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Expression and Evaluation of Immuno-Reactivity of Recombinant VP2 Protein of Bluetongue Virus Serotype-23

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A B S T R A C T

Bluetongue is an arthropod-borne, non-contagious viral disease of domestic and wild ruminants. In the present study, truncated VP2 protein of BTV serotype 23 (BTV-23) was expressed in a prokaryotic expression system and assessed for its potential use as a candidate for the development of serotype-specific BTV diagnostics assay. The expression of the VP2 protein was carried out in *E. coli* BL21 Codon Plus (DE3) cells using the pET32a expression vector. The purity of recombinant protein VP2 (rVP2) was analyzed in SDS-PAGE and Western blotting. To evaluate the immune reactivity and cross-reactivity of rVP2, homologous and heterologous BTV hyperimmune serum (HIS) were used respectively. There was no cross-reactivity of rVP2 BTV-23 protein with HIS against BTV-1. The expressed rVP2 protein may be used as a diagnostic antigen for the development of serotype-specific ELISA to detect antibody against BTV-23 serotypes in serum samples.

Introduction

Bluetongue virus (BTV), the causative agent of bluetongue (BT) is an arthropod-borne disease of domestic and wild ruminants (Mertens *et al.,* 2005). The disease is transmitted between domestic and wild ruminants by *Culicoides* species which are most abundant and active in hot and humid climate (Mellor, 1990). At present, worldwide 27 distinct BTV serotypes have been identified (Zientara *et al.,* 2014). The BTV genome consists of ten segmented double-stranded RNA (dsRNA) which code for seven structural (VP1- VP7) and four non-structural (NS1, NS2, NS3/NS3A and NS4) proteins (Belhouchet *et al.,* 2011). BTV is enzootic in India and 26 serotypes of the virus have been reported based on serology and virus isolation. So far, at least 13 different serotypes (BTV-1-4, 6, 9, 10, 12 16-18, 21 and 23) of BTV were isolated from the country (Chand *et al.,* 2015).

The diagnosis of BT requires isolation of virus, standard serological methods and other nucleic acid and antigen detection assays (Afshar, 1994; Clavijo *et al.,* 2000). Competitive ELISA (c-ELISA), indirect ELISA (i-ELISA) and agar gel immunodiffusion (AGID) assay can be used

for the detection of serogroup-specific antibodies (Afshar *et al.,* 1989; Chand *et al.,* 2017). Molecular tools such as reverse-transcriptase polymerase chain reaction (RT-PCR), Real-time PCR and Loop-Mediated Isothermal Amplification (LAMP) have also been demonstrated for detection of viral nucleic acid in the clinical samples (Shaw *et al.,* 2007; Mohandas *et al.,* 2015). Due to the presence of a large number of susceptible hosts, BTV serotypes and *Culicoides* vector, the control of BT is difficult. Vaccination is the most effective control strategy for developing countries like India. Rapid and reliable identification of virus serotype can, therefore, play an important part in the design and implementation of appropriate control measures. Identification of BTV 'type' also demonstrates conclusively that the virus belongs to the BTV species, confirming any initial diagnoses by other means, and can help to map the origins, movement and spread of individual virus strains (Hamblin *et al.,* 2004). In India, an inactivated pentavalent vaccine containing most prevalent serotypes (BTV-1, 2, 10, 16 and 23) was developed and commercialized (Reddy *et al.,* 2010). Detection of serotypes is necessary for taking up effective control measures including vaccination. VP2 is the most variable non-glycosylated BTV proteins and is a major protective antigen. The specificity of its interactions with neutralizing antibodies determines the identity of the known BTV serotypes (Maan *et al.,* 2011). BTV serotypes can be identified by virus neutralization test (VNT), RT-PCR followed by sequencing of VP2 gene and type-specific real-time RT-PCR (Breard *et al.,* 2016; Maan *et al.,* 2016). These entire assays are tedious, time taking and only small number of samples can be tested at a time. Serological typing methods like VNT require access to standardized reagents, including reference antiserum, or reference strains for all 26 BTV serotypes, which can be difficult to produce and could be a potential infection risk (Maan *et al.,* 2012). In addition, for VNT, live and characterized BTV serotype strains are needed and it must be performed in a BSL3 laboratory (Breard *et al.,* 2016). Therefore alternative assay like serotype-specific ELISAs will be a good choice for detection of BTV antigen and antibody. A serotype-specific ELISA will be much faster, economical and easier to perform compared to VNT or other nucleic acid-based assays. Moreover, a large number of clinical or laboratory samples could be screened in a less time in epidemiological studies and also the assay can be completed within 4-5 hr. Therefore, in present study VP2 protein of BTV-23 serotype (prevalent serotype in India) was expressed in prokaryotic system and assessed for its potential use as a candidate for the development of serotype-specific BTV diagnostic assay.

