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Development of a novel real-time RT-PCR assay with LUX primer for the detection of swine transmissible gastroenteritis virus

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Abstract

Real-time RT-PCR assay, based on light upon extension (LUX) fluorogenic primer and LightCycle technology, was developed for rapid detection of transmissible gastroenteritis virus (TGEV). Viral RNA from different TGEV isolates and clinical specimens was detected. To evaluate the sensitivity of the assay, a gel-based RT-PCR method targeted at the same 101 bp sequence was also developed. Serial 10-fold dilutions of TGEV RNA were detected by the two methods. Although the real time method used only 2 μl RNA for each reaction, a 10-fold increase of sensitivity over that of the gel-based method, which used 10 μl RNA was demonstrated. The study indicates that the LUX assay reported below is rapid, reliable and sensitive and it has the potential for use as an alternative molecular method for TGEV diagnosis.
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Keywords: LUX fluorogenic primers; Transmissible gastroenteritis virus; Real-time RT-PCR; Rapid detection

1. Introduction

Transmissible gastroenteritis (TGE) is an acute enteric viral disease of pigs resulting in vomiting, diarrhea in all ages of pigs and with high mortality rate in piglets (Garwes, 1988). The disease was first reported in 1933 in the Unite States, and has spread throughout many parts of the world including America, Europe and Asia (Yu Dahai et al., 1997). TGE virus (TGEV) was first isolated in 1946 (Doyle and Hutchings, 1946), and is a member of the *Cornaviridae* family, a group of enveloped viruses with a large, single positive-strand RNA genome (Lai, 1990; Page et al., 1991).

Rapid diagnostic methods for TGE are very important because of the highly contagious nature of the disease. Various methods have been developed for the diagnosis of the disease including virus isolation in tissue culture (Dulac et al., 1977) and immunodiagnostic methods, particularly enzyme-linked immunosorbent assay (ELISA) for detection of virus in faeces (Bernard et al., 1986; Van Nieuwstadt et al., 1988) and fluorescent antibody tests (FAT) on cryostat sections of intestine (Pensaert et al., 1968). Virus isolation is slow and the virus is often difficult to adapt to growth in cell culture (Paton et al., 1997). Immunoassays for antigen required fresh clinical samples and may fail to detect virus present at very low levels. Serological methods such as virus neutralization test (Witte, 1971) and ELISAs on TGEV antibody (Huang et al., 1988; Rukhadze et al., 1989; Brown and Paton, 1991; Liu et al., 2001) are used widely but lack advantage in terms of rapid diagnosis because of the time needed for antibody development. Nucleic acid recognition methods including in situ hybridization (Sirinarumitr et al., 1996) and RT-PCR (Paton et al., 1997; Kim et al., 2000; Woods, 1997) have also been described for the direct detection of TGEV RNA. RT-PCR tests are convenient to conduct compared to hybridization methods and increasingly used. More recently, real time fluorescent nucleic acid amplification techniques were developed for detection of disease agents and proved to be rapid and sensitive (Stram et al., 2004; King et al., 2003). However, there are no reports so far on real-time RT-PCR methods for TGEV.

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The aim of this study was to develop a practical and reliable molecular diagnostic method for TGEV by using real-time techniques principles.

A real-time one-step RT-PCR assay with light upon extension fluorogenic primer (LUX RT-PCR) is described for detecting TGEV. It includes a single-labeled primer with a FAM fluorophore at the 3′ end in a hairpin structure and a corresponding unlabeled primer, designed to amply the 5′ end of the gene encoding the S protein of TGEV. The configuration of the labeled primer enables the fluorescence quenching capability. When the primer is incorporated into double-stranded RT-PCR product, the fluorophore is dequenched, resulting in a significant increase in fluorescent signal (http://www.invitrogen.com/lux). The target region is located within the large deletion found exclusively in isolates of porcine respiratory coronavirus (PRCV) that is probably a deletion mutant of TGEV (Rasschaert et al., 1990; Page et al., 1991; Laude et al., 1993). Thus the assay that was developed can differentiate TGEV from PRCV, which is important for disease surveillance.

A comparison between this real-time assay and a gel-based RT-PCR method was also carried out, which demonstrated increase sensitivity in real-time assay.

