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Establishment and Application of the Method of Molecular Diagnostic of Porcine Epidemic Diarrhea

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Porcine epidemic diarrhea (PED) is caused by porcine epidemic diarrhea virus in pigs, which is a highly contagious enteric disease with vomiting, diarrhea, and loss of appetite and of all ages can be susceptible to this disease. The current diagnosis of PED was immune electron microscopy, immunofluorescence, indirect hemagglutination test, ELISA, RT-PCR, neutralization test. With the development of molecular biology, the method of ELISA and RT-PCR are specific diagnostic methods for detecting PED and currently the most widely used. The biggest advantage of ELISA method is can directly check PEDV antigen from feces, and can also be used for the detection of PED antibody. TR-PCR has good sensitivity and specificity. In this study, according to the PED virus genome database sequences of S gene nucleotide sequence specific amplification, a pair of primers were designed, through optimizing concentration of primers and the template concentration and PCR amplification conditions, determine the optimum conditions for optimal RT-PCR system: primer concentration: 1.0-1.2mol/L, $MgCl_2$: 2mmol/L annealing temperature of 56!. The best cycle condition: 95! 3min, 94! 30S, 56! 30S, 72! 1min, 35 cycles, the last 72! 10min. This study provides a simple method for the clinical diagnosis of PED, can be measured in 6 hours, can be used for the diagnosis and epidemiological survey of PED.

Key words: Porcine epidemic diarrhea, RT-PCR, Molecular diagnosis.

PED is a highly contagious enteric disease caused by porcine epidemic diarrhea virus. The first found PED was in the UK in 1971, which was an acute diarrhea of cause of suckling piglets and fattening pigs, feeder pigs[1-5]. This was endemic in many countries and regions[6]. The incidence can up to 100% of suckling piglets and fattening pigs, feeder pigs, especially in suckling piglets was the most serious[7]. The incidence was 15~90% in sows. The disease mainly occur in winter and summer.The immune electron microscopy, immunofluorescence, indirect hemagglutination test, ELISA, RT-PCR and neutralization test are the mainly PED diagnosis methods[8-10]. RT-PCR method with the better sensitivity and specificity, which is a common clinical pathogenic diagnosis in PED[11].This study was optimized PED-RT-PCR reaction system and conditions, which was provides an effective method for molecular diagnosis of PED in clinical diagnosis and molecular epidemiological survey.

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MATERIALS AND METHODS

Reagents

AMV reverse transcriptase, Taq DNA polymerase, RNA enzyme inhibitor, 100bp DNA Ladder, dNTP, $MgCl_2$, RT-PCR Buffer, DEPC-treated ddH_2O, RNase-free centrifuge tube were purchased from TaKaRa Biological Engineering Co Ltd.

Sample collection and processing

Aseptic collection fresh stool sick animal, diluted 5 times with saline, vortex stirring, 8000

rpm/min for 10min, the supernatant was placed in -20 ! saving or immediate extraction RNA.

Primers were designed

According to the PED virus genome database sequences of S gene nucleotide sequence specific amplification, a pair of primers were designed, upstream primer: 5 '-TATTTTGGTGTAATCGTATGGC-3', a downstream primer 3 '-GGCTGTTTTTCCA CGTAATCGGAA-5'. The expected amplification fragment is 200 bp, primer were designed by Sanggon Biotech. The primer were dilution of 25 pmol/µl.

Extraction of RNA

RNA extraction according to the extraction kit (SK86932, Sangon Biotech), with the following steps:

(1) 450µl Buffer AG add to the 1.5ml RNase-free centrifugation tube.

(2) 25~50mg animal tissues was grinded by liquid nitrogen, added to the 1.5ml centrifuge tube, immediately turbulence mixing, vibrating for 2min, and then placed at room temperature for 3min.

(3) 12 000rpm, 4 ! for 4 min, the supernatant moved to 1.5ml RNase-free tube.

(4) Add 250 µl absolute alcohol mixing.

(5) Put silica column into the collection tube, transfer the 4 step solution to the silica column, 12 000 rpm, 4 ! for 1 min, discard the collection tube liquid.

(6) The silica column put a new collection tube, add 500 µl GT solution, 10 000 rpm, 4 ! for 1 min, discard the collection tube liquid.

(7) Add 500 µl NT Solution, 10 000 rpm, 4 ! for 1 min, discard the collection tube liquid. Then 12 000 rpm, 4 ! for 2 min.

(8) Add 50 µl DEPC-treated ddH_2O to the center of the silica column, 12 000 rpm, 4 ! for 2 min. The obtained RNA solution preserved in -70 !or immediately used for subsequent test.

Reverse transcription (RT)

For first-stand Cdna synthesis, 1 µg total RNA was reversed transcribed using the Primescript 1st Strand Synthesis kti, as per manufacture2 s instructions (Takara Bio Inc., China).The reaction system is 25 µl, RNA 5µl, downstream primer 2µl, 5×RT Buffer 5µl, 2.5mmol/ L dNTP 5µl, RNA enzyme inhibitor 1µl , AMV reverse transcriptase 1 µl, DEPE H_2O 1µl, uniformly mixture, 42 ! for 1h.

