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Application of nucleic acid based techniques for detection of Classical swine fever virus: A comparative study

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Abstract: *Classical swine fever (CSF) is a contagious and devastating viral disease, causing serious losses in the pig industry worldwide. In the control programmes of CSF, rapid detection and identification of the causative agent is a crucial step. Various PCR based techniques like nested RT-PCR, SYBRGreen based Real-Time PCR and TaqMan based Real-time PCR were used for detection of CSFV nucleic acid in clinical as well as tissue samples. In our study three detection systems were tested for classical swine fever virus (CSFV) detection and for its discrimination from other pestiviruses; Nested PCR, non-specific dsDNA-binding SYBR Green dye based and specific fluorogenic TaqMan MGB probe based Real-Time PCR. However, one-step TaqMan Real-Time PCR assay was shown to be the most appropriate for pestivirus discrimination in comparison to the other two assays, moreover, it reduces the risk of contamination and is less time consuming.*

Keywords: *Classical swine fever virus, Nested PCR, Pestivirus, Real-time PCR, SYBR Green, TaqMan probe*

I. Introduction

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a disease notifiable to the Office International des Epizooties (OIE) according to the Terrestrial Animal Health Code (www.oie.int). CSFV is a member of the genus Pestivirus in the family Flaviviridae. The genus also includes Bovine viral diarrhoea virus 1 (BVDV-1), Bovine viral diarrhoea virus 2 (BVDV-2), Border disease virus (BDV), and a tentative species, Pestivirus of giraffe [1]. CSFV is an enveloped virus with a single stranded, positive-sense RNA genome of approximately 12.5 kb in size. The genome contains two conserved untranslated regions(UTR) at the 5' and 3' ends, and a single open reading frame (ORF) encoding a polyprotein of about 4000 amino acid residues. The polyprotein is processed into 12 polypeptides by viral and cellular proteases [2, 3].The envelope of the CSFV contains three glycoproteins Erns, E1, and E2 [4]. The E2 protein in CSFV-infected animals is a major immunogen and contains conserved antigenic determinant regions.

CSF is a highly contagious disease that is mainly spread by contacts between pigs or by feeding pigs with contaminated meat. It is very important to differentiate between CSFV and BVDV or BDV infections in pig herds. This is why rapid, sensitive and specific laboratory diagnostic methods are needed to confirm outbreaks of CSF.

II. Materials And Methods

2.1. Collection of samples: Different outbreaks that occurred in Assam and adjoining states during 2013-14 were attended and whole blood were collected from clinically affected pigs in EDTA containing vacutainer. A total of 65 blood samples were processed with three different detection assays for CSFV.

2.2. Detection of CSFV nucleic acid in clinical samples

2.2.1. RNA extraction: Viral RNA was extracted using the QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.2.2. Reverse transcription (RT): cDNA synthesis was done by using High Capacity cDNA Reverse Transcription Kit(Invitrogen) as per manufacturer's instructions. The cDNA was further used for E2 Nested RT-PCR and SYBR Green based Real-Time PCR.

2.2.3. Nested RT-PCR: For Nested RT-PCR four sets of primers described earlier [5] were used targeting E2 gene of CSFV. The primary and nested PCR reaction was carried out in standard 50 µl reaction mixture as per the method described in European Union Diagnostic Manual. For confirmation of Nested RT-PCR amplicons gel electrophoresis was carried out in 1.7% agarose gel containing ethidium bromide in 0.5X Tris borate EDTA and visualized on a UV transilluminator as per standard procedures. For size comparision, a 100bp DNA ladder marker was run parallel to the PCR amplicons**.**

2.2.4. SYBR Green based Real-Time PCR: SYBR Green based Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) as per manufacturer's instructions in a 7300 Real Time PCR system (Applied Biosystems) with Pan pesti specific primer sets (Table 1).

2.2.5. TaqMan MGB probe based Real-Time RT-PCR: The TaqMan Real-Time assay was carried out using SuperScript III Platinum One-step Quantitative RT-PCR kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions in a 7300 Real Time PCR system (Applied Biosystems). A set of primers and a probe described earlier [6] targeting the highly conserved CSF sequence was selected for the Single Step TaqMan Real-Time RT-PCR assay.

Positive and negative extraction and amplification controls were included in each evaluation.

