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Note

Establishment of method for dual simultaneous detection of PEDV and TGEV by combination of magnetic micro-particles and nanoparticles☆

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ABSTRACT

Transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) are the main pathogens causing viral diarrhea in pig, mixed infections of these two viruses are very common in intensive pig rearing. However, there is a lack of a method to simultaneously detect and distinguish PEDV and TGEV in preclinical levels. In this study, we aimed to establish a dual ultrasensitive nanoparticle DNA probe-based PCR assay (dual UNDP-PCR) based on functionalized magnetic bead enrichment and specific nano-technology amplification for simultaneous detection and distinguish diagnosis of PEDV and TGEV. The detection limit of dual UNDP-PCR for single or multiple infections of PEDV and TGEV is 25 copies/g, which is 400 times more sensitive than the currently known duplex RT-PCR, showing better specificity and sensitivity without cross-reaction with other viruses. For pre-clinical fecal samples, the dual UNDP-PCR showed a markedly higher positive detection rate (52.08%) than conventional duplex RT-PCR (13.21%), can rapidly and accurately identify targeted pathogens whenever simple virus infection or co-infection. In summary, this study provides a technique for detecting and distinguishing PEDV and TGEV in preclinical levels, which is high sensitivity, specificity, repeatability, low cost and broad application prospect.

In recent years, with the rapid development of large-scale pig industry, the number of cases of swine viral diarrhea has shown a sharp increasing trend, causing huge economic losses to pig farmers, which serious hinders the healthy and orderly development of the pig industry. Among them, porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV) are the main pathogens causing viral diarrhea in piglets. TGEV and PEDV, as the main members of the coronavirus family, can cause highly contagious intestinal infections in piglets [1]. Because transmissible gastroenteritis (TGE) and porcine epidemic diarrhea (PED) show great similarities in clinical symptoms, pathological changes and epidemiology, therefore, it is difficult to diagnose and distinguish one from another only depending on clinical features and histopathology. If there is not a timely method to control their infection, TGEV and PEDV will spread rapidly across the whole swine farm. At present, the prevention and control of TGE and PED are mainly carried out from two aspects: vaccination and purification of the farm environment. Among them, early detection is the key to decontamination of the farm. Therefore, to establish a pre-clinical laboratory detection technology with high sensitivity and specificity for these two pathogens is urgently needed, which will be of great significance for the pre-clinical diagnosis to purify the environment as soon as possible, preventing large-scale infection and reducing economic losses.

Porcine epidemic diarrhea (PED), one of the most severe and globally widespread infectious diseases in all ages of swine is caused by the porcine epidemic diarrhea virus (PEDV) [2]. As a member of the coronaviridae family, PEDV infects the epithelial cells mainly of the porcine intestine, leading to acute diarrhea, vomiting, and dehydration, which cause the high morbidity and mortality in newborn piglets [3]. Pigs of all ages can be infected with PEDV, and the severity of clinical symptoms is inversely related to the age of the pigs. Generally, the younger pigs, especially suckling piglets show more higher incidence rate and severe

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symptoms after infection [4]. For PEDV, once the infection reaches a certain level, it will spread in the whole pig farm and into surrounding areas if the prevention is not timely [5]. According to recent reports, PED is widely spread around the world, and the disease has spread to China, Japan, South Korea, Germany, Belgium, France and other countries or regions, the prevalence and incidence of the disease in Asia is far more serious than that in Europe [6].

Transmissible gastroenteritis (TGE) is a highly-contact enteric infectious disease. Pigs of all ages are the main susceptible host of the disease, but the incidence of piglets within 10 days of age generally show a high mortality rate [7]. When healthy pigs are exposed to air, drinking water, feed, utensils, etc. contaminated by viruses, TGEV easily enters the piglets from the outside through the respiratory or digestive tract, eventually reaches the small intestine of the target organ, and proliferates in the intestinal epithelial cells, causing the small intestine villi to shrink or even fall off [8].

