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Production of recombinant non-structural protein-3 hydrophobic domain deletion (NS3ΔHD) protein of bluetongue virus from prokaryotic expression system as an efficient diagnostic reagent

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ABSTRACT

Serological diagnostics for bluetongue (BT), which is an infectious, non-contagious and arthropod-borne virus disease of ruminants, are primarily dependent on availability of high quality native or recombinant antigen(s) based on either structural/non-structural proteins in sufficient quantity. Non-structural proteins (NS1-NS4) of BT virus are presumed candidate antigens in development of DIVA diagnostics. In the present study, NS3 fusion gene encoding for NS3 protein containing the N- and C-termini with a deletion of two hydrophobic domains ($_{118}$A to S_{141} aa and $_{162}$S to A_{182} aa) and intervening variable central domain ($_{142}$D to K_{161} aa) of bluetongue virus 23 was constructed, cloned and over-expressed using prokaryotic expression system. The recombinant NS3ΔHD fusion protein (~38 kDa) including hexa-histidine tag on its both termini was found to be non-cytotoxic to recombinant *Escherichia coli* cells and purified by affinity chromatography. The purified rNS3ΔHD fusion protein was found to efficiently detect BTV-NS3 specific antibodies in indirect-ELISA format with diagnostic sensitivity (DSn = 94.4%) and specificity (DSp = 93.9%). The study indicated the potential utility of rNS3ΔHD fusion protein as candidate diagnostic reagent in developing an indirect-ELISA for sero-surveillance of animals for BTV antibodies under DIVA strategy, wherever monovalent/polyvalent killed BT vaccine formulations devoid of NS proteins are being practiced for immunization.

1. Introduction

Bluetongue (BT), a vector-borne viral disease of domestic and wild ruminants, is caused by Bluetongue virus (BTV), which is the type species of the genus *Orbivirus* within the family *Reoviridae* [1]. BTV, a non-enveloped virion with icosahedral capsid comprising double-stranded RNA (dsRNA) with ten segments, primarily infects sheep, however, goats and cattle are also susceptible. BTV genome encodes for seven structural proteins (VP1-VP7) and four non-structural (NS) proteins (NS1-NS4) [2,3]. BTV is known to undergo mutations and reassortment with a high frequency which lead to genetic changes and emergence of new serotypes or antigenic variants of the same serotype [4]. Clinical diagnosis of BT is based primarily on clinical signs and lesions in sheep. However, confirmatory diagnosis is based on number of laboratory tests such as virus isolation, fluorescent antibody test (FAT), molecular tools like RT-PCR, real time RT-PCR, LAMP assay, whole genome sequencing and antibody detection assays [5–7].

Basically, antibody detection in serum samples relied on complement fixation, agar gel immunodiffusion (AGID), serum neutralization tests (SNT) and enzyme-linked immunosorbent assays (ELISA) [8]. Highly sensitive antibody ELISAs could recognize the humoral response to BTV as early as one week after infection [9,10]. Several commercially available ELISA formats (i-ELISA, c-ELISA, and s-ELISA) are mostly based on VP7 protein produced either in native/recombinant form [11–14], which is largely

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conserved across all serotypes. It has been noted that in convalescent animals, antibodies to BTV are detectable for much longer periods, which presumably precludes diagnostic and control strategies for BTV since the reliable differentiation diagnostic assays under DIVA strategy to discriminate infected and vaccinated animals, are currently not available. Earlier attempts in DIVA diagnostics demonstrated the discriminatory potential of non-structural protein based ELISAs [15,16], however, it is highly dependent on the purity of the vaccine. Nevertheless, in a situation wherein monovalent or polyvalent BTV killed vaccine devoid of NS proteins is being practiced for immunization, complimentary NS protein based immuno-diagnostics could be potentially developed under DIVA strategy.

Non-structural proteins, NS1 (~64 kDa), NS2 (~41 kDa), NS3/NS3A (~25.5–24 kDa) and NS4 (~10 kDa) encoded by BTV genome, are involved in either replication, maturation, or export of virions from infected cells [3,17–19]. The genes encoding for NS proteins and internal proteins are found to be highly conserved among the 27 serotypes of BTV reported worldwide [17,20]. NS3, which is synthesized in larger amounts in insect cells is considered as one of the candidate antigens for diagnostic assay. Earlier attempts in production of recombinant full length NS3 protein using different expression systems resulted in limited success due to presence of two hydrophobic domains (HD-I, $_{118}$A to S_{141} aa and HD-II, $_{162}$S to A_{182} aa), which were found to cause cytotoxicity in host cells [16,20]. Hence, there is a need for methodology to produce recombinant NS3 using appropriate expression system and optimization of purification protocol. Therefore, in this study, we describe a strategic design of construct, cloning, over-expression and optimized purification protocol for recombinant NS3 hydrophobic domain deletion/mutant protein, which could be potentially used as efficient diagnostic reagent in development of immuno-diagnostic assays such as ELISA for bluetongue in ruminants.

