

Detection of United States Orbivirus serogroup using a multiplex RT-PCR

Imadeldin Aradaib,^{1*} Abdelrahim Karrar¹, Mohammed Abdalla¹,
and Bennie Osburn²

¹Department of Medicine, Pharmacology and Toxicology, Faculty of Veterinary Medicine,
University of Khartoum, Khartoum North, Sudan

²Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine,
University of California, Davis, USA

ARADAIB, I., A. KARRAR, M. ABDALLA, B. OSBURN: Detection of United States Orbivirus serogroup using a multiplex RT-PCR. Vet. arhiv 73, 63-71, 2003.

ABSTRACT

A multiplex RT-PCR assay, for simultaneous detection and differentiation of United States serogroup of Orbiviruses, including bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) in cell culture, was developed. Sets of primers were designed to hybridize to genome segment six of EHDV-2 and to genome segment 10 of BTV-10. The RT-PCR assay utilized a single-tube PCR amplification in which EHDV and BTV primers were used simultaneously in a multiplex format. The EHDV primers produced a 387 base pair (bp) specific PCR product from RNA samples of cell culture-adapted EHDV serotypes 1 and 2, but not from BTV serotypes 2, 10, 11, 13, 17; or from total nucleic acid extract of baby hamster kidney (BHK) cells controls. Likewise, the BTV primers generated a 251-bp amplicon from RNA samples of BTV serotypes 2, 10, 11, 13, and 17, whereas EHDV-1 and EHDV-2; and BHK-21 cells total nucleic acid extract failed to demonstrate the 251-bp specific BTV PCR product. EHDV and BTV PCR amplification products were easily identified on the basis of size differences on ethidium bromide-stained agarose gels. This multiplex RT-PCR assay provides supportive diagnostic method for rapid detection of BTV and/or EHDV-infections among susceptible ruminants.

Key words: orbiviruses, diagnosis, RT-PCR

* Contact address:

Prof. Dr. Imadeldin E. Aradaib, Department of Medicine, Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Khartoum, P.O. Box 32, Khartoum North, Sudan, Phone: +249 13 316 283; E-mail: aradaib@yahoo.com

Introduction

Bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) are insect-transmitted double-stranded (ds) RNA Orbiviruses of the family *Reoviridae* (BORDEN et al., 1971). BTV and EHDV infect domestic and wild ruminants (SHOPE et al., 1955; HOFF and TAILER, 1974; ARADAIB et al., 1994). These orbiviruses are the most important cause of highly infectious non-contagious diseases in white-tailed deer populations in the United States (SHOPE et al., 1960). Sudanese zebu cattle are susceptible to infection with both viruses but the association EHDV or BTV with clinical hemorrhagic disease is rare (MOHAMMED, 1987; ARADAIB et al., 1994; ARADAIB et al., 2000). Twenty-five serotypes of BTV (DAVIES et al., 1992) and ten serotypes of EHDV are recognized worldwide (GORMAN, 1992). In the United States BTV serotypes 2, 10, 11, 13 and 17; and EHDV serotypes 1 and 2 are enzootic. Clinical hemorrhagic disease in North American white-tailed 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 (OSBURN et al., 1994). In addition, clinical signs and pathological lesions induced by BTV in white-tailed deer are indistinguishable from those induced by EHDV and hence these viral infections are of interest to veterinary diagnosticians (PEARSON et al., 1992; ARADAIB et al., 2000). Therefore, it was thought of interest to optimize a rapid diagnostic assay for detection and differentiation of these hemorrhagic infections among susceptible animal populations.