At present, the main laboratory testing techniques for detecting PEDV and TGEV include: isolation and identification of viruses, serological assays, immunological and molecular biology methods. However, the method of isolation and identification of viruses has a long period of time and the process is cumbersome, the serological assays have low detection sensitivity and cannot be early warning, the cost of immunological methods is high, the detection range is limited, and the application range is not extensive. In addition, the molecular biology methods such as duplex RT-PCR are inconvenient to operate and costly, which required the process of viral nucleic acid extraction, purification and reverse transcription. Therefore, it is an urgent need to develop new detection methods with the characteristics of high sensitivity, high specificity and low cost [9–11]. In this study, dual ultrasensitive nanoparticle DNA probe-based PCR (dual UNDP-PCR) assay for both PEDV and TGEV uses magnetic beads and gold nanoparticles to improve the detection sensitivity, and uses specific probes and labels to reduce the probability of false positives; meanwhile, the whole operation does not require reverse transcription and professional research personnel, which save the detection cost.

In our previous research, we have separately established detection methods for PEDV or TGEV. The principle of magnetic particle enrichment and specific nanoprobe amplification has achieved a strong specificity and convenient operation [12,13]. However, it is inconvenient to detect and distinguish these two viruses at same time using our previous PEDV or TGEV specific detection method. In this study, we further established a dual UNDP-PCR assay for both PEDV and TGEV, which effectively solves this problem, and can simultaneously and rapidly detect and distinguish TGEV and PEDV in the same reaction system at the preclinical level.

To test the sensitivity of dual UNDP-PCR assay for PEDV and TGEV, the field samples containing PEDV and TGEV were diluted serially from 10^5 to 1 copies/gram respectively. The diluted samples containing same viral copy numbers of PEDV and TGEV per g were mixed, and then tested by conventional duplex RT-PCR, and specific probe-coated magnetic microparticles (MMPs) and specific probe barcode-coated Gold nanoparticles (Au-NPs)-based dual UNDP-PCR. The inter-assay and intra-assay tests were carried out in triplicate by detecting three different concentrations of mixed field containing serial diluted PEDV and TGEV (10^4, 10^3, 10^2 copies/g) to measure the reproducibility of this assay. In the study of evaluating the specificity of dual UNDP-PCR, Porcine parvovirus (PPV), Porcine circovirus type 2 (PCV2), Pseudorabies virus (PRV), Porcine reproductive and respiratory syndrome virus (PRRSV) and Classical swine fever virus (CSFV) were tested by the uniformed detection method.

Fecal samples were collected from epidemic farms with diseased pigs were detected for PEDV and TGEV have been detected by dual UNDP-PCR and conventional duplex RT-PCR, statistics and analysis of the detection results of the collected fecal samples to calculate the positive rate of single virus infection and mixed infection detected. In addition, the sensitivity of clinical application of these two methods was compared by comparing the positive detection rates of pre-clinical samples and clinical samples. The samples with inconsistent detection results of dual UNDP-PCR and conventional duplex RT-PCR were re-examined, and the results were verified by separation and purification. Consequently, the sensitivity of dual UNDP-PCR assay can be demonstrated further.

Serial 10-fold dilutions of template in samples were tested to assess the sensitivity. PEDV and TGEV genomic RNA were released by boiling with lysis buffer containing RNase inhibitors, then were used to form AuNP-RNA-MMP complexes, mixed by magnetic separation and oligonucleotide elution. The oligonucleotides were then purified and detected by UNDP-PCR. As shown in Fig. 1A, visible targeted bands around 628 bp and 501 bp could be seen in lanes representing fecal samples with viral concentrations ranging from 10^3 copies/g to 25 copies/g respectively. However, at least 10^4 copies/g of TGEV and PEDV in samples, visible targeted bands around 451 bp and 275 bp could be seen in lanes when detected by conventional duplex RT-PCR assay (Fig. 1B), suggesting that the sensitivity of dual UNDP-PCR specific for PEDV and TGEV was 400-fold that of the conventional duplex RT-PCR.

Inter-assay and intra-assay test were executed to estimate the reproducibility of the dual UNDP-PCR method, triplicates of each concentration (10^4 copies/gram, 10^3 copies/gram, 10^2 copies/gram) by three independent tests for three consecutive days. The results of the independent triplicates assay showed highly consistency as our expected. (Fig. 2A).

