Development of PCR-based Tests for the Identification of North American Isolates of Epizootic Haemorrhagic Disease Virus

Martha J. Harding, Isabelle Prud'homme, Jerzy Rola, and Gilles C. Dulac

ABSTRACT

A serogroup-specific polymerase chain reaction (PCR) assay and isolate identification strategies (restriction endonuclease analysis (REA) and nucleotide sequencing) were developed for the detection of North American isolates of epizootic haemorrhagic disease virus (EHDV). PCR primers (EHDV-pr4, EHDV-pr5) were designed to hybridize to the L3 gene of a North American isolate of EHDV serotype 1. Total nucleic acid was extracted from preparations of infected tissue culture and PCR was performed using a cDNA-PCR kit, according to the manufacturer's specifications. The PCR assay generated a 459 base pair product from North American isolates of EHDV serotypes 1 and 2, while bluetongue virus (BTV) serotypes 10, 11, 13, and 17, and cell controls, failed to demonstrate PCR products. Slight modifications allowed for the PCR detection of EHDV-1 and -2 in white-tailed deer blood (*Odocoileus virginiatus*); PCR fragments were not amplified from uninfected deer blood. A number of restriction endonucleases and sequencing primers were evaluated for their utility in isolate identification experiments. Specifically, REA employing *Hin*cII and cycle sequencing with an internal primer (EHDV-1-pr3) proved most successful for identifying isolate-specific genome markers. The techniques presented are expected to prove valuable for rapid and specific detection of possible future EHDV incursions in wild and domestic animal species.

RÉSUMÉ

Un test d'amplification en chaîne par la polymérase (ACP) et des stragégies d'identification (analyse des fragments de restriction et séquençage) ont été développés pour détecter différents sérotypes du virus de la maladie hémorragique du cerf (VMHC). Des amorces pour ACP, (EHDV-pr4, EHDV-pr5) ont été créées de façon à ce qu'elles s'hybrident au gène L3 de l'isolat nord-américain VMHC sérotype 1. Des extraits d'acides nucléiques provenant de préparation de cultures cellulaires infectées ont subi le test ACP utilisant une trousse d'amplification d'ADN complémentaire selon les recommendations du manufacturier. Le test a généré un fragment d'ADN long de 459 paires de bases pour VMHC-1 et -2, mais aucun produit n'á été amplifié à partir du virus de la fièvre catarrhale (sérotypes 10, 11, 13 et 17) ni dans les témoins cellulaires. Quelques modifications au test ont permis la détection du VMHC types 1 et 2 dans le sang de cerf de virginie (*Odocoileus virginiatus*). La performance de plusieurs enzymes de restriction et amorces de séquençage a été évaluée lors d'expériences d'identification des isolats. L'analyse par restriction utilisant l'enzyme *Hin*cII et le séquençage à l'aide de l'amorce interne EHDV-1-pr3 se sont avérés des plus utiles pour l'identification de marqueurs génomiques spécifiques aux isolats. Les techniques proposées se montreront probablement utiles pour la détection rapide et spécifique du VMHC lors d'incursions chez les animaux sauvages et domestiques.

INTRODUCTION

Epizootic haemorrhagic disease virus (EHDV) is a member of the *Orbivirus* genus of the family Reoviridae. EHDV possesses typical orbivirus morphology (1,2), consisting of an amorphous outer layer of structural proteins VP2 and VP5, and an inner capsid layer containing 2 major structural proteins, VP3 and VP7, and 3 minor proteins, VP1, VP4, and VP6. Three nonstructural proteins have been identified; NS1, NS2, and NS3. Within the inner core are 10 segments of double-stranded RNA, each of which encodes for a major polypeptide (3).

Eight EHDV serotypes are recognized by the serum neutralization test (4). In North America, EHDV types 1 and 2 are present; however in Canada, only the type 2 virus has been recognized (5). Canadian outbreaks of EHDV occur occasionally; the last outbreak was reportedly transported northwards by wind currents which carried infected gnats of the genus *Culicoides* (6). Under natural conditions, EHDV infects several species of ruminants, but clinical signs occur mostly in wild ungulates such as white-tailed deer, mule deer, and antelope. In these species, the infection is very debilitating and often fatal (7–9). In livestock species, the infection is generally inapparent or very mild (10).