2. Materials and methods

2.1. Viruses and tissue samples

Six virus isolates were used, including two standard strains TGEV-Miller6, & TGEV-Purdue115, and TGEV-020PDV8601 (from NVSL, Ames, IA, USA), TGEV-H1988 (isolated in China), TGEV-VAC (Vaccine from Ambico, INC, USA), TGEV-AG1 (isolated in China). All these viruses except the vaccine strain were propagated in PK-15 cell line; cell culture materials were collected 48hrs after infection. The vaccine liquid was used directly for RNA extraction. The TGEV-Miller6 and TGEV-Purdue115 strain was titrated as 1×10^4 $TCID_{50}$/ml and 1×10^5 $TCID_{50}$/ml, respectively. The tissue samples were small intestine specimens from TGEV-Miller6 inoculated piglets and healthy piglets, respectively. TGEV positive specimens from three inoculated piglets were available. These materials were kept at −20 °C for 4 years.

2.2. Preparation of RNA samples

The virus infected cell harvest and vaccine liquid were frozen and thawed, and 140 μl of each liquid sample was used for RNA extraction by QIAamp Viral RNA methods (QIAgene Cat. No. 52904), each RNA sample was eluted and kept in 60 μl buffer provided in the kit. RNA from the blank PK-15 cell was prepared in the same way.

The tissue samples were grinded with PBS and marinated overnight at 4 °C, then centrifuged at 3000 rpm for 5 min, 140 μl of the supernatant was used for RNA extraction. RNA from small intestine specimens of healthy piglets was also prepared as the blank control and for specificity tests.

2.3. The LUX RT-PCR assay for TGEV

2.3.1. Primers

The fluorogenic forward primer and the corresponding unlabeled reverse primer were designed and ordered using LUX™ Designer Software online. The target sequence was based on TGEV spike protein gene (Genebank accession number X53128). The sequence of the primers: forward primer 5′-caacaaGGTTTGCAGATGCGGTTGtTG-FAM-3′, reverse primer 5′-CGC ACG CAT ATC ACC AAG TGT-3′. The sequences of the two primers were checked using the NCBI Blast Software, and no significant alignment with any other animal virus gene was found. The target sequence is from 2138 to 2238 bp in the gene.

2.3.2. Detection of TGEV

The LUX RT-PCR reactions were prepared using the commercial quantitative RT-PCR system (Invitrogen Cat. No. 11731-015). The reaction was carried out in 20 μl in a single tube, containing 3 mM $MgSO_4$, 0.2 mM of each of dNTPs, 500 nM of each primer, 20 U of RNaseOUT Ribonuclease inhibitor (Invitrogen), 5 μg bovine serum albumin (SERVA), 0.8 μl ThermoScript™ Plus/Platinum® Taq enzyme mix, and 2 μl RNA template. We used the Roche LightCycler® with the optimized cycling program: 60 °C, 20 min (1 cycle); 95 °C, 2 min (1 cycle); 45 cycles of 94 °C, 5 s; 60 °C, 20 s (single acquire); 40 °C, 0 s.

All six virus isolates and three tissue specimens were tested together with cell blank or tissue blank sample. Six swine RNA viruses were also tested, including PRCV, Porcine epidemic diarrhea virus (PEDV), Porcine pseudorabies virus (PRV), Hemagglutinating encephalomyelitis virus of pigs (HEV), Porcine reproductive and respiratory syndrome virus (PRRSV) and swine rotavirus.

2.4. Gel-based RT-PCR for TGEV

The primers were the same as the LUX assay, except that the forward primer was not labeled and did not have the six hairpin forming bases at the 5′ end. This method also used reagents from Invitrogen (Cat. No. 10928-034). The reaction was carried out in 50 μl, containing 1.2 mM $MgSO_4$, 0.2 mM of each dNTP, 0.2 μM of each primer, 20 U of RNaseOUT Ribonuclease inhibitor (Invitrogen), 1 μl RT/Platinum *Taq* mix, and 10 μl RNA template. The amplification was carried out in a PE 2400 PCR system (Perkin Elmer) under the condition: 50 °C, 30 min; 94 °C, 2 min; 35cycles of 94 °C, 20 s; 60 °C, 30 s; 72 °C, 30 s; 72 °C, 2 min. The 101 bp product was examined by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

2.5. Sensitivity testing and comparison between the two methods

The sensitivities of the LUX RT-PCR and gel-based assay were determined by using a 10-fold dilutions (10^{-1}–10^{-7}) series of the RNA from the six TGEV isolates. The diluent, Dnase/Rnase free distilled water, was run at each reaction panel as the negative control.