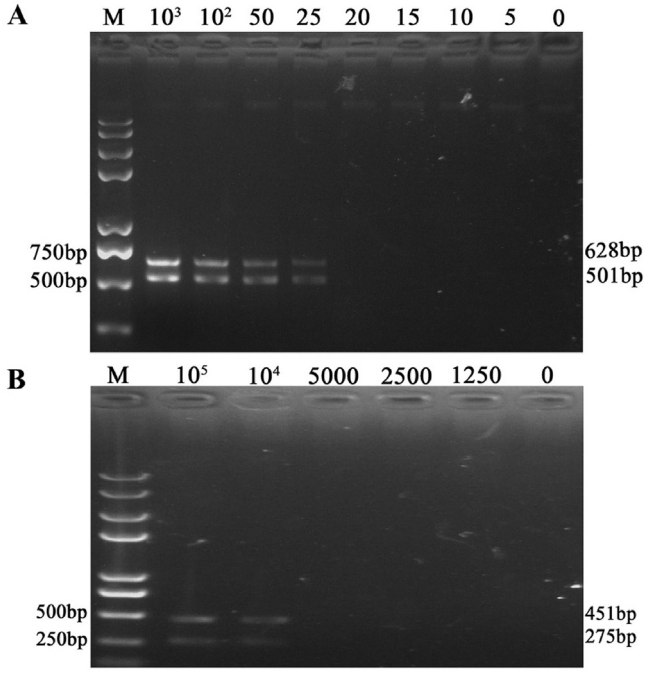

Fig. 1. Analysis of the sensitivity of dual UNDP-PCR for PEDV and TGEV. (A) Serial dilutions of PEDV and TGEV fecal samples were tested by dual UNDP-PCR assay. M: Trans 2K Plus DNA Marker; other lanes represent different viral concentration of fecal samples were detected by UNDP-PCR assay. 10^3:10^3 copies/g; 10^2: 10^2 copies/g; 50: 50 copies/g; 25: 25 copies/g; 20: 20 copies/g; 15: 15 copies/g; 10: 10 copies/g; 0: negative samples. (B) Serial dilutions of PEDV and TGEV fecal samples were tested by duplex RT-PCR assay. M: Trans 2K Plus DNA Marker; other lanes are the viral concentration of fecal samples. 10^5:10^5 copies/g; 10^4: 10^4 copies/g; 5000: 5000 copies/g; 2500: 2500 copies/g; 1250: 1250 copies/g; 0: negative samples.

