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Effect of silver nanoparticles on antibody response against recombinant VP2 protein of Infectious bursal disease virus

Sudhakar Awandkar*, Prabhakar A. Tembhurne, Nitin V. Kurkure, Sandeep P. Chaudhari, Sachin W. Bonde & Vijay C. Ingle

[1]Department of Veterinary Microbiology; [2]Department of Veterinary Pathology;
[3]Department of Veterinary Public Health; [4]Department of Veterinary Biochemistry, Nagpur Veterinary College, Nagpur – 440 006, Maharashtra, India

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A strong humoral response against Infectious bursal disease virus (IBDV) in breeder chickens transfers protective maternal immunity in chicks, and protect them from the disease. The present study explains the modulatory effect of silver nanoparticles on humoral immune response against recombinant VP2 (rVP2) protein of IBDV. The hypervariable VP2 gene segment of field IBDV was amplified and cloned into pGEM-T Easy plasmid followed by subcloning into pET32a plasmid vector. Truncated rVP2 protein expressed in *E. coli* BL32DE3 cells, showed reactivity with specific anti VP2 chicken antibodies. The results of immunoblot revealed its utility in serological diagnosis. The rVP2 protein was evaluated for immunogenic potential by vaccinating the chickens with and without silver nanoparticles (AgNP). The titers induced by rVP2 protein blended with montanide oil were non-significant when compared with titers induced by the conventional vaccines. The IgY response was highly significant in chickens vaccinated with rVP2 protein blended with montanide oil and AgNP than in chickens vaccinated with conventional vaccines or rVP2 protein in montanide oil without AgNP. The results represent Infectious bursal disease virus rVP2 protein as a promising candidate for the differentiation of vaccinated versus infected and sero-diagnostic tools. The current study elucidated the adjuvanticity effect of AgNP on IBDV rVP2 protein potency for the first time.

Keywords: AgNP, VP2, Montanide oil, IgY

The Infectious bursal disease (IBD) is a highly contagious disease of chickens[1,2] and transient immuno-suppression and decreased productivity due to the infection result in substantial direct and indirect economic losses to the poultry industry worldwide[3-5]. The conventional live vaccines derived of *Infectious bursal disease virus* (IBDV) have better efficacy[6]. However, these vaccines induce moderate to severe bursal lesions leading to mild immunosuppression with low mortality[7]. Increased morbidity and mortality were documented in vaccinated flocks indicating that these vaccines may not fully protect chickens from very virulent IBDV[8]. The reports suggested that conventional vaccines do not offer complete protection against new IBDV variants[9,10]. Thus, the safety and efficacy of these vaccines remain a significant concern[11].

The structural VP2 protein is considered the best candidate for developing diagnostics and subunit vaccines as it induces a specific immune response in chickens[12]. The virus neutralizing humoral immune response is generated against this protein[8]. Therefore, it is considered for the development of recombinant vaccine candidates. A limited number of antigenic determinants can trigger the B and T cell responses, and the epitopes present in VP2 protein produce virus-neutralizing antibodies[13]. Two conformational virus-neutralizing epitopes were suggested and mapped in the 204- 359 amino acids on VP2 protein[14]. The recombinant vaccine candidates are considered to be weak immunogens in producing antiviral responses. Therefore, adjuvants are used to enhance their immunogenicity. An ideal adjuvant should stimulate a long-term immune response with minimum side effects. Various adjuvants are used in veterinary vaccines. The newer developments in metal nanotechnology exhibited a variety of different applications. The nanoparticles can trap proteins[15]. Therefore, they can be used for antigen delivery and immune system stimulation by targeting antigen-presenting cells (APC). Antigen presentation to the APCs is an essential issue in the improvement of

*Correspondence:
Phone: +91 95033 97929 (Mob.)
E-Mail: drspawandkar@gmail.com

vaccine potency. The nanoparticles have been widely used in antigen delivery[15]. Recent studies showed the adjuvanticity of silver, gold, polylactide-co-glycolide, and calcium phosphate nanoparticles in enhancing the immunogenicity of antigens[12,16]. Despite other applications of nanoparticles, they are used as immune potentiators. Moreover, few recent studies elucidated the immunomodulatory effects of silver nanoparticles (AgNPs) on the immune response to weaker antigens like albumin and Rabies veterinary vaccine[17,18].

The safety and efficacy of existing live IBD vaccines and weaker immune response to a recombinant immunogen need to be addressed. The immunomodulatory effects of nanoparticles may modulate host immune responses to the recombinant immunogens. Therefore, in the present study, we assessed the potential role of the AgNPs to enhance the humoral immune response against truncated recombinant VP2 protein (rVP2) of IBDV and its diagnostic potential.

