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Novel multiplex PCR assay using locked nucleic acid (LNA)-based universal primers for the simultaneous detection of five swine viruses

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A B S T R A C T

A novel multiplex PCR assay using non-homologous oligonucleotides with locked nucleic acid (LNA) modifications as universal primers was developed and validated for the simultaneous detection of five swine viruses. The assay utilizes five virus-specific primer pairs modified at the 5′ end through the addition of the universal primer sequence. In the reaction, small amounts of target templates with the 5′ tail were generated and subsequently amplified through the extension of a LNA universal primer set. To validate the specificity of this assay, 27 viral target strains and 12 non-target pathogens were tested. The lower limit of detection of viral nucleic acids was 1.1–1.9 pg per reaction or 11–32 pg in a five-plex viral nucleic acid mixture. The LNA mPCR assay displayed higher analytical sensitivity and efficiency for the detection of plasmid standards compared with the conventional assay, which uses standard primers without the 5′ tail. A total of 207 field samples were tested using both assays. The LNA mPCR assay provided numerically higher detection rates for all pathogens in independent samples. Moreover, the LNA mPCR assay had significantly higher detection rates in independent samples compared with the conventional assay.

1. Introduction

Swine infectious diseases are major problems in the pig industry worldwide. Classical swine fever virus (CSFV), porcine circovirus type 2 (PCV2), porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV) and porcine pseudorabies virus (PRV) are common and economically important pathogens that cause reproductive disorders in pigs. PRRSV and PCV2 have also been associated with respiratory symptoms (Lin et al., 2011). Multiple infection with two or more of these viruses commonly occurs in pigs (Ellis et al., 2000; Yue et al., 2009; Burgara-Estrella et al., 2012). These virus infections result in viral shedding in boar semen (Guérin and Pozzi, 2005; Maes et al., 2008). The effective monitoring of etiological agents in animals and semen samples destined for artificial insemination is crucial for swine disease prevention. Multiplex methods for the simultaneous detection of several targets offer increased test capacity and reduce overall cost and time, which is desirable for swine disease surveillance.

Multiplex PCR or RT-PCR assays have been developed worldwide for the simultaneous detection of swine viruses (Giammarioli et al., 2008; Ogawa et al., 2009; Jiang et al., 2010; Xu et al., 2012; Liu et al., 2013). Current methods use multiple viral primer pairs in a single assay to conduct multiplex PCR. However, avoiding the reduction of PCR efficiency through the interactions of multiple primers in an assay is a major challenge.

In the present study, a novel multiplex amplification assay was developed for the simultaneous detection of CSFV, PCV2, PPV, PRRSV, and PRV using a non-homologous universal primer set containing locked nucleic acids. Locked nucleic acids (LNAs) are nucleic acid analogs that increase the strength and specificity of oligonucleotide hybridization (Singh et al., 1998; Vester and Wengel, 2004; Ballantyne et al., 2008). The incorporation of LNA bases into standard PCR primers or real-time PCR probes increases the specificity and sensitivity of DNA amplification and detection (Ugozzoli et al., 2004; Reynisson et al., 2006; Ballantyne et al., 2008, 2011).

In the present study, instead of directly incorporating LNAs into virus-specific primers, universal primers modified with LNA sequences and viral primer pairs modified at the 5′ end were used in multiplex PCR (mPCR). Specifically, two oligonucleotides not homologous to the virus sequence were designed, incorporated with LNA bases and used as the universal primer set in the mPCR assay. The sequence of the universal primer was added to the

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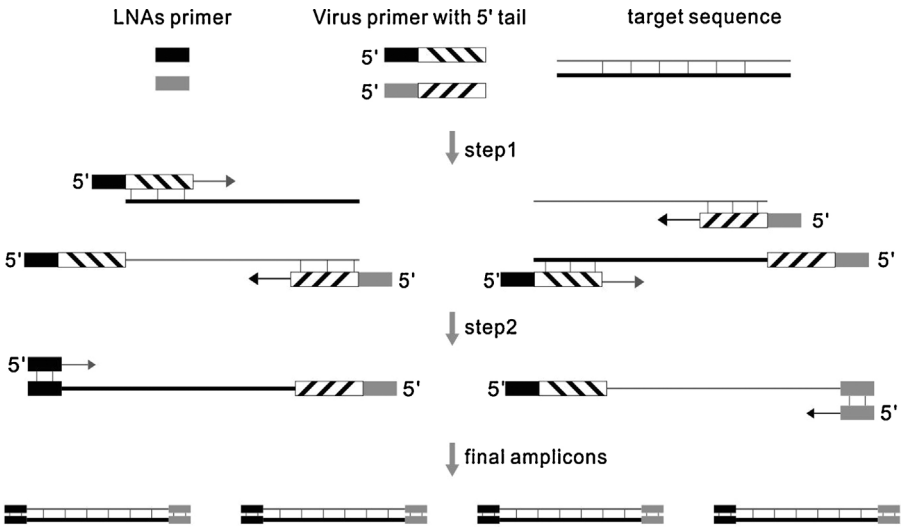

