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Comparative evaluation of single step real-time RT-PCR and gel based RT-PCR assay for detection of classical swine fever

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ABSTRACT

To control classical swine fever (CSF), a highly contagious viral disease causing serious losses in the pig industry worldwide, rapid detection and identification of the causative agent is a crucial step. In the present study, real-time RT-PCR and gel based RT-PCR techniques were compared for detection of CSF virus nucleic acid in both clinical as well as tissue samples. Clinical and tissue samples (325) were collected from CSF suspected outbreaks from different part of north eastern region of India. In gel based RT-PCR, % positivity was 44.61% while in real-time RT-PCR it was 57.23%. Highest per cent positivity was recorded in tonsil followed by mesenteric lymph node, blood, nasal swab, spleen, kidney and ileum. The study indicated that probe based RT-PCR could specifically detect CSF virus genome and detection limit was about one log higher than a gel based PCR assay targeting the non translated region. Total time required to complete the gel based RT-PCR including extraction of viral RNA was about 6 h. On the other hand, real-time RT-PCR assay can be performed in 2 to 3 h, thus providing a rapid detection tool.

Key words: Classical swine fever, CSFV, Gel based RT-PCR, Real-time RT-PCR

Classical swine fever (CSF), a highly contagious and enzootic disease in most of the pig producing states, particularly in the north eastern states of India, is caused by the classical swine fever virus (CSFV), under the genus *Pestivirus*. *Pestivirus* consists of one large open reading frame flanked by highly conserved 52 and 32 non-translated regions (NTR) and the CSFV, BVDV and BDV can be differentiated by the sequences of their 52 NTR (Hoffmann *et al*. 1994).

The clinical picture of CSF infection is highly variable and rapid and highly sensitive laboratory methods are essential for detection of CSF. Detection of viral nucleic acid by polymerase chain reaction (PCR) meets the criteria of speed and high sensitivity and a gel based nested reverse transcription-PCR (nRT-PCR) assay for the diagnosis of CSF was found sensitive and specific, but its performance was not compared to other molecular assays. While with fluorogenic probe, detection of sequence specific templates can be achieved in real-time, specificity is ensured by an inherent hybridization reaction and cross contaminations are largely avoided (Heid *et al.* 1996). Therefore in the present study, a gel based nested RT-PCR and real-time RT-PCR targeting the 52 NTR of CSFV genome were compared in detecting CSF virus in both clinical and tissue samples.

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MATERIALS AND METHODS

Collection of samples: Clinical samples like anti-coagulant added blood and nasal swab and postmortem tissue samples like tonsil, mesenteric lymph node, spleen, kidney and ileum from dead pigs suspected for CSF infection were collected from different parts of north eastern regions without using any preservative and stored at –80°C till further processing. Tissue samples collected from dead pigs were processed in the laboratory as per European Union Diagnostic Manual (Anon 2007).

RNA extraction: Classical swine fever viral RNA was extracted from clinical and postmortem tissue samples, cell culture propagated CSF virus, positive and negative controls using viral RNA kit according to the manufacturer's instructions. Quantity of fluid sample (140 ml) was used for RNA extraction. Finally, the RNA pellet was eluted using 60 ml elution buffer and stored at –20°C till further use.

Reverse transcription: The 6 ml of RNA was subjected to cDNA synthesis using random primer (50 ng/ml) and nuclease free water to make 13 ml reaction volume and subjected to 70°C for 5 min and later hold on 4°C and briefly spun down. Thereafter, 4 ml of 5 × RT buffer, 1 ml of RNAse

inhibitor (40 U/ml), 1 ml of 10 mM dNTP mix and 1 ml of reverse transcriptase (200 U/ml) were added and incubated at 25°C for 10 min followed by 42°C for 1 h. The reaction was stopped by incubating the reaction mixture at 70°C for 10 min. RNA and cDNA concentration was measured using nano drop spectrophotometer in ng/l and quality was checked as a ratio of OD 260/280.

