

A nanoparticle-assisted PCR assay for the detection of encephalomyocarditis virus

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ABSTRACT

The encephalomyocarditis virus (EMCV) can cause acute myocarditis in young pigs or reproductive failure in sows. Diseases caused by EMCV currently affect the swine industry worldwide. In this study, a nano polymerase chain reaction (nanoPCR) assay, targeting the 3D gene of EMCV, was developed and its sensitivities and specificities were investigated. The results indicated that the nanoPCR assay is highly sensitive and able to detect 1.2×10^2 copies/ μ L of EMCV RNA, as no cross-reaction was observed with other viruses. This is the first report to demonstrate the application of a nanoPCR assay for the detection of EMCV. The sensitive, and specific nanoPCR assay developed in this study can be applied widely in clinical diagnosis and field surveillance of EMCV-infection.

Key words: encephalomyocarditis virus, nanoPCR, RT-PCR, detection

Introduction

The encephalomyocarditis virus (EMCV) is a member of the genus *Cardiovirus* of the family *Picornaviridae*, and has worldwide distribution (MINOR et al., 1995). The virus was first isolated from pigs in Panama in 1958 (MURNANE et al., 1960), and EMCV has been recognized worldwide as a pathogen that can infect several host species, including pigs, rodents, cattle, elephants, raccoons, marsupials, and primates, such as baboons,

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monkeys, chimpanzees, and even humans (AN et al., 2009; GELMETTI et al., 2006; KRYLOVA and DZHIKIDZE, 2005; SPYROU et al., 2004). For pigs, EMCV isolates induce myocardial or reproductive failure, or both (KOENEN and VANDERHALLEN, 1997). In China, EMCV infection has been confirmed in several pig farms by etiology and serology (GE et al., 2010; LIN et al., 2012; YUAN et al., 2014a).

The current methods of detecting EMCV include virus isolation, serology, reverse transcription-polymerase chain reaction (RT-PCR), reverse transcription loop-mediated isothermal amplification (RT-LAMP), and TaqMan-based real-time RT-PCR (GE et al., 2007; JIA et al., 2008; YUAN et al., 2014b; YUAN et al., 2014c). No nanoPCR assay, however, has been developed for EMCV. Relative to the conventional PCR assay, a nanoPCR assay has the potential for increased sensitivity and specificity. NanoPCR is an advanced form of conventional PCR in which solid gold nanometal particles (1-100 nm) form colloidal nanofluids, which increase thermal conductivity. Therefore, PCR assays with nanofluids reach the target temperature more quickly than PCR assays with original liquids, and this reduces the time at non-target temperatures, thereby reducing non-specific amplification and increasing specific amplification (LI and ROTHBERG, 2004; SHEN et al., 2009). In addition, the nanoPCR assay does not require specialized instruments beyond standard PCR equipment (MA et al., 2013).

In this study, we developed a highly sensitive and specific nanoPCR method to target the 3D gene for the rapid detection of EMCV in clinical specimens.

Materials and methods

Virus and sample preparation. The EMCV BD2 strain used for the study was isolated and identified in the author's laboratory (YUAN et al., 2014a). BHK-21 cells used for virus propagation were maintained in GIBCO™ Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Auckland, NY), supplemented with 10 % fetal bovine serum (FBS; Hyclone Laboratories Inc., South Logan, UT) at 37 °C under 5 % CO₂. The Nano PCR Kit (NPK02) was purchased from GREDBIO (Weihai, China)

Design of primers. The genome of EMCV consists of a large open reading frame (ORF). The 3D gene is highly conserved. Primers were selected and designed from the conserved 3D gene, using Primer premier 5 software to generate a 425 bp amplicon (Table 1).

