

Cloning and Expression of VP2 Outer Coat Protein Gene of Bluetongue Serotype1 Virus, Indian Isolate

S.N. Nagesha*¹, H.M. Mahadeva Swamy¹, S.M. Byre Gowda²
and V.V.S. Suryanarayana³

¹University of Agricultural Sciences, GKVK, Bangalore-560065, Karnataka, India

²Institute of Animal Health and Veterinary Biologicals,
Bangalore -560024 Karnataka, India

³Indian Veterinary Research Institute, Hebbal, Bangalore-560024, Karnataka, India

*Corresponding Author E-mail: nagisn@gmail.com

Abstract

Bluetongue (BT) is a viral disease causing morbidity and mortality in sheep, cattle and wild ruminants, including deer, sambar and bluebull. In this study we present the internal gene of outer coat protein, VP2 of bluetongue virus serotype 1 of Indian isolate was amplified from cDNA synthesized through RT-PCR using total RNA with specific forward and reverse primers. The PCR product was cloned to pBKS⁺ cloning vector and sequenced. It was found that 880bp VP2 gene was 87% homology to Australian BTV VP2 and 85% homology to BTV serotype 1. The VP2 gene was released from pBKS⁺ cloning vector and sub cloned into expression vector pET32a and expressed protein of size 32.89 KDa was confirmed by SDS-PAGE and Western blot analysis further the expressed antigen could be used for diagnosis of BT without cross reaction and for vaccination once the antigen evaluated through clinical trials.

Keywords: Bluetongue virus; RT-PCR; Western blot; serotype

Introduction

Amongst the numerous diseases of ruminants, bluetongue (BT) has gained considerable importance in recent years of the tropical and subtropical countries of the world. (Mellor *et al.*, 2000 and 2008; Purse *et al.*, 2005 and Wilson *et al.*, 2008). As an arbovirus, Bluetongue virus (BTV), the etiological agent of BT, depends almost entirely on the presence of competent *Culicoides* biting midges to be transmitted to the local host population. Among ruminant's cattle, goats and buffaloes harbor the

virus with out any clinical manifestation for quite a long period. Currently, 24 serotypes of BTV have been identified worldwide, with most infected countries confronted with the challenge of dealing with multiple serotypes circulating in their ruminant populations (Mellor *et al.*, 2009). The disease is caused by Bluetongue virus (BTV), which is the type species of Orbivirus genus of Reoviridae family. The virus is non-enveloped and icosahedral in symmetry with ten double stranded RNA segments.

Each RNA segment codes for different proteins which include seven structural (VP1 to VP7) and three non structural (NS1, NS2, NS3) proteins (Roy, 1991, Francki, *et al.*, 1991).

The outer coat proteins that determine serotype specificity of BTV are VP2 and VP5 encoded by RNA segment 2 and segment 5 respectively. These two proteins vary significantly among different serotypes both by amino acid sequence and genome sequence of corresponding genes; study of the variations in the genome sequences of VP2 and VP5 will facilitate identification of variations in BTV isolates. It is known that VP2 is the main serotype specific antigen (Huisman and Erasmus, 1981; Kahlon *et al.*, 1983) and that solubilized VP2 induces neutralizing antibodies in sheep and is capable of protecting against viral infection (Huisman *et al.*, 1983).

There are at least 24 serotypes have been identified of which 21 have been identified in India (Prasad, 2002). In a disease outbreak identifying the serotype involved is necessary. At present the BTV serotyping is done by serum neutralization. Serological tests like AGID test or by ELISA are not useful for serotyping. Detection of serotypes is necessary for taking up effective control measures including vaccination. For rising the serotype specific sera one of recombinant protein specific to VP2 protein will be of use. The recombinant protein could be expressed in suitable expression system like *E.coli* or yeast to express serotype specific antigen (BTV VP2 serotype 1) for using it as a standard antigen for raising serotype specific sera and for vaccination.

Materials and methods

Isolation of BTV VP2 gene fragment

Total RNA from BTV infected BHK-21 cells was extracted using Trizol reagent (Gibco BRL, USA) method and was further subjected to cDNA synthesis at 42°C for 60 min using Murine Moloney Leukemia Virus (M-MLV) reverse transcriptase enzyme with VP2 specific primers. The cDNA was amplified through PCR employing the same primers. PCR was carried out under the following conditions: 94°C for 45 s, 59°C for 40 s, 72°C for 50 s, for 30 cycles, and finally 72°C for 5 min. The amplified product was electrophoresed across a 1.2% agarose gel stained with ethidium bromide and visualized under UV transilluminator.

