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Evaluation of Single Step TaqMan Real-time PCR Assay Lateral to Conventional RT-PCR and Antigen-Capture ELISA for Pre-Clinical Detection of *Classical swine fever virus*

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A B S T R A C T

Classical swine fever (CSF) is a highly contagious and devastating viral disease, causing serious losses in the pig industry worldwide. Rapid detection and identification of the causative agent is a crucial step in controlling CSF infection in pig population. In the present study, a fluorogenic-probe hydrolysis (TaqMan)-reverse transcriptase real time PCR assay (RT-qPCR)was evaluated parallel to conventional RT-PCR and antigen capture ELISA to detect Classical swine fever virus (CSFV) in the pre-clinical phase of the disease. In addition, hematological analysis was performed at different clinical phases in order to diagnose CSF pre-clinically. Thrombocytopenia and leucopenia were early clinical clues recorded in CSFV infected pigs. Single step RT-qPCR confirmed the presence of CSFV nucleic acid in blood, nasal swabs, ocular swabs as well as in tonsillar scrapings in the pre-clinical phase. CSFV nucleic acid was detected with maximum positivity in blood and tonsillar scrapings (70-73%) using RT-qPCR as compared to 60% and 33.33-40% positivity in conventional RT-PCR and Ag-ELISA, respectively. Thus, TaqMan based RT-qPCR assay can be used as an efficient assay for rapid CSFV detection at pre-clinical phase of the disease to contain the disease from in-contact infected pigs to susceptible population.

Introduction

Classical swine fever (CSF)is a highly contagious, economically devastating disease of domestic pigs, wild boars and pygmy hogs notifiable to the World Organization for Animal Health (OIE)(Depner *et al.,* 1994; Dewulf *et al.,* 2004). The disease is caused by *Classical Swine Fever virus* (CSFV), a positive-sense, enveloped virus belonging to the genus *Pestivirus* of the family *Flaviviridae* (https://talk.ictvonline.org/ictv-). CSFV can be transmitted horizontally from an infected animal to susceptible populations through direct contact as well as vertically from an infected sow to off springs through transplacental transmission (Barman, 2018).Indirectly, CSFV can be transmitted through biological vectors (wild boars), artificial insemination, contaminated garbage/swill feed and mechanical transmission via humans or agricultural and veterinary equipments(De Smit *et al.,* 1999; Ribbens *et al.,* 2004; Blome *et al.,* 2017). The principal mode of entry of CSFV in pigs under natural infections is the oro-nasal route although other possible routes such as the conjunctival, genital mucous membranes and skin abrasions have been described(Floegel *et al.,* 2000; Blome *et al.,* 2017). The incubation period of CSFV is typically 3-10 days following an infection (Postel *et al.,* 2018).

The primary site of CSFV replication is tonsils through which it reaches the peripheral blood causing a high level of viraemia (Stewart, 1981; Van Oirschot, 1999; Barman, 2018). Depending upon the virulence of CSFV, host age, status of individual or herd immunity, CSFV exhibits anacute, chronic and persistent disease mode in host animals (Isoda *et al.,* 2020).Typical clinical signs of CSFV in natural infections include high fever (>40℃), respiratory distress, neurological symptoms (convulsions, uncoordinated movement and staggering gait) and skin haemorrhages (Postel *et al.,* 2018). However, these symptoms are seldom visible in animals infected with strains of varied virulence and infected animals might develop a mild, chronic or unapparent form of the disease (Tarradas *et al.,* 2014). Such uncharacteristic profiles of clinical symptoms complicate with the proper diagnosis of the disease.

Rapid, sensitive and specific pre-clinical diagnostic methods are necessary for early identification of infected herds to contain further spread of the disease and to control CSF epidemics. Detection of CSFV in live animals has been performed traditionally by a combination of antigen detection and virus isolation using blood samples (Kaden *et al.,* 1999). Antibody against CSFV can be detected by virus neutralization test or by antibody-ELISA but infected antibody appears 2-3 weeks of post infection (Ganges *et al.,* 2020).Therefore, to provide a precise diagnosis in the face of an outbreak, conventional methods of antigen detection (antigen capture ELISA)is practically not always feasible because of its low sensitivity. As a routine diagnostic tool, RT-PCR targeting a highly conserved viral gene is more sensitive in early detection of CSFV during the incubation period (OIE 2019). However, RT-PCR technique may provide false positive results due to laboratory contamination as well as false negative result due to inhibitors contained in the sample (OIE, 2019). In such situations, real-time PCR protocols (RT-qPCR) helps to increase the throughput, reduces the chance of carryover contamination and disables post-PCR processing as a potential source of error (Hoffmann *et al.,* 2005; Ciglenečki *et al.,* 2008).