2. Materials and methods

2.1. Virus, plasmids, primers and bacterial strains

Bluetongue virus serotype 23 (Dehradun isolate) maintained in the Division of Virology, ICAR-Indian Veterinary Research Institute (IVRI), Mukteswar, Uttarakhand (UK), India, was used. Prokaryotic expression vector (pET32a) and bacterial strains such as *E. coli* TOP10 (DH5α) and *E. coli* BL21-CodonPlus(DE3)-RIPL cells procured from Novagen (USA), were used for initial cloning/propagation of plasmid and subsequently for expression of recombinant fusion protein. Two sets of primers required for target gene fragment amplification and cloning were synthesized from Eurofin Genomics (India) and procured.

2.2. Construction of plasmid (pNS3ΔHD)

Initially, multiple sequence alignment was carried out using representative NS3 protein sequences from all the 26 serotypes of BTV available in the GenBank using ClustalW method of DNASTAR and the regions of N- and C- terminus along with hydrophobic domain regions were identified. Subsequently, two primer sets targeting N- and C-terminus of *NS3* gene devoid of hydrophobic domain (HD) regions including intervening central region (total nucleotide region-Nt: 352 to 546 [~195 bp] and amino acid region: $_{118}$A to A_{182} [~65 aa]) encoding for hydrophobic domain (HD) deletion mutant non-structural protein-3 were designed based on its available gene sequence (GenBank Acc. # HQ719213). A gene fragment encoding for N- and C-terminus were amplified separately using respective primer sets as described in Table 1.

RNA was isolated from BTV 23 Dehradun isolate as per the standard procedure and subsequently cDNA was prepared for use as template in PCR reactions. For each gene fragment amplification, PCR mixture consisted of 50 ng of template, 25 pmol of respective primers and 2× Dream*Taq* PCR Master Mix (Fermentas, USA) along with NFW to a final volume of 25 μl. For both fragments, PCR was performed with following conditions: Initial denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 8 min. The PCR amplified N-fragment gene and C-fragment gene products (50 μg) were digested with *Bam*HI/*Hind*III and *Hind*III/*Xho*I respectively, and were gel eluted using QIAquick gel extraction kit (Qiagen, Germany). Both N- and C-fragments were fused through *Hind*III overhangs by ligation as per manufacturer's instructions (Fermentas, USA) and was followed by PCR amplification using N-term forward and C-term reverse

Table 1
List of primers used in construction of recombinant NS3 HD deletion (rNS3ΔHD) protein of BTV-23.

NS3 Fragments	Primer name	Oligonucleotide sequence (5'----------3')	Gene region	Protein region
NS3-N fragment	BT-NS3F (For)	cgcGGATCCCCTGGTGGAATGCTATCCGGGCTGA	Nt: 1 to 351 (~384 bp)	$_{1}$M to R_{117} (13.2 kDa)
	BT-NS3R1*Hind* (Rev)	gacAAGCTT*CGCCGA*TCTCTTTTTCTTTAAACCA		
NS3-C fragment	BT-NS3F1*Hind* (For)	gacAAGCTTATGAAGAGTGAGAGAGGGCTA	Nt: 547 to 687 (~162 bp)	$_{183}$K to T_{229} (5.4 kDa)
	BT-NS3R (Rev)	gtgCTCGAGGGTTAATGGTAGTTCGA		

Note: The sequences of the oligonucleotides had an added restriction sites (underlined) for *Bam*HI (BT-NS3F), *Hind*III (BT-NS3R1*Hind* and BT-NS3F1*Hind*) and *Xho*I (BT-NS3R) at 5' end along with primer tags (small letters).

A linker sequence (in italics) coding for 'SAKL' was incorporated at 5' end of NS3R1*Hind* primer before RE site for *Hind*III.

Abbreviations: NS3, non-structural protein 3; For, forward primer; Rev, reverse primer; N, amino terminus, C, carboxyl terminus; HD, hydrophobic domain.

primers (BT-NS3F and BT-NS3R) to derive full length NS3 fusion gene product without HDs and central region. Further, NS3 fusion gene was ligated in to a linearized pET32a vector (50 ng/μl) in 10 μl ligation reaction at 16 °C with overnight incubation. Subsequently, pNS3ΔHD was used for initial transformation in to *E. coli* TOP10 cells and its presence was confirmed by restriction endonuclease (RE) analysis, colony PCR and target fusion gene sequencing. Later, pNS3ΔHD was transformed in to expression host cells (*E. coli* BL21-CodonPlus[DE3]-RIPL cells) using appropriate antibiotics such as ampicillin (50 mg/ml) and chloramphenicol (35 mg/ml).

