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# Immuno-reactivity of recombinant non-structural protein 3 N-terminus (rNS3Nt) in indirect-ELISA for detection of bluetongue viral antibodies in serum samples

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ABSTRACT

Bluetongue, an arthropod borne non-contagious disease of ruminants especially sheep, is caused by bluetongue virus (BTV). Detection of BTV antibodies in susceptible hosts is considered to be of significance in disease diagnosis and differentiation. In the present study, a partial NS3 gene encoding for non-structural protein-3 N-terminus ($_{1}$M-$T_{117}$ aa) of BTV-23, produced as purified recombinant NS3Nt fusion protein (~32 kDa) using prokaryotic expression system (*Escherichia coli*), was evaluated as a candidate antigen in an indirect-ELISA (rNS3Nt-ELISA) to measure the serologic response to NS3 protein in small ruminants. The rNS3Nt fusion protein obtained in sufficient quantity and quality has good reactivity in detecting NS3 specific antibodies in field serum samples by indirect-ELISA. As NS3 protein is highly conserved, rNS3Nt-ELISA has potential for NS3 specific detection of antibodies in BTV affected animals irrespective of different viral serotypes. In comparison to structural protein (VP7) based c-ELISA kit and i-ELISA kit, the diagnostic sensitivity (85.1%, 86.2%) and specificity (92.5%, 93.2%) of rNS3Nt-ELISA were found to be relatively lower, respectively. Nevertheless, the study indicated the potential utility of rNS3Nt-ELISA as an alternate assay in routine sero-diagnosis of BTV infection and possible sero-surveillance of ruminants under DIVA strategy.

**Key words**: Antibody titers, Bluetongue virus, Immuno-reactivity, Indirect-ELISA, Recombinant non-structural protein 3 N-terminus (rNS3Nt)

Bluetongue, a vector-borne viral disease of domestic and wild ruminants affecting mainly sheep followed by goats and cattle, is caused by bluetongue virus (BTV) which is a type species of genus *Orbivirus* in the family *Reoviridae* (Mertens *et al.* 2004). BTV is a non-enveloped virion with genome consisting of ten (1–10) double-stranded RNA (dsRNA) segments packaged within a three-layered icosahedral capsid of seven structural proteins (VP1 to VP7). At present, 27 distinct serotypes of BTV have been recognized worldwide (Prasad *et al.* 1992, Hofman *et al.* 2008, Maan *et al*. 2011, Zientara *et al.* 2014, Schulz *et al.* 2016, Hemadri *et al.* 2017).

A preliminary disease diagnosis is usually based on clinical signs and lesions in sheep, but the disease is mostly subclinical in goats and cattle; however, further confirmatory diagnosis is based on number of laboratory tests (Afshar 1994). It includes virus isolation in embryonated chicken egg or in various cell types such as BHK-21/Vero/insect cell line etc. (Afshar 1994, Clavijo *et al*. 2000, Eaton and White 2004) and serum neutralization tests (SNT) (Bulut *et al*. 2006). Several other tests such as agar gel immunodiffusion (AGID), enzyme-linked immunosorbent assay (ELISA), and fluorescent antibody (FAT) test are also employed. Molecular tools such as the reverse-transcriptase polymerase chain reaction (RT-PCR), real time RT-PCR and LAMP assay to identify BTV directly in blood samples or cultured cells have also been developed (Shad *et al.* 1997, Aradaib *et al.* 1998, Eaton and White 2004). Although, SNT is highly sensitive and specific, it is not routinely used as it is time consuming and expensive (Hamblin 2004).Various types of ELISAs such as competitive ELISA (c-ELISA), sandwich-ELISA (s-ELISA) and an indirect-ELISA (i-ELISA) have been developed in the recent past using either native (Afshar *et al*. 1987, Chand *et al.* 2009) and/or recombinant VP7 antigen (Martyn *et al.* 1990, Afshar *et al.* 1992, Pathak *et al.* 2008) for detection of BTV. However, a limited effort was made to evaluate the potential utility of non-structural (NS) proteins of BTV as alternate diagnostic antigen, which could also assist in developing immune-diagnostics under DIVA strategy (Anderson *et al.* 1993, Barros *et al.* 2009). In view of already developed non-structural proteins based DIVA

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assays for several viral infections in animals, the strategies for differentiating infected from vaccinated animals under DIVA are possible with inactivated BTV vaccines and NS proteins (Barros *et al*. 2009, Sperlova and Zendulkova 2011).

