-time reverse transcription polymerase chain reaction (RT-PCR) assay for the early detection of indigenous and exotic BTV and EHDV is described. This sequence data foundation focused on 2 conserved target genes: one that is highly expressed in infected mammalian cells, and the other is highly expressed in infected insect cells. The analysis of all BTV and EHDV prototype strains indicated that a complex primer design was necessary for both a virus group–comprehensive and virus group–specific gene amplification diagnostic test. This information has been used as the basis for the development of a rapid multiplex BTV-EHDV real-time RT-PCR that detects all known serotypes of both viruses and distinguishes between BTV and EHDV serogroups. The sensitivity of this rapid, single-tube, real-time RT-PCR assay is sufficient for diagnostic application, without the contamination problems associated with standard gel-based RT-PCR, especially nested RT-PCR tests.

Key words: *Bluetongue virus*; *Epizootic hemorrhagic disease virus*; real-time reverse transcription polymerase chain reaction.

Introduction

The recent European outbreaks of bluetongue disease in cattle and sheep have demonstrated the economic impact of this disease.[4] In a 1989 estimate, the annual loss to the livestock industry from this disease in the United States and worldwide was significant (Bath GF: 1989, Bluetongue. *In:* 2nd International Congress for Sheep Veterinarians, pp. 349–357. Massey University, Palmerston North, New Zealand, February 12–16, 1989). The causative agent, *Bluetongue virus* (BTV; family *Reoviridae*, genus *Orbivirus*) is closely related to another orbivirus in the same family, *Epizootic hemorrhagic disease virus* (EHDV). These viruses are transmitted by biting midges of the genus *Culicoides*.[44] EHDV has been associated with bluetongue-like disease in cattle,[11] but experimental inoculation of cattle with North American EHDV serotype 2 did not result in clinical disease.[1] Although sheep are susceptible to EHDV, clinical disease has not been observed with U.S. serotypes.[35] Severe clinical disease in cattle, however, was reported with an outbreak of EHDV serotype 7 in Israel.[45] It is difficult to predict how exotic serotypes of these viruses could affect North American livestock and wildlife. The outbreak of BTV serotype 8 in Europe has had a greater impact than previous experience would predict.[26] The need for a differential diagnostic assay is emphasized by the serologic evidence of widespread EHDV infection in U.S. cattle.[22]

An understanding of the molecular genetics of these viruses is needed for the development of rapid and sensitive molecular diagnostic assays. The protein coding assignments for BTV and EHDV are identical, consisting of 10 genome segments that encode 7 structural and 4 nonstructural proteins.[20] Developing molecular diagnostics is complicated by the multiple serotypes of these viruses: 24 for BTV and 8 for EHDV.[19] The outer capsid protein, VP2, is the

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primary serotype determinant, but the second outer capsid protein VP5 can also influence serotypic epitopes.[21,25,40] The structural genes that are fairly conserved within the virus serogroup encode the inner core proteins VP3 and VP7,[23,42] and this information has been used to develop reverse transcription polymerase chain reaction (RT-PCR) assays.[2] The VP7 protein is commonly used as the antigen for enzyme-linked immunosorbent assays.[24]

The nonstructural (NS) proteins are highly conserved among North American orbiviruses.[3,27,28] The conservation of these viral proteins is likely because of their various roles in viral replication. The NS1 protein produces tubules in infected cells[37] and is thought to be important in virus assembly. Binding of single-stranded RNA has been demonstrated with the BTV NS2 protein.[8] Viral egress has been shown to be facilitated by the BTV NS3 protein.[12]

A number of real-time RT-PCR assays were recently developed for BTV.[7,14,29,32,36] The genome segments are commonly denoted as L1–3, M4–6, and S7–10, based on sequence size, similar to the *Reoviridae* nomenclature. The coding assignments are based on sequence size and protein produced, with structural proteins denoted as VP1–7 and nonstructural protein denoted as NS1–3. The serogroup-specific BTV real-time RT-PCR assays are based on L1 (VP1) and M5 (NS1). The authors' laboratory also has been developing real-time RT-PCR based on 2 conserved target genes[13,39]: one that is highly expressed in infected mammalian cells, M5 (NS1),[38] and another that is highly expressed in infected insect cells, S10 (NS3).[5] These target genes were used to develop a robust EHDV real-time RT-PCR assay that detects RNA from all 8 EHDV serotypes.[43] In the current report, a rapid BTV real-time RT-PCR is described, based on sequence analysis of the M5 and S10 BTV genome segments that was multiplexed with the EHDV assay. This multiplex assay detects all known BTV and EHDV serotypes in a single tube.