Table 1. Primers used in nanoPCR assay for Encephalomyocarditis Virus

Type	Sequence (5'-3')	Position ^a
Forward	CAG AGG CTG ATG TAG ATG AAG TGG C	6357-6381
Reverse	CAG AAT GCA ATG CTC AAA TGG TGG A	6757-6781

^aNumbers represents the nucleotide position within the genome of BD2 (GenBank accession number: KF709977.1)

RNA extraction. Viral RNA was extracted from 150 μL of supernatant from virus-infected BHK-21 cells or tissue samples, using the RNA extraction kit (Qiagen Inc., USA) following the manufacturer's instructions. The extracts were resuspended in 20 μL of distilled water, aliquoted and stored at $-80\text{ }^{\circ}\text{C}$ before nanoPCR amplification was carried out.

NanoPCR assay. The RNA was extracted as described above. RNA was used as a template and serially diluted 10-fold in 10 mM Tris-EDTA buffer (pH 8.0) to produce 5.7 to 5.7×10^{-6} ng/ μL . The NanoPCR assay was performed using EMCV-specific primers (Table 1). Briefly, 11 μL RNA extracted from either virus stocks or clinical samples was transcribed into cDNA, using 1 μL (10 μM) of P2 (5'-CAG AAT GCA ATG CTC AAA TGG TGG A-3') primer in a 20- μL reaction volume. The reverse transcription (RT) reaction was performed at $42\text{ }^{\circ}\text{C}$ for 50 min. The cDNA was amplified in a 25 μL reaction mixture containing 1 μL cDNA, 12.5 μL nanoPCR Mixture (GREDBIO, Weihai, China), 0.5 μL Taq DNA polymerase (GREDBIO, Weihai, China), and 10 μM each of forward and reverse primers by following the manufacturer's protocol, with the following cycling times and temperatures: $94\text{ }^{\circ}\text{C}$ for 3 min and 30 cycles of $94\text{ }^{\circ}\text{C}$ for 30 sec, $56\text{ }^{\circ}\text{C}$ for 30 sec, and $72\text{ }^{\circ}\text{C}$ for 25 sec. Five microliters of nanoPCR product was analyzed by agarose gel electrophoresis and subjected subsequently to automated sequencing reactions (Invitrogen, Beijing, China).

Sensitivity of nanoPCR. To examine the sensitivity of nanoPCR for EMCV amplification, RT-PCR, and nanoPCR reaction were conducted using various concentrations of EMCV RNA as template. The RNA was quantified by NanoDrop 1000 (Thermo Scientific, USA) and was diluted serially 10-fold from 1.2×10^7 to 1.2×10^1 copies/ μL as template for the two methods. NanoPCR were performed using the optimized reaction parameters. RT-PCR was performed using the same primers and reaction parameters as nanoPCR. Five microliters of RT-PCR products were analyzed by agarose gel electrophoresis. The size of the EMCV fragment amplified by RT-PCR was 425 bp.

Specificity of nanoPCR. Reactions with different viruses, including EMCV, classical swine fever virus (CSFV), porcine enterovirus (PEV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine teschovirus (PTV), and porcine epidemic diarrhea virus (PEDV) were performed to determine the specificity of the nanoPCR assay. PEV, PRRSV, PTV, and PEDV were identified by our laboratory. CSFV was purchased from the Chinese preservation center for veterinary microorganisms.

Clinical specimens. Clinical specimens were collected from different pig farms in the Hebei province. The samples included sera, and the tonsils of diseased pigs. Collecting clinical specimens met the International Guiding Principles for Biomedical Research Involving Animals. Tissue samples were homogenized and centrifuged at $4000 \times g$ for 15 min to obtain a cell-free supernatant. The sample RNAs were extracted as described in

the section “RNA extraction”. The nanoPCR were performed as described in the section “NanoPCR assay”. Conventional PCR was performed simultaneously.

Results

The optimization of the nanoPCR reaction was performed by evaluating different concentrations of components and cycling conditions using the EMCV BD2 strain. Primers were titrated to determine optimum concentrations, and different annealing and data acquisition temperatures were also evaluated (data not shown). The optimized reaction parameters are given in Table 2. With these parameters, EMCV RNA was amplified adequately by nanoPCR (Fig. 1). The sequence analysis showed high similarity (100 %) between the products obtained with the nanoPCR amplification of the 3D gene of EMCV (the objective sequences) and the reference sequence of EMCV. The results indicated that the nanoPCR method for the detection of EMCV is specific.