BTV genome is also known to encode for four non-structural proteins; NS1(~64 kDa), NS2(~41 kDa), NS3/ NS3A (~25.5 kDa) and NS4 (~10 to 22.5 kDa), which are involved in either replication, maturation, export from infected cells and/or interaction with host (Schwartz-Cornil *et al*. 2008, Ratinier *et al*. 2011, Belhouchet *et al*. 2011). However, the host antibody response kinetics to different BTV-NS proteins is not yet fully understood. There is a need to evaluate the efficacy of BTV-NS proteins to detect the circulating antibodies in livestock. In the present study, we evaluated the potential utility of recombinant NS3 N-terminus fragment (rNS3Nt) fusion protein in detection of BTV antibodies against NS3 protein in serum samples by indirect-ELISA.

## MATERIALS AND METHODS

*Virus, clone and host cells:* BTV 23 Dehradun strain, an isolate from a natural bluetongue outbreak in India, maintained in the Division of Virology, ICAR-Indian Veterinary Research Institute (IVRI), Mukteswar, Uttarakhand (UK), India, was used in target NS3 gene fragment amplification and clone construction as described earlier (Chacko *et al*. 2015). A previously constructed recombinant *E. coli* BL21-CodonPlus(DE3)-RIPL cells (Novagen, USA) harbouring plasmid (pNS3Nt) in a pET32a vector were used for production of recombinant antigen (rNS3Nt) in bulk (Chacko *et al.* 2015).

*Production of recombinant antigen (rNS3Nt) of BTV:* A recombinant NS3Nt fusion protein was produced in bulk as per the standard procedures described previously (Ahuja *et al*. 2012, Chacko *et al*. 2015, Shivachandra *et al.* 2012, 2015, 2017, Yogisharadhya *et al*. 2017). Briefly, *E. coli* BL21-CodonPlus(DE3)-RIPL cells harbouring recombinant plasmid pNS3Nt were grown in 1 L Luria Bertani (LB) broth using appropriate antibiotics (ampicillin [50 mg/ml] and chloramphenicol [35 mg/ml]) and kept in shaking incubator at 37°C. Subsequent to chemical induction with 1mM IPTG (Sigma-Aldrich, USA), cells were harvested, resuspended in buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl and lysozyme) before lysis by sonication (Sonics, USA). Purification was carried out by affinity chromatography using Ni-NTA superflow cartridges (Qiagen, Germany). Following column binding, renaturation on column and washing with buffer (50 mM Tris-HCl, pH 6.0, 100 mM NaCl and 50 mM Imidazole), recombinant NS3Nt was eluted with elution buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl and 300 mM Imidazole) before dialysis of pooled fractions in a buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl) and quantification by standard method. Purified rNS3Nt proteins were aliquoted and stored at –80°C until further use.

*Serum samples:* Randomly collected field serum samples (314) were used for evaluation of reactivity of rNS3Nt protein/ optimization of rNS3Nt based i-ELISA. Initially, all the field serum samples were screened with commercially available c-ELISA kit (Bluetongue antibody test kit, VMRD, Inc., Pullman, USA) and rVP7 based indirect-ELISA kit (Pathak *et al.* 2008) before segregation as 'positive' and 'negative' on the basis of detectable antibodies to BTV. These positive and negative samples were tested along with the standard negative and positive control serum for validation of the assay. Then, the rNS3Nt protein based indirect-ELISA was used to screen random field serum samples. Positive and negative serum available at Bluetongue Virus Laboratory, ICAR-IVRI, Mukteswar, UK, India, were used as controls.

*Optimization of indirect ELISA using rNS3Nt protein:* The optimal concentration of antigen and dilution of the serum were determined by performing a checkerboard titration of the rNS3Nt antigen along with positive and negative caprine control serum. On the basis of initial titration, 100 ng/well of antigen and 1:10 serum dilution were used in all further process of rNS3Nt-ELISA. The coating of ELISA plate (MaxiSorp, Nunc A/S, Rosklide, Denmark) with purified recombinant protein diluted in 0.05 M carbonate bicarbonate buffer pH 9.6 (Sigma, UK), was carried out at 37°C for 1 h on a plate shaker. The plate was washed three times with PBST (0.03% Tween 20 in PBS) to remove the unbound antigen. The same washing procedure was followed throughout the study, if not mentioned otherwise. Primary blocking was done with 100 µl of blocking buffer (2% gelatin and 3% skim milk powder in PBS) at 37°C for 1 h. Following three washes, diluted test and control serum (1:10 in blocking buffer) were added to the respective wells of the plate and incubated for 1 h at 37°C. The plate was washed and 50 µl of diluted (1:10000) rabbit anti-goat IgG HRPO conjugate (Sigma, UK) in blocking buffer was added to all the wells. Following incubation and washing, 50 µl of OPD-substrate (Sigma, UK) solution was added to each well and incubated in dark for 8–10 min. The reaction was stopped by 50 µl of 1 M $H_2SO_4$ and absorbance was measured at 492 nm in a microplate reader (Biorad). The optimized rNS3Nt based indirect ELISA was further used for screening of sheep serum (118) samples.

