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A TaqMan real time probe assay for specific detection and quantification of lapinized vaccine strain of classical swine fever virus

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Classical swine fever (CSF) is a highly contagious viral disease having a tremendous socio-economic impact. The high variability of clinical and pathological signs necessitates the use of laboratory tests for confirmation of suspected outbreaks. Nowadays, the detection of viral RNA by reverse transcription polymerase chain reaction (RT-PCR) has become an important and very useful diagnostic tool. Some recent reports suggest that RT-PCR assays have been developed that distinguish between wild-type CSFV and several attenuated lapinized vaccine strains (Liu *et al*. 2011). In countries such as India, where pigs are vaccinated with a live attenuated virus, an assay should be important to detect lapinized classical swine fever virus (L-CSFV). Compared with RT-PCR, real time PCR assays is more sensitive and less time consuming. By this assay, quantification of L-CSFV is also possible, which can be used for post-vaccination studies, in quality control and also for checking the vaccine efficiency. In this present study, we designed a real time TaqMan probe assay for rapid, sensitive and specific detection and quantification of lapinized classical swine fever virus.

Achieved frozen tissue specimens of tonsil, cervical and mesenteric lymph nodes, spleen, kidney, pancreas, lung, liver, intestine and blood in EDTA were collected from six rabbits inoculated with LCSFV strain from vaccine production unit. Samples for viral RNA extraction were collected and transported to the laboratory on ice with proper labeling and was stored at –700°C until further processing.

The TaqMan probe and primers for specific detection of L-CSFV (Vaccine strain used in India), the nucleotide sequence available in the GenBank database was used (EU857642). To find out a unique region in the genome of the L-CSFV, nucleotide sequences pertaining to many field strains of CSFV, were downloaded from the NCBI database and aligned using ClustalV of MegAlign 5.00 software. The sequences were scanned using Genscript real time PCR (TaqMan) primer designer for finding probe and primer oligonucleotides which matched to the set parameters of 150 bp length, melting temperature (59±5°C) and GC content (30–80%).

The primers [LCSFVF-5'- GAACTGGTGTTGGCAA GAAA-3' (11939–11956) and LCSFVR-5' TCTCAGCC TCCTTAACACCA-3' (12015–12034)] and probe [5'-FAM CCTGTCTGTCCCTTGAAAGTTGCTGA3'BHQ' (11964-11989)] which was expected to produce an amplicon of 96 nucleotides was selected and used.

The optimum annealing temperature of the newly designed primers was determined by running a gradient PCR using known positive vaccine cDNA. The PCR amplicons (10 µl) were electrophoresed along with 100 bp molecular weight marker on a 1.5% agarose gel in Tris borate EDTA (TBE) buffer containing 0.5 µg/ml ethidium bromide for 50 min at 70 V. Following electrophoresis, the gel was visualized under UV illumination at 254 nm and photographed in a gel documentation system. The temperature at which the bands were thickest and sharp was taken as the optimum annealing temperature of the primers.

Real-time PCR was performed on the Cepheid Smart Cycler machine using Probe Fast qPCR Kit following manufacturer's instructions. Standard procedures such as use of separate rooms for extraction of RNA, preparation of cDNA, preparation of Mastermix, cloning, dilutions of plasmid to avoid false-positive result because of contamination were followed. Total RNA was extracted from all the tissues using Trizol reagent as per manufacturer's instructions. RNA was eluted with 20 µl of RNase free water and after spectrophotometric quantification, stored at –70° C. Following RNA extraction, first strand cDNA synthesis was carried out in a 20 µl reaction mixture using 1.5 µg of total RNA, by random primer using RevertAid H Minus Reverse Transcriptase. The reaction was carried out in duplicate for each sample using 12.5 µl of (2×) Probe Fast qPCR Master Mix, 0.5 µl each of 10 pmole/µl forward, reverse and probe, 3 µl of cDNA and nuclease free water up to 25 µl. Amplification was performed for 35 cycles consisting of initial denaturation at 95ºC for 120 sec, followed by denaturation at 95ºC for 20 sec and annealing at 52ºC for 45 sec. Cycle threshold value was determined for each sample using the

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log linear phase of each reaction.