Materials and Methods

Extraction of viral dsRNA

The bursal samples were aseptically collected from broiler birds exhibiting Infectious bursal disease (IBD) from central India. The bursa of Fabricius was frozen at −80°C and minced in fine powder to obtain 10% suspension. The clarified suspension was subjected to total RNA extraction using TRI reagent (Sigma Aldrich, USA) following the manufacturer's instructions. The resultant RNA pellet was resuspended in 200 µL DEPC treated water. Lithium chloride and 3M Sodium acetate (Sigma Aldrich, USA) precipitation was followed to extract viral dsRNA[19]. The RNA pellet was resuspended in 25 µL of 1X TE buffer and quantified using nanodrop 1000 (Eppendorf, Germany).

Amplification of hypervariable segment of VP2 gene

The cDNA was synthesized using a Superscript TM III First-Strand Synthesis kit (Invitrogen USA). The polymerase chain reaction was set up in 50 µL volume (5.0 µL of 10X PCR buffer, 1.5 µL of 50 mM $MgCl_2$, 1.0 µL of 10 mM dNTPs, 2.0 µL of each ten pmol primers and 1.0 unit of Taq DNA polymerase (Promega, USA). The F 5'- ACT GTC CTC AGC TTA CCC ACA T-3' and R 5'- TCT GTG ACC AGG TTC TTT GCT A-3' primers specific to a 678 bp VP2 gene segment, consisting of the hypervariable region, were used for amplification[2]. The amplification was carried out in 35 cycles at an annealing temperature of 49°C with a final extension at 72°C for 10 min. The amplicon was sequenced using commercial sequencing services (Eurofins, India). The sequence was deposited in GenBank (Accession No. MK172062). The forward and reverse nucleotide sequences were subjected to base call in Chromas software. The reverse sequence was reverse complemented and analyzed in the NCBI BLASTn interface. The homologous deduced amino acid sequences of the IBDVs from India and other countries were retrieved from GenBank. These sequences were aligned with the deduced amino acid sequence of the IBDV isolate.

Cloning of VP2 gene segment

The *EcoR*I (Fermentas, USA) digested VP2 amplicon was cloned into pGEM-T Easy vector (Promega, USA) using T4 DNA ligase (Promega, USA). The JM109 *Escherichia coli* cells were transfected with a recombinant plasmid (50 ng) and plated onto the Luria-Bertani agar (Sigma, USA) with 5-bromo-4-chloro-3-indyol β-D galactoside (X-Gal, 100 µg/mL), isopropylthio-β-galactoside (IPTG, 40 µg/mL), and ampicillin (50 µg/mL)[19]. The plates were incubated at 37°C for 48 h. The transformed bacteria with self-ligated vector produced blue colonies and were eliminated. The transformed bacteria with recombinant pGEM-T-Easy plasmid had white colonies. The colonies were inoculated into Luria-Bertani broth containing ampicillin (50 µg/mL). The recombinant plasmid DNA was extracted from the overnight grown cultures using QIA Prep plasmid isolation kit (Qiagen, Germany). The recombinant plasmid was subjected for confirmation of the insert by restriction digestion.

Sub-cloning of VP2 gene segment

The agar gel-purified VP2 gene insert (12 ng/µL) from recombinant pGEM-T Easy plasmid was isolated after *EcoR*I digestion. The insert was ligated into *EcoR*I restriction sites of pET32a (40 ng/µL, Novagen, USA) plasmid. The recombinant plasmid DNA was transformed into BL32DE3 *Escherichia coli* cells[19]. The insert orientation in recombinant clones was determined by restriction endonuclease digestion using *BamH*I (Fermentas, USA).

Expression and purification of rVP2

The rVP2 protein was expressed into a prokaryotic expression system using BL21DE3 *E. coli* cells[19]. The overnight grown culture (100 µL) of a single transformed colony was transferred to 10 mL LBAmp

broth in a 100 mL conical flask. The flask was incubated in an orbital shaker incubator. The expression was induced at the mid-exponential phase with one mM IPTG for 3 h at 37°C. Bacterial pellets were resuspended and boiled in LaemmLi buffer for five minutes. The expression of rVP2 protein was confirmed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). Large-scale protein expression was carried out at an optimum concentration of 0.5 mM IPTG for 6 h. The bacterial pellet was resuspended in a binding buffer and subjected to three cycles of sonication at 5 µM amplitude for 10 s pulse each in an ice bath with 30 s gap between each cycle. The slurry was centrifuged at 10000 g for 30 min at 4°C. The supernatant was subjected to the purification of rVP2 protein using charged Ni-NTA resin (Thermo, USA). The Ni-NTA resin column was equilibrated by adding a five-column volume of equilibration buffer with one mL per minute flow rate. The supernatant from the above step was loaded onto the column, and flow-through was collected. The washing was done with a 10-column volume of washing buffer at one mL per minute flow rate. The protein was eluted with a 5-column volume of elution buffer. The rVP2 protein was quantified by the Bradford method.

Immunoblot

The purified rVP2 protