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Nested Reverse Transcriptase-PCR (NRT-PCR) Assay for detection of Classical Swine Fever Virus

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Abstract :*Classical Swine Fever(CSF) or Hog Cholera is one of the most feared and devastating disease of pigs. The disease has become main threat to pig industry in counrties with a dense pig population and is known to cause more deaths in pigs as compared to many other infectious agents. The disease is endemic in many parts of India including Assam. The virus belongs to genus Pestivirus and family Flaviviridae and has close antigenic similarity with the two other members of this genus ie. Border disease virus (BDV) and Bovine viral diarrhea virus (BVDV). Out of these CSFV induces severe illness in young piglets and thereby cause severe economic loss in pig industry.It is important to differentiate CSF from other pestiviruses. nRT-PCR is a suitable approach for screening of suspected cases of disease and is now accepted by many countries and the European Union. Among several genes tested E2 is found to be more effective for genotyping of CSFV isolates. The envelope glycoprotein E2 is highly conserved and CSFV specific.The present investigation was conducted to detect CSFV from Tissue culture fluid using Nested Reverse Transcriptase- Ploymerase Chain Reaction (nRT-PCR) assay by amplifying the CSFV specific E2 gene fragment.*

Keywords: *CSFV, E2 glycoprotein, Flaviviridae, Hog cholera Virus, Pestivirus, nRT-PCR.*

I. Introduction

A major portion of the national economy of India is contributed by pig industry. Keeping the pace with other part of India, the North Eastern region of India specially Assam has also made a significant progress in pig industry in the recent years. A vast majority of the population being of tribal origin, pig rearing and consumption of pork have been traditionally popular in this region. In recent times the popularity of pork among the non-tribal particularly in urban areas is also increasing steadily.

Among various infectious agents associated with diseases of pig is the virus. Out of these viral agents, Classical Swine Fever Virus (CSFV)is responsible for the most devastating disease that causes stillbirth, abortion, persistent infection [1] .Also compared to any other infectious disease CSF causes more number of deaths in pigs. Therefore, it is a cause of fear or threat to pig industry.

The etiological agent of CSF is the Classical Swine Fever Virus (CSFV), a member of the genus Pestivirus, of the family Flaviviridae. The genome of the CSFV is single stranded RNA. The RNA is positive sense, the number of nucleotides in the RNA is approximately 12300bp and GC content is 46%.The CSFV measures approximately 40±3 nm in diameter and the inner core or nucleocapsid alone is about 29±3nm.The virus has an envelope with glycosylated membrane proteins and icosahedral symmetry [2].The virus has a non-translated region at either end (5′ NTR and 3′ NTR), encompassing a single open reading frame encoding a large protein that is cleaved into smaller fragments. The genes encoding the structural proteins (Cprotein, Erns, E1 and E2) are found towards the 5′ end of the genome, and include the major envelope glycoprotein gene E2.The genes encoding non-structural proteins (N^{pro}, P^7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) are located mainly in the 3′ two thirds of the genome, and include the polymerase gene NS5B .The glycoprotein E2 is most immunogenic protein and neutralizing antibodies are directed to it during CSFV infection [3, 4]. For detection of CSF viral nucleic acid, the highly conserved E2 gene fragment of CSFV (Lowing et al., 1996) was used in the present study. Specific primers were used to amplify E2 gene fragment of CSFV isolates from Assam using nRT-PCR.

II. Materials and Methods

The study area of the present study was in the Assam, India. A total of ten (10) classical swine fever isolates was taken from the repository of the ICAR National Fellow Project (ICAR), Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-22.

The total RNA was extracted from the cell culture suspension with QIAamp Viral Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. RT-PCR was carried out to amplify highly conserved E2 gene fragment using published primer [5, 6]. For synthesizing the complementary DNA (cDNA) 6µl of total RNA was mixed with 1µl random primer and the total volume was made upto 13µl using nuclease free water. The mixture in a PCR tube was heated to 70^0C for 5min, 25^0C for 10 min and hold at 4^0C. Thereafter 4µl of 5XRT buffer, 40Uof RNAsin, 10mM of dNTP mix, 200U of MMLV-RT were added. The amplification was carried out at 25^0C for 5 min, 42^0C for 1hr, the enzyme was heat inactivated at 70^0C for 10min. The cDNA thus formed was stored at -20^0C till further use.

The cDNA was further used for amplification of E2 gene fragment using the primer sequences - (F) 2228-2250bp, 5' AGR CCA GAC TGG TGG CCN TAY GA 3' and (R) 2898-2880bp, 5' TTY ACC ACT TCT GTT CTC A 3'. The amplification was carried out in 50µl reaction volume containing 10X buffer, 10mMdNTP mix, forward and reverse primer 20pmol,25mM $MgCl_2$,5µl of cDNA,1U of Taq DNA polymerase and nuclease free water to make 50µl. After initial denaturation at 95^0C for 2min,the amplification was carried out for 34 cycles each of 95^0C for 30sec, 56^0C for 45sec, 72^0C for 1min with final extension of 10 min at 72^0C.For nested PCR , the procedure was essentially the same except that the template cDNA was replaced by 1µl of primary PCR amplicons and the internal primers (F) 2477-2497bp, 5' TCR WCA ACC AAY GAG GGG 3'and (R)2748-2726bp, 5' CAC AGY CCR AAY CCR AAG TCA TC 3' were used. The annealing temperature was kept 58^0C.

