International Journal of Advanced Biotechnology and Research
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 4, Issue 1, 2013, pp 8-14
http://www.bipublication.com

EXPRESSION OF INTEGRATED BLUETONGUE VP7VP5 PROTEINS IN PROKARYOTIC SYSTEM

Purushotham K.M[*], Pawan Kumar* Byregowda S.M[***], Gopinath S. M**

*Dept. Of Biotechnology, Singhania University, Pacheri Bari, Jhunjhunu (Rajasthan).
[***]Institute of Animal Health and Veterinary Biologicals, Hebbal, Bangalore-560024
[**]Acharya Institute of Technology, Soldevanahalli, Hesaraghatta Road, Bangalore-560090
**Email Id: researchgopinath@gmail.com

[Received-17/12/2012, Accepted-02/01/2013]

ABSTRACT

This study describes the expression of integrated VP7 (group specific) VP5 (Type specific) recombinant protein of BTV16. The primers were designed to amplify complete VP7 and VP5 encoding gene of Bluetongue virus serotype 16 (BTV-16). The suitable RE sites were included in the primers for pET32a insertion. Both genes were amplified by reverse transcription polymerase chain reaction (RT-PCR) using gene specific BTV16 VP5 and VP7 primers. Complete VP7 of 1109bp and VP5 gene of 1625bp was inserted into pET32a vector. Recombinant plasmid DNA was integrated into the genome of *Escherichia coli* (E. coli) DH5 alpha cells by $CaCl_2$ method. Colonies were selected on LB plate containing 50µg/ml ampicillin. Selected colonies were confirmed through colony PCR and Restriction Enzyme analysis. Conformed positive recombinant BTVVP7VP5 plasmid was expressed in a prokaryotic expression system, BL21 (DE3) pLysS E. coli . The expressed protein was separated on 12% SDS PAGE, which showed 117kDa band. The actual size of integrated protein VP7+VP5 is 98kDa. The extra 20 kDa proteins are from the fusion tag of the vector comprising thioredoxin (Trx) tag and GSSG-6 His tag. The protein was confirmed by Western Blot Analysis.

Key Words : VP7, VP5, recombinant protein BTV16, pET32a, thioredoxin (Trx) tag, GSSG-6 His tag.

INTRODUCTION

Bluetongue virus (BT) is the causative agent of Bluetongue disease, an arthropod-transmitted disease of wild and domestic ruminants. BTV is the prototype virus of the genus *Orbivirus* in the family *Reoviridae*. BT occurs throughout the temperate and tropical regions of the world, in an area that parallels the distribution of the competent vector, *Culicoides* spp. There are mandatory restrictions on the movement of ruminants from BTV endemic countries to BTV free countries as it is listed under 'List A' diseases by Office International des Epizooties [9]. Bluetongue is endemic in India and with large livestock population and it is one of the major diseases to be considered as a National level for control. The virus is architecturally complex and has the ability to attach and replicate in a variety of vertebrate and invertebrate hosts. BTV double stranded RNA composed of 10 discrete segments of genome surrounded by two layers of protein capsid. Each RNA segment code for different proteins,

which include seven structural (VP1 to VP7) and three nonstructural proteins (NS1 to NS3). Among the viral proteins VP5 structural proteins which are serotype specific and it could enhance the ability of neutralizing antibody induction. VP7 is the major capsid protein and contributes for the group specificity antigen among all the serotypes which reacts with antibodies of all 26 serotypes so far recognized. The recent developments in the recombinant DNA technology have been reported for use of expressed VP7 protein as diagnostic reagent in c-ELISA [1]. Both VP7 and VP5 could be used for the assembly of virus like particles and for the development of a genetically engineered vaccine. [15]. The recombinant DNA technology is being applied for cloning and expression of integrated VP7 VP5protein which can be used as candidate vaccine and diagnostic reagent for ELISA.

MATERIALS AND METHODS

Amplification of BTV16 VP7 and VP5 genes.

