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Short communication

Reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay for the visual detection of European and North American porcine reproductive and respiratory syndrome viruses

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A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for visual detection of European (EU) and North American (NA) porcine reproductive and respiratory syndrome viruses (PRRSVs) were established and evaluated with reference PRRSV strains and clinical samples. The assay was performed in two reaction tubes containing each set of primers specific for EU or NA-PRRSV at 58 °C for 40 min, and the results could be visually detected by the naked eye, using hydroxynaphthol blue dye. The detection limit of the assay was 1 or 0.1 $TCID_{50}$/0.1 mL for EU or NA PRRSV, respectively, which was comparable to that of the previously described real-time RT-PCR (qRT-PCR). The detection rate of the assay on 130 field samples was 72.3%, relatively higher than that of qRT-PCR (70.8%), and there was high overall percentage agreement between the two assays. The high specificity, sensitivity, and reliability of the RT-LAMP assay described in this study renders it useful for the rapid and differential diagnosis of EU and NA PRRSVs, even in under-equipped laboratories.

Porcine reproductive and respiratory syndrome virus (PRRSV) is an important swine pathogen that causes tremendous economic losses in swine production worldwide. This virus divided into two genetically and antigenically distinct genotypes: European (EU) and North American (NA) (Nelsen et al., 1999). Although each genotype had independently circulated and evolved in their corresponding geographical regions, co-infection with both genotypes has recently been increasing in Europe, North America, and Asia, resulting in a significant impact on PRRSV diagnostics and management (Corzo et al., 2010; Murtaugh et al., 2010). For the effective control and management of PRRSV infection, it is desirable to have a sensitive and specific diagnostic assay that can detect the virus in a simple, rapid, and cost-effective manner. The highly sensitive and specific reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR (qRT-PCR) are considered as the best tools for PRRSV detection during the acute or persistent phase of infection (Gilbert et al., 1997; Kleiboeker et al., 2005). However, these PCR-based assays require sophisticated equipment, specialized labor, and complicated procedures for the detection of amplified products, making them unsuitable for on-spot rapid diagnostics in field situations or under-equipped laboratories in developing countries. Therefore, the development of a simple, rapid, and cost-effective assay with desirable specificity and sensitivity is imperative for detecting PRRSVs in field samples.

Since the development of loop-mediated isothermal amplification (LAMP) in 2000, the assay has been recognized as a valuable tool for the detection of various pathogens, with high sensitivity, specificity, rapidity, and simplicity (Mori and Notomi, 2009). Several RT-LAMP assays have been described for the detection of NA PRRSV or its highly pathogenic variants (Chen et al., 2008; Chen

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et al., 2009; Gao et al., 2012; Gou et al., 2014; Li et al., 2009; Zhang et al., 2011). However, considering that co-circulation of the EU and NA genotypes is increasing in pig farms, a RT-LAMP assay for the simultaneous and differential detection of both PRRSV genotypes is required (Murtaugh et al., 2010). Recently, Chen et al. (2010) developed an RT-LAMP assay for the simultaneous detection of EU and NA PRRSVs using a common primer set specific for the virus's N gene. Although the assay was rapid and sensitive enough for detecting both EU and NA PRRSVs simultaneously, it could not differentiate between the two, limiting its usefulness for PRRS control and management in pig farms co-infected with both genotypes. Rovira et al. (2009) have also described an RT-LAMP assay for simultaneous detection of PRRSV, using two primer sets, which to the best of our knowledge is the only RT-LAMP-based assay for differential detection of EU and NA PRRSVs. However, the limit of detection (LOD) of their assay ranged between 10^2 and 10^4 $TCID_{50}$/mL, significantly lower than that of conventional RT-PCR, thus rendering their method inadequate as a routine diagnostic tool. Therefore, in this study, we developed a more sensitive RT-LAMP assay for the differential detection of EU and NA PRRSVs using newly designed primer sets and evaluated the usefulness of the assay with reference strains and clinical samples.

For the rapid and differential detection of EU and NA PRRSV, RT-LAMP performed in two reaction tubes containing each set of primers specific for the N gene of EU or NA PRRSV was attempted in this study. The Web-based primer design software Primer Explorer V4 (Eiken chemical, Japan; http://www.primerexplorer.jp/e/) was used to design the primers targeting the conserved regions of the nucleocapsid (N) gene of EU and NA PRRSVs, as recommended by the software developer. Six primers (two inner, two outer, and two loop primers) that hybridize with eight specific sequences of the targeted N gene of EU or NA PRRSV were used in the RT-LAMP assay (Table 1). The reaction mixture contained 40 pmol each of the inner primers FIP and BIP, 5 pmol each of the outer primers F3 and B3, 20 pmol each of the loop primers LF and LB, 10 mM of dNTPs (Takara Bio, Japan), 8 U of *Bst* DNA polymerase (New England Biolabs, USA), 1 × the supplied *Bst* DNA polymerase buffer, 10 U of AMV reverse transcriptase (Promega, USA), 250 mM of betaine (Sigma, USA), 150 mM of $MgSO_4$, 3 mM of hydroxynaphthol blue (HNB; Lemon-Green, China), and 5 μL of template RNA in a final volume of 25 μL. Amplification was carried out at 58 °C for 40 min and then the mixture was heat inactivated at 80 °C for 5 min to terminate the reaction. After the RT-LAMP reaction, the positive results were visually confirmed by color change (from an initial purple to a positive sky blue) in the reaction tubes. The change in color is due to HNB, a metal ion indicator (Goto et al., 2009). The amplicons were also detected by observing LAMP-specific ladder-like DNA bands with an ultraviolet light transilluminator (Bio-Rad Lab., USA), after 1.5% agarose gel electrophoresis and staining with NEO green dye (Neoscience, Korea).

