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Quantification of classical swine fever virus load by one-step TaqMan real-time RT-PCR assay

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A fluorogenic-probe hydrolysis (TaqMan) real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay based on amplification of a 93 bp fragment from 5'un-translated region (5'UTR) of classical swine fever virus was used for detection and absolute quantification of the virus in clinical and tissue samples. For determining analytical sensitivity, an *in vitro* transcript RNA containing 5'UTR of classical swine fever virus (CSFV) strain Alfort187 from plasmid pCRXLV324-6 was used as a positive control and a standard for quantification of CSFV genomic RNA copies. The real-time quantitative RT-PCR (qRT-PCR) assay was used to assess the CSFV shedding from naturally infected pigs in whole blood, nasal swab and also in tissue samples. Used qRT-PCR was specific and sensitive as it could detect as low as 16.3 copies of CSFV genomic RNA. The assay was also reproducible as shown by satisfactory low intra-assay (0.80 % to 1.87 %) and inter-assay (1.00% to 3.80%) coefficient of variation with an efficiency of 102.3% and R^2 of 0.993. Thus, the real-time qRT-PCR assay described here allows rapid, specific and sensitive laboratory detection and quantification of CSFV genomic RNA copies.

Keywords: Classical swine fever virus, real time PCR, TaqMan probe, qRT-PCR

Introduction

Classical swine fever (CSF) is a highly contagious and multi-systemic haemorrhagic disease that results in economic losses in the swine industry worldwide and is a notifiable disease to the World Health Organization for animal health[1]. The causative agent, classical swine fever virus (CSFV) is a member of the genus pestivirus within the family flaviviridae. The other members of the genus pestivirus are bovine viral diarrhoea virus (BVDV) of cattle and border disease virus (BDV) of sheep[2]. The positive sense CSFV genome is about 12.3 kb in length which contains highly conserved untranslated regions (UTR) at 5' and 3' ends and encodes a single polyprotein that is both co- and post, translationally processed to yield four structural (C, E0, E1 and E2) and 8 non-structural (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) viral proteins[3]. The genus pestivirus, including CSFV, BVDV and BDV can be differentiated by the sequences of their 5' UTR fragments[4].

Rapid and precise detection of CSFV is critical for disease containment. A diagnostic method used increasingly for the detection of viral pathogens, is the polymerase chain reaction (PCR). Now-a-days, real-time reverse transcriptase polymerase chain reaction (RT-PCR) has become one of the most widely used method in the field of molecular diagnostic and research. With the introduction of fluorogenic probes, detection of sequence specific templates can be achieved in real-time PCR, specificity is ensured by an inherent hybridization reaction and cross-contaminations are largely avoided[5]. TaqMan based quantitative real-time PCR (qRT-PCR) has shown increased sensitivity and specificity to the measurement of the amplification product in the exponential phase of the reaction and the initial concentration can be measured in a definite volume (viral load)[6-7]. The additional information of viral load can be a useful diagnostic tool to predict virus-associated diseases, assess disease status, identify different states of viral infection or monitor the efficacy of cell culture adapted vaccine[8]. The results of qRT-PCR experiments usually needs 2 h and can be analyzed directly without any post-PCR steps. Data generated by qRT-PCR can be analyzed using either absolute or relative quantification[9]. Absolute quantification requires construction of a standard curve using relevant standards such as a known copy number of plasmid

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DNA (pDNA) or *in vitro* transcribed RNA[10]. Moreover, absolute quantification analysis is useful in determining absolute viral RNA copies based on a constant, allowing straight forward comparison of data from different PCR runs on the same day or on different days and more importantly between different laboratories.

Until recently, there were a few studies that performed real dynamic quantification of CSF viral load and little is known about the virus load in relation to disease progression. In this study, we aimed to quantify the CSF viral load in different clinical and tissue samples by single step 5'UTR based TaqMan real-time RT-PCR.