Materials and methods

Prototype viruses

EHDV-1 (New Jersey), EHDV-2 (Alberta), BTV- 2 (Ona B), BTV-10 (BT-8), BTV-11 (Texas Station Strain), BTV-13 (67-41B), and BTV-17 (63-66B) were obtained from the Arthropod-Borne Animal Diseases Research Laboratory (ABADRL) virus collection. Prototype strains of BTV-1, 3–9, 12, 14–16, 18, and 19 previously provided by the Onderstepoort Veterinary Institute (OVI; Pretoria, Republic of South Africa) were obtained from the ABADRL virus collection. National Veterinary Services Laboratories (NVSL; Ames, IA) provided prototype strains of BTV-20–24 (originally from OVI). Prototype strains of EHDV-3–8 were also kindly provided by the NVSL. EHDV types 3 and 4 were isolated in Nigeria and provided to NVSL by the Institute for Animal Health (Pirbright, UK). The Australian Animal Health Laboratory originally provided prototype strains of EHDV-5 (CSIRO 157), EHDV-6 (CS753), EHDV-7 (CS775), and EHDV-8 (DPP059) to NVSL. The prototype viruses were propagated in baby hamster kidney (BHK)-21 or African green monkey kidney epithelial (Vero Middle America Research Unit) cell cultures. Cells were infected by using 0.01 multiplicity of infection, and RNA extractions were performed when approximately 80% of the infected cells showed cytopathology.

RNA extraction from infected cells or blood

Total RNA was extracted from cells by using a commercial RNA isolation kit[a] as per the manufacturer's protocol, with the following exceptions: for cell lysis, the initial centrifugation was carried out for 5 min at 15,000 × *g* to pellet the cells; for RNA precipitation, RNA containing isopropanol was centrifuged for 10 min at 15,000 × *g* to pellet the RNA. Ethanol precipitation was omitted, and the RNA pellet was lyophilized and resuspended in nuclease-free water. Double-stranded RNA (dsRNA) was purified by using the lithium chloride differential precipitation described previously.[41] The dsRNA was lyophilized and resuspended in the appropriate volume of nuclease-free water. The RNA was quantitated by absorbance at 260 nm by using a nanophotometer.[b]

High-throughput RNA extractions from blood were compared by using 2 different magnetic-bead capture kits: MagMAX™-96 for Microarrays Total RNA Isolation[c] (hereafter, MagMAX-96 kit) and MagMAX™ Viral RNA Isolation[c] (hereafter, MagMAX kit). Blood samples from sheep were spiked with BTV-17 and EHDV-2 with 10-fold dilutions from 10^6 to 1 $CCID_{50}$/ml ([50% cell culture infective dose]/ml). The MagMAX kit was slightly modified. Briefly, 20 µl of bead mix was added to 100 µl of blood and mixed by shaking in a 96-well plate. Lysis-binding solution (400 µl) was then added, and the mixture was shaken for 5 min, after which it was centrifuged for 5 min, and the supernatant was discarded. The original protocol was followed for the wash steps; however, elution was done by using 100 µl of 0.1 mM ethylenediamine tetraacetic acid (EDTA) at 65°C, instead of the kit elution solution. The MagMAX-96 kit used 200 µl of blood starting material, mixed with 750 µl TRI reagent,[c] followed by a spin procedure and elution in 100 µl of elution buffer. RNA was quantitated as stated previously.

M5 and S10 cloning and sequence analysis

Amplification of the M5 and S10 genes was performed as described previously, with primer modifications for the M5 gene.[3,13] The amplification primers used for the S10 gene were the following: BTV S10+1D (GTTAAAAAGTG TCGCTGYCA) and BTV S10-822D (GTAAGTGTRTA GYRYCGC) or BTV S10-822ND (GTAAGTGTG TAGGGCCGC). The amplification primers for the M5 gene were the following: BTV10NS1-N1-5 (atccgatccGT

TAAAAAAGTTCTCT) and BTV10NS1-N1-3 (atccggatccGTAAGTTGAAAAGTT; lower case bases are not complementary to the gene). The amplicons were then cloned into the TOPO (topoisomerase I) 2.1 vector[d] and transformed into TOP10 F' chemically competent cells.[d] Sequencing was performed by using standard automated protocols[e] on at least 3 individual clones, and on PCR products to resolve any discrepancies in consensus sequences. SeqMan software[f] was used for sequence assembly. Viral gene sequences, minus the primer sequences (except for sequences in the coding region of S10), were aligned by using either ClustalX[34] or MUSCLE[6] with default settings. Phylogenetic trees were calculated from these multiple sequence alignments by using MrBayes,[31] with the standard nucleotide substitution model (4 × 4) with GTR (nst = 6). Substitution rates were set to invgamma (gamma-shaped rate variation with a proportion of invariable sites); default values were used for all other settings. A total of 750,000 generations were calculated by sampling every 100th tree, and a consensus tree was calculated after a burn-in of 2,500 trees by using the allcompat setting. Phylogenetic trees were also generated by using ClustalW alignment based on absolute number of differences with gaps distributed proportionally, and Unweighted Pair Group Method with Arithmetic mean (UPGMA; bootstrap [1,000 reps]; tie breaking = systematic).[g]

BTV primer-probe sets or signatures

A panel of signatures (set of primers and probe with specificity for a distinct target sequence) was generated by using bioinformatics as previously described[16] on published sequence data and data generated in the current study for gene segments L1, M5, S8 (NS2), and S10. These signatures were prescreened in silico and further screened to demonstrate no cross-reactivity with more than 2,500 potential background samples, as described previously.[16] The remaining signatures were then further evaluated for their ability to detect BTV strains but not the closely related EHDV RNA.

