

Development of a new LAMP assay for the detection of CSFV strains from Cuba: a proof-of-concept study

Alexander Postel¹ · Lester J. Pérez² · Carmen L. Perera² · Stefanie Schmeiser¹ · Denise Meyer¹ · Alexandra Meindl-Boehmer¹ · Liliam Rios² · Sophia Austermann-Busch¹ · Maria T. Frias-Lepoureau² · Paul Becher¹

Received: 27 November 2014 / Accepted: 22 March 2015 / Published online: 1 April 2015
© Springer-Verlag Wien 2015

Abstract Classical swine fever (CSF) is a devastating animal disease of great economic impact worldwide. In many countries, CSF has been endemic for decades, and vaccination of domestic pigs is one of the measures to control the disease. Consequently, differentiating infected from vaccinated animals by antibody ELISA screening is not applicable. In some countries, such as Cuba, lack of molecular techniques for sensitive, rapid and reliable detection of virus genomes is a critical point. To overcome this problem, an easy-to-use one-tube assay based on the loop-mediated isothermal amplification (LAMP) principle has been developed for detection of the genome of CSF virus (CSFV) of endemic Cuban genotype 1.4 isolates. The assay reliably detected recent isolates from three different regions of Cuba with an analytical sensitivity 10–100 times lower than that of quantitative reverse transcription RT-qPCR. Diagnostic test sensitivity was examined using reference sera from two groups of pigs experimentally infected with Cuban virulent strain CSF0705 “Margarita” and the recent field isolate CSF1058 “Pinar del Rio”. Differences in pathogenicity of the two viruses were reflected in the clinical course of disease as well as in virus loads of blood samples. Low viral RNA loads in samples from pigs infected with the field isolate caused serious detection problems in RT-LAMP as well as in RT-qPCR.

Thus, it will be necessary in future research to focus on targeted sampling of diseased animals and to restrict diagnosis to the herd level in order to establish LAMP as an efficient tool for diagnosing CSF under field conditions.

Introduction

Worldwide, great efforts have been undertaken in order to control and to eradicate classical swine fever (CSF). Severe outbreaks, as well as subsequent control measures based on a strict stamping-out strategy, have a severe socio-economic impact, in particular for developing countries [1]. CSF is endemic in several countries of South America, Central America and the Caribbean, and information about numerous cases has been published [2–5]. In 2012, 227 CSF outbreaks were reported to the OIE (World Organisation for Animal Health) from the Americas, comprising outbreaks in Bolivia (n = 15), Ecuador (n = 82), Guatemala (n = 9), Peru (n = 40) and Cuba (n = 81) [6]. Viruses of subgenotypes 1.1 and 1.3 of CSF virus (CSFV) were most frequently identified as the causative agents of the respective outbreaks [3–5]. So far, genetically characterised CSFV isolates have been divided into three genotypes, which can be further divided into eleven subgenotypes, namely, 1.1-1.4, 2.1-2.3, and 3.1-3.4 [7, 8]. CSFV isolates from Cuba are genetically highly similar to each other, while they differ considerably from other isolates from the Caribbean, South and Central America; accordingly, they form a separate subgenotype, 1.4 [3, 8–10].

A prominent obstacle in the control of the disease is the presence of field isolates in the vaccinated pig population, as clinical signs are generally hard to recognise. Insufficient vaccination coverage in the field is mostly due to a combination of distinct problems, e.g., quality and

✉ Paul Becher
Paul.Becher@TiHo-Hannover.de

¹ EU and OIE Reference Laboratory for Classical Swine Fever, Institute of Virology, Department of Infectious Diseases, University of Veterinary Medicine Hannover, Buenteweg 17, 30559 Hannover, Germany

² Centro Nacional de Sanidad Agropecuaria (CENSA), San Jose de las Lajas, Apdo. 10, Mayabeque, Cuba

availability of the vaccine, gaps in the cold chain after vaccine production until the time point of application, and suboptimal cooperation between vaccinating personnel and farmers. In Cuba, during the past few years, an increasing incidence of mild or even subclinical courses of CSFV infection has been observed, making clinical and post-mortem diagnosis of the disease even more difficult [9, 11]. Many countries with endemic status for CSF are facing similar problems. Until now, CSF diagnosis in regional laboratories of many developing countries has relied exclusively on clinical examination and necropsy. Against this background, one aim of a twinning project between the European Union and OIE Reference Laboratory (EU and OIE RL) for CSF in Hannover, Germany, and the National Center for Animal and Plant Health in Cuba (CENSA) was the establishment of an easy-to-perform molecular biological method to enable Cuban regional laboratories to perform rapid and highly sensitive CSF diagnosis. Loop-mediated isothermal amplification (LAMP) was chosen as the genome detection method, as it can be performed without highly sophisticated laboratory equipment [12]. LAMP runs at a single temperature, and therefore no PCR thermocycler is required. In addition, results can be interpreted directly by the naked eye without further steps such as gel electrophoresis. A crucial feature of this method is that a set of four to six primers is required, recognising six or eight highly conserved regions in the target genome. The high variability of RNA viruses like CSFV makes it difficult to find suitable LAMP primers with appropriate characteristics that enable the detection of diverse CSF isolates of different genotypes and subgenotypes with appropriate sensitivity. Several CSFV-specific LAMPs have been developed, differing in terms of methodological design (one-step/two-step) and intended purpose (broadly reacting/C-strain (vaccine) specific) [13–18]. However, none of the LAMP assays developed so far have been reported to be used routinely for diagnostic purposes under field conditions, implying that their overall performance and implementation under field conditions are problematic. The unique epidemiological situation in Cuba, namely, low diversity of known Cuban isolates as well as low likelihood of an introduction of new CSFV strains belonging to other subgenotypes, can be regarded as favourable for the development of a LAMP-based assay and its application for first-line diagnosis of CSF in Cuba.

The aim of our study was to establish a one-step CSFV-specific RT-LAMP assay with high sensitivity for detecting Cuban CSFV isolates. The strategy of a single-tube protocol was favoured, as this would simplify handling and reduce the risk of cross-contamination. The adaptation of a previously published one-step CSFV RT-LAMP protocol had not been possible due to a failure to fulfil requirements of adequate diagnostic performance [13, 14, 16]. Hence, a

new one-step real-time RT-LAMP assay that specifically detects the endemic Cuban CSFV isolates was developed, and proof of principle of the assay was demonstrated in the present study. Reference sample material was obtained from an animal experiment performed within the tasks of the EU and OIE RL for CSF, including two different CSFV isolates/strains from Cuba, which were analysed with regard to their pathogenicity.

Materials and methods

Sample preparation

RNA preparation was performed with 140 µl of serum or PK15 cell culture supernatants, using a QIAamp Viral RNA Mini Kit as recommended by the manufacturer. Two µl of this RNA preparation was added to the RT-LAMP mastermix, and 5 µl of the RNA preparation was used for CSFV-specific RT-qPCR.

Primers for RT-LAMP

CSFV-specific RT-LAMP primers were obtained at the highest available purity (PAGE, Hypur) from Sigma-Aldrich and MWG, respectively (Table 1). Primer sets 1–3 and 7–9 were constructed based on NS5B and 5'NTR sequences, respectively. For this purpose, sequences of the 1.1 isolates CSF0902 (Alfort187), CSF0940 (C-strain “Riems”), and CSF0947 (Brescia) as well as the five available Cuban isolates were analysed using the LAMP primer design software “PrimerExplorerV4” (<https://primerexplorer.jp/e/>). Primer sequences of sets 4, 5 and 6 had been published previously [13, 14, 16]. The published sequence of the backward inner primer (BIP) of set 6 had been erroneous and was corrected [13]. The BIP primer sequence of set 4 was corrected for one missing nucleotide according to the reference sequences used by the author (e.g., AF091661, AF326963, U90951) [14]. Primer set 3 was a modified version of set 1, with changes based on the NS5B sequences of the Cuban isolates determined in this study (Fig. 1). Genome fragments of the partial NS5B-encoding sequences were amplified by RT-PCR and sequenced using the primers CSF-Alf_11674 fw (5'-CCCGATATCTGCCTACAAGGA-3') and CSF-LOM_12126 rev (5'-TGTTATTTACAATAGGGTCCTAC-3'). Sequences were deposited in the GenBank database under accession numbers KM000008–KM000012.

Quantitative reverse transcription PCR (RT-qPCR)

CSFV-specific RT-qPCR was performed according to the accredited method of the EU and OIE RL for CSF as published previously [19]. Run-off RNA transcripts of

Table 1 LAMP primer sets

| Primer set | Target region | Reference | Primer sequences |
|------------|---------------|------------------------|---|
| Set 1 | NS5B | This study | F: GGCTAGTTAATGCAGACAGA B: ATGACTCTCAGCCTCCTT FIP: AATGTCTCCCTTGCAGGGTGCTAGTGAGTAGCAAGACTGG BIP: ATGAAGAAGCTGGTGTGGCAAGATGTTGACTATTGGGCCTAG |
| Set 2 | NS5B | This study | F: AAGAGGGCAACTGGCTAG B: GACTATTGGGCCTAGATTGT FIP: GGCCCTCTCCAGGTATGTACTTAATGCAGACAGACTAGTGAG BIP: CACCCTGCAAGGGAGACATTCTGTCTGTCCCTTGAAAGT |
| Set 3 | NS5B | This study | F: GGCTGGTTAACGCAGACAGA B: ATGACTCTCARCCTCCTT FIP: AGTGTCTACCTTGCAGGGTGCTGGTGAGTAGTAAGACTGG BIP: ATGAAGAAGCTGGTGTGACAAGATGTTGACTATTGGGCCTAG |
| Set 4 | NS5A | Chen et al., 2010 [14] | F: CGGAGATGCAGGAGGAGAT B: CCTCTGTGGATTAGTCCCGG FIP: AGGGGCTCCCTACCACAGCTTGCAGCCTGCTGTAGTG BIP ^a : GGAGACT <u>CCG</u> ACAGCGTTCACCCTTACCTACCCCCAGT LF: CGGTCACGTCAGTTGTATCCA LB: CAGCTCAGGTTGACACCCG |
| Set 5 | E2 | Yin et al., 2010 [16] | F: AACCACGATTTGCAACTGAA B: TATCAAACGGGCACAGCC FIP: CCTCCTACTGACCACATTAGCCATTTGCGTGGCAGGTTCC BIP: CTCCGTGACATTCGAGCTCCTTCTCCATTTCTCAGTTG LF: CCTACTGACCACATTAAGTGCTG LB: GTTCGACGGGACCAACCCAT |
| Set 6 | 5'NTR | Chen et al., 2009 [13] | F: ACTAGCAAACGGAGGGACTA B: CTCCATGTGCCATGTACAGC FIP: AGCTCCCTGGGTGGTCTAAGTTTTTCGTGCAACTACTGACGACTG BIP ^b : <u>CATGCCCAAGACGCACCTTAA</u> TTTTGCCTCTGCAGCGCCCTATCA |
| Set 7 | 5'NTR | This study | F: GGGTGGTCTAAGTCCTGAGT B: AGCCTAATAGTGGGCCTCTG FIP: GTCTTGGGCATGCCCTCGTCCGTCAGTAGTTTCGACGTGAG BIP: ACACCTTAACCCTAGCGGGGGCAGCACCCCTATCAGGTCGTA |
| Set 8 | 5'NTR | This study | F: AGGACTAGCCGTAGTGG B: ATCAGGTCTGACTCCCATCA FIP: GGCTTCTGCTCACGTCGAACTAAGCTCCCTGGGTGGTCTA BIP: ATGCTATGTGGACGAGGGCATGGTGTGATTTACCCTAGCGA |
| Set 9 | 5'NTR | This study | F: AGCTCCCTGGGTGGTCTA B: AGCCTAATAGTGGGCCTCTG FIP: GTCTTGGGCATGCCCTCGTCAGTACAGGACAGTCGTCAGT BIP: ACACCTTAACCCTAGCGGGGGCAGCACCCCTATCAGGTCGTA |

^a One additional nucleotide was added according to GenBank sequence (underlined)

^b A mistake in the published BIP primer sequence was corrected (underlined)

genotype 1.1 strain Alfort187 (CSF0902) were used as copy standards for determining assay sensitivity in genome equivalents per reaction (5 µl). In each run, samples as well as copy standards were tested in duplicate, and test results always represented the mean of both reactions.

Evaluation of published CSF-specific LAMP assays

A literature survey revealed that three different CSF-specific RT-LAMP assays (LAMP primer sets 4-6, Table 1) had been developed as one-tube assays to detect

Table 2 Test performance of CSFV-specific RT-LAMP assays used in this study

| Primer set | Reference strains subgenotype 1.1 | | | Cuban isolates (n = 4) |
|------------|-----------------------------------|--------------------|-------------------|------------------------|
| | CSF0902 (Alfort187) | CSF0940 (C-strain) | CSF0947 (Brescia) | |
| Set 1 | + | + | + | (+) |
| Set 2 | + | (+) | (+) | (+) |
| Set 3 | + | + | + | + |
| Set 4 | + | + | (+) | - |
| Set 5 | + | - | + | (+) ^a |
| Set 6 | + | - | - | - |
| Set 7 | - | - | - | nt |
| Set 8 | - | - | - | nt |
| Set 9 | - | - | - | nt |

-, negative (no fluorescence); (+), weak positive; +, positive; nt, not tested

^a only CSF0705 and CSF1058 detected

Table 3 Preparation of real-time RT-LAMP mix

| Reagent | Volume [μ l] | Final concentration | Company |
|-------------------------------|-------------------|---------------------|---------------------|
| Inner primers (FIP, BIP) | 1.0 | 2.0 μ M | MWG/Sigma-Aldrich |
| Outer primers (F, B) | 1.0 | 0.2 μ M | MWG/Sigma-Aldrich |
| dNTP | 2.5 | 1.0 mM each | Roche |
| AMV reverse transcriptase | 0.1 | 1 U | Promega |
| <i>Bst</i> DNA polymerase | 1.0 | 8 U | New England Biolabs |
| 10x ThermoPol Reaction Buffer | 2.5 | 1x | New England Biolabs |
| Betaine | 4.0 | 0.4 M | Sigma-Aldrich |
| RNase inhibitor | 0.5 | 5 U | Life Technologies |
| Syto-9 (1:250 diluted) | 0.5 | 0.4 μ M | Life Technologies |
| Aqua dest. | 7.9 | - | Thermo Scientific |
| Σ | 23.0 | - | - |

diagnostic range of the real-time RT-LAMP assay was evaluated with RNA from different CSFV genotypes. Twenty-five isolates representing the variability of CSFV were selected from the CSF virus collection of the EU and OIE RL in Hannover, comprising 11 isolates of genotype 1, 12 isolates of genotype 2, and two isolates of the uncommon genotype 3 (Table 4). Genotype 1 isolates included the 1.4 reference strain “Margarita” and the recent 1.4 isolate “Pinar del Rio” from Cuba, isolates of subgenotype 1.1 and 1.2 comprising three vaccine strains and two representatives of subgenotype 1.3, which is of relevance in South and Central America. Real-time RT-LAMP was performed in duplicate and in three independent runs. To determine the analytical sensitivity for the Cuban genotype 1.4 isolates, serial tenfold dilutions of RNA prepared from supernatants of infected cell cultures were analysed, as well as negative pig sera spiked with cell culture supernatants of PK15 cells infected with CSF1058 “Pinar del Rio” or CSF0705 “Margarita”.

Animal experiments

Due to the lack of suitable and well-characterised sample material from the field, diagnostic sample material was obtained from two independent infection experiments. These were carried out as part of the official function of the EU and OIE RL to study pathogenicity and gain reference material of recent CSFV isolates. Animal experiments were performed according to national and EU legislation. Six- to ten-week-old piglets were obtained from a commercial pig breeder and were hosted in the high containment unit of the EU and OIE RL for 1-2 weeks before infection. Prior to infection, all animals tested negative for pestivirus antibodies and genomes and were clinically healthy on the day of infection. For each trial, five animals were infected oronasally with 1×10^6 TCID₅₀ of either reference strain “Margarita” or field strain “Pinar del Rio”. Pigs were sampled (full blood for serum preparation and anti-coagulated blood samples for haematology analysis) at 0, 4,

Table 4 Diagnostic range and repeatability of real-time RT-LAMP assay

| Isolate characteristics | | | Results of real-time RT-LAMP | | | RT-qPCR | |
|-------------------------|-------------------|-----|------------------------------|---------------|---------------|--------------------------|------------------------|
| Cat. number | Name | gt | LAMP #1 [min] | LAMP #2 [min] | LAMP #3 [min] | Result [Cq] ¹ | Quantity [copies/5 µl] |
| CSF0705 | Margarita | 1.4 | 14.6/14.7 | 13.6/13.7 | 14.0/14.0 | 19.0 | 2.8E+06 |
| CSF1058 | Pinar del Rio | 1.4 | 15.1/14.9 | 14.8/14.4 | 14.7/14.6 | 20.0 | 1.4E+06 |
| CSF0902 | Alfort187 | 1.1 | 17.0/17.2 | 15.9/16.1 | 16.1/16.0 | 18.2 | 4.5E+06 |
| CSF0940 | C-strain vacc. | 1.1 | 20.8/20.4 | 19.1/18.6 | 18.7/19.0 | 16.9 | 1.1E+07 |
| CSF0947 | Brescia | 1.1 | 28.3/27.9 | 27.3/25.9 | 26.4/26.6 | 16.7 | 1.3E+07 |
| CSF0383 | TVM vaccine | 1.2 | 17.3/17.3 | 16.1/16.4 | 16.3/16.3 | 19.6 | 1.8E+06 |
| CSF0697 | RU vaccine | 1.2 | 19.0/19.2 | 17.7/17.7 | 18.1/18.3 | 22.2 | 3.3E+05 |
| CSF0929 | Brescia | 1.2 | 18.9/18.5 | 17.1/17.6 | 17.9/17.9 | 16.2 | 1.8E+07 |
| CSF0932 | Baker A | 1.2 | 17.8/17.9 | 16.8/16.6 | 16.7/17.0 | 18.4 | 4.1E+06 |
| CSF0306 | | 1.3 | 27.4/26.8 | 24.5/25.5 | 24.4/24.8 | 17.0 | 1.0E+07 |
| CSF0650 | | 1.3 | 19.6/20.2 | 18.5/18.7 | 19.0/18.7 | 22.3 | 2.9E+05 |
| CSF0021 | | 2.1 | -/- | -/- | -/- | 20.8 | 8.1E+05 |
| CSF0277 | Paderborn | 2.1 | -/- | -/19.2 | -/- | 19.4 | 2.0E+06 |
| CSF1048 | Panevezys | 2.1 | -/- | -/- | -/- | 17.4 | 7.7E+06 |
| CSF0014 | | 2.2 | -/- | -/- | -/- | 16.5 | 1.5E+07 |
| CSF0378 | | 2.2 | -/- | -/- | -/- | 18.8 | 3.0E+06 |
| CSF0573 | Parma | 2.2 | -/- | -/- | -/- | 17.7 | 6.6E+06 |
| CSF0906 | Bergen | 2.2 | -/- | -/- | -/- | 18.3 | 4.2E+06 |
| CSF0496 | | 2.3 | 30.7/- | -/- | -/- | 18.5 | 3.9E+06 |
| CSF0729 | | 2.3 | -/22.4 | -/- | -/- | 19.8 | 1.6E+06 |
| CSF0867 | | 2.3 | -/- | -/- | -/- | 18.2 | 4.5E+06 |
| CSF0904 | Alfort-Tueb. | 2.3 | -/- | 25.7/- | -/- | 21.3 | 5.9E+05 |
| CSF1027 | | 2.3 | -/- | -/- | 22.3/- | 16.8 | 1.2E+07 |
| CSF0309 | Kanagawa | 3.4 | -/- | -/- | -/- | 18.5 | 3.7E+06 |
| CSF0410 | Congenital Tremor | 3.1 | -/- | -/- | -/- | 18.9 | 2.9E+06 |

gt, genotype; -, no RT-LAMP amplification or amplification times of ≥ 35 min

7, 11 and 14 days postinfection (dpi) in order to obtain defined material for evaluating the RT-LAMP (Fig. 3). The clinical course of the disease was documented by body temperature and clinical scores according to Mittelholzer et al. [21] (scoring with slight modifications). In addition, haematological parameters (leucocyte and thrombocyte counts) were determined using a haematology analyser (Abacus Junior vet/130464, Guder Medizintechnik, Bad Oeynhausen, Germany). On the day of euthanasia, each animal was subjected to post-mortem examination, and reference material (organ and serum samples) were taken and archived. For final sera, neutralising antibodies were quantified by virus neutralisation assay [22].

Diagnostic test sensitivity and repeatability of the one-step real-time RT-LAMP

For determining diagnostic test sensitivity, defined material from two animal infection experiments was used. Three independent real-time RT-LAMP runs were performed

with samples in duplicate and compared to duplicates of an approved RT-qPCR. In total, 45 sera were analysed, comprising 17 sera of animals infected with CSF0705, 18 sera of animals infected with CSF1058, and ten sera of the same pigs taken prior to infection.

Results

Performance of previously published CSFV-specific RT-LAMP protocols

None of the previously published one-step RT-LAMP assays were able to detect all three investigated reference isolates of subgenotype 1.1, even in RNA preparations obtained from strongly CSFV-positive cell culture supernatants [13, 14, 16]. In addition, none of the RT-LAMPs were able to detect all of the Cuban isolates (Table 2). The overall poor performance of these one-step RT-LAMP protocols demonstrated that none of these assays were

promising candidates for diagnostic purposes and for adaptation to Cuban CSFV isolates. Only the E2-specific RT-LAMP (primer set 5) was able to detect two (CSF0705 and CSF1058) out of four tested Cuban isolates [16]. As expected, analysis of the respective E2 coding region showed that there was a low degree of conservation in the target sequences of the Cuban CSFV isolates. Therefore, primer set 5 was deemed unsuitable for further development (data not shown). Against this background, development of a new one-step RT-LAMP became necessary.

Identification of a suitable primer set

New RT-LAMP primer sets were designed, targeting conserved sequences in the NS5B region (sets 1 and 2) and the 5'NTR (sets 7-9) (Table 1). Primer sequences were based on sequences of subgenotype 1.1 isolates, as the corresponding sequences of Cuban isolates were not available. These primer sets were tested with a defined CSFV panel containing three reference CSFV strains of subgenotype 1.1 and four Cuban isolates. Strikingly, the three 5'NTR-based RT-LAMPs were unable to detect any of the three selected subgenotype 1.1 reference strains. Due to this failure, Cuban isolates were not tested with those primer sets (Table 2). In contrast, LAMP primer sets 1 and 2 were able to detect all three subgenotype 1.1 strains as well as the four Cuban isolates. Primer set 1 gave the best amplification for all initially tested strains/isolates (Table 2). In order to achieve the best possible detection of Cuban isolates, the NS5B sequences of the target region were determined (Fig. 1). Accordingly, the primer sequences of set 1 were adjusted to the corresponding Cuban sequences, and for further development of the real-time RT-LAMP assay, the resulting primer set 3 was applied (Table 1, Fig. 1). For recent isolates there was only one variable position in the sequence targeted by the outer backward primer (B primer), and therefore, degeneracy was introduced at one nucleotide position (Fig. 1). Only primers of the highest possible purity allowed sensitive detection of viral RNA, and some primer batches of certified high quality proved unsuitable for LAMP. The best amplification results for real-time RT-LAMP were obtained with primer set 3 at 57 °C for 35 minutes using 2 µl of the sample in a 25-µl reaction mix.

Determination of the diagnostic range using primer set 3 for real-time RT-LAMP was performed with a panel of infectious cell culture supernatants containing 25 strains/isolates of distinct genotypes and subgenotypes. The results revealed a broad detection of genotype 1 isolates (Table 4). In contrast, isolates of genotypes 2 and 3 were not detected by this real-time RT-LAMP. The highly positive result for the two Cuban isolates ("early" detection after 13-15 min in the real-time setup) indicated a high sensitivity for

subgenotype 1.4 isolates. These results demonstrate that the newly developed real-time RT-LAMP assay is able to detect other subgenotypes within genotype 1 as well, albeit with decreased sensitivity for some of them (e.g., CSF0947, CSF0306).

Analytical test performance

To determine the analytical sensitivity for detecting the Cuban isolates, serial tenfold dilutions of RNA isolated from supernatants of infected cell cultures were analysed (Fig. 2). Depending on the CSFV isolate, the detection limit was similar or up to 100 times lower when compared to the RT-qPCR gold standard. RNA dilutions containing 40-140 genome copies per LAMP reaction (2 µl RNA preparation) were reliably detected for all isolates, with amplification times below 25 minutes (Fig. 2). In the following RNA dilution, three out of eight duplicate samples remained negative, but one of these duplicates was also negative in the RT-qPCR (CSF0705). The two LAMP-negative RNA dilutions were PCR positive (5 µl RNA preparation/PCR) in both duplicates with quantification cycle (Cq) values ranging between 32.4 (CSF1056) and 34.8 (CSF1057). RNA dilutions containing about 1-20 viral genome copies/LAMP are obviously close to the detection limit. The final RNA dilutions that tested positive in both duplicates by real-time RT-LAMP contained between six (CSF1058) and 140 copies (CSF1056) per LAMP reaction and tested positive in RT-qPCR, with Cq values between Ct 29.8 (CSF1056) and 34.4 (CSF1058). Accordingly, RNA of isolate CSF1058 was detected with sensitivity comparable to that of RT-qPCR (Fig. 2).

Additionally, the test sensitivity was estimated using negative pig serum spiked with serial tenfold dilutions of infectious cell culture supernatants of CSF0705 or CSF1058 (Table 5). The spiked sera revealed a detection limit of approximately 24 copies for strain CSF0705 and approximately 72 copies per LAMP reaction for isolate CSF1058. Final real-time RT-LAMP positive results in both duplicate samples were obtained with samples that tested positive in RT-qPCR with Cq values of 34.6 (CSF0705) and 33.1 (CSF1058), respectively. Based on the fact that dilutions of samples with expected Cq values between 35 and 40 remained negative in the LAMP assay, real-time RT-LAMP for detection of isolates CSF0705 and CSF1058 is up to 100 times (six Cq values) less sensitive than the gold standard RT-qPCR protocol.

Clinical disease in pigs infected with Cuban CSFV isolates

In order to obtain information on the pathogenicity of Cuban strains/isolates and to obtain defined diagnostic

Fig. 2 Analytical sensitivity of real-time RT-LAMP compared to RT-qPCR. Symbols represent the mean Cq value of duplicates obtained from serial tenfold RNA dilutions of the four Cuban CSFV isolates. Columns represent duplicates of amplification time required for giving a LAMP-positive result. LAMP results were judged positive when the amplification time was ≤ 35 minutes. RT-qPCR results were classified as negative when Cq values were >40 . Ranges of copy numbers per RNA dilution of the real-time RT-LAMP are given below the graphs. For each real-time RT-LAMP reaction, 2 μ l RNA was used

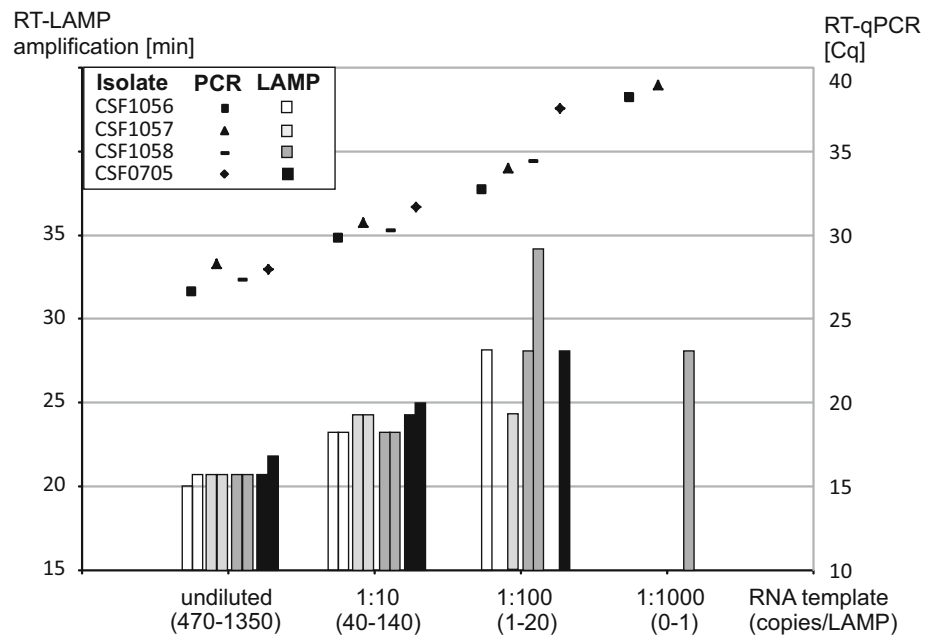


Table 5 Analytical sensitivity of the real-time RT-LAMP assay determined with CSFV spiked pig serum

| Sample characteristics | | Real-time RT-LAMP | | RT-qPCR ^a | |
|------------------------|----------------------------|-------------------|------------------------|----------------------|-----------------------|
| Virus | Dilution in negative serum | Result [min] | Quantity [copies/LAMP] | Result [Cq] | Quantity [copies/PCR] |
| CSF0705 | 10^{-3} | 21.6/22.2 | 2960 | 27.7 | 7400 |
| | 10^{-4} | 26.8/25.2 | 396 | 30.6 | 990 |
| | 10^{-5} | 25.3/30.2 | 24 | 34.6 | 60 |
| | 10^{-6} | 23.1/- | 2 | 38.1 | 6 |
| | 10^{-7} | -/- | - | - | - |
| | 10^{-8} | -/- | - | - | - |
| CSF1058 | 10^{-3} | 21.0/21.1 | 3360 | 27.5 | 8400 |
| | 10^{-4} | 22.2/25.4 | 480 | 30.3 | 1200 |
| | 10^{-5} | 22.3/32.4 | 72 | 33.1 | 180 |
| | 10^{-6} | -/- | - | - | - |
| | 10^{-7} | -/28.2 | - | - | - |
| | 10^{-8} | -/- | - | - | - |

-, negative: no real-time RT-LAMP amplification or amplification times of ≥ 35 min, no amplification in RT-qPCR; ^a mean value of duplicate results

reference material, infection experiments were carried as part of the mission of the EU and OIE RL. Groups of five pigs each were inoculated with reference strain “Margarita” (CSF0705) or the recent field isolate “Pinar del Rio” (CSF1058). CSFV strain “Margarita” displayed moderate to high virulence. Starting from day 4 postinfection until the end of the experiment (up to 31 dpi) all pigs showed fever (peaks between 41.4 °C and 41.8 °C) and clinical scores of 11-12 points. Infected pigs showed considerable clinical signs, including reduced liveliness, non-physiological body tension, reduced appetite, profuse diarrhoea, loss of body mass, walking abnormalities, and

conjunctivitis. Two out of five infected animals had to be euthanised on day 11 postinfection for animal welfare reasons. In contrast to reference strain “Margarita”, results from an infection experiment with recent Cuban isolate “Pinar del Rio” are indicative of the low pathogenicity of this isolate. Only on day nine postinfection was body temperature slightly elevated (39.7 °C-40 °C), and clinical symptoms scoring 3 points were detected. The observed symptoms were nonspecific; pigs displayed reduced liveliness, reduced appetite and mild conjunctivitis. None of the infected pigs showed fever or clinical signs after day 9 postinfection. Nevertheless, all pigs of the “Pinar del Rio”

group seroconverted after infection, reaching titers of 60–1920 (50 % neutralizing dose; ND₅₀) in a neutralisation assay carried out with the homologous virus. Haematological parameters underlined the observed differences between the isolates. Animals infected with strain CSF0705 “Margarita” showed a significant drop in leucocyte and thrombocyte counts, whereas these parameters were only slightly affected in the group of pigs infected with the field isolate “Pinar del Rio” (data not shown).

Diagnostic test sensitivity with defined pig sera from an animal experiment

A diagnostic test panel comprising sera from a total of 10 infected pigs (sampled at different days postinfection) was compiled. All of the 17 samples derived from pigs infected with CSF0705 “Margarita” tested positive in RT-qPCR and also in real-time RT-LAMP, independent of the time point of infection (Table 6), whereas five sera taken prior to infection tested negative. Viral RNA loads were calculated to be between 250 and 880 copies per PCR reaction (5 µl RNA preparation) at early time points postinfection (4 dpi), whereas at later time points (14 dpi), viral RNA loads of up to 7.5×10^6 copies per reaction were detected. As the CSFV-LAMP performed with 2 µl of the RNA preparation reliably detected all samples taken on day 4 dpi, it can be concluded that the assay is capable of detecting 100–352 copies (Table 6). CSF1058 “Pinar del Rio” proved to be a CSF isolate of low virulence, as it caused only mild symptoms and only slightly elevated rectal temperatures (Fig. 3). Analysis of sera (n = 18) from pigs infected with CSF1058 “Pinar del Rio” revealed considerably lower amounts of viral RNA. In the RNA prepared from samples taken at different time points postinfection, there were generally fewer than 880 copies per PCR reaction (352 copies/LAMP), and only in a single sample were slightly higher amounts of RNA detected (animal 387, 11 dpi: 2500 copies/PCR or 1000 copies/LAMP). Analysis by RT-qPCR gave either negative results or Cq values higher than 34.0 with the exception of four samples taken on 7 dpi or 11 dpi from animals 397, 398 and 400 (Table 6). With regard to the real-time RT-LAMP assay, the amounts of viral RNA present in the majority of samples were close to or at the detection limit. This was clearly shown by the fact that in six sera with high Cq values, the LAMP assay was only capable of detecting viral RNA in three or fewer runs out of the six runs performed. The four samples with the slightly higher genome loads (Ct 29.8–32.2) contained approximately 132–1000 genome equivalents per LAMP reaction and repeatedly tested positive by RT-LAMP. Out of these four sera, the serum with the highest Cq value (Cq 32.2, 132 copies per LAMP) originating from animal 400 (11 dpi) gave a false negative

result in one out of six LAMP runs. Taken together, the test results of RT-PCR and real-time RT-LAMP analyses clearly indicate that the replication rate of isolate CSF1058 “Pinar del Rio” in the host is very low. This finding is in line with the mild clinical symptoms in the infected animals. In addition, viremia caused by this low-virulent isolate turned out to be very short, as six out of 18 samples (taken at 4 dpi and 14 dpi) did not contain detectable amounts of viral RNA (Table 6, Fig. 3).

Discussion

Development of LAMP technology introduced a promising easy-to-use molecular biological method for sensitive and rapid detection of CSFV. The major aim of the present study was to establish a CSFV-specific RT-LAMP one-tube assay for detecting CSFV isolates in Cuba. As none of the previously published CSFV-specific RT-LAMP one-tube assays provided satisfactory test performance, adaptation of an already established test was not possible. The reason for the failure or bad performance of some of the previously published CSFV RT-LAMP assays, in particular the one using primer set 6 [13], is still unknown. For all LAMP primer sets, the same standardised protocol was used, with only minor variations from some of the published protocols. Significantly better performance can be achieved when these LAMP assays are not performed in a one-step protocol but in a two-step protocol where cDNA is added directly to the LAMP reaction (data not shown). In contrast, the newly developed one-step RT-LAMP assay allowed specific detection of all available Cuban CSFV isolates, regardless of the year or region of isolation. Broad reactivity within genotype 1 showed that this RT-LAMP assay is sufficiently robust to tolerate a certain number of mutations in the target region (NS5B). With regard to Cuban CSFV isolates, the average detection limit ranged from 100 to 1000 copies per reaction. Thus, it can be concluded that RT-LAMP is only about 10–100 times less sensitive than the RT-qPCR applied at the EU and OIE RL for CSF. RT-qPCR was chosen as the reference assay, as its sensitivity is superior to virus isolation and antigen ELISAs and is considered to be the gold standard for viral genome detection. In addition, diagnostic kits like antigen-capture ELISAs are often not available in Cuba (due to import restrictions), and therefore, comparison of the performance with RT-qPCR was most suitable and of particular interest. Only recently, a one-step CSFV-LAMP with a broader detection range was developed that was reported to provide a sensitivity comparable to that of RT-qPCR when amplicons were detected by lateral flow devices [15]. *In silico* analysis revealed that only a few mismatches are present in the targeted regions of the 5′NTR when comparing the

Table 6 Diagnostic sensitivity and repeatability of the real-time RT-LAMP assay

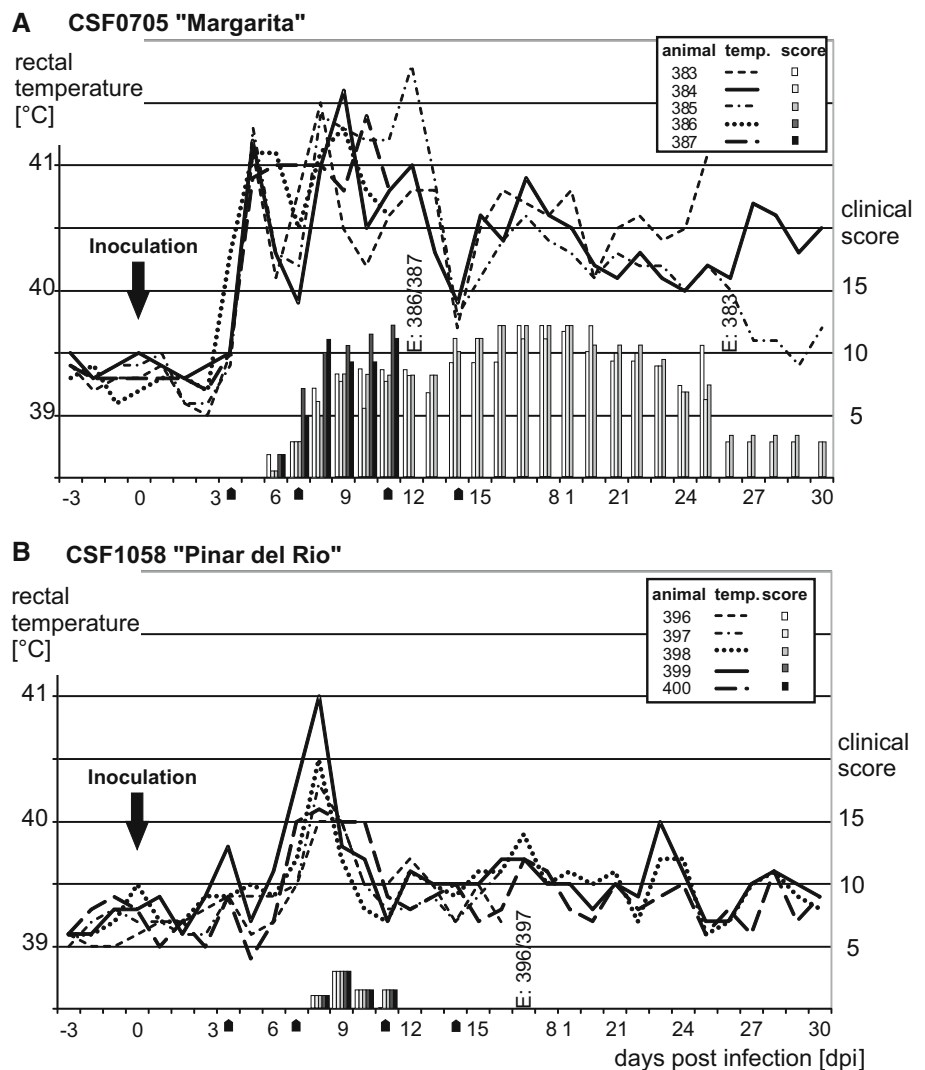
| Sera | | | Real-time RT-LAMP | | | | RT-qPCR ^a | |
|-------------------------|-----------|--------------------|-------------------|-----------------|-----------------|------------------------|--------------------------|------------------------|
| Inoculum | Animal ID | Sampling day [dpi] | Result #1 [min] | Result #2 [min] | Result #3 [min] | Quantity [copies/2 µl] | Result [Cq] ¹ | Quantity [copies/5 µl] |
| CSF0705 “Margarita” | 383-387 | 0 | -/- | -/- | -/- | - | - | - |
| | 383 | 4 | 27.3/24.8 | 27.0/32.3 | 26.8/22.4 | 232 | 30.9 | 580 |
| | 384 | | 25.7/30.6 | 25.5/24.8 | 24.0/25.1 | 132 | 31.7 | 330 |
| | 385 | | 24.2/28.9 | 25.1/25.6 | 25.4/23.7 | 100 | 32.1 | 250 |
| | 386 | | 23.4/25.0 | 24.1/24.7 | 24.3/22.4 | 352 | 30.3 | 880 |
| | 387 | | 23.5/25.7 | 21.6/23.7 | 23.7/22.4 | 276 | 30.2 | 690 |
| | 383 | 7 | 16.9/17.0 | 16.7/17.0 | 16.4/16.4 | 1.8E+05 | 21.0 | 4.5E+05 |
| | 384 | | 17.3/17.7 | 17.0/17.3 | 16.7/16.7 | 1.0E+05 | 21.9 | 2.6E+05 |
| | 385 | | 16.5/16.7 | 16.0/16.2 | 16.0/15.9 | 1.9E+05 | 20.9 | 4.8E+05 |
| | 386 | | 17.8/17.7 | 17.4/17.4 | 16.9/17.0 | 7.2E+04 | 22.3 | 1.8E+05 |
| | 387 | | 16.6/16.8 | 16.6/16.4 | 15.9/16.1 | 1.6E+05 | 21.2 | 3.9E+05 |
| | 383 | 11 | 14.5/14.7 | 14.7/14.4 | 13.9/14.0 | 1.2E+06 | 18.3 | 2.9E+06 |
| | 384 | | 14.5/14.6 | 14.4/14.5 | 13.7/14.0 | 1.4E+06 | 17.9 | 3.6E+06 |
| | 385 | | 13.9/14.2 | 13.8/14.0 | 13.3/13.9 | 1.6E+06 | 17.8 | 3.9E+06 |
| | 386 | | 15.4/15.6 | 15.6/15.6 | 14.8/15.2 | 6.4E+05 | 19.1 | 1.6E+06 |
| | 387 | | 14.9/15.3 | 14.8/14.8 | 14.1/14.4 | 8.4E+05 | 18.7 | 2.1E+06 |
| | 384 | 14 | 14.5/15.3 | 14.6/14.7 | 13.8/14.4 | 1.4E+06 | 18.0 | 3.4E+06 |
| | 385 | | 13.8/13.9 | 13.7/13.6 | 13.5/13.5 | 3.0E+06 | 16.8 | 7.5E+06 |
| CSF1058 “Pinar del Rio” | 396-400 | 0 | -/- | -/- | -/- | - | - | - |
| | 396 | 4 | -/- | -/- | -/- | - | - | - |
| | 397 | | -/- | -/- | 29.4/- | 13 | 35.9 | 33 |
| | 398 | | -/- | -/- | -/- | - | - | - |
| | 399 | | -/- | -/- | -/- | - | - | - |
| | 400 | | -/- | -/- | -/- | - | - | - |
| | 396 | 7 | -/- | 27.5/- | -/- | 1 | 39.5 | 3 |
| | 397 | | 26.5/- | 25.3/- | -/- | 28 | 34.9 | 69 |
| | 398 | | 21.4/22.4 | 19.8/21.3 | 19.6/19.8 | 352 | 31.7 | 880 |
| | 399 | | -/- | -/- | -/- | - | - | - |
| | 400 | | 22.0/23.9 | 22.8/22.5 | 21.6/22.0 | 140 | 32.1 | 350 |
| | 396 | 11 | -/- | -/- | -/33.7 | 31 | 34.7 | 77 |
| | 397 | | 22.9/22.3 | 22.1/21.7 | 20.7/21.5 | 1000 | 29.8 | 2500 |
| | 398 | | 33.2/- | -/- | -/- | 3 | 37.8 | 7 |
| | 399 | | 28.9/- | 24.6/- | 30.9/- | 6 | 36.7 | 16 |
| | 400 | | 26.8/20.9 | 20.8/22.4 | 26.7/- | 132 | 32.2 | 330 |
| | 398 | 14 | -/- | -/- | -/- | - | - | - |
| | 399 | | -/- | -/- | -/- | - | - | - |
| 400 | | -/- | -/- | -/- | 10 | 35.9 | 26 | |

-, negative: no real-time RT-LAMP amplification or amplification times of ≥ 35 min, no amplification in RT-qPCR; ^a mean of duplicates

published primers with the sequences of the Cuban isolates [8]. A second CSF-specific LAMP capable of detecting Cuban CSFV isolates would be highly desirable, as it might be a valuable alternative assay in cases whether there is a need for further confirmation, e.g., when there are doubtful results or when problems with amplicon-borne contamination occur.

So far, CSFV-specific LAMP has not been applied for routine diagnosis of CSF. The unique CSF situation in Cuba, namely the low diversity of Cuban CSFV isolates in combination with a low risk for new CSFV introduction (due to the geographical and political situation and the lack of significant trade of pigs and pork meat with other countries) enhance the chances of successful

Fig. 3 Clinical course of infection of pigs experimentally infected with CSF0705 “Margarita” (n = 5) and CSF1058 “Pinar del Rio” (n = 5). Lines represent rectal temperatures and columns depict clinical scores of pigs infected with CSF0705 “Margarita” (A) and CSF1058 “Pinar del Rio” (B). Black arrows indicate time points of infection (top) and sampling (bottom). For animals 383, 386, and 387, the time points of euthanasia are indicated (E: identifier of animal)



implementation of a LAMP assay as a supportive diagnostic tool for regional laboratories. Currently, further testing in suspected CSF cases in Cuba can be performed by immunoperoxidase assay on organ sections by the National Reference Laboratory (NRL) in Havana. Samples that have to be characterised further by conventional or RT-qPCR are forwarded to CENSA. The lack of modern molecular biological techniques in the regional laboratories and in the NRL has been identified as being one critical point for rapid and reliable CSF diagnosis. In particular, the need for several conserved regions in the target sequence hampers development and successful application of a LAMP-based diagnostic assay for detecting highly variable RNA viruses like CSFV. RT-LAMP is more vulnerable to isolate-specific sequence variation than, for example, RT-qPCR. The main argument for implementation of a LAMP assay instead of an RT-PCR-based technique in Cuban regional laboratories is the possibility to perform CSF

diagnosis without sophisticated equipment, which is expensive and requires regular maintenance and technical support. In addition, the developed assay has the capacity to speed up CSF diagnosis at the local level significantly, as test results can already be obtained after 35 minutes of amplification.

Despite these advantages of LAMP-based methods, their use for diagnostic purposes in the field requires a thorough understanding of assay limitations, which have been reasonable for limited application for diagnostic purposes so far. In general, one observed method-related limitation of LAMP assays seems to be a certain intrinsic risk of producing false positive results [23]. For this reason, the amplification process of the developed assay must be terminated on time (for the developed CSFV-specific RT-LAMP, after 35 min), and results need to be interpreted immediately. Furthermore, there is a general risk of cross-contamination, in particular, when highly sensitive

amplification-based assays are performed on-site at premises not optimised for molecular biology techniques (even when the assay is performed in a one-tube format). Hence, it is highly recommended to include more than one non-template control and, in addition, several negative samples as extraction controls per run, with the total number of controls depending on the number of samples analysed. Assay runs without appropriate controls cannot provide valid results, as they cannot be interpreted properly. RT-LAMP implementation requires well-trained staff regarding molecular biology techniques in general (e.g., RNA preparation) and LAMP assay in particular. The implementation of a LAMP-based assay for first-line diagnosis of CSF in Cuba is an achievable but challenging and ambitious aim. First steps of method transfer to the Cuban NRL and the regional laboratories are planned. Once the general implementation of LAMP technology in the Cuban laboratories is successfully completed, it will be tested whether, in addition to the newly developed assay tailored to the endemic CSFV isolates, further new developments, like the more generic approach of Chowdry and co-workers are suitable under Cuban conditions as well [15].

According to the data presented in this study, a set of recommendations can be listed for successful implementation and application of the developed assay in Cuba and for LAMP-based assays in general. First, CSF-specific RT-LAMP should not be used as a stand-alone assay. Results need confirmation by another test with comparable sensitivity (e.g., by RT-qPCR performed at CENSA) and must be interpreted in the context of clinical signs and the epidemiological situation. Second, RT-LAMP should be used at herd level, and not for individual samples, to ensure reliability of results. It is of utmost importance that samples are taken from diseased animals, preferably with strong clinical signs. Third, test performance still needs to be monitored under real “field” conditions in Cuba. One critical factor of test performance is a consistent high primer quality, which might be problematic under “field conditions”. This phenomenon is probably due to the complex folding of the primers and amplicons required for LAMP reaction. The crucial role of primer purity was already mentioned in the first description of the LAMP method, and HPLC-grade primers were therefore strongly recommended [12].

Against this background, a long-term available sensitivity control should be used, and the respective results should be documented over time to reveal problems of sensitivity, for example, caused by primer degeneration or insufficient reagent quality. After implementation in the regional laboratories, a field trial must be organised for testing CSF-suspected herds by RT-LAMP and RT-qPCR in parallel. This trial can help to evaluate whether a proper

performance of RT-LAMP is possible under field conditions in regional laboratories. Sustainable technical and scientific assistance should be implemented to ensure that RT-LAMP is used properly and test performance fulfils the requirements with regard to sensitivity and specificity. For this purpose, a regular (annual) ring trial for CSFV-LAMP assay at the national level would be a valuable tool.

When discussing possible limitations of CSFV-LAMP, the risk of failure to detect new virus variants or newly introduced CSFV subgenotypes must be mentioned. Therefore, the RT-LAMP assay is not applicable for the purpose of CSF surveillance and is only to be used for rapid confirmation of outbreaks. For surveillance, well-established RT-PCR assays are required, as these assays are generally able to detect new virus variants or the introduction of new virus subgenotypes. As RT-LAMP assays are vulnerable to mutations in the target region, primers need to be adapted regularly based on currently circulating isolates and the epidemiological situation.

The infection experiment showed that CSF0705 “Margarita” is a moderately to highly pathogenic CSFV strain, confirming previous descriptions of the strain [24–27]. In contrast, CSFV isolate “Pinar del Rio” induced only mild symptoms in the infected animals. This finding is in line with the field observation that there is a tendency towards a milder course of the disease in Cuba [9, 11]. Testing of serum samples obtained from the animal experiments demonstrated that the recent CSFV isolate “Pinar del Rio” caused only short-term viremia with very low levels of viral RNA present in the blood. Interestingly, a study dealing with the current CSF situation in Cuba indicated that current CSF isolates cause nonspecific clinical symptoms and are often combined with low genome loads [11]. Samples from three Cuban pig herds have been tested by RT-qPCR and most of the positive results displayed Cq values ≥ 35 [11]. The three positive herds were found in the regions Holguin (2009), Santiago de Cuba (2011) and Pinar del Rio (2010). The respective pig herds had been subjected to the Cuban CSF vaccination programme, which could be an explanation for the low replication rate of the field viruses. However, results of the infection experiment with CSF1058 “Pinar del Rio” isolated in 2010 (representative of current Cuban isolates) presented in this study also clearly demonstrated a mild course of disease. Short viremia and low genome loads in serum samples are not only due to previous vaccination but also have been observed in the experimentally infected unvaccinated pigs. Nevertheless, vaccination can further reduce the number of acute clinical cases, thus making CSF diagnosis even more difficult and challenging. With regard to the newly developed RT-LAMP assay, RNA levels determined after infection with field isolate CSF1058 “Pinar del Rio” were below or close to the detection limit. The detection limits

estimated with the help of CSF0705- and CSF1058-spiked serum samples (Table 5) are in line with results of samples obtained from the infection experiment (Table 6). The detection limit of samples originating from infection with isolate CSF1058 “Pinar del Rio” corresponds to viral RNA loads, resulting in Cq values of 32–34 determined by RT-qPCR. Taken together, detection of such isolates is problematic and highly dependent on optimal LAMP assay performance and a good sampling strategy.

The characteristics of isolate CSF1058 “Pinar del Rio” determined in this study resemble those of a vaccine strain. Based on the data obtained with experimentally infected animals, there is no indication of a chronic course of infection. In contrast, chronic courses of CSFV infection have been reported to occur frequently in Cuba [11]. Further characterisation of other currently circulating CSFV isolates (e.g., from the regions Santiago de Cuba and Holguin) with respect to pathogenicity is required in order to decide whether a change in CSF control strategy would be beneficial. At the moment, vaccination in Cuba is performed with a C-strain live-attenuated vaccine produced under governmental management in the country. So far, the vaccination programme implemented in Cuba has not led to the expected success to control clinical disease. It was observed that even in vaccinated herds, the induction of antibodies was unsatisfactory. The reason for this observation is still under investigation. Accordingly, the CSF control programme is currently under review, including evaluation of vaccine quality, maintenance of the cold chain until the time point of application, vaccination coverage, and other concerted control measures. Due to trade and import restrictions, the pig-production sector in Cuba in the near future will not have access to vaccines like the novel marker vaccine licensed in Europe. Nevertheless, the application of marker vaccines and implementation of the corresponding serological assays would be of great value and an important pillar of a powerful CSF control programme.

As long as establishment of PCR techniques in regional laboratories of countries like Cuba is not possible and CSF diagnosis is mainly based on pathological findings and immunostaining with polyclonal CSFV antiserum, assays like the CSFV-LAMP assay described in this study could be a valuable diagnostic tool when applied carefully and the limitations of the method are taken into consideration. The intended purpose of the newly developed CSFV-LAMP is to serve as a supportive tool for the establishment of diagnostic procedures and to provide rapid information in the event of a suspected outbreak. Although in this study we demonstrate good performance with RNA prepared from well-defined experimental serum samples, a detailed validation under real “field conditions” in Cuba is still required, including RNA preparations of different sample

materials obtained from different CSFV outbreaks. It is undisputable that strong diagnostic capacities have to be developed step-by-step, focusing on the establishment of a powerful CSF surveillance in Cuba. A possible future stop of mass-vaccination will allow the implementation of serological assays to detect CSF in an unvaccinated pig population.

Acknowledgments This study is dedicated to our deceased colleague Irene Greiser-Wilke. She initiated the OIE twinning project between CENSA, Cuba, and the EU and OIE RL for CSF, Germany, and had the vision to implement a LAMP assay for CSF diagnosis in Cuba. The study was financed by the OIE in the framework of the twinning project “Support to the CENSA Laboratory for CSF for the Planned Establishment of an OIE Reference Laboratory” and by the Institute of Virology, University of Veterinary Medicine in Hannover. We wish to thank the entire staff of the EU and OIE RL for CSF. We are grateful to have with Monika Berg, Holger Mosch and Günter Thiem an outstanding animal care team. We thank Benjamin Ostermann for his support in the initial phase of the study, and Inga Grotha for her tireless efforts and excellent technical assistance.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Moennig V, Floegel-Niesmann G, Greiser-Wilke I (2003) Clinical signs and epidemiology of classical swine fever: a review of new knowledge. *Vet J* 165(1):11–20
2. Edwards S, Fukusho A, Lefevre PC, Lipowski A, Pejsak Z, Roehe P, Westergaard J (2000) Classical swine fever: the global situation. *Vet Microbiol* 73(2–3):103–119
3. Pereda AJ, Greiser-Wilke I, Schmitt B, Rincon MA, Mogollon JD, Sabogal ZY, Lora AM, Sanguinetti H, Piccone ME (2005) Phylogenetic analysis of classical swine fever virus (CSFV) field isolates from outbreaks in South and Central America. *Virus Res* 110(1–2):111–118
4. Sabogal ZY, Mogollon JD, Rincon MA, Clavijo A (2006) Phylogenetic analysis of recent isolates of classical swine fever virus from Colombia. *Virus Res* 115(1):99–103
5. Arainga M, Hisanaga T, Hills K, Handel K, Rivera H, Pasick J (2010) Phylogenetic analysis of classical swine fever virus isolates from Peru. *Transbound Emerg Dis* 57(4):262–270
6. OIE (2013) WAHID home page. http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasetimes
7. Paton DJ, McGoldrick A, Greiser-Wilke I, Parchariyanon S, Song JY, Liou PP, Stadejek T, Lowings JP, Bjorklund H, Belak S (2000) Genetic typing of classical swine fever virus. *Vet Microbiol* 73(2–3):137–157
8. Postel A, Schmeiser S, Perera CL, Rodriguez LJ, Frias-Le-poureau MT, Becher P (2013) Classical swine fever virus isolates from Cuba form a new subgenotype 1.4. *Vet Microbiol* 161(3–4):334–338
9. de Arce HD, Ganges L, Barrera M, Naranjo D, Sobrino F, Frias MT, Nunez JI (2005) Origin and evolution of viruses causing classical swine fever in Cuba. *Virus Res* 112(1–2):123–131
10. de Arce HD, Nunez JI, Ganges L, Barreras M, Teresa Frias M, Sobrino F (1999) Molecular epidemiology of classical swine fever in Cuba. *Virus Res* 64(1):61–67
11. Perez LJ, Diaz de Arce H, Perera CL, Rosell R, Frias MT, Percedo MI, Tarradas J, Dominguez P, Nunez JI, Ganges L

- (2012) Positive selection pressure on the B/C domains of the E2-gene of classical swine fever virus in endemic areas under C-strain vaccination. *Infect Genet Evol* 12(7):1405–1412
12. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28(12):E63
 13. Chen HT, Zhang J, Ma LN, Ma YP, Ding YZ, Liu XT, Chen L, Ma LQ, Zhang YG, Liu YS (2009) Rapid pre-clinical detection of classical swine fever by reverse transcription loop-mediated isothermal amplification. *Mol Cell Probes* 23(2):71–74
 14. Chen L, Fan XZ, Wang Q, Xu L, Zhao QZ, Zhou YC, Liu J, Tang B, Zou XQ (2010) A novel RT-LAMP assay for rapid and simple detection of classical swine fever virus. *Virol Sin* 25(1):59–64
 15. Chowdry VK, Luo Y, Widen F, Qiu HJ, Shan H, Belak S, Liu L (2014) 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. *J Virol Methods* 197:14–18
 16. Yin S, Shang Y, Zhou G, Tian H, Liu Y, Cai X, Liu X (2010) Development and evaluation of rapid detection of classical swine fever virus by reverse transcription loop-mediated isothermal amplification (RT-LAMP). *J Biotechnol* 146(4):147–150
 17. Zhang XJ, Han QY, Sun Y, Belak S, Liu L, Qiu HJ (2011) Development of a loop-mediated isothermal amplification for visual detection of the HCLV vaccine against classical swine fever in China. *J Virol Methods* 171(1):200–205
 18. Zhang XJ, Sun Y, Liu L, Belak S, Qiu HJ (2010) Validation of a loop-mediated isothermal amplification assay for visualised detection of wild-type classical swine fever virus. *J Virol Methods* 167(1):74–78
 19. Hoffmann B, Beer M, Schelp C, Schirmeier H, Depner K (2005) Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. *J Virol Methods* 130(1–2):36–44
 20. The Classical Swine Fever Database—Version 3 (2013) EU and OIE Reference Laboratory for Classical Swine Fever. <http://viro08.tiho-hannover.de/eg/csf/>
 21. Mittelholzer C, Moser C, Tratschin JD, Hofmann MA (2000) Analysis of classical swine fever virus replication kinetics allows differentiation of highly virulent from avirulent strains. *Vet Microbiol* 74(4):293–308
 22. Anonymous (2007) EU Diagnostic Manual for Classical swine fever (CSF) diagnosis: Technical part. EU and OIE Reference Laboratory for CSF, Homepage. http://viro08.tiho-hannover.de/eg/Technical_Annex_Draft_2007.pdf. Accessed 1 June 2014
 23. Postel A, Letzel T, Frischmann S, Grund C, Beer M, Harder T (2010) Evaluation of two commercial loop-mediated isothermal amplification assays for detection of avian influenza H5 and H7 hemagglutinin genes. *J Vet Diagn Invest* 22(1):61–66
 24. Tarradas J, Argilaguuet JM, Rosell R, Nofrarias M, Crisci E, Cordoba L, Perez-Martin E, Diaz I, Rodriguez F, Domingo M, Montoya M, Ganges L (2010) Interferon-gamma induction correlates with protection by DNA vaccine expressing E2 glycoprotein against classical swine fever virus infection in domestic pigs. *Vet Microbiol* 142(1–2):51–58
 25. Ganges L, Barrera M, Diaz de Arce H, Vega A, Nunez JI, Sobrino F, Frias MT (2007) Antigenic, biological and molecular characterization of the Cuban CSFV isolate “Margarita”. *Rev Salud Anim* 29(3):182–192
 26. Sanchez O, Barrera M, Rodriguez MP, Frias MT, Figueroa NE, Naranjo P, Montesino R, Farnos O, Castell S, Venereo A, Ganges L, Borroto C, Toledo JR (2008) Classical swine fever virus E2 glycoprotein antigen produced in adenovirally transduced PK-15 cells confers complete protection in pigs upon viral challenge. *Vaccine* 26(7):988–997
 27. Barrera M, Sanchez O, Farnos O, Rodriguez MP, Dominguez P, Tait H, Frias M, Avila M, Vega E, Toledo JR (2010) Early onset and long lasting protection in pigs provided by a classical swine fever E2-vaccine candidate produced in the milk of goats. *Vet Immunol Immunopathol* 133(1):25–32