2.3. Expression of rNS3ΔHD fusion protein

Initially, to determine the time-course of rNS3ΔHD expression, a pilot expression study was carried out. One colony of *E. coli* BL21-CodonPlus(DE3)-RIPL cells harboring recombinant plasmid pNS3ΔHD was picked and used to inoculate 5 mL LB medium containing ampicillin (50 mg/ml) and chloramphenicol (35 mg/ml). The culture was grown overnight at 37 °C with shaking. The overnight culture was inoculated on to fresh LB medium containing appropriate antibiotics, to obtain 10 mL of a new culture with OD_{600} ~0.05–0.1. The new culture grown at 37 °C with shaking was induced with 1 mM IPTG (Sigma–Aldrich, USA) during bacterial mid-log phase growth (OD_{600} ~0.4) to express recombinant fusion protein. One milliliter aliquots were collected at 0, 1, 2, 3 and 4 h intervals subsequent to IPTG induction for quantitative/qualitative analysis on 10% SDS-PAGE. For bulk expression, the overnight grown culture was used to inoculate 1 L fresh LB medium containing antibiotics at mid-log phase, the protein expression was induced by 1 mM IPTG addition. After induction, the culture was allowed to grow in a shaking incubator at 37 °C for 4 h, and then the cells were harvested by centrifugation at 6000 rpm for 10 min and stored at −80 °C prior to protein purification.

2.4. Standardization of purification protocol for rNS3ΔHD fusion protein

The above harvested *E. coli* cells pellet was resuspended in buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl and lysozyme) before lysis by repeated freeze-thawing and sonication (Sonics, USA). The cell lysate was centrifuged at 18,000 rpm for 30 min to separate soluble and insoluble fractions. The supernatant containing soluble fraction was removed and pellet containing inclusion bodies were solubilized by treatment with the 25 mL of denaturing buffer (8 M Urea, 50 mM Tris-HCl, pH 7.8, 100 mM NaCl and 10 mM Imidazole) by incubating at room temperature for 30 min. The resuspended solution was centrifuged at 18,000 rpm for 30 min and supernatant containing soluble protein was used for further purification. The rNS3ΔHD fusion protein was purified by affinity chromatography using Ni-NTA Superflow Cartridges (Qiagen, Germany) as per the procedure described previously [21–25]. Briefly, following column binding and washing, the protein was refolded on column using renaturating buffer containing decreasing gradient of Urea buffers (6, 4, 2 and 1 M Urea buffers). After renaturation, column was washed with washing buffer (50 mM Tris-HCl, pH 6.0, 100 mM NaCl and 50 mM Imidazole) thrice before final elution of rNS3ΔHD protein with elution buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl and 300 mM Imidazole). Eluted rNS3ΔHD fusion protein peak fractions were pooled and dialysed at 4 °C overnight in a buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl). The dialysed protein was concentrated (Vivaspin, Germany), quantified and aliquoted before storage at −80 °C for further analysis.

2.5. Qualitative analysis by SDS-PAGE, native-PAGE and Western blot

The rNS3ΔHD fusion protein was analyzed on 10% SDS-PAGE as well as 10% native-PAGE. For Western blot, the purified protein was initially electrophoresed on 10% SDS-PAGE gel and subsequently transferred on to the nitrocellulose membrane using semi-dry immunoblot system (Amersham pharmacia, USA). The proteins were detected by chromogenic method using QIAexpress detection kit (Qiagen, Germany) as per the manufacturer's instructions. Briefly, the nitrocellulose membrane with transferred proteins was blocked using TBS-Tween buffer at room temperature. After thorough washing, membrane was incubated in blocking buffer containing mouse monoclonal anti-His IgG horseradish peroxidase (HRP) conjugate (1:2000 diluted) at room temperature for 1 h. Following several washes with TBS-Tween buffer, the membrane was incubated for 1–5 min in freshly prepared substrate solution (10 mg diaminobenzidine tetrahydrochloride) for development of color, which was later stopped and the blots were dried before recording of results.

2.6. Prediction of protein characteristics of rNS3ΔHD fusion protein

The prediction of primary/secondary characteristics of rNS3ΔHD fusion protein was carried out using the PROTEAN program (DNASTAR) as well as proteomics tools from the ExPASy website (www.expasy.org).