Materials and Methods

Cloning of the VP2 protein of BTV-23 in transfer vector

The total RNA of BTV-23 serotype (Parbhani/Ind) was extracted by Tri-Reagent (Sigma, St. Louis, USA). BTV dsRNA was purified by sequential precipitation by lithium chloride (LiCl). RT-PCR of purified viral dsRNA was carried out using VP2 gene-specific primers. The forward and reverse primers with *BamHI* restriction enzyme (RE) recognition site [BTV23/S2/F: 5'-*CGCGGATCCTGGTTACAGTGGATGATCG*-3') and *Xho*I RE site (BTV23/S2/R: 5'-*GTGCTCGAGGTCATGAGCGTATAGTAGG*-3') respectively were designed using published sequences. The amplification was carried out using GoTaq® Long PCR Master Mix (Promega, Madison, USA) and the cycling conditions followed were: initial denaturation at 94 °C for 5 min, denaturation at 95 °C for 30 sec, annealing at 59 °C for 30 sec and extension at 68 °C for 2 min for 35

cycles with a final extension of 68 °C for 10 min. The PCR amplicon was gel purified and cloned directionally into pET32a vector (Novagen, Madison, WI, USA) with *Bam*HI and *Xho*I restriction sites. The recombinant clones were selected on LB agar plates containing ampicillin (50 µg/ml) and were verified by restriction enzyme analysis, and sequencing.

Expression and purification of recombinant VP2 protein

E. coli BL21 – CodonPlus (DE3) – RIPL cells were transformed with recombinant plasmid and grown at 37 °C overnight, on LB agar plates containing chloramphenicol (34 µg/ml) and amplicillin (50 µg/ml). *E. coli* BL21 – CodonPlus (DE3) – RIPL with pET32a-VP2 were grown in LB medium supplemented with antibiotics at 37 °C to an optical density at 600 nm of approximately 0.6. Protein expression was induced by adding IPTG to 1 mM final concentration. Then, additional 5 h incubation at 37 °C with vigorous shaking at 180 rpm. After the cells were harvested by centrifugation at 5000 ×g for 10 min at 4 °C, cell lysate was sonicated, centrifuged and purified using nickel affinity columns according to the manufacturer's instructions with some modification (M/s Qiagen, Hilden, Germany). Briefly, after centrifugation the supernatant was discarded while the pellet was resuspended in Tris-HCl (50 mM; pH 7.6). The cells were disrupted by lysozyme, freeze-thawing and ultrasonication for 10 min with 20 sec intervals between pulses. The cell lysate was then centrifuged at 18000 rpm at 4 °C for 20 min. The pellet was resuspended with denaturing binding buffer (0.01 M Na2HPO4, 0.01 M NaH2PO4, 0.5 M NaCl, 8 M urea, pH 8.0). The denatured pellet was centrifuged at 18000 rpm for 20 min at 4 °C. Supernatant was filtered using 0.45 µm membrane filters and transferred to a fresh tube. To the Ni–NTA agarose superflow column pre-equilibrated with the binding buffer, supernatant was passed slowly (1 ml/ minute) and column was sequentially washed with the wash buffer (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.5 M NaCl, 6 M urea, pH 6.5). Then recombinant protein was eluted using elution buffer (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.5 M NaCl, 4 M urea, pH 4.0) and dialyzed. The final concentration of purified protein was estimated colorimetrically using Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The harvested post-induced samples at 2-5 h, uninduced samples, and eluted fractions were mixed separately with SDS Laemmli sample buffer and analysed by polyacrylamide gel (5% stacking and 10% resolving). The resolved protein was transferred on to a nitrocellulose membrane (M/s Pharmacia Biotech, USA using semi-dry blot system (M/s ATTO corporation, Japan). The recombinant protein on the blot was detected by incubation with Ni-NTA HRP conjugate (1:2000 dilution) as per the standard procedure.