3. Results

3.1. Performance of the LUX RT-PCR assay for TGEV

The assay with LUX Primer successfully detected RNA from the six TGEV isolates and virus infected tissue samples as shown in Table 1.

3.2. Specificity of the LUX RT-PCR assay

The reaction was carried out with the following RNA virus, PRCV, PRV, PEDV, HEV, PRRSV and swine rotavirus. Small intestine specimens from twenty healthy piglets were also tested. All gave negative results. All these samples were tested together with the TGEV-Miller 6 virus RNA as the positive control.

3.3. Comparison of the sensitivity of the LUX RT-PCR and the gel-based RT-PCR assay

To evaluate the sensitivity of the LUX RT-PCR assay, serial 10-fold dilutions of the RNA extracted from each of the six TGEV isolates were tested by both methods. The limit of detection is shown in Table 2. Fig. 1 illustrates the real-time fluorescence curve obtained by the LUX method and the bands on gel electrophoresis for the conventional RT-PCR. The LUX RT-PCR assay demonstrated 10-fold higher limit of detection than the gel-based method. Whereas the LUX method used only 2 μl of RNA template in each reaction compared to the amount of 10 μl used in the gel-based method.

Table 1
The detection of TGEV infected cell and tissue samples by the LUX RT-PCR assay

Virus & intestine specimens	C_T values of the test	Result
TGEV-Miller 6	22.65	+
TGEV-Purdue 115	22.60	+
TGEV-020PDV8601	22.30	+
TGEV-H1988	26.22	+
TGEV-VAC	25.74	+
TGEV-AG1	21.04	+
PK-15 cell blank control	37.93	–
Water blank control	38.78	–
Specimen 1	26.99	+
Specimen 2	24.85	+
Specimen 3	28.05	+
Specimen control	38.05	–

Table 2
The detection limits for TGEV by the LUX and gel-based method

TGEV isolates	Limit of detection		
	LUX RT-PCR	Gel-based RT-PCR	Virus $TCID_{50}$/ml
Miller6	10^{-4}	10^{-3}	10^4
Purdue115	10^{-5}	10^{-4}	10^5
020PDV8601	10^{-4}	10^{-3}	N.D
H1988	10^{-5}	10^{-4}	N.D
VAC	10^{-3}	10^{-2}	N.D
AG1	10^{-4}	10^{-3}	N.D

N.D., not determined.

4. Discussion

A novel real-time RT-PCR assay for TGEV detection was developed by using LUX fluorogenic primers technology that was established in 2002 by scientists in the Invitrogen Corporation. The LUX fluorogenic primers technology is of high-performance and cost-effective (*Invitrogen Press Room* online, http://www.invitrogen.com). Unlike the current well known real-time technology that relies on a synthetic DNA probe labeled with two different fluorescent dyes, LUX primers technology does not require an expensive probe so is more suitable for routine laboratory diagnosis. What a LUX assay needs is a specific primer set with a single labeled, self-quenched primer and a corresponding unlabeled one, it is more reliable than the real-time method using DNA binding dyes that may produce potentially misleading results due to the lack of specificity of the dyes. A previous study also indicates that the LUX primers technology is reliable for quantitation of gene expression and the result is similar to the probe-based quantitative assay (Brian et al., 2003). LUX fluorogenic primers can be designed and ordered via online software. (http://www.invitrogen.com/lux).

The LUX assay developed in the present study was targeted at a 101 bp gene sequence found only within TGEV RNA, which is confirmed by online NCBI blast examination. The good specificity is also verified by the test of other swine virus and pig tissue samples. The assay was 10-fold more sensitive than the conventional RT-PCR method as demonstrated in the study, while the amount of RNA used in the LUX method is only one-fifth of the gel-based method. Due to the 20 μl volume limit of the Roche LightCycler® instrument that uses capillaries, the maximum volume of RNA template for the LUX test is 2 μl. If an instrument using PCR tubes or plates is available, the LUX test can be carried out in 50 μl by using 10 μl RNA template and shall be more sensitive.