2.7. Evaluation of immuno-reactivity of rNS3ΔHD fusion protein

An indirect-ELISA was carried out to evaluate the efficacy of rNS3ΔHD fusion protein to detect the BTV-NS3 specific antibodies in sera samples of ruminants. A standardized indirect-ELISA format as described previously [13,14] was employed to evaluate the immuno-reactivity potential of rNS3ΔHD antigen. Briefly, the optimal concentrations of antigen and serum dilution were determined by performing a checkerboard titration of the rNS3ΔHD fusion antigen and positive/negative control sera. An ELISA plate (96 well) was coated with rNS3ΔHD fusion protein (~100 ng/well) in coating buffer (pH 9.4) and incubated at 37 °C for 1hr. Following blocking, 50 μl of serum samples diluted at 1:10 was added to each well and incubated. The secondary antibody conjugate (rabbit anti-goat-HRPO, 1:8000 dilution or rabbit anti-sheep-HRPO, 1:3000 dilution) was added (50 μl) to all the wells. Incubation of plates at 37 °C for 1hr and three times washing in phosphate buffer saline containing 0.03% Tween-20 was followed after each step of blocking, sample and conjugate addition. Finally, 50 μl OPD substrate solution was added to each well and incubated at 37 °C for 10–15 min till color development. The plates were read at 492 nm wavelength on an iMarK microplate absorbance reader (Biorad, USA).

i) Cut-off estimation and statistical validation of rNS3ΔHD based indirect-ELISA

Sheep and goat serum samples (n = 239), as detailed in Table 2, were used to determine the diagnostic sensitivity and specificity of the assay. Initially, all the field sera samples were screened with commercially available c-ELISA kit (Bluetongue antibody test kit, VMRD Inc., Pullman, USA) and status of the samples were designated based on (recommended cut-off value of the kit) the test result. Mean OD values of the each sample tested by NS33ΔHD based I-ELISA were calculated as percentage positivity (PP). Receiver operating characteristic curve (ROC) analysis was performed by the MedCalc software following DeLong et al. [26]

Table 2
Comparative reactivity between rNS3ΔHD-ELISA and commercial c-ELISA.

Serum source	Number of samples tested	rNS3ΔHD-ELISA		c-ELISA		Concordance rate (%)
		Positive	Negative	Positive	Negative	
Sheep	125	51	74	49(46)	76(71)	93.60%
Goat	114	44	70	41(40)	73(69)	95.61%
Total	239	95	144	90(86)*	149(140)*	94.56%

Note: *Parentheses indicate number of the samples, which gave similar results in both the assays.

procedure available in the software. The Diagnostic sensitivity (DSn), diagnostic specificity (DSp), cut-off value and other optimal test parameters like Youden index, Area under curve (AUC) along with confidence interval and standard errors for the assay were calculated by receiver operator characteristic (ROC) analysis.

3. Results

3.1. Construction of pNS3ΔHD expression vector

The result of multiple sequence alignment of BTV-NS3 protein sequences along with marked region for deletion is depicted in Fig. 1. A high degree of sequence conservation was noticed at both N- and C-terminal regions, whereas heterogeneity was noticed in centrally located region between HD regions among all BTV serotypes. Primers were designed to amplify N- and C-termius separately, leaving the target deletion sequence (65 aa) in the central of NS3 gene. PCR amplification of *NS3* gene encoding for N-terminus and C-terminus of NS3 protein using their specific primer set and cDNA of BTV 23 resulted in an amplicon of ~384 bp and ~162 bp in size respectively (Fig. 2, Panel C, Lane 1 and 2). A ligation of N- and C- fragments through a RE digested site, *Hind*III resulted in a fusion NS3ΔHD gene fragment (Fig. 2, Panel C, Lane 3). Further, cloning of fusion NS3ΔHD gene fragment in to a pET32a vector resulted in generation of recombinant clone pNS3ΔHD (Fig. 2, Panel C, Lane 4).

3.2. Expression of rNS3ΔHD fusion protein

A recombinant *E. coli* cells harboring pNS3ΔHD plasmid following induction with 1 mM IPTG, resulted in expression of rNS3ΔHD protein along with hexa-histidine tags on its both termini, which accounted for a total molecular weight ~38 kDa as observed on 10% SDS-PAGE (Fig. 2, Panel D, Lane 2). The bacterial growth curve analysis subsequent to induction revealed absence of cytotoxicity, which was indicated by proper growth curve without inhibition/cell lysis with optimum expression of target protein.

3.3. Purification of rNS3ΔHD fusion protein

Solubility analysis of over-expressed rNS3ΔHD fusion protein following cell lysis indicated the presence of high amount of expressed protein in insoluble fraction (>90%). Further, the rNS3ΔHD fusion protein was purified under denaturing condition and renatured on column before elution and dialysis. An optimized purification protocol yielded high purity of target protein following

Fig. 1. Multiple sequence alignment of NS3 protein of bluetongue virus (BTV) serotypes. The representative NS3 protein sequences from all the 26 serotypes of BTV available in the GenBank were aligned by ClustalW method of DNASTAR. The regions of N- and C-terminus along with hydrophobic domains (HD1 and HD2) are indicated with schematic tubes. The region selected for deletion in the complete NS3 sequence is marked with red colored box. The predicted antigenic index by PROTEAN (DNASTAR) is shown on top of the NS3 consensus sequence.

