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Detection of infectious bursal disease virus (IBDV) antibodies using chimeric plant virus-like particles

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ABSTRACT

The aim of the present study is to use *Physalis mottle virus* (PhMV) coat protein (CP) as a scaffold to display the neutralizing epitopes of *Infectious bursal disease virus* (IBDV) VP2. For this, three different chimeric constructs were synthesized by replacing the N-terminus of PhMV CP with tandem repeats of neutralizing epitopes of IBDV VP2 and expressed in *Escherichia coli*. Expression analysis revealed that all the three recombinant chimeric coat protein subunits are soluble in nature and self-assembled into virus-like particles (VLPs) as evidenced through sucrose density gradient ultracentrifugation. The chimeric VLPs were characterized by various biochemical and biophysical techniques and found that they are stable and structurally sound. When the chimeric VLPs were used as coating antigen, they were able to detect IBDV antibodies. These results indicated that the chimeric VLPs can be used as potential vaccine candidates for the control of IBDV, which needs to be further evaluated in animal models.

1. Introduction

Infectious bursal disease (IBD) is an economically important disease of chickens worldwide and it is caused by *Infectious bursal disease virus* (IBDV) (Arega, 2018). IBDV attacks and destroys the developing B-lymphocytes in the bursa of Fabricius. IBDV is a small (~70 nm in diameter), non-enveloped icosahedral virus belonging to the genus *Avibirnavirus* in the family *Birnaviridae*. IBDV consists of bisegmented dsRNA (A and B) as genome and the larger segment A encodes a 110 kDa precursor protein in a single large open reading frame (ORF), which is cleaved by auto proteolysis to yield mature VP2, VP3 and VP4 structural proteins (Qin and Zheng, 2017). Among the structural proteins of IBDV, VP2 contains major molecular determinants for virulence and is responsible for neutralizing antibody response in the host (Pikuła et al., 2018).

IBD is endemic in India and control is possible only by means of vaccination. Determination of maternally derived antibody levels and monitoring of immune response before and after vaccination in the chickens are important along with the identification of virus antigen in case of infections (Besseboua et al., 2015). Commercial enzyme-linked immunosorbent assay (ELISA) kits and "in-house" ELISAs were developed using purified IBDV and recombinant full length or partial IBDV VP2 protein as coating antigens (Jackwood et al., 1996; Dey et al., 2009; Singh et al., 2010). Besides ELISA, lateral flow immunoassay (LFIA) has been developed as a rapid diagnostic tool for the detection of IBDV antibodies due to its sensitivity, specificity, low cost and quick detection (Nurulfiza et al., 2011).

Epitope mapping studies of IBDV VP2 revealed that many of the neutralizing epitopes have been mapped into a major immunogenic variable domain of VP2 (vVP2) (Azad et al., 1987; Yamaguchi et al., 1996). Within vVP2, four amino acid stretches are of critical importance to antigenicity and these are amino acid positions 210–225 (major peak A), 249–252 (minor peak 1), 281–292 (minor peak 2) and 313–324 (major peak B) (Bayliss et al., 1990; Heine et al., 1991). Mapping of VP2 conformational epitopes was carried out by phage-displayed heptapeptide library screening using synthetic overlapping peptides and the neutralizing linear B-cell epitopes on VP2 (197–209 aa, 210–225 aa and 329–337 aa) were identified (Cui et al., 2003; Wang et al., 2005). Using neutralizing epitopes of VP2 as diagnostic antigens may be advantageous due to their immunodominant nature and devoid of non-specific moieties that are present in whole protein preparations.

Physalis mottle virus (PhMV), a spherical plant tymovirus consisting of ssRNA genome of size 6.3 Kb encapsidated in an icosahedral shell of 180 coat protein (CP) subunits of size 21 kDa, arranged in T = 3

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symmetry. In earlier studies, the PhMV coat protein expressed in *E. coli* was shown to self-assemble into stable virus-like particles (VLPs or capsids) that were nearly identical to the native viruses formed *in vivo* (Sastri et al., 1997). Previously, PhMV coat protein was genetically engineered to display infection-related and immunodominant epitopes of *Foot and mouth disease virus (*FMDV*)*, *Canine parvovirus* (CPV), *Canine distemper virus* (CDV) and *Japanese encephalitis virus* (JEV) and the chimeric capsids were used as diagnostic and vaccine candidates (Hema et al., 2007; Chandran et al., 2009; Shahana et al., 2015). The objective of the present study is to display the neutralizing epitopes of IBDV VP2 on the surface of PhMV capsids and to demonstrate the use of chimeric VLPs to detect IBDV antibodies by indirect (ELISA) and (LFIA).

