Available online at www.sciencedirect.com
ScienceDirect
Journal of Virological Methods 140 (2007) 140–147
Journal of Virological Methods
www.elsevier.com/locate/jviromet

An improved, high-throughput method for detection of bluetongue virus RNA in *Culicoides* midges utilizing infrared-dye-labeled primers for reverse transcriptase PCR

Cecilia Y. Kato *, Richard T. Mayer

U.S. Department of Agriculture, Agricultural Research Service, Arthropod-Borne Animal Diseases Research Laboratory, College of Agriculture D3354, 1000 East University Avenue, Laramie, WY 82072, USA

Received 22 August 2006; received in revised form 6 November 2006; accepted 8 November 2006
Available online 21 December 2006

Abstract

A new rapid (less than 6 h from insect-to-results) high-throughput assay that is sensitive and specific for detecting BTV RNA in *Culicoides* biting midges is reported. Homogenization and extraction of nucleic acids from individual *Culicoides* specimens were performed in a 96-well plate format using specialized beads in a homogenization buffer compatible with cell culture and RNA extraction. A portion of homogenate (10%) from each specimen was retained for confirmatory infectious virus isolation, while the remaining 90% was used for RNA extraction. The RNA was used in a single step reverse transcriptase PCR (RT-PCR) reaction with infrared (IR)-dye-labeled primers. The RT-PCR products were visualized in agarose gels with an infrared scanner. The adaptation of IR-dye-labeled primers in combination with a one step RT-PCR resulted in a detection limit of 0.5 pfu of purified BTV RNA. All 24 serotypes of BTV prototype strains and none of the 8 serotypes of the closely related epizootic hemorrhagic disease virus (EHDV) prototype strains were detected.
Published by Elsevier B.V.

Keywords: Bluetongue virus; Infrared-dye; Reverse transcriptase PCR; *Culicoides sonorensis*

1. Introduction

Bluetongue virus (BTV) is the prototype *Orbivirus* within the *Reoviridae* family. The double stranded, segmented RNA arbovirus is vectored by several *Culicoides* spp. biting midges. BTV and *Culicoides* vectors exist in subtropical, tropical and temperate regions throughout the world. Changes in climatic conditions have allowed the spread of exotic strains of BTV into Europe, Asia, and North America (Gibbs and Greiner, 1994; Mellor and Wittmann, 2002; Purse et al., 2005; Tabachnick et al., 1996). BTV is on the Office International des Epizooties (OIE) list of notifiable diseases because of the severe socio-economic impacts caused by BTV outbreaks. BTV vectors have been identified as the critical component in the spread of the virus and the changing global epidemiology of BTV. OIE therefore emphasizes the need for surveillance of competent *Culicoides* vectors in areas at high risk for expanded habitation based on historical, geographic and climatic factors (MacLachlan and Osburn, 2006; OIE, 2004). The recent OIE recommendations necessitate the development of effective, reliable, high-throughput methods to survey competent *Culicoides* and potential vectors for all BTV serotypes.

Current protocols for BTV detection in biting midges involve time consuming steps for sample (single insect) homogenization, preparation, and assay (Wieser-Schimpf et al., 1993). For example cell culture techniques for BTV identification may not rapidly detect all BTV particles within a sample. Standard RT-PCR and nested PCR assays for BTV RNA require multiple steps for reverse transcriptase reaction, and primary and/or secondary PCR amplification (Akita et al., 1992; Aradaib et al., 1998, 2003; Dangler et al., 1990a; Katz et al., 1993; Wilson and Chase, 1993). Finally, real time PCR protocols do not detect all 24 serotypes (Jimenez-Clavero et al., 2006; Orru et al., 2004; Wilson et al., 2004).

Initial PCR detection assays for BTV involved either standard or single amplification reverse transcriptase PCR (RT-PCR) and have detection sensitivities of 1 pg to 17 fg BTV RNA (Akita et al., 1992; Dangler et al., 1990b; McColl and Gould, 1991; Wade-

* Corresponding author. Tel.: +1 307 766 3624; fax: +1 307 766 3500.
E-mail addresses: ckato@uwyo.edu (C.Y. Kato), dmayer@uwyo.edu (R.T. Mayer).

0166-0934/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jviromet.2006.11.009

Evans et al., 1990). The incorporation of a second amplification step, and the development of nested PCR protocols, increased detection sensitivities to 0.1 fg and one cell culture infective dose ($CCID_{50}$)/ml. However, nested PCR often involves five to seven product manipulations that increase the possibility of cross-contamination and false positives (Aradaib et al., 1998; Katz et al., 1993; Parsonson and McColl, 1995; Shad et al., 1997; Wilson, 1999; Wilson and Chase, 1993). Real time PCR for arbovirus detection is gaining popularity (Mackay et al., 2002). BTV detection by real time PCR is efficient and can be very sensitive (Jimenez-Clavero et al., 2006; Orru et al., 2004; Wilson et al., 2004). Jimenez-Clavero et al. (2006) recently reported high-throughput real time PCR detection of BTV in blood samples with detection sensitivities equivalent to nested PCR. The assay targets BTV segment 5 (NS1 protein) which is involved in virus replication. In the study, 9 of 24 reference strain serotypes could not be detected (serotypes 4, 7, 10, 13, 16, 19, 20, 21, and 24), because of nucleotide sequence variations in the regions of probe and the 5′-primer binding site.