Materials and Methods

Primer and Probe

Specific oligonucleotide primers and the fluorogenic probe[11] to target a highly conserved region within the 5' UTR of the CSFV genome were used in this study. The locations and sequences of the primers and probe were as follows: forward primer, starting at base position 100, 5' ATG CCC AYA GTA GGA CTA GCA; reverse primer, starting at base position 172, 5'CTA CTG ACG ACT GTC CTG TAC ; and probe, starting at base position 141, 5' TGG CGA GCT CCC TGG GTG GTC TAA GT. The TaqMan probe was labeled with a 5' reporter dye, 6-carboxyfluorescein (FAM), and a 3' quencher dye, 6-carboxy-N, N, N, N'- tetramethylrhodamine (TAMRA).

Standard Curve of Real Time RT-PCR

The plasmid pCRXLV324-6 which contains region of the 5'UTR of CSFV strain Alfort187, flanked by primers V324 and V326[12] was kindly provided by Helen Crooke from the Veterinary Laboratory Agency, New Haw, United Kingdom. The real time PCR standard consisted of RNA transcribed *in vitro* from the plasmid pCRXLV324-326 with the help of MEGA short script kit (Ambion, Applied Biosystems, USA). To make *in vitro* transcript RNA, 20 µl reaction was prepared by adding 2 µl T7 10X reaction buffer, 2 µl T7 ATP solution (75 mM), 2 µl T7 CTP solution (75 mM), 2 µl GTP solution (75 mM), 2 µl T7 UTP solution (75 mM), 2 µl T7 enzyme mixture and 8 µl template plasmid (pCRXLV324-6) DNA. After incubation the reaction at 37°C for 4 h, 1 µl TURBO DNase was added to the reaction and then again incubated at 37°C for 15 min. Termination of the reaction was done by adding 115 µl nuclease free water and 15 µl ammonium acetate stop solution. RNA was precipitated by adding 2 volumes of ethanol and mixing well and then chilled the mixture at -20°C for 15 min. The reaction mixture was centrifuged at 4°C for 15 min at 10,000 g to pellet the RNA and then store the RNA at -70°C. Finally concentration of RNA was determined by spectrophotometrically at 260 nm. The viral copy of the extracted *in vitro* transcript (IVT) RNA was calculated using the formula:

$$\frac{X\ (g/\mu l\ RNA) \times 6.02 \times 10^{23}}{\text{Transcript length in nucleotides (bp)} \times 340} = Y \text{ viral copy}/\mu l$$

Following optimization of concentration, the IVT RNA was used to develop standard curve and evaluate the diagnostic sensitivity of the real time RT-PCR.

qRT-PCR

To minimize the risk of contamination, a one step RT-PCR protocol was carried out using the commercially available Superscript III Platinum one-step quantitative RT-PCR kit (Invitrogen, USA). The real time RT-PCR assay was optimized using a total volume of 25 µl. Briefly, for a single well 4 µl nuclease free water, 12.5 µl 2X reaction buffer, 1 µl 25 mM $MgCl_2$, 1 µl RNase inhibitor, 1 µl FAM labeled TaqMan probe, 1 µl forward primer, 1 µl reverse primer and 0.5 µl Superscript III RT/Platinum® *Taq* mix was pooled as a master mix. Finally, 3 µl of RNA template were added and real time RT-PCR was carried out in an ABI 7300 real time PCR system (Applied Biosystems, USA). The temperature profile was reverse transcription at 50°C for 15 min, inactivation of reverse transcriptase/ activation of *Taq* polymerase at 95°C for 2 min, followed by 50 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. During amplification, the ABI 7300 real time PCR system monitored PCR amplification by quantitative analysis of the fluorescence emissions. The reporter dye (FAM) signal was measured against the internal reference dye (ROX) signal to normalize the signals for non-PCR related fluorescence fluctuations that occur from well to well. The complete run takes approximately 2 and 1/2 h. Though real-time PCR does not require running of gel for PCR products, as a mean of authenticity, the

real time PCR products were analyzed by agarose gel electrophoresis.