Cloning and sequencing of BTV VP2 sequence

The BTV VP2 gene was PCR amplified with left and right specific primers VP2LI 5' GCCGATCCATGGATGAGTTAGGCAT 3' and VP2RI 5' GGCCTCGAGCCAATCGTTGACAATATCTTACATA 3' and cloned into pBKS⁺

cloning vector (Fermentas, USA) with T₄ DNA ligase. Competent DH5 α cells were transformed with 2 μ l of the ligated DNA product and plated on LB Ampicillin (20 μ g) plates. The positive colonies were screened by colony lysis and enzymatic digestion with *Bam*HI and *Xho*I restriction enzymes (Fermentas, USA) and the inserted gene was sequenced with Sequencing was done in both directions using T7 and reverse primer used in the PCR amplification of gene. The partial gene sequence was submitted to NCBI GenBank (Accession No.GQ233041).

Comparison and Sub-cloning of BTV VP2 gene into pET32a expression vector

The sequence data of BTV1 VP2 gene obtained was analyzed with the published sequence available in the gene bank and comparison was made. The VP2 gene insert of BTV serotype 1 was released from the cloned vector pBKS⁺ DNA by restriction digestion with *Bam*HI and *Xho*I was purified through LMP agarose gels and used for sub cloning. It was ligated into *Bam*HI and *Xho*I sites in pET32a vector, at the vector: insert ratio of 1: 4 with T4 DNA ligase. Competent DH5 α cells were transformed with 2 μ l of the ligated DNA product and plated on LB Ampicillin (20 μ g) plates. The positive colonies were screened by colony lysis and enzymatic digestion with *Bam*HI and *Xho*I restriction enzymes and transformed the positive pET32a vector with VP2 insert into *E.coli* BL21DE3 P-lys S, cells.

Expression and Determination of BTV VP2 gene expression through SDS-PAGE

The colonies harboring the pET32a+ VP2 were inoculated into a 10ml LB broth containing antibiotics (20 μ g/ml ampicillin) and incubated in a shaker at 250 rpm and 37°C for 16 hours. The bacterial cells were pelleted and suspended in 10ml LB containing appropriate antibiotics and incubated with shaking for 5 more hours until the OD reached 1.0 at 600nm. The cells were pelleted and resuspended in a 10ml LB without antibiotics. IPTG was added to a final conc. of 0.2mM to the culture for induction and was incubated for a further 6 hours at 30°C with shaking. The bacteria were pelleted at 6000rpm for 10min and the pellet was suspended in sterile TE (pH-8) containing PMSF at 1mM conc. The suspended pellet was freezed at -80°C overnight. Appropriate negative controls were used for expression in each case. SDS-PAGE was done to confirm the presence of the protein as per the procedure (Sambrook *et al.*, 2001). To confirm the expression of the gene in *E.coli* BL21DE3 P-lys S, cells the total protein from the bacterial cells was extracted, concentrated and subjected to SDS-PAGE to obtain the protein profile, based on their molecular weight. The protein bands were visualized by Coomassie blue staining of the gel. (Data not shown).

Affinity purification of protein and Western blot analysis of expressed protein

The protein purification was done to obtain specific protein for further western blot analysis using regenerated column (Nickel-CL Agarose column, Bangalore Genei.).

Western blot analysis of the expressed protein was done to detect the specific protein by its immuno reactivity with known antibody against the protein. In brief, after the SDS-PAGE, the proteins were transferred on to nitrocellulose membrane using electrophoretic transfer apparatus according to the manufacturer's instructions. The electrophoretic transferred protein was subjected to immunological detection.

Before adding the specific antibodies the membrane was incubated in blocking buffer (5% skimmed milk powder in PBS) at 37°C for 1 hr. The membrane was washed for three times in PBST (PBS with 0.05% Tween –20), the membrane was transferred into fresh blocking buffer to which bovine serum against BTV-1 at 1:200 dilution was added and incubated further with gentle shaking. After 1hr of incubation, the membrane was washed three times in PBST and anti bovine rabbit IgG with HRPO conjugate at a concentration of 1:1000 was added in blocking buffer. The membrane was incubated at 37°C for 1hr. After three times washing with PBST the antigen antibody reaction was detected by incubating the membrane with substrate (Ortho dianaisidine dihydrochloride) ODD/H₂O₂.

