International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2018; 6(3): 148-151

Received: 04-03-2018
Accepted: 08-04-2018

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Molecular cloning of e2 gene fragment of classical swine fever virus (*CSFV*) from Assam

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Abstract
Classical swine fever (CSF) also known as hog cholera is one of the most economically important diseases of swine including domestic pigs and wild boars that results in great losses in the population of these animal species worldwide. In the present study the full length E2 gene fragment which is a structural glycoprotein of Classical Swine fever Virus was detected in ten (10) numbers of *CSFV* isolates by nRT-PCR using specific primers. One of these samples was cloned into pTZ57R/T, a TA cloning vector. Cloned product was sequenced that confirmed the proper orientation of the desired insert.

Keywords: classical swine fever, cloning, nrt-pcr, e2 gene, glycoprotein, cloning vector

1. Introduction
Classical swine fever is endemic in much of Asia, where the greatest diversity of viruses is now found [1]. In India CSF has been first reported in 1944 at Aligarh, Uttar Pradesh and later on the virus has been spread to other parts of India [2]. Assam is highly endemic to CSF. Several workers have reported the disease from different places in Assam [3, 4, 5, 6, 7, 8].
Classical swine fever virus belongs to the Pestivirus genus and the Flaviviridae family [9]. The virus is small and enveloped with a single stranded RNA genome of positive polarity [10].The size of the RNA genome is 12.3 Kb that codes for a unique polyprotein of about 3898 amino acids that, upon proteolytic processing, yields the mature proteins [11]. The mature proteins include four structural (C protein, Erns, E1 and E2) and seven non-structural proteins (N^{pro}, P^7, NS2, NS3, NS4A, NS4B, NS5A and NS5B).
E2 is a transmembrane protein made up of four antigenic domains A-D, located at the N-terminus, among which B, C and D vary between isolates, whereas domain A is conserved between different CSF virus strains [12]. Also it plays a key role in viral attachment [13] [14]. Glycoprotein E2 contains most of the known humoral and cell mediated protective determinants of *CSFV* [15] and highly immunogenic against which most of the neutralizing antibodies are induced [16, 17, 18, 19]. Therefore, E2 has been the main component in the design of marker vaccines of *CSFV* [20]. Hence, specific amplification of E2 gene fragment of *CSFV* followed by cloning and sequencing forms a basis in the diagnosis of *CSFV*.

2. Materials and Methods
2.1 Source of the sample or isolates
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.

2.2 RNA isolation and RT-PCR
The total RNA was extracted from the cell culture suspension with QIAamp Viral Mini Kit (Qiagen, Germany) according to the manufacturer's instruction and further subjected to nRT-PCR amplification. Total RNA was reverse transcribed using random hexamer primers with MMLV reverse transcriptase at 42°C for 1 hr.

2.3 Polymerase Chain Reaction (PCR) and nested PCR
The cDNA obtained from RT-PCR was further used for amplification of full length E2 gene fragment using the published primer sequences - (F) 2268-2288bp, 5' GTT ATT TGA AGA

GGC AGG ACA GG 3' and (R) 3763-3746bp, 5' GGC AAC CCC GCT AAC CAT 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, 52^0C 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) 2359-2404bp, 5'GTG AGG ATC CGC CAC CAT GAT AAA AGT ATT AAG AGG ACA GGT CGT 3'and (R) 3647-3687bp, 5' TAG CTC TAG ATT AAT GGA ACA GCA GTA GTA TCC ATT TCT T 3' were used. The annealing temperature was kept 52^0C and final extension was done for 15 minutes. 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 one of the sample was used for cloning.

3. Cloning of full length E2 gene fragment

After purification the nPCR product of full length E2 gene fragment was cloned in pTZ57R/T, a TA Cloning Vector (Ins TAclone PCR Cloning Kit, Cat No. #K1213, Thermo Scientific) according to the manufacturer's instruction.

3.1 Transformation

The transformation was carried out as per the Transformation Aid™ Bacterial transformation Kit (MBI, Fermentus) protocol using Ligation Master Mix and competent DH5-α and immediately plating was done on LB-ampicillin X-Gal/ IPTG agar plates.

3.2 Screening of recombinant clones

The recombinant clones were selected based on blue/white colony screening. White recombinant colonies appearing by 12-16 hours on LB ampicillin X-Gal/ IPTG agar plates were picked up and grown overnight in 2ml LB broth containing 100µg/ml ampicillin. Subsequently PCR was carried out using appropriate primers to detect the presence of desired insert.

3.3 Sequencing and analysis of cloned product

One positive clone was sent for sequencing by Primer Walking to ascertain the orientation of desired insert to DNA Sequencing Facility, Department of Biochemistry, South Campus, and Delhi University. Sequence analysis was done by using Mega 5 software.