Imuno-reactivity of BTV23- rVP2 with HIS

The immuno-reactivity of the recombinant BTV-23 rVP2 protein was determined using BTV-23 HIS raised in rabbit in Indirect ELISA. The cross-reactivity of rVP2 BTV-23 with HIS against BTV-1 was also evaluated. Breifly, the recombinant VP2 antigen in 100-1000 ng conc. were diluted in carbonate-bicarbonate buffer and 50 µl of each dilution was coated in duplicates in a 96 well plate and incubated for 1 h at 37 °C. Then unbound areas on the plate were blocked with 1%BSA+1%SMP and the plate was washed three times with washing buffer (PBST). Then two fold dilutions of HIS (against BTV-23 and BTV-1) and negative serum were added. After incubation and washing, anti-Rabbit HRPO conjugate (Sigma-Aldrich, St. Louis, USA) was added for 1 h at 37 °C. After washing, substrate (OPD + H_2O_2) solution was added to

all the wells and incubated in dark for 15 minutes. The reaction was stopped using 1 M H_2SO_4. The optical density (OD490 value) was measured at 492 nm using a Microplate Reader (Bio-Rad, USA).

Results and Discussion

For amplification of segment-2 gene, BTV (23)-VP2 (aa662) at N-terminal of VP2 protein was targeted. The amplified product with serotype-specific primers was visualized by resolving in 1% agarose gel and the sizes of the amplified product was~1986 bp. Both gel purified PCR product and vector DNA were double digested with *Bam*HI and *Xho*I for directional cloning.

The fragments at their appropriate ratios were ligated with digested linearized vector using T_4 DNA ligase. The *E. coli* TOP 10F' competent cells were transformed with the ligated mixture and transformants were screened on LB agar plates containing ampicillin. The plasmids subjected to double digestion with *Bam*HI and *Xho*I showed the insert release of the recombinant plasmid in agarose gel. The identity of sequence data thus generated was confirmed by nucleotide BLAST (NCBI) analysis. BLAST (NCBI) analysis confirmed that serotype (BTV- 23) was having sequence homology with published sequences of BTV-23 serotype available in the database (GenBank: BTV-23/ AY643506).

The positive clone was transformed into bacterial expression host so as to over-express the target protein. During expression studies, the optimum level of expression of VP2 protein was seen at 37 °C after 5 h of induction with 1 mM IPTG. The expression study revealed that the expressed protein was insoluble and found in the insoluble fraction of cell lysate. The protein expressed was analyzed in SDS-PAGE and protein of expected size (~90 kDa) was observed in the induced positive clone (Fig. 1). The recombinant VP2 protein, in the insoluble fraction of the bacterial cell lysate was purified under denaturing condition. Confirmation of the expressed protein was done by Western blot analysis using Penta-his HRP conjugate. The purity of protein analysed in SDS-PAGE and Western blotting revealed homogenous purity of the protein with the appearance of respective protein size product (~90 kDa) (Fig. 2a and b). The immuno-reactivity and cross-reactivity of rVP2 protein was assessed with homologous and heterologous HIS against BTV-23 and BTV-1 respectively. An indirect ELISA format was used to test the diagnostic potential of the expressed rVP2 protein. In order to find out the single sera dilution and antigen concentration, a chequer board titration was done. The optimum antigen concentrations for rVP2 BTV-23 was found to be 250 ng/well at BTV-23 HIS dilution of 1:50. At this concentration, the OD490 value of BTV-23 HIS, BTV-1 HIS and negative serum were 1.1, 0.32 and 0.4 respectively. There was no cross-reactivity of rVP2 BTV-23 with HIS against BTV-1 (Fig. 3).

