



## Short communication

# Comparison of primer sets and one-step reverse transcription polymerase chain reaction kits for the detection of bluetongue viral RNA



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Bluetongue virus is the etiological agent of bluetongue, one of the most important insect-transmitted animal diseases in the world. To establish a feasible diagnostic procedure for detecting the viral RNA, seven commercially available one-step RT-PCR kits in combination with three primer sets were evaluated. Results of this study showed remarkable differences in analytical sensitivity between the examined RT-PCR kits. In addition, it was found that a World Organization for Animal Health-recommended primer set may not be effective in detecting most BTV RNA.

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Bluetongue is one of the most important insect-transmitted diseases globally. Bluetongue virus (BTV), a member of the genus *Orbivirus* within the family *Reoviridae*, is the etiological agent of bluetongue, and 26 BTV serotypes have been identified (Maan et al., 2011). The virus is transmitted by *Culicoides* biting midges (Mellor and Boorman, 1995), and the disease affects primarily domestic and wild ruminants. In most ruminants, BTV infection is asymptomatic or mild, whereas the infection in some breeds of sheep may result in severe illness, characterized by facial edema, dermatitis, corinitis, abortion, and even death (Goltz, 1978; Luedke et al., 1977; MacLachlan et al., 2009).

Infection of BTV can cause prolonged viremia in infected animals. After the infection, the virus can be sheltered by erythrocytes and continues to circulate in the blood for more than one month (Barratt-Boyes and MacLachlan, 1994; Bonneau et al., 2002; Koumbati et al., 1999; MacLachlan, 2004; Vöggtlin et al., 2013). Therefore, for bluetongue diagnosis, detection of BTV or its genomic RNA within blood samples is meaningful; moreover, for quarantine and international trade, the detection is also helpful to identify livestock which carries BTV.

Reverse transcription polymerase chain reaction (RT-PCR) has been employed widely in the rapid diagnosis of diseases caused

by RNA viruses, including bluetongue. One-step RT-PCR, which combines reverse transcription and thermo-cycling amplification in one test tube, has the advantages of not only decreasing the number of pipetting steps but also minimizing the risk of contamination between samples. For a diagnostic laboratory that receives a large number of samples frequently, one-step RT-PCR is extremely convenient for sample processing. Moreover, with respect to the detection ability of the assay, one-step RT-PCR can be comparable to real-time RT-PCRs (Batten et al., 2008, 2009). However, our experiences have implied that the efficacy of commercially available one-step RT-PCR kits can be varied and that the selection of the kits for a given reaction is crucial because the kit chosen influences the efficacy and also the diagnostic sensitivity heavily.

The aim of the present study was to compare analytical sensitivity of several commercially available one-step RT-PCR kits in combination with different primer sets to detect BTV RNA.

To measure the analytical sensitivity of one-step RT-PCR kits, at least three rounds of testing were carried out. In each testing round, seven RT-PCR kits were used to amplify a batch of serially diluted extracted RNA of each tested BTV strain with a given primer set. To avoid bias caused by possible RNA degradation following its dilution, the sequence of the seven RT-PCR kits in a given testing round was arranged with a random table.

Two BTV strains, BTV2/KM/2003 (Ting et al., 2005) and BTV12/PT/2003 (Lee et al., 2010), were employed in this study. The

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two BTV strains were propagated in a baby hamster kidney cell line, and the titer of the multiplied BTV in the virus suspension was  $10^{4.5}$  TCID<sub>50</sub>/ml.

Viral RNA was extracted from 140 µl of the BTV suspension (equivalent to approximately  $10^{3.6}$  TCID<sub>50</sub> of BTV), using QIAamp Viral RNA Mini Kit (QIAGEN Inc., Valencia, CA, USA). The extracted BTV RNA was dissolved in RNase-free double-distilled water and stored at  $-20^{\circ}\text{C}$  until use. To prepare RNA samples for the comparison, the BTV RNA extracted from each strain was diluted serially 10-fold to a  $10^7$  dilution with RNase-free double-distilled water. The dilution was performed immediately before each round of testing and was used only for the round within two to three days.

Three sets of RT-PCR primers were used in this study. The VP7F/VP7R primer pair was designed to amplify full-length BTV segment 7 that encodes structural protein VP7 (Lee et al., 2011). The BTVL3-1/BTVL3-2 primer pair was designed to amplify a 708-base pair region within segment 3 that encodes structural protein VP3 (Ohashi et al., 2004). The primer set of Primers A/B/C/D was used to amplify a 101-base pair region within nonstructural protein NS1 gene (Dangler et al., 1990).

Seven commercially available one-step RT-PCR kits were evaluated in this study. The SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (SS-III kit; Invitrogen, Life Technologies, Carlsbad, CA, USA) is based on a version of M-MLV reverse transcriptase with reduced RNase H activity and a recombinant *Taq* DNA polymerase. The SuperScript III One-Step RT-PCR System with Platinum *Taq* High Fidelity (SS-III-HF kit; Invitrogen, Life Technologies, Carlsbad, CA, USA) is based on the M-MLV reverse transcriptase and a recombinant DNA polymerase, *Pyrococcus* species GB-D polymerase, with proofreading capability. The AccessQuick RT-PCR System (AccessQuick kit; Promega, Madison, WI, USA) is based on AMV reverse transcriptase and *Tfi* DNA polymerase. The QIAGEN OneStep RT-PCR Kit (QIAGEN kit; QIAGEN, Valencia, CA, USA) is based on two reverse transcriptases, Omniscript Reverse Transcriptase and Sensiscript Reverse Transcriptase, and HotStarTaq DNA polymerase. The One-Step RT-PCR Master Mix Kit (Novagen kit; Novagen, Merck Group, Madison, WI, USA) utilizes a recombinant *Thermus thermophilus* DNA polymerase which has both RNA-dependent and DNA-dependent DNA polymerase activities. The Fast-Run Hot Start RT-PCR (AMV) Kit (Fast-Run kit; Protech Technology Enterprise, Taipei City, Taiwan) is based on AMV reverse transcriptase and *Taq* DNA polymerase, and all the reactants are pre-mixed and lyophilized. The MasterAmp RT-PCR Kit for High Sensitivity (MasterAmp kit; EPICENTRE Biotechnologies, Madison, WI, USA) utilizes RetroAmp RT DNA Polymerase which is capable of catalyzing reverse transcription and DNA polymerization.