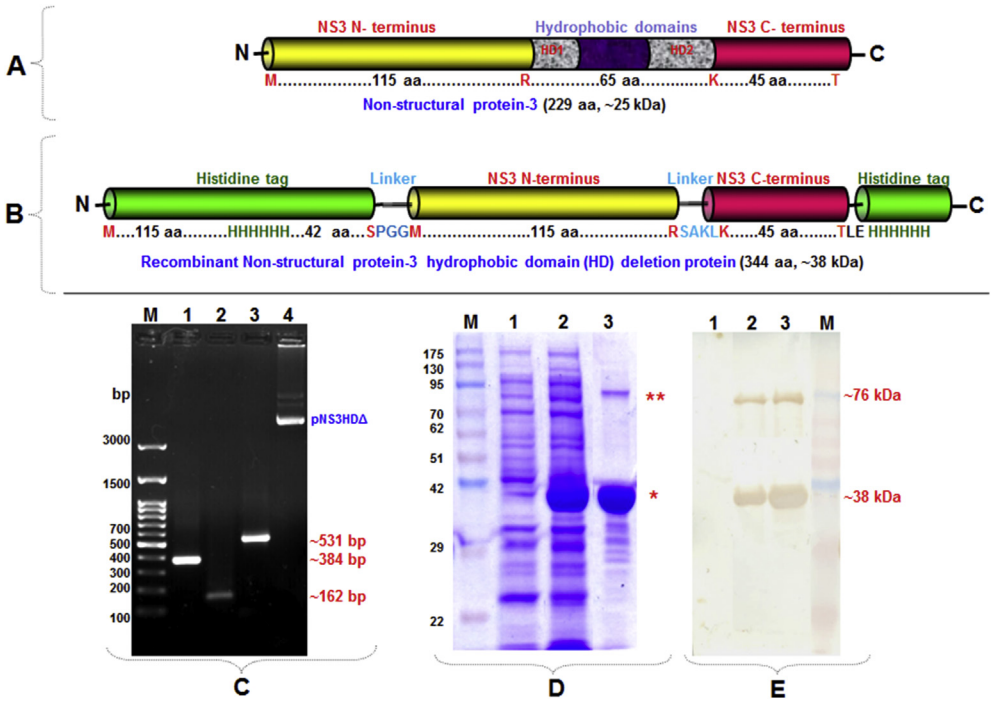

Fig. 2. Schematic of rNS3ΔHD construct, expression, purification and detection. Panel A: Full length NS3 protein of Bluetongue virus 23. Panel B: Recombinant NS3ΔHD fusion protein with hexa-histidine tags at both N-/C- termini. Panel C: PCR amplification of NS3 gene fragments from Bluetongue virus 23. Lane M: DNA standard ladder; Lane 1: NS3 N-terminus gene amplified product (~384 bp); Lane 2: NS3 C-terminus gene amplified product (~162 bp); Lane 3: NS3 N- and C-terminus gene fusion product (~531 bp); Lane 4: Uncut pET32a vector with ligated NS3ΔHD fusion gene. Panel D. Over-expression of recombinant NS3ΔHD fusion protein. Lane M: Protein standard marker; Lane 1: Uninduced *E. coli* cell lysate; Lane 2: Induced *E. coli* cell lysate showing expressed rNS3ΔHD protein (~38 kDa); Lane 3: Purified rNS3ΔHD fusion protein fractions by affinity chromatography showing monomer (~38 kDa) and dimer (~76 kDa); The positions of monomer and dimer are indicated by red colored single and double stars respectively. Panel E. Immunoblot using anti-His IgG antibodies. Lanes 1 to 3: as described in Panel D, the brown color development after immunoblot using QIAexpress detection kit containing mouse monoclonal anti-His IgG HRP conjugate and DAB substrate in chromogenic reaction observed on nitrocellulose membrane. Lane M: Protein standard marker.

one step affinity chromatography. The purified recombinant protein yield was found to be in the range of 15–20 mg/L.

3.4. SDS-PAGE and Western blot of rNS3ΔHD fusion protein

The purification process resulted in purified two bands of rNS3ΔHD fusion protein corresponding to monomer and dimer on 10% SDS-PAGE at expected size of ~38 kDa and ~76 kDa respectively (Fig. 2, Panel D, Lane 3). Immunoblot using anti-His antibodies detected the presence of hexa-histidine tag in both forms (monomer and dimer) of rNS3ΔHD fusion protein by development of brown color in a chromogenic reaction on nitrocellulose membrane (Fig. 2, Panel E, Lane 2 and 3). No color was developed in uninduced *E. coli* culture.

3.5. Predicted characteristics of rNS3ΔHD fusion protein

The predicted primary characteristics of rNS3ΔHD fusion protein revealed the presence of total 344 aa (38,035 Da), which included majority of hydrophobic amino acids (107) along with basic (44), acidic (49) and polar (71) amino acids. The rNS3ΔHD fusion protein was found to have an isoelectric point of 6.687. Out of total 344 aa, NS3 protein region had 164 aa, whereas vector encoded region including hexa-histidine tag and linker region accounted for 180 aa.

