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BRIEF REPORT

Establishment of a nanoparticle-assisted RT-PCR assay to distinguish field strains and attenuated strains of porcine epidemic diarrhea virus

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Abstract Porcine epidemic diarrhea virus (PEDV) can cause serious disease and even death in neonatal piglets, resulting in serious damage to the swine industry worldwide. Open reading frame 3 (ORF3) is the only accessory gene in the PEDV genome. Previous studies have indicated that PEDV vaccine strains have a partial deletion in ORF3. In this study, a nanoparticle-assisted polymerase chain reaction (nanoparticle-assisted RT-PCR) assay targeting the ORF3 of PEDV was developed to distinguish PEDV field strains from attenuated strains by using a specific pair of primers. The PCR products of field strains and attenuated strains were 264 bp and 215 bp in length, respectively. The sensitivity and specificity of this assay were also assessed. The nanoparticle-assisted RT-PCR assay was 10-100 times more sensitive than the conventional RT-PCR assay, with no cross-reactions when amplifying porcine pseudorabies virus (PRV), porcine circovirus type 2 (PCV2), classical swine fever virus (CSFV), porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine rotavirus (RV), and porcine transmissible gastroenteritis virus (TGEV). The nanoparticle-assisted RT-PCR assay we describe here can be used to distinguish field strains from vaccine strains of PEDV, and it shows promise for reducing economic loss due to PEDV infection.

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Introduction

Porcine epidemic diarrhea virus (PEDV) is a highly pathogenic and lethal virus. It belongs to the genus *Alphacoronavirus* of the family *Coronavirus*. Its main clinical features are vomiting, diarrhea, dehydration and high mortality in suckling piglets [1]. In the 1970s, PEDV first appeared in the United Kingdom [2]. Subsequently, it was reported in Europe and Asia [3, 4]. Since 2010, PEDV has caused large-scale diarrhea outbreaks in pigs and a 80-100 % fatality rate in suckling piglets in China [5, 6]. Recently, a PEDV outbreak has rapidly swept throughout the United States in less than a year [7, 8]. PEDV has lead to significant economic losses worldwide, but there has been no specific or effective method available to keep the spread of PEDV under control [9].

PEDV is an enveloped, positive single-strand RNA virus. It has a large genome of approximately 28 kb in size, which is composed of 5′ UTR-ORF1-S-ORF3-E-M-N-3′ UTR, encoding four structural and four non-structural proteins [10]. Open reading frame 3 (ORF3) is the only accessory gene in the PEDV genome. The ORF3 of PEDV has an important role in virulence. Some studies have shown that the coronavirus ORF3-encoded protein forms an ion channel and modulates virus release, and ORF3 gene silencing can reduce viral titers in Vero cells [11, 12]. Further research has shown that deletions are present in ORF3 of cell-adapted PEDV strains when compared to field strains [13, 14]. In Asia, vaccines based on attenuated PEDV strains have been approved [13, 15], including the

CV777 and ZJ08 strains in China, the P-5V strain in Japan, and the KPED-9 and DR13 strains in South Korea. These cell-adapted vaccine strains have a 49-bp deletion in the ORF3 gene. This feature can be used to distinguish natural field strains from attenuated vaccine strains.

The current methods of detecting PEDV include electron microscopy, virus isolation, reverse transcription polymerase chain reaction (RT-PCR), reverse transcription loop-mediated isothermal amplification (RT-LAMP), and real-time RT-PCR [16–19]. These approaches have their own disadvantages. Electron microscopy and virus isolation are highly specific and sensitive, but they are time-consuming. Samples get contaminated easily in the LAMP assays, and real-time RT-PCR requires special instruments. In this study, a nanoparticle-assisted RT-PCR assay was developed to distinguish PEDV field strains from vaccine strains. Similar to conventional RT-PCR assays, this method showed improved sensitivity and specificity. The advanced nanoparticle-assisted RT-PCR uses metal particles to create a better thermal conductivity environment, thereby reducing nonspecific amplification and facilitating specific amplification. Moreover, the nanoparticle-assisted RT-PCR assay does not require specialized instruments, in contrast to the real-time RT-PCR assay [20].

In this study, a pair of primers targeting the ORF3 was designed, with binding sites at both ends of the ORF3 gene deletion. The nanoparticle-assisted RT-PCR described in this study was developed to identify whether the virus strains in the samples are field PEDV or vaccine strains, and it was also used to detect the virus in clinical samples.