Analysis of new BTV signatures for real-time RT-PCR

All 24 BTV serotypes were run against all signatures individually by using a commercial real-time PCR detection system.[h] The real-time RT-PCR procedure was performed as described previously.[16] Cycle threshold (Ct) values were recorded for each signature. Of the signatures that amplified all 24 BTV serotypes, a limit of detection (LOD) was then determined for each signature on each serotype. For LOD, samples were run in triplicate with 10-fold dilutions from 2 pg to 2 fg of dsRNA, and Ct values were recorded and averaged. The signatures that detected all serotypes were also tested on prototype EHDV 1–8 serotypes to validate lack of cross-reactivity. The selected signatures were then used to detect RNA (200-pg run in duplicate) from 45 random BTV strains and 60 BTV- and EHDV-suspect clinical samples from the authors' inventory.

Real-time RT-PCR

Initial experiments were conducted with only the BTV signatures generated in the current study. Final analysis was done after combining the BTV signatures with the previously published EHDV signatures[43] (Table 1) by using a multiplex format. Two different quencher and/or reporters were used to differentiate between BTV and EHDV. The quencher and/or reporter choices varied depending on the filter sets of the instrument available at the time of the experiment. Initial experiments on RNA from cell cultures were conducted with 3 different thermocyclers[e,h,i] by using the TaqMan EZ RT-PCR core reagents[e] and the cycling parameters based on the authors' previous EHDV real-time PCR assay[43] of 55°C for 25 min, 95°C for 2 min, followed by 40 cycles of 95°C for 10 sec and 55°C for 1 min. The BTV probes used with the Bio-Rad iCycler[h] was dual labeled with fluorophore 6-carboxyfluorescein (FAM) and Black Hole Quencher (BHQ)-1[j] during the initial primer evaluation studies. In the multiplex assay development, when the Applied Biosystems SDS7000 was used, the probes were dual labeled with 5' CAL Fluor Orange 560 and BHQ-1.[j] The BTV probes for use with the Cepheid SmartCycler II[i] was dual labeled with 5' CAL Fluor Gold 540 and BHQ-1.[j] The EHDV probes were dual labeled with FAM and BHQ-1.[j] Two real-time RT-PCR protocols were compared by using 2 different kits (AgPath-ID kit[e] and TaqMan EZ[e]) and 2 different cycling parameters on the Applied Biosystem SDS7000 real-time PCR instrument.[e] For both kits, 8 µl of template was used from 100 µl of the blood extraction procedure. The template was denatured at 95°C for 8 min, and 2 cycling parameters were compared. In some cases noted in the text, the template was heated to 95°C for 3 min with 10% dimethyl sulfoxide, as described previously.[36] The first set of parameters was Applied Biosystems's thermal profile parameters (manufacturer recommendation): 1 cycle of 48°C for 10 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The second set of parameters was based on the authors' previous EHDV real-time PCR assay[43] as stated above.

Results

Sequence and phylogenetic analysis

The M5 (NS1) and S10 (NS3) genes from BTV prototype strains, where sequence information was not available, were amplified by using terminal primers and the sequences were determined. The primer sequences, except for the first 5 bases on the 5' end that included the ATG start codon for the S10, were removed for data analysis. The 40 new sequences generated were reported to GenBank (accession nos. FJ713316–FJ713355). Unfortunately, the origins of these prototype viruses were not able to be confirmed. The BTV M5 gene sequences had a percent identity that ranged from 78.7% to 99.7%. The percent identity range between BTV and EHDV M5 gene sequences was 56.3–58.4%. The BTV S10 gene sequences had a percent identity range from 81.5% to 99.9%. The percent identity range between BTV and EHDV S10 gene sequences was 58.3–61.8%. The BTV M5 sequences separated into 2 major

Table 1. *Bluetongue virus* real-time reverse transcription polymerase chain reaction primer and probe sets or signatures.

Name	KPATH*	Probe	Forward primer	Reverse primer	Gene
A5	1759932	ACAGAAGATGATTGGCCCACGAGTTAG	GCACCCTATATGTTTCCAGACCA	CAGCTAACTCTTCAGCCACACG	L1
A6	1759933	CCATCACACCATTATACTGTACCCGCGTAGC	AGAATTCAGGATGGGCAGGA	GCACAATTCCCATCCCCTTA	S8
A7	1810199	ACGAAACGCTTCCGCGTACGATG	CACATGTCGCTTAATTTGTCTTAACC	GCGGAGAAGGCTGCATT	S10
A8	1810200	CACATCATCACGAAACGCTTCTGCG	TTAAGCCTCCTAGGTCACTTTTCAA	AAAGCTGCATTCGCATCGT	S10
A9	1810201	AGTCCCGCTAGATGGTTTCGAATTACCATTA	TAATGATGCGGTGAGGATGAGT	CGCCACTCTACCTACTGATCTTAGG	S10
B1	1810205	ACGAAACGCTTCCGCGTACGATG	TCAATTTTGGTAGAATTTGTTCATTCA	GCGGAGAAGGCTGCATTC	S10
B3	1810207	TCGCATCGTACGCAGAAGCGTTTC	CAAACACAAAAGGCGGAGAAG	GGCGTTTAATCTGTCTTAGTCTTACGT	S10
B5	1810182	TGCACCCCCGCACCGCTTC	GATTGCTTCACGGCCTCAT	TTGGCAAAGGAGGCAATGT	M5
B6	1810183	CGCGTCCGAGCATGAAAATACCCTC	CTTCGTCAGCTCCCATCTCA	GGCCTTGATTACAACTGCGATT	M5
B11	1810188	TCACGGCCTCATCCATCATTCCACT	TCTTTCTGAATTAGCCAGTGCAGAT	AGCGGTGTGGAGTGCAACT	M5

* www.llnl.gov/str/April04/Slezak.html

lineages, with a very high degree of confidence. Phylogenetic analyses of M5 and S10 sequences were performed by using several algorithms, and all showed essentially the same profiles (Figs. 1, 2).

