Rev. Salud Anim. Vol. 30 No. 2 (2008): 133-136

Short Communication

RAPID DETECTION OF SWINE TRANSMISSIBLE GASTROENTERITIS VIRUS BY NESTED POLYMERASE CHAIN REACTION

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ABSTRACT: **The aim of this study was to develop a nested PCR for the rapid detection of TGEv. The primers were designed from the highly conserved regions of several sequences of this virus included in the analysis. With the purpose of achieving a good technical specificity, sequences of porcine epidemic diarrhoea virus (PEDv) were included in the design. This virus belongs to the same viral family and it has a close genetic relationship with TGEv. Since both viruses produce the same clinical signs, it was very important to get primers which not amplify PEDv. With the external primers, the amplification of a fragment of the expected size (441 pb) in all the samples evaluated by RT-PCR was obtained, but there was a very low intensity. In the second round (nested PCR), with the internal primers, the amplification of a fragment of the expected size of 168 pb was shown, with a very good concentration. It has been considered that when using nested PCR, TGEv detection sensitivity increased in isolates with low viral concentration.**

(Key words: Transmissible Gastroenteritis Virus (TGEv); RT-PCR; nested PCR)

DETECCIÓN RÁPIDA DEL VIRUS DE LA GASTROENTERITIS TRANSMISIBLE EN CERDO A TRAVÉS DE LA REACCIÓN EN CADENA DE LA POLIMERASA

RESUMEN: **El objetivo de este estudio fue desarrollar una PCR anidada para la detección rápida del virus de TGE. Los cebadores fueron diseñados a partir de regiones altamente conservadas de varias secuencias de este virus incluidas en el análisis. Con el fin de lograr una buena especificidad de la técnica se incluyeron en el diseño, secuencias del virus de la diarrea epidémica porcina (PEDv), el cual pertenece a la misma familia viral y posee una estrecha relación genética con el virus de TGE. Como ambos virus producen los mismos signos clínicos fue importante obtener cebadores que no amplificaran el PEDv. Con los cebadores externos se obtuvo la amplificación de un fragmento de la talla esperada de 441 pb en todas las muestras evaluadas por RT-PCR, pero de muy baja intensidad. En la segunda amplificación (PCR anidado), con los cebadores internos, se mostró la amplificación de un fragmento de la talla esperada de 168 pb, con una buena concentración. Consideramos que con el PCR anidado se incrementó la sensibilidad de la detección del virus de TGE en aislados con baja concentración viral.**

(Palabras clave: Virus de la Gastroenteritis Transmisible del cerdo; RT-PCR; PCR anidado)

Transmissible gastroenteritis (TGE) is a highly contagious enteric disease affecting pigs, caused by a virus (TGEv), which belongs to *Coronaviridae* family, *coronavirus* genus (7). This disease produces relevant economic losses due to the serious dehydration and mortality rates close to 100% in newborn piglets (14, 11). Rapid diagnostic methods for TGE are very important because of the highly contagious nature of the disease (2). Various methods have been developed for the diagnosis of the disease, including virus isolation in tissue culture (10), immunodiagnostic methods: particularly enzyme-linked immunosorbent

assay (ELISA) for detection of virus in faeces (13) and fluorescent antibody tests (FAT) on cryostat sections of intestine (4). Virus isolation is slow and the virus is often difficult to adapt to growth in cell culture (12). Immunoassays required fresh clinical samples and may fail to detect virus present at very low levels. Serological methods such as virus neutralization test and ELISA on TGEV antibody (9) are used widely but there is lack advantage in terms of rapid diagnosis because of the time needed for the antibody development. Nucleic acid recognition methods including *in situ* hybridization (8) and RT-PCR (15) have been described for the direct detection of TGEV RNA. RT-PCR test is useful to detect small quantities of nucleic acids (3). The purpose of this study was to develop a nested PCR for the diagnosis of TGE virus since having a rapid result allows the application of the quarantine measures thus it prevents the dissemination of the virus, reducing the losses by delayed diagnosis.

Twelve Cuban isolates of swine TGEv grown two times into secondary cultures of pig kidney cell monolayer (PKC) were used. The cells were maintained under Dulbeco's modified Eagle's medium supplemented with two percents of inactivated fetal serum.

