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Simultaneous Detection and Identification of Epizootic Hemorrhagic Disease Virus Serotype 1 and 2 using A Multiplex RT PCR

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Abstract: A multiplex reverse transcriptase (RT) polymerase chain reaction (RT-PCR)-based assay, for simultaneous serogroup-specific detection and serotype-specific identification of North American serotypes of epizootic hemorrhagic disease virus (EHDV) in cell culture and clinical samples, was developed. For detection of EHDV serogroup-specific, a pair of primers (EG1 & EG4) was designed from a conserve region of non-structural protein 1 (NS1) genome of EHDV serotype 2 (EHDV-2). For serotype-specific identification, two pairs of primers (ES1 and ES4) and (ESa and ESb) were designed from variable regions of genome segment 2 (L2) of EHDV-1 and that of EHDV-2. The multiplex RT-PCR-based assay utilized a single tube-PCR amplification in which EHDV serogroup-specific and serotype-specific primers were used simultaneously in a multiplex format. The EHDV serogroup-specific primers generated a 387 base pair (bp) PCR product from RNA samples of EHDV-1 and EHDV-2. The EHDV serotype-specific primers generated a 821-bp PCR product and a 1054-bp PCR product from RNA samples of EHDV-1 and EHDV-2, respectively. However, RNAs from BTV serotypes 2, 10, 11, 13 and 17; or total nucleic acid extract from non infected Vero cells failed to demonstrate the specific EHDV PCR products. The described multiplex RT-PCR-based assay could be used to facilitate rapid detection and differentiation of EHDV serotypes 1 and 2. In addition, it could also be used to monitor incursion of new serotypes of EHDV in North American continent

Key words: Epizootic hemorrhagic disease virus, PCR, assay

Introduction

Epizootic hemorrhagic disease virus (EHDV) is an insect-transmitted double-stranded (ds) RNA orbivirus in the family Reoviridae (Borden *et al.*, 1971). EHDV infects domestic, captive and free-ranging ruminants (Shope *et al.*, 1960; Metcalf *et al.*, 1992 and Aradaib *et al.*, 2002). These orbiviruses are the most important cause of highly infectious non-contagious diseases in wild deer populations in the United States (Shope *et al.*, 1960 and Osburn *et al.*, 1994). Cattle are susceptible to infection but the association of EHDV with clinical hemorrhagic disease is rare (Osburn *et al.*, 1994). Ten serotypes of EHDV are recognized worldwide, of which EHDV serotypes 1, 2 are enzootic in United States. Clinical hemorrhagic disease in deer populations and restrictions on the sale of livestock and associated germplasm in the international markets are of concern to wildlife managers and dairy producers (Aradaib *et al.*, 1994b). In addition, clinical signs and pathological lesions induced by EHDV in susceptible animals are indistinguishable from those induced by bluetongue virus (BTV) and hence these viral infections are of interest to veterinary diagnosticians (Wilson, 1994). Therefore, the development of a rapid, reliable, reproducible, sensitive, specific and inexpensive diagnostic assay for simultaneous detection and differentiation of these hemorrhagic infections among susceptible North American deer population would be advantageous (Aradaib *et al.*, 1994a). In a previous report, we described a single EHDV RT-PCR amplification based on NS1 genome of EHDV-2 (Aradaib *et al.*, 1994a; Aradaib *et al.*, 1994b and Aradaib *et al.*, 1995a). A nested PCR, based on NS1 genome sequence analysis of EHDV-1, was also developed and evaluated for detection of EHDV in cell culture and clinical samples (Wilson, 1994). Previous studies showed that NS1 genome is the most highly conserved genome among the 10 dsRNA of EHDV serogroup (Aradaib *et al.*, 1994). Recently, we have developed PCR-based assays for detection of EHDV serogroup and serotypes in cell culture and tissue samples (Aradaib *et al.*, 1995b and Aradaib *et al.*, 1995c). Although these EHDV PCR-based detection assays proved highly sensitive and specific, they do require individual testing of each submitted sample for the presence of EHDV-1 or EHDV-2 ribonucleic acids. This limitation renders these PCR-based detection assays rather expensive and time consuming. To address these problems, a pilot study was conducted to differentiate between the 2 serotypes of EHDV 1 and 2 (Aradaib *et al.*, 2002). However, in that study the specific identification was made possible by a

chemilumimescent hybridization assay which is tedious, laborius and time cosuming (Aradaib *et al.*, 1998). In the present study, we describe a reproducible, sensitiveand specific assay for simultaneous detection and differentiation of North American serotypes of EHDV-1 and EHDV-2, in cell culture and clinical samples using a multiplex RT-PCR amplification technology.