Evaluation of Specificity, Sensitivity and Reproducibility of qRT-PCR

The CSFV along with BVDV I, BVDV II and BDV were used to determine the analytical specificity of the primers and the TaqMan probe used in the experiment. Ten fold serial dilutions of the *in vitro* transcript (IVT) RNA from 10^{11} to 1 viral copy were tested to determine the detection limits and efficiency of qRT-PCR. The serially diluted IVT RNA was also used to establish a standard curve by plotting the threshold cycle (C_T) and the viral RNA copy numbers. The standard IVT, including 10^5, 10^4, 10^3 and 10^2 viral copies were used to evaluate the intra-assay and inter-assay reproducibility of qRT-PCR. In case of intra-assay variation, each concentration was detected in 5 repeats at the same time and same condition. Inter-assay variation was determined by performing real time PCR on 5 consecutive days but at same condition. Five assays were used to determine the mean, standard deviation (S.D.) and coefficient of variation (CV). The coefficient of variation denotes the precision of qRT-PCR. Appropriate negative control (NTC) was maintained in the test.

Conventional Nested RT-PCR

To compare the sensitivity of the real-time RT-PCR and conventional nested RT-PCR (nRT-PCR), 10-fold serial dilutions of the IVT RNA was carried out and tested subsequently by both real-time and nRT-PCR. The nRT-PCR was performed on Veriti 96 well thermal cycler (Applied Biosystems, USA). For synthesizing the complementary DNA (cDNA) total RNA 5 µl, random primer (50 ng/ µl) 1 µl and nuclease free water to make 13 µl were used. The mixture was heated to 70°C for 10 min and 25°C for 10 min. Thereafter, 4 µl 5X RT buffer, RNase inhibitor 1 µl, 10 mM dNTP mix 1 µl and M-MuLV RT 1 µl were added. The PCR protocol consisted of primer annealing at 25°C (10 min), extension at 37°C (1 h) and inactivation of enzyme at 70°C (15 min) and hold at 4°C. The cDNA thus formed was stored at -20°C till further use.

For amplification of partial 5'UTR gene**,** forward primer (5'CTAGCCATGCCCWYAGTAGG 3') and reverse primer (5'CAGCTTCARYGTTGATTGT 3')[13], hybridized to 5'UTR genomic areas were amplified by using 10X buffer 5 µl, 25 mM $MgCl_2$ 3 µl, forward primer 1 µl, reverse primer 1 µl, 10 mM dNTPs 1 µl, cDNA 5 µl, *Taq* DNA polymerase 0.5 µl and nuclease free water to make a total volume of 50 µl and subjected to a thermal cycler at 95°C for 2 min (1 cycle), 95°C for 30 s, 58°C for 45 s, 72°C for 1 min (34 cycles) and 72°C for 1 min. The primers used in nested PCR were internal forward primer (5'AGCTCCCTGGGTGGTCTA 3') and reverse primer (5'TGTTTGCTTGTGTTGTATA 3')[13]. For nested PCR, the procedure was essentially the same except that the template cDNA was replaced by 5 µl of primary PCR amplicon and also the annealing temperature was kept at 56°C. The resulting nested-PCR amplified products were visualized after electrophoresis on an ethidium bromide stained agarose gel.

Field Samples

In this study, two different unorganized pig farms located in and around Guwahati, Assam were identified. Farm A kept cross-bred pigs of < 6 months of age and animals were unvaccinated for CSF. Most of the grower pigs died in a span of 10-14 days and clinical and postmortem changes as recorded were suggestive of acute form of CSF. In farm B, grower to finisher age groups (> 1 year) of cross-bred pigs were maintained. Affected animals exhibited fever, loss of appetite, diarrhoea and debilitating conditions for 20-35 days. Symptoms were suggestive of chronic form of CSF. Six animals from pig farm A and B were selected for collection of samples. Clinical samples like blood and nasal swab were collected from clinically affected pigs and tissue samples collected after death of pigs. Swabs were collected into 2 ml of sterile Hank's balanced salt solution containing antibiotics and stored at -80°C until processed. To eliminate risks of cross contamination, substantial precautions were taken during collection of blood, swab and tissue samples for PCR analysis. For viral RNA extraction, QIAamp viral RNA kit (Qiagen, Germany) was used to extract total RNA from blood, nasal swab and tissue samples according to the manufacturer′s instruction. To do this, 140 µl of sample was added to 560 µl lysis buffer, vortexed and incubated for 10 min at room temperature. After 10 min, the solution was mixed with 560 µl ethanol and the mixture was centrifuged through a QIAamp spin column. After washing the column twice with the appropriate buffer, the RNA was eluted using 60 µl elution buffer and stored at -80°C.