The major thrust of the current research conducted in our laboratory is directed toward the application of biotechnology in diagnostic veterinary medicine using the modern techniques in cellular immunology and molecular biology (ARADAIB et al., 2000). In previous studies, we developed RT-PCR single-tube amplification for detection of BTV serogroup in cell culture (AKITA et al., 1992; ARADAIB et al., 1998a). We have also described RT-PCR for detection of EHDV serogroup in cell culture and tissue samples (ARADAIB et al., 1994; ARADAIB et al., 1995a; ARADAIB et al., 1998a). Application of RT-PCR assays for specific identification of EHDV-1 and EHDV-2 in cell culture was also reported (ARADAIB et al., 1995b; ARADAIB et al., 1995c). Although these BTV and EHDV PCR-based detection assays proved highly sensitive and specific, they do require individual testing of each submitted

sample for the presence of EHDV or BTV ribonucleic acids. This limitation renders these PCR-based detection assays rather expensive and time consuming. In addition, no work has yet been carried out to evaluate the potential use of PCR technology for simultaneous detection and differentiation of BTV and EHDV infections in a multiplex format. To address these problems, in the present study we have developed a multiplex RT-PCR assay, in which primers derived from EHDV non-structural proteins 1 (NS1) and BTV NS3 genome segments were used, for simultaneous detection and differentiation of BTV and EHDV infections.

Materials and methods

Virus isolation and nucleic acid extraction from infected BHK cell cultures. The United States EHDV prototype serotypes 1 and 2; and BTV prototype serotypes 2, 10, 11, 13 and 17 were received (Arthropod-borne Animal Diseases Research Laboratory, Laramie, Wyoming, USA). BHK-21 cell line was used for propagation of the viruses. Virus isolation and RNA extraction methods were described previously (ARADAIB et al., 1994).

Primer selection for PCR amplification. EHDV primers (E1 and E2) and BTV primers (B1 and B2) were designed based on the nucleotide sequences of genome segment 6 (NS1) of EHDV serotype 2 (EHDV-2) and nucleotide sequences of segment 10 (NS3) of BTV serotype 10 (BTV-10), respectively. Primers derived from these genome segments were reported to be highly conserved (AKITA et al., 1992; ARADAIB et al., 1994). E1 primer included bases 175-194 of the positive sense strand of NS1 genome (5): -TCGAAGAGGTGATGAATCGC (3). E2 primer included bases 543-562 of the complementary strand (5): -TCATCTACTGCATCTGGCTG (3). B1 primer included bases 12-31 of the positive sense strand of NS3 genome (5): -TCGCTGCCATGCTATCCG (3). B2 primer included bases 245-264 of the complementary strand (5): -CGTACGATGCGAATGCAG (3) (AKITA et al., 1992). EHDV primers would result in amplification of a 387-bp PCR product whereas those of BTV would result in amplification of a 251 bp PCR product.

EHDV-BTV reverse transcriptase polymerase chain reaction (RT-PCR). The protocol used in this study was a modification of our previously

reported protocol (ARADAIB et al., 1998b). One microliter (μl) of 80 millimole (mM) methyl mercuric hydroxide was used to denature a mixture of 5 μl of target RNA and 4 μl of pooled BTV and EHDV primers, such that concentrations of 8.0 mM methyl mercuric hydroxide and 1.0 mM of each primer in a total volume of 10 μl per tube were obtained. The denaturation mixture was then incubated at 25 °C for 10 minutes. 10 μl of neutralization mixture containing 1 μl of 1 M 2-mercaptoethanol, 1 μl of RNase inhibitor at a concentration of 20 units/microliter (U/ μl), and 2 μl of each dNTP (dATP, dTTP, dGTP, dCTP) at a concentration of 10 micromole (μM) were added. A reverse transcriptase mixture of 8.8 μl containing (5 μl of 25 mM magnesium chloride, 2.7 μl of 10 \times PCR buffer and 1.1 μl of 50 U/ μl M-MLV reverse transcriptase (RT) enzyme) was added immediately after neutralization and the reaction was incubated at 42 °C for 30 minutes followed by incubation at 99 °C for 5 minutes. A PCR reaction mixture containing (7.3 μl of 10 \times PCR buffer, 8 μl MgCl_2 , 54.9 μl double distilled water, and 1 μl of Tag DNA polymerase at a concentration of 5.0 U) was added to each PCR tube. All PCR reactions were carried out at a final volume of 100 μl per tube. A drop of mineral oil was used to prevent evaporation. The thermal cycling profiles were as follows: an initial 2-min incubation at 95 °C, followed by 40 cycles of 95 °C for 1 min, 55 °C for 30 sec and 72 °C for 45 sec, and a final incubation at 72 °C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (USA). Following amplification, 20 μl of the PCR products were electrophoresed on a 1.5% agarose gel. The agarose gels were stained with ethidium bromide and the PCR products were visualized under UV light.

