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EXPRESSION OF BTV-16 VP5 GENE IN PROKARYOTIC EXPRESSION SYSTEM

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ABSTRACT

Bluetongue virus belongs to *Orbivirus* genus of the family *Reoviridae* having 10 discrete segments of double stranded RNA, each RNA segment code for seven structural (VP1 to VP7) and three nonstructural proteins (NS1 to NS3). VP5 is a serotype specific structural protein. The complete VP5-encoding gene of Bluetongue virus serotype 16 (BTV-16) was amplified by reverse transcription polymerase chain reaction (RT-PCR) using gene specific BTV16 VP5 primers. Complete VP5 gene of 1625bp was inserted into pET32a vector in presence of restriction sites. Recombinant plasmid DNA was integrated into the genome of *Escherichia coli* (*E. coli*) DH5α cells by $CaCl_2$ method. Colonies were selected on LB plate containing Ampicillin. Selected colonies were confirmed through colony PCR, sequencing and restriction enzyme analysis. Positive recombinant BTVVP5 plasmid was expressed in a prokaryotic expression system, i.e. *E. coli* BL21cells. The expressed protein was subjected for SDS page and Western blot Analysis.

Keywords: BTV (Bluetongue virus), VP5 (Viral protein), SDS-PAGE, Western Blotting, prokaryotic expression, Reverse transcription, PCR.

INTRODUCTION

Bluetongue (BT) is an infectious, non-contagious, insect borne viral disease of domestic and wild ruminants, such as sheep, goats, cattle, deer, buffaloes, camels, antelopes. The disease will affect more severe in sheep and less frequently in other ruminants, which is widespread in many parts of the world causing considerable harm to livestock. Biting midges belonging to the species *Culicoides* are implicated in transmission [1].

Bluetongue virus belongs to *Orbivirus* genus of the family *Reoviridae*, with the double stranded RNA. The Bluetongue VP5 is a structural protein which is serotype specific and it could enhance the ability of neutralizing antibody induction in sheep and are capable of protecting against the infection [15]. Genetic engineering techniques offer the possibility of producing recombinant proteins from the immunogenic gene of the virus by cloning and expression in good expression systems

[13]. The main objective is to express protein and to generate a new kind of DNA based vaccine specific for BTV serotype 16 and it is used to identify BTV-16 serotype by ELISA. A Complete BTV VP5 gene has been cloned and characterized. The current work is for standardizing method for the production of a recombinant BTV VP5 preparing the necessary background for commercial production of subunit vaccine for BT. The main objective of the study involves the complete procedure of VP5 gene amplification, transformation, selection of recombinant colonies, and expression of the target protein in *E. coli* BL21.

MATERIALS AND METHODS:

Amplification of BTV16 VP5 gene.

Bluetongue virus (BTV) serotype 16 was propagated in Baby Hamster Kidney (BHK-21) cell lines and used for total RNA isolation. The complete BTV 16 VP5 genes were amplified using the following gene-specific primers employing reverse transcription polymerase chain reaction (RT-PCR). To clone the gene in frame with pET32a vector, the restriction sites were added to the following designed primer.

BTV16VP5F (BamHI):
5'GCGGATCCGGTACCCAGATCCCTACGATTACGGAAGATG **3'** and

BTV16VP5R (NotI):
5'CGGGCGGCCGCCGCGTTTTTTAGGAAGAGCGGGAC **3'**

Total RNA was extracted from 200µl of BTV infected BHK-21 cell culture fluid using Trizol reagent (Invitrogen, USA) and was further subjected to cDNA synthesis with BTV16 VP5 primers (Bioserve Biotechnologies, Hyderabad, India) at 47°C for 30 min using High Retro transcriptase enzyme (Biotools, Spain). The 2µl of cDNA was amplified through PCR from the above mentioned primers. PCR temperature conditions were standardized with the following conditions, initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30sec, annealing at 51°C for 1min, extension at 72°C for 2min and a final extension of 72°C for 10 min. The amplicon was analyzed by 1% agarose gel electrophoresis using 1kb DNA molecular weight marker (Fermentas, USA).