Results and Discussion

To determine the absolute quantity of the viral load in clinical and tissue samples, *in vitro* transcript (IVT) RNA transcribed from plasmid pCRXLV324-6 which contains the region of the 5'UTR of CSFV strain Alfort187 was used. The concentration of IVT RNA was determined in spectrophotometer at 260 nm and finally IVT RNA was optimized to 1.63 x 10^{11} copy/µl and used as known standard RNA copy. The C_T value and RNA copy number of serially diluted IVT RNA was determined by qRT-PCR. For generation of a standard curve, ten fold serial dilutions of IVT RNA were used and these corresponded to copy numbers of 1.63 x 10^6 to 1.63 x 10^1. Analysis of the copy numbers and linear regression curve was performed with sequence detector version 1.4 software of ABI 7300 real time PCR system (Applied Biosystems, USA). For absolute quantification of viral genome, the standard curve was designed by plotting the C_T values (the cycle in which a target sequence is first detected) on Y axis and the IVT RNA copies per reaction on X axis (Fig. 1). The real time qRT-PCR was able to distinguish 10-fold differences in concentration over a range from 1.63 X 10^1 (C_T = 35.80) to 1.63 X 10^6 (C_T = 19.16) copies per reaction mixture.

Experiments were undertaken to assess diagnostic criteria such as specificity, sensitivity, and reproducibility. In order to exclude nonspecific results, the reaction was carried out against BVDV I, BVDV II and BDV were subjected to amplification in the real-time qRT-PCR at the annealing temperature of 60°C in order to establish the specificity of the assay and no nonspecific amplification was obtained. The analytical detection limit (sensitivity) of qRT-PCR was determined by assaying 5 replicates of IVT RNA standard containing 1.63 x 10^1, 1.63 x 10^2, 1.63 x 10^3, 1.63 x 10^4, 1.63 x 10^5 and 1.63 x 10^6 copies per reaction mixture. For each concentration level, the proportion of positive samples was calculated. Detection limit of 1.63 x 10^1 copies (C_T value 35.80) of IVT RNA was established in all 5 replicates in qRT-PCR assay with no amplification of no template control (NTC) (Fig. 2). To assess the intra-assay reproducibility of qRT-PCR assay, dilutions (10^{-5} to 10^{-8}) of IVT RNA standard ranging from 1.63 x 10^6 to 1.63 x 10^3 copies per reaction mixture were analyzed in five replicates in each run under same time and same conditions. Mean C_T values, standard deviations (SD) and coefficients

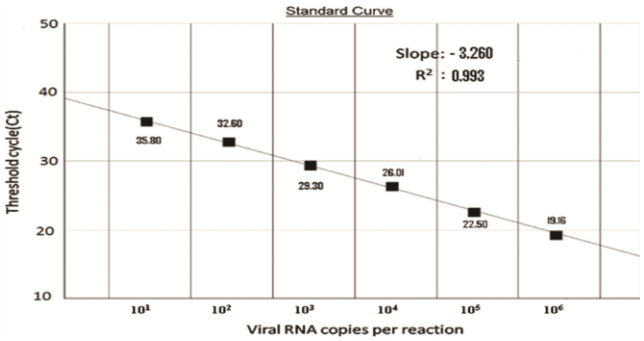

Fig. 1 — Standard curve for qRT-PCR generated by using C_T values on the *Y* axis and *in vitro* transcript RNA copy number on the *X* axis.