In present study, truncated VP2 protein of BTV serotype 23 was expressed in prokaryotic system and its potential use as a candidate for the development of serotype-specific BTV diagnostics assay was assessed. Bacterial system was used for the production of recombinant antigen, as large quantities of viral antigen with good purity can be obtained. One of the main advantages of bacterial expression is the low cost associated, with rapid and high productivity of heterologous proteins (Georgiou and Valax, 1996). It might also be necessary to mention that the lack of a post-translational modification pathway in the prokaryotic expression system should not be of a disadvantage since BTV-VP2 is a non-glycosylated protein (Roy, 2008).

Fig.1 SDS–PAGE analysis of BTV-23 VP2 protein expressed at 37°C with 1mM IPTG at 5 hour post induction. Lanes; (1): pET-32a/BL21 without induction; (2): Recombinant BTV-23 VP2 protein expressed in *E. Coli* BL21 [insoluble fraction]; (3): Soluble fraction (M): Protein Marker (puregene)

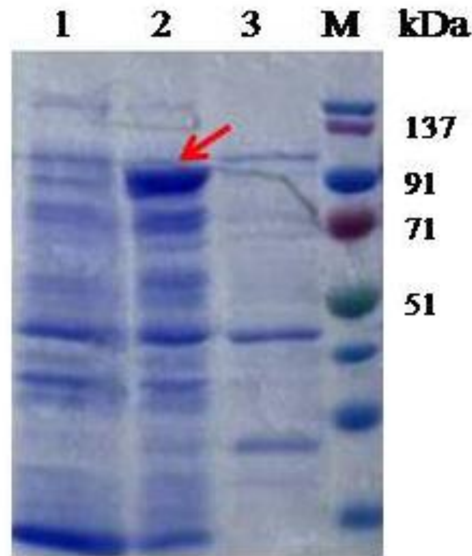
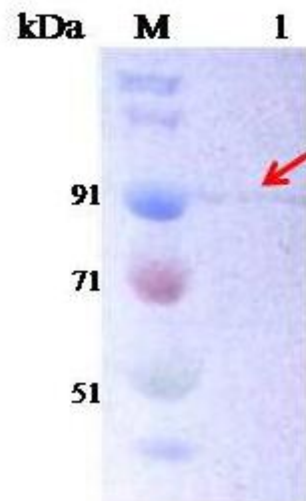