Increasing the sensitivity of a standard RT-PCR assay eliminates the need to identify additional primer or probe binding sites. IR-dye-labeled primers have been used in place of radioisotope labeled primers for various applications where enhanced sensitivity is needed. Applications include electrophoretic mobility shift (EMS) assays, amplified fragment length polymorphism (AFLP) analysis, single nucleotide polymorphism (SNP) mapping, DNA sequencing, short tandem repeat (STR) analysis, and DNA footprinting (Ford et al., 2000; Machida et al., 1997; Middendorf et al., 1992; Myburg et al., 2001; Ricci et al., 2003; Somboonthum et al., 2005). The spectral characteristics of these dyes allow for detection in the near IR (700–800 nm) range. At this spectral range, background fluorescence is low, therefore, detection of minute amounts of product can be differentiated from background (Middendorf et al., 1992). Primer extension and DNA sequencing applications previously described with IR dye labeled primers indicate that the dye conjugates do not interfere with reverse transcriptase and DNA polymerase activity, while retaining a high level of selectivity and sensitivity (Ricci et al., 2003; Somboonthum et al., 2005). In coupling this highly sensitive dye with a one step RT-PCR assay, a sensitive assay for pathogen detection was developed. This infrared reverse transcriptase PCR (IR-RT-PCR) assay is suitable for high-throughput analysis of individual *Culicoides* for BTV. The IR-RT-PCR assay is sensitive, rapid, economical, reduces false positives (due to cross-contamination), and is specific to all 24 BTV serotypes. Method development also focused on efficiency and economy of sample preparation so that the processing time and effort was decreased and the limited sample volume could easily and effectively be used in both cell culture and IR-RT-PCR assays.

2. Materials and methods

2.1. Virus

BTV serotype 11 (station strain; 6.8 × 106 plaque forming units (pfu)/ml), was used in all assays for IR-RT-PCR sensitivity, cell culture detection, and oral infection of *Culicoides*. Double stranded RNA from all 24 serotypes of BTV (prototypes) and all 8 epizootic hemorrhagic disease virus (EHDV) serotypes were obtained from the Arthropod-Borne Animal Diseases Research Laboratory (ABADRL) collection (USDA, ARS, ABADRL, Laramie, WY) and were used for determining primer specificity.

2.2. IR-RT-PCR assay sensitivity

BTV serotype 11 RNA was isolated from stock cell culture (400 μl) containing 6.8×10^6 pfu/ml with the Mag Attract Virus Mini M48 Kit and a BioRobot M48 (Qiagen, Inc., Valencia, CA, USA). The extraction was performed as specified by the manufacturer and eluted in a final volume of 50 μl.

IR-RT-PCR assay sensitivity tests were performed by the analysis of three different dilution sets each carried out in triplicate. Set 1: 500, 50, 5, 0.5, and 0.05 pfu/reaction. Set 2: 250, 25, 2.5, 0.25, and 0.025 pfu/reaction. Set 3: 100, 10, 1, 0.1, and 0.01 pfu/reaction. A non-template control was included in each replicate.

BTV specific primers BTV 12F (5′-TCGCTGCCATGCTATCCG-3′) and BTV 246R (5′-CGTACGATGCGAATGCAG-3′) (Akita et al., 1992) were synthesized with a 5′ IR-Dye 800 chromophore (LI-COR, Inc., Lincoln, NE). These primers are specific to the highly conserved regions of the S10 gene (nonstructural protein 3), located at nucleotide positions 12 and 246, for forward and reverse primers, respectively (251 bp product). Five hundred nanomoles of each of the IR-dye-labeled primers was added (1 μl) to each well of a 96-well thin walled PCR plate (Applied Biosystems, Foster City, CA). Each nucleic acid sample (5 μl) was then added to the PCR plate. The RNA/primer mixture was heat denatured at 95 °C for 3 min then quickly quenched in an ethanol ice bath for at least 5 min before a 1 min centrifugation at 2000 × *g* at 4 °C. The plate was placed on ice and the RT-PCR reagent mix (1× Taqman One-Step RT-PCR master mix and multiscribe) was added to each well. Thermal cycler conditions (GeneAmp 9600, Applied Biosystems) were as follows: 48 °C for 30 min, 95 °C for 10 min, 40 PCR cycles of 95 °C for 15 s and 60 °C for 1 min, followed by 4 °C hold.

All 96 RT-PCR products were run with 1.4× orange loading dye (LI-COR Inc.) with an impact, 8-channel pipette (Matrix Technologies Corp., Hudson, NH, USA) on a 3% agarose gel (Owl Separation Systems, Inc., Portsmouth, NH, USA) at 100 V for 45 min. The gels were loaded quickly, and the gel box was covered with aluminum foil to reduce chromophore exposure to light. Bands were visualized by scanning with an Odyssey (LI-COR) instrument with the 800 channel at medium sensitivity.

2.3. IR-RT-PCR assay specificity

Assay specificity tests were preformed as described above (Section 2.2), except using purified dsRNA obtained from the ABADRL BTV reference collection. Samples included 1 ng each of the 24 reference strains of BTV and the 8 known reference strains of EHDV. These assays were preformed in triplicate and included a non-template control for each replicate.