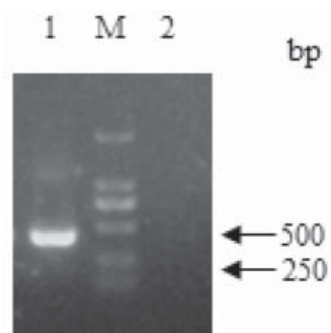


Fig. 1. Data graph of the nanoPCR assay for EMCV detection. M: DL2000; Lane 1: EMCV; Lane 2: negative control

Table 2. Optimized parameters for nanoPCR reaction

Component (concentration)	Volume (μL) per 25 μL reaction
nanoPCR Mixture (2 \times)	12.5
Forward primer (10 μm)	1.0
Reverse primer (10 μm)	1.0
cDNA/DNA	2.0
Taq DNA polymerase (5 U/ μL)	0.5
dd H ₂ O	8.0

The sensitivity of nanoPCR assay was evaluated by testing 10-fold serial dilution of RNA templates (1.2×10^7 to 1.2×10^1 copies/ μL). The detection limit of nanoPCR was 1.2×10^2 copies/ μL , whereas that of RT-PCR was 1.2×10^4 copies/ μL (Fig. 2). Comparisons between the nanoPCR and RT-PCR amplifications indicated that nanoPCR is 100-fold more sensitive than RT-PCR. The specificity of the nanoPCR assay was evaluated using other animal viruses, and a water negative control. The nanoPCR assay amplified EMCV but none of the other viruses (Fig. 3).

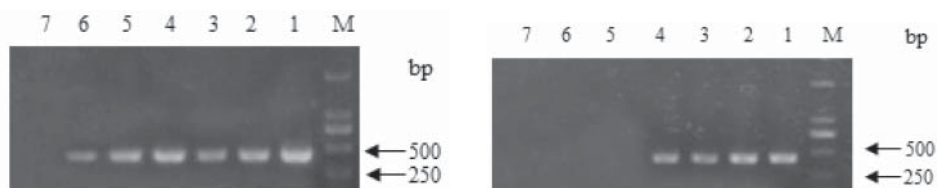


Fig. 2. The sensitivities of nanoPCR and RT-PCR assays. Serial 10-fold dilution of EMCV RNA derived from BD2 strain was used in the nanoPCR and RT-PCR assays. (A) Analysis of nanoPCR assay by agarose gel electrophoresis. (B) Analysis of the RT-PCR assay by agarose gel electrophoresis. M: DL2000; Lane 1: 1.2×10^7 copies/ μL ; Lane 2: 1.2×10^6 copies/ μL ; Lane 3: 1.2×10^5 copies/ μL ; Lane 4: 1.2×10^4 copies/ μL ; Lane 5: 1.2×10^3 copies/ μL ; Lane 6: 1.2×10^2 copies/ μL ; Lane 7: 1.2×10^1 copies/ μL . All experiments were repeated three times and similar results were obtained.



Fig. 3. Specific evaluation of the nanoPCR assay for the detection of EMCV. The specificity of the EMCV nanoPCR assay was determined by testing EMCV and five other viruses including PRRSV, PEV, CSFV, PTV, and PEDV. The control reaction using DEPC-treated water as template was also included. M: DL2000; Lane 1: PRRSV; Lane 2: PEV; Lane 3: CSFV; Lane 4: PTV; Lane 5: PEDV; Lane 6: negative control.

NanoPCR assay and conventional PCR were performed simultaneously on 100 clinical samples. The results are shown in Table 3. Eight of 100 samples (8 %) were positive by RT-PCR analysis, whereas 13 of 100 samples (13 %) were positive by nanoPCR (Table 2). Eight samples (8 %) were positive by both methods. Five samples (5 %) were positive by nanoPCR, but negative by RT-PCR. No sample (0 %) was positive by RT-PCR and negative by nanoPCR. The results showed that nanoPCR was more sensitive than the conventional RT-PCR assay.