The Northeastern states of India are known for pig rearing and the region possesses one third of country's pig population. CSF has attained an endemic status in this region and till date

diagnosis mostly relies on necropsy analysis and antigen detection by antigen-capture ELISAs. There is a growing demand for techniques which are simpler, sensitive and fast to detect CSFV and control further outbreaks. In the present study, we evaluated a single step TaqMan based real-time RT-PCR (RT-qPCR) in parallel to conventional RT-PCR and antigen capture ELISA(Ag-ELISA)for pre-clinical detection of CSFV in infected pigs from natural outbreaks reported from Assam.

Materials and Methods

Outbreak information

A total of 16 CSF outbreaks, consisting of six in organized Government pig farms and ten in small private owned pig units occurring in and around Guwahati, Assam, India was attended. Outbreaks were confirmed by CSFV E2 gene-based nested RT-PCR. In each CSFV affected farm/unit, animals were categorized as per pre-clinical (Group I), early clinical (Group II) and late clinical phase (Group III) of the disease based on clinical parameters(Table 1)previously described by Mittelholzer *et al.,* (2000)with some modifications.

Collection of biological samples and hematological investigation

Biological samples such as nasal and ocular swabs, tonsillar scrapings and whole blood collected from each animal at pre-clinical and late clinical phase of the disease were processed and tested by single step RT-qPCR, nested RT-PCR and Ag-ELISA. Haematological analysis was carried out in a total of 70 blood samples collected at pre-clinical (n=30) and late clinical phase (n=30) of the disease. Blood samples collected from unaffected healthy pigs (n=10) were analyzed to compare as normal hematological data. The hematological parameters like total leukocytic count (TLC), differential leukocyte count (DLC) and platelet count were determined in automated blood cell analyzer (Model: MetelSchloesing, MS-4e, France).

Detection of CSFV antigen and nucleic acid

Detection of CSFV antigen in clinical samples was done using CSFV antigen test kit (IDEXXCSFVAg Serum Plus Test, IDEXX Laboratories, USA) following manufacturer's instruction. For detection of CSFV nucleic acid, viral RNA was extracted using the QIAamp RNA Kit (QIAgen, Hilden, Germany) according to the manufacturer's instructions. RNA concentrations were found in the range between 100-300 ng/μl. To obtain cDNA 1μg of total RNA from each sample was used. The cDNA synthesis was performed using cDNA synthesis kit (Invitrogen, Carlsbad, USA) and initially used for amplification of CSFV specific E2 gene using a nested RT-PCR with a set of external and internal primers as described earlier (Lowings *et al*, 1996). Positive and non-template controls (NTC) were included in all the reactions. Single-step TaqManRT-qPCR was performed to detect CSFV genome as per the method described by Hoffmann *et al.*, (Hoffmann *et al.,* 2005). The TaqMan real-time assay was carried out using SuperScript III Platinum One-step Quantitative RT-PCR kit (Invitrogen, Carlsbad, USA) in a 7300 Real Time PCR system (Applied Biosystems, USA).

Results and Discussion

Animals in the pre-clinical phase was categorized as Group I and consists of in-contact pigs that have not exhibited any CSFV specific clinical symptoms post outbreak. Pigs in the early-clinical phase that presented an acute infection within day 1-4 post CSFV infection was categorized as Group II. Infected pigs presenting a late clinical phase

exhibiting clinical symptoms up to 5-10 days and/or >10 days post infection was categorized as Group III.

In group I, no clinical symptoms were apparent other than loss of appetite and liveliness in the in-contact pigs. In group II, early clinical symptoms between 1-4 day post infection (dpi) was recorded in 57 out of 92 young pigs. The early clinical symptoms observed were loss of appetite along with rise of temperature (104-106^{o}F), depression, reddened skin, frequent respiration and reduced dry faeces. In group III, 39 out of 45 grower to adult pigs that survived 5-10 days post infection exhibited clinical signs such as high rise of temperature (106-108^{o}F), tendency to lie down, lameness, emaciation, red eye with ocular discharge, purple discoloration of skin and dry faeces with fibrin coat. Out of 71 pigs infected with CSFV, 41 animals that survived >10 days post infection were mostly adult pigs exhibiting deep abdominal breathing, few to extensive petechial hemorrhages, sticky eye lids with turbid discharge, paralysis, scanty faeces or diarrhoea, emaciation with visible ribs and low body temperature (101-96^{o}F).

Hematological examination revealed marked leucopaenia and thrombocytopaenia in the blood samples collected from in-contact animals at pre-clinical phase of the disease (Table 2). In the pre-clinical phase, all the in-contact pigs apparently seemed to be healthy showed drop in TLC, total platelet count and lymphocyte percentage in comparison to healthy pigs; while granulocyte count was found to be increased in infected pigs compared to healthy pigs (Fig. 1 and 2).In late clinical phase, moderate increase in TLC, total platelet count and lymphocyte percentage was observed in the CSFV infected pigs compared to pre-clinical phase while the granulocyte count dropped in the late clinical phase compared to pre-clinical phase (Fig. 1 and 2).

Maximum samples (73% in pre-clinical; 53% in late clinical) were found positive in single step RT-qPCR, followed by E2 gene based nested RT-PCR (60% in pre-clinical; 26% in late clinical) and CSFV Ag-ELISA (40% in pre-clinical; 13% in late clinical)(Table 3). In clinical samples, CSFV was detected maximum in tonsillar scrapings followed by whole blood, nasal and ocular swabs. Again, maximum CSFV positive cases (70-73%) were detected in samples collected in the pre-clinical phase as compared to 40-53% positive samples in the late clinical phase.

In whole blood, CSFV RNA was detected by single step RT-qPCR upto late clinical phase (40%) but at pre-clinical phase maximum samples (73%) were found to be positive (Fig. 3). Whereas, in tonsillar scrapings, nasal and ocular swabs, CSFV nucleic acid was detected at pre-clinical phase and percent positive was 70%, 53% and 60% respectively. At late clinical phase, RT-qPCR detected 53% was found positive for CSFV in tonsillar scrapping, 20% in ocular and 16% was in the nasal swab. Although nested RT-PCR could detect biological samples collected at the pre-clinical phase of the disease (Fig. 4), the percent positivity was found to be comparatively lower (16-60%) than RT-qPCR (Table 3).

Laboratory investigation results of the present study clearly showed that both viral antigen and nucleic acid could be detected in all clinical samples (blood, nasal swabs, ocular swabs and tonsillar scrapings) collected at pre-clinical phase. However, at late clinical phase CSFV nucleic acid could be detected only in blood and in tonsillar scrapings.

Table.1 Categorization of clinical symptoms of CSF in pre-clinical, early clinical and late clinical phase as per days post infection (dpi)

Parameters	Group 1 Pre-clinical	Group 2 Early clinical (1-4 dpi)	Group 3 Late clinical (≥10 dpi)
Body temp.	102-104^{o}F	104-106^{o}F	101-96^{o}F
Liveliness	Less active	Fatigue; Walk on forced	Fatigue; Unable to stand up
Appetite	Reduced, feed left in bowl/trough	Decreased appetite, No intake of feed	Intermittent intake of water
Body shape	No changes observed	Empty stomach	Prominent back bone, ribs visible
Respiration	Normal	Frequent, watery nasal discharge	Deep and abdominal, muco-purulent discharge
Skin	No changes observed	Reddened	Few petechial, reddish blackdiscoloration
Eye	No abnormality visible	Reddened	Pasting eye lids, Turbid ocular secretion
Defecation	Reduced	Reduced and dry	Diarrhea

Table.2 Mean± SE of different hematological parameters in CSFV affected and healthy pigs

Group	No. of animals	Platelet 10^3/mm^3	TLC 10^3/mm^3	Differential leukocyte count (%)		
				Lymphocyte	Monocyte	Granulocyte
Reference range		300-500	10.0-22.0	35-64	2-10	28-62
Unaffected healthy pigs	10	311.300 ±6.475	13.333 ±0.589	46.633 ±2.804	3.250 ±1.645	34.119 ±6.475
Pre-clinical phase	30	97.100 ±7.476	8.209 ±0.195	17.617 ±1.139	3.330 ±0.148	56.482 ±1.006
Late clinical phase	30	218.342 ±6.895	9.334 ±0.590	32.633 ±2.805	3.125 ±0.213	40.117 ±2.681

Table.3 Detection of CSFV in various clinical samples in pre-clinical and late clinical phases

Animal groups	Type of test performed	No. of samples tested positive (% positive)			
		Whole blood	Nasal swabs	Ocular swabs	Tonsillar scrapings
Pre-clinical phase (No. tested 30)	Antigen detection by Ag-ELISA	10 (33.33)	4 (13.33)	2 (6.66)	12 (40.00)
	Nested RT-PCR	18 (60.00)	10 (33.33)	5 (16.66)	18 (60.00)
	Single step TaqMan RT-qPCR	22 (73.33)	16 (53.33)	18 (60.00)	21 (70.00)
Late clinical phase (No. tested 30)	Antigen detection by Ag-ELISA	4 (13.33)	-ve	-ve	4 (13.33)
	Nested RT-PCR	7 (23.33)	-ve	-ve	8 (26.66)
	Single step TaqMan RT-qPCR	12 (40.00)	5 (16.66)	6 (20.00)	16 (53.33)

Fig.1 Total Platelets and leukocyte count (TLC) in control healthy pigs and CSFV infected pigs in pre-clinical and late clinical phase. The values are presented as total count x $10^3/mm^3$.

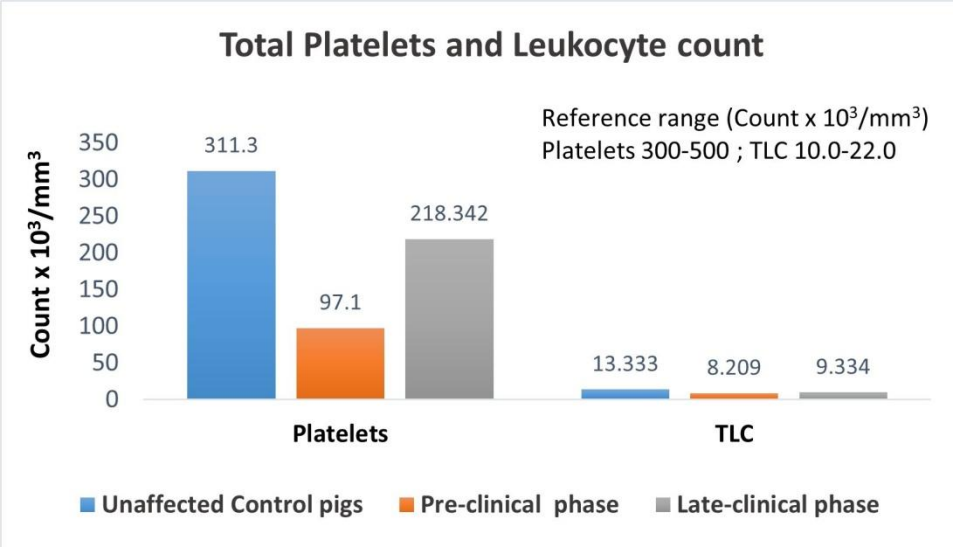


Fig.2 Differential leukocyte count (DLC) in healthy pigs and CSFV infected pigs in pre-clinical and late clinical phase. The values are presented as % of total count.

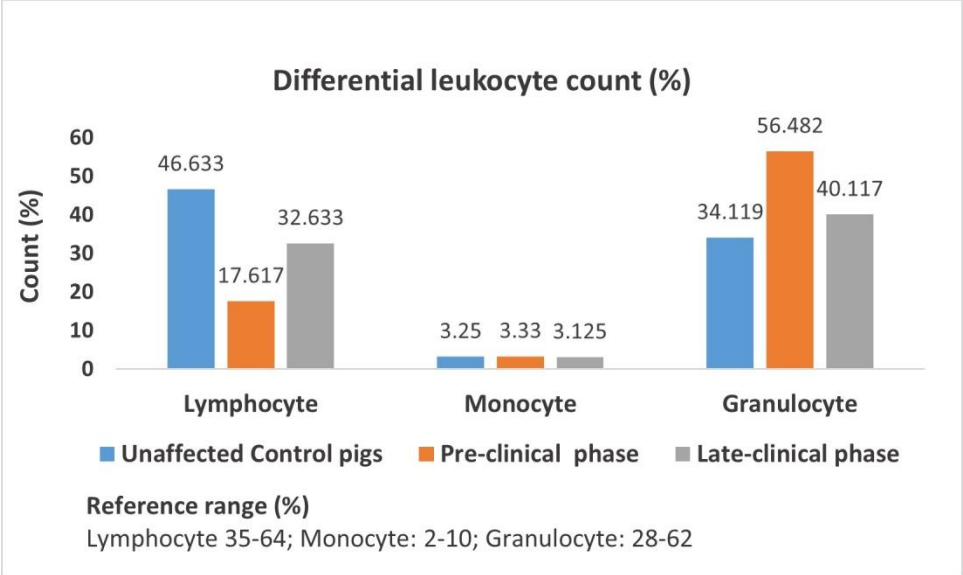


Fig.3 Amplification curves of CSFV using TaqMan RT-qPCR from blood samples and tonsillar scrapings collected at pre-clinical phase of the disease. Curves that crossed the threshold ΔRn value were considered positive.

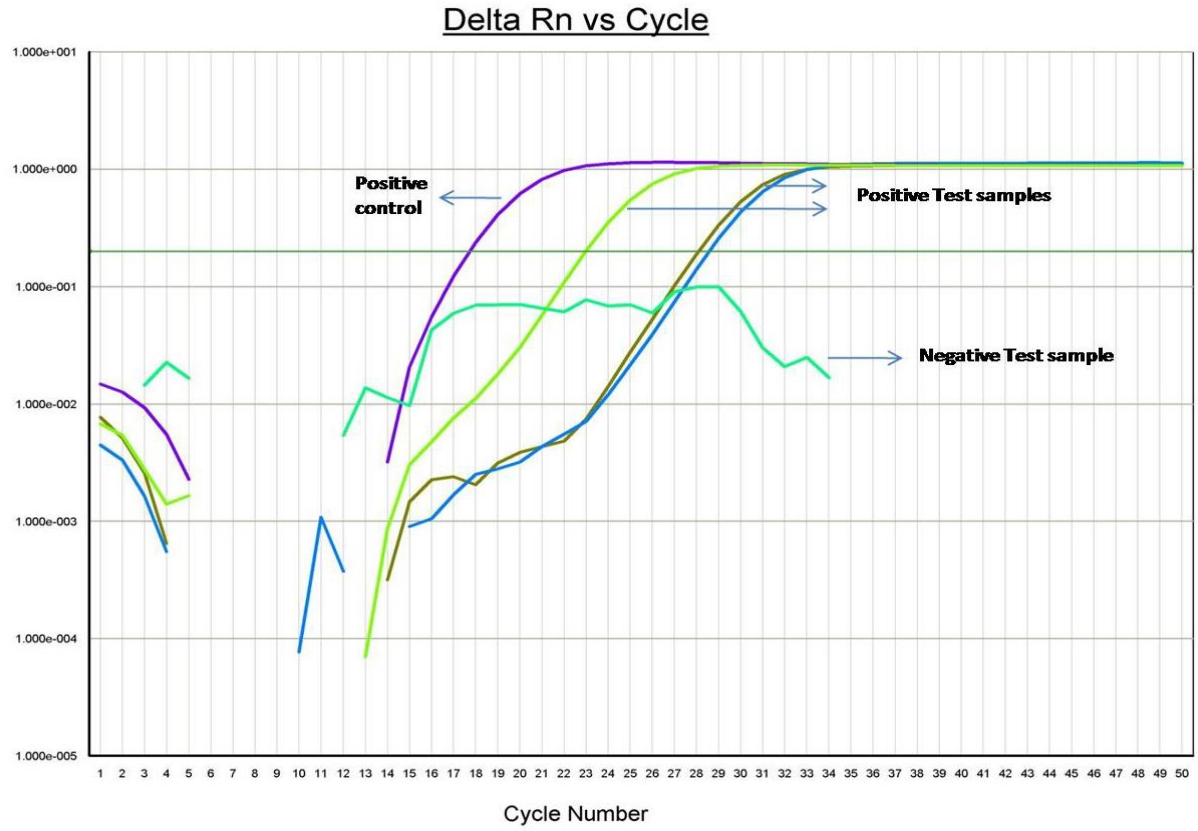
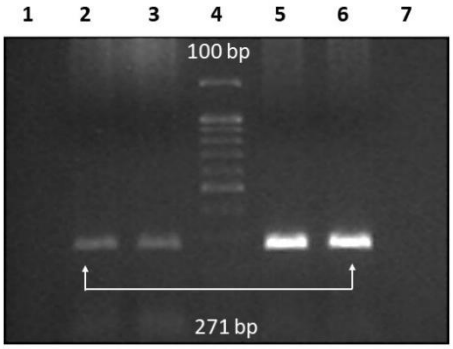


Fig.4 Nested RT-PCR amplification of CSFV-E2 gene from different clinical samples collected at pre-clinical phase.