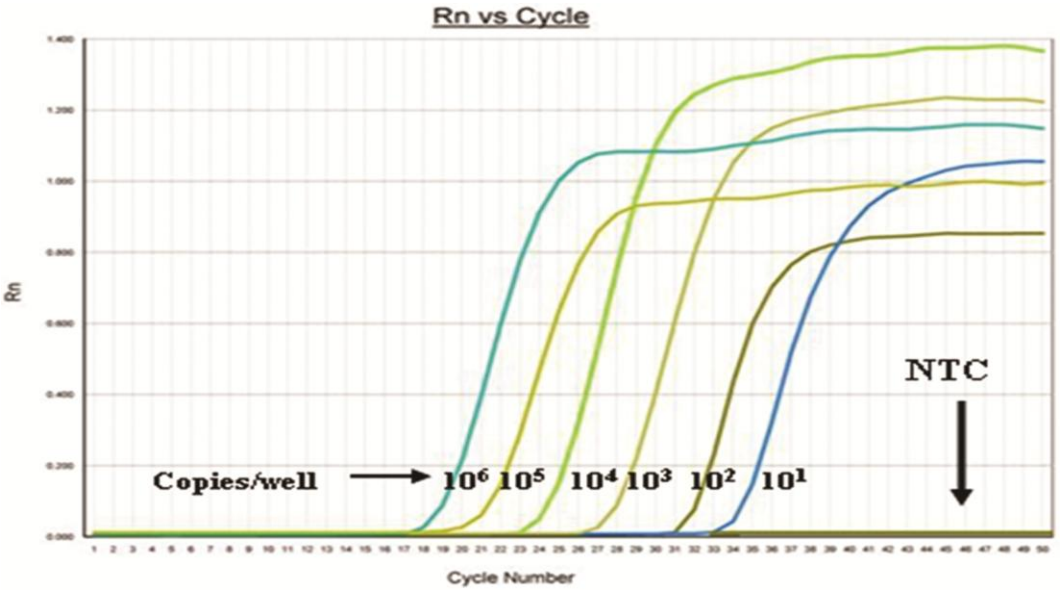

Fig. 2 — TaqMan probe based real time RT-PCR showing the analytical sensitivity of the reaction.

Table 1 — Intra–assay performance of TaqMan qRT-PCR with four different dilutions of *in vitro* transcript RNA

Dilution	C_T value					Result of intra- assay qRT-PCR		
	1	2	3	4	5	Mean	SD	CV (%)
10^{-5}	19.26	19.32	19.48	19.01	19.99	19.41	0.364	1.87
10^{-6}	22.53	22.43	22.80	22.96	22.10	22.56	0.334	1.48
10^{-7}	26.01	26.15	26.38	26.70	27.09	26.46	0.435	1.64
10^{-8}	29.30	29.15	29.62	29.55	29.82	29.48	0.261	0.80

5 repeats assay performed at the same time and under the same conditions.

SD = standard deviation

CV = coefficient of variation

Table 2 — Inter– assay performance of TaqMan qRT-PCR with four different dilutions of *in vitro* transcript RNA

Dilution	C_T value					Result of inter- assay qRT-PCR		
	1	2	3	4	5	Mean	SD	CV (%)
10^{-5}	19.20	19.80	20.02	21.11	20.88	20.20	0.787	3.80
10^{-6}	22.89	23.61	22.80	22.99	23.02	23.01	0.317	1.30
10^{-7}	26.01	26.52	27.99	27.01	27.32	26.97	0.756	2.80
10^{-8}	29.38	29.66	29.87	30.01	29.96	29.77	0.325	1.00

5 repeats assay performed at different times and under the same conditions.