In susceptible species, infection with EHDV needs to be differentiated from infection with bluetongue virus (BTV), a related orbivirus, which causes disease in sheep and also causes serious effects on the livestock industry due to limitations on export of animals, semen, and embryos from

Animal Diseases Research Institute, P.O. Box 11300, Station H, Nepean, Canada K2H 8P9.

Correspondence and reprint requests to Dr. M.J. Harding: James A. Baker Research Institute, College of Veterinary Medicine, Cornell University, Ithaca, New York 14 853 USA.

Submitted November 10, 1994.

infected regions. Current diagnostic protocols for detection of these viruses involve the isolation of virus in cell culture (EHDV), or in embryonated chicken eggs, or sheep (BTV), followed by virus typing using the serum neutralization test (11). The above procedures are costly, time-consuming and require animal usage.

Polymerase chain reaction (PCR) assays (12) have been developed for the detection of many pathogens with the intent of circumventing the inherent drawbacks of conventional diagnostic methods. Recent reports describe the detection of EHDV-1 and -2 using PCR primers derived from NS-1 gene sequences (13,14). Nucleic acid sequence comparisons of the L3 gene, which encodes the serogroup-reactive protein VP3 (15), reveal that this gene is also highly conserved in their respective serogroup (16–20). Indeed, Australian isolates of EHDV (serotypes 2,5,6, and 7) have been amplified using a pair of L3 gene-based primers (21). This report illustrates the application of PCR amplification of North American isolates of EHDV, and the development of timely confirmatory and isolate identification protocols for use in the event of an outbreak.

MATERIALS AND METHODS

VIRUSES

Tissue culture EHDV and BTV reference viruses, originally donated by Dr. J.E. Pearson, (National Veterinary Services Laboratory (NVSL), Ames, Iowa) were propagated either in BHK-21 or Vero cells. Cells were grown in minimum essential medium in Earle's balanced salt solution supplemented with 10% fetal bovine serum (FBS), 200 m ML-glutamine, 10 mM of nonessential amino acids, and 110 mg/mL Na pyruvate. Penicillin (100 IU/mL) and streptomycin (100μg/mL) were included in the medium. The FBS was shown to be free of bovine viral diarrhea virus (BVDV), by immunoperoxidase analysis of bovine turbinate cells following 5 tissue culture passages in complete medium, as described above. The FBS was also negative for antibodies to BTV, EHDV, and BVDV, using either ELISA (BTV), or serum neutralization (EHDV and BVDV), tests.

Blood from 2 EHDV-infected *Cervidae* (white-tailed deer, *Odocoileus virginiatus*; EHDV-1: 5 d postinfection, EHDV-2: 9 d postinfection) was collected using EDTA as the anticoagulant, and the blood cells were washed with PBS prior to storage at 4°C. The deer blood was donated by Dr. E. Howerth, (Department of Pathology, College of Medicine, University of Georgia, Athens, Georgia). To confirm the presence of EHDV in the deer blood specimens, the virus was passaged twice on BHK-21 monolayers, then identified as EHDV or BTV using specific monoclonal antibodies in an indirect immunoperoxidase assay (11). Noninfected *Cervidae* blood was used as a negative control sample.

HARVESTING OF NUCLEIC ACID

Tissue cultures were infected according to established protocols. Following complete destruction of the monolayer, cells were harvested by scraping and centrifugation. Cells were lysed by 2 freeze/thaw cycles, followed by treatment with 0.1 mg/mL proteinase K and 1% (final concentration) SDS at 37°C for 1 h. Total cell nucleic acid was extracted with a single treatment with phenol:chloroform: isoamyl alcohol (25:24:1) and concentrated by ethanol precipitation in the presence of sodium acetate (final concentration of 0.3 M, pH 5.5). Nucleic acid pellets were resuspended in H_2O and stored at −70°C. Retrieval of nucleic acid from specimens of washed *Cervidae* blood cells was performed essentially as above. However, the red blood cells were initially concentrated by centrifugation at 12 000 × *g* for 5 min. The pellet was resuspended in 500 μL phosphate buffered saline (pH 7.2), then subjected to proteinase K treatment for 3 h, followed by 2 cycles of phenol extraction. Prior to PCR testing, blood samples were treated at 95°C for 10 min, to reduce porphyrin inhibition.