2. Materials and methods

2.1. Construction of wild-type and chimeric PhMV coat protein (CP) constructs

Wild-type PhMV CP gene of size 564 bp (GenBank Accession No: S97776) was used as a synthetic construct with *Nde* I and *Hind* III sites at 5′ and 3′ ends, respectively with a stop codon at the end of the reading frame (Jacob et al., 1992). A Kpn I site was introduced as silent mutation at 19 and 20 amino acids of the CP for the insertion of heterologous sequences. PhMV CP was cloned at Nde I and Hind III sites of the pRSET-A vector (Invitrogen, USA). Sequence confirmed (Bioserve Biotechnologies India Pvt. Ltd, Hyderabad) recombinant vector harboring wild-type coat protein gene was named as pR-Ph-CP. Three DNA constructs coding for tandem repeats of IBDV VP2 neutralizing epitopes (Heine et al., 1991; Cui et al., 2003; Wang et al., 2005), linked by GGS linker sequence of lengths 165, 153 and 132 nucleotides were made synthetically with Nde I site at 5′ end and Kpn I site at the 3′ end. These heterologous sequences were swapped in-frame into the Nde I and Kpn I digested pR-Ph-CP (Fig. 1). Plasmid DNA isolations were carried out by the alkaline lysis method (Sambrook and Russell, 2001). The inserts in the recombinant constructs were confirmed by automated DNA sequencing and were named as pR-Ph-IB1, pR-Ph-IB2 and pR-Ph-IB3.

2.2. Expression and purification of wild-type and chimeric PhMV virus-like particles (VLPs)

Competent *E. coli* BL21(DE3)pLysS cells (Invitrogen, USA) were transformed with recombinant vector constructs; pR-Ph-CP, pR-Ph-IB1, pR-Ph-IB2 and pR-Ph-IB3. The expression of recombinant constructs and purification of chimeric VLPs were carried out according to Sastri et al. (1997). Briefly, a single colony from the respective transformed plates were inoculated into 2 ml of Luria-Bertani (LB) broth medium containing ampicillin and chloramphenicol and grown at 30 °C overnight. 2% of the cultures were inoculated in 5 ml of LB medium and grown until the optical density reached 0.5 (A_{600}). Then the cultures were induced with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 30 °C for 4 h.

Total and soluble *E. coli* proteins were analyzed through 12% SDS-PAGE. For purification of chimeric VLPs, each of 1 l culture was induced by following the procedure as described above, cell pellets were suspended in 50 mM sodium citrate buffer (SCB) pH 5.5 and sonicated using Vibra-Cell sonicator (Sonics, USA) at 3 s pulse with 2 s interval for 30 min at 40% amplitude. The sonicated suspensions were centrifuged at 10,000 rpm for 30 min. The supernatants were then centrifuged at 28,000 rpm at 4 °C for 3 h using SW 32 Ti rotor (Beckman Coulter Inc.). The pellets were resuspended in SCB and layered onto a 10–40% linear sucrose gradient and centrifuged at 26,000 rpm at 4 °C for 3 h using SW 32 Ti rotor. The light scattering zones were collected with a Pasteur pipette, diluted with SCB, and centrifuged at 35,000 rpm at 4 °C for 3 h using Ti 70 rotor (Beckman Coulter Inc.). The final pellets were resuspended in SCB and stored at 4 °C. The protein concentrations were estimated using Bradford reagent (Sigma-Aldrich, USA).

2.3. Characterization of chimeric PhMV VLPs

2.3.1. Agarose and denaturing gel electrophoresis

Wild-type (WT) and chimeric VLPs (referred as IB1, IB2 and IB3) (10 µg per lane) were analyzed by 1% (w/v) agarose gel electrophoresis in TBE buffer (pH 8.3). Denatured coat protein subunits (10 µg per lane) were analyzed by 12% SDS-PAGE. Gels were photographed under white

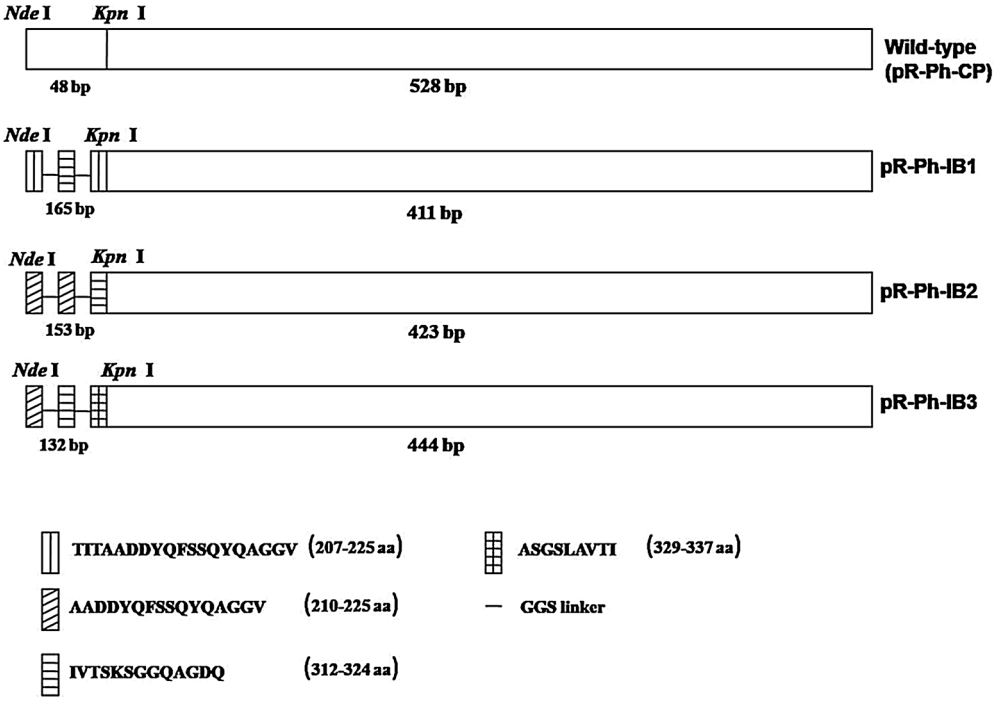

Fig. 1. Schematic representation of chimeric PhMV CP constructs pR-Ph-IB1, pR-Ph-IB2 and pR-Ph-IB3.
(Source of IBDV neutralizing epitopes: Heine et al., 1991; Cui et al., 2003; Wang et al., 2005)

light after staining with Coomassie Blue by using a Gel Imager system (Life Technologies, USA).

