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Cloning of 6.5 kb Segment of Cell Culture Adapted Classical Swine Fever Virus

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In this study, a 6.5 Kb fragment of cell culture adapted Lapinized Classical Swine Fever Virus (CSFV) was cloned into pTZ57R/T vector using the TA molecular cloning technique. Briefly, viral RNA was isolated using a modified RNA isolation protocol using Ribozol and RNeasy followed by cDNA synthesis using Clontech superscript RT. The cDNA obtained was used to synthesize 6.5 kb PCR products using (Long acting) LA-PCR. Gel purified PCR product was cloned with pTZ57R/T vector which was confirmed by RE digestion and partial sequencing. The same protocol can be utilized for cloning of similar sized amplified products in molecular biology laboratories.

Key words: Classical swine fever, LA-PCR, TA Cloning.

Cloning of smaller fragments is a routine technique used in the molecular biological laboratories, but cloning of larger segments is a challenging task due to reduced transformation efficiency with increasing size of plasmid[1]. Although cloning of larger fragments is reported in low copy number plasmid vectors (pBR322, pACYC177, pACNR1180, pSC vectors etc), cosmid vectors, BAC and YAC but in our knowledge cloning in high copy no. cloning vectors (pTZ57R/T, pJET 1.2, pGEMT etc) is not reported. In this study, we are reporting cloning of a 6.5 kb fragment of CSF virus into pTZ57R/T vector.

In present study 5' end 6.5 kb region of lapinized vaccine adapted in PK-15 cell culture was used. The 6.5 kb region codes for all four structural proteins viz. C protein, Erns, E1, E2 and nonstructural proteins viz. Npro, P7, NS2 and partial NS3 coding regions and a non translated region (NTR) on 5' end was cloned into pTZ57R/T vector and confirmed by RE digestion and sequencing. This clone of 6.5 kb along with another clone of 5.8 kb segment generated in our laboratory would be used for generation of full length (12.3 kb) genomic backbone of CSFV.

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MATERIALS AND METHODS

Virus and vector

Lapinized CSF virus adapted in PK-15 cell line (passage 6), pTZ57R/T vector (Thermoscientific, USA) and *E.Coli* DH5α (Invitrogen, USA) were used in this study.

RT-PCR amplification of 6.5 kb region of CSFV:

RNA was isolated using combined Ribozol/RNeasy protocol to obtain high quality larger length RNA. Total RNA was isolated from 25cc flask of PK-15 cell monolayer infected with cell culture adapted lapinized CSF virus. At 4 days post infection, the medium was removed and washed once with DEPC treated PBS. The PBS was then removed and 1ml of the Ribozol reagent was added to the infected cell monolayer and incubated for 5 minutes at room temperature. The lysed cell suspension was transferred to 1.5 ml eppendorf tube and centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was transferred into a new eppendorf tube and 200 µl of chloroform was added followed by incubated for 15 minutes at room temperature. The eppendorf tube was

centrifuged at 12000 rpm for 15 min and the aqueous phase was transferred carefully to a fresh tube. Equal amount of 70% ethanol was added to the aqueous phase, mixed thoroughly and transferred to RNeasy column (Qiagen, USA). The column was used for RNA isolation using the manufacturer's protocol.

Primer designing:

Primers were designed on the basis of complete CSFV sequences available in genbank. RE sites (underlined) were incorporated viz. *Vsp*I in forward (PF-5' aag cc<u>A TTA AT</u>**T AAT ACG ACT CAC TAT A**GT ATA CGA GGT TAG TTC ATTC TC-3') and *BamH*I in reverse (PR- 5'CTC AGG TTA GAT <u>GGA TCC</u> TCT CCA CTA TA-3') primers respectively. Additionally T7 promoter (bold) was incorporated in PF for scope of RNA transcription studies.

Preparation of long cDNA and LA-PCR:

Reverse primer (PR) primer was used to synthesize cDNA using Clontech Superscribe™ Reverse transcriptase (Takara, Japan) in 10 µl volume. Initial primer- template annealing was done in 5 µl final volume with ~ 1 µg of total RNA with PR (final concentration 2 µM) at 72°C for 3 minutes. This was followed by addition of 5X first strand buffer (final conc. 1X), dNTP mix (final conc. 1 mM), DTT (final conc. 2 mM) and 100 units of Smart scribe RT. The mixture was incubated at 42°C for 90 minutes and reaction was terminated by heating at 70°C for 15 min. 4 µl of the synthesized cDNA was used for PCR amplification of 5.8 kb region of CSFV by Takara LA Taq (Takara, Japan) using primers PF and PR. PCR conditions were optimized to be 1.5mM $MgCl_2$, 0.4mM each dNTP, 0.4µM of each primer, with 1X PCR buffer II and 2.5 units of Takara LA Taq™ in a total volume of 50 µl.

PCR was performed in thermocycler (Mastercycler personal, Eppendorf, 5330, Germany) using a initial denaturation temperature of 94°C for 1 minute followed by 30 cycles of denaturation for 30 sec. at 95°C, annealing temperature of 57 °C for 30 sec. and extension of 68°C for 6.5 min. Final extension was carried out at 72°C for 10 minutes. The amplified products were visualized by electrophoresis in 0.8 % agarose gel with ethidium bromide (0.5 µg/ml).

Cloning of 6.5 kb segment and confirmation of recombinant clones:

The amplified PCR product was gel purified using QIAquick Gel Extraction Kit (Qiagen Inc. Valencia, CA, USA) as per the manufacturer's protocol. The gel purified PCR product was cloned using InsTAclone PCR Cloning Kit into pTZ57R/T vector system (ThermoScientific, USA). The ligation reaction was performed with 5X ligation buffer (final conc. 1X), pTZ57R/T vector (110 ng), purified PCR product (~510 ng) i.e. insert vector ratio of 2:1, T4 DNA ligase (5 U) and nuclease free water to make total 20 ml reaction. The ligation mixture was incubated at 16 °C for 16 hours. 20 ml of ligation mixture was diluted 10X with 180 ml of freshly prepared 1.1X TCM solution (1.1 mM Tris HCl, 1.1 mM $CaCl_2$ and 1.1 mM $MgCl_2$) and incubated at 4°C for 30 min.

Preparation of competent cells and transformation:

In order to transform DH5α cells (*E. coli* cells) with the ligated DNA, fresh competent cells were prepared. Fresh cultures of DH5α cells were grown in 5ml LB medium at 37°C overnight. The cells were diluted 100 times in SOB medium and incubated in shaking incubator at 37°C with moderate agitation for about 2-3 hrs until cell density of 4-7 X 10^7 viable cells/ml (OD_{550} = 0.5) was observed. The cells were pelleted at 750-1000 g (2000-3000 rpm) for 10 min at 4°C and the pellet was resuspended in 1/10 volume of chilled TSS solution and incubated on ice for 1 hour.

200 µl of freshly prepared competent cells were gently added to the 200µl ligation DNA (diluted TCM), mixed and incubated on ice for 1 hour. The DNA-competent cell mixture were subjected to heat shock at 42°C for 90 sec. and were immediately snap-chilled on ice for 5 min [2]. 600 ml SOC medium was added to the transformants and incubated at 37°C for 1 hr with vigorous shaking. Transformants were then plated on LB/Amp/X-gal/IPTG plates at 37°C overnight. The white colonies were picked up on the next day and grown in LB/Amp broth for plasmid extraction.

Isolation of recombinant plasmids and their confirmation

The plasmid DNA was extracted using GeneJET Plasmid Miniprep Kit (Thermoscientific, USA) using manufacture's protocol and confirmed by digestion using *BamH*I and *Vsp*I REs (Thermoscientific, USA). The extracted plasmid was treated with *Vsp*I and *BamH*I in a reaction mixture containing 1unit of each enzyme, 2X Tango buffer, 5 µl of recombinant plasmid and nuclease free water

to make volume 20 µl. The digestion mixture was incubated for 16 hours at 37°C. Then, the insert release was confirmed in 0.8% agarose gel along with 1 kb DNA ladder (Thermoscientific, USA). The recombinant clones were further confirmed by partial sequencing of both ends using vector specific primers.

RESULTS AND DISCUSSION

RNA isolation and cDNA synthesis

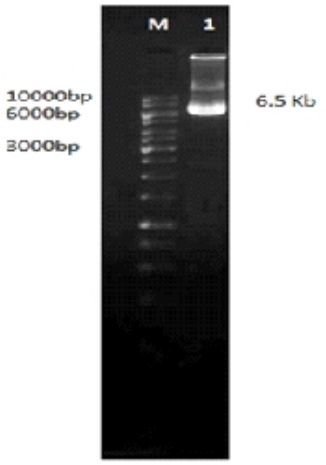
Classical swine fever viral RNA isolated from modified Ribozol/RNeasy method was used to generate 6.5 kb amplified fragment by RT-LA PCR which was cloned into pTZ57R/T vector by TA cloning strategy. For the amplification of longer genomic segments, high purity RNA is required so RNA isolation method described by Rasmussen et al., (2010) was used with some modifications as described before[3]. Viral RNA was isolated from three different sources viz. spleen tissue stored at -20 °C, freeze dried virus and fresh tissue culture flask. Although, RNA from all three sources were able to amplify PCR of 5'NTR and NS5B, while amplification of 6.5 kb segment was only obtained from RNA isolated from fresh tissue culture flask. This may be due to loss of RNA integrity on storage, while actively growing CSFV could provide the desired length RNA template in ample quantity[4]. Also, the spleen tissue is highly susceptible to degradation and contains high level of endogenous RNases[5]. Long cDNA preparation from Clontech Superscribe™ (Takara, Japan) was synthesized.

Amplification of 6.5 kb segment

Primers designed for LA PCR were having length of 51 and 29 nucleotide out of which 23 and 29 nucleotides were complementary to the template sequence for PF and PR, respectively. Higher primer length is expected to increase specificity of amplified product of longer PCR products. On LA PCR specific 6.5 kb amplified product was obtained along with nonspecific products which needs to be removed using gel extraction. This amplification was optimized at 1.5mM $MgCl_2$, 0.4mM each dNTP, 0.2mM of each primers. $MgCl_2$ and dNTP conc. were found to be critical for specific and efficient amplification[6]. Annealing temperature was optimized to be 57°C at these optimized ingredient concentrations. LA Taq™ is having both proofreading activity (3' to 5' exonuclease activity) and adding one non- template Adenine (A) nucleotide residue at 3' termini (terminal transferase like activity) of Taq. So amplified product could be directly used in TA cloning with least errors in amplification.

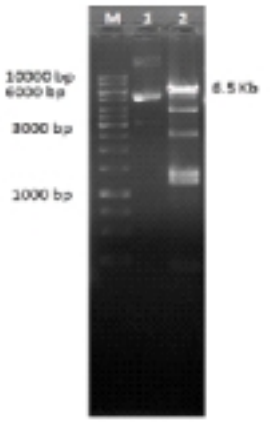
TA cloning method

TA cloning is very useful and time saving method of cloning of PCR products especially when compatible restriction sites are not available due to possibility of presence of RE sequences in the larger size of insert. TA cloning strategy is both simple and much more efficient than blunt-ended



Lane M – 1 kb ladder (Thermoscientiifc, USA)
Lane 1 – 6.5 kb PCR product

Fig. 1. Amplified PCR product of 6.5 kb



Lane M – 1 kb ladder (Thermoscientiifc, USA)
Lane 1 – pTZ/CSF/6.5
Lane 2 – pTZ/CSF/6.5 digested with *Vsp*I and *BamH*I RE

Fig. 2. RE digestion of recombinant plasmid with *BamH*I and *Pvu*I

ligation for cloning of PCR products[7, 8]. For ligation of 6.5 kb purified PCR product with pTZ57R/T vector, 1:2 vector insert molar ratio was used. Ligation reaction was incubated for 16°C for 16 hrs[8]. It was found that incubation temperature and time are critical for ligation reaction and for larger size insert longer incubation is required.

It has been found that transformation efficiency decreases linearly with increasing size of plasmids[1], so competent cell formation and transformation protocol was standardized. Freshly prepared competent cells were used to retain maximum transformation efficiency as transformation efficiency decreases dramatically on storage of the competent cells[1, 9] and ligated insert vector mixture diluted in TCM solution was used for transformation. SOC media was used instead of LB after transformation for faster growth of transformants and higher selection efficiency as described by Tu et al. (2005). Transformants were plated on LB agar/Ampilcillin/IPTG/X-Gal plates and white recombinant colonies were picked out for confirmation. Recombinant colonies appeared comparatively smaller in size. On successive sub culturing, it was observed that recombinant plasmid in the *E. coli* was stable only up to 3 passages. This may be due to ~ 9.3 kb size of recombinant plasmid. Although recombinant plasmid was found stable up to three generations, but seems to be first report of cloning of 6.5 kb viral segment into cloning vector (pTZ57R/T).

Plasmid isolation and confirmation:

Plasmids were isolated using GeneJET plasmid purification kit (Thermoscientific, USA) and designated as pTZ/CSF/6.5. On digestion with *BamH*I and *Pvu*I RE, 6.5 kb insert was released (Fig. 2) although some smaller fragments also appeared due to presence of RE sites for *Pvu*I (2 sites) and *BamH*I (1 site) in pTZ57R/T vector sequence. This confirms the cloning of expected 6.5 kb segment into pTZ57R/T vector. Recombinant plasmid was further confirmed by partial sequencing of the both ends using sequencing primers of the vector. Sequence obtained was confirmed by nucleotide blast.

CONCLUSION

The present study describes a modified method for amplification and cloning of 6.5 kb segment of CSFV adapted in cell culture into pTZ57R/T vector using TA cloning strategy and the recombinant plasmid (pTZ/CSF/6.5) was confirmed by RE digestion and partial sequencing. This cloning strategy can be used for cloning of longer amplified products without restriction enzyme selection.

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