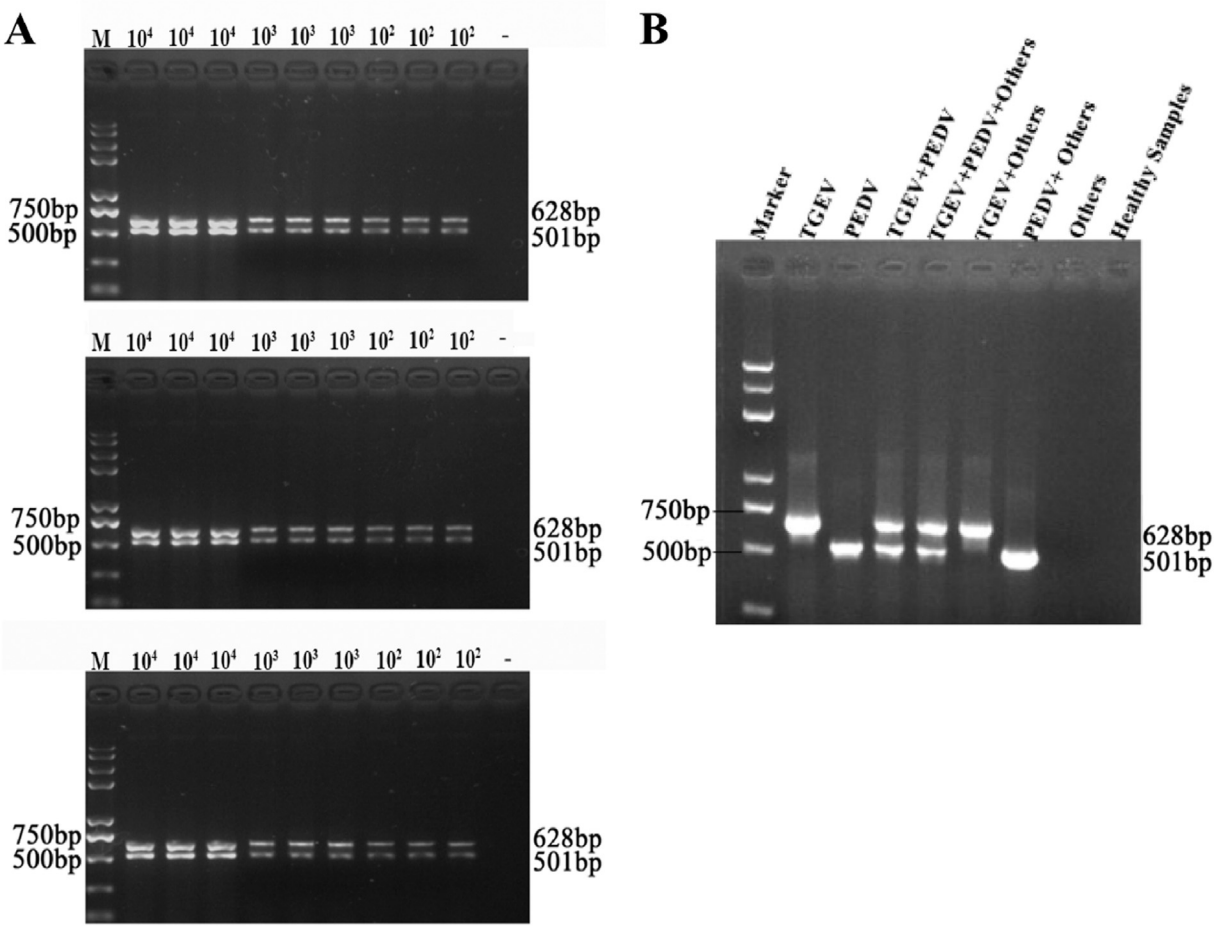

Fig. 2. Analysis of the reproducibility and specificity of dual UNDP-PCR for PEDV and TGEV. (A)Three repeated test of each concentration in three independent assays for three consecutive days were detected by dual UNDP-PCR for PEDV and TGEV. M: Trans 2K Plus DNA Marker; other lanes are the viral concentration of fecal samples. 10^4: 10^4 copies/g; 10^3: 10^3 copies/g; 10^2: 10^2 copies/g; -: negative samples. (B)Samples infected with PPV, PRV, PCV2, CSFV, PRRSV and healthy samples were detected by dual UNDP-PCR for PEDV and TGEV as control. M:Trans 2K Plus DNA Marker; other lanes indicate samples contained with different viruses or healthy samples, among them "Others" represents the sample contained with PPV, PRV, PCV2, CSFV and PRRSV.

The specificity of the dual UNDP-PCR assay for PEDV and TGEV was measured by fecal samples collected from healthy pigs and others infected with PPV, PRV, CSFV, PCV2 or PRRSV. As our predicted, specific PCR products with bands of 628 bp and 501 bp were detected in the sample with PEDV and TGEV whenever other viruses exist or non-exist. As shown in Fig. 2B, the result demonstrated that the detection results of TGEV and PEDV do not interfere with each other and were not disturbed by other viruses, revealing the specificity and independence of the established dual UNDP-PCR for PEDV and TGEV.

Fecal samples from epidemic farms with diseased pigs were detected for PEDV and TGEV using dual UNDP-PCR assay and conventional duplex RT-PCR. As shown in Table 1, using conventional duplex RT-PCR detection found 53 positive samples and 219 negative samples, while using dual UNDP-PCR assay detection found 96 positive samples and 176 negative samples among 272 samples; the detection positive rate of dual UNDP-PCR assay (35.29%) is markedly higher than that of conventional duplex RT-PCR (19.49%). We found 46 fecal sample were collected from the piglets with clinical diarrhea symptoms in all of 53 positive samples detected by conventional duplex RT-PCR, while 50 fecal sample were collected from the piglets without apparent clinical symptoms in all of 96 positive samples detected by dual UNDP-PCR assay. Notably, all of 53 positive samples detected by conventional duplex RT-PCR were also positive in dual UNDP-PCR assay, and TGEV positive rate (9.43%), PEDV positive rate (56.61%) and double positive rate of co-infection (33.96) were completely identity when these 53 positive samples were detected by dual UNDP-PCR assay

Table.1
Comparison of the detection results of 272 fecal samples collected tested by dual UNDP-PCR and conventional duplex RT-PCR.

