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Development of a loop-mediated isothermal amplification assay combined with a lateral flow dipstick for rapid and simple detection of classical swine fever virus in the field

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A B S T R A C T

Classical swine fever (CSF) is a highly contagious viral disease and may cause heavy economic loss to farmers. The rapid, simple and accurate diagnosis of the disease at the frontline, for example on the farms of concern is crucial for disease control. This study describes the development and evaluation of a new loop-mediated isothermal amplification (LAMP) assay coupled with lateral flow dipstick (LFD) for the detection of classical swine fever virus (CSFV). This RT-LAMP-LFD assay combines the efficient one-step isothermal amplification of CSF viral RNA and the simplicity of the LFD to read the results within two to five minutes. Seven genotypes (1.1, 1.2, 1.3, 2.1, 2.2, 2.3 and 3.1), but not genotype 3.4, were successfully detected by the RT-LAMP-LFD assay, indicating that the method has a broad range of detection and can be applied in different geographical areas where CSFV strains belonging to these genotypes are present. The performance of this RT-LAMP-LFD assay was similar to that of the real-time RT-PCR. The analytical sensitivity was about 100 copies per reaction when testing two genotypes (1.1 and 2.3). No cross-reactivity to non-CSFV pestiviruses was observed. This RT-LAMP-LFD assay can be a useful novel tool for the rapid, simple and economic diagnosis of classical swine fever in the field.

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1. Introduction

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a highly contagious viral disease. It is a small, enveloped, positive-sense, single-stranded RNA virus belonging to the genus *Pestivirus* of the family *Flaviviridae* (Pletnev et al., 2011). The other member of the genus include *bovine viral diarrhea virus* 1 (BVDV-1), *bovine viral diarrhea virus* 2 (BVDV-2) and *border disease virus* (BDV). The virus is categorized into three major genetic groups (Lowings et al., 1996), which are further divided into 10 sub-groups: 1.1, 1.2, 1.3, 2.1, 2.2, 2.3, 3.1, 3.2, 3.3, and 3.4 (Paton et al., 2000). Both domestic pigs and wild boars are natural host of CSFV and the disease can be mild, moderate or severe. CSF may cause heavy economic losses to farms. For example, outbreaks in The Netherlands during 1997/98 resulted in the slaughter of over 10 million pigs at a cost of over 2 billion US dollars (Pluimers et al., 1999).

As the clinical signs vary from case to case, laboratory diagnosis utilizing different techniques is needed in order to either confirm or exclude the infection. While conventional virus isolation remains the "gold standard" method, novel molecular approaches, such as real-time RT-PCR are widely used in national and reference laboratories. A ring trial among 10 European laboratories has demonstrated reliability of the CSF diagnosis using real-time RT-PCR on an international level (Hoffmann et al., 2011). In the meantime, simple and rapid methods such as loop-mediated isothermal amplification (LAMP), published originally by Notomi et al. (2000), have been developed and evaluated for the detection of CSF viral nucleic acids (Chen et al., 2009; Yin et al., 2010; Zhang et al., 2010). Compared to virus isolation and real-time RT-PCR, the LAMP method is more suitable for field applications, since it does not require specialized and/or expensive equipment. In addition, LAMP results can be read by the naked eye, based on the change of turbidity. Alternatively, the LAMP products can be labeled with small molecules such as fluorescein isothiocyanate (FITC) and digoxigenin (DIG), and then separated on lateral flow strips by chromatographic force. Upon specific binding between the molecules

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Table 1
Virus isolates and comparison of the RT-LAMP-LFD and real-time RT-PCR assays. The results are the last dilution of the viruses and Ct values from real-time RT-PCR are presented in brackets.

Isolates	Genotype	Country	RT-LAMP-LFD	Real-time RT-PCR
31240/96	1.1	Slovak Republic	10^{-1}	10^{-2} (35.92)
Romania I 01	1.1	Romania	10^{-2}	10^{-3} (40.08)
Alfort 187	1.1	France	10^{-3}	10^{-3} (42.52)
Argentina	1.1	Argentina	10^{-2}	10^{-3} (38.05)
742/Ru	1.1	Russia	10^{-2}	10^{-2} (36.54)
7/3	1.2	Poland	10^{-2}	10^{-4} (41.34)
No. 3/brescia	1.2	Romania	10^{-2}	10^{-3} (38.93)
JIK/Ru	1.2	Russia	10^{-1}	10^{-1} (39.43)
VRI 4167	1.3	Malaysia	10^{-2}	10^{-2} (37.45)
94-14901	1.3	Costa Rica	Original	10^{-1} (40.74)
97-7446/#4	1.3	Honduras	Negative	10^{-1} (37.59)
907/1	2.1	Germany	10^{-1}	10^{-2} (40.22)
VRI 2277	2.1	Malaysia	10^{-2}	10^{-2} (36.99)
2000/8	2.1	Great Britain	10^{-1}	10^{-2} (35.89)
V2/97	2.1	Germany	10^{-2}	10^{-3} (39.50)
4905 I 97/03	2.1	Italy	10^{-3}	10^{-4} (41.72)
V 273/89	2.2	Germany	10^{-3}	10^{-4} (37.89)
P40/07/87	2.2	Singapore	10^{-2}	10^{-3} (38.89)
VA 531	2.2	Italy	10^{-3}	10^{-3} (38.88)
2213/97	2.2	Czech Republic	10^{-3}	10^{-3} (37.47)
2699/Osterode	2.3	Germany	10^{-1}	10^{-3} (36.62)
Pomi/2004	2.3*Rostock	Romania	10^{-2}	10^{-2} (39.95)
30853	2.3	Israel	10^{-2}	10^{-3} (39.73)
591/02	2.3*Uelzen	Bulgaria	10^{-2}	10^{-2} (37.35)
M7 19928/60	2.3*Slovakia	Hungary	10^{-2}	10^{-1} (32.53)