Polymerase chain reaction (PCR)

The cDNA as template, according to the following system for PCR amplification reaction: the total volume is 25 µl, DEPC H_2O 7 µl, L, 2×Buffer (Mg^+) 4.5µl, $dNTP_S$ 4 µl, the upstream primer 2 µl (25 pmol/ L), downstream primer 2 µl, Taq enzyme 0.5 25 µl (5U/µl), cDNA 5 µl. The initial denaturation step (95 ! for 5 min) in PCR reaction was followed by 32 cycles of 94 ! for 30 s, 56 ! for 1 min, 72 ! for 1 min, and a final extension period at 72 ! for 10 min. The amplified product was visualized by gel electrophoresis in a 1% (w/v) agarose gel.

RT-PCR specific test

Collect clinical samples disease of swine manure, according to the above method of RT-PCR.

Analysis of RT-PCR product sequence

RT-PCR product was purified, send to company for sequence(Takara Bio Inc., China).The obtained sequence were compared with the database in NCBI.

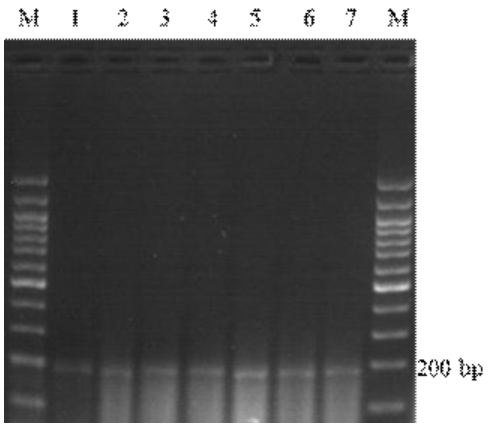

Fig. 1. PED RT-PCR

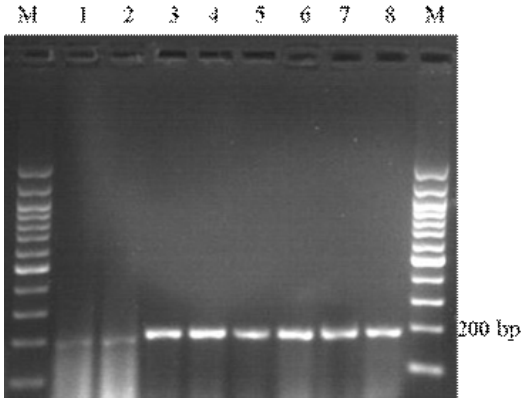

Fig. 2. PED RT-PCR

RESULTS

Detection of RT-PCR products

The PCR product electrophoresis results see Figure 1. The Marker was 100 bp. From top to bottom as follows:1500 bp, 1200 bp, 1000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp, 200 bp, 100 bp.

As can be seen from Figure 1, the PED template RT-PCR amplified fragment of approximately 200 bp. It demonstrated that the amplified fragment was the purpose expansion fragment, which consistent with the expected size of the design.

RT-PCR specific test

As you can see from Figure 2, pig fecal samples collected from clinical, RT-PCR according to the above method, all could amplify the fragment with the expected.

Analysis of RT-PCR product sequence

According to the conventional method, the PCR product was cloned into pMD18-T vector, the recombinant plasmid was sequenced by (Takara Bio Inc., China). The sequence was more than 99% homology compared with sequence in the NCBI database of PED.

DISCUSSION

PED is a kind of common diseases of digestive system diseases in pig farms, upon the occurrence of the disease, it is difficult to eradicate and purification[12-14]. Disease and infected pigs are the major source of infection[15]. It was transmitted through the digestive tract. PED often single or mixed infection with transmissible gastroenteritis of pigs, sometimes with porcine circovirus infection or bacterial infection[17-19].

Upon this disease occurs, there is no good treatment, porcine interferon can decrease the weight loss, cooperate with the monoclonal antibody can reduce the mortality of piglets, the treatment of pigs to replenish the glucose, glycine and electrolyte solution and oral rehydration salts. Vaccination is the primary means to prevent PED. The disease with the age of small, acute onset, high mortality characteristics, relying on their own immunity is often too late, so mostly through to sow vaccinated, depending on the specific antibodies in breast milk to provide good protection of piglets[20]. The sow before childbirth 20~30d vaccine injection, piglets by feeding colostrum and passive immune protection. To prevent the disease is to do the work of feeding and management of the usual. Promptly isolation and disinfection when found to have this disease occurs, reduce the flow of personnel, with all in and all out system to prevent and control.This study established PDE RT-PCR molecular diagnosis method, has good specificity. This study provides a simple method for the rapid detection of PED in clinical, and laid the foundation for PED epidemiology and molecular diagnosis.

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