Assay	Stage	Number of each component	Rate of each component (%)	Total number of tested samples
dual UNDP-PCR	pre-clinical positive	50	18.38	272
	clinical positive	46	16.91	
	Negative	176	64.71	
duplex RT-PCR	pre-clinical positive	7	2.57	272
	clinical positive	46	16.91	
	Negative	219	80.52	

and conventional duplex RT-PCR (Table S3). These results suggested that dual UNDP-PCR assay not only possesses great diagnostic accuracy as well as conventional duplex RT-PCR, but showed a higher positive detection rate than conventional duplex RT-PCR.

For 43 positive samples, 3 samples were TGEV positive, 27 samples were PEDV positive, 13 samples were double positive, which were further confirmed by the amplification of isolated pathogens. These data further show the higher sensitivity of dual UNDP-PCR assay, and demonstrate that dual UNDP-PCR assay can rapidly and accurately identify targeted pathogens from preclinical samples whenever single virus infection or co-infection.

Currently, although single or dual RT-PCR assays for TGEV and PEDV viruses have been established, they still undergo complex processes of viral nucleic acid extraction and RNA reverse transcription [14,15]. In addition, traditional RT-PCR methods can not detect the presence of low-concentration viruses in the early stages of infection. Therefore, the virus infection could not be discovered in time, and the virus began to spread rapidly throughout the piggery, causing serious harm to the pig industry. With the development of nanotechnology, nanoparticles have been widely used in pathogen detection. In this study, based on nanoparticle and DNA tags, we established a dual UNDP-PCR technology to simultaneously detect and distinguish TGEV and PEDV in the same system. This combined method not only greatly shortens the detection time and reduces the detection cost, but also show reliable reproducibility, specificity and higher sensitivity than the conventional duplex RT-PCR.

In summary, compared with traditional virus detection methods, the advantages of dual UNDP-PCR are mainly reflected in the following aspects: first of all, compared with viral isolation and identification method and other serological methods, this method does not require cell culture and antibody production, saving testing costs and labor. Secondly, compared with the duplex RT-PCR technology, real-time RT-PCR and other molecular biology methods, dual UNDP-PCR saves detection time and testing costs by eliminating the need for process of viral nucleic acid extraction, purification and reverse transcription. Thirdly, based on the two kinds of special gold nanoparticles and functionalized magnetic beads, dual UNDP-PCR allows simultaneous preclinical testing of TGEV and PEDV in low-dose viral stool samples and distinguishes each other in one test, which is more convenient and quick than the single UNDP-PCR assay established previously. In addition, the experimental results show that the method can detect 25 copies/g of TGEV and PEDV with 400 times the sensitivity of traditional duplex RT-PCR. Finally, in terms of its specificity, specific nucleic acid probes for labeling magnetic particles and DNA labels for modifying nano-gold particles are two completely different specific sequences designed based on the conserved region of the viral gene, and by evaluating their ability to capture viral nucleic acids optimized screening for specificity. In the clinical sample application, the detection technology established in this study can easily identify whether it is pure PEDV or TGEV infection or mixed infection, which provides a basis for clinical medication and early control of infection to reduce the economic loss of pig farms.

In conclusion, the dual UNDP-PCR assay established in this study allows simultaneous detection of TGEV and PEDV in large-scale fecal samples in the same reaction system without the need for viral nucleic acid extraction, purification and reverse transcription. This method can markedly improve the current detection technological ability in preclinical phase, achieving early diagnosis and early prevention, thus reducing the morbidity and mortality of TGE and PED in newborn piglets. In summary, dual UNDP-PCR is a fast and economical assay with high specificity, sensitivity and repeatability.

Ethics statement

All animal procedures and study design were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006), and were approved by the animals ethics committee of Northwest A&F University.

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Conflict of interests

The authors have no financial conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jiac.2020.01.008.

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