Prior to performing the TaqMan quantitative rRT-PCR on unknown tissue samples, the assay was performed on serial 10 fold dilutions from workable stock concentration that is 6.6 e-8 µg/µl of purified plasmid of 796 bp fragment from the NS5B region of CSF. The copy(s) no. of target gene was calculated as 108/3µl, 107/3µl, 106/3µl, 105/3µl, 104/3µl, 103/3µl copies in dilution no.1 to 6, respectively. Serial dilutions of plasmid with estimated copy number (108 –103) were used to generate the standard curve and dilutions from 108 –101 were used to access the sensitivity of the test. The gradient PCR yielded optimum product at a temperature of 52°C, which was provisionally considered for testing with real-time PCR. The product size was 96 bp. In the present study, all the tissue samples tested were found positive by rRT-PCR using TaqMan probe. The run-time of this assay was 1 h and the mininmum detection limit of this assay is up to 102 dilution. BLAST analysis of the probe sequence used in the present assay showed 100% identity with a few 1.1 genotype viruses from India, China, Sweden and Germany with query coverage of only 80–85%. However, since many of the recent outbreaks of CSF are caused by genotype 2, this probe based assay may be suitable for specific detection of the lapinized vaccine strain of CSFV.

Quantification of virus load in the tissues was done after creating a standard curve with different dilutions of plasmid DNA containing a small fragment of the CSFV genome targeted by the primers and probe. Plotting of the Ct values against initial copy number of corresponding dilutions resulted in the generation of a standard curve with R squared (Rsq) value of 0.99, an indication of the fit of the standard curve to the standard data points plotted and regression curve with $Y = –0.334x + 12.842$.

From the observation of LCSFV load in different organs of rabbits, it appears that blood, lymph nodes, spleen, kidneys, pancreas contained the highest viral loads, while, intestine, lungs and liver contained relatively lesser quantity of the virus.

Somewhat similar results were obtained when virus load was estimated in experimentally infected pigs assessed by fluorogenic real-time PCR assay. Highest viral load was found in blood, lymphoid tissue, pancreas and ileum, while heart, duodenum and brain had relatively low viral loads (Liu *et al*. 2011). Also by this assay post-vaccination study, its quality control and vaccine efficiency can be analysed. rRT-PCR methods for CSFV detection are rapid, are not influenced by neutralizing antibody, have very large dynamic range and allow precise quantification of as little as two fold differences of viral RNA amounts (Uttenthal *et al*. 2003). The sensitivity of the fluorogenic probe hydrolysis reverse transcriptase (RT) PCR assay for CSFV was found to be equal or more than the sensitivity of virus isolation (Risatti *et al*. 2003). In CSFV infected pigs, quantitative TaqMan PCR assay has been used to study sequential spread of virus to different organs and its excretion at different days post infection (Ophius *et al*. 2006). The assay has also been adopted for differential detection of wild-type viruses and C-strain vaccine of CSFV (Zhao *et al*. 2008).

SUMMARY

Classical swine fever (CSF) is a highly contagious viral disease having a tremendous socio-economic impact. In CSF endemic countries such as India, control strategies depend heavily on the use of a live attenuated vaccine. The persistence of vaccine virus in tissue/blood of vaccinated animals may interfere with the diagnosis when RT-PCR assay is used for CSF diagnosis. A TaqMan based real-time RT-PCR assay was developed with primers and probe targeting the NS5B region of the lapinized CSFV (vaccine strain) used in India for detection and quantification of lapinized CSFV. The real-time TaqMan RT-PCR assay presented in the current study provided a rapid, specific and sensitive diagnostic tool for the detection of LCSFV and for quantification of virus load in various organs. This real time RT-PCR assay can differentiate the infected from vaccinated animals and quantification assay can be used for post-vaccination studies.

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