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Cloning of classical swine fever virus E_2 gene in replicase based eukaryotic pSinCMV vector

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Summary: The CSFV E_2 gene has been cloned in replicase based eukaryotic vector pSinCMV. The recombinant plasmid pSinCMV.csfv.E_2 containing gene in right orientation was selected using RE analysis, PCR and sequencing. This can be used for immunological studies.

Key words: Classical Swine Fever Virus, cloning, PCR, E_2 gene.

Introduction

Classical swine fever (CSF), also known as hog cholera, is a highly contagious, multisystemic and hemorrhagic viral disease, included in the list of diseases notifiable to the OIE (www.oie.int), distributed almost world-wide and is considered the most economically important but vaccinepreventable disease of swine in areas of intensive pig farming. Natural host of classical swine fever virus are members of the family Suidae, which include domestic pigs and wild boars (Depner et al., 1995; Laddomada, 2000). A considerable problem is the survival CSF virus in wild pig population, which is considered to be the potential source of the infection.

Genomic RNA possess single ORF. Initially a polyprotein is formed which is further cleaved cotranslationally and post-tanslationally by the host and viral genome encoded proteases to yield four structural (C protein, Erns, E1 and E2) and seven non structural proteins. Erns, E1 and E2 are the viral envelope glycoprotein. After infection with CSFV, antibodies are raised against the structural glycoproteins E2 and Erns and non structural protein NS3 (Paton et al., 1991; Terpstra, 1988). Antibodies against Erns and NS3 have only low neutralizing capacity or none at all (Rumenapf et al., 1991; Konig et al., 1995). E2 glycoprotein contains most of the known humoral and cell mediated

protective determinants of CSFV (Ceppi et al., 2005), highly immunogenic against which most of the neutralizing antibodies are induced and the only one capable of conferring protection against CSFV challenge (Rumenapf et al., 1991; Hulst et al., 1993).

Materials and Methods

Vector: The replicase based pSinCMV vector (Nagarajan, 2005), was used.

CSFV E_2 gene: The E_2 gene already cloned in pVAX1 vector was used as the candidate gene, the sequence accession no. EU857642.

Host Bacterial strains: Escherichia coli (E.coli) DH5α (Proteges,Madison,USA) host strain was used for transformation experiments.

Preparation for E_2 gene: pVAX1.CSFV.E_2 recombinant plasmid containing E_2 gene insert was revived in LB broth and plasmid DNA was isolated. Restriction endonuclease digestion of recombinent plasmid pVAX1.csfv.E_2 with PmeI enzyme was done to release the E_2 gene insert. This enzyme created blunt end. The size of E_2 gene ORF is 1.125 Kb.

pSinCMV vector preparation: StuI was use to create blunt end using 50 µl reaction mix. A 10 µl reaction mixture was for blunt end ligation containing T4 DNA ligase (Fermentas) 1 µl, pSinCMV Vector 2 µl, CSFV E_2 gene 5 µl, ligation buffer (10X) 1µl, nuclease free water 1 µl. The reaction mixture was incubated overnight at 14°C. The linearised plasmid was checked and quantitated on 1% agarose gel electrophoresis. The size of pSinCMV vector is 10.779 Kb.

Transformation of E.Coli DH5α cells with the ligated product was done as per Sambrook and Russell (2001).

Screening of recombinant clones: A large number of colonies (approximately 25) were picked from the overnight grown transformants. The individual colonies were inoculated in fresh ampicillin (50 µg/ml) containing LB broth and allowed to grow for 18 to 24 hours. Plasmid was isolated from these colonies by TELT method (Ausubel et al., 1990). After that all plasmids were checked on 1% agarose gel electrophoresis for presence of DNA.

Digestion with KpnI, AluI and HindIII to check the presence of CSFV E_2 insert: Isolated plasmids were checked for the presence of insert by digestion with enzyme KpnI, AluI and HindIII. A 10 µl reaction mixture was prepared containing Plasmid 5 µl, Enzyme (Fermentas, 10 U/µl) 1 µl, buffer (10X) 1 µl, nuclease free water 3 µl. The

reaction mixture was vortexed and spinned and incubated in a waterbath at 37°C overnight. The digested mixture was then electrophoresed in 1% agarose.

Digestion of plasmids with enzyme BsrGI to identify the clones with insert in correct orientation: A 10 µl reaction mixture was prepared for digestion containing plasmid DNA (300 ng/µl) 5 µl, BsrGI enzyme (Fermentas, 10 U/µl) 1 µl, Tango buffer (10X) 1 µl, nuclease free water 3 µl. Digestion was done at 37°C overnight.

Polymerase chain reaction: The recombinant plasmid was used as a template in PCR using gene specific forward primer and BGH reverse primer. The reaction mixture of 50 µl contained recombinant plasmid 2 µl, 10 pmol E_2 gene forward primer 0.5 µl, 10 pmol BGH reverse primer 0.5 µl, 50 mM MgCl2 1.25 µl, 2.5 mM dNTP mix 2.0 µl, 10 X PCR buffer 5.0 µl, nuclease free water 38.5 µl, Taq DNA polymerase (5 unit/µl) 0.2 µl. The contents were mixed by moderate vortexing and microcentrifuged. The cyclic conditions used for PCRwere, initial denaturation 94^0C for 5 min. and 30 cycles of denaturation 94^0C for 30 sec., annealing 52^0C for 30 sec., extension 72^0C for 45 sec and final extension 72^0C for 10 minute.Then an aliquot of 10 µl was subjected to agarose gel electrophoresis for the analysis of PCR product.

Sequencing: Once the recombinant plasmids were identified containing CSFV E_2 gene in correct orientation, one plasmid was sequenced with BGH reverse primer. The obtained sequence was analysed by DNAstar.

Results and Discussion

The csfv E_2 gene cloned in pVAX1 vector was released by digesting with PmeI enzyme which produces blunt ended products. The pSinCMV vector was linearized by digesting with StuI enzyme which also produces blunt ended products. Blunt end ligation was done and ligated vector was transformed in E.coli DH5α cells. The colonies were screened by PCR amplification of isolated plasmids with CSFV E_2 gene specific forward primer and BGH reverse primer. It resulted in amplification of E_2 gene giving a product of length of 1350 bp on 1% agarose gel electrophoresis (Fig.1). The orientation of insert in these plasmids were confirmed by specific restriction enzyme BsrGI which has two sites in the recombinant plasmid, one in the insert and the other in the vector. The fragment size from this digestion was predicted by analysis on Mapdraw (DNASTAR). The digestion with BsrGI enzyme released two fragment of size 6.637 kb and 5.267 kb if the gene is in right orientation. The respective length of fragments size confirmed that plasmid contained insert in right orientation (Fig.2). The recombinant plasmid clone with insert in right orientation (pSinCMV.csfv.E_2) was sent for sequencing using BGH primer and the results showed that the gene was in right orientation.

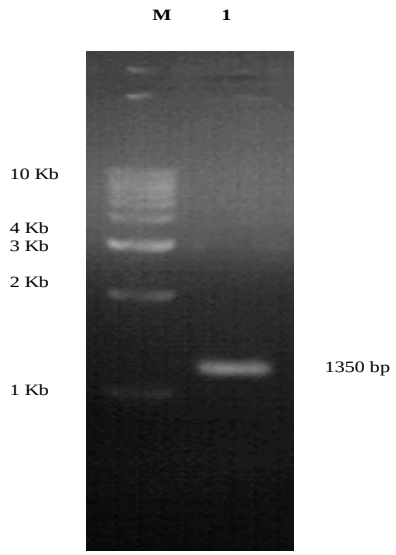

Fig.1: PCR amplification of rplasmid pSinCMV.csfv.E2 for presence of insert

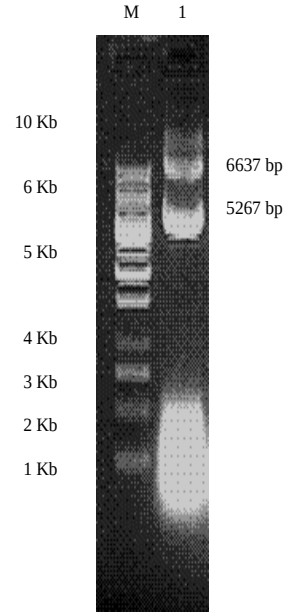

Fig.2: Confirmation of rplasmid pSinCMV.csfv.E_2 in right orientation by RE analysis. Lane M: Marker 1 Kb DNA ladder, **1:** Digestion with BsrGI enzyme

E_2 glycoprotein contains most of the known humoral and cell mediated protective determinants of CSFV (Ceppi et al.,2005), highly immunogenic against which most of the neutralizing antibodies are induced and the only one capable of conferring protection against CSFV challenge (Rumenapf et al.,1991; Hulst et al.,1993). Thus E_2 has become the main candidate for the development of DNA vaccine against CSFV. We cloned the E_2 gene in replicase based eukaryotic vector pSinCMV.

pSinCMV is an alpha virus (Sindbis virus) based plasmid vector which serves as negative strand once it enters into the host cell. There after entering into the nucleus it is transcribed by host RNA polymerase enzymes from CMV promoter into a full length RNA transcript. This full length transcript then acts as positive sense alpha virus which in turn is translated in cytoplasm to form replicase protein. This protein serves as RNA

dependent RNA polymerase enzyme and forms negative sense RNA from positive sense transcript. From this negative sense strand full length as well as smaller fragments from subgenomic promoters are transcribed which in-turn is translated into proteins. Since the cloned insert is downstream to the subgenomic promoter, the translated proteins represents our target proteins. The subgenomic promoter of alpha virus is very strong so that it makes large number of target mRNA from the sequence downstream to it. Replicase based vectors are superior over other conventional vectors in terms of its lower requirement of dose of immunization (Xiong et al., 1989; Hariharan et al.,1998, Berglund et al., 1998, Leitner et al., 2000).

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