4. Results and Discussion

4.1 Isolation of Viral RNA

In the present study viral RNA was extracted from *CSFV* field isolates (10) 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, [21] 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. Agarose gel electrophoresis yielded a single band of specific size of 271bp and is in good agreement with the result reported by [22, 23, 24, 25]. 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 [26] have reported nPCR as the most sensitive method for detection of *CSFV*. Thus nRT-PCR would be the suitable technique for detection of *CSFV* in the samples.

4.2 Detection of E2 gene fragment by nested reverse transcriptase - polymerase Chain reaction (nRT-PCR)

The full length E2 gene fragment was amplified by nested RT-PCR using specific primers. A specific band of 1328 bp was obtained after performing agarose gel electrophoresis (Fig.1).Similar findings were also obtained by [27] [28].

4.3 Purification of PCR product

After confirmation of the size, the PCR products were purified using PCR product purification kit (QIA quick PCR Purification Kit, Cat No. 2814, Qiagen, Germany).

4.4 Cloning of full length E2 gene fragment

After purification the nPCR product of full length E2 gene fragment was cloned in TA Cloning Vector (Ins TAclone PCR Cloning Kit, Cat No. #K1213, Thermo Scientific) according to the manufacturer's instruction. DH5-α strain of *E.coli* was used for transformation using Transformation Aid™ Bacterial transformation Kit (MBI, Fermentus). A sufficient number of white colonies appeared on LB-ampicillin X-Gal/ IPTG agar plates after 18 hours of incubation which were suspected as recombinant colonies with E2 full length gene insert (Fig. 2). These suspected recombinant colonies were checked for the possession of the recombinant plasmid

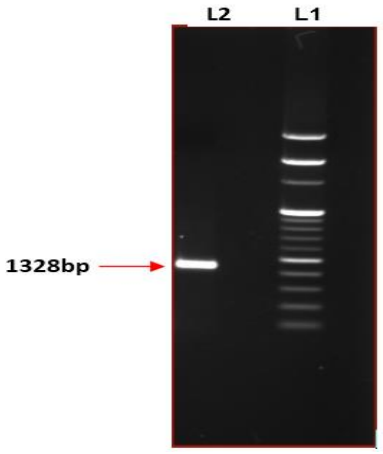

Fig. 1: 1.7% agarose gel electrophoresis of amplified full-length E2 gene fragment of CSFV fragment

L1=1000bp Ladder

L2= amplified full-length E2 gene fragment (1328bp)

With desired insert using PCR assay. The positive clone showed the PCR products approximately 1328bp.

This finding is in good agreement with the findings reported by [27, 28]. Also in several studies cloning of genetic fragments in pGEMT, pUC19 vectors have been carried out before sequencing [29].

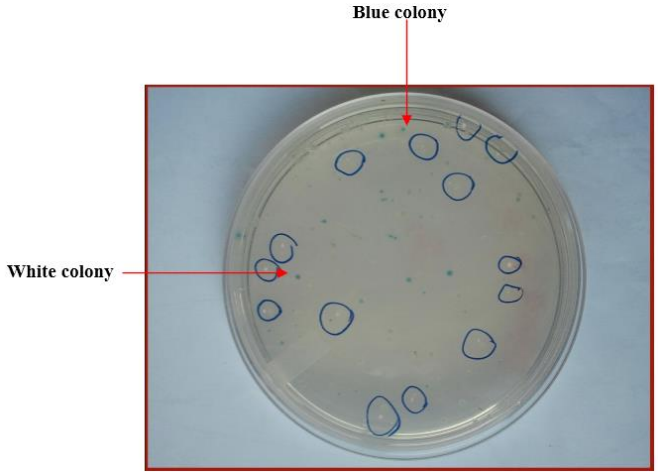

Fig 2: Blue white colony screening on LB-ampicillin X-Gal/ IPTG agar plates after 18 hours of incubation

4.5 Sequencing and analysis of cloned product

One positive clone was sent for sequencing to ascertain the orientation of desired insert to DNA Sequencing Facility, Department of Biochemistry, South Campus, Delhi University and submitted to GenBank (Accession No. KF007919). Cloned product was sequenced that confirmed the proper orientation of the desired insert.

5. Conclusions

In the present study, a total of ten (10) classical swine fever isolates were used for extraction of viral RNA. It was followed by synthesis of cDNA using RT-PCR and specific amplification of 1328 bp by nPCR. One of the samples was successfully cloned into pTZ57R/T, a TA Cloning vector. The result of the present study would be useful for the development of marker vaccine by ex-pressing E2 protein in suitable expression vector. This marker vaccine will be helpfull to differentiate infected and vaccinated animals

6. Acknowledgement

We are highly thankful to ICAR National Fellow Project (ICAR), Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, and Guwahati-22 for their financial support and help during the research work.

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