The specificity of the primer pairs for the RT-LAMP assay was tested with EU and NA PRRSV reference strains (Lelystad virus and LMY strain, respectively) and five other viral pathogens, including two subtypes of swine influenza virus [A/Korea/103/2009(H1N2) and A/Korea/A18/2011(H3N2)], classical swine fever virus (LOM strain), porcine parvovirus (NADL-2 strain), and porcine circovirus type 2 (PCK0201 strain), provided from the Animal and Plant Quarantine Agency in Korea. As shown in Fig. 1, the RT-LAMP assay with two defined primer sets, successfully amplified the N gene of EU and NA PRRSVs, whereas no amplification occurred with the other tested viruses. This demonstrated that the RT-LAMP assay was specific and, more importantly, that the assay differentially detected EU and NA PRRSVs in the same reaction time (Fig. 1).

The sensitivity of the RT-LAMP assay was analyzed 3 times and compared with that of qRT-PCR assays (Kleiboeker et al., 2005) for detecting EU or NA PRRSV, using 10-fold serial dilutions of RNA template extracted from primary porcine alveolar macrophages and MARC-145 cell cultures infected with the Lelystad virus and LMY strain, respectively, at the initial viral titer of 10^4 50% tissue culture infection dose ($TCID_{50}$)/0.1 mL. The RT-LAMP assay had an LOD of 1 or 0.1 $TCID_{50}$/0.1 mL for EU or NA PRRSV, respectively, which was comparable to that obtained by qRT-PCR (Fig. 2). Several researchers have described RT-LAMP assays targeted matrix genes (Chen et al., 2009; Gao et al., 2012; Gou et al., 2014; Li et al., 2009; Rovira et al., 2009), open reading frame 1a (Chen et al., 2008), nonstructural protein 2 (Zhang et al., 2011), or the N gene (Chen et al., 2010) of PRRSV, the sensitivity of which ranged from 0.1 to 10^4 $TCID_{50}$ of viral titer. As described above, the RT-LAMP method of Rovira et al. (2009) was the only one that could differentially detect EU and NA PRRSVs; however, the low sensitivity of the assay limited its usefulness as a routine diagnostic tool. The sensitivity of our assay was a minimum 100-fold higher than that of Rovira's method and comparable to that of previously reported qRT-PCR assays that can differentially detect EU and NA PRRSVs (Kleiboeker et al., 2005). Therefore, the specificity and sensitivity of the RT-LAMP assay in this study should be sufficient for the routine diagnosis of PRRSV in swine disease diagnostic laboratories.

To further evaluate the potential use of the RT-LAMP assay for the differential detect of EU and NA PRRSVs, 130 field samples (60 sera and 70 lung tissues) from PRRSV-infected pig farms were analyzed using the new method, and the detection rate was compared with that obtained with the qRT-PCR assay (Kleiboeker et al., 2005). EU, NA, and EU and NA PRRSVs were detected in 37.7% (49), 21.5% (28), and 13.1% (17) of the 130 samples, respectively, by the RT-LAMP assay, with a detection rate of 72.3% (94/130). The qRT-PCR detected EU, NA, and EU and NA PRRSVs in 37.7% (49), 20.8% (27),

Table 1
RT-LAMP primers designed for the detection of European and North American PRRSVs.

Genotype	Primer	Sequence (5′–3′)[a]	Genome position[b]
EU	F3	GCCTGAGAAGCCACATTT	14747–14764
	B3	AATCAGGCGCACTGTATG	14928–14945
	LF	GYTCRGTCTGGGTGAGRTG	14796–14814
	LB	GCTTTCATCCAGYGGGAA	14873–14890
	FIP (F1c-F2)	CTGRATCGATTGCAAGCARAG- CTSCTGAAGATGACRTYCG	14820–1484014773–14791
	BIP (B1-B2c)	TTYAATCAAGGCGCWGGAACTG-GCAGCATRAACTCAACCT	14847–1486814902–14919
NA	F3	CCAAATAACAACGGCAAGC	14892–14910
	B3	GGTCTGGATYGACGACAG	15114–15131
	LF	TGYTGRGCGATGATCTTACC	14967–14986
	LB	CTRGCGACTGAAGATGAYGTCA	15057–15078
	FIP (F1c-F2)	TTTCTTYCCCGGTCCCTTGCC- AATCAGCTGTGCCARAT	15000–1502015946–14962
	BIP (B1-B2c)	CGGAGAAGCCCCATTTYCCTC- ACAATTGCCGCTCACTA	15095–1511115037–15057

[a] R, A and G; Y, C and T; S, C and G; W, A and T.
[b] The position of the primer-binding sequence is marked according to the genetic sequence of the EU and NA reference PRRSV Lelystad and LMY strains (GenBank accession numbers M96262 and DQ473474.1).