BTV primer and/or probe (PCR signatures) design

The sequence data from the current study and previous publications were used for BTV signature generation based on 27 sequences for the M5 gene and 52 sequences for the S10 gene (unpublished data). An in silico analysis, by using a previously described alogorithm,[16] was used to identify multiple candidate signatures of highly conserved genome regions. This analysis provided 11 PCR signatures for M5 and 16 PCR signatures for S10 that were discriminatory by the in silico analysis. These signatures were tested against 50 soil, 60 prokaryotes, 21 eukaryotes, 2,000 aerosol samples, and 4 U.S. strains of EHDV. The specificity testing by gel RT-PCR screening, followed by real-time RT-PCR assay screening against these potential background samples, resulted in 8 potential segment M5 signatures and 10 potential segment S10 signatures. Preliminary target detection with 5 domestic serotypes by using known amounts of BTV RNA eliminated 4 of the signatures. Further screening on the remaining seven M5 and seven S10 signatures resulted in three M5 and five S10 signatures that were further screened against all 24 BTV serotypes. The specificity of these signatures, along with previously designed L1 and S8 signatures, is shown in Table 1.

The 4 signatures that detected all 24 BTV serotypes, at 200 pg dsRNA, were A7, A8, B1, and B3, which all targeted the S10 gene (Table 2). The LOD was determined for each of these 4 signatures, and Ct values were recorded and sensitivity analyzed (Table 3). Although the linearity between Ct and dilution is not always consistent, the general trend was and the results were consistent with the experimental error of real-time RT-PCR assays around the LOD (data not shown). In some cases, there was no amplification of specific targets that were amplified in the initial screening. This was likely because of experimental error around the LOD for the target. The signatures A7, B1, and B3 were selected for future inclusion in the multiplex vesicular disease panel.[16] The intent was to use separate signatures in the real-time RT-PCR and the multiplex vesicular disease panel,[10] so that the real-time RT-PCR assay could be a confirmatory test based on an independent target sequence. Therefore, the remaining signature, A8 was used for the real-time RT-PCR BTV assay. After further analysis of inventoried strains, some of the strains were not detected when using A8, notably, BTV-2 Ona A. The other signatures that did not