Results

The multiplex RT-PCR assay afforded a rapid method for simultaneous detection and differentiation of United States BTV and EHDV serotypes. The EHDV specific 387-bp PCR product was visualized on ethidium bromide-stained agarose gels from 1.0 pg RNA of EHDV serotypes 1 and 2 but not from BTV serotypes 2, 10, 11, 13 and 17 or total nucleic acid extract from BHK cell control. The BTV specific 251 bp PCR product was visualized on ethidium bromide-stained agarose gel from 1.0 pg RNA



Fig. 1. Detection of the 387-bp specific-EHDV and the 251-bp specific BTV PCR products from infected BHK-21 cell cultures. Lane M: molecular weight marker; Lane A, B, C, D, and E: RNA from BHK-21 cell cultures infected with BTV serotypes 2, 10, 11, 13 and 17; respectively. Lane F and G: RNA extracted from BHK-21 cell cultures infected with EHDV serotypes 1 and 2, respectively; Lane H, I and J: Total nucleic acids extracted from non infected BHK cell cultures (negative controls).

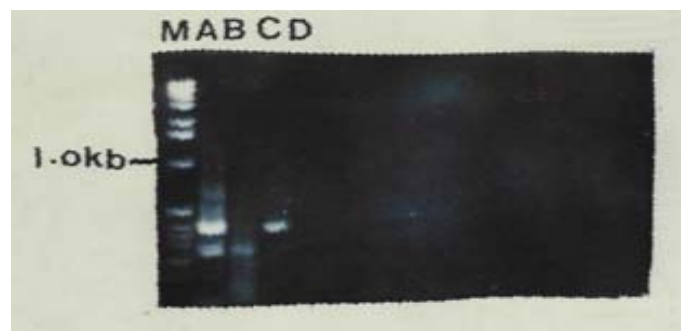


Fig. 2. Simultaneous detection of the 387-bp specific-EHDV and the 251 bp specific BTV PCR products on ethidium bromide-stained agarose gel from 1.0 pg of RNA extracted from BHK-21 cell cultures infected with BTV and EHDV. Lane M: molecular weight marker; Lane A: RNA extracted from cell culture infected with both EHDV serotype 2 (EHDV-2) and BTV serotype 10 (BTV-10); Lane B: BHK-21 cell culture infected with BTV-10; Lane C: BHK-21 cell culture infected with EHDV-10; Lane D: Total nucleic acids extracted from non-infected BHK-21 cell culture (negative control).

of BTV serotypes 2, 10, 11, 13, 17 but not from EHDV serotypes 1 and 2 or BHK-21 cell controls (Fig. 1).

A sample of non-infected total nucleic acid, extracted from BHK-21 cells, was spiked with EHDV and BTV RNAs. The spiked sample produced two bands representing the 387-bp and the 251-bp PCR products for EHDV and BTV, respectively. Amplification products were visualized on ethidium bromide-stained agarose gel (Fig. 2).

Discussion

The described multiplex RT-PCR assay, using primers derived from genome segment 10 of BTV-10 and genome segment 6 of EHDV-2, reproducibly and specifically detected BTV and EHDV RNAs in infected cell cultures. The multiplex RT-PCR assay was a simple procedure that utilized a single amplification in which EHDV and BTV primers were used simultaneously. PCR buffer and $MgCl_2$ concentrations, and the number of cycles of the three temperatures per time segments were very important for maintaining sensitivity and specificity of the multiplex RT-PCR assay. EHDV and BTV primers were designed to have the same annealing temperature.