PCR PRIMERS

Published nucleic sequence information for the L3 gene of a North American isolate of EHDV-1 (20) was analysed for the derivation of PCR primers (EHDV-pr4 and EHDV-pr5). Furthermore, it was predicted that these primers were suitable for the amplification of an Australian EHDV-2, based on published data (20). The primer pair was complementary to sequences at nucleotide positions 648 to 668 (5′-GTATATCGCATATACAATCGG-3′) and 1086 to 1106 (5′-CATTCTTTCACCCGGATCTAT-3′). Prior to primer synthesis, nucleotide sequences contained in GenBank (Intelligenetics, Mountain View, California) using the Pustell Sequence Analysis Program (IBI, New Haven, Connecticut) were reviewed to ensure that cross-hybridization with other viruses was unlikely. Primers were synthesized on a PCR-Mate Model 391 (Applied Biosystems, Foster City, California) then eluted from the columns with fresh ammonium hydroxide, concentrated with butanol, and dried to a pellet in a speed-vac (Savant, Farmingdale, New York). Primer concentrations were determined using the "Saran-wrap" method (22), by comparing to an MS2 primer control (Invitrogen, San Diego, California). Analysis of the biochemical properties of the primers utilized the Gene Runner software package (Hastings Software Inc., Hastings on Hudson, New York).

REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT-PCR)

To avoid test compromise due to false-positive reactions, separate work areas and supplies for reagent preparation, sample set-up and reaction analysis were designated. A single tube RT-PCR was performed using reagents supplied in a cDNA Cycle kit (Invitrogen), according to the manufacturer's specifications. Briefly, nucleic acid was denatured at room temperature for 5 min with 20 mM (final concentration) methyl-mercury hydroxide, then inactivated with 140 mM β-mercaptoethanol (final concentration). An RT mix containing 0.5 units/μL RNase inhibitor, 50 ng/ μL random primers, 1.25 mM dNTPs, 0.25 units/μL avian reverse transcriptase, and 5× RT buffer was added. Final buffer concentration in this reaction step was 10 mM Tris-HCl, pH 8.3, 40 mM KCl and 10 mM $MgCl_2$. The solution volume during the RT step was 20 μL. Tubes were

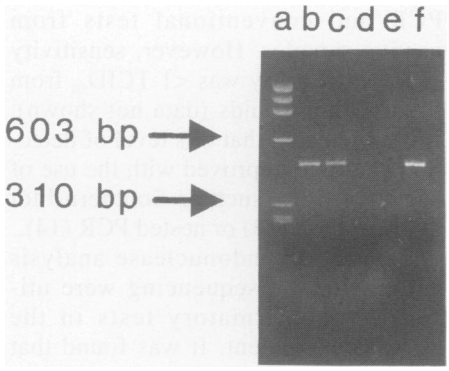

Figure 1. Detection of EHDV in deer blood using PCR. White-tailed deer blood infected with EHDV-1 and -2 were subjected to EHDV PCR as described in Materials and methods. Lane a: ϕX174/*Hae*III size standard; lanes b, c: EHDV-1 and -2 infected deer blood; lanes d, e: uninfected deer blood and reagent negative controls; lane f: EHDV-1 positive control.

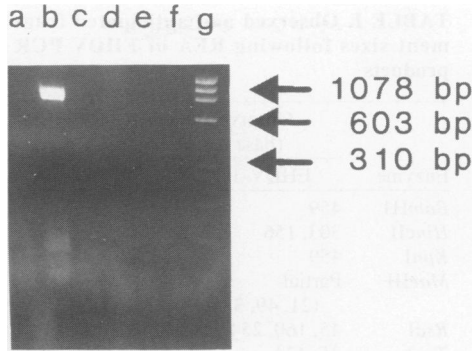

Figure 2. EHDV PCR Specificity Assay. EHDV primers were assessed for serogroup specificity, using the PCR assays described in Materials and methods. Lane a: Vero cell control; lane b: BTV positive control with BTV primers; lanes c–f: BTV serotypes 10, 11, 13 and 17, respectively, with EHDV primers; lane g: ϕX174/*Hae*III size standard.