Materials and methods

Viruses and sample template preparation

The PEDV vaccine strain (ZJ08), field strain (HEBEI08), and the following viruses used in this experiment – porcine circovirus type 2 (PCV-2), porcine productive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), transmissible gastroenteritis virus (TGEV), rotavirus (RV), porcine parvovirus (PPV), and porcine pseudorabies virus (PRV) – were obtained from the Animal Medical Center DBN Technology Group.

Clinical samples from the small intestine were collected from 14 diseased piglets in Hebei. Clinical samples were collected in accordance with the International Guiding Principles for Biomedical Research Involving Animals.

DNA and RNA were extracted from the viruses and clinical samples using a TIANamp Virus Genomic DNA/RNA Kit (Beijing Tiangen Biotech Company, Beijing, China) according to the manufacturer's instructions. cDNA synthesis was performed using TranScript First-Strand cDNA Synthesis SuperMix (Beijing TransGen Biotech Company, Beijing, China). The DNA and cDNA samples were stored at -20 °C.

Primer design and preparation of the recombinant plasmid

Primer Premier 5.0 software was used to design the PEDV nanoparticle-assisted RT-PCR primers. The full-length ORF3 genes of the PEDV vaccine strain (CV777) and wild strain (JSTS2012) were chosen based on published sequences in the GenBank database (GenBank accession numbers: GU372744 and KC161198) (Table 1). The amplified products of primers were 264 bp in size for the field strain and 215 bp in size for the attenuated vaccine strain. The sequences of the ORF3 gene were inserted into the vector pMD19-T (TaKaRa Biotechnology Company, Dalian, China) as standards. The recombinant plasmids were amplified in *E. coli* DH5α and purified using an AxyPrepTM Plasmid Miniprep Kit (Axygen Biotechnology Company, Hangzhou, China). The plasmids were kept at -20 °C.

Optimization of the conditions for PEDV nanoparticle-assisted RT-PCR

A Nano PCR Kit (NPK02) was purchased from GREDBIO (Weihai, China). The nanoparticle-assisted RT-PCR system was used according to the manufacturer's instructions. The annealing temperature and primer concentration were optimized for the nanoparticle-assisted RT-PCR assay. The annealing temperature ranged from 48 to 56 °C. The forward and reverse primers (E1 and E2 at 10 μM) were tested at volumes ranging from 0.2 to 1.6 μL in increments of 0.2 μL. The cycling conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 48 to 56 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. Amplified products were analyzed on 2 % agarose gels.

Analysis of the sensitivity and specificity of the PEDV nanoparticle-assisted RT-PCR assay

To assess the sensitivity of the method compared with conventional PCR, the recombinant plasmids containing inserts from PEDV vaccine strains were quantified and serially diluted tenfold from 7.30×10^9 to 7.30×10^1 copies/μL. Plasmids containing inserts from PEDV field strains were quantified and serially diluted tenfold from 6.10×10^9 to 6.10×10^1 copies/μL. Each diluted sample was used as a template and was tested using the optimized nanoparticle-assisted RT-PCR assay reaction parameters. The conventional RT-PCR assay was performed using the

Table 1 Primers used in this study

Virus	Gene	Primer	Primer sequence (5′-3′)	Product size (bp)
PEDV	ORF3	P1	5′-TAGACAAGCTTCAAATGTGAC-3′	264 (field strains)
		P1	5′-GTATTAAAGATAATAAAGAGCGC-3′	215 (attenuated strains)

same primers and reaction parameters. Amplified products were then analyzed on 2 % agarose gels.

DNA or cDNA samples from the following viruses were used to evaluate the specificity of the nanoparticle-assisted RT-PCR assay: PPV, PCV2, PRRSV, CSFV, RV, TGEV, and ?PRV?. Recombinant plasmids were used as positive controls. Amplified products were analyzed on 2 % agarose gels.

PEDV clinical sample detection

Clinical samples were also tested using the nanoparticle-assisted RT-PCR assay. The samples of small intestine from 14 piglets from Hebei were tested. Amplified products were analyzed on 2 % agarose gels.

