Transboundary and Emerging Diseases

SHORT COMMUNICATION

Pre-Clinical Evaluation of a Real-Time PCR Assay on a Portable Instrument as a Possible Field Diagnostic Tool: Experiences from the Testing of Clinical Samples for African and Classical Swine Fever Viruses

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Summary

African swine fever (ASF) and classical swine fever (CSF) are two highly infectious transboundary animal diseases (TADs) that are serious threats to the pig industry worldwide, including in China, the world's largest pork producer. In this study, a duplex real-time PCR assay was developed for the rapid detection and differentiation of African swine fever virus (ASFV) and classical swine fever virus (CSFV). The assay was performed on a portable, battery-powered PCR thermocycler with a low sample throughput (termed as 'T-COR4 assay'). The feasibility and reliability of the T-COR4 assay as a possible field method was investigated by testing clinical samples collected in China. When evaluated with reference materials or samples from experimental infections, the assay performed in a reliable manner, producing results comparable to those obtained from stationary PCR platforms. Of 59 clinical samples, 41 had results identical to a two-step CSFV real-time PCR assay. No ASFV was detected in these samples. The T-COR4 assay was technically easy to perform and produced results within 3 h, including sample preparation. In combination with a simple sample preparation method, the T-COR4 assay provides a new tool for the field diagnosis and differentiation of ASF and CSF, which could be of particular value in remote areas.

Introduction

Field tests, similar to 'point-of-care (POC)' testing in human medicine, generally refer to the rapid assays that are performed either on a simple device, such as a lateral flow dipstick (LFD), or on a simple-to-use, portable (or at least transportable) instrument under field conditions. These assays can be carried out in a basic regional laboratory or in the farms or slaughterhouses of concern. Results are available within minutes or hours, depending on the type of tests, and decisions can be made rapidly. For example, a veterinarian can use field tests to confirm the clinical diagnosis of infectious animal diseases and immediately initiate treatment or implement necessary control actions to prevent further spread, depending on the kind of infectious agent. Field tests have the potential to enable earlier diagnosis of both transboundary animal diseases (TADs) and local endemic diseases prior to confirmation at central or

reference laboratories, enabling the implementation of proper control measures in a timely manner before the diseases become widespread.

Different diagnostic tools have been developed for field applications. LFDs are inexpensive devices for the simple and rapid detection of pathogens or antibodies. Isothermal amplification methods, such as recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP), are often used to amplify nucleic acids, which are then detected without the need for an expensive thermal cycler. Nucleic acid amplified by LAMP can be visualized by coloured lines displayed in specific positions on an LFD, as illustrated by the authors' previous study for the detection of CSFV (Chowdry et al., 2014). In general, RPA and LAMP are regarded as less sensitive and robust than real-time RT-PCR methods, as shown by the detection of Schmallenberg virus and bovine viral diarrhoea virus (Aebischer et al., 2014) and of H5N1 highly pathogenic avian influenza virus (Soliman et al., 2010). In contrast, real-time PCR-based tests have a higher performance and results can be read directly on the display without any post-amplification processing of the PCR products. This type of tests requires a portable or at least a transportable PCR cycler with an integrated optical detection system. Recently, a probe-based insulated isothermal PCR (iiPCR) on a compact device (POCKIT™ Nucleic Acid Analyzer) showed promising results for field application (Lung et al., 2015).

African swine fever (ASF) and classical swine fever (CSF) are two major infectious diseases of swine that are of significant concern to governments, veterinarians and farmers, as well as to the World Organisation for Animal Health (OIE, www.oie.int). ASF is caused by a large complex DNA virus, African swine fever virus (ASFV), which belongs to the genus *Asfivirus* in the family *Asfiviridae*. ASFV is very stable in the environment and in biological materials. As clinical signs of ASF vary greatly from subclinical infection to sudden death with few or no other signs in its acute form, differential diagnosis is needed to exclude other diseases such as CSF, which is caused by an RNA virus, classical swine fever virus (CSFV) of the genus *Pestivirus* of the family *Flaviviridae*. A duplex ASFV/CSFV assay would be very useful in assisting diagnosticians and veterinarians with differential diagnosis of the two diseases. An ASFV LAMP assay described by James et al. (2010) was able to detect a panel of 38 isolates representing ten genotypes (I~VI, VIII~X and XVIII) with an analytic sensitivity of at least 330 genome copies. A CSFV RT-LAMP assay coupled with LFD was able to detect subgenotypes 1.1, 1.2, 1.3, 2.1, 2.2, 2.3 and 3.1, but not the most divergent 3.4, and had an analytic sensitivity of 100 genome copies for the genotypes 1.1 and 2.3 tested (Chowdry et al., 2014). In a previous study, duplexing of the two LAMP assays was found to have a reduced performance when compared with the two assays (V. Chowdry, S. Belák, and L. Liu, unpublished data). Therefore, the objective of this study was to evaluate the feasibility and reliability of a duplex real-time ASFV/CSFV PCR assay, performed on a portable thermocycler T-COR4 (termed hereafter as 'T-COR4 assay'), as a field diagnostic tool for the detection and differentiation of the two fever-inducing swine viruses in clinical samples.