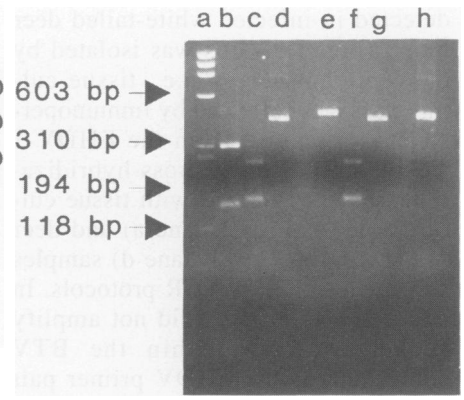

Figure 3. Representative REA of EHDV PCR products. EHDV-1 and -2 PCR products were digested as described in Material and methods. Lane a: ϕX174/*Hae*III size standard; lanes b–d: EHDV-1 digested with *Hin*cII, *Rsa*I and *Taq*I, respectively; e–g: EHDV-2 digested with *Hin*cII, *Rsa*I and *Taq*I, respectively; lane h: uncut EHDV control.

transferred to a Coy thermal cycler (Diamed Lab Supplies, Mississauga, Ontario) and the mixture was incubated at 42°C for 1 h, followed by enzyme denaturation at 95°C for 5 min. While the mixture remained at 80°C, PCR reagents were added to produce a mixture with a final volume of 50 μL and final concentrations of 10 mM Tris-HCl, pH 8.3, 46 mM KCl, 6 mM $MgCl_2$, 1.3 μg/μL gelatin, 0.2 mM dNTPs, 2 ng/μL EHDV-specific primers and 0.5 units/μL Amplitaq polymerase (Perkin-Elmer, Mississauga, Ontario). The thermal-cycling program included 40 cycles of 1 min denaturation at 94°C, 1 min of primer annealing at 53°C, and 2 min of extension at 72°C, and a similar final cycle, except with a 10 min extension period. Subsequently, 20% of the amplification product was analyzed on 3% NuSieve/1% SeaKem agarose (FMC/Mandel, Guelph, Ontario) containing 0.5 μg/mL ethidium bromide. Gels were electrophoresed at 50 V for 2 h in tris-acetate-EDTA buffer, also containing 0.5 μg/mL ethidium bromide. Bands were visualized with a UV light source and compared to ϕX174-*Hae*III size standards (BRL, Burlington, Ontario).

RESTRICTION ENDONUCLEASE ANALYSIS (REA) OF PCR PRODUCTS

Putative restriction maps were produced by the Pustell Sequence Analysis Program (IBI), utilizing the published L3 gene sequence for EHDV (19,20). A spectrum of restriction endonucleases (BRL) was employed; however, digestion of the PCR products with *Hin*cII, *Kpn*I, *Mae*III and *Taq*I were expected to allow differentiation of EHDV-1 and -2. Unpurified PCR product (representing approximately 200 ng EHDV-specific fragment) was used in these experiments. All reactions were performed for 2 h at 37°C, except that *Taq*I incubations were held at 65°C.

NUCLEIC ACID SEQUENCING OF PCR PRODUCTS

Definitive confirmation of the amplification products was pursued by DNA sequencing using the EHDV PCR (EHDV-pr4, EHDV-pr5) primers, or from an internal primer (EHDV-1-pr3; 5′-TGG TCC AGT TGT AGT AAT-3′), which hybridized to nucleotides 963–980 of the L3 gene of EHDV serotype 1. PCR templates that had been selected for cloning were purified by electrophoresis in 1% LMP agarose (BRL, Burlington, Ontario) followed by DNA extraction using the Magic PCR-Prep system (Promega, Nepean, Ontario) according to the manufacturer's instructions. Nucleic acid concentration was determined by comparing to a set of known standards using ethidium bromide quantitation (22). Sequencing data were generated using the fmol DNA sequencing system (Promega), according to the manufacturer's specifications. Briefly, 10 pmol of primer was radiolabelled with γ-^{32}P- dATP in the presence of 50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine and 8 units polynucleotide kinase. Extension products were produced in a reaction mixture containing 40 pmol template, 1.5 pmol labelled primer, 50 mM Tris-HCl, 2 mM $MgCl_2$ and 5 units sequencing grade *Taq* polymerase (Promega). One-fourth of the mixture was transferred to the appropriate dd/dNTP mix and extension/termination products were generated using a thermal cycler. Annealing temperatures of 53°C (EHDV-pr4, 5), or 42°C (EHDV-1-pr3) were used in the cycling program. Sequencing data was determined by autoradiography following electrophoresis in 6% polyacrylamide/8 M urea gel, using tris-borate-EDTA buffer, and transfer to 3MM Whatmann filter paper. Nucleotides sequences were analyzed with the IBI Pustell sequence analysis program.