SD = standard deviation

CV = coefficient of variation

of variation (CV%) were determined. In qRT-PCR, the intra-assay coefficient of variation was ranged from 0.80 to 1.87% (Table 1). The inter-assay precision, which expresses the variability from run to run, was assessed by testing 1.63 x 10^6, 1.63 x 10^5, 1.63 x 10^4 and 1.63 x 10^3 copies of the IVT RNA standard in five replicates per run at different time but under the same conditions. The coefficient of variation of the precision from run to run was in range of 1.00 to 3.80% in qRT-PCR (Table 2). Tenfold dilution series of IVT RNA standard were tested in parallel in the qRT-PCR assay as well as in the nRT-PCR. An amplified product of 93 bp (Fig. 3) and 271 bp (Fig. 4) fragments located in the 5'UTR region of CSFV genome was generated in qRT-PCR and in nRT-PCR, respectively. The detection limits of both the assays were compared and in terms of sensitivity, one log unit (10 times) was increased in qRT-PCR as compared to the nRT-PCR (Fig. 4).

The qRT-PCR used for absolute quantification of CSF viral load in clinical and tissue sample was highly sensitive and able to detect as low as 16.3 copies of viral genome. In optimization of qRT-PCR, the standard curve established showed an efficiency of 102.3% with a slope of -3.260 and R^2 0.993. According to previous workers, the slope of the qRT-PCR should be between -3.6 to -3.1 and PCR efficiency should be between 90 – 110 percent[14]. The real time qRT-PCR used in present study was within

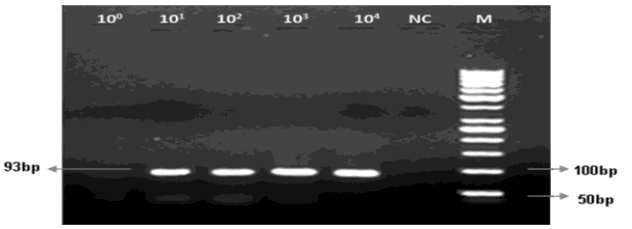

Fig. 3 — Electrophoresis of real time qRT-PCR product of ten-fold serial dilutions of *in vitro* transcript RNA which amplifies 93 bp fragment of 5'UTR region of CSF virus, M:100 bp ladder; NC: negative control.

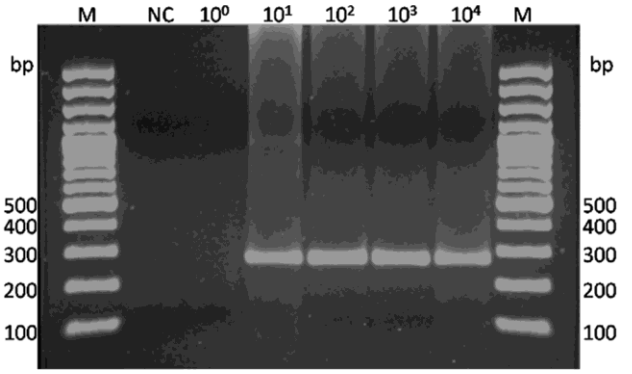

Fig. 4 — Electrophoresis of nested RT-PCR product of ten-fold serial dilutions of *in vitro* transcript RNA which amplifies 271 bp fragment of 5' UTR region of CSF virus, M: 100 bp ladder; NC: negative control.

the acceptable range. A real time RT-PCR assay with PCR efficiency of 99.8% and slope of -3.327 was developed by earlier worker[11]. In qRT-PCR, the intra-

assay coefficient of variation range was found between 0.80 to 1.87% and the inter-assay coefficient of variation of the precision from run to run was found in the range of 1.00 to 3.80%. For quantification of virus, the intra-assay variation should be < 2.7% and inter-assay variation should be < 4.5%. Therefore, the real time qRT-PCR used in the present study was within the acceptable range. This describes better repeatability and reproducibility of the qRT-PCR assay aiding in accurate diagnosis of CSFV infections.

In this study, samples were collected from two groups of CSF suspected pigs. All the pigs were categorized into two different groups based on age of the pigs, clinical symptoms and postmortem findings. Group A pigs represented acute form and group B pigs represented chronic form of suspected CSFV infection and each group consisted of six pigs. From these 12 pigs, a total of 84 samples consisted of whole blood (12), nasal swab (12), tonsil (12), mesenteric lymph node (12), spleen (12), kidney (12) and ileum (12) were collected. All the clinical and tissue samples were finally analyzed to evaluate the use of the qRT-PCR for the detection and quantification of 5' UTR of CSF virus.