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 and VP7gene 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 primers.[Table-1]

[**Table-1** Gene-specific primers employing for reverse transcription polymerase chain reaction]

Sl. No.	Primer code	Primer sequence	Primer length	Product size
1	BTV-16F VP7 (BglII)	**5'**GC**AGATCT**GCCGGCCGGGTAGAGATGGACACTATCGC**3'**	37	1109bp
2	BTV-16R VP7 (BamHI)	**5'**GC**GGATCC**GGTACCACATAGGCGGCGCGTGCAAT**3'**	34	
3	BTV-16F VP5 (BamHI)	**5'**GC**GGATCC**GGTACCCAGATCCCTACGATTACGGAAGATG**3'**	39	1625bp
4	BTV-16R VP5 (NotI)	**5'**CGG**GCGGCCGC**CGCGTTTTTTAGGAAGAGCGGGAC**3'**	35	

200µl of BTV infected BHK-21 cell culture fluid was used for total RNA extraction from Trizol reagent (Invitrogen, USA) and was further subjected to cDNA synthesis with BTV16 above mentioned gene specific primers (Bioserve Biotechnologies, Hyderabad, India) at 47^0C for 30 min using High Retro transcriptase enzyme (Bio tools, Spain). The 2µl of cDNA was amplified through PCR from the same primers. PCR temperature conditions for BTVVP7 were standardized, the cycling parameters consisted of a 5 minute initial denaturing step at 94^0C followed by 30 cycles of denaturation 94^0C for 30 seconds, annealing at 56^0C for 45 seconds, extension at 72^0C for 1.5 minutes and a final extension for 10 minutes at 72^0C, where as for VP5 gene amplification, initial denaturation at 94^0C for 5 min, followed by 30 cycles of denaturation at 94^0C for 30sec, annealing at 51^0C for 1min, extension at 72^0C for 2min and a final extension of 72^0C for 10 min. The amplicon was analyzed by 1% agarose gel electrophoresis using 1kb DNA molecular weight marker (Fermentas, USA).

Cloning of VP7 and VP5 gene to pET32a vector.

PCR amplified product and plasmid DNA were subjected to restriction endonuclease digestion using restriction enzymes BglII-BamHI for VP7 gene, BamHI-NotI for VP5 and BglII-NotI for combined VP7 and VP5. By phenol extraction

method the digested products were purified [12]. The concentration and purity were checked by spectrophotometer. Ligation was carried by using T4 DNA ligase enzyme and buffer (Fermentas) approximately 60 ng of VP7, VP5, gene and pET32a was used.

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 $CaCl_2$ (Sambrook and Russel 2001). 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^{o}C for 50 sec followed by snap cooling on ice. Further 1 ml of LB broth was added and incubated at 37^{o}C with constantly shaking for 45 min. The cells were plated on LB agar containing 50µg/ml of Ampicillin and incubated at 37^{o}C for 16hrs.

Conformation of transformants by PCR and RE digestion.

The recombinant clones of pET32aVP7VP5 suspected colonies were inoculated into LB broth with 50µg/ml Ampicillin at 37^{O}C for overnight. PCR was carried with above mentioned primers i.e BTV-16F VP7 and BTV-16R VP5 for confirmation. PCR temperature conditions were standardized, the cycling parameters consisted of a 5 minute initial denaturing step at 94^{0}C followed by 30 cycles of denaturation 94^{0}C for 30 seconds, annealing at 56^{0}C for 1 minute, extension at 72^{0}C for 2.5 minutes and a final extension for 10 minutes at 72^{0}C. Recombinant plasmid DNA was isolated by the alkali lysis method as described in [12] with slight modification. The isolated recombinant plasmid DNA was subjected to RE digestion with BglII-NotI.

Expression of recombinant pET32a VP7VP5

The recombinant pET32aVP7VP5 was transformed into Bl21 (DE3) P lysis and positive transformants were selected on the 50µg / ml of the Ampicillin LB agar plate. The positive clones were inoculated into 10ml LB containing 50µg/ml Ampicillin and incubated for 16hrs at 250 rpm at 37^{o} 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^{O}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 [10].

Analysis of the expressed protein through SDS PAGE and confirmation of the expressed BTV16 VP7+VP5 protein through western blotting.

The cell lysate was separated on 12% SDS PAGE using protein molecular weight markers as standard [12]. For confirmation by Western blot, the protein was transformed to the nitrocellulose membrane and coated with BTV 16 specific rabbit serum at 1:2000 dilution. The bounded IgG was detected by using anti rabbit HRPO conjugate at a concentration of 1:2000. The protein was visualized with OOD and H_2O_2 (Ortho dianisidine dihydrochloride-hydrogen peroxide) chromogen-substrate solution [4].

RESULTS

Amplification of BTV16 VP7 and VP5gene.

After 48hrs of post infectious with BTV16, BHK21 cells showed characteristic CPE with bunch of grapes like aggregation, finally leading to complete destruction of the cell sheath at 72hrs with the visual observation of the medium showed a drop in the pH due to cellular destruction. 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. To check purity by spectrophotometry the OD was read at 260nm and 280nm. The A260: A280 ratio was found to be greater than 2 indicating pure preparation. cDNA

was synthesized by specific primers and subjected for further amplification. PCR amplification of VP7 and VP5 encoding a gene fragment was analyzed by agarose gel electrophoresis along with 1kb DNA molecular weight marker showing a band size of 1.1kb for VP7 and 1.6kb for VP5 (Fig.1 and 2).