Cloning of VP5 gene to pET32a vector.

The amplified VP5 gene as well as pET32a plasmid was subjected for restriction endonuclease digestion with BamHI and NotI. The digested products were further purified by phenol extraction method. Concentration and purity of the purified products were determined by spectrophotometrically, approximate 60 ng of VP5 gene and pET32a was ligated using T4 DNA ligase enzyme and buffer (Fermentas, USA). The *E-coli* DH5α strain, E coli BL21 (Invitrogen) used as host for DNA manipulation and expression respectively, cultured in LB media (Himedia) and were made competent for transformation by treating with $CaCl_2$. DH5α competent cells were mixed with 20-30 ng of ligated mixture and incubated on ice for 30 min. The cells were subjected to heat shock at 42°C for 50 Sec followed by snap cooling on ice. Further 1 ml of LB broth was added and incubated at 37°C with constantly shaking for 45 min. The cells were plated on LB agar containing 50µg/ml of Ampicillin and incubated at 37°C for 16hrs.

Conformation of transformation by PCR and RE digestion.

The colonies suspected for recombinant clones of pET32aVP5 were inoculated into LB broth with 50µg/ml Ampicillin at 37°C for overnight. PCR was carried with above mentioned primers for confirmation. Recombinant plasmid DNA was isolated by the alkali lysis method. The isolated recombinant plasmid DNA was subjected to RE digestion with BamHI and NotI. The amplified PCR product was sequenced.

Expression of recombinant pET32a VP5

About 20-30ng of recombinant pET32aVP5 was transformed into Bl21 (DE3) P lysis and positive transformants were selected on LB agar plate containing 50µg/ml of Ampicillin. The positive clones were inoculated into 10ml LB containing

50µg/ml Ampicillin and incubated for 16hrs at 250 rpm at 37°C. Incubated culture was inoculated into fresh medium (1:50) without Ampicillin and incubated until OD reaches 1 at 600nm. The cells were pelleted at 5000rpm for five minutes and resuspended into 25ml fresh LB medium containing 1mM IPTG and incubated for 5 hours at 30°C at 250 rpm with constant shaking. The cells were pelleted at 6000rpm for 5min and suspended in TE (pH 8) with 1 mM PMSF and freeze thaw was done for lysing the cells. The lysate is treated with DNAse to shear genomic DNA completely and was used to analyze the expression of protein on SDS page [11].

Analysis of the expressed protein through SDS PAGE and confirmation of the expressed BTV VP5 protein through western blotting.

The cell lysate was mixed with SDS PAGE loading buffer and analyzed on 12% SDS PAGE using protein molecular weight markers as standard. In case of western blot the protein was transformed to the nitrocellulose membrane and coated with BTV VP5 specific rabbit serum at 1:2000 dilutions. The bounded IgG was detected by using anti rabbit HRPO conjugate at a concentration of 1:2000. The protein was visualized with ODD and H2O2 (Ortho dianisidine dihydrochloride-hydrogen peroxide) chromogen-substrate solution [4].

RESULTS

Amplification of BTV16 VP5 gene.

BHK21 cells infected with BTV16 showed CPE after 48hrs of post infectious with characteristic bunch of grapes like aggregation finally at 72hrs leading to complete destruction of the cell sheath with the visual observation of the medium showed a drop in the pH due to cellular destruction. On the other hand the control BHK-21 cell monolayers were healthy, intact with no apparent changes in morphology. The RNA extracted from infected cell culture using Trizol® reagent, was checked for purity by spectrophotometry, reading O.D at 260nm and 280nm. The A260: A280 ratio was found to be greater than 2 indicating pure preparation. Simultaneously, RNA from healthy uninfected BHK-21 cells was extracted for negative control. The RNA was subjected to cDNA synthesis and PCR amplification of VP5 encoding gene fragment was analyzed by agarose gel electrophoresis along with 1kb DNA molecular weight marker showing a band size of 1.6kb (Fig.1).