III. Result And Discussion

Nested RT-PCR: Out of total 65 clinical samples screened 39 samples were detected positive in E2 Nested PCR. The Nested PCR amplicons of E2 shows a product size of 271 bp (Fig. 1). The detection time was calculated to be approximately 6 hour and 30 minutes.

Real-Time PCR using SYBR Green method: 50 out of 65 samples were found positive by SYBR Green Real-Time PCR. Samples showing threshold cycle (Ct) values below 35 were designated positive, and samples with Ct values above 35 were considered negative. SYBR Green Real-Time assay can be non-specific and less accurate as intercalating dyes (SYBR Green I dye) generate fluorescence when bound to any dsDNA products including primer dimers. Therefore, to avoid false positive signals non specific product formation were again checked using dissociation curve or gel analysis. Amplification from specific product is displayed with a Tm of 84 ^{0}C, while primer dimer product has a characteristically lower Tm of 75 ^{0}C (Fig. 2). For SYBR Green Real-Time PCR detection time was calculated to be approximately 4 hours.

Real-time RT-PCR using TaqMan MGB probe: 45 out of 65 samples were found positive by TaqMan Real-Time assay. The samples showing threshold cycle (Ct) values below 35 were designated positive, and samples with Ct values above 35 were considered negative (Fig. 3). Since single step TaqMan assay is sequence-specific probe-based detection system where the probe is designed to bind within the amplified PCR fragment, competing side reactions such as primer dimerization and mispriming were significantly avoided and the no template controls and negative samples did not result in an increase in fluorescence above the baseline. For single step TaqMan Real-Time Assay detection time was approximately 2 hour and 20 minutes.

IV. Figures And Tables

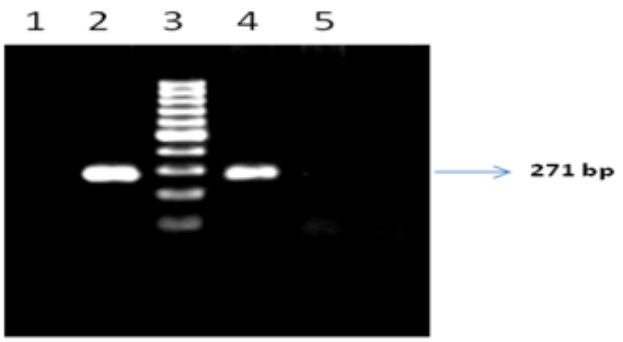

Fig. 1. Gel electrophoresis of E2 PCR amplicons

Lane1: Negative control; Lane2: Positive control; Lane 3: 100 bp marker; Lane 4: Positive sample

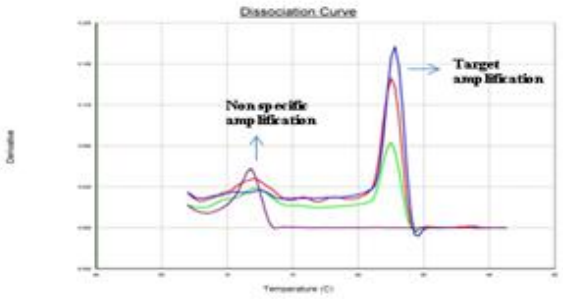

Fig. 2 Dissociation curve analysis using SYBR Green Real-Time PCR Assay

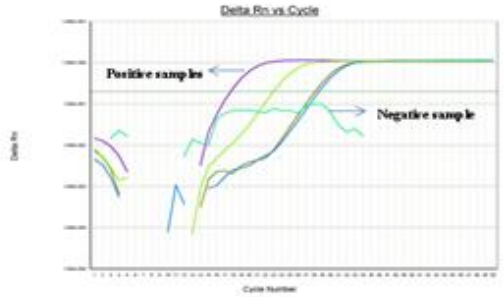

Fig. 3. Amplification plot using TaqMan Real-Time PCR Assay

Table 1. Pan pesti specific primer sets used in SYBR Green based Real-time PCR

Primer Identity	Sequence (5'-3')	Reference
Forward Primer	TGG GTG GTC TAA GTC CTG AGT	G. Saikumar
Reverse Primer	GTG TGA TTT CAC CCT AGC GA	

V. Conclusion

In this study, the performances of a gel-based nested RT-PCR assay and two Real-Time based assays were evaluated for detection of CSFV.