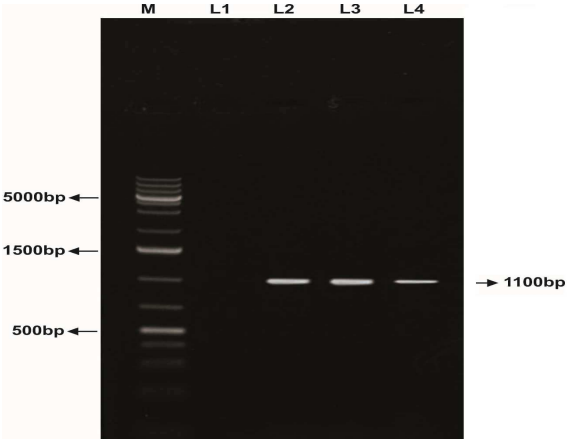

Fig. 1. Agarose gel electrophoresis of VP7 gene amplification
Lane M - 1kb plus molecular marker
Lane 1 - Negative control
Lane 2-5 - VP7 showing 1.1kb band

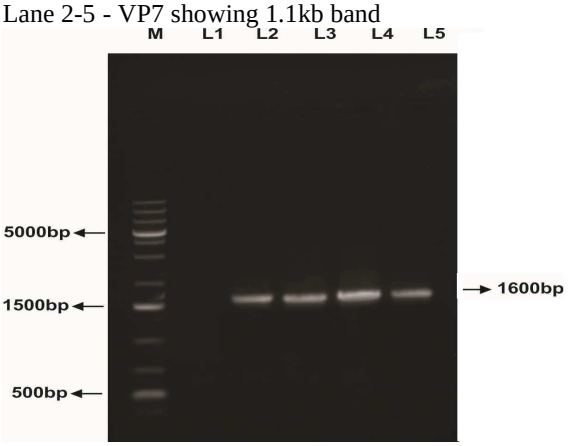

Fig. 2. Agarose gel electrophoresis of VP5 gene amplification
Lane M - 1 kb plus molecular marker
Lane 1 - Negative control
Lane 2-5 - VP5 showing 1.6kb band

Cloning of VP7 and VP5 gene into pET32a vector

The restricted products were ligated and inserted into pET32a vector and transformed into DH5α cells. The transformants were selected on LB agar plates containing Ampicillin, about 25 colonies appeared after 16 hours of incubation.

Conformation of transformation by PCR and RE digestion.

Random 5 colonies were selected and colony PCR carried out, the products were analyzed on 1% agarose gel electrophoresis. Out of 5 colonies, 2 showed the amplification at 2.7kb band indicating the presence of insert, no amplification was observed with pET32a vector (without insert) as a negative control (Fig. 3).

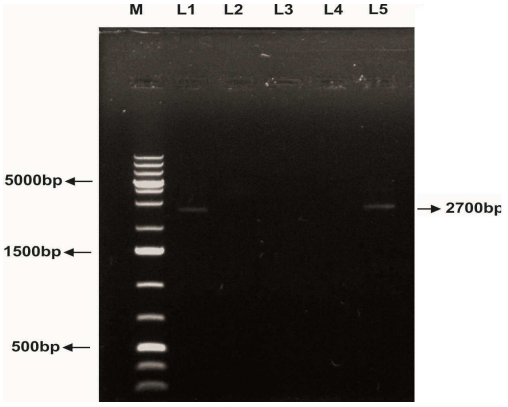

Fig 3. Agarose gel electrophoresis of colony PCR amplified products from positive colonies in colony lysis
Lane M - 1kb plus molecular weight marker
Lane 1&5 - 2.7 kb PCR amplified VP7VP5 gene from recombinant vector
Lane 2, 3& 4 - colonies without VP7VP5 insert