The LUX assay also has the advantage of increase speed and is less laborious over the gel-based RT-PCR technique that is currently the routine gene analysis tool for TGEV. The

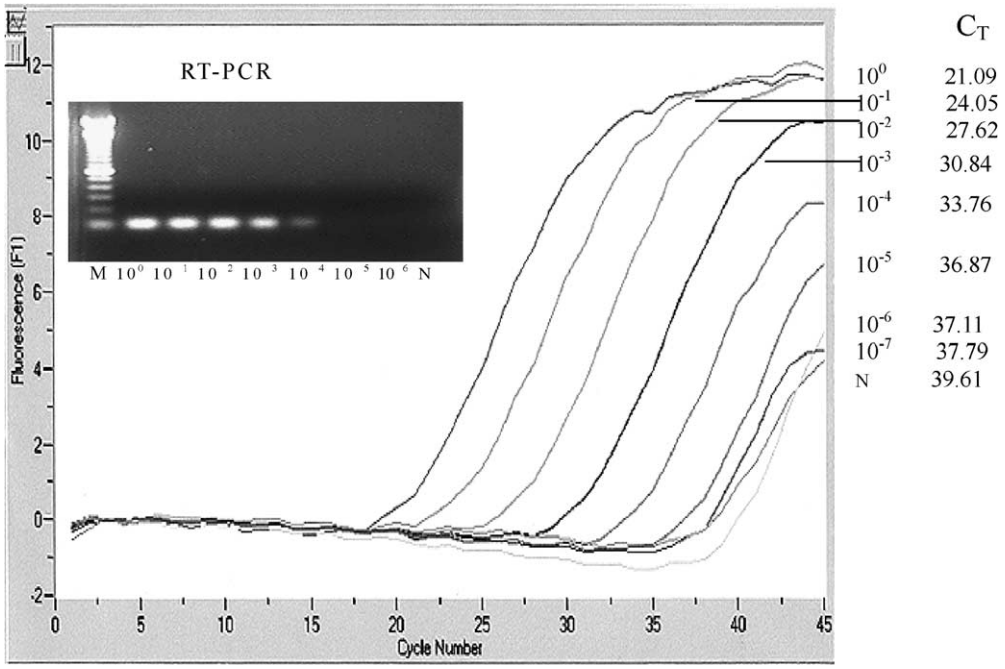

Fig. 1. The results obtained by the LUX and RT-PCR assays on the dilution series of TGEV-Purduce115 RNA. LightCycle real-time fluorescence signal curve and the C_T value for each dilution is shown. The inset depicts agarose gel electrophoresis of the RT-PCR products (101 bp). M, 100 bp DNA ladder; N, negative control.

LUX assay took less than an hour to complete the amplification reaction and the process was viewed in real time, while conventional RT-PCR methods usually take more than 1 h for gene amplification and half an hour or more to run the gel and examine the result. The advantage of speed of the LUX assay is more apparent when compared to other routine diagnostic methods for TGE. For example, virus isolation and neutralization tests require several days to declare a negative result, while ELISAs and FAT take several hours, even overnight incubation to finished a test. Furthermore, the LUX assay is closed-tube and one-step technique, which reduces the risk of contamination and reaction variability. This sensitive and specific test complements existing gene methods for the detection of TGEV. The method shall prove to be a valuable tool in the laboratory diagnosis of TGEV, especially as a means of confirming positive results from serological tests.

LUX primers technology supports multiplex amplification (http://www.invitrogen.com/lux) that makes detecting different pathogens in a single assay possible. By using two sets of primers, each labeled with a different dye, a single LUX assay can detected two different viruses.

LUX primers are compatible with a wide variety of real-time PCR instruments (http://www.invitrogen.com/lux). More assays can be developed for the detection of other pathogens. By reducing the cost of real-time gene detection and with high performance, LUX fluorogenic primers technology may has the potential to be used widely in the field of animal disease surveillance and control as well as import and export animal quarantine management.

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