RESULTS

PCR primers amplified both EHDV North American serotypes 1 and 2 propagated in vitro (data not shown). In addition, viral nucleic acid was

detected in infected white-tailed deer blood (Fig. 1). Virus was isolated by conventional means (i.e., tissue culture passages followed by immunoperoxidase staining) from the EHDV-1 sample only. Primer cross-hybridization was not apparent with tissue culture control (Fig. 2, lane a) and deer blood control (Fig. 1, lane d) samples subjected to EHDV PCR protocols. In addition, the primers did not amplify sequences from within the BTV serogroup, as the EHDV primer pair failed to amplify the BTV serotypes 10, 11, 13, and 17 (Fig. 2). The presence of amplifiable BTV template was confirmed by subjecting a similar sample to a BTV PCR assay (26; Fig. 2, lane b).

Utilizing a variety of restriction endonucleases, REA of the EHDV-1 PCR product was consistent with what was anticipated (Fig. 3, Table I), based on previous EHDV-1 nucleotide sequence data (20). In addition, the EHDV-2 *Bam*HI, *Hin*cII and *Rsa*I digestion patterns were expected (19). However, *Taq*I and *Kpn*I did not digest EHDV-2 in the manner which was suggested by the sequence information for an Australian isolate of EHDV-2. Isolates of the EHDV serotypes could be distinguished by the *Hin*cII (Fig. 3, lanes b and e) pattern. The restriction endonuclease, *Mae*III repeatedly generated a partial digest, in spite of extended incubation times. Specimens of in vitro- and in vivo-propagated viruses revealed identical PCR product restriction maps (data not shown).

Portions of EHDV-1 (nucleotides 679–1062) and -2 (nucleotides 688–1062) PCR products were sequenced (Table II) with the downstream PCR primer (EHDV-pr5) and the internal primer (EHDV-1-pr3). Autoradiograms were not legible using the upstream PCR primer (EHDV-pr4), in spite of lowering the annealing temperature to 50°C. The nucleotide sequence of the EHDV-1 PCR product was identical to that previously published (20). In the region sequenced, comparisons between the EHDV-1 and -2 PCR sequences revealed nucleotide and amino acid homologies of 97% and 98% respectively. In spite of a 78% nucleotide sequence homology between the EHDV-2 PCR product and published Australian EHDV-2 data (19), the majority of mutations were silent, resulting in amino acid sequence homology of 96%. The sequence information generated from the particular North American EHDV-2 isolate studied was identical to an Alberta isolate of EHDV-2, presented in the EMBL database under the identifier E2L3US, submitted by Cheney et al. (23).

TABLE I. Observed and anticipated fragment sizes following REA of EHDV PCR products

Enzyme	Observed fragment sizes (base pairs), see Fig. 3	
	EHDV-1	EHDV-2
*Bam*H1	459	459
*Hin*cII	303, 156	459
*Kpn*I	459	459 (204, 255)[a]
*Mae*III	Partial (21, 49, 389)	Partial (57, 117, 285)
*Rsa*I	45, 160, 254	45, 162, 254
*Taq*I	35, 424	35, 424 (107, 352)

[a] Fragment size predicted from published sequence data (19, 20)