2.3.2. Electroblot immunoassay (EBIA)

Chimeric VLPs were resolved in 12% SDS-PAGE (Laemmli, 1970) and transferred onto nitrocellulose membrane (GE, Amersham pharmacia Biotech AB). WT PhMV VLPs and IBDV Georgia vaccine were used as negative and positive controls, respectively. Hyperimmune IBDV chicken serum in 1:250 dilution was used as primary antibody. Horseradish peroxidase (HRP) labeled rabbit anti-chicken Ig-G (Invitrogen, USA) in 1:2000 dilution was used as secondary antibody. The blot was developed using 3,3′-diaminobenzidine (DAB) and hydrogen peroxide (H_2O_2) in SCB.

2.3.3. Size exclusion chromatography

The purity of the wild-type and chimeric VLPs was analyzed by size exclusion chromatography using Sepharose 6 10/300 GL column on AKTA Purifier system (GE Healthcare Life Sciences, USA) by loading 100 µg of sample at a flow rate of 0.5 ml/min.

2.3.4. Dynamic light scattering (DLS)

The particle size distribution of wild-type and chimeric VLPs was analyzed using HORIBA scientific nanoparticle analyzer (Nanoparticle SZ-100) at 660 nm by keeping the scattered angle at 90°.

2.3.5. Transmission electron microscopy

Wild-type and chimeric VLPs were diluted to a concentration of 0.1 mg/ml in SCB and 10 µl of the samples were deposited onto formvar coated copper grids (SPI supplies) for 2 min at room temperature followed by washing twice with deionized water for 60 s. 2% (w/v) uranyl acetate (UAc) was used to negatively stain the samples for 45 s followed by a wash with deionized water for 30 s. FEI Tecnai transmission electron microscope was used for sample analysis at 120 kV at a magnification of 105000x.

2.4. IBDV cultivation and purification

Virus was propagated in primary culture of chicken embryo fibroblast (CEF) cells by inoculating 22nd passage of MB11 strain (GenBank Accession number: KM065458) of IBDV and allowed to adsorb for 45 min. The unadsorbed virus was washed with Dulbecco's modified Eagle's medium (DMEM) and maintenance medium [DMEM with 2% fetal bovine serum (FBS)] was added to the flask. The cell culture flask was incubated at 37 °C with 5% CO_2 for 72 h–96 h. When the cytopathic effect reached to 80–90%, the cells were harvested by three cycles of freezing and thawing. Then the cells were subjected to sucrose density gradient centrifugation using Beckman Coulter Optima XPN 100U centrifuge (USA) according to Das et al. (1997). Obtained IBDV purified virus was used as positive control in the indirect ELISA and LFIA tests.

2.5. Production of hyperimmune IBDV chicken serum

IBDV vaccine (Intermediate Plus strain type of SPF origin, Live Vaccine; Ventri Biologicals, Chennai) was administered intra-occularly into 35 days old chicken at two weeks interval for a total of 8 weeks and the serum was collected. The hyperimmune IBDV chicken serum was used to test the specificity of chimeric VLPs through indirect ELISA and LFIA.

2.6. Direct antigen coating-Enzyme-linked immunosorbent assay (DAC-ELISA)

The reactivity of chimeric VLPs (IB1, IB2 and IB3; 1 ng/50 µl) was determined using hyperimmune IBDV chicken serum (1:100 dilution) by DAC-ELISA according to Hobbs et al. (1987). The A_{405} values were determined for each sample in triplicates using ELISA plate reader (Bio-Rad, USA) and the graph was plotted by taking the average values. Purified IBDV was used as positive control and wild-type PhMV VLPs were used as negative control.

To further validate the chimeric VLP-based DAC-ELISA, 44 IBDV suspected chicken sera samples (U1-U16, S1-S25, N1, N9, and N10) were collected from commercial poultry farms in Namakkal, Tamil Nadu and DAC-ELISA was performed using the IB1, IB2 and IB3 at a concentration of 1 ng/50 µl. The diagnostic sensitivity (DSn) and diagnostic specificity (DSp) values along with the confidence intervals (CI) were determined by receiver-operating characteristic (ROC) curve analysis. The ROC analysis was performed using the GraphPad Prism 7.0 software and the results were compared with commercial IDEXX-ELISA kit.

2.7. Lateral flow immunoassay (LFIA)

LFIA was performed as per the manufacturer instructions (Sigma, USA). Colloidal gold (1 ml) solution was centrifuged at 17,000 rpm for 2 h at 4 °C. The pellet was suspended in residual 100 µl of the supernatant and the pH was adjusted to 9.0 with amine-free HEPES

(4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) buffer. Then 3 µl of dialyzed goat anti-chicken Ig-Y (100 µg/ml) (Sigma, USA) was added to the gold solution and vortexed gently for 5 min. Further, it was blocked with 10 µl of 10% BSA to get a final concentration of 1% BSA and vortexed gently for 30 min at room temperature. Finally, it was centrifuged at 8000 rpm for 1 h at 4 °C and the pellet was suspended in 100 µl of 20 mM HEPES buffer with 1% BSA. Purified IBDV was used as positive control and wild-type PhMV VLPs were used as negative control.

The membrane strips (sample pad, conjugate pad, nitrocellulose membrane and absorbent pad) were arranged from left to right (Supplementary data - S1) and the prepared colloidal gold conjugate (1:10) was added on to the conjugate pad at the appropriate site and air dried. Then each of the test antigens: IBDV, IB1, IB2 and IB3 (12 mg/ml) and hyperimmune IBDV chicken serum (1:10) were added at the test and control lines on the NC membrane. The immobilized strip was then allowed to air dry in a non-humid incubator at 37 °C for 5 min and the reactivity was observed by comparing with positive control. Fifteen IBDV suspected field chicken sera samples that are positive in IB2-ELISA were tested through LFIA using IB1 and IB2 as coating antigens.

3. Results

3.1. Production of chimeric PhMV VLPs in E. coli

The cloning of wild-type (WT) PhMV coat protein (CP) gene into pRSET-A vector and its expression resulted in a capsid protein of 21 kDa size, which self-assembled into VLPs in *E. coli* system. The chimeric gene constructs containing IBDV VP2 neutralizing epitopes arranged in tandem joined with GGS linkers were replaced at the N-terminus (between *Nde* I and *Kpn* I) of wild-type PhMV CP (Fig. 1). *E. coli* BL21(DE3) pLysS cells were transformed with wild-type and chimeric PhMV CP constructs (pR-Ph-CP, pR-Ph-IB1, pR-Ph-IB2 and pR-Ph-IB3) and 12% SDS-PAGE analysis revealed the high expression of recombinant chimeric coat proteins in the induced total protein fractions of *E. coli* (Fig. 2A). The total proteins were semi-quantitatively analyzed (ImageJ software) and the significant portion of recombinant chimeric proteins was found to be in the soluble fraction (87% IB1, 77% IB2 and 84% IB3) (Fig. 2B). No expression was observed in *E. coli* transformed with pRSET-A (negative control). The chimeric proteins were purified through sucrose density gradient centrifugation as described in the methods section. Light scattering zone in the sucrose linear density gradients (20–30%) indicated that the replacement of N-terminal region of PhMV CP with IBDV VP2 neutralizing epitopes did not affected the assembly of chimeric CP subunits into T = 3 VLPs (capsids).

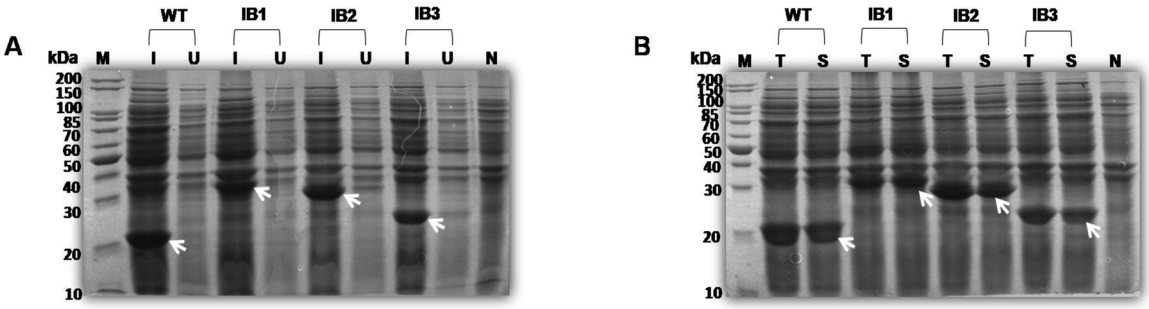

Fig. 2. Expression analysis of chimeric PhMV coat protein constructs by SDS-PAGE. **A.** Induced (I) and un-induced (U) protein fractions of *E. coli* transformed with WT: pR-Ph-CP, IB1: pR-Ph-IB1, IB2: pR-Ph-IB2, IB3: pR-Ph-IB3 and NC: negative control (pRSET-A vector). **B.** Induced total (T) and soluble (S) protein fractions of *E. coli* transformed with WT: pR-Ph-CP, IB1: pR-Ph-IB1, IB2: pR-Ph-IB2 and IB3: pR-Ph-IB3. Lane M represents standard molecular weight markers (Fermentas, USA).

3.2. Stability of chimeric PhMV VLPs

Characterization of the chimeric VLPs (referred as IB1, IB2 and IB3) was carried out using a combination of SDS-PAGE, agarose gel electrophoresis, electroblot immunoassay, size exclusion chromatography, dynamic light scattering and transmission electron microscopy. SDS-PAGE analysis of each of the chimeric VLPs showed a single band (Fig. 3A), indicating their homogeneity and the molecular weights of chimeric coat protein subunits of IB1, IB2 and IB3 are 27, 26 and 25 kDa respectively, while the wild-type coat protein corresponds to 21 kDa. Agarose gel electrophoresis revealed that the mobility of IB1, IB2 and IB3 was similar to wild-type PhMV VLPs (Fig. 3B). In EBIA, denatured chimeric VLPs and positive control (IBDV Georgia vaccine) showed reactivity with hyperimmune IBDV chicken serum, while WT PhMV CP did not showed any reaction (Fig. 3C), indicating the association of IBDV VP2 neutralizing epitopes with chimeric VLPs. Each of the chimeric VLPs were eluted as a single peak at 8 ml when passed through a Superose-6 column and this confirms that these VLPs were intact and stable (Fig. 3D). Electron microscopy analysis revealed the presence of spherical particles of diameter 30 (± 3.0) nm for IB1, 30 (± 4.0) nm for IB2, 31 (± 2.0) nm for IB3, indicating the sound nature of chimeric capsids similar to wild-type VLPs (28 ± 3.0 nm) (Fig. 3E). In accordance with the electron microscopy results, the particle size analysis by DLS at 660 nm showed mean size of IB1, IB2 and IB3 as 38 ± 10 nm, 29 ± 0.7 nm and 42 ± 12.8 nm, respectively (Supplementary data - S2 A–C), indicating no obvious difference in the size distribution.

3.3. Development of DAC-ELISA and LFIA

IBDV was isolated from chicken embryo fibroblast cells as described in methods section and the cytopathogenic changes were characterized by formation of multinucleated cells, rounding of cells and cell death (Supplementary data - S3 A–D).

The reactivity of IB1, IB2 and IB3 antigens with hyperimmune IBDV chicken serum was assessed by DAC-ELISA. The absorbance values that are 2 times higher than wild-type (negative control) were considered as positive and 3, 6 and 1 fold increase was observed while using IB1, IB2 and IB3 antigens, respectively when compared to purified IBDV (Fig. 4A). This indicated that the IBDV neutralizing epitopes were successfully displayed on the surface of PhMV capsid. Further, DAC-ELISA was validated using 44 IBDV suspected chicken sera samples (U1-U16, S1-S25, N1, N9, and N10) by comparing with IDEXX ELISA kit. Out of 44 chicken sera samples, 13, 15, 8 and 16 showed positive reaction with IB1, IB2, IB3 and purified IBDV antigens respectively, whereas 18 samples showed positive reaction in IDEXX ELISA kit (Supplementary data - Table S1). The sensitivity and specificity of chimeric VLP-based ELISA were determined through ROC curve analysis (GraphPad Prism 7.0 software). At the decided cut-off of > 0.2, the diagnostic sensitivity (DSn) of IB1, IB2 and IB3-based ELISA's were estimated to be 61% (95% CI: 35.75%–82.7%), 78% (95% CI: 52.36%–93.59%) and 39% (95% CI: 17.3–64.25%) respectively as compared to 89% for IDEXX ELISA kit (95% CI: 65.29%–98.62%). Similarly, the estimated diagnostic specificity (DSp) of IB1-ELISA was 100% (95% CI: 88.6%–100%), IB2-ELISA was 100% (95% CI: 88.6%–100%) and IB3-ELISA was 100% (95% CI: 87.6%–100%) as compared to 96% for IDEXX ELISA kit (95% CI: 81.65%–99.9%) (Fig. 4B).

LFIA was carried out with IB1, IB2 and IB3 as coating antigens and hyperimmune IBDV chicken serum. Positive result was indicated by a maroon line at the test and control line after incubation of the test strips. IB1 and IB2 showed a significant reaction with hyperimmune IBDV chicken serum when compared to IB3 (Fig. 5A). Wild-type VLPs and purified IBDV were taken as negative and positive controls respectively, where purified IBDV showed positive reaction with hyperimmune IBDV chicken serum (Fig. 5A). The test antigens did not showed any reaction with IBDV negative chicken serum (Fig. 5B). The sensitivity of LFIA was determined by testing 15 field chicken sera samples that were shown positive in ELISA. Among the 15 samples tested (supplementary data-Table S1), U1, U4 and U8 showed significant reaction with IB1 and IB2 (Fig. 5C). When compared to purified IBDV, IB1 and IB2 showed similar sensitivity to detect IBDV antibodies (data not shown).

4. Discussion

Fusion of small peptides to plant viral structural proteins is an important approach for the creation of novel vaccines for many animal and human viruses (Tissot et al., 2010; Tyler et al., 2014; Ye et al., 2013; Caldeira et al., 2015; Balke and Zeltins, 2018). The sites of insertion were chosen such that the resulting peptides will be displayed on the surface of virus-like particles (VLPs or capsids) (Grasso and Santi, 2010; Soria-Guerra et al., 2011). *E. coli* expressed fusion protein containing multi-mimotope peptides of IBDV VP2, which were identified by phage-displayed peptide library, reacted with IBDV antiserum (Wang et al., 2007). In another study, these peptides were presented onto the hepatitis B virus (HBV) VLPs and found that it is a promising approach for improving mimotope vaccines for IBDV (Wang et al., 2012). A highly variable region (207–350 aa) containing neutralizing epitopes of IBDV VP2 was displayed on the *Bamboo mosaic virus* (BaMV) coat protein and the chimeric VLPs can be used as sub-unit vaccines against IBDV (Chen et al., 2012).

In the present study, PhMV CP gene was modified through genetic engineering to display tandem repeats of neutralizing epitopes of IBDV VP2 and the chimeric CP genes were expressed in *E. coli* system. The chimeric PhMV CP subunits were found to be self-assembled to form T = 3 capsids (VLPs). Characterization of chimeric VLPs revealed their sound nature upon modification and EBIA analysis indicated the association of IBDV-specific epitopes with the chimeric VLPs. The stability of chimeric VLPs upon storage was confirmed through fast protein

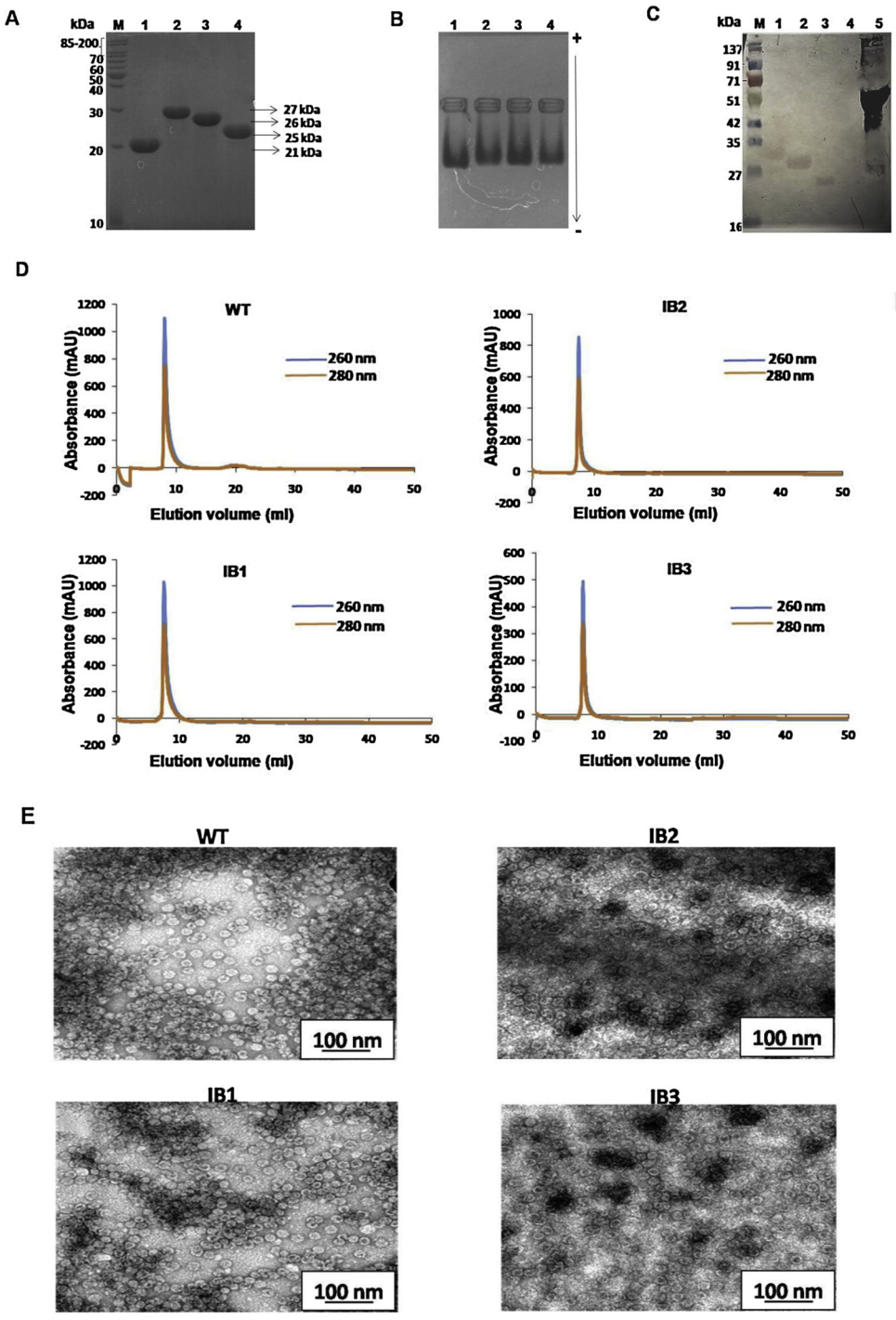

Fig. 3. Characterization of chimeric PhMV VLPs. **A**. SDS-PAGE analysis of 1-WT, 2-IB1, 3-IB2 and 4-IB3, visualized under white light after Coomassie blue staining. Lane M represents standard molecular weight marker (Fermentas, USA). **B**. Agarose gel electrophoresis of 1-WT, 2-IB1, 3-IB2 and 4-IB3 visualized under white light after Coomassie blue staining. **C**. Electroblot immunoassay of chimeric PhMV VLPs M- Prestained protein marker (Puregene); 1-IB1, 2-IB2, 3-IB3, 4-WT (negative control) and 5-IBDV Georgia vaccine (positive control). **D**. Size exclusion chromatograms of WT, IB1, IB2 and IB3 (monitored at 260 nm and 280 nm). **E**. Transmission electron micrographs of negatively stained (UAc) WT, IB1, IB2 and IB3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

liquid chromatography (FPLC) and they were found to be intact for one year in 50 mM sodium citrate buffer (SCB) pH 5.5 at 4 °C (Supplementary data - S4 A–D).

The reactivity of chimeric VLPs through DAC-ELISA indicated that IB2 (6 fold increase when compared to positive control) showed a higher reaction when compared to IB1 and IB3. Further, the DAC-ELISA test was validated using 44 IBDV suspected field chicken sera. The sensitivity and specificity of chimeric VLP-based ELISA was determined through ROC curve analysis and the results showed that the DSn of IB2 ELISA was higher (78%) than that of IB1 ELISA (61%) and IB3 ELISA (39%), which may be due to the presence of tandem repeats of highly immunogenic epitope covering 210–225 amino acids (Cui et al., 2003). The detection limit for antigen using IB2 ELISA was 1 ng/well, which is much less when compared to the antigen used in the tests that are developed in the earlier studies based on recombinant IBDV VP2 protein (0.5–1.5 mg/well, Dey et al., 2009; 100 ng/well, Pradhan et al., 2012). Since the chimeric VLPs are displaying the highly conserved epitope regions of IBDV VP2 in each of the CP subunit, where 180 subunits were accounted for the formation of VLPs, the polyvalent nature is an advantage and the developed chimeric VLP based DAC-ELISA is found to be highly specific and equally sensitive when compared to the IDEXX ELISA kit.

LFIA results demonstrated that among the three VLPs, IB1 and IB2 were more reactive than IB3 with hyperimmune IBDV chicken serum and they did not showed reaction with IBDV negative serum. Hence, these two VLPs were further used for testing the field chicken sera