The authenticity of the amplicons were verified by its size in 1.7% Agarose gel and visualized on a UV trans-illuminator.PCR products were purified using gel extraction kit and sequencing was done under Delhi University by automated DNA sequencer. The nucleotide sequence determined in this study was deposited in the NCBI GenBank sequence database with accession number fromKC623544 to KC623553.

III. Results and Discussions

In the present study viral RNA was extracted from ten CSFV field isolates from different places of Assam (taken from the repository of National Fellow Laboratory, Department of Microbiology, College of Veterinary Science, AAU, Khanapara, Guwahati) using QIAamp Viral Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. Sufficient quantity of viral RNA was obtained from the samples. Random hexamer primer was used for reverse transcription PCR (RT-PCR) for the synthesis of cDNA. Further, Mc Coll and Gould (1994) [7] reported that random hexamer primer increases the sensitivity of RT-PCR. Specific primers were used for primary and nested PCR for amplification of E2 gene fragment. All the ten samples tested were positive for Hog Cholera or CSFV as evidenced by a 271bp product (Fig.1) in 1.7 % Agarose gel electrophoresis as expected indicating that the nPCR step increased the sensitivity of the diagnosis. The findings were in close agreement with the earlier results [8, 9, 10, and 11]. Nested RT-PCR was more sensitive than primary RT-PCR. In nRT-PCR, the larger fragment produced by the first round of primary RT-PCR amplification 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 enhanced due to elimination of spurious amplification product. Several workers [5, 6] have reported nPCR as the most sensitive method for detection of CSFV. Thus this type of rapid and sensitive methods provide useful insight on genome of the virus circulating in the country which in turn helps in drawing up an effective control strategy for the disease. This is highly essential as CSF being an economically important disease, steps for its control needs to be taken as early as possible.

IV. Conclusion

In the present study ten suspected viral isolates were taken from the repository of National Fellow Laboratory, Department of Microbiology, College of Veterinary Science, AAU, Khanapara, Guwahati, Assam, India. After viral RNA isolation and cDNA synthesis nRT-PCR was performed using specific published primers. The highly conserved E2 gene fragment of 272 bp of CSFV was successfully amplified in all the ten isolates. This was evidenced by the appearance of clear DNA band of 272bp in 1.7% agarose gel against 100bp ladder (Fig.1).

Hog Cholera is predominantly restricted to pigs. Infections in swine with pestiviruses other than CSFV/HCV can give rise to a clinical disease that is indistinguishable from CSF. It might, therefore be concluded that the nRT-PCR , a rapid and sensitive method can be used for differentiation of CSFV/HCV from other members of the pestivirus of pigs as it demands strict control measures to prevent vast economic losses. Therefore, it could be used in the diagnosis of Classical Swine Fever in phase of outbreaks to decide control strategies.

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References

[1]. V. Moennig, and P.G. Plagemann, The pestiviruses, Adv Virus Research, 1992, 41: 53-98.
[2]. V. Moennig, G. Floegel-Niesmann, I. Greiser-Wilke, Clinical signs and epidemiology of classical swine fever virus: a review of new knowledge, Veterinary Journal ,2003,165:11-20.
[3]. M. Konig, T. Lengsfeld, T. Pauly, R. Stark, H. J. Thiel, Classical swine fever virus: independent induction of protective immunity by two structural glycoproteins, J. Virol., 1995. 69:6479-86.
[4]. T. Rumenapf, R. Stark, G. Meyers, H.J. Thiel, Structural proteins of hog cholera virus expressed by vaccine virus: further characterization and induction of protective immunity, J. Virol., 1991, 65: 589-59.
[5]. P. Lowings, G. Ibata, J. Needham, and, D. Paton ,Classical swine fever virus diversity and evolution.,J. Gen. Virol., 1996, 77(6): 1311-1321.
[6]. D.J. Paton , A. McGoldrick, I. Greiser-Wilke, S. Parchariyanon, J.Y. Song, P.P. Liou, T. Stadejek, J.P. Lowings, H. Bjorklund, and S. Belak, Genetic typing of classical swine fever virus, Vet, Microbiology, 2000,73:137-157.
[7]. K. A. McColl, and A. R. Gould, Detection and differentiation of blue tongue virus using polymerase chain reaction,Virus Res., 1994, 21: 19-24.
[8]. N. H. Chen, Z. Hongxia, S. Zhanfeng, J. L. Jiangbing, W. Fang, Genetic diversity of the envelope glycoprotein E2 of classical swine fever virus: Recent isolates branched away from historical and vaccine strains, 2008, Vet. microbiol., 127 (3):286-299**.**
[9]. N. A. Chopade, N. V. Kurkure, P. A. Tembhurne, A. G. Bhandarkar, S. S. Rautmare and D. R. Kalorey, Detection of Swine Fever Virus from Field Outbreaks in Maharashtra, Indian Vet. J., 2011, 88 (10):62-63.
[10]. G. Rajbongshi, N.N. Barman, R.S. Gupt, S.K. Das, Diagnostic Tests for Classical Swine Fever Virus, Indian Vet, J., 2011,88 (12):22-24.
[11]. D.K. Sarma, N. Mishra, S. Vilcek, K. Rajkumar, S.P. Behra, R.K. Nema, P. Dubey, and S.C. Dubey, Phylogenetic analysis of recent classical swine fever virus (CSFV) isolates from Assam, India. Comp. Immunol. Microbial, Infect. Dis., 2011, 34(1): 11-15.

Fig.1: 1.7% Agarose Gel Electrophoresis Of Amplified Partial E2 Gene Fragments of CSFV. Here lane indicates L1= 100 bp ladder, L2-L11= Amplified Partial E2 Gene Fragment (271 bp)