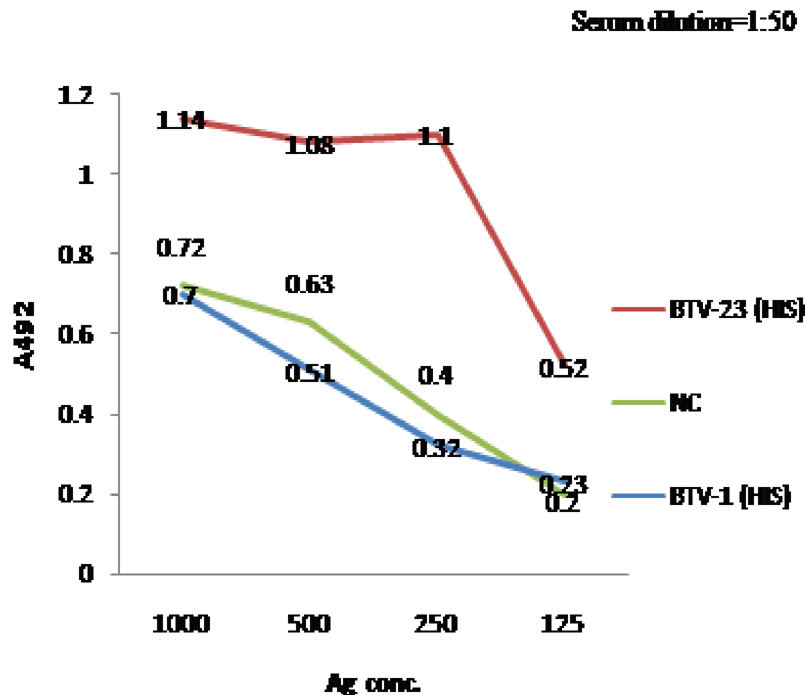
Fig.2 a] Western blot assay using Ni-NTA HRP conjugate at 1:2000 dilution for the detection of protein expression. Lanes; **(M)**: Protein marker (Puregene) (1) Induced recombinant BL21 CodonPlus (DE3) expressing BTV (23)-VP2 protein; b] Purified fractions of BTV (23)-VP2 in 10% SDS-PAGE. Lanes; (M): Protein marker (Puregene). (1) Purified fraction of recombinant BTV(23)-VP2 protein

a)



b)

Fig.3 Determination of optimum conc. /dilution of r VP2 BTV-23 protein and HIS (BTV-23) by chequer board titration method and detection of cross reactivity of r vP2 BTV-23 protein with HIS of BTV-1. The optimum antigen concentrations for rVP2 BTV-23 was found to be 250 ng/well at BTV-23 HIS dilution of 1: 50



The VP2 protein was targeted as truncated fragment (N-terminal) as the expression of full length VP2 proved difficult, probably because expression of recombinant proteins over larger residues is problematic in prokaryotic system, since these proteins are often unstable or form insoluble inclusion bodies (Sambrook and Russell, 2001). The expression of VP2 protein was carried out in *E.coli* BL21 CodonPlus (DE3) cells using the pET32a expression vector, as protein with high purity and solubility can easily be obtained by affinity chromatography due to the two histidine tags on both sides of the protein. During expression studies the optimum level of expression of VP2 proteins were seen at 37 °C after 5 h of induction with 1 mM IPTG. The expression study revealed that, all of the expressed proteins were insoluble since they could only be found in the insoluble fraction. This is in agreement with a previous study by Han *et al.,* (2017), where truncated rVP2 proteins were purified from the insoluble fraction. In the bacterial system, the presence of the recombinant proteins in the insoluble fraction is a common occurrence during cytoplasmic over-expression in prokaryotic expression systems. An indirect ELISA format was used to test the diagnostic potential of the proteins. Though other immuno-assays can be applied, ELISA is one of the most sensitive methods to evaluate expressed proteins and has been extensively applied in the diagnosis of BTV (Inumaru *et al.,* 1987; Pathak *et al.,* 2008; Barros *et al.,* 2009). To evaluate the immuno-reactivity and cross-reactivity of rVP2, homologous and heterologous HIS against BTV-23 and BTV-1 respectively were used. The rVP2 protein gave good immuno-reactivity with homologous HIS and there was no cross reactivity with heterologous HIS. The generated rVP2 protein will be promising candidate for the development of a serological

diagnostic test for detection of serotype-specific antibody against BTV-23.

In conclusion, the present study has successfully produced a recombinant VP2 protein of BTV-23 and immuno-reactivity and cross-reactivity were evaluated homologous and heterologous HIS against BTV-23 and BTV-1 respectively. The expressed rVP2 protein will be potential candidate for the development of serotype specific diagnostic assay for detection of type specific antibody against BTV-23. Further, the cross- reactivity of the rVP2 protein to all serotype-specific anti-BTV sera is needed to be evaluated.

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Conflict of Interest

The authors declare no conflict of interest

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