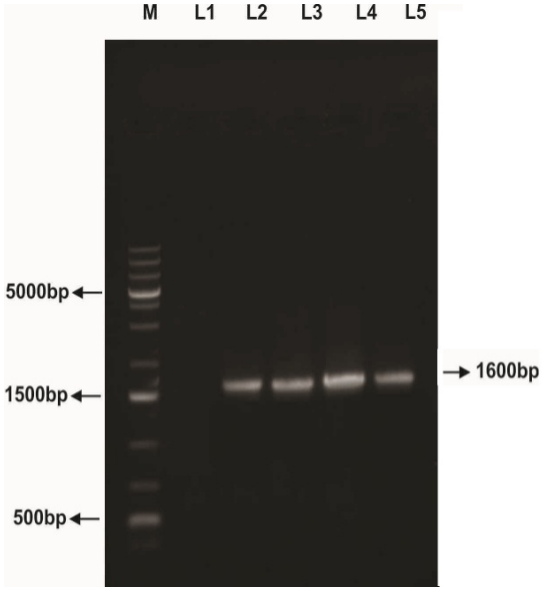

Fig. 1. Agarose gel electrophoresis of VP5 gene amplification

Lane M - one Kb plus molecular marker, **Lane 1**- Negative control, **Lane 2-5** - VP5 positive

Cloning of VP5 gene to pET32a vector

PCR product and pET32a vector was subjected to RE digestion with BamHI-NotI. The restricted products were ligated and inserted into expression vector pET32a and transformed into DH5α cells. The transformants were selected on LB agar plates containing Ampicillin, about 30-55 colonies appeared after 16 hrs of incubation.

Conformation of transformation by PCR and RE digestion.

Randomly 5 colonies were selected and colony PCR carried out, the products analysed on 1% agarose gel electrophoresis. Out of 5 colonies, 2 showed the amplification at 1.6kb band indicating the presence of inserts with pET32a vector (without insert) as a negative control, no amplification was observed. (Fig 2).

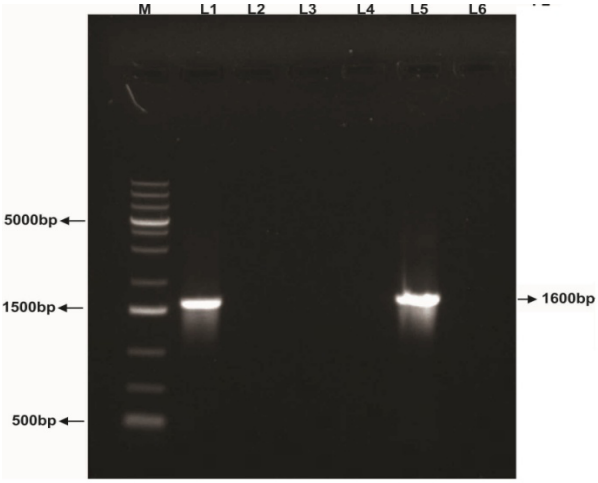

Fig 2. Agarose gel electrophoresis of colony PCR amplified products from positive clones

Lane M - one Kb plus molecular weight marker,**Lane 1&5** - 1.6 Kb PCR amplified VP5 gene from recombinant vector, **Lane 2,3& 4** - Negative colony without VP5 insert, **Lane 6** - Negative control.

The recombinant plasmid DNA was isolated from the colonies positive for PCR were subjected to RE digestion using BamHI-NotI for pET32aVP5. On agarose gel electrophoresis, two bands were observed of which, one is specific to linearized vector at 5.9 kb and the other VP5 at 1.6kb (Fig. 3).