Fig. 1. A schematic diagram of the LNA universal primer assay.

5′ end of each forward or reverse virus-specific primer, so that virus specific primers were modified with a 5′ universal sequence tail of normal bases. The LNA mPCR assay contained the LNA universal primer set and small amounts of the five modified virus-specific primer pairs. A two-stage amplification process was applied. First, a 10-cycle-amplification was performed at the optimum annealing temperature to yield a small amount of the target amplicons carrying the 5′ tail. Subsequently, a 30-cycle amplification was performed, and the annealing temperature was increased to 70 °C. Multiplex amplification was subsequently performed through the extension of the LNA universal primer set, using the target amplicons carrying the 5′ tail as templates. Fig. 1 provides a schematic diagram of the LNA universal primer assay.

The aim of the present study was to determine a new method for improving multiplex PCR using a non-homologous universal primer set containing LNA bases. This method is based on the enhanced oligonucleotide affinity and increased melting temperature of a LNA-modified DNA duplex (Singh et al., 1998; Vester and Wengel, 2004). This strategy generates a few virus templates with the 5′ universal sequence tail at the beginning of the amplification reactions using small amounts of modified virus-specific primers at a typical annealing temperature, and subsequent multiplex amplification through the extension of a LNA universal primer set is conducted at a significantly increased annealing temperature. Through this strategy, it is possible to use small quantities of multiple primers and high annealing temperatures without a negative impact on PCR amplification.

The LNA mPCR assay reported here was evaluated for specificity, sensitivity and clinical performance compared with conventional multiplex amplification assays, which use virus-specific primers without the 5′ tail.

2. Materials and methods

2.1. Viruses and vaccine products

The swine target viruses, non-target pathogens and vaccine products used in the present study are listed in Table 2. The RNA purified from the wild type CSFV Shimen strain was a kind gift from Professor Chen JD of South China Agriculture University. The virus strains were maintained in the laboratory. The vaccine products were purchased from manufacturers in China.

2.2. Recombinant plasmids

Recombinant plasmids containing the target sequences for CSFV, PCV2, PPV, PRRSV or PRV were constructed. Each virus target sequence was cloned into vector pMD-19T (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions. The recombinant plasmids were verified through PCR and sequencing. Recombinant plasmid DNA was extracted and purified using an AxyPrep Plasmid Miniprep Kit (Axygen Biotechnology, Hangzhou, China). The concentrations of recombinant plasmid DNA were measured using a spectrophotometer (NanoDrop, Wilmington, DE, USA).

2.3. Swine samples

Swine semen ($n = 164$) and tissue (inguinal lymphatic glands, $n = 43$) samples were collected from 40 pig farms in south China from 2013 to 2014. The tissue samples and 34/164 semen samples were obtained from farms with clinical disease. The remaining 130 semen samples were randomly collected from pig farms without clinical symptoms at the time of sample collection. Each sample corresponds to one pig. The samples were freshly processed or stored at −30 °C until further analysis.

2.4. Sample treatment and nucleic acid extraction

Pig semen, tissue homogenates and virus-infected cells were frozen and thawed three times, followed by centrifugation at low speed. The supernatants were immediately used or stored at −30 °C. Viral genomic DNA or RNA was extracted from the supernatants using the TIANamp Virus DNA/RNA kit (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. Each extracted sample was eluted in 50 μL of RNase-free ddH_2O and stored at −20 °C until subsequent analysis. The concentration of purified viral nucleic acids was determined based on the absorbance at A_{260} using a spectrophotometer (NanoDrop, Wilmington, DE, USA).

2.5. Primer design and selection

The basic sequences of the universal primers were designed based on common primer design rules with the following specifications: the oligonucleotide should not contain the homologous