Results

cDNA synthesis and PCR amplification of BTV

The cDNA was synthesized from the total RNA using reverse transcriptase with primers specific to VP2 genome of BTV. To optimize the conditions for dsRNA denaturation, RNA was heated at 85°C for 5min then the denatured RNA was then snap cooled and subjected to RT-PCR. Amplification of BTV RNA segment 2 encoding VP2, corresponding to the nucleotides 19 to 898 was carried out by RT-PCR using VP2 LI and VP2RI primers. The amplified product was analyzed by 1.2% agarose gel electrophoresis along with DNA molecular weight markers. As expected the DNA of 0.88kb could be visualized in ethidium bromide stained agarose gel .The amplification yielded single band indicating the specific amplification of BTV genome. (Fig.1)

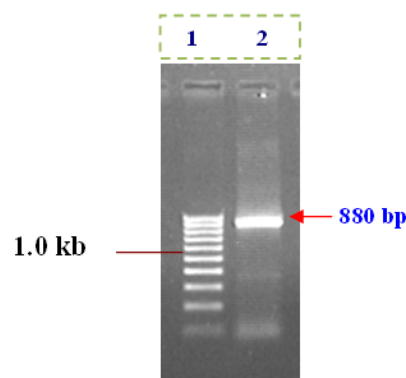


Figure 1: RT-PCR amplification of BTV -1 VP2 gene. Lane 1: 100 bp marker. Lane 2: BTV VP2 gene after RT-PCR amplification.

Cloning of VP2 amplified product in pBKS⁺ plasmid vector and sequencing

In order to express the VP2 encoding gene of BTV-1 in *E.coli*. The purified DNA of VP2 gene was cloned into pBKS⁺ plasmid using cohesive end ligation as per material and methods. Two µl of ligated mixture was transferred into DH5α *E coli* competent

cells and plated on to LB agar plates with ampicillin. On over night incubation 100 colonies were seen on the plate. All the colonies were streaked on to a new ampicillin plate for further screening then the positive colonies were screened by colony lysis and insert release with restriction enzymes. (Fig.2)

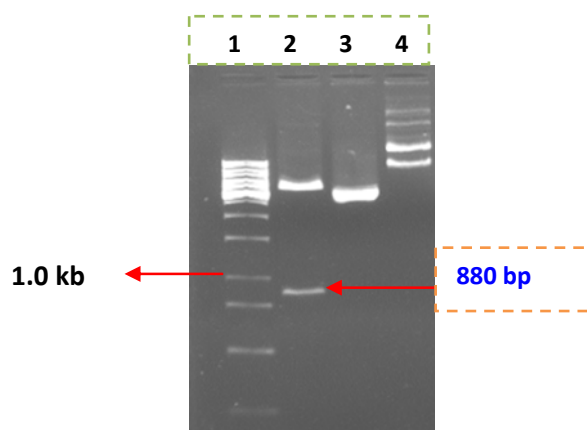


Figure 2: Agarose gel electrophoresis of enzyme digested recombinant plasmid of pBKS⁺

Lane 1: 1kb molecular marker (MBI FERMENTAS 1Kb gene ruler)

Lane 2: pBKS⁺ plasmid with insert after digestion with *Bam*HI and *Xho*I enzymes.

Lane 3: pBKS⁺ plasmid without insert digested with *Bam*HI and *Xho*I.

Lane 4: pBKS⁺ plasmid with insert which is undigested

Comparison of nucleotide sequence homology of VP2 gene of BTV-1 with available reported genes in the nucleotide databases through BLASTn search.

After complete alignment of nucleotide sequence into a single strand, the data was submitted to BLASTn search to find out the homology and novelty of the sequenced gene. BLASTn results showed homology to 3 BTV in the Gen Bank, EMBL, DDBT and PDB sequences (but no EST, STS, GSS or Phase 0, 1 or 2 HTGS sequences). There were 855 nucleotide sequences in the query sequence; percent nucleotide homology of query sequence to BTV serotype 1 was calculated. It was found that the query sequence was 88% homology to BTV1 VP2, 87% homology to AUS BTV VP2. Details are given in the table 1.