*Comparison with commercial c-ELISA and rVP7-ELISA kits:* Bluetongue virus antibody test kit (VMRD Inc., Pullman, WA, USA), a competitive ELISA (c-ELISA) for detection of antibodies specific to the highly conserved VP7 protein of BTV, was used as a standard for comparison. Further, an in-house developed rVP7 based indirect-ELISA kit (Pathak *et al*. 2008) was also used for initial screening of serum samples. The data generated from screening of total 314 serum samples using rNS3Nt-ELISA, c-ELISA and rVP7- ELISA were subjected to ROC analysis.

*Statistical analysis:* For determination of cutoff values, the gross $OD_{492}$ value of test samples was expressed as percent positivity [percent positivity, $PP= \{(OD_{sample}-OD_{neg\ control})/ (OD_{pos\ control}-OD_{neg\ control})\} \times 100$] and the area under

curve (AUC) was calculated by Receiver Operating Characteristic (ROC) analysis using MedCalc software package version 13.2.2.0 (MedCalc Software, Belgium). The diagnostic sensitivity and specificity of assays were estimated at different-cut off value using ROC analysis. Variability and reproducibility of the rNS3Nt-ELISA were determined by calculation of intra-assay and inter-assay variation. The $OD_{492}$ value of pooled positive serum was measured ten times in different runs for calculation of inter-assay variation. For intra-assay variation, $OD_{492}$ values of ten replicates of the same pooled serum were measured in ten columns of the plate in a single run. For comparison of data between different ELISA plates, OD values were routinely normalized against a positive control serum and expressed as percent positivity (mean corrected test OD value/corrected positive control OD value × 100).

## RESULTS AND DISCUSSION

Detection of antibodies for a particular pathogen especially BTV is of importance for both routine surveillance and trade purpose as well as to differentiate naturally infected and vaccinated animals. In the past, various formats of ELISA for detecting antibodies to structural proteins especially VP7 of BTV were developed (Afshar *et al.* 1987, Pathak *et al.* 2008). Presumably, antibodies to non-structural BTV proteins are less likely to be present in animals immunized with inactivated vaccines which help in the DIVA strategy. Further, the higher levels of NS3 antibodies recorded in BTV infected animals in comparison to the levels induced in animals vaccinated with inactivated BTV vaccines (Barros *et al.* 2009) indicated the possibilities to develop an immunodiagnostic assay under DIVA strategy. Hence, we evaluated the potential utility of recombinant NS3Nt antigen based indirect-ELISA to detect levels of antibody response to NS3 in random field serum samples.

*Production of recombinant antigen (rNS3Nt):* Following chemical induction, the recombinant *E. coli* expressed NS3Nt protein along with hexa-histidine tags on its both termini accounting for a total molecular weight ~32 kDa (293 aa), was purified under denaturing/renaturing condition. The purified protein was a mixture of two forms of proteins, viz. monomer and dimer of rNS3Nt fusion protein on 10% SDS-PAGE at expected sizes of ~32 kDa and ~64 kDa, respectively (data not shown). Amongst all the 27 serotypes of BTV reported worldwide, the genes coding for the non-structural proteins and the internal proteins are highly conserved (Roy 1992, Mumtsidu *et al.* 2007). NS1 and NS2 are the most highly expressed NS proteins in BTV infected cells whereas in insect cells, NS3/NS3A is synthesized in larger amounts (Schwartz-Cornil *et al.* 2008). NS3 (229 aa) and NS3A (216 aa, ~24 kDa) are membrane proteins which function as viroporin helping in viral exist by assisting in budding of virus from infected cells (Schwartz-Cornil *et al.* 2008). NS3 of BTV, similar to rotavirus NSP4 and African horse sickness virus (AHSV) NS3, has two hydrophobic domains, viz. TM1 (118–141 aa) and TM2 (162–182 aa) with both the amino- and carboxyl-terminal ends of the protein appearing to be cytoplasmic (Bansal *et al.* 1998, Huismans *et al.* 2004). Recently, we identified a conserved α-helical heptad sequences (coiled-coil motifs) at 14 to 26 aa (CCM-I), 185 to 198 aa (CCM-II), and 94 to 116 aa (CCM-III). Of which, CCM-I found close to the N-terminus of NS3 which was presumed to be involved in oligomerization (Chacko *et al.* 2015). We also noticed a SDS-resistant monomer (~32 kDa), a possible dimer (~64 kDa) and/or oligomers of rNS3Nt fusion protein produced from prokaryotic (*E. coli*) expression system (Chacko *et al.* 2015). In the past, homo-oligomerization properties of NS3-WT as well as its deletion mutant forms producing tetramer (~100 to 110 kDa) protein species of NS3 were detected following analysis of cell extracts (Han and Harty 2004). Till date, almost all the characterized viroporins are known to possess the ability to oligomerize and form aggregates in mammalian cells. In our prokaryotic expression system as well, we noticed aggregates of rNS3Nt following over-expression, which were further purified under denaturing and on column renaturation procedure to get near native conformation.