The isolates were obtained from different provinces and times during the epizootic (Table 1). Also TGEv reference strain (Purdue 115) was used as a positive control of the reaction, gently donated by the Dr. Dongwan Yoo, University of Guelph, Ontario, Canada.

The oligonucleotide primers for the RT-PCR and nested PCR were design. There were based on a fragment of the nucleocapsid gene (N) of TGEv. The sequences were obtained from the GenBank database, accession numbers: AJ271965, NC002306, AY587884, AY335549, AF104420, DQ201447, DQ443743, DQ811785, DQ811786, DQ811788 and DQ811789. The oligonucleotide primers were design from the highly conserved regions, with a 100% identity between all the sequences of TGEv included in the analysis. Also, with the aim of obtainig specific primers, which not amplify the porcine epidemic diarrhoea virus (PEDv), sequences of N gene of this virus: AF237764, AF353511, AY653206, DQ072726, DQ355221, DQ355223, DQ355224 and NC_003436) were included in the design. PEDv as well as TGEv belong to the group 1 of the coronavirus genus. They have a close genetic relationship (5, 1). The sequences of primers were as follows: forward primer for RT-PCR: (TGENF): 5' CTGGAAGAGAACTGCAGGTA 3' and reverse primer for RT-PCR: (TGENR): 5' TTAGTTCGTTACCTCATC 3' (441pb), forward primer for nested PCR: (TGENFI): 5' GGCGACCAGATAGAAGTCACG 3' y reverse primer for nested PCR: (TGENRI): 5' CTTGCTCTGACCTTTCTGCAG 3' (168pb). The softwares used in the analysis were: Gene Runner Version 3.05, CLUSTAL W Multiple Sequence Alignment Program, BLAST and Oligo Analyser version 1.2.

TABLE 1. Isolates used in the RT-PCR assay./ *Aislados utilizados en el ensayo de RT-PCR*

Isolate	Province	Date
VB 479	Cienfuegos	31-3-03
VB 481	Ciudad Habana	4-4-03
VB 546	Villa Clara	17-4-03
VB 623	La Habana	16-5-03
VB 625	Holguín	18-5-03
Vto 12-15	Matanzas	20-6-03
Vto 17	Isla de la Juventud	1-7-03
Vto 19	Holguín	1-7-03
Vto 25	Villa Clara	15-8-03
Vto 32	Villa Clara	10-9-03
VB 3/05	La Habana	25-1-05
VB 5/05	La Habana	25-1-05

The RNA used for the reverse transcriptase reaction (RT-PCR) was isolated by the QIAamp® Viral RNA Mini Kit (QIAGEN) from cell-culture supernatants. The reaction was conducted as follows. In the tube, 8.5 µl of RNA sample were added to 2 µl of the TGENR primer [100 ng/ µl]. The tube was incubated at 70ºC for 10 min and then quenched on ice for 5 min. Subsequently, mixtures of 4 µl of 5x RT buffer, 2 µl of 0.1M dithiothereitol, 2 µl of 10mM dNTPs, 0.5 µl of RNAsin, and 1 µl of M-MLV RT (200 U/ µL) (Invitrogen Corporation) were added and incubated at 42ºC for 60 min. Finally an inactivation step was made at 95ºC for 10 min.

Then 5 µl of the RT reaction samples were added to 45 µl of the PCR mixture. The PCR mixture consisted of 5 µl of 10x buffer, 2.5 µl of $MgCl_2$ (50 mM), 1 µl of 10mM dNTPs, 1 µl of each primer (TGENF and TGENR) [100 ng/ µl], 34 µl of water and 0.5 µl of Taq DNA Polymerase (5 U/ml) (Invitrogen Corporation). The amplification program of the PCR was as follows: 5 min at 94º C, 35 cycles (45 s at 94º C, 45 s at 50º C, and 1 min at 72 º C) and 10 min at 72º C.