Materials and Methods

Virus and Clinical Samples: The prototype viruses including EHDV serotypes 1 and 2and bluetongue virus prototype serotypes 2, 10, 11, 13 and 17 were received from the Arthropod-borne Animal Diseases Research Laboratory, Laramie, Wyoming. The clinical samples including (blood, spleen and lung suspension) were obtained from naturally infected deer and from EHDV-1and EHDV-2 experimentally infected calves. Heparinized blood samples were also collected from clinically normal calves. After virologic and serologic screening to eliminate the possibility of EHDV infection, the samples were used as negative controls.

Processing of Blood Samples: Processing of the blood samples for virus isolation was described previously (Aradaib *et al.*, 1994a).

Cells Culture and Virus Isolation: BHK-21 cells were prepared in minimal essential media (MEM) (GIBCO BRL, Gaitherburg, MD.) containing 100 units penicillin/ml and 100 mcg streptomycin/ml, 10% tryptose phosphate broth and 10% fetal bovine serum (FBS) that has been heat inactivated at 56 C for 30 minutes. Cell cultures were incubated at 37 ^{0} C in a humidified incubator with 5.0 % carbon dioxide until confluent monolayers were obtained (usually 2-3 days).

The BHK-21 cell monolayers were inoculated with lysed blood diluted 1:10 in MEM. After incubation at 37 C for 1 hour, the inoculated cell cultures were supplemented with MEM, 2% tryptose phosphate brothand 2% FBS. The cell cultures were again incubated at 37 ^{0}C and observed daily until cytopathic effect was 80% complete. Cultures with no cytopathic effect were blind passaged. The remaining lysed blood samples were stored at 4 ^{0}C for further analysis by PCR assay.

Plaque Inhibition Test: After subsequent passages on BHK-21 cell monolayers, all cytopathic agents were identified by plaque inhibition test (Aradaib *et al.*, 1994a).

Viral Nucleic Acid Extraction from Clinical Samples: Viral nucleic acid extraction from the clinical samples was as previously described (Aradaib *et al.*, 1994b). Five microliters of the resuspended nucleic acid was used in the PCR assay.

Primer Selection: Primer selection and synthesis of the probe for serogroup-specific EHDV PCR was based on the NS1 genome sequence analysis of EHDV-2 (Alberta strain). Primer selection and synthesis of the probes for specific identification of EHDV-1 and EHDV-2 were based on L2 gene of EHDV-1 (Roy *et al.*, 1992) and that of EHDV-2 (Aradaib *et al.*, 1995b) as shown in (Table 1).

All primers were synthesized on a DNA synthesizer (Milligen/Biosearch, A division of Millipore, Burlington, MA). and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, VA). as per manufacturer's instructions. The PCR-generated probes were purified using DNA binding beads (Mermaid Kit, Bio 101, La Jolla, CA). according to the manufacturer's instructions and used for chemiluminescent hybridization.

EHDV Reverse Transcriptase (RT) Polymerase Chain Reaction(RT-PCR): The protocol used in this study was a modification of our previously reported protocol (Aradaib *et al.*, 1994a). One ul of 80 mM methyl mercuric hydroxide was used to denature a mixture of 5 ul of target EHDV RNA and 6 ul of a pooled EHDV serogroup, EHDV-1 and EHDV-2 primers, such that concentrations of 6.67 methyl mercuric hydroxide, 1.75 uM of each primer in a total volume of 12 ul per tube were obtained. The denaturation mixture was then incubated at 25 $^{\circ}$C for 10 minutes. 10 ul of neutralization mixture containing 1 ul of 1 M 2-mercaptoethanol, 1 ul of 20 U ul^{-1} RNAse inhibitionand 2ul of each dNTP (10 uM dATP, 10 uM dTTP,10 uM dGTP, 10 uM dCTP), were added. A reverse transcriptase mixture of 8.8 ul containing 5 ul of 25 mM magnesium chloride, 2.7 ul of 10 X PCR buffer and 1.1 ul of 50 U ul^{-1} M-MLV reverse transcriptase was added immediately after neutralization and the reaction was incubated at 42 ^{0}C for 30 minutes followed by incubation at 99 ^{0}C for 5 minutes. 69 ul of a PCR reaction mixture containing 7.3 ul of 10 X PCR buffer, 8 ul Mg cl, 52.7 double distilled water and 1 ul of Tag DNA polymerase (Perkin-Elmer Cetus, The Perkin Elmer Corporation, Norwalk, CT.) at a concentration of 5.0 U ul^{-1} was added to each PCR tube. All PCR reactions were carried out at a 100 ul volume per tube. A drop of mineral oil was used to prevent evaporation. The thermal cycling profiles were as follows: a 2-min incubation at 95 $^{\circ}$C, followed by 40 cycles of 95 $^{\circ}$C for 1 min , 55 $^{\circ}$C for 30 sec and 72 $^{\circ}$C for 45 secand a final incubation at 60 $^{\circ}$C for 10 min. Thermal profiles were performed on a Techne