*Optimization of indirect-ELISA:* The rNS3Nt protein based indirect-ELISA was initially optimized using positive and negative serum samples, which were pre-screened with standard commercial c-ELISA kit. The mean OD of known positive and negative serum was 1.0±0.05 and 0.16 ±0.06, respectively. The optimum concentration/dilution of rNS3Nt protein and test serum was 100 ng and 1.10 per well, respectively. Based on ROC analysis, the cutoff was set as a PP value >20.3 at which diagnostic sensitivity and diagnostic specificity of 85.1% and 92.5% with 95% confidence interval respectively was observed (Fig. 1, Panel A), when compared to c-ELISA kit. Area under curve (AUC) which quantifies the diagnostic accuracy of the test was determined to be 0.916. Inter-assay coefficient of variation (CV%) calculated from ten different runs was

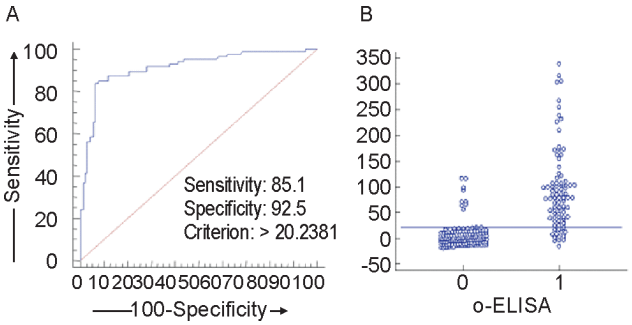


Fig. 1. ROC curve and interactive dot diagram. Panel A: ROC curve. The true positive rate (Sensitivity) is plotted in function of the false positive rate (100–Specificity) for different cut-off points. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. Panel B: Interactive dot diagram. The data of negative and positive groups are displayed as dots on the vertical axis. The horizontal line indicates the cut-off and corresponding sensitivity and specificity is displayed on the side.

5.04±1.10 and 3.16±0.80, whereas, the coefficient of variation for intra-assay calculated from replicates of same samples in a single run was 3.16±0.51 and 3.92±0.45 for positive and negative serum control, respectively. An optimized rNS3Nt-ELISA employed for further testing of goat and sheep (118) serum samples indicated 25.4% and 52.5% positive, respectively.