The sensitivity of multiplex RT-PCR assay was at least 1.0 pg of EHDV or BTV RNAs (equivalent to 6×10^3 EHDV and 5×10^3 BTV viral particles, respectively). This level of sensitivity is comparable to virus isolation and could be increased using more sensitive chemiluminescent hybridization techniques (ARADAIB et al., 1994; ARADAIB et al., 1995a; MOHAMMED et al., 1996; ARADAIB et al., 1998a). Nested amplification using internal primers could also be used to increase the sensitivity of multiplex RT-PCR amplification technology (ARADAIB et al., 1998b; ARADAIB et al., 2000). Excellent correlation of results from agarose gels indicated that diagnosis of EHD and BT could be based on visualization of the amplified PCR products on an ethidium bromide-stained agarose gel, since it is a simple procedure, which requires only 1 hour after amplification.

Simultaneous detection and differentiation of BTV and EHDV will simplify the assay, save time and above all save on cost, because each clinical sample will be tested once instead of individual testing for BTV and

EHDV viral nucleic acids. Because of its rapidity, sensitivity and specificity, the multiplex RT-PCR assay described in this study would be used for detection of field isolates, and to determine the prevalence and frequency of Orbivirus infections in susceptible animal populations. It is worth mentioning that this RT-PCR could also be used for evidence of viral incursion in a particular geographical location.

This multiplex PCR assay could be used as supportive diagnostic techniques to the lengthy, cumbersome conventional virus isolation procedures currently used for detection of BTV and EHDV, and could be used for the purpose of export regulation to certify animals free of BTV and/or EHDV infections (ARADAIB et al., 2000). Extraction of EHDV and BTV dsRNAs should be carried out in separate rooms to avoid contamination.

In conclusion, the scientific data presented in this communication indicated that the described multiplex RT-PCR provides a simple, rapid, and inexpensive method for simultaneous detection and differentiation of BTV and EHDV infections in cell culture.

Further studies are currently under way to determine the capacity of the described multiplex RT-PCR assay for direct detection of dual infections with BTV and EHDV in clinical samples from naturally or experimentally infected animals, and to evaluate its potential as a sensitive and specific diagnostic assay through comparison with current diagnostic techniques used for detection of these Orbiviruses.

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Received: 10 January 2003

Accepted: 15 April 2003

ARADAIB, I., A. KARRAR, M. ABDALLA, B. OSBURN: Dokaz američke serološke skupine orbivirusa upotrebom višestruke RT-PCR. Vet. arhiv 73, 63-71, 2003.

SAŽETAK

Rabljen je višestruki RT-PCR (lančana reakcija polimerazom uz prethodnu reverznu transkripciju) za dokazivanje i razlikovanje serološke skupine virusa bolesti plavog jezika (BPJ) i virus epizootske hemoragijske bolesti jelena (EHB) izdvojenih u SAD-u te uzgojenih na staničnoj kulturi. Određeni su parovi početnica za hibridizaciju na odsječak 6 genoma virusa EHB-2 te na odsječak 10 genoma virusa BPJ-10. Za RT-PCR korištena je PCR amplifikacija u jednoj epruveti u kojoj su početnice za virus EHB i virus BPJ istodobno upotrijebljene u višestrukome obliku. Pomoću početnica virusa EHB proizvedeno je 387 parova baza (pb) specifičnog proizvoda PCR iz uzoraka RNA serotipova 1 i 2 toga virusa prilagođenog na staničnu kulturu, ali ne i iz serotipova 2, 10, 11, 13 i 17 virusa BPJ ili iz ekstrakta ukupne nukleinske kiseline kontrolne stanične kulture bubrega hrčka (BHK). Početnice za virus BPJ dovele su do proizvoda od 251 para baza iz uzoraka RNA serotipova 2, 10, 11, 13 i 17 toga virusa, dok serotipovi 1 i 2 virusa EHB i ukupni ekstrakt nukleinske kiseline stanične kulture BHK-21 nisu doveli do specifičnog proizvoda od 251 para baza. Umnoženi proizvodi PCR za viruse EBH i BPJ mogli su se jednostavno identificirati na osnovi razlika u veličini na agaroznom gelu obojenom etidij-bromidom. Postupak višestruke RT-PCR pruža pomoćnu dijagnostičku metodu za brzo dokazivanje infekcija uzrokovanih virusima BPJ i EHB među prijemljivim preživačima.