Table 1: Comparison of BTV VP2 sequence to other published sequences in the data Bank.

Sl. No.	Gene bank Acc.No.	Homology gene	Size of the gene (bp)	Nucleotide sharing homology	Percentage homology
1	XO6464.1	BTV1 VP2	2940	736/836	88
2	M21844.1	AUS BTV VP2	2940	731/837	87

Cloning of vp2 gene for expression

In order to achieve higher expression levels the released insert gene from pBKS⁺ vector was sub-cloned into the expression vector pET32a as described under material and methods. The Two μ l ligation mix was transferred into *E. coli* DH5 α cells and plated on LB plate containing X-gal and IPTG for screening for positive colonies. On overnight incubation 40 colonies were observed on the plate. The colonies were streaked on to a LB agar plate with X-gal and 0.4 mM IPTG for further characterization and the positive colonies were confirmed through colony lysis and insert release with restriction enzymes. (Fig.3)

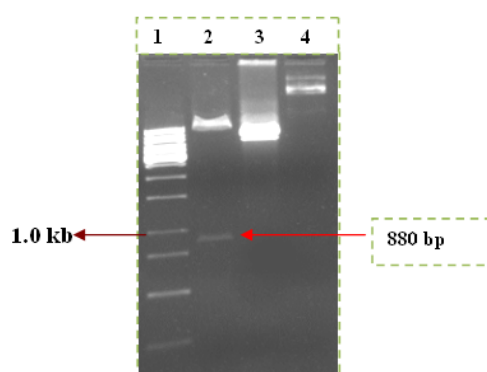


Figure 3: Agarose gel electrophoresis of enzyme digested recombinant plasmid of pET 32a. Lane 1: 1Kb molecular marker. (MBI FERMENTAS 1Kb gene ruler) Lane 2: pET 32a plasmid with insert after digestion with BamHI and XhoI. Lane 3: pET 32a plasmid without insert digested with BamHI and XhoI. Lane 4: pET 32a plasmid with insert which is undigested.

Expression of VP2 Specific Protein In pET32a Vector

One of the positive clone having the VP2 gene was further subjected for expression studies. Cells from the positive colonies were inoculated into 50 ml of LB containing 50 μ g per ml of ampicillin and incubated at 37°C with vigorous shaking till the OD reached 0.8 at A₅₆₀ nm. An aliquot of 25 ml was collected to serve as uninduced cell control. The remaining culture was induced with IPTG for expression at the final concentration of 0.2 mM and allowed to grow at 37°C with shaking after 6 hrs of incubation the cells were pelleted and were studied for VP2 expressed protein by SDS- PAGE.

Characterization of E coli Expressed proteins.

SDS PAGE analysis

Analysis of *E. coli* lysate with commassie blue stained SDS PAGE gels revealed several protein bands of host and vector with a thick band approximately 32.89 KDa. The 32.89 KDa protein is a fusion protein carrying of 6X His tag protein from the vector for the better protein purification. The total size as per the deduced amino acid sequence matches with the observed size of the protein in the SDS PAGE gel. This

indicated that 32.23 KDa expressed protein is from the insert and 0.66 KDa is from 6XHis tag. The protein band was intense in the sample collected after IPTG induction, *E coli* cultures with vector without insert grown and induced along with the test did not show such specific band observed in case of *E coli* vector with insert suggesting that the intense protein may be an expressed protein from the inserted gene. (Figure not shown)

Affinity purification of protein and western blot analysis of the expressed protein

The protein was purified using Nickel-Cl agarose column from Genei a thick protein band was seen corresponding to the expected size of the protein in SDS-PAGE with standard protein marker. Further to confirm the expression of the protein, the protein was subjected to the western blot analysis.