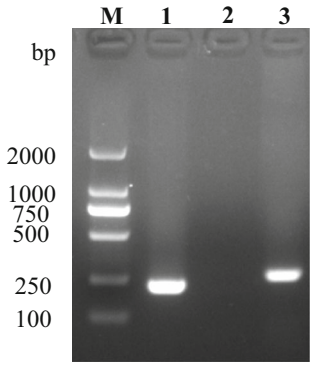

Fig. 1 The size of the product of nano PCR amplification of the target gene, analysed by agarose gel electrophoresis. M, DL2000 marker; 1, attenuated vaccine strain ZJ08; 2, negative control; 3, The field strain HEBEI08

Results

Optimizing the nanoparticle-assisted RT-PCR assay

Recombinant plasmids were used as templates in this nanoparticle-assisted RT-PCR assay. First, the optimal annealing temperature was determined and found to be 51 °C. Then, the optimal annealing temperature was used to determine the optimal primer volume, and the best volume of upstream and downstream primers (10 μM) was 0.8 μL. The final 20-μL reaction system included 10 μL of 2× nano buffer, 0.8 μL each of the upstream and downstream primers (10 μM), 0.5 μL of Taq DNA polymerase (5 U/μL), 1 μL of template DNA, and ddH_2O to 20 μL. The PCR reaction conditions were as follows: 94 °C for 5 min, followed 30 cycles of 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. The final amplification (Fig. 1) could clearly distinguish the field and vaccine strains. Sequence analysis showed approximately 99 % sequence identity between the products of nanoparticle-assisted RT-PCR amplification and the reference sequence of PEDV. The vaccine strain ZJ08 had an obvious sequence deletion in ORF3 gene.

Sensitivity of the nanoparticle-assisted RT-PCR assay

The sensitivity of the nanoparticle-assisted RT-PCR assay was compared with that of the conventional RT-PCR by using a dilution series of recombinant plasmids as the amplification templates. The detection limit of the nanoparticle-assisted RT-PCR for the attenuated strain was 7.30×10^5 copies/μL (Fig. 2A), and that of the conventional RT-PCR assay was 7.30×10^6 copies/μL (Fig. 2B). For the field strains, the detection limit of the nanoparticle-assisted RT-PCR was 6.10×10^4 copies/ μL (Fig. 2C), and that of the conventional RT-PCR assay was 6.10×10^6 copies/ μL (Fig. 2D). These results indicated that the nanoparticle-assisted RT-PCR assay was 10-100 times more sensitive than the conventional RT-PCR.

Specificity of the nanoparticle-assisted RT-PCR assay

The specificity of the upstream and downstream primers was evaluated using other viruses, and the amplification results showed that there was no cross-reaction (Fig. 3), indicating that the assay was specific.

Use of the nanoparticle-assisted RT-PCR assay to detect PEDV cDNA in clinical samples

Fourteen pig diarrhea clinical samples in total tested positive in the nanoparticle-assisted RT-PCR assay (Fig. 4). The amplification products of four samples had the same size as that of the PEDV vaccine strain, and PEDV field strains were detected in nine samples. One sample was found to contain both the PEDV vaccine strain and a field strain. Since the farm animals were immunized with the inactivated PEDV vaccine, we had reason to believe that

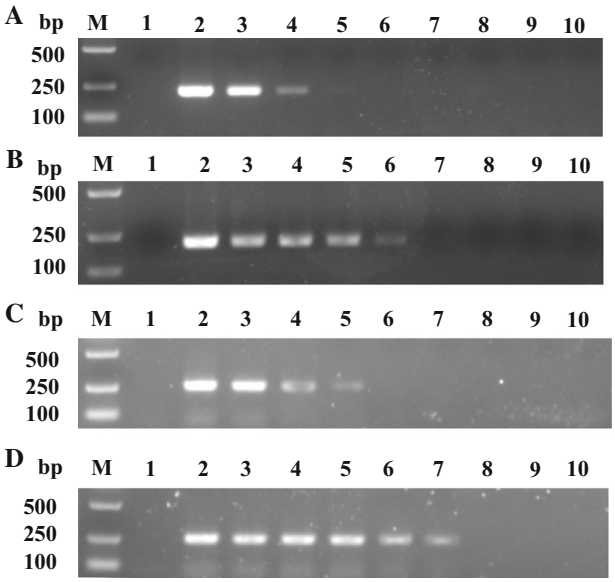

Fig. 2 Sensitivity of the conventional RT-PCR assay (A) and nanoparticle-assisted RT-PCR assay (B) for detection of the attenuated vaccine strain ZJ08. A serial tenfold dilution was used and analyzed by agarose gel electrophoresis. M, DL2000 marker; 1, negative control; 2, 7.30×10^9 copies/μL; 3, 7.30×10^8 copies/μL; 4, 7.30×10^7 copies/μL; 5, 7.30×10^6 copies/μL; 6, 7.30×10^5 copies/μL; 7, 7.30×10^4 copies/μL; 8, 7.30×10^3 copies/μL; 9, 7.30×10^2copies/μL; 10, 7.30×10^1 copies/μL. Sensitivity of the conventional RT-PCR assay (C) and nanoparticle-assisted RT-PCR assay (D) for detection of the field strain HEBEI08. A serial tenfold dilution was used and analyzed by agarose gel electrophoresis. M, DL2000 marker; 1, negative control; 2, 6.10×10^9 copies/μL; 3, 6.10×10^8 copies/μL; 4, 6.10×10^7 copies/μL; 5, 6.10×10^6 copies/μL; 6, 6.10×10^5 copies/μL; 7, 6.10×10^4 copies/μL; 8, 6.10×10^3 copies/μL; 9, 6.10×10^2 copies/μL; 10, 6.10×10^1 copies/μL