DISCUSSION

A North American serotype-specific assay for detection of EHDV isolates was developed and found suitable for amplification from tissue culture fluids or directly from deer blood. Supportive technologies (REA and nucleotide sequencing) were devised for confirmation of the identity of the PCR product and as a means of isolate identification. Classical isolation techniques revealed presence of EHDV infection in the blood from an EHDV-1-inoculated deer, while the presence of an orbivirus in the EHDV-2 blood sample could not be confirmed in our laboratory. Both the infected and control deer specimens were contaminated with yeast, most likely due to the length of time the blood had been stored at 4°C. Therefore, prior to isolation attempts, the blood was filtered, which perhaps hindered the ability to isolate virus. This finding highlights an advantage of PCR methods: samples contaminated with other microbes, such as bacteria or yeast, are still amenable to pathogen detection via PCR. The titer of EHDV in the deer blood was too low to allow for sensitivity comparisons between PCR and conventional tests from in vivo samples. However, sensitivity of the PCR assay was <1 $TCID_{50}$ from tissue culture fluids (data not shown). It is anticipated that this level of detection could be improved with the use of ancillary tests, such as Southern blot hybridization (13) or nested PCR (14).

Restriction endonuclease analysis and nucleotide sequencing were utilized as confirmatory tests in the assay development. It was found that these methods could also discriminate between isolates of EHDV-1 and -2. The partial digest observed following digestion with *Mae*III may be indicative of residual endonuclease binding to the DNA. Evaluation of procedures designed to disrupt the protein-nucleic acid bonds, such as phenol extraction or addition of SDS, may be warranted. The *Kpn*I and *Taq*I restriction patterns for EHDV-2 were not anticipated. The fact that the published sequence information relates to an Australian isolate of EHDV-2 may explain these deviations in the REA and sequencing data generated with a North American EHDV-2 isolate. Indeed, phylogenetic analyses reveal that an American isolate of EHDV-1 differs from the Australian EHDV group (21). It is likely that North American EHDV-2 is somewhat phylogenetically removed from its Australian counterpart. In the VP3 region analyzed, the North American EHDV-2 was more similar to the North American EHDV-1 than to an Australian EHDV-2 (19). These observations support the hypothesis that viruses within a given topotype evolve from a common ancestor.

Experiments that utilized the downstream PCR and internal primers as a cycle sequencing primer demonstrated that both EHDV-1 and -2 PCR products were genuine. The upstream primer was not a suitable reagent for generating nucleotide sequences. This oligonucleotide possessed 4 hairpin, 3 bulge and 3 internal loops, including 1 internal loop with a T_m of 92.8°C, which could have prevented primer annealing during sequencing procedures. In contrast, analysis revealed no significant secondary structures in the downstream primer. It is suggested that the differences in the molecular behavior between the PCR primers

TABLE II. Nucleotide sequence of a portion of EHDV-2 and -1 PCR products, and comparison with an Australian isolate of EHDV-2 (EHD2VP3AUS). Nonsilent mutations, represented by lower case letters, are depicted relative to the EHDV-2 PCR product sequence

	688													
EHDV-2 PCR	CC	GTT	CAA	TTG	GAA	GAG	TTG	CGC	GGA	GCG	GTG	ACA	TGG	CTT
EHDV-1 PCR						A			c			G		
EHD2VP3AUS[a]	G	A			G		A	G	c g		a c	G		
	729													
EHDV-2 PCR	GAG	CGT	CTG	GGG	AAA	CGA	AAA	CGC	ATG	ACT	TTT	TCA	CAA	GAA
EHDV-1 PCR		C												
EHD2VP3AUS	A	A A		A	g	T				C		G	G	G
	771													
EHDV-2 PCR	TTT	CTA	ACA	GAT	TTT	AGA	CGT	GCA	GAT	ACG	ATT	TGG	GTA	CTG
EHDV-1 PCR								t		A				
EHD2VP3AUS		C		C			A	G			A			T A
	813													
EHDV-2 PCR	GCG	TTG	AGG	CTG	CCA	GCC	AAC	CCA	CGC	GTG	ATT	TGG	GAT	GTA
EHDV-1 PCR														
EHD2VP3AUS	A	A	c a	T A		A	T	C	T	A			c g	
	855													
EHDV-2 PCR	CCG	AGG	TGT	TCA	ATT	GCA	AAT	TTG	ATA	ATG	AAT	ATA	GCG	ACG
EHDV-1 PCR														
EHD2VP3AUS	C	A			A		C	C A	C		C		A	
	897													
EHDV-2 PCR	TGC	TTA	CCG	ACA	GGA	GAA	TAT	GTT	TCA	CCC	AAT	CCT	CGT	ATA
EHDV-1 PCR									G				C	
EHD2VP3AUS			A					A	T	A		A	A	T
	939													
EHDV-2 PCR	GCT	TCA	ATC	ACA	TTG	ACA	CAA	AGA	ATT	ACT	ACA	ACT	GGA	CCA
EHDV-1 PCR				G		G								
EHD2VP3AUS	A		A	G	C T	C	G		A	G	G	C	T	T
	981													
EHDV-2 PCR	TTT	GCT	ATA	TTA	ACG	GGA	TCA	ACG	CCA	ACG	GCA	CAA	CAA	TTA
EHDV-1 PCR														
EHD2VP3AUS			T		T		G	C	T	C	T			
	1023													1062
EHDV-2 PCR	GAT	GAC	GTA	AGA	AAG	ATA	TAC	TTA	GCG	TTG	ATG	TTT	CCA	G
EHDV-1 PCR							T	G						
EHD2VP3AUS	C	T	G	G	A		T		A	A				

[a] Based on Gould and Pritchard (1991)

caused the variation in utility in cycle sequencing experiments.

This report outlines a PCR-based technique for rapid and specific detection and identification of North American isolates of EHDV and the rapid differentiation from BTV. The rapidity of amplification and supportive technologies, such as REA or sequencing, makes these tests attractive to veterinary diagnosticians as confirmatory tests for the PCR assay, as well as generating novel sequence data for isolate identification. These protocols could serve as valuable tools for expeditious isolate identification, especially in evaluations of the index case in any future incursions. These methods have the potential to be an alternative to the time-consuming amplification and identification of virus in cell culture. Minimizing the delay between sample submission to a diagnostic laboratory and acquisition of results is especially of benefit during epizootics. Due to gene segment reassortment among orbivirus of various serotypes (24,25), an inference of serotype can not be made based on VP3 REA and/or sequencing analysis; rather an additional PCR based on serotype-specific VP2 sequences will be required. Additional studies with a larger number of North American field isolates are in progress prior to transferring these methods to a diagnostic setting.

ACKNOWLEDGMENTS

The authors wish to thank Mr. A. Tremblay for technical assistance and Dr. G. Alvarado, University of Ottawa, for his assistance with the analysis of primer sequences.

REFERENCES

1. **THOMAS FC, MILLER J.** A comparison of bluetongue virus and EHD virus: electron microscopy and serology. Can J Comp Med 1971; 35: 22–27.
2. **TSAI KS, KARSTAD L.** Ultrastructural characterization of the genome of epizootic hemorrhagic disease virus. Infect Immun 1973; 8: 463–474.
3. **MECHAM JO, DEAN VC.** Protein coding assignment for the genome of epizootic