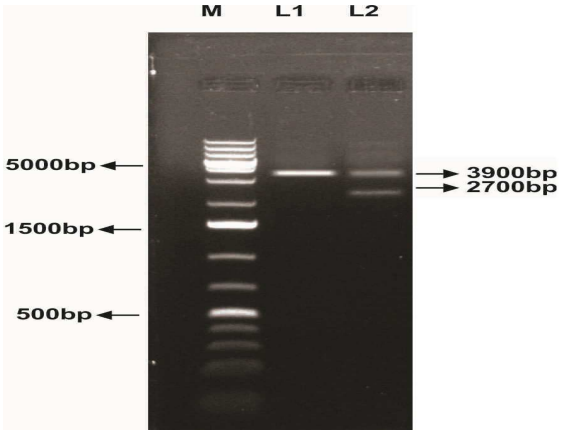

Fig. 4 Agarose gel electrophoresis of pET32aBTVP7VP5 plasmid
DNAs digested with BglII and NotI
Lane M - one Kb plus molecular weight marker
Lane 1- Linearized pET32a vector control

Lane 2 - BglII and NotI digested recombinant pET32aBTVP7VP5 plasmids showing a release of 2.7 kbp VP7VP5 gene fragment

The recombinant plasmid DNA was isolated from the colonies that were positive for PCR, were subjected to RE digestion using BglII-NotI for pET32aVP7VP5. On agarose gel electrophoresis, two bands were observed of which one is specific to linearized vector at 5.9 kb and the other VP7VP5 at 2.7kb (Fig. 4).

Expression of recombinant pET32aVP7VP5, SDS PAGE analysis and confirmed by Western blotting.

Lysate containing recombinant pET32aVP7VP5 plasmid and pET32a vector alone (without insert) was run along the side of protein molecular weight marker. In recombinant pET32aVP7VP5 protein 117kDa protein band was observed, where 20kda band was observed in negative control (Fig. 5).

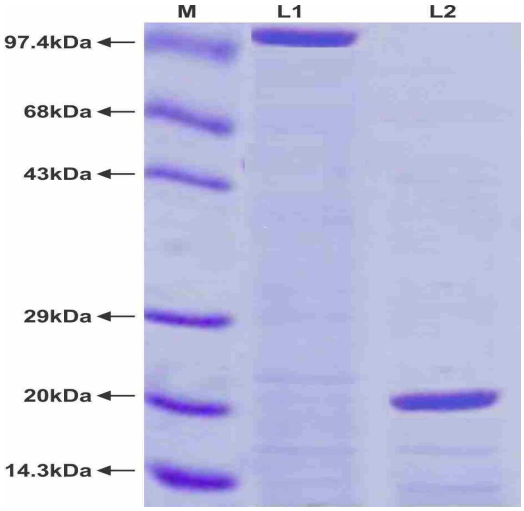

Fig. 5. SDS-PAGE analysis of *E. coli* BL21(DE3)PLysS expressed cell lysate

Lane M - protien marker

Lane 1 - Cell lysate of induced colonies with pET32aBTVP7VP5 plasmid transformant

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

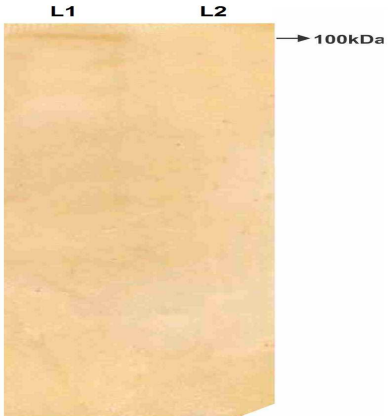

Fig 6. Western blot analysis of expressed *E. coli* (BL21(DE3)pLysS) transformed with recombinant pET32aBTVP7VP5 plasmid

Lane 1 - Cell lysate of induced colonies with pET32aBTVP7VP5 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 97kDa of VP7VP5 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. The proteins were detected by BTV16 specific rabbit serum, the reaction showed a 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 of BTV were specific (Fig.6).

DISSCUSSION

Bluetongue is an economically significant vector borne, non contagious, viral disease of domestic and wild ruminants in many parts of the world. Presently, diagnosis of BT relies on serological assays, which are both cross reactive and expensive. Although, monoclonal antibody based ELISA kits overcome cross reactivity, they are expensive and need to be imported. At present, the only preventive method is the use of vaccines. 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 trying 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 [1].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 [2]. RNA of BTV is double stranded, initial denaturation for primer annealing was found to be critical. Researchers have used different temperature conditions with or without denaturing agent. In the present study the optimum temperature conditions of 94^0C for 5min standardized by [1] was found ideal for denaturation of BTV RNA

For RT-PCR, the primers were designed in the 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 quantities of the desired product by PCR, it was essential to optimize different annealing temperature and the number of cycles. In the present study, the optimum annealing temperature was found to be 30 seconds at 56^0C for VP7 gene and 1 minute at 51^0C for VP5 gene, wherein a clear amplified product band was visible on electrophoresis. The number of cycles was optimized and it was found that 30 cycles for both VP 7 and 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 hours [13],[5]. VP7 and 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 under the control of the lacUV5 promoter [8]. The lysate containing expressed protein was subjected to SDS-PAGE analysis. A protein band of 117kDa integrated fused protein was observed. Similar protein band in the cell lysate of induced *E. coli* cells carrying only pET32a plasmid were not seen. However, one extra protein band around 20 kDa size was seen in the case of control which represent the fusion tag. The actual protein size of VP7VP5 is 97kDa. This 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 VP7VP5 showed a positive colour reaction with BTV 16 rabbit serum and goat anti-rabbit HRPO conjugate (Sigma, USA) and chromogen substrate.