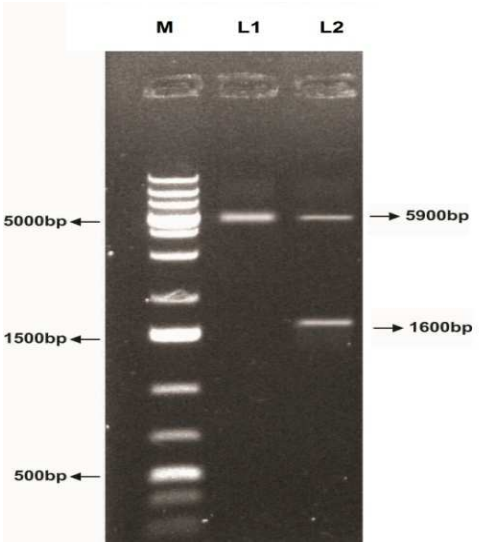

Fig. 3. Agarose gel electrophoresis of pET32aBTVP5 plasmid

DNAs digested with BamHI and NotI

Lane M - one Kb plus molecular weight marker,**Lane 1** - Linearized pET32a vector control, **Lane 2** - BamHI and NotI digested recombinant pET32aBTVP5 plasmids showing a release of 1.6 kbp VP5 gene fragment.

The obtained sequence of BTV16 confirmed the cloned VP5 gene as with that of the BTV gene sequences available in the gene bank with accession no.AB6862289 with 97% similarity.

Expression of recombinant pET32a VP5, analysis through SDS PAGE and confirmation by western blotting.

The positive recombinant plasmids pET32a VP5 was subjected for transformation in BL21competent cells. Recombinant clones were induced with one mM IPTG for five hrs. The lysate of cells collected after induction was subjected to SDS-PAGE analysis. The colonies positive for recombinant pET32aVP5 plasmid and pET32a vector alone (without insert) were run along the side of protein molecular weight marker. 79kDa protein band was observed for pET32aVP5, where as in case of negative control above noted bands were absent (Fig. 4).

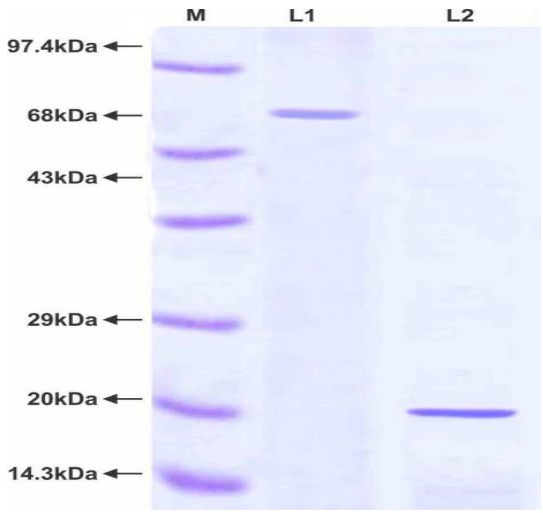

Fig.4.SDS-PAGE analysis of *E.coli* BL21(DE3)PLysS expressed cell lysate

Lane M - protien marker,

Lane 1 - Cell lysate of induced colonies with pET32aBTVP5 plasmid transformant
Lane 2 - Cell lysate of induced colonies with pET32a vector transformants (without insert)
The expressed proteins containing N-terminal Trx tag which add about 20 kDa size to the 59 kDa of VP5 protein and also contain His tag and S tag for detection and purification. Expression of the protein from the cloned gene was further confirmed by Western Blot analysis of SDS-PAGE separated proteins.

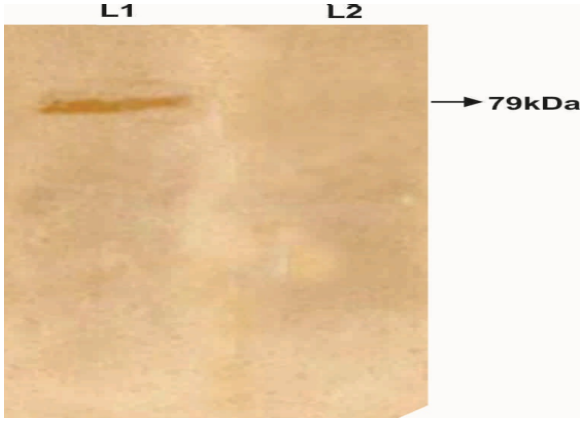

Fig 5. Western blot analysis of expressed VP5 from *E. coli* (BL21(DE3)pLysS) transformed with recombinant pET32aBTVP5 plasmid.