Table 1: Positions and sequences of the oligonucleotide primers used in the multiplex EHDV PCR-based detection assay

Primer	Position	Sequence	Size(bp)product
EHDV group			
	175-194	(5)TCGAAGAGGTGATGAATCGC	
	543-562	(5)TCATCTACTGCATCTGGCTG	387-bp product
EHDV-1			
	1071-1090	(5)AACGCGCTATCTGGAGCAAT	
	1914- 1933	(5)GAAGATCGTCAACTTCTGCC	862-bp product
EHDV-2			
	901-918	(5)CATTATTAGAGCGGCTGG	
	1936-1952	(5)TCTCCGCTGTCTCTATA	1051-bp product

PHC-2 thermal cycler (Techne, Princeton, NJ).
Following amplification, 20 ul of the PCR product was electrophoresed on a 1.5% agarose gel. The agarose gels were stained with ethidium bromide and the PCR products were visualized under UV light or detected by chemiluminescent hybridization (ECL direct nucleic acid labeling and detection system, Amersham Corporation, Arlington Heights, IL).

Results

The multiplex PCR-based assay afforded a rapid, sensitive , specific and inexpensive method for simultaneous serogroup-specific detection and serotype-specific identification of North American EHDV isolates.
The specific 862 bp EHDV-1 and the specific 1051 bp EHDV-2 PCR products were amplified from twelve EHDV-1 and EHDV-2 field isolates, respectively. The serogroup-specific 387 bp PCR product was amplified from all EHDV field isolates used in this study. Amplification products were visualized on ethidium bromide-stained agarose gel (Fig. 1). Application of this PCR assay to clinical samples from infected animals, including unfractionated lysed blood, lung suspension and spleen samples, resulted in amplification and visualization of the serogroup-specific 387 bp PCR products from all EHDV RNA samples. The specific 862 bp product was amplified and detected from EHDV-1 RNA only. Likewise the specific 1050 bp PCR product was amplified and detected from EHDV-2 RNA but not EHDV-1 RNA (Fig. 2).

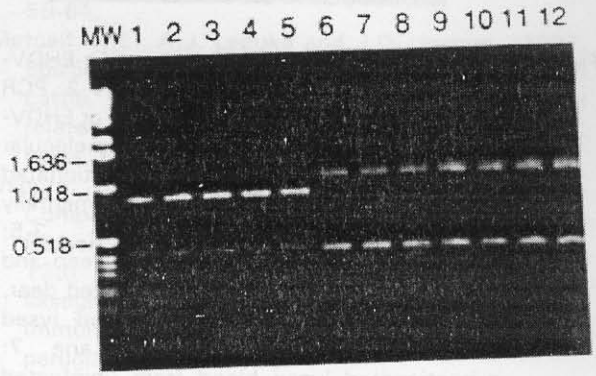

Fig. 1: Visualization of the 387-bp serogroup-specific EHDV from both EHDV-1 and EHDV-2 field isolates, the 862-bp specific PCR product from EHDV-1 and the 1050-bp PCR products from EHDV-2 on ethidium bromide-stained agarose gel from 1.0 pg of RNA from twelves samples of EHDV-1 and EHDV-2 field isolates. Lane MW: molecular weight marker; Lane 1-5: EHDV-1 field isolates; Lane 6-12: EHDV-2 feild isolates

Discussion

Orbivirus infections in domestic and wild ruminants, particularly epizootic hemorrhagic disease, constitute one of the major unresolved veterinary problems in many parts of the world inciuding the united States (Shope *et al.*, 1960; Metcalf 1992; Osburn *et al.*, 1994; Aradaib *et al.*, 1994b; Aradaib *et al.*, 1997 and Aradaib *et al.*, 2002). To advance beyond the current knowledge on the epidemiology of EHDV, we developed a multiplex PCR-based assay for simultaneous detection and identification of EHDV serotypes 1 and 2 in cell culture and a variety of clinical samples. In the present study, the multiplex PCR assay was optimized to detect and differentiate between EHDV serotypes 1 and 2 RNA targets. This was achieved by using a pool of BTV and EHDV primers in a single-tube PCR amplification. The EHDV and BTV primers were used simultaneously in a multiplex format.
This multiplex PCR assay would facilitate clinical disease investigation, epidemiological investigation, ease diagnosis and should enhance herd health monitoring.
Specific identification of EHDV-1 (Aradaib *et al.*, 1995c) and EHDV-2 (Aradaib *et al.*, 1995b) using PCR assays were also described. These PCR assays proved