and antibodies embedded in the strip, a colored line appears indicating a positive reaction. A combination of LAMP and lateral flow dipstick (LFD) would make rapid diagnosis in the field or close to farms possible.

The objective of this study was to develop and evaluate a one-step RT-LAMP assay combined with LFD as readout for the rapid detection of CSFV. The new assay offers a rapid, economic and simple-to-use novel diagnostic tool suitable for field application.

2. Materials and methods

2.1. Samples and RNA preparation

A total of 105 samples were used in this study. Those included 25 CSF virus isolates of cell culture passages (Table 1) and 27 reference sera, kindly provided by the OIE and EU reference laboratory for classical swine fever, Hannover, Germany; 47 RNA preparations (Table 2) from a pestivirus ring trial panel (Hoffmann et al., 2011); and 6 pooled wild boar sera, kindly supplied by ANSES, Maisons Alfort, France. The samples covered eight subtypes (1.1, 1.2, 1.3, 2.1, 2.2, 2.3, 3.1, and 3.4). All the sera were positive for CSFV E2 antibody ELISA. RNA extraction from the 25 virus isolates was performed as described previously (Liu et al., 2011). RNeasy mini kit (Qiagen, Hilden, Germany) was used to extract RNA from serum samples, according to the manufacturer's protocol. Ten-fold serial dilutions of RNA from virus isolates were prepared. Isolate Argentina (genotype 1.1) was used to optimize initially the RT-LAMP assay.

2.2. Primer design

Six specific primers targeting the 5′UTR of viral genome were designed using the online program Primer Explorer version 4 (http://primerexplorer.jp/elamp4.0.0/index.html). The primer set included two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers labeled with DIG and FITC, respectively (Table 3).

2.3. Optimization of the RT-LAMP-LFD assay

Experiments were performed to optimize the RT-LAMP assay by testing different incubation times, Betaine concentrations and amplification temperature. The optimized CSFV RT-LAMP assay in a total reaction volume of 25 μl consisted of 1× Thermo buffer, 0.33 mM dNTPs, 0.2 μM F3, B3, Floop, and Bloop primers, 2 μM FIP and BIP primers, 0.4 M Betaine (Sigma, St. Louis, MO), 16 U of *Bst* DNA polymerase (New England Biolabs, Herts, UK), 9 U of Cloned AMV-RT (Invitrogen, Carlsberg, CA) and 3 μl of viral RNA. The reaction mixture was incubated on a thermocycler at 60 °C for 60 min followed by 80 °C for 2 min. Eight microliters of the RT-LAMP products were run on a 1.5% agarose gel to visualize the results, while 10–12 μl were used in the lateral flow dipstick, HybriDetect 2T (Milenia Biotec, Gissen, Germany), for the detection of amplified products according to manufacturer's instructions.

2.4. Comparison with CSFV real-time RT-PCR

The RNA preparations from the serum samples were tested by a published CSFV real-time RT-PCR (Hoffmann et al., 2005) using SuperScript® III One-Step RT-PCR System (Invitrogen, Carlsberg, CA) in a RotorGene3000 instrument. For detection of CSF viral RNA from virus isolates and RNA panel, AgPath-ID One-step master mix (Applied Biosystems, Foster city, CA) was used. The reaction mixture consisted of 12.5 μl of 2× RT-PCR buffer, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 0.5 μl of 10 μM probe, 1 μl of 25× RT-PCR enzyme mix and 3 μl of RNA template. Cycling steps were 45 °C for 10 min, 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 45 s.

3. Results

3.1. Development of an RT-LAMP-LFD assay

A simple, one-step RT-LAMP assay was developed in this study to amplify CSF viral RNA under constant temperature, which was followed by detection of the products in a lateral flow dipstick.

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