Materials and Methods

Samples

A total of 134 samples were tested in this study (Table 1). These included three types of specimens: ready-to-use DNA (ASFV) and RNA (CSFV) preparations, serum samples collected from experimental infections, and whole blood, sera and homogenized tissues from clinically diseased pigs investigated for CSFV infections. The RNA preparations were part of a ring-trial panel (Hoffmann et al., 2011), the experimental serum samples were from the Centre de Recerca en Sanitat Animal (CReSA) in Barcelona, Spain, and the clinical samples were originated from China.

Nucleic acid preparations

Nucleic acids were extracted from 50 μl of each blood/serum sample or the homogenates by a MagMAX™-96 Total RNA Isolation Kit, according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). A DynaMag™-2 magnet (Life Technologies) was used as the only equipment. For biosafety reasons, samples were handled in a Laminar flow cabinet at SVA, CReSA and HVRI. For comparison, the RNeasy mini kit (Qiagen, Hilden, Germany) was used to prepare the nucleic acids from CSFV-positive samples at SVA and the NucleoSpin blood kit (Macherey-Nagel, Düren, Germany) was used to prepare ASFV DNA at CReSA. Ten-fold dilutions of these

Table 1. List of samples

Source[a]	Target	Type	Number
SVA	ASFV	DNA preparation	1
CReSA	ASFV	Sera from experimental infections[b]	58
FLI	CSFV	RNA preparation	9
TiHo	CSFV	Sera from experimental infections	5
SVA	–	Negative serum	1
HVRI	CSFV	Blood, sera and homogenized tissues	59

[a]SVA, National Veterinary Institute, Uppsala, Sweden; CReSA, Centre de Recerca en Sanitat Animal, Barcelona, Spain; FLI, Friedrich-Loeffler-Institut, Greifswald – Insel Riems, Germany; TiHo, Veterinary University of Hannover, Hannover, Germany; HVRI, Harbin Veterinary Research Institute, Harbin, China.
[b]Including 18 10-fold dilutions of six sera.

samples in a negative serum were prepared prior to extraction. The ASFV DNA preparations were transported from CReSA on dry ice to a biosafety level 3 (BSL3) laboratory at SVA and heat-inactivated at 70°C for 30 min prior to being taken to a BSL2 laboratory for testing.

Real-time PCR assay

An AgPath-ID One-Step RT-PCR kit and primers and probes (Haines et al., 2013) were used in the real-time PCR assay. The reaction contained 0.8 μM ASFV primers, 0.6 μM CSFV primers, 0.2 μM FAM-labelled CSFV probe and Cy5-labelled ASFV probe, 1 μl enzyme mix, 12.5 μl 2 × buffer, 4 μl water and 3 μl viral nucleic acids. Cycling steps for the duplex assay included a reverse transcription at 45°C for 10 min, activation of DNA polymerase at 95°C for 10 min and 48 cycles of 45°C for 15 s and 60°C for 45 s. All the samples were tested on the portable real-time PCR cycler T-COR4, which has a weight of 2.81 kg, dimensions of 22.86 cm × 19.05 cm × 5.72 cm, and a rechargeable battery for 8 h of continuous PCR on all four wells (Tetracore, Inc., Rockville, MD, USA). The assay was evaluated in a three-step process as follows: (i) initial testing of the ready-to-use RNA/DNA preparations, (ii) selection of nucleic acid preparation methods using the samples from experimental infections and (iii) subsequent testing of clinical samples including sample preparations. For comparison, the seven CSFV sera and the dilutions were also tested by the same assay performed on RotorGene 3000 and/or Bio-Rad CFX at SVA; the ASFV sera were tested in an ABI 7500 Fast system at CReSA; and at HVRI, the CSFV RNA preparations were first reverse-transcribed into cDNA, and then, 2 μl cDNA was used as a template in a real-time PCR assay (Zhao et al., 2008). A cut-off C_t value of 38 was set for this two-step PCR assay. Virus isolation at HVRI was attempted for nine samples on PK-15 cells for three passages.

Results

Initial evaluation of the duplex real-time PCR assay on T-COR4

During the initial evaluation step, CSFV-positive RNA was detected up to the 1000-fold dilution in the T-COR4 assay, which was similar to the previous results given by real-time RT-PCR and RT-LAMP-LFD (Chowdry et al., 2014). Further evaluation of the assay was performed using two strains, Alfort/187 (genotype 1.1) and the most divergent Kanagawa (genotype 3.4) at different dilutions, which were part of a CSFV RNA panel of a known copy number ($1.28 \times 10^0 \sim 1 \times 10^4$ copies/μl). Results from the T-COR4 assay showed a detection limit close to 100 copies per reaction, at the C_t value of 35.6 for Kanagawa. The C_t values for other dilutions of the strain were 33.5 (1.6×10^2 copies/μl), 32.0 (8×10^2 copies/μl) and 30.5 (4×10^3 copies/μl), and for the strain Alfort/187, 33.7 (1.6×10^2 copies/μl), 31.0 (8×10^2 copies/μl) and 30.3 (4×10^3 copies/μl). For ASFV detection, only serial dilutions of the strain E75 were used for the initial evaluation. The assay detected the 10 000-fold dilution with a C_t value of 34.3. The C_t values ranged between 35 and 37 when the same dilution was tested on RotorGene 3000 and Bio-Rad CFX systems.

Selection of a nucleic acid preparation method

The CSFV viral RNAs prepared using the MagMAX kit gave consistent results when tested on the T-COR4 and Bio-Rad CFX system (Table S1). However, the viral RNAs that were extracted from the same samples using the RNeasy kit were found to contain a smaller number of viral genome copies (Table S1). The same trend was found when detecting ASFV DNA in sera using the T-COR4 and ABI 7500 Fast systems, where samples prepared by the spin-column method gave slightly higher C_t values (Table S2). In addition to the six ASFV-positive swine serum samples, two sera sampled prior to the experimental ASF infection and an extraction control tested negative on T-COR4 at CReSA in Spain. After transport to SVA, the remaining 32 ASFV DNA preparations were tested by the same real-time PCR assay on T-COR4 and the Bio-Rad platform. Twenty-nine samples had consistent results, whereas three samples were negative on T-COR4 but positive on the Bio-Rad system, with high C_t values of 37.89, 39.75 and 39.77, respectively (Table S3).

Pre-clinical evaluation of the duplex real-time PCR assay using clinical samples

Testing of a total of 59 clinical samples on the T-COR4 instrument at HVRI in China found 33 to be positive and 26 negative, with 41 samples matching the results from the two-step real-time PCR assay. A breakdown of the test results (Table 2) showed that 17 strong positive samples were readily detected by both assays, and of the 16 T-COR4 assay weak positive samples, three were positive with high

Table 2. Breakdown of CSFV PCR results of testing field samples from HVRI, China

T-COR4 results	No. of samples	In-house PCR results	No. of samples
<31	17	18.09–33.06	17
31.1–35.4	16	33.06–35.07	3
		Negative (>38/no C_t)	13
Negative	26	35.32–37.52	5
		Negative (>38/no C_t)	21

C_t values by the real-time PCR. However, five of 26 samples found to be negative by the T-COR4 assay were found to be positive by the real-time PCR assay, albeit with high C_t values ranging from 35.32 to 37.52. Virus isolation failed for all nine of 18 samples that had discordant T-COR4 and the two-step real-time PCR results. An indirect immunofluorescence assay (IFA) of the discordant samples was also negative. No ASFV was detected in the 59 clinical samples collected from China.

Discussion

Although a fairly high number of field tests employing different technologies such as isothermal amplification and lateral flow devices have been developed and evaluated for field applications, only a handful of these assays have been found to be sufficiently suitable and reliable for rapid diagnosis in a simple laboratory or under field conditions. Among many factors limiting transition from laboratory assays to field application, feasibility and reliability are two important criteria determining the applicability of field tests. To overcome limitations in the ability of the CSFV RT-LAMP-LFD assay to detect different genotypes (Chowdry et al., 2014), this study evaluated the feasibility and reliability of a real-time PCR assay performed on a portable real-time PCR thermocycler as a field diagnostic tool for the early and rapid detection of ASFV and CSFV in clinical samples. Field testing of clinical samples would be a valuable tool in remote areas where logistical difficulties prevent the rapid transportation of samples to central laboratories for the diagnosis of transboundary animal diseases.

For this field test, the primers and probes have already been extensively evaluated (Hoffmann et al., 2005; Haines et al., 2013); therefore, this study started with an adaptation of the assay to the portable T-COR4. The AgPath-ID One-Step RT-PCR kit was used in this assay as it has previously been shown to give high sensitivity (Stephens et al., 2010). An initial evaluation of the real-time ASFV/CSFV duplex assay on T-COR4 using ready-to-use nucleic acid preparations showed promising results, comparable to those of the same assay performed on stationary real-time PCR cyclers RotorGene 3000 and Bio-Rad CFX systems. In particular, the assay detected the most divergent CSFV strain Kanagawa, which was negative by the RT-LAMP-LFD assay (Chowdry et al., 2014). A better performance of a Newcastle disease virus real-time PCR has been observed in the testing of reference virus isolates on the same T-COR4 cycler (Liu et al., 2016). Therefore, it was concluded that the portable real-time PCR cycler T-COR4 has a comparable performance to the stationary systems in laboratory settings. The low throughput capacity of this portable system might be a weak point for field application, as veterinarians might prefer to test more samples to ensure accurate diagnosis. In addition, compared with stationary thermocyclers, T-COR4 uses an algorithm to calculate C_t values that cannot be adjusted by the user. This might explain the higher C_t values obtained on T-COR4 than on ABI 7500 Fast for testing the same samples.

It was observed that proper sample preparation is a prerequisite for use of the T-COR4 assay in pen-side conditions. In laboratory settings, infectious materials are handled inside a Laminar hood, nucleic acids bound to a membrane are separated from debris and solutions by centrifugation and, after washing steps, are eluted from the membrane again by centrifugation. Such protocols would not work in a simple laboratory without the required equipment or under field conditions. Compared with the other two spin-column-based kits, the MagMAX kit seemingly extracted a higher amount or better quality of nucleic acids from serum samples collected from experimentally infected pigs, leading to better results. The current nucleic acid extraction protocol takes less than 1 h to complete the preparation of four samples, the maximum number of samples that can be tested in one run on a single T-COR4. Therefore, sample preparation using the MagMAX kit was regarded as very practical for application in a simple laboratory and to a lesser degree under field conditions. It would be highly desirable to further simplify this protocol by optimizing solutions and reducing the number of washing steps.

Discrepancies were observed when detecting CSFV in clinical samples by the T-COR4 assay compared to the two-step real-time PCR assay. The differences mainly concerned 18 samples that either had high C_t values ($n = 13$) or were regarded as negative ($n = 5$) by the two-step real-time PCR assay (Table 2). These samples most likely had a very low viral load, if positive at all, that was close to the detection limit of the T-COR4 assay, which corresponded to approximately 10^2 copies/μl or C_t values above 31. The two-step real-time PCR might be less sensitive than the T-COR4 assay and encountered difficulties in detecting viral nucleic acids in these samples. In contrast, when the viral load was high, containing at least 10^3 to 10^4 genome copies/μl or C_t values below 31, both the T-COR4 assay and two-step real-time PCR produced reliable results. As it would be hard to know the viral load beforehand in clinical cases suspected of CSF, the T-COR4 assay might be unable to accurately identify infected pigs with a low viremia. Therefore, as a whole diagnostic process, further improvements are needed such as: (i) the simplification of sample preparation procedures to be more applicable for field veterinarians and to reduce sample-to-result time, (ii) the use of dried-down reagents in the PCR to avoid the need for a cold chain and (iii) the increased robustness of the portable thermocycler under different environmental conditions. One such example is the insulated isothermal PCR assay on a POCKIT™

Nucleic Acid Analyzer, which is easy to use and has a short turnaround time for detection of CSFV in serum samples (Lung et al., 2015).

In summary, the feasibility and reliability of a real-time PCR performed on the portable PCR thermocycler T-COR4 was evaluated as a field diagnostic tool for the rapid detection of ASFV and CSFV in clinical samples. Although the T-COR4 real-time PCR assay was technically easy to perform and produced results within 2 h using extracted nucleic acids, the performance was more moderate when testing clinical samples compared to reference materials or experimental samples. These preliminary results indicate that the assay has the capacity to provide a new and important tool for field diagnosis. By making further improvements and optimizations to the portable thermocycler as well as to the assay conditions, the practical applicability of the new assay will be further increased, supporting programmes to eradicate ASF and CSF, two devastating diseases in swine.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Comparison of Ct values for detection of ASFV in samples from experimentally inoculated animals on T-COR4 and Bio-Rad systems.

Table S1. Comparison of sample preparation methods for detection of CSFV on T-COR4 and Bio-Rad CFX system.

Table S2. The portable PCR assay for ASFV detection in sera on two different real-time PCR platforms.

Table S3. Detection of ASFV in samples from experimentally inoculated animals on T-COR4 and Bio-Rad systems.