3.6. Immuno-reactivity of rNS3ΔHD fusion protein

An indirect-ELISA using rNS3ΔHD fusion protein as coating antigen (100 ng/well) and serum dilution at 1:10 showed reactivity by detecting NS3 specific antibodies in field sera samples. The plot of ROC estimation is presented in Fig. 3. The results of comparative immuno-reactivity between rNS3ΔHD-ELISA and commercial c-ELISA are presented in Table 2. Out of 239 sera samples screened, 86 samples were found positive for BTV antibodies by both the ELISA formats (rNS3ΔHD-ELISA and commercial c-ELISA). However, additionally, four sera samples, which were negative by c-ELISA, were also found to be positive in rNS3ΔHD based indirect-ELISA. Percent positivity (PP) values were used for the estimation of cut-off values for rNS3ΔHD based indirect-ELISA. On ROC analysis, different cut-off values along with their DSn and DSp were obtained. Among these, >20% PI value was accepted as cut-off for indirect-ELISA at which DSn 94.4 0% at 95% confidence interval and DSp of 93.9% at 95% confidence interval was observed. The Youden index (*J*), the maximum potential effectiveness of an antigen was found to be 0.9042. Area under curve (AUC) in the indirect-ELISA was determined to be 0.982 (SE = 0.00945) and 0.955 to 0.995 confidence interval.

4. Discussion

Protein engineering either by gene fragment deletion, fusion or insertion using recombinant DNA technology focused at production of efficient recombinant protein molecules suitable for their utility either as novel vaccine or diagnostic reagent in biomedical applications, are being considered as robust alternative strategy over conventional methodologies in recent times [24,25,27–29]. We applied this strategy to produce efficient rNS3ΔHD antigen and evaluated its potential suitability for developing diagnostics of bluetongue in small ruminants.

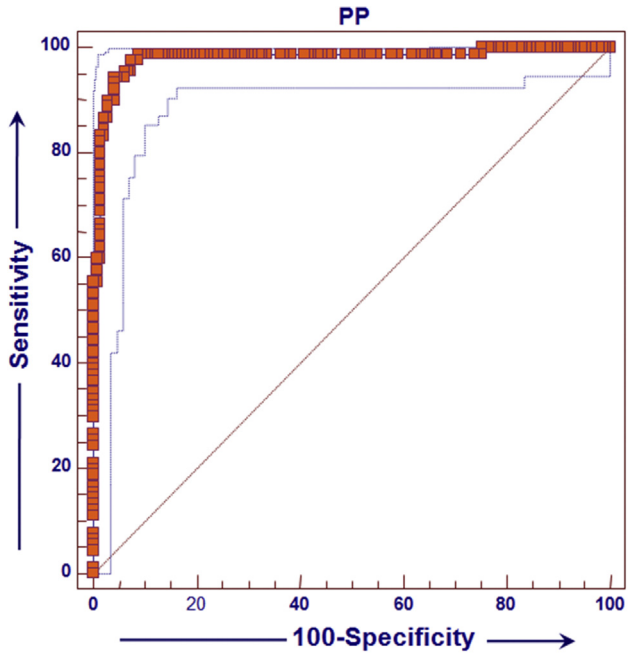

Fig. 3. ROC for estimation of cut-off values of rNS3ΔHD based indirect-ELISA. Sensitivity over (100-specificity); at different cut-off values. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. The intersection point of the line indicates the cut-off point (>20%) at which sensitivity (DSn) 94.4. and specificity (DSp) 93.9% are noted.

NS3/NS3A is the only membrane protein encoded by Orbiviruses and is associated with smooth intracellular membranes and at the plasma membrane [30]. The conserved and variable domains/regions of BTV-NS3 such as amphipathic helix region, CCMs, proline rich region, and two hydrophobic domains (HD1/TM1 and HD2/TM2) with central variable region have been very well characterized in the past [2,20,22,31]. As observed in our study as well as in others [32], NS3 protein sequence is strikingly conserved with minimal variation (1% and 10%) among various BTV strains originated from different livestock-ecosystems [20]. The most variable region is situated between the two transmembrane hydrophobic domains with only 64% identity. In the case of BTV-NS3, the conserved N-terminal domain has 96% identity across different serotypes and the sequences of HD1 are 73% conserved [20]. On the basis of studies on NS3 protein expressed in mammalian cells, Han and Harty [33] suggested that the protein has a viroporin-like property and is capable of permeabilizing or destabilizing lipid membrane and self-oligomerization.