The C_T value of the 42 clinical and tissue samples collected from acute CSFV infected pigs (group A) were ranged from 17.02 to 35.18 and copy number ranged from 1.34×10^1 to 7.97×10^6. Out of 42 samples, 9 samples showed C_T values between 17 and 20, 12 samples between 21 and 25, 13 samples between 26 and 30, and 8 samples between 31 and 35. In terms of absolute copy number, it was found that tonsil / spleen samples contained 10^5- 10^6 genome copies of virus followed by lymph node and whole blood (10^4- 10^5), nasal swab (10^3- 10^5) and kidney (10^2- 10^4) genome copies of virus. However, ileum contained only 10^1- 10^2 genome copies of virus. All the 42 samples were also tested by nRT-PCR and found that 38 samples which showed C_T value between 17 and 32 were positive for CSFV nucleic acid. However, four ileum samples which showed C_T value more than 33 were found negative for CSFV nucleic acid.

Absolute quantification of the clinical and tissue samples collected from chronic CSFV infected pigs (group B) showed that the C_T values were ranged from 22.13 to 37.56 and copy number ranged from 1.54×10^0 to 1.44×10^5. Out of 42 samples, 36 samples were found positive for CSFV infection and 6 samples showed C_T values between 22 and 25, 23 samples between 25 and 30, 7 samples between 31 and 35. Considering virus copy number, it was found that tonsil, lymph node and spleen samples contained 10^3 - 10^4 copies, kidney contained 10^2 - 10^3 copies and whole blood contained 10^2 genome copies of virus. On the other hand, ileum samples contained 10^3 - 10^5 genome copies of virus which was higher in comparison to group A pigs. However, in nRT-PCR all the tissue samples from group B pigs were found positive for CSFV nucleic acid. On the other hand, all the nasal swabs were found negative for CSFV nucleic acid in both nRT-PCR and qRT-PCR.

The precise CSFV genomic RNA copy numbers (absolute quantification) were calculated in the field samples by the standard curve generated by analysis of 10-fold dilutions of an *in vitro* transcript RNA. In this study, TaqMan qRT-PCR was used to detect and quantify the viral genomic RNA in clinical samples viz. blood and nasal swab and in tissue samples viz. tonsil, mesenteric lymph node, kidney, spleen and ileum in both acute and chronic CSF infection. In quantification by qRT-PCR, variation in distribution of virus load in both clinical and tissue samples were found. In acute CSFV infection, lymphoid tissue usually contained the highest viral loads between 10^5 and 10^6. However, kidney contained relatively low viral loads between 10^2 to 10^4 and ileum contained very low viral load between 10^1 to 10^2. It has been earlier been reported that lymphoid tissue is the ideal tissue sample for diagnostic testing by real time PCR based assay[15]. On the other hand, earlier worker used qRT-PCR and reported that the CSF virus was detected in 21 internal organs and blood collected from pigs at day 1 to day 8 post infections and virus load was increased from day 1 to day 8 post infection[16]. However, there was variation in distribution of virus load in most internal organs during first 2 days post infection. Blood, lymphoid tissue, pancreas and ileum usually contained the highest viral loads while heart, duodenum and brain showed relatively low viral loads.

In the present study, the maximum viral load was detected in lymphoid tissues in both acute and chronic cases of CSFV infection. On the otherhand, present findings suggested clear discrepancies between acute and chronic CSFV infection. In acute CSFV infection 100 to 1000-fold more copy number was found in whole blood, nasal swab, tonsil and spleen. Similarly in chronic CSFV infection 100 to 1000-fold more