Expression of cloned gene was further confirmed by western blot analysis of SDS-PAGE separated proteins. The proteins were detected by specific chick HRPO conjugate as described under Material and Methods. The reaction showed light brown coloured band at position corresponding to the intense band observed on Commassie blue stained SDS-PAGE gel indicating that the 32.89 KDa protein is BTV specific and expressed in *E.coli*. (Fig.4)

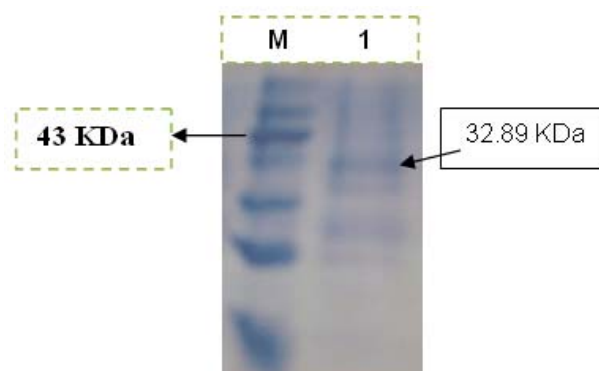


Figure 4: SDS-PAGE analysis of affinity purified BTV-1 VP2 protein. Lane. M: Protein molecular weight marker. Lane. 1: Affinity purified protein of BTV-1 VP2.

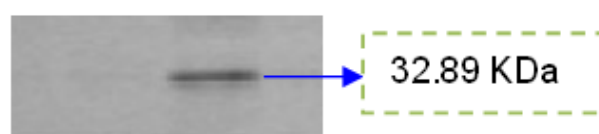


Figure 5: Western blot analysis of expressed BTV-1 VP2 protein.

Discussion

In India suitable vaccines are not available against Bluetongue. Presently inactivated and modified live vaccines are being used in some of the countries where the disease

is endemic, there is a scope for development of recombinant DNA based vaccines in BT by making use of VP2 gene which is responsible for serotype specificity and eliciting neutralizing antibodies in animals. Keeping in view of the above the present study was taken up as an initial study towards the development of a recombinant vaccine for BT in *E coli* and its possibility of expression of 880 bp gene of VP2 in plant expression system. The expressed protein could also be used as a diagnostic tool as it could serve as a type specific protein and sera raised against it as a serotype specific sera which could be of use in identifying the serotype in serological methods without cross reactivity.

Blue tongue disease although was first noticed more than a century ago, is still persisting in most of the countries. India with 90 million sheep population is harboring the disease in many states except in North eastern states. After the first report of serological evidence by Spare during 1964, several workers identified the disease by serology and virus isolation (Jain *et al.*, 1986; Babu *et al.* 1988 ; Sreenivasalu *et al.*, 1999) and many confirmed the prevalence of as many as 21 serotypes in India (Prasad, 2002). The significant economic losses associated with the disease are due to mortality and morbidity, reduced reproductive efficiency and in the national interest, restrictions on trade. Presently diagnosis for BT depends on serological assay, which have got limitations of cross reactivity and non-availability of suitable reagents. Although monoclonal antibody based ELISA tests, to some extent over come cross reactivity.

Several methods of RNA extraction from the samples are in use. However Trizol method of RNA extraction has been found to be simple as the whole process can be completed in less than an hour. In addition total RNA extracted will be intact and found to be efficient for cDNA synthesis. The effectiveness of Trizol in controlling RNA degradation was shown by Hofmann *et al.*, (2000) in their studies with Foot and Mouth disease viral RNA where the infectivity of the RNA was found to be unaffected even after one month when accidentally left at room temperature in Trizol reagent. This method appears to be very useful compared to differential precipitation by lithium chloride method as used by other worker (Bandyopadhyaya *et al.*, 1998), which requires three days for complete extraction process. In the present study the RNA extracted was used cDNA synthesis and for further amplification by PCR.

In order to clone the VP2 gene for expression studies the regions corresponding to 19 and 898bp was selected for primer synthesis. The Primers were designed using sequences available in the Gene bank. Sequence alignments were deduced using Omega software (Oxford technologies, USA). The primers were designed with little modification to insert the *Bam*HI site in the left primer and *Xho*I site in the 5' end of right primer. One ATG codon was also included along with the restriction site in the 5' end of left primer as an advantage for expression studies of the amplified gene.

Bluetongue viral RNA being double stranded, initial denaturation for primer annealing was found to be critical. Researchers have used different temperature conditions with or without denaturing agents. Akita *et al.*, (1992) have used methyl mercuric hydroxide along with heating which is highly toxic. Formamide plus heating has been used by Bandyopadyaya *et al.*, (1998) have denatured the RNA by keeping in boiling water for 3 min.

In present study the optimum temperature conditions were standardized and found that 85^o C for 5 min is ideal for synthesis of cDNA with BTV RNA and better amplification was achieved by using the above temperature alone for RNA denaturation, hence use of chemical denaturants were not tried.