All the RT-PCRs were performed with DNA Engine PTC-200 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). Manipulation of the seven RT-PCR kits followed the instruction manuals provided by their manufacturers, and the number of thermocycles was 40 for all kits.

After RT-PCR was performed, 10 µl of each reaction product was run out using 2% agarose gel electrophoresis with SYBR Safe DNA Gel Stain (Invitrogen). The reaction products were visualized by ultraviolet transillumination. The analytical sensitivity of the RT-PCR kit was determined as the lowest dilution at which amplified BTV RNA could be visualized following the agarose gel electrophoresis.

To analyze the sequence variation of primer-annealed regions, available BTV nucleotide sequences from the GenBank Database were aligned by Molecular Evolutionary Genetic Analysis version 5.1, or MEGA 5.1 (Tamura et al., 2011).

Before comparing the analytical sensitivity of the one-step RT-PCR kits, the reaction conditions of each kit were optimized, and the

optimal conditions were determined as shown in Table 1. The optimal reaction concentrations of each primer were as follows: 0.2 µM for the AccessQuick and Novagen kits; 0.25 µM for the MasterAmp kit; 0.5 µM for the SS-III, SS-III-HF, and Fast-Run kits; and 0.6 µM for the QIAGEN kit. Using the MasterAmp kit, no product was generated. With Primers A/B/C/D, no product was generated by any of the RT-PCR kits employed in the present study.

The analytical sensitivity of each one-step RT-PCR kit for detecting the two Taiwanese BTV strains with the two primer pairs, VP7F/VP7R and BTVL3-1/BTVL3-2, are shown in Table 2. The SS-III and QIAGEN kits demonstrated the best analytical sensitivities among the kits tested.

Reverse transcription polymerase chain reaction is one of the most familiar tools supporting diagnoses of RNA viral infections. In addition to conventional RT-PCR, real-time RT-PCR has been also applied widely, including those to detect BTV RNA (Chatzinasiou et al., 2010; de Santis et al., 2004; Vanbinst et al., 2010). Meanwhile, although advanced, the machines and reagents for real-time RT-PCR are rather expensive and may not be affordable for every laboratory. Furthermore, conventional RT-PCRs have demonstrated a capacity comparable to that of real-time RT-PCRs (Batten et al., 2008, 2009). Conventional RT-PCR has therefore been accepted in many laboratories as a crucial part of diagnostic procedures. To this end, a quantitative comparison between the amplification abilities of commercially available conventional RT-PCR kits, in special reference to the one-step format, was conducted in this study to aid the selection of feasible kits.

The abilities of the tested one-step RT-PCR kits to amplify BTV RNA varied. The differences in the analytical sensitivities of the kits were remarkable (Table 2). With the VP7F/VP7R primer pair for the detection of BTV12 RNA, for instance, a  $10^4$ -fold difference between the kits was observed. The SS-III, SS-III-HF, and QIAGEN kits offered superior performance, regardless of the primers that were used and the virus strains targeted. This variation in analytical sensitivities suggested that the selection of the RT-PCR system is critical and extensively influences the interpretation of diagnosis.

The differences in analytical sensitivity might result from the enzymes used. In each RT-PCR kit, two enzyme activities are involved in the reaction: a reverse transcriptase activity and a thermostable DNA polymerase activity. For reverse transcriptase, there is no significant difference in the RNA-dependent DNA polymerase activities of the reverse transcriptases derived from Moloney murine leukemia virus (M-MLV) and avian myeloblastosis virus (AMV) (Verma, 1975). However, when reacting in the same tube with DNA polymerase, previous studies suggested that M-MLV and AMV can affect the activity of *Taq* polymerase (Lee et al., 1994; Sellner et al., 1992). Although information on the concentration and the extension rate of the enzymes used was not provided by the manufacturers, results of the present study echoed the study demonstrating that the SuperScript III reverse transcriptase is one of the recommended enzymes for detecting viral RNA (Okello et al., 2010) and the study reporting that the extension rate of Platinum *Taq* polymerase is higher than that of *Pfu* DNA polymerase (Markoulatos et al., 2003). Both SuperScript III reverse transcriptase and Platinum *Taq* polymerase are used in the SS-III kit. Additionally, the two kits with single-enzyme formulas (the Novagen kit and the MasterAmp kit), which employ *T. thermophilus* DNA polymerase, did not perform well in the present study. In contrast, a previous article reporting that the amplification ability of this enzyme surpassed that of *Taq* DNA polymerase (Shames et al., 1995) is inconsistent with the findings of this study.

The primer set recommended by the World Organization for Animal Health, Primers A/B/C/D (Dangler et al., 1990), was unable to detect the Taiwanese BTV strains. With Primers A/B, no RT-PCR product was observed in the present study regardless of the

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