It has been shown that molecular based technique using E2 specific primer could specifically detect CSFV genome in higher percentage comparing with that of the polyclonal antibody based techniques like direct FAT and S-ELISA [7]. In addition, it has been shown that the order of sensitivity for different tests in detection of CSFV varies as RT-nested PCR>RT-PCR>Virus isolation>ELISA [8].The gel-based RT-PCR assay is the method of choice for rapid detection of CSFV especially for laboratories that cannot afford a Real-Time machine.

Pan pesti specific SYBR Green Real-Time PCR detected the highest number of positive samples followed by TaqMan Probe based Real-Time PCR and E2 Nested PCR. Samples with very low viral load detected negative in E2 Nested PCR were detected positive by both the Real time assays because of their higher sensitivity. Comparing the two Real-Time PCR detection systems, both non-specific dsDNA-binding dye SYBR Green and specific fluorogenic TaqMan MGB probes reduced the risk of contamination and was less time consuming. However, SYBR Green based Real-Time PCR being pan pesti specific could detect samples other than CSFV i.e. BVDV or BDV. ButTaqMan Probe based Real-Time PCR being sequence-specific probe-based detection system detected only CSFV specific positive samples. Therefore, Real-Time RT-PCR with TaqMan MGB probes is more suitable for CSFV detection and for its discrimination from other pestiviruses than the Real-Time assay with SYBR Green.

Unlike a nested, or two-step SYBR Green RT-PCR assay, Single step TaqMan assay is much simpler, more convenient and easier to undertake. The combination of the reverse transcription (RT) and PCR steps into one step greatly reduces time-consuming procedures and eliminates additional manipulations that are normally required for a two-step reaction. The risk of carryover contamination can be minimized since reaction tubes are not required to open for RT step or at the end of the run for gel electrophoresis.

In summary, the one-step TaqMan Probe based Real-Time PCR assay provides a simple, fast, highly sensitive and specific method for detection of CSFV in clinical samples or as confirmatory tests for other assays, such as virus isolation, or antigen ELISA. Moreover this diagnostic tool, using EDTA or Heparin treated blood from live animals, allowed for rapid identification of CSF infected pigs and could save a lot of time when control measures have to be applied to prevent virus dissemination between herds, after the first detection of the virus.

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References

[1]. H.-J. Thiel, M.S. Collett, E.A. Gould, F.X. Heinz, M. Houghton, G. Meyers, R.H. Purcell, C.M. Rice, Family flaviviridae,In C.M. Fauquet, M.A. Mayo,J. Maniloff, U. Desselberger, L.A. Ball (Eds.), Virus Taxonomy, (VIIIth Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, 2005) 979-996.

[2]. G. Meyers, H.-J. Thiel, T. Rümenapf, Classical swine fever virus: recovery of infectious viruses from cDNA constructs and generation of recombinant cytopathogenic defective interfering particles. J. Virol., 70, 1996,1588-1595.

[3]. T. Rümenapf, H.-J. Thiel, Molecular biology of pestiviruses. In T.C. Mettenleiter, F. Sobrino (Eds.), Animal Viruses: Molecular Biology,(CaisterAcademic Press, Norfolk, UK, 2008) 39-96.

[4]. H.J. Thiel, R. Stark, E. Weiland, T. Rumenapf, G. Meyers, Hog cholera virus: Molecular composition of virions from a pestivirus. J Virol., 65(9), 1991, 4705–4712

[5]. P. Lowings, G. Ibata, J. Needham and D. Paton, D, Classical swine fever virus diversity and evolution. Journal of General Virology, 77, 1996, 1311-1321.

[6]. B. Hoffmann, M. Beer, Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. J Virol Methods, 130 (1-2), 2005, 36-44.

[7]. N.N. Barman, R.S. Gupt, N.K. Singh, A.K. Tiwari, R.K. Singh, S.K. Das, Comparative evaluation of molecular and antibody based technique for detection of classical swine fever virus infecting pigs of NE region, India. The Indian Journal of Animal Sciences, 79, 2009, 10

[8]. D.J. Paton, A. McGoldrick, S. Belak, C. Mittelholzer, F. Koenen, H. Vanderhallen, M. Biagetti, G. De Mia, T. Stadejek, M.A. Hofmann, B. Thuer. Classical swine fever virus: a ring test to evaluate RT-PCR detection methods. Vet. Microbiol., 73, 2009b, 159-174.