copy number was observed in ileum. However, copy number in kidney and lymph node could not differentiate the acute and chronic CSFV infection. Previous study showed a direct correlation between clinical signs and viral RNA load in serum samples. They reported that affected pigs with none or mild clinical signs, the viral RNA in serum reached levels of almost around 30 C_T values[17]. In contrast, in pigs which exhibited more pronounced clinical signs, viral RNA in serum reached levels of up to 20 C_T values which were approximately 1000-fold higher serum viral RNA load than pigs without clinical symptoms. It was reported that pigs had viral RNA in serum as soon as 2 days post-infection and after 1-2 weeks viral RNA was cleared from most of the experimentally infected pigs. On the other hand, few workers suggested that viral load is of minor importance in determining the clinical course of CSFV infection[18]. In another study, pigs was infected with high or low doses of CSFV, produced almost identical clinical scores and body temperature values suggesting the importance of virulence factors in the type of clinical disease manifested in infected pigs[19]. It was reported that the moderately virulent strains of CSFV associated with chronic forms of the diseases are detected more frequently in field[20]. However, further studies will be required to correlate the viral load in pigs infected with mild, moderate and highly virulent CSF virus.

CSF is one of the major viral diseases of pigs prevalent in North Eastern states of India and a rapid, presumptive diagnosis at the site of a suspected disease outbreak would be extremely useful for controlling the disease. The currently available techniques either do not meet the desired characteristics or are not yet suitable for testing large numbers of samples in a short period. To address this need, in the present study a CSFV specific fluorogenic-probe hydrolysis (TaqMan) quantitative RT-PCR was applied for detection of the clinical and tissue samples. Real-time laser scanning coupled with a fluorogenic probe is a new technique which enables us to quantify a large number of amplified products rapidly and accurately. The main advantages are improved sensitivity that is based on very short amplification products and results in increased specificity. The ability to do multi-colour analysis for simultaneous detection of relevant PCR products, and the prevention of cross contaminations through the absence of post-PCR handling. Moreover, the combination of reverse transcription and polymerase chain reaction steps into one step greatly reduces time-consuming procedures and eliminates additional manipulations that are normally required for a two-step reaction[11]. Using this system, it is possible to analyze more than 40 samples in 2 to 3 h even if they are tested in duplicate. The real-time RT-PCR assay detected the presence of the virus before the appearance of the disease. The early detection of CSFV by real time PCR assays suggested its potential use in disease control, as screening assay for monitoring a disease outbreak in real time[21]. The 5'UTR of the CSF viral genome is frequently used as a target for detection of CSFV, due to its high conservation among all the pestiviruses. Any real-time PCR targeting this part of 5'UTR of CSFV will amplify this type of viruses if present in the samples[22].

Nested RT-PCR is especially suitable for early diagnosis on individual pigs, which can be very useful for the animal trade. By using nested RT-PCR, CSFV can be detected in whole blood samples of the infected pigs on average 2.8 days earlier than when using virus isolation in whole blood[16]. But major drawback of the nested RT-PCR is the risk of carryover contamination and the test is time consuming which takes 6 to 7 h to complete the assay. However, rapid detection of infected pig holdings is of paramount importance in stopping the further spread of CSF. The real-time qRT-PCR, on the other hand, generates complete result within 2.5 h. The reaction is performed in a closed-tube system and requires no additional manipulations. Another major advantage of real-time qRT-PCR is the ability to quantify the viral load in clinical as well as postmortem specimens, whereas conventional RT-PCR allows only qualitative analysis[23]. The qRT-PCR assays described here allow absolute viral load measurement and can be used as rapid diagnostic and prognostic tools. Compared to the sensitivity of the nested RT-PCR, a one-log unit increase in sensitivity is obtained in the real-time assay.

In conclusion, the real-time qRT-PCR used in this study is a rapid, reproducible, specific and sensitive assay for the detection and quantification of CSFV. Additionally, real-time RT-PCR can also be used in various study areas, including epidemiology, pathogenesis and vaccine quality evaluation. Real-time qRT-PCR appears to be more functional than all of the existing assays and may be suitable for routine laboratory diagnosis, both for the detection and the

quantification of CSFV. Importantly, the assay detected the presence of the virus before the appearance of the disease. This early detection of CSFV suggests the potential uses in disease control for the assay, as a surveillance tool in areas free of the disease and as a screening assay for monitoring a disease outbreak in real time. However, further studies should be performed to quantify the CSF virus load in all the internal organs in experimentally infected pigs.

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