Lane 1 - Cell lysate of induced colonies with pET32aBTVP5 plasmid transformant
Lane 2 - Cell lysate of induced colonies with pET32a vector transformants (without insert)

The proteins were detected by BTV16 specific rabbit serum, the reaction showed strong positive band at the position corresponding to the band observed on Commassie brilliant blue stained SDS-PAGE gel and absent in control indicating that the proteins for BTV were specific (Fig.5).

DISSCUSSION

Bluetongue is an economically significant vector borne, non contagious, viral disease of domestic and wild ruminants in many parts of the world. Research for finding an effective control measure is being carried out in the area of new generation vaccines like recombinant proteins virus like particles [3] and Baculovirus expressed triple and quadruple type recombinant vaccines have been tried the BTV core like particles and were found to be successful in eliciting immune response under laboratory conditions at various levels. Cloning and expression is necessary for the production of specific proteins which can be used as a specific vaccine.

In the present study BHK-21 cell lines are successfully used to propagate the BTV and the CPE was characteristic as described by earlier works (Byregowda 2000). Trizol method of RNA extraction has been found to be simple. In addition, the total RNA extracted was intact and found to be efficient for cDNA synthesis. Researchers have used different temperature conditions with or without denaturing agent. In the present study the optimum temperature conditions of 94^0C for 5min standardize [2]was found ideal for denaturation of BTV RNA

For RT-PCR, the primers were designed in lab using the consensus sequences of BTV 16 gene available in the GenBank. The suitable RE sites were included which were suitable for pET32a vector insertion and to align in frame which is suitable for expression. For specific amplification of the target sequence in the complementary DNA and for obtaining sufficient quantity of the desired product by PCR, it was essential to optimize different annealing temperature and number of cycles. In the present study, the optimum annealing temperature was found to be 51^0C for 1 minute for VP5, wherein a clear amplified band was visible on electrophoresis. The number of cycles was optimized and it was found that 30 cycles for VP5 gave optimum results.

pET system was used by several workers for the expression of different genes in *E. coli* BL21(DE3)pLysS cells and noticed very high levels of expression upon induction at one mM IPTG concentration for three to four hrs [14],[5] VP5 gene of BTV16 was amplified and used for cloning into pET32a plasmid. Confirmed clones were transformed into BL21(DE3)pLysS cells

and induced for expression. It is based on the T7 and T7 lac promoters driven system originally. After plasmids were established in non-expressing host they were transferred into a host bearing the T7 RNA polymerase gene (BL21(DE3)pLysS) for expression of target proteins. In BL21(DE3)pLysS cells the T7 polymerase gene in under the control of the lacUV5 promoter [8].The lysate containing expressed protein was subjected for SDS-PAGE analysis. A protein band of 79kDa was observed. Similar protein band in the cell lysate of induced *E. coli* cells carrying only pET32a plasmid was not seen. However, one extra protein band around 20 kDa size was seen in case of control which represent the fusion tag. The actual protein size of VP5 is 59kDa. Thus can conclude that the extra 20 kDa protein is from the fusion tag of the vector comprising thioredoxin (Trx) tag and His tag. The protein expressed was further subjected to immunological detection after electrotransfer of SDS-PAGE separated protein onto nitrocellulose membrane. The VP5 showed a positive colour reaction with BTV16 rabbit serum and goat anti-rabbit HRPO conjugate (Sigma, USA) and chromogen substrate.

The BTV VP5 protein contains neutralizing epitopes [6],[7] and is a potential target for developing a subunit vaccine. Although the expressed BTV VP5 protein can elicit an immune response in rabbits. BT viral challenges in sheep are required to confirm its action as a potential subunit vaccine. The recombinant protein expressed may have potential applications in developing BTV16 serotype-specific diagnostics and also as a subunit vaccine, which needs further studies.

CONCLUSION

In conclusion, the BTV VP5 recombinant protein has been expressed. The pET32a BTVVP5 recombinant vector is suitable for expression of target protein. Further studies as to be taken up for purification of the recombinant protein, studies like serum neutralization, and BT viral challenges in sheep are required to confirm its action as a potential subunit vaccine. For the large scale production it can be aligned in the secretory expression system namely the yeast, *Pichia pastoris.*

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