Ours as well as others earlier attempts [20,22] to produce a recombinant full length NS3 in *E. coli* were unsuccessful possibly due to its cytotoxicity. A similar cytotoxicity exhibited by NS3 was observed following expression in mammalian or *Spodoptera frugiperda* cells [34]. Previously, in order to analyze the effect of NS3 on bacterial cells, the gene encoding for full-length NS3 protein (218aa) of AHSV-3 was serially fragmented to produce a series of NS3 N-/C- terminal truncated mutants including deletions of either one or both hydrophobic domains HD1 and HD2 such as M1 (1–92aa), M2(1–120aa), M3 (1–140aa), M4 (150–218aa), M5 (112–218aa) and M6 (92–218aa) and cloned in pET41c vectors [20]. Subsequently, expression of the wild-type as well as mutant proteins in *E. coli* cells and their effect on cell growth was monitored by growth curve which clearly indicated that the full-length NS3 protein was detrimental to *E. coli* growth [20]. The growth of *E. coli* cells expressing full-length NS3 was inhibited by 35%, whereas C-terminal truncated M1 and M2 mutants (without HD1 and HD2) did not inhibit cell growth compared M3 and M4 mutants (with HD1 or HD2) which inhibited growth by 10% and 5%, respectively [20]. Notably, a significant growth inhibitory effect was seen in the case of the M5 (92%) and M6 (71%) mutants that each contained both hydrophobic domains (HD1 and HD2). These studies revealed that NS3 cytotoxicity requires both HD1 and HD2 [20].

Hence, we designed a construct so as to eliminate both HD1 and HD2 along with central variable region. Only highly conserved N- and C-terminus regions of NS3 as noted in multiple sequence alignment (Fig. 1) were selected for generation of fusion gene. We amplified regions encoding only for NS3 N-terminus and C-terminus separately without involving HD1/HD2 regions and gene fusion was carried out by ligation of two fragments via *Hind*III site created by RE digestion. An added structureless linker sequences coding for 'PGG' and 'SAKL' were incorporated between vector encoded sequence/NS3 N- terminus; and NS3 N-/C-terminus fusion, respectively (Fig. 2, Panel B), to provide flexibility as well as to enhance native conformity/proper folding of protein. The entire NS3ΔHD fusion was sandwiched between vector encoded sequences comprising 6x-histidine tags on both termini. This strategy was used to simplify the protein folding and efficient purification of rNS3ΔHD fusion protein so as to achieve good quantity as well as quality of target antigen.

We chose prokaryotic expression vector (pET32a), which has tightly regulated inducible system particularly amenable for the synthesis of toxic proteins [35], for cloning of NS3ΔHD fusion gene as it is considered to be ideal for cost effective bulk production of recombinant protein followed by easy/simple purification protocol under affinity chromatography. Previously, many researchers have noticed the positive effect of the hydrophilic nature of the histidine tag on enhanced expression of recombinant fusion proteins [36]. Moreover, post-translation modifications were not a concern as NS3 was found to possess a single glycosylation site in the extracellular domain at 150th amino acid residue [2,20], which was deleted in our construct design.

Naturally, NS3 protein is expressed in very low levels in mammalian cells whereas more protein is made in insect cells. Previously, attempts were made to express NS3 protein in *E. coli* [16], yeast cells [37] and baculovirus expression system, all of which indicated cytotoxic effect of NS3 on host cells [20]. Upon chemical induction, we noticed a successful over-expression of rNS3ΔHD fusion protein which was accounting for more than 20% of total *E. coli* cell protein. Absence of bacterial growth inhibition upon induction reflected the fact that our construct was devoid of HD1/HD2 domains. The time course analysis revealed an optimum expression noticed near 3–4 h subsequent to induction. There was neither leaky expression nor degradation/fragmentation of expressed fusion protein noticed as evidenced by SDS-PAGE (Fig. 2, Panel D, Lane 2). Solubility of expressed fusion protein indicated partitioning of rNS3ΔHD fusion protein in to insoluble fraction of the cell lysate. It could be due to presence of higher hydrophobic residues (107 aa) in the complete fusion protein.

Generally, it was recognized that inclusion bodies facilitate overproduction of recombinant proteins by the *E. coli* cells and can be isolated in highly pure form. Hence, in the present study, the overexpressed rNS3ΔHD fusion protein which was stored as inclusion bodies inside the cytoplasma of bacterial cell was successfully purified to higher homogeneity (>95%) by employing a standardized denaturation and on-column renaturation protocol. This methodology is known to maximize the stability, native conformity, high quantity as well as purity of protein. The purification procedure was simple and efficient to produce nearly conformationally native NS3 protein. Moreover, there were no Cysteine residues in our designed construct, which eliminated the probability of forming high order intra-molecular di-sulfide bonds which are known to contribute

for protein aggregation. Further, neither precipitation nor aggregation of purified rNS3ΔHD fusion protein was noted upon storage. In the present study, rNS3ΔHD fusion protein was readily renatured to its dimeric state following denaturation/renaturation (Fig. 2, Panel D, Lane 3), which might have resulted in a more compact oligomeric structure.