haemorrhagic disease virus. J Gen Virol 1988; 69: 1255–1262.
4. **ST. GEORGE TD, CYBINSKI DH, STANDFAST HA, GARD GP, DELLAPORTA AJ.** The isolation of five different viruses of the epizootic haemorrhagic disease of deer serogroup. Aust Vet J 1983; 60: 216–217.
5. **DULAC GC, STERRITT WG, DUBUC C, AFSHAR A, MYERS DJ, TAYLOR EA, JAMIESON BR, MARTIN MW.** Incursions of orbiviruses in Canada and their serologic monitoring in the native animal population between 1962 and 1991. In: Walton TE, Osburn BI, eds. Bluetongue, African horse sickness, and related orbiviruses, 2nd ed. London: CRC Press, 1992; 120–127.
6. **SELLERS RF, MAAROUF AR.** Possible introduction of epizootic hemorrhagic disease of deer virus (serotype 2) and bluetongue virus (serotype 11) into British Columbia in 1987 and 1988 by infected Culicoides carried on the wind. Can J Vet Res 1991; 55: 367–370.
7. **SHOPE RE, MacNAMARA LG, MANGOLD R.** A virus-induced epizootic hemorrhagic disease of the Virginia white-tailed deer (Odocoileus virginianus). J Exp Med 1960; 111: 155–170.
8. **CHALMERS GA, VANCE HN, MITCHELL GJ.** An outbreak of epizootic hemorrhagic disease of wild ungulates in Alberta. Wildl Dis 1964; 42: 15–20.
9. **HOFF GL, TRAINER DO.** Bluetongue and epizootic hemorrhagic viruses: their relationship to wildlife species. Adv Vet Sci Comp Med 1978; 22: 111–132.
10. **FOSTER NM, METCALF HE, BARBER TL.** Bluetongue and epizootic hemorrhagic disease virus isolations from vertebrate and invertebrate hosts at a common geographic site. J Am Vet Med Assoc 1980; 176: 126–129.
11. **PEARSON JE, CARBREY EA, GUSTAFSON GA.** Bluetongue and related orbivirus diagnosis in the United States. Progress Clin and Biol Res 1985; 178: 469–475.
12. **SAIKI RK, GELFAND DH, STOFFEL S, SCHARF SJ, HIGUCHI R, HORN GT, MULLIS KB, ERLICH HA.** Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988; 239: 487–491.
13. **ARADAIB IE, AKITA GY, OSBURN BI.** Detection of epizootic hemorrhagic disease virus serotypes 1 and 2 in cell culture and clinical samples using polymerase chain reaction. J Vet Diag Invest 1994; 6: 143–147.
14. **WILSON WC.** Development of a nested-PCR test based on sequence analysis of epizootic hemorrhagic disease viruses nonstructural protein 1 (NS-1). Virus Res 1994; 31: 357–365.
15. **HUISMANS H, ERASMUS BJ.** Identification of the serotype-specific and group specific antigens of bluetongue virus. Onderstepoort J Vet Res 1981; 48: 51–58.
16. **PURDY M, PETRE J, ROY P.** Cloning of the bluetongue virus L3 gene. J Virol 1984; 51: 754–759.
17. **GHIASI H, PURDY MA, ROY P.** The complete sequence of bluetongue virus serotype 10 segment 3 and its predicted VP3 polypeptide compared with those of BTV serotype 17. Vir Res 1985; 3: 181–190.
18. **GOULD AR.** The complete nucleotide sequence of bluetongue virus serotype 1 RNA3 and a comparison with other geographic serotypes from Australia, South Africa and the United States of America, and with other orbivirus isolates. Vir Res 1987; 7: 169–183.
19. **GOULD AR, PRITCHARD LI.** Phylogenetic analyses of the complete nucleotide sequence of the capsid protein (VP3) of Australian epizootic haemorrhagic disease of deer virus (serotype 2) and cognate genes from other orbiviruses. Vir Res 1991; 21: 1–18.
20. **WILSON WC.** Molecular comparison of VP3 from bluetongue and epizootic hemorrhagic disease viruses. Vir Res 1991; 21: 225–236.
21. **GOULD AR, McCOLL KA, PRITCHARD LI.** Phylogenetic relationships between bluetongue viruses and other orbiviruses. In: Walton TE, Osburn BI, eds. Bluetongue, African horse sickness, and related orbiviruses, 2nd ed. London: CRC Press, 1992; 452–460.
22. **SAMBROOK J, FRITSCH EF, MANIATIS T.** Molecular Cloning. A laboratory manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
23. **CHENEY IW, LARSON MD, MECHAM JO, WILSON WC.** Geographical genetic variation in the gene encoding VP3 from the Alberta isolate of epizootic hemorrhagic disease virus. Vir Res 1995; 36: 279–286.
24. **SAMAL SK, LIVINGSTON CW, McCONNELL S, RAMIG RF.** Analysis of mixed infection of sheep with bluetongue virus serotype 10 and 17: Evidence for genetic reassortment in the vertebrate host. J Virol 1987; 61: 1086–1091.
25. **STOTT JL, OBERST RD, CHANNELL MB, OSBURN BI.** Genome segment reassortment between two serotypes of bluetongue virus in a natural host. J Virol 1987; 61: 2670–2674.
26. **HARDING MJ, PRUD'HOMME I, ROLA J.** Identification of the major North American bluetongue viruses using nucleic acid amplification techniques. Mol Cell Probes 1995; 9: 223.