Gel based nested RT-PCR assay: The primers used for primary and nested RT-PCR targeting 5¢ NTR were designed from the genome of Alfort-187 strain of CSFV (Greiser-Wilke *et al*. 1998). The primary PCR was performed in 50 ml reaction volume containing 10X *Taq* buffer, 25 mM $MgCl_2$, 10 mM dNTPs mix, 5 ml of cDNA, 20 pmol of each Primer I (52 CTAGCCATGCCCWYA GTAGG 32, nucleotide position 94–113) and Primer II (52 CAGCTTCARYGTTGATTGT 32, nucleotide position 514–496) and 2.5 U of *Taq* DNA polymerase and nuclease free water. The reaction was carried out for 34 cycles in a thermal cycler. Each PCR cycle consisted of 30 sec of denaturation at 95°C, 45 sec annealing at 58°C and 1 min extension at 72°C. The CSFV vaccine strain RNA and RNA extracted from tonsil of caesarean derived old piglet was used as positive and negative control respectively.

For nested PCR, 5 ml of primary PCR product was amplified using Primer III (52 AGCTCCCTGGGTGG TCTA 32, nucleotide position 146–163) and Primer IV (52 TGTTTGCTTGTGTTGTATA 32, nucleotide position 417–399) and subjected to thermo cycling conditions as primary PCR except annealing at 56°C. The nested PCR products along with positive control and negative control were subjected to electrophoresis in 1.7 % agarose gel containing ethidium bromide at 78 V for 1 h in 0.5 X Tris borate EDTA buffer. The DNA bands were visualized on a UV transilluminator. For size comparison, a 100 bp DNA ladder was run parallel to the PCR amplicons.

Single step TaqMan real-time RT-PCR assay: Published oligonucleotide primers and the fluorogenic probe to target a highly conserved region within the 52 NTR of the CSFV genome were used in this study. The locations and sequences of the primers and probe according to CSFV Alfort/187 were as follows: forward primer (5' ATGCCCAYAGTAG GACTAGCA 3', nucleotide position 100–120), reverse primer (5' CTACTGACGACTGTCCTGTAC 3', nucleotide position 192–172) and TaqMan probe (5' TGGCGAGCTC CCTGGGTGGTCTAAGT 3', nucleotide position 141–166). 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). To minimize the risk of contamination, a single step RT-PCR protocol was carried out using the commercially available one-step quantitative RT-PCR kit.

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 sec and annealing and extension at 60°C for 1 min. Amplification, data acquisition and analysis were carried out by using ABI 7300 real time PCR system and ABI prism SDS software which determines the cycle threshold (C_T). 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.

To compare the sensitivity of gel based nested RT-PCR and real-time RT-PCR, 10-fold dilutions of cell culture adapted CSF vaccine virus was used and tested subsequently by both gel based and real-time RT-PCR.

RESULTS AND DISCUSSION

The various clinical and tissue samples were subjected for gel based nested RT-PCR analysis. The nested RT-PCR was done using primary PCR product as template yielded a very precise band of 271 bp for 52 NTR region of CSF virus. In nested RT-PCR, the larger fragment produced by the first round of primary RT-PCR was used as a template for the second amplification. The primers for the second amplification were different from the first sets and located within the amplified region. The specificity was particularly enhanced because this technique almost and always eliminates any spurious non-specific amplification product. Thus the desired target sequence alone was amplified. Again, no amplification in the primary or nested PCR in negative control tissue indicated specificity of PCR product obtained in clinical and post mortem samples. Barman *et al*. (2012) used same primers for confirmation of CSF virus in the pygmy hog.

In nested RT-PCR 325, clinical and postmortem tissue samples were processed for amplification of 52 NTR fragment of CSFV. About 44.61 % samples were found positive for CSFV and highest percent of samples positivity was demonstrated in tonsil (60.00%) followed by mesenteric lymph node, blood, spleen, nasal swab, kidney and ileum (Table 1). Van Oirschot (1992) stated that in the first viraemic phase, almost all the lymphoid organs including tonsil, mesenteric lymph node and spleen were infected and propagated to highest concentration. Present study also justified frequent demonstration of virus in the lymphoid organs. The CSF virus is in general detectable in blood and organ samples for weeks after infection.

Table 1. Detection of classical swine fever virus in clinical and tissue samples by Gel based RT-PCR and real-time RT-PCR

Type of sample	No. of sample tested	No. of sample positive in	
		Gel based RT-PCR (%)	Real-time RT-PCR (%)
Blood	66	30 (45.45)	39 (59.09)
Nasal swab	28	12 (42.85)	16 (57.14)
Tonsil	25	15 (60.00)	18 (72.00)
Spleen	86	38 (44.18)	48 (55.81)
Mesenteric lymph node	28	16 (57.14)	18 (64.28)
Kidney	81	30 (37.03)	42 (51.85)
Ileum	11	4 (36.36)	5 (45.45)
Total	325	145 (44.61)	186 (57.23)

Irrespectively of the strain specific virulence, CSF wild type virus was routinely amplified in blood samples for more than 3 weeks by Handel *et al*. (2004), who recommended RT-PCR assay for the detection of pigs infected with low virulent field virus strains.