On a routine basis for a positive BTV diagnosis, either BTV antigen/RNA or antibodies need to be detected in a sample by virus isolation, molecular or serological methods. ELISAs with high sensitivity could detect BTV antibodies produced by host humoral responses as early as one week after infection (Batten *et al*. 2008, Oura *et al*. 2009) and longer persistence of neutralizing antibodies as long as 4 years in cattle and 2.5 years in sheep (Batten *et al*. 2013). Previously, recombinant full length NS3 protein was used as an antigen in an indirect ELISA (NS3-ELISA) to measure the serologic response to NS3 protein in cattle and sheep under DIVA strategy (Barros *et al*. 2009). In cattle, NS3 antibodies were detected at approximately 15 days post BTV infection, whereas animals vaccinated with a bivalent inactivated BTV 2–4 failed to develop detectable NS3 antibodies. The amount of NS3 expression is generally high in insect cells, and low in cells of vertebrate origin (French *et al*. 1989, Guirakhoo *et al*. 1995). Nevertheless, there is a need to study the dynamics of the appearance of NS3 antibodies in naturally infected animals as well as vaccinated animals. In a study by Barros *et al*. (2009), the animals inoculated with BTV-1 were found to seroconvert to NS3 on 10 dpi and develop higher levels of NS3 antibodies in comparison to the response elicited by inactivated BTV vaccines, which induced little or no detectable antibodies to NS3 protein. Since, the currently used BTV inactivated vaccines are most likely to be contaminated with NS proteins, the purity of the BTV vaccines seems to be of crucial importance in order to avoid the false positive results due to presence of antibodies against NS3 and others. It was noted in the recent past that NS3 protein is highly conserved amongst global strains of BTV despite originating from divergent ecosystems (Balasuriya *et al*. 2008) and further, all the BT viruses from Asia and Australia were grouped in one clad. Since it was indicated that NS3 is not evolving by adoptive (positive) selection imposed by different selection pressures such as vector species (Balasuriya *et al.* 2008), there is a limited chance for NS3 to undergo variation in the future. Although, NS3 protein is produced in large quantities in infected cells, it is not packaged in the virion.

*Comparative reactivity of rNS3Nt-ELISA with c-ELISA and rVP7-ELISA for BTV antibodies:* In rNS3Nt-ELISA, the mean OD of known negative and positive serum was 0.16 ±0.03 and 1.02±0.06 respectively, and OD value was expressed into per cent positivity (PP). In ROC analysis, a PP value >20.3 was set as criteria, which gave a sensitivity and specificity of 85.1 and 92.5% respectively, as compared to c-ELISA (Fig. 1, Panel A) and it was 86.2 and 92.5%, respectively, with rVP7-ELISA.

Animals infected with BTV are known to develop a high titerd antibody response to a variety of viral proteins. Apart from serogroup specific antibodies induced by VP7 protein, the presence of antibodies against other structural and non-structural proteins in serum of infected animals have been reported (MacLachlan *et al.* 1987, MacLachlan 2004). We evaluated an indirect ELISA format using rNS3Nt fusion protein for detecting antibodies to BTV in the serum of sheep and goat, which were initially screened by c-ELISA kit and were used as the positive and negative control. The cut off value was determined by ROC analysis, as PP >20.3 at which diagnostic sensitivity was found to be 85.1% and diagnostic specificity was 92.5%. The CV calculated from the normalized data obtained from replicate of the positive and negative serum were all below 6%, which is below the acceptable value of 10% (Maree and Paweska 2005). BT c-ELISA kit is a monoclonal antibody based test to detect BTV structural protein VP7. Studies on experimentally infected sheep showed that the antibodies to VP7 protein developed one week prior to that of NS3 and it can be detected even after 330 days post infection, unlike that of NS3 (Barros *et al*. 2009). This may account for the lower sensitivity of rNS3Nt-ELISA as non-structural protein based assay may not be able detect early and very late infection. Further, an indirect-ELISA using recombinant NS3ΔHD protein may enhance immuno-reactivity as it contains additional C-terminal epitopes (Mohanty *et al*. 2016). Nevertheless, the kinetics of antibodies to non-structural proteins has to be studied in infected and vaccinated animals, so as to further evaluate the efficiency of recombinant non-structural protein based immunodiagnostics. Recombinant non-structural proteins based DIVA tests have been successfully developed in the past for various diseases such as foot-and-mouth disease virus (Clavijo *et al.* 2004, Lu *et al.* 2007), African horse sickness virus (Laviada *et al.* 1995), and equine influenza virus infections (Birch-Machin *et al.* 1997). A total of around 314 random field serum samples of sheep and goat were tested using rNS3Nt-ELISA to detect antibodies against the non-structural protein. It was found that around 52.5% of sheep samples and 25.4% of goat samples were positive. This indicates that the assay can be used for serological monitoring of antibodies to BTV. However, screening of more number of field serum samples of both large and small ruminants need to be carried out to further validate the tests.

Akin to other antibody detection ELISAs, rNS3Nt-ELISA is capable of detecting NS3 specific antibodies in all serotypes. Although, the current assay is standardized for testing sheep and goat serum samples, it would be equally suitable for testing sera from other susceptible animal species. Further, evaluation and validation of reported assay require screening of more number of serum samples from other susceptible animals. Conclusively, our preliminary results suggested that rNS3Nt-ELISA is able to detect BTV NS3 antibodies and can be used as a reliable tool for routine serological survey to detect BTV infection

in the animal population, irrespective of different viral serotype.

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