For the nested PCR, 5 µl of RT-PCR products were added to a tube containing 45 µl of the same PCR mixture mentioned before but with the internal primers (TGENFI and TGENRI) 1 µl of each primer [100 ng/ µl]. The amplification program was 3 min at 94º C, 35

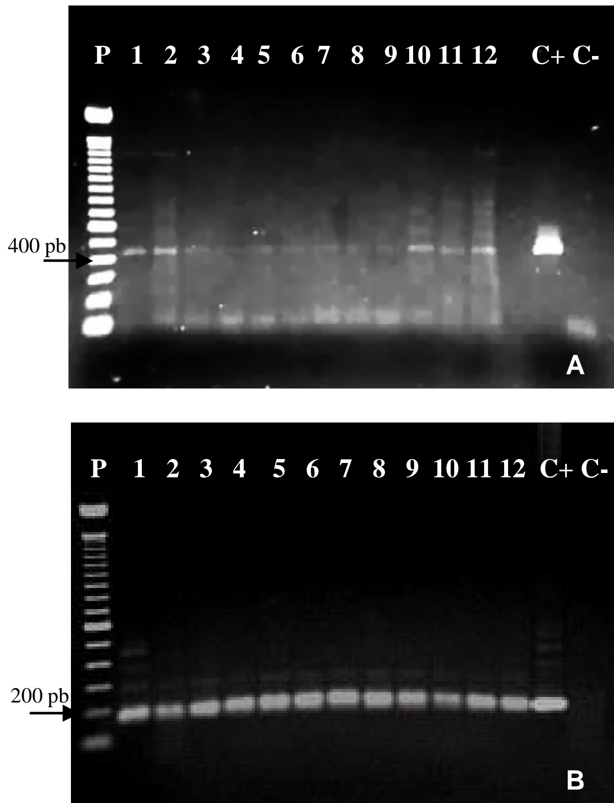

FIGURE 1. A: RT-PCR and B: Nested PCR. P: Ladder molecular marker 1Kb plus (Invitrogen), 1-12 different isolates (VB 481, Vto 25, Vto 32, Vto 19, Vto 17, VB 3/05, VB 5/05, VB 479, VB 623, VB 625, VB 546, Vto 12-15), C+: Positive control TGEv strain (Purdue 115), C-: Negative control./ *A: RT-PCR y B: Nested PCR. P: Patrón de peso molecular 1Kb plus (invitrogen), 1-12 diferentes aislados (VB 481, Vto 25, Vto 32, Vto 19, Vto 17, VB 3/05, VB 5/05, VB 479, VB 623, VB 625, VB 546, Vto 12-15), C+: Control positivo Cepa de TGE (Purdue 115), C-: Control negativo.*

cycles of (45s at 94ºC, 45s at 58ºC, and 1 min at 72ºC) and 7 min at 72ºC. Both PCR reactions were carried out in an Eppendorf thermal cycler.

PCR products were analysed by elecrophoresis in a 2% agarose gel containing ethidium bromide.

The N gene was chosen because it is highly conserved among TGEv strains. The N protein is the most abundant antigen in coronavirus-infected cells since its RNA template is the smallest and it has the most abundant sgRNA (subgenomic RNA) during transcription (6). This indicates that there is more available RNA for the N gene than for the other TGEv protein genes. Consequently, detection of the N gene RNA might be advantageous due to its high abundance in cells, facilitating a high sensitivity of the diagnostic technique (16).

With the external primers designed for the detection of TGEv, the amplification of a fragment of the expected size (441 pb) was obtained in all the samples evaluated by RT-PCR (Figure 1A), but there was a great difference among the amplification of the positive control (Purdue strain) and the rest of the samples, in which the intensity of the band was very weak, while the positive control showed to be very strong. This result showed the necessity of developing a nested PCR to increase the sensitivity of the assay.

In the second round (nested PCR), with the internal primers, a fragment of the expected size of 168 pb was shown, and at this time the amplicons for all the samples had a very good concentration (Figure 1B). With the nested PCR, the sensitivity of TGEv detection increased in isolates with low passages.

It has been concluded that the nested PCR described here is a useful tool for the detection and identification of TGEv in cell cultures with low passages. Differences in the ability to amplify and the characteristics of PCR systems available for TGEv detection in clinical samples should be considered before using test in future epidemiological studies. The availability of a sensitive and specific diagnostic technique such as the nested PCR described in this work will make possible to undertake larger epidemiological studies of TGEv involved in the porcine diarrhoea aetiology.

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(Recibido 12-11-2006; Aceptado 15-6-2007)