Although McColl and Gould (1991), have used single primer (5') in RT reaction, both 5' (upstream) and 3' (down stream) end primers VP2 LI and VP2RI were used in our studies in the reverse transcription reaction for cDNA synthesis as use of single primer for cDNA synthesis did not result in optimum amplification by PCR by Byregowda *et al.*, 2000. In order to express VP2 protein in bacterial system the amplified gene was first cloned into cloning vector pBKS⁺ to facilitate easy screening. The amplified product of VP2 after digesting with *Bam*HI and *Xho*I enzymes was cloned into plasmid pBKS⁺ vector digested with same enzymes. Transformation efficiency was found to be high with DH5 α competent cells as it was forced cloning with cohesive ends. The presence of gene in the vector was confirmed by colony lysis and checked for the insert release. Expected size of the gene 0.88 Kb was obtained from positive colonies by restriction digestion analysis using same set of enzymes which were used earlier. On screening of all the 30 colonies, 16 were found to be positive for the presence of the insert. Plasmid DNA was prepared from one of the characterized clone and insert was released for further expression studies.

In order to confirm whether the gene, which was amplified and cloned, was VP2 or not the vector plasmid, which was isolated after restriction digestion, was given to sequencing with T₇ and reverse primer used in the PCR. The sequence obtained was good and confirmed as VP2 gene. The aligned gene sequence was submitted to NCBI GenBank (Accession No.GQ233041).

There were 855 nucleotide sequences in the query sequence, percent nucleotide homology of query sequence to BTV serotype 1 was calculated. It was found that the query sequence was 88% homology to BTV1 VP2, 87% homology to AUS BTV VP2.

Virus specific proteins have their role in diagnosis in both serological (ELISA, Immunoblot assay) and molecular methods (antigen capture PCR). In order to have a group specific protein BTV VP2 gene was cloned into pET32a expression vector. The insert was released from the pBKS⁺ vector clone with *Bam*HI and *Xho*I enzymes further cloned into pET32a vector. As it was forced cloning with cohesive end ligation cloning efficiency was high on X-gal, IPTG plate. The positive colonies were screened by colony lysis and release check and 6 colonies were found to be positive out of 15 colonies indicating high cloning efficiency. The colonies were further confirmed by restriction digestion and expected size 0.88kb insert was obtained.

Expression conditions were optimized in order to get high expression. The characterized clones were grown to OD 0.6 (at A₅₆₀) and induced with IPTG at 0.2 mM concentration (*E.coli* with gene clone). The protein produced may be fusion protein and the expected size of protein could be 32.89 KDa in which 32.23 KDa from the VP2 gene and 0.66 KDa from 6X His tag fusion protein of the vector.

A few cells from the positive colonies were inoculated into 50 ml of LB containing 50 μ g per ml of Ampicillin and incubated at 37^oC with vigorous shaking till the OD reached 0.8 at A₅₆₀ nm. An aliquat of 25 ml was collected to serve as

uninduced cell control. The remaining culture was induced with IPTG for expression at the final concentration of 0.2 mM and allowed to grow at 37°C with shaking 6 hrs. Aliquot of 25 ml was collected and pelleted cells were studied for VP2 protein expression. The pellet was suspended in 0.5ml of sterile TE (pH-8) containing PMSF at 1mM conc. On analysis of SDS-PAGE gels, it was found that an intense band of size 32.89 KDa was seen along with the other host proteins bands. The protein expressed may carry 293 aminoacids from 880bp VP2 insert accounting for 32.23 KDa with fusion 6X His tag from vector.

The protein was purified using Nickel-Cl agarose column when analysed in SDS-PAGE a thick band of protein corresponding to the expected size was seen and subjected to western blot analysis. The protein expressed was further subjected to immunological detection by western blotting. The proteins separated by SDS-PAGE were electro-transferred on to nitro cellulose membrane and then treated with anti BTV bovine serum. Bound bovine antibodies were detected by anti chick HRPO conjugate. A light brown band developed with ODD indicates that the protein expressed is VP2 of BTV1 on stained gel.

Conclusion

In conclusion, the expressed protein can be used as an antigen for diagnosing the specific virus in India and the bacterial expressed purified antigen can be used as vaccine after evaluating its efficiency as an antigen which can elucidate the neutralizing antibodies to prevent the viral infection.

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