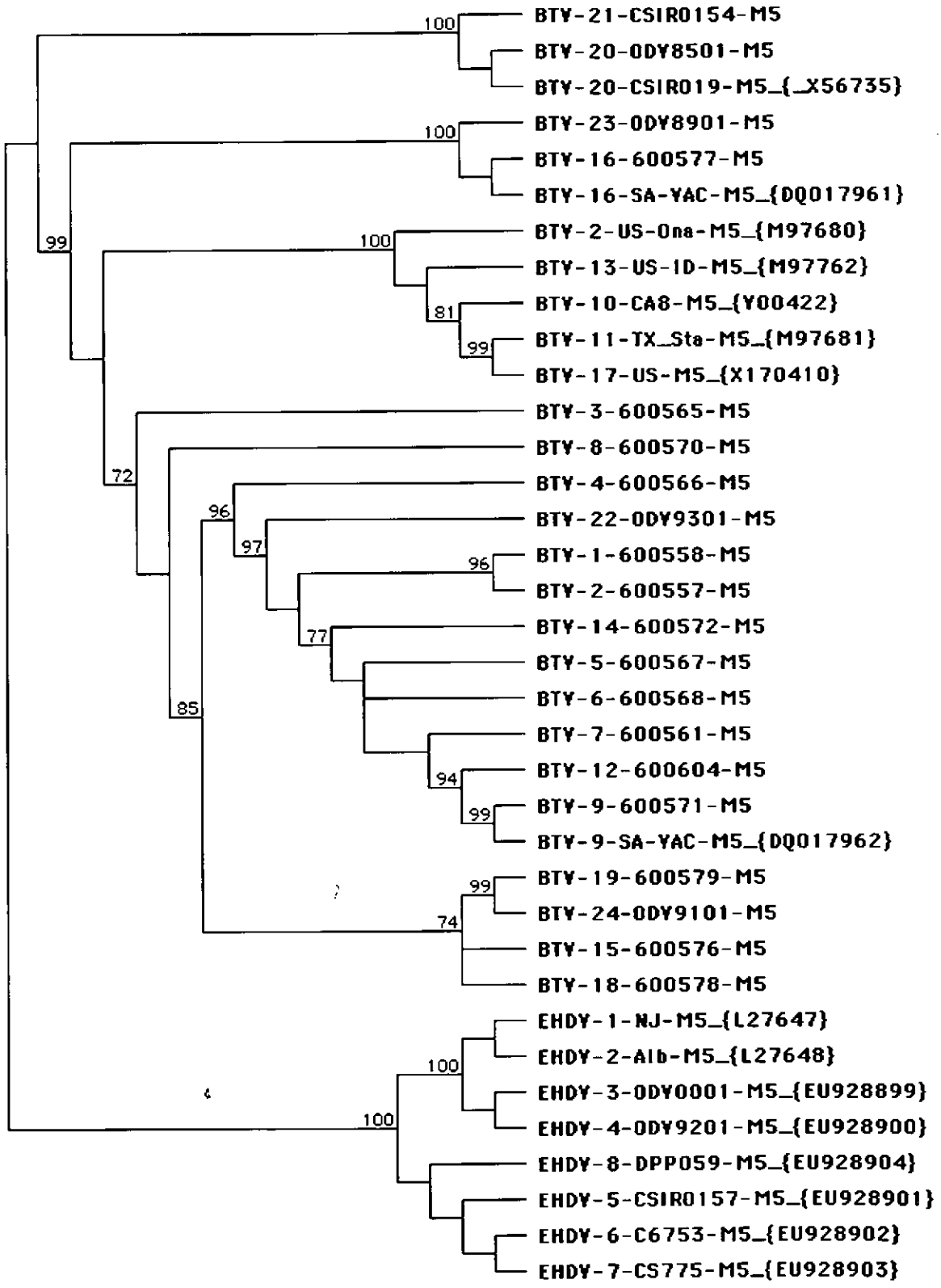

Figure 1. Example of phylogenetic analysis of *Bluetongue virus* (BTV) and *Epizootic hemorrhagic disease virus* (EHDV) M5 (NS1) gene sequences by using ClustalW alignment based on absolute number of differences, with gaps distributed proportionally and UPGMA (bootstrap [1,000 reps]; tie breaking = systematic).

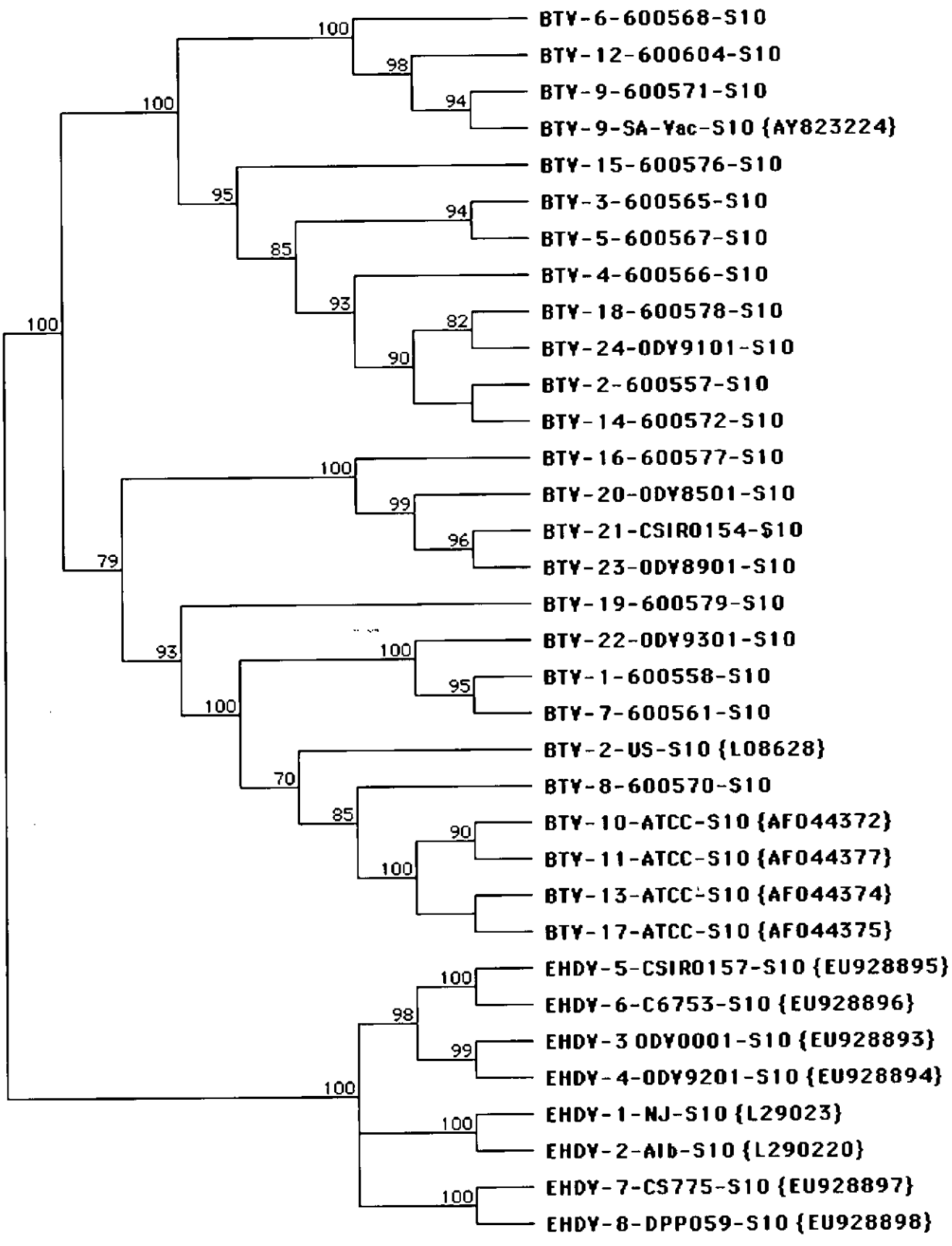

Figure 2. Example of phylogenetic analysis of *Bluetongue virus* (BTV) and *Epizootic hemorrhagic disease virus* (EHDV) S10 (NS3) gene sequences by using ClustalW alignment based on absolute number of differences, with gaps distributed proportionally and UPGMA (bootstrap [1,000 reps]; tie breaking = systematic).