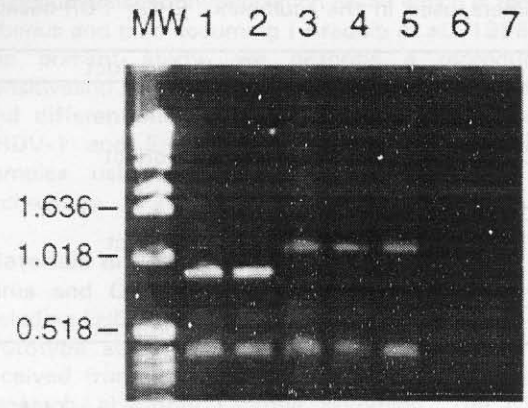

Fig. 2: Detection of either the 862-bp specific-EHDV-1 or the 1050 bp specific EHDV-2 PCR product on agarose gel from EHDV-1 or EHDV-2 clinical samples. Lane MW: molecular weight marker; Lane 1 and 2: unfractionated lysed blood cells from a calf experimentally infected with EHDV-1; Lane 3-5: unfractionated lysed blood cells, spleen and lung suspension from EHDV-2 infected deer, respectively; Lane 6: unfractionated lysed blood from uninfected deer; Lane 7: unfractionated lysed blood from uninfected calves. Amplification of the serogroup-specific 387 bp PCR product was achieved from all clinical samples tested.

superior for detection and identification of EHDV infection in susceptible ruminants when compared to conventional virus isolation and serotyping (Osburn *et al.*, 1994). In previous studies, optimal primers for one serotype showed primer-primer interaction with primers for another serotype (Wilson, 1994). Therefore, selection of the primers requires design and empirical testing. In this study, the primers were also designed in such a way that they would have the same annealing temperature to insure maximum amplification for the PCR products. The primers worked well as a mixture and afforded optimal amplification for serogroup-specific and serotype-specific EHDV.

In the present study, the PCR assay was modified to detect and differentiate between EHDV-1 and EHDV-2. This was achieved by using a pool of primers designed from VP2 gene sequence analysis of EHDV-1 and that of EHDV-2 (Roy *et al.*, 1992). After visualization of the amplification product on an ethidium bromide-stained agarose gel, the specific PCR product can be used as diagnostic procedure for detection of EHDV serogroup. confirmatory diagnosis could be made based on the appropriate size of the specific 826 and 1054 bp PCR products for EHDV-1 and EHDV-2, respectively. Simultaneous visualization of the serogroup-specific and the serotype-specific EHDV PCR products indicated that there is no need for nested amplification or nucleic acid hybridization. Nested amplification has disadvantage of contamination and hybridization assay is tedious, laborious and time consuming (Aradaib *et al.*, 1997).

The multiplex EHDV RT-PCR, described in this study, for detection and differentiation of EHDV-1 and EHDV-2 in clinical samples from naturally or experimentally infected animals will simplify the assay, save time and above all save on cost. This is because each clinical sample will be tested once instead of individual testing for the presence of EHDV-1 or EHDV-2. Further more, this assay can be modified in the future to include primers to new or previously unrecognized serotypes. In addition, it could also be used for detection of EHDV in focal areas of endemicity.

Because of its rapidity, sensitivity and specificity, the multiplex PCR assay described in this study would be advantageous in epidemiological investigations, where field isolates can be tested to determine the prevalence and frequency of EHDV-1 or EHDV-2 infections in susceptible herds. The modifications described in this communication should serve as a superior diagnostic alternative or at least as complement to the existing techniques currently used for detection and identification of EHDV-1 and EHDV-2 . Genome segment 3 of EHDV was found to be conserve among cognates of EHDV serogroup (Harding *et al.*, 1996). However, non-structural proteins 1 (NS1) genome requires small number of infected cells to produce positive hybridization signal compared to other nucleic acid probes (Aradaib *et al.*, 1994a). However, selection of the serotype-specific primers was based on segment 2 (L2) which is the most variable gene among cognates of EHDV serotypes (Roy *et al.*, 1992).

In previous studies, using ethidium bromide-stained agarose gel, the sensitivity of the BTV\ EHDV multiplex PCR assay was found to be at least 100 fg of EHDV or BTV RNAs (equivalent to 6x 10^3 BTV or EHDV viral particles (Aradaib *et al.*, 1994b). This level of sensitivity is comparable to or even more sensitive than virus isolation (Aradaib *et al.*, 1997). It is suggested that this multiplex EHDV RT-PCR can also be used for evidence of viral incursion in a particular geographical region during a sentinel study.

Negative and positive controls should be included in each PCR amplification to estimate the lower limit of specificity and the higher limit of sensitivity. Further studies are currently under way to determine the capacity of the described multiplex EHDV RT-PCR for direct detection of dual infections with EHDV-1 and

EHDV-2 in clinical samples from naturally infected animals and to evaluate its potential as a sensitive and specific diagnostic assay.

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