The TaqMan based real-time RT PCR for the clinical and tissue sample was carried out using the set of primers and probe designed by Hoffmann *et al*. (2005). To minimize the risk of contamination, one step RT-PCR protocol was carried out and the cut off C_T value was taken after 50 cycles. The complete run takes approximately 2 h. The real-time PCR analysis showed that out of the total 325 samples, detection of CSFV was possible in 57.23 % samples (Table 1). Out of the 57.23 % positive samples, highest percent positivity was recorded in tonsil (72.00%) followed by mesenteric lymph node, blood, nasal swab, spleen, kidney and ileum. No false negative result was obtained with real-time RT-PCR since all the samples assigned negative by real-time RT-PCR were also negative by nested RT-PCR. On statistical analysis significant difference was observed between both the tests ($P<0.05$). Ten-fold dilution series of cell culture adapted CSF vaccine virus containing 5.5 $TCID_{50}$ were tested in parallel in the real-time RT-PCR as well as in nested RT-PCR assay. An amplified product of 93 bp and 271 bp fragments located in the 52 NTR region of CSFV genome was generated in real-time RT-PCR and in nested RT-PCR respectively. The detection limits of both the assays were compared. In terms of sensitivity a one log unit (10 times) was increased in real-time RT-PCR as compared to the nested RT- PCR. The amplification plot of test sample in real-time PCR is shown in Fig 1.

Thus PCR methods offer a promising alternative in the diagnosis of CSF and differentiation of the virus from other Pestiviruses (Hoffmann *et al*. 2005). Due to the high proportion of detected animals, nested RT-PCR is especially suitable for early diagnosis on individual pigs, which can be very useful for the animal trade.

But major drawback of the nested RT-PCR is that the risk of carryover contamination and time consumed as it 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. To overcome this problem real-time RT-PCR is used, which is an accurate, rapid and reliable method for the detection of CSFV. It also eliminates post-PCR processing of PCR products. This helps to increase the throughput, reduces the chance of carryover contamination and disables post-PCR processing. In the present study, real-time RT-PCR alone could additionally detect about 12.62 % more positive samples than that of nested RT-PCR. The real-time PCR can be carried out even with a poor quality sample in which the pH has altered or even putrefaction has initiated as such sample may still have sufficient intact RNA. The real time RT-PCR protocol used was rapid and specific in detection of CSFV genome where results could be obtained within 2 hours. Jamnikar Ciglenecki *et al*. (2008) also reported that TaqMan real time PCR is more sensitive for virus detection in clinical samples from naturally infected animals. This is an important feature when definitive diagnostic results are required in a short timescale during emergencies.

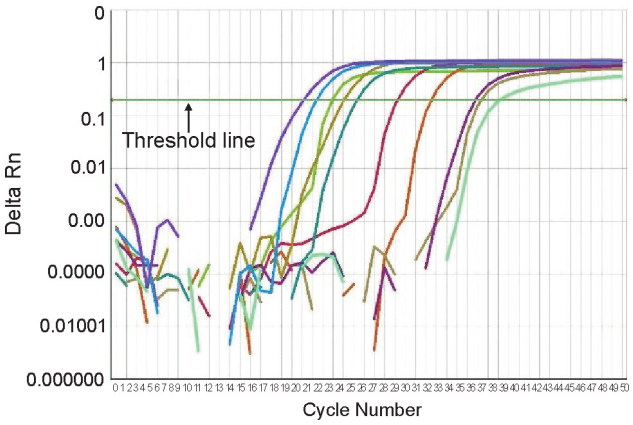

Fig. 1. Amplification plot of clinical and tissue samples in real-time RT-PCR. Amplification plot that crossed the threshold line was considered as positive for CSF virus.

Based on the results, it was concluded that the real-time RT-PCR assay provides a rapid, highly sensitive and specific method for detection of CSFV in both clinical and tissue samples. The early detection of CSFV by real-time RT-PCR suggested potential use in disease control, as screening assay for monitoring a disease outbreak in real time. However, the real-time RT-PCR assays enable handling with several samples at the same time and provide results with greater sensitivity in comparison to gel based RT-PCR.

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