The BTV VP7 and VP5 protein contains neutralizing epitopes [6],[7] and is a potential target for developing a subunit vaccine. BT viral challenges in sheep are required to confirm its action as a potential subunit vaccine. Since the ultimate goal of our study was in the production of recombinant BTV VP7VP5 in *E. coli* that could be taken up for up scaling, we did not carry out extensive studies on vaccination in sheep. However, the recombinant protein expressed may have potential applications in developing group-

specific diagnostics and also as a subunit vaccine, which needs further studies.

CONCLUSION

In conclusion, the BTV VP7VP5 recombinant protein has been expressed. The pET32a BTV VP7VP5 recombinant vector is suitable for expression of target proteins. 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 larger scale production it can be aligned in the secretory expression system namely the yeast, *Pichia pastoris.*

ACKNOWLEDGMENTS

- This work was supported by AINP-BT, Indian Council of Agricultural Research (ICAR)
- Indian Animal Health and Veterinary Biologicals, Hebbal, Bangalore.

REFERENCES

[1] Byregowda (2000). Development of molecular methods of Bluetongue diagnosis and characterization of selected isolates. Ph.D. Thesis, Univ. Agric. Sci., Bangalore.

[2] Chomezynski, P. (1993). Biotecniques 15:532.

[3] French, T.J. and Roy, P. (1990). Synthesis of Bluetongue virus (BTV) core like particles by a recombinant *Baculovirus* expressing the two major structural core proteins of BTV. *J. Virol*., **Apr.**: 1530-1536.

[4] Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press). 726 pg

[5] Lin-Fa Wang, Kattenblelt, J.A., Gould, A.R., Pritchard, L.I., Crameri, G.S. and Eaton, B.T., (1994). Major core protein VP7 of Australian Bluetongue virus serotype 15: sequence and antigenicity divergence from other BTV serotype. *J. Gen. Virol.*, **75**: 2421-2425.

[6] Mertens, P.P.C., Brown, F. and Sangar, D.V. (1984). Assignment of the genome segments of bluetongue virus type 1 to proteins which they encode. *Virology*, **135**: 207-217.

[7] Mertens, P.P.C., Burrough, J.N. and Andersson, J. (1987). Purification and properties of the virus particles, infectious sub virus particles and cores of bluetongue virus serotype 1 and 4. *Virology*, **157**: 375-386.

[8] Novagen. (2001). Protein Expression: Prokaryotic Expression: pET System Overview. 2001 Novagen Catalog. p 68-72

[9] Office International des Epizooties, (1998). Supporting document for the OIE International Animal Health Code chapter 2.1.9 on bluetongue. OIE Adhoc working group on bluetongue, September 1998.

[10] Ramanagowda, R. B. (2005). Cloning and expression of VP7 gene of bluetongue virus in *Pichia pastoris*. M.V.Sc. Thesis, Univ. Agric. Sci., Bangalore.

[11] Roy P, French T, Erasmus BJ. (1992) Protective efficacy of virus-like particles for bluetongue disease. *Vaccine;* 10:28–32.

[12] Sambrook, J. and Russell, D.W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd Edn., Cold Spring Harbour Laboratory Press, New York.

[13] Suryanarayana, V.V.S., Madanmohan, B., Bist, P., Natarajan, C. and Tratschin, J.D. (1999). Serotyping of FMD virus by antigen capture RT-PCR. *J. Virol. Methods*, **80**: 45-52.

[14] Simms, D., Cizdziel, P.E and Chomezynski, P. (1993). *Focus*. 15(4):99

[15] Wang-JianWei, Jiang HuiYing, Li XiaoCheng, Li YunGang, Zhao TongXing, Hung Tao, Wang, J.W., Jiang, H.Y., Li, X.C., Li, Y.G., Zhao, T.X. and Hung, T. (1999). Expression of bluetongue virus VP7 in insect cells and its application for diagnosis of group specific antigen. Chinese J. Virol., **15**: 238-243.