Table 3. Comparison of nanoPCR and RT-PCR assays for detection of Encephalomyocarditis Virus from clinical samples

Animal	Type of tissue or samples	No. positive/no. tested samples (%)	
		nanoPCR	RT-PCR
Pigs	Serum	6/90	4/90
	Tonsils	7/10	4/10
	Total	13/100	8/100

Discussion

EMCV can cause acute myocarditis in young pigs or reproductive failure in sows. Serological studies have shown the presence of EMCV antibodies in the human population (OBERSTE et al., 2009). It was demonstrated that an EMCV strain, isolated from an aborted swine fetus, productively infected human cardiomyocytes *in vitro* (BREWER et al., 2001). The significance for public health of EMCV is also worthy of concern.

The current methods for detecting EMCV include virus isolation, serology, reverse RT-PCR, RT-LAMP, and TaqMan-based real-time RT-PCR (GE et al., 2007; JIA et al., 2008; YUAN et al., 2014b; YUAN et al., 2014c). Virus isolation is one of the conventional methods, but it is time-consuming and laborious. Serology is the technique used most widely, however, this method has some disadvantages because antibody titers can fall rapidly after infection. In addition, real-time PCR assays require expensive instruments, and LAMP assays are easily contaminated. NanoPCR is an advanced form of PCR in which solid gold nanometal particles (1-100 nm) form colloidal nanofluids, which increase thermal conductivity. Therefore, PCR assays with nanofluids reach the target temperature more quickly than PCR assays with original liquids, reducing the time at non-target temperatures, thereby reducing non-specific amplification and increasing specific amplification (LI and ROTHBERG, 2004; SHEN et al., 2009). In addition, the nanoPCR assay does not require specialized instruments beyond standard PCR equipment (MA et al., 2013). The nanoPCR assay has been used in detecting viral RNA molecules from animals due to its simplicity and high sensitivity, including pseudorabies (MA et al., 2013), and porcine bocavirus (WANG et al., 2014).

In this study, a highly efficient and practical method for the detection of EMCV was established. Since the 3D gene of EMCV is among the most conserved regions and has been chosen as a preferred target region for the detection of EMCV RNA by RT-PCR (JIA et al., 2008; YUAN et al., 2014b), primers were designed to amplify target sequences at the 3D gene region of the EMCV genome for nanoPCR assay. The nanoPCR assay is highly sensitive and able to detect 1.2×10^2 copies/ μL of EMCV RNA, as no cross-reaction was observed with other viruses. The sensitivity of nanoPCR for EMCV was 100-fold greater than RT-PCR. This is the first report to demonstrate the application of a nanoPCR amplification technique for the detection of EMCV.

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SAŽETAK

Virus encefalomiokarditisa može uzrokovati akutni miokarditis u prasadi ili reproduksijske poremećaje u krmača. Poremećaji uzrokovani virusom encefalomiokarditisa prošireni su u svinja u industrijskoj proizvodnji diljem svijeta. U ovom je istraživanju razvijena nano-lančana reakcija polimerazom (nanoPCR) za dokaz gena 3D virusa encefalomiokarditisa te je istražena njezina osjetljivost i specifičnost. Rezultati su pokazali da je nanoPCR visoko osjetljiva metoda kojom se može dokazati $1,2 \times 10^2$ kopija RNA virusa encefalomiokarditisa na μL te da nisu dokazane križne reakcije s drugim virusima. Ovo je prvo izvješće u kojem je dokazana primjena nanoPCR-a za dokaz virusa encefalomiokarditisa. Njezina osjetljivost i specifičnost upućuju na zaključak da se može naširoko rabiti u kliničkoj dijagnostici i terenskim istraživanjima encefalomiokarditisa svinja.

Ključne riječi: virus encefalomiokarditisa, nanoPCR, RT-PCR