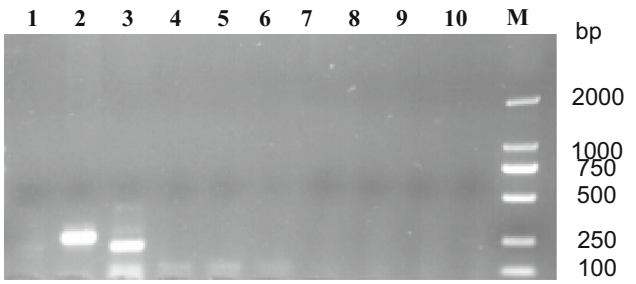

Fig. 3 Specificity of the nanoparticle-assisted RT-PCR assay. M, DL2000 marker; 1, negative control; 2, field strain HEBEI08; 3, attenuated vaccine strain ZJ083: 4, PCV-2; 5, PRRSV; 6, PPV; 7, PRV; 8, PRoV; 9, CSFV; 10, TGEV

they were infected with PEDV after immunization. The results showed that the nanoparticle-assisted RT-PCR assay could distinguish PEDV field strains from the attenuated strain in samples from infected pigs.

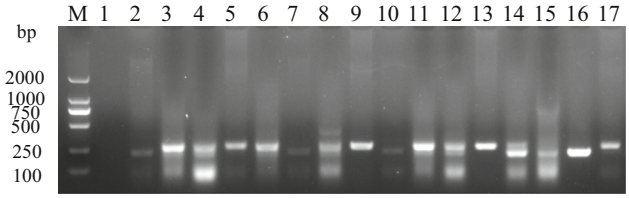

Fig. 4 PEDV gene fragments amplified by nanoparticle-assisted RT-PCR from clinical samples. M, DL2000 marker; 1, negative control; 2, 7, 10, 15, attenuated vaccine strain; 3, 4, 5, 6, 8, 9, 11, 12, 13, field strain; 14, mixture of the field and attenuated vaccine strain; 16, attenuated vaccine strain ZJ08; 17, field strain HEBEI08

Discussion

PEDV causes serious disease and economic loss in the pig industry [21]. Inactivated vaccines are effective for controlling the disease. However, PEDV has been found to appear in immunized pigs since 2006 [21]. Since 2010, PEDV outbreaks have led to 100 % mortality in newborn piglets throughout southern China, during which time more than 1 million pigs have died [22]. In diarrheal piglets, the positive rate for PEDV is significantly higher than that for porcine transmissible gastroenteritis virus and porcine rotavirus [5, 23], suggesting that PEDV is the main pathogen causing the diarrhea in China.

Attenuated strains are applied for vaccination in many Asian countries, including China. The traditional identification methods may not provide accurate distinction of the attenuated vaccine strain for the field strain. The nanoparticle-assisted RT-PCR assay has been used for rapid and accurate detection of other pig viruses [24–26]. The present study demonstrated that an effective nanoparticle-assisted RT-PCR assay targeting the ORF3 gene of PEDV was able to distinguish field strains from attenuated vaccine strains. This method was more sensitive than the conventional RT-PCR assay and suitable for testing clinical samples with low viral loads. The assay was specific for PEDV, exhibiting no cross-reactivity against other viruses. The method could effectively distinguish the PEDV field strains and the attenuated vaccine strains in the clinical samples. When testing clinical samples, in one case, a vaccine strain was found to coexist with a virulent strain. The reason for this phenomenon might be the failure of vaccine immunization, suggesting that there were potential threats of PEDV infection after vaccination. In addition to this, there are concerns about the safety of the live attenuated PED vaccine for use in pregnant gilts and sows because a live attenuated vaccine virus might be able to revert to virulence under field conditions.

The application of the nanoparticle-assisted RT-PCR amplification technique is reported here for the first time to distinguish PEDV field strains from vaccine strains. This approach will facilitate the identification of piglets that are infected with PEDV field strains, the detection of latent infections, and the prevention of PEDV outbreaks.

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Compliance with ethical standards

Conflict of interest None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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