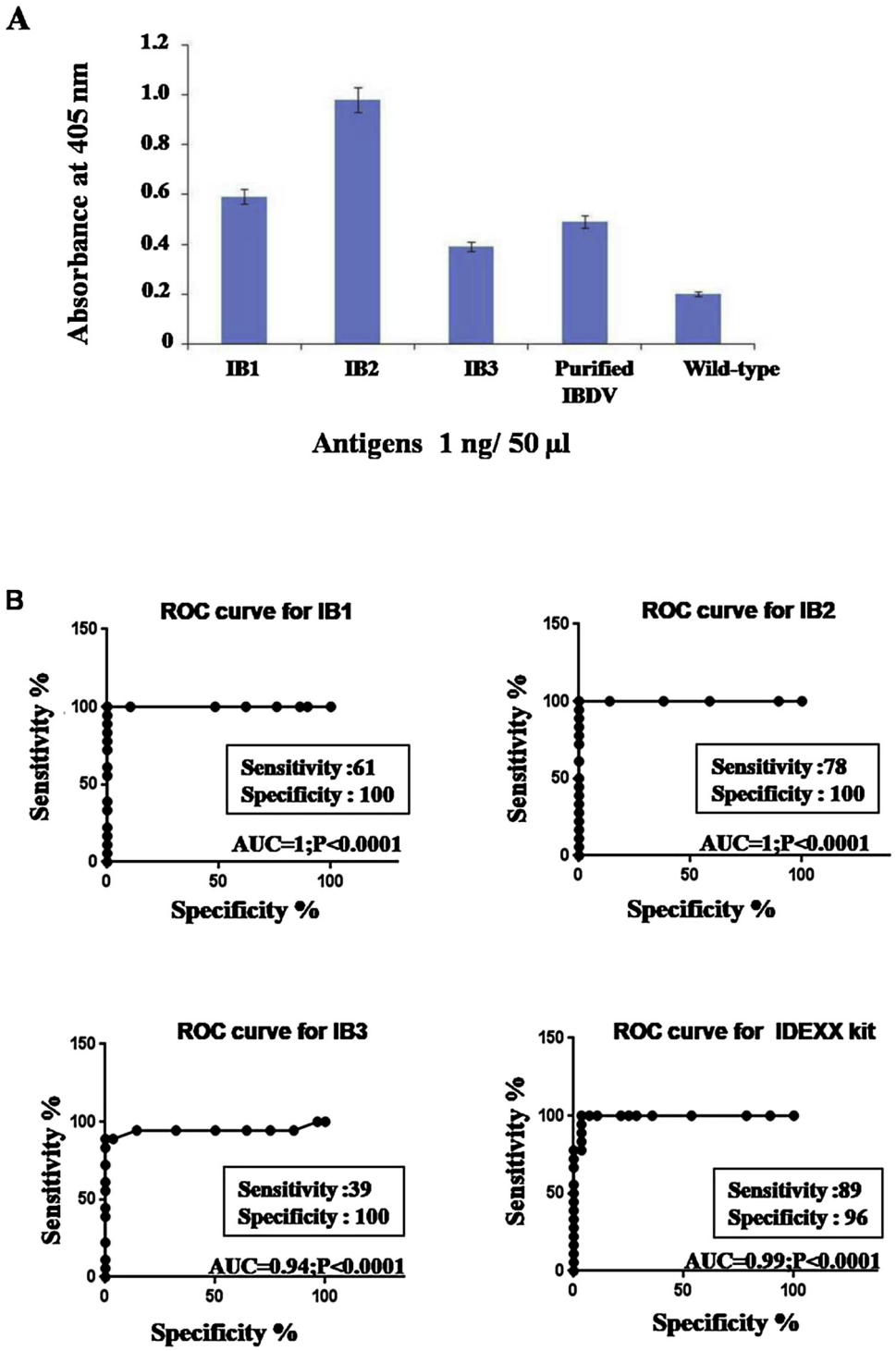

Fig. 4. DAC-ELISA analysis. **A**. Specificity of chimeric PhMV VLPs (IB1, IB2 and IB3), purified IBDV (positive control) and wild-type PhMV VLPs (negative control) using hyperimmune IBDV chicken serum. **B**. Validation of VLP-based DAC-ELISA using field chicken sera samples in comparison with commercial IDEXX ELISA kit. Each point on the ROC plot represents a sensitivity-specificity pair corresponding to a particular decision threshold.

samples that are positive in ELISA and the results indicated that IB1 and IB2 showed positive reaction with U1, U4 and U8 samples similar to purified IBDV. Immuno chromatographic strip test that was developed earlier by Nurulfiza et al. (2011) was able to detect all the 6 IBDV samples that were shown positive reaction in ELISA and hence the sensitivity was found to be 100%. In our study, work is under progress to improve the sensitivity of chimeric VLP-based LFIA by optimizing the concentration of reagents and by using combination of mixed chimeric VLPs.

5. Conclusion

In conclusion, we were successful in displaying IBDV epitopes on the surface of PhMV VLPs. Further, chimeric VLP-based ELISA and LFIA tests have the potential to screen IBDV sera samples collected from commercial poultry farms. Preparation of chimeric VLP antigens is simple, cost-effective and can be obtained in large amounts (15–20 mg/ litre of Luria-Bertani medium) and can be easily scaled up by using fermenters. The chimeric VLPs are polyvalent, non-infectious, non-

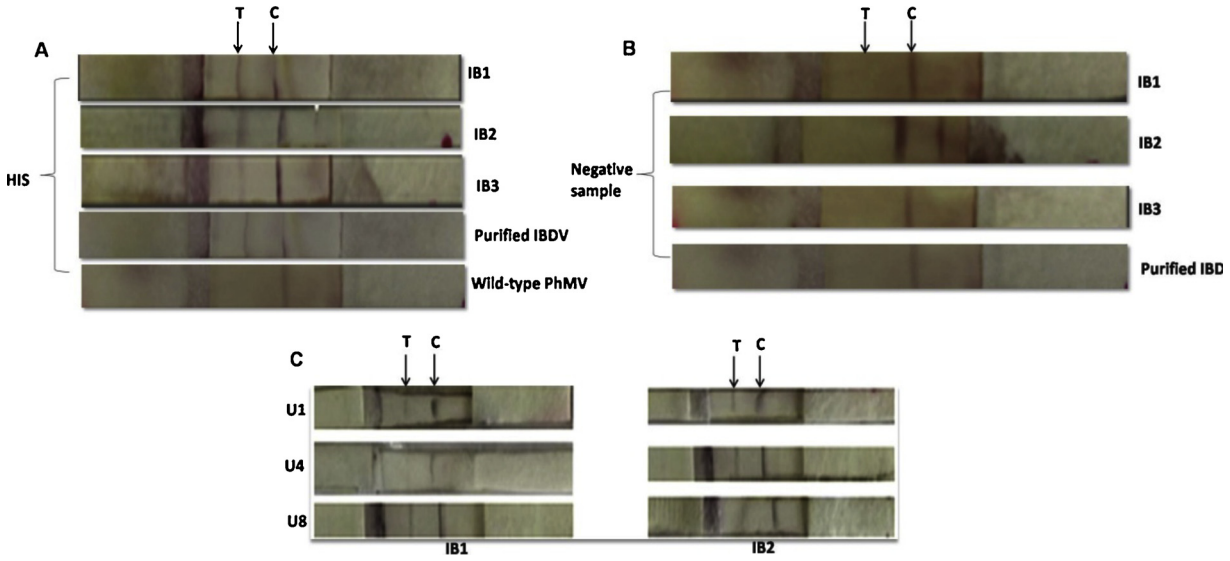

Fig. 5. Lateral flow immunoassay using chimeric PhMV VLPs **A**. Positive reaction indicated by two maroon lines at the test and control lines by employing IB1/IB2/IB3/purified IBDV/wild-type PhMV VLPs at the test line using hyperimmune IBDV chicken serum. T and C represent test line and control line respectively. **B**. Negative reaction indicated by no color at the test line by employing IB1/IB2/IB3/purified IBDV at the test line using IBDV negative chicken serum. **C**. Positive reaction with IB1 and IB2 antigens at the test line using U1, U4 and U8 field chicken sera samples.

toxic, non-hazardous, biodegradable and monodisperse (no batch to batch variation). The stability of VLPs is an added advantage to maintain the shelf-life of reagents. Chimeric VLP-based DAC-ELISA can be used for large-scale screening of IBDV suspected sera samples from the commercial poultry farms in a cost-effective manner. LFIA test is expensive when compared to ELISA, but it can be used for the on-site quick detection. The sensitivity of chimeric VLP based LFIA can be improved by optimizing the antigen/antibody concentrations and using mixed antigens.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.12.008.

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