Lane 1: Non template control
Lane 2 and 3: CSFV(+) from blood
Lane 4: 100bp DNA Marker (ThermoFisher)
Lane 5 and 6: CSFV (+) from tonsillar scrapings
Lane 7: CSFV negative amplification

Dewulf *et al.*, (2004) stated that the nested RT-PCR is suitable for early detection of CSFV in blood on average 2.8 days earlier than the isolation of CSF virus. But the test is not free from the risk of carry over contamination and is time consuming taking around 6-7 hours to complete the assay. In contrast, TaqMan probe-based RT-qPCR is an accurate, rapid and reliable assay for the detection of CSFV. Experimental study carried out by Everett *et al.*, (2010) demonstrated detection of viral nucleic acid in blood at preclinical phase prior to excretion of virus at nasal secretions. It is interesting to note from the present study that viral RNA could be efficiently detected in blood, tonsillar scrapping, nasal and ocular swabs using Rt-qPCR with maximum positivity (70-73%) from the CSFV infected animal in the pre-clinical phase prior revealing any prominent CSFV specific clinical signs. In blood as well as in tonsil high percentage of virus positive samples was identified up to mid clinical phase of CSF infection. Present findings thus emphasize the importance of blood, tonsillar scrapings, nasal and ocular swabs as suitable clinical samples for pre-clinical diagnosis of CSFV infection by TaqMan RT-qPCR assays. The RT-qPCR assay has high sensitivity without loss of specificity and possessed higher predictive value. The assay allowed rapid identification of CSF affected in-contact pigs and thus provided opportunity to take instant decision regarding the infection status to contain disease transmission. Blood samples and tonsillar scrapings could be the clinical material of choice for detecting CSF wild type virus infection at pre-clinical or early stage of the disease (Donahue *et al.*, 2012). On the other hand, low percentage of CSFV antigen detected by Ag-ELISA in blood further proved that Ag-ELISA is not suitable diagnostic tool for early clinical detection (Shivaraj *et al.*, 2013).Negative result in Ag-ELISA does not eliminate true positive cases. Therefore, the Ag ELISA could be used as a screening technique for diagnosis of individual pig where nucleic acid-based facilities are limited.

Along with early clinical signs, hematological examination in-contact pigs can be an indirect way for presumptive diagnosis of CSFV infection. The present study showed two-to-three-fold decrease in leukocyte and thrombocyte counts in pre-clinical phase in all age groups of affected pigs. There was significant increase of granulocyte count. Remarkably, thrombocytopenia appeared quite ahead of clinical manifestation of the disease and even 4-5 days prior to detection of CSFV in blood. It was postulated that peripheral platelet depletion is due to disseminated intravascular coagulation and phagocytosis of activated/damaged platelets(Calderón *et al.*, 1998; Bautista *et al.*, 2002). Nevertheless, macrophage mediated cytokines are contributing factors in early thrombocytopenia with co-incidence of fever. Finding of the present study suggests that no marked thrombocytopenia and leukopenia persist at late clinical phase of CSFV infection but can be distinctive at the pre or early clinical phase of the disease. Presumably, thrombocytopenia, leukocytopenia and hyperthermia can be used first as screening test for early presumptive diagnosis of CSFV and further confirmed by demonstration of the virus.

In summary, the single-step RT-qPCR assay is a simple, fast, highly sensitive and specific method for the pre-clinical detection of CSFV in clinical samples. The protocol presented is easy to perform and can be used for rapid detection of CSFV infected pigs or wild boars.

The tool would prove useful during CSF outbreaks when diagnosis needs to be made rapidly at preclinical phase to curtail disease transmission. Our findings could be valuable in order to diagnose the pre-clinical cases of CSF infection in an endemic locality.

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