Ključne riječi: orbivirusi, dijagnosticiranje, RT-PCR

Book review

HELMUT MEYER, MANFRED COENEN: **Horse Feeding**, Blackwell Wissenschafts - Verlag, Berlin - Wien, 2002. 244 pages, 47 pictures, 115 tables, 25x18 cm. Hard cover. ISBN 3-8263-3398-5. Price: •29.95 / CHF 52.-

This book comprises 11 chapters, each chapter encompassing a large number of topic units. In the introduction the authors describe, in a very interesting and educational way, the evolution and domestication of the horse, further expanding through Figure 1 on page 2 the textual part of this chapter. The second chapter describes the total number of horses in the world, from 1950 until the present day. Subsequently, the authors consider the body weight of the most significant world breeds and present, through tables, part of the muscle mass, the bone system of some single organs, water and electrolytes in the horse organism. This chapter also describes the horse profile as a productive animal. The third chapter presents a classic approach to basic feeding, as well as the build and physiology of the horse digestive system. In the fourth chapter energy needs regarding nutritive matter and minerals are described, together with the authors' detailed explanation of energy needs, further describing in detail, on page 45, the work of the muscle and its physique, explaining these facts and extending as far as the constitution of histological muscle fibre. In the fifth chapter, next to water, the types of food for the horse are described, primarily green grass, hay, root crops, tuberous plants and cereals. The role of fruit in the feeding of horses is also referred to, a factor hitherto not taken into consideration by books on this topic. The sixth chapter describes the manner of horse feeding, in which the authors consider microclimate in stables, the positioning of troughs, the space necessary for different types of feeding, feeding frequency and watering techniques. The seventh chapter describes the feeding of different horse categories, such as riding horses and working horses. Here, the authors' specifically consider the feeding of older horses. The eighth chapter describes poisoning by fungi - contaminated food, as well as diseases of the digestive tract. In the ninth chapter special emphasis is placed on dietary regime on those occasions when the horse is suffering from various disorders and diseases of the organic system. Thus, the feeding of a horse with a kidney function disorder is described on page 200, which is supplemented by a detailed table regarding the presentation of meals for a horse with protein deficiency. The final part of the book contains an annex with tables on the share of raw fibre, fats, ash and other ingredients, in percentages and g/kg of some certain types of food.

Considering the horse as a whole we may conclude that this book represents an interdisciplinary approach to the problems of horse feeding. In certain chapters the connection with animal hygiene, physiology, pathology, internal diseases and toxicology is considered, which has not been the case to date with other, similar publications. Furthermore, it is unnecessary to possess an in-depth knowledge of horses in order to understand the themes communicated in this book, due to the fact that within each chapter a gradual approach to the main topic is presented. Consequently, the reader receives an explanation of certain single concepts through understanding. This book does not therefore represent a classical textbook but rather a more modern and simplified approach to problems involved in horse feeding and the reader is not expected to have previous knowledge of the physiology and anatomy of a horse, as even the most basic elements are explained in detail. Overall, the book is theoretically well treated and founded on concrete data and results from the scientific literature, referred to at the end of the book for each chapter.

Finally, we are able to conclude that this book represents a valuable contribution with regard to the presentation of horse feeding, even more so considering the fact that the reader will not need to search through other textbooks in order to be appraised of the field of veterinary medicine essential for feeding. Accordingly, the book is designed for students of veterinary medicine, veterinary professionals, agricultural practitioners and horse fans alike, that is to say for all who, in their professional and breeding work constantly come into contact with horses, offering them breeding conditions founded on the most contemporary scientific and professional knowledge in the sphere of horse feeding.

•eljko Pavičić