We also observed rNS3ΔHD fusion protein forming monomer (~38 kDa) as well as dimers (~76 kDa) subsequent to analysis of purified proteins on SDS-PAGE. The role of coding region found within the pET32a vector in formation of oligomers was ruled out as it was shown to have no effect on the formation of oligomers in previous studies [23–25,38]. Rather, it is probably due to the presence of coiled-coil motifs (CCMs) at its N-terminus. Recently, bio-informatic analysis of NS3 sequences of all 26 BTV serotypes revealed the presence of highly conserved α-helical heptad sequence, especially at 14 to 26 aa identified as CCMs (CCM-I) to presumably be involved in oligomerization [22]. The purified rNS3Nt fusion protein ($_{1}$M-R$_{117}$ aa) accounting for ~32 kDa was found to form dimers (~64 kDa) and high order oligomers (hexamer and octamer) under reducing or non-reducing conditions [22]. Therefore, an *in silico* predicted centrally located CCM ($_{182}$-MKHNQDRVEELSLV-$_{195}$) in the rNS3ΔHD fusion protein might have contributed significantly in the inter-helical interactions leading to stable dimers. A conserved CCM with predicted heptad region as a highly versatile oligomerization motif in several proteins with a common structural and functional theme has been noted [39]. Despite our attempts to predict the tertiary structure of newly constructed rNS3ΔHD fusion protein by homology modeling, the features in terms of native conformity/oligmoerization etc., were inconspicuous. Interestingly, we did not notice high order oligomers of rNS3ΔHD fusion protein during the course of study, which needs further investigation especially in the absence of precise stoichiometry as well as inconspicuous features of NS3 protein in natural scenario.

Immune responses, both humoral and cell mediated mechanism are attributed to be involved in the protection of host against BTV infection as evidenced by extensive earlier studies [16,40]. Nevertheless, there is a need for systematic study to analyze the antibody kinetics to viral structural and non-structural proteins. For evaluating the diagnostic suitability of rNS3ΔHD antigen in indirect-ELISA format, the random field sera samples segregated as positive/negative based on the commercial c-ELISA kit results were used in rNS3ΔHD-ELISA. Our construct, rNS3ΔHD fusion protein, was also found to detect NS3 specific antibodies in sera samples in an indirect-ELISA, the results of which were comparable with that of detection by rNS3Nt-ELISA (data not shown) and other ELISA formats. In our current study, out of 239 sera samples screened, 86 samples found to be positive by both ELISAs (rNS3ΔHD- ELISA and c-ELISA). However, additionally, four more sera samples from sheep (3) and goat (1) were found positive by rNS3ΔHD-ELISA, despite negative detection by c-ELISA (Table 2). In earlier studies, it was noted that persistence of antibodies to structural proteins are generally higher in animals compared to non-structural proteins. The reason for four sera samples which were negative by c-ELISA, were found positive based on NS3 antibodies in rNS3ΔHD-ELISA could not be clearly ascertained. Nevertheless, on the basis of recent studies [41], it is presumed that there could be other cross-reactive Orbiviral (e.g., Epizootic Hemorrhagic Disease Virus [EHDV]) antibodies circulating in small ruminants, which warrants further investigation in domestic as well as wild animals of India. Antibodies to non-structural proteins are found to develop at a later stage than that against structural proteins and tend to wane off earlier also during an infection [16]. The lower sensitivity of the non-structural protein based ELISA as compared to the structural protein VP7 based c-ELISA may be due this factor as it may not be able detect early and very late infection. The same variation being reflected in indirect-ELISA depending on coating antigen. These results indicate the potential utility of rNS3ΔHD fusion protein as a coating diagnostic antigen in further development and evaluation of DIVA diagnostic assay for BT infection. Nevertheless, an assay using rNS3ΔHD fusion protein needs to be further investigated for its specificity/cross-reactivity in closely related Orbivirus disease (epizootic hemorrhagic disease) and others such as foot-and-mouth disease (FMD), vesicular diseases, bovine viral diarrhea (BVD), bovine herpesvirus type-1 (BHV-1) infection and malignant catarrhal fever (MCF) infection under differential diagnosis.

Conclusively, the current study described a strategic design of construct for efficient over-expression of BTV-NS3 fusion protein devoid of hydrophobic domains (HD1 and HD2) including a centrally located variable region. It also described a methodology for efficient purification of biologically active rNS3ΔHD fusion protein which could be potentially incorporated as diagnostic reagent in further development and evaluation of immuno-diagnostic assay either under DIVA strategy or alternative routine sero-surveillance assay for BT diagnosis.

Conflict of interest

There was no conflict of interest among the authors.

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