Table 2. Threshold cycle values from 10 signatures tested against 24 *Bluetongue virus* serotypes at 200-pg double-stranded RNA. Four signatures detected all 24 serotypes (A7, A8, B1, and B3).*

	L1 (A5)	S8 (A6)	A7	A8	A9	B1	B3	B5	B6	B11
BT1	11.8	16.0†	20.1	19.2	18.7	20.4	19.5	18.6	17.7	23.6
BT2	23.0	24.7	27.2	24.0	23.1	21.0	21.0	17.9	18.0	23.9
BT3	23.6	NA	20.4	21.6	NA	22.0	20.4	19.3	24.2	19.6
BT4	23.2	NA	25.1	23.3	NA	24.1	21.2	16.2	18.8	22.0
BT5	24.8	NA	23.1	23.7	35.1	22.2	20.2	17.9	18.2	22.5
BT6	26.8	NA	20.5	23.9	NA	22.5	20.5	17.9	18.3	24.5
BT7	21.3	NA	18.1	14.8	14.3	15.1	17.4	13.0	15.4	16.6
BT8	24.0	26.0†	20.3	18.4	18.3	19.8	21.0	NA	20.1	18.8
BT9	23.2	13.15†	18.2	23.6	NA	21.9	18.8	15.8	17.0	21.8
BT10	20.7	17.1	25.5	16.5	NA	19.2	15.8	24.5	16.8	20.3
BT11	16.8	14.7	15.6	14.8	16.6	16.4	24.3	24.7	15.9	20.2
BT12	19.0	NA	23.8	19.9	19.1	18.3	19.3	14.3	14.6	19.2
BT13	22.9	18.1	16.9	17.0	20.6	18.9	23.5	25.4†	22.5	22.9
BT14	23.8	NA	22.3	19.1	NA	17.7	20.2	16.4	16.7	22.7
BT15	28.6	NA	25.5	33.3	25.3	25.0	24.7	19.7	23.5	23.6
BT16	NA	NA	30.3	35.5	18.7	26.5	21.7	NA	NA	NA
BT17	22.6	18.5	15.7	16.5	18.0	18.6	26.5	NA	20.2	NA
BT18	24.8	36.6†	22.8	25.1	NA	20.7	19.2	16.7	20.2	21.8
BT19	26.8	NA	29.3	24.4	24.7	27.0	30.0	23.0	29.9	25.9
BT20	NA	NA	26.4	26.7	21.1	26.0	25.1	NA	NA	NA
BT21	37.1†	NA	32.1	29.6	23.5	28.3	32.5	31.8†	NA	33.6
BT22	24.4	NA	22.7	21.1	21.3	21.7	24.2	19.9	23.9	23.5
BT23	34.0†	34.1	30.4	30.4	21.6	24.6	26.1	35.0†	NA	31.9
BT24	30.5	NA	28.8	28.8	NA	27.1	24.4	23.1	28.8	25.5

* NA = no amplification.
† Only 1 duplicate amplified.

detect all serotypes were tested again to determine if they were able to detect the BTV-2 Ona A strain. One of the 6 remaining signatures (B5 from M5) was able to detect BTV-2 Ona A. The BTV real-time RT-PCR assay thus consists of 2 signatures (A8 and B5), or 2 probes and 4 primers, and detected all 24 BTV prototype serotype strains. In addition, it detected 45 random BTV strains and 4 BTV virus isolation positive clinical samples from our inventory.

BTV-EHDV multiplex real-time RT-PCR

In the final design, the BTV A8 and B5 primers and respective probes were combined with the previously published EHDV real-time RT-PCR assay.[43] The probes for BTV and EHDV contained different quencher and reporter dyes. The BTV probes contained CAL Fluor Orange 560[j] on the 5′-end and BHQ-1[j] on the 3′ end. The EHDV probes had FAM on the 5′ end and BHQ-1 on the 3′ end. Initial tests with this design detected and differentiated cell culture source BTV and EHDV RNA; however, the assay lacked sensitivity with clinical samples. To address the sensitivity, heat or heat plus dimethyl sulfoxide denaturation of the dsRNA target was evaluated. The assay was able to detect 1,000 copies of BTV-10, BTV-17, EHDV-1, and EHDV-2, with Ct values in the mid 20 range. At 100 copies of EHDV-1 or -2, a slightly, but not significantly lower, Ct value was found when denaturing the template alone before reagent addition. The BTV-10 and BTV-17 RNA were not detected at 100 copies. To further increase the sensitivity, various commercial RNA extraction and real-time RT-PCR reagents were evaluated. This evaluation was done by using sheep blood spiked with known quantities of BTV or EHDV. Of the blood extraction kits, the MagMAX Viral RNA Isolation kit[e] used with Applied Biosystem cycling parameters was more sensitive, with a LOD of 10 $CCID_{50/ml}$ for BTV and 1 $CCID_{50/ml}$ for EHDV in comparison with the original EHDV parameters. This extraction kit was also easy to use, because it did not require phenol extraction or individual tubes when processing high-volume samples. The AgPath-ID kit[e] provided greater sensitivity for real-time RT-PCR and uses fewer reagents, so it is a faster option, with less chance of error. A comparison of the original assay reaction, consisting of the TaqMan EZ[e] versus Ag-Path ID,[e] indicated that the Ag-Path ID reagents had a 1,000-fold increase in sensitivity for both BTV and EHDV RNA detection (Table 4).

Discussion

Exotic serotypes of BTV continue to be introduced into countries where there are naïve livestock. This

Table 3. The limit of detection (LOD) was determined for signatures A7, A8, B1, and B3 by running each serotype in at least triplicates from 2 pg to 2 fg.*

	B1		B3		A7		A8	
	LOD (copy no.)	Mean Ct value	LOD (copy no.)	Mean Ct value	LOD (copy no.)	Mean Ct value	LOD (copy no.)	Mean Ct value
BTV-1	10,000	30.8 ± 1†	100	35.4 ± 1.7	1,000	35.9 ± 1.3	1,000	35.5 ± 1.3
BTV-2	1,000	31.9 ± 0.4	100	35.3 ± 0.4	1,000	36.8 ± 0.3	1,000	31.2 ± 5.9
BTV-3	1,000	34.5 ± 2.3	100	34.1 ± 1.0	10,000	34.3 ± 1.8†	10,000	35.5 ± 1.2
BTV-4	10,000	34.4 ± 0.6†	100	36.4 ± 0.4	100,000	34.6 ± 1.7†	100,000	34.4 ± 1.3†
BTV-5	1,000	35.3 ± 1.7†	100	35.3 ± 1.1	1,000	36.0 ± 1.0	100,000	34.9 ± 0.4
BTV-6	1,000	32.7 ± 0.1	1,000	35.9 ± 1.3	10,000	33.2 ± 0.5†	100,000	36.7 ± 0.8
BTV-7	100	25.1 ± 0.5	100	27.2 ± 1.3	100	33.2 ± 0.5	100	30.6 ± 0.5
BTV-8	100	25.8 ± 0.1	1,000	32.6 ± 0.4†	100	36.7 ± 0.9	1,000	35.2 ± 1.7
BTV-9	100	25.8 ± 0.1	1,000	33.4 ± 1.5†	1,000	34.2 ± 1.0	10,000	35.1 ± 1.9†
BTV-10	100	26.0 ± 0.2	100	31.9 ± 1.3	100,000	33.8 ± 1.2†	100	33.3 ± 1.4
BTV-11	1,000	32.2 ± 1.6	1,000	33.4 ± 1.8†	100	36.0 ± 0.6	100	34.3 ± 1.4
BTV-12	100	33.4 ± 2.4	100	32.9 ± 0.6	1,000	34.3 ± 0.4	100,000	33.2 ± 0.2
BTV-13	100	34.6 ± 1.0	1,000	35.4 ± 0.7	100	33.2 ± 2.0	100	34.0 ± 1.2
BTV-14	1,000	31.2 ± 0.9†	1,000	32.5 ± 0.6†	10,000	34.9 ± 1.2	1,000	35.4 ± 1.3†
BTV-15	100	32.1 ± 5.5	1,000	34.6 ± 0.3†	10,000	33.9 ± 0.7†	100,000	NA
BTV-16	100	29.6 ± 2.6	100	34.4 ± 2.3	100,000	36.8‡	100,000	NA
BTV-17	100	29.2 ± 0.3	1,000	35.3 ± 1.1†	100	29.3 ± 1.7	100	29.0 ± 1.5
BTV-18	1,000	34.3 ± 1.1†	100	36.5 ± 0.8	1,000	36.3 ± 1.7†	10,000	34.5 ± 1.6
BTV-19	100	31.3 ± 0.01	10,000	36.5 ± 0.5	10,000	34.2 ± 2.2†	100	35.2 ± 2.2
BTV-20	10,000	35.6 ± 1.7†	10,000	35.4 ± 1.7	10,000	36.3 ± 1.2	100,000	33.8‡
BTV-21	1,000,000	32.9 ± 2†	10,000,000	34.6 ± 2	10,000,000	34 ± 2†	10,000	35.4 ± 0.4
BTV-22	100	32.6 ± 0.4	1,000	33.4 ± 0.5†	100	31.5 ± 1.9	1,000	25.6 ± 8.0†
BTV-23	1,000	23.0 ± 8.2†	10,000	37.0 ± 0.2	100,000	36.6 ± 1	100,000	35.4 ± 1.1†
BTV-24	10,000	27.5 ± 7.8	1,000	35.3 ± 1.2	100,000	36.9 ± 0.1	100,000	36.4 ± 1.2†

* BTV = *Bluetongue virus*. Data shown are the LOD in RNA copy no. and mean threshold cycle (Ct) value ± standard deviation of the last dilution, where all 3 replicates were amplified. NA = no amplification.

† At least one replicate amplified at the 10-fold lower dilution.

‡ Less than 3 of the replicates amplified, and the standard deviations were not calculated.

Table 4. Comparison of the TaqMan EZ RT-PCR core reagents[c] and the AgPath-ID One-Step RT-PCR reagents[c] paired with the MagMAX Viral RNA Isolation.[c]*

	$CCID_{50}$/ml	TaqMan EZ RT-PCR	AgPath-ID	Paired *t*-test
BTV-17	1000	34.9 ± 1.0	29.6 ± 0.2	0.09
BTV-17	100		32.3 ± 0.2	
BTV-17	10		34.8 ± 2.1	
BTV-17	1		34.9 ± .06	
EHDV-2	1,000	29.1 + 0.2	29.6 + 0.4	0.05
EHDV-2	100	32.6 + 1.1	33.1 + 0.8	0.28
EHDV-2	10	33.9 + 1.5	33.8 + 0.5	0.46
EHDV-2	1	NA	NA	

* $CCID_{50}$ = 50% cell culture infective dose; RT-PCR = reverse transcription polymerase chain reaction; BTV = *Bluetongue virus*; EHDV = *Epizootic hemorrhagic disease virus*; NA = no amplification.

has been a significant issue in Europe with the introduction of BTV into southern Europe,[17] and, more recently, the introduction of BTV-8 in northern Europe, which is causing significant disease in cattle.[17] The introduction of exotic BTV or EHDV serotypes, and the potential effect on livestock health and trade, is a concern to the U.S. livestock industry. *Bluetongue virus* serotype 2 was first isolated in the United States in 1982[9]; BTV-1 was isolated in Louisiana in 2004,[15] and multiple isolations of exotic BTV serotypes were made from Florida during 1999–2006 (Johnson DJ, Mertens PPC, Maan S, Ostlund E: 2007, Exotic bluetongue viruses identified from ruminants in the south-eastern U.S. from 1999–2006. *In:* American Association Veterinary Laboratory Diagnosticians, Reno, NV, October 18, 2007). In addition, the isolation of EHDV-6 in the United States was recently reported.[33] These recent exotic serotype introductions clearly demonstrate the need for robust assays that rapidly detect all known orbiviral strains.

The sequence analysis performed in this study was done to assist in primer design and not for use in a comprehensive phylogenic analysis; however, sophisticated analysis was performed. The prototype viruses used in the current study were provided by or received by previous investigators, and their origins were unable to be confirmed. It was presumed that BTV prototype serotypes 1–9, 12, 14–16, 18, 19, 23, and 24 are of African, primarily southern African, origin. Serotypes 20 and 21 likely originated from Australia. These assumptions fit the phylogenetic analysis in that viruses tend to group according to geographic origin. This tendency has previously been shown for the S10 gene in a more comprehensive phylogenetic study.[3] A similar tendency for geographic topotypes was demonstrated for the S3 and S7 genes as well.[30,42] It is unfortunate that the origins of these viruses could not be confirmed, but the data suggest that the M5 gene may also display a tendency for geographical topotypes. The current approach for BTV and EHDV phylogenetic analysis is to use whole genome sequencing approaches.[18] This technology will rapidly advance our understanding of the molecular epidemiology of these viruses.

The sequence and phylogenetic analyses reported here demonstrated greater variability of the 2 target nonstructural genes than was shown in previous studies.[39] Understanding genetic diversity of potential viral genome targets is essential in designing more robust real-time RT-PCR assays that rapidly detect both domestic and exotic BTV and EHDV strains. The multiplex BTV and/or EHDV real-time RT-PCR assay reported in this study incorporates multiple RNA targets, thus it is less likely to be adversely affected by genetic variation than previous real-time RT-PCR assays for BTV or EHDV. Use of a sophisticated bioinformatic design approach has allowed the development of an assay that detects an estimated 10 $CCID_{50/ml}$ for BTV and 1 $CCID_{50/ml}$ for EHDV in spiked blood. A future improvement of this assay would be to include an internal or positive RNA control as in the multiplex vesicular disease panel.[16] However, to do so at this time would significantly delay release of the assay. The current assay is advantageous over previously reported assays, because it is the first reported real-time RT-PCR assay to detect and distinguish both BTV and EHDV in a single-tube format. In fact, the presence of both BTV and EHDV RNA in a clinical sample was detected. The assay was proven to be adaptable to genetic variation discovered in field isolates; thus, it provides a convenient and robust tool to orbivirus researches as well as veterinary diagnostic laboratories.

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Sources and manufacturers

a. Purescript®, Gentra Systems Inc., Minneapolis, MN.
b. Implen Inc., Westlake Village, CA
c. Ambion® MagMAX™-96 for Microarrays Total RNA Isolation, Ambion® MagMAX™ Viral RNA Isolation, Ambion® AgPath-ID™ One Step RT-PCR Reagents; Applied Biosystems, Foster City, CA.
d. Invitrogen Inc., Carlsbad, CA.
e. TaqMan EZ RT-PCR core reagents, Applied Biosystems, Foster City, CA.
f. DNA Star Inc., Madison, WI.
g. MacVector Inc., Cary, NC.
h. MyiQ Single-Color Real-Time PCR Detection System, Bio-Rad Laboratories, Hercules, CA.
i. Cepheid, Sunnyvale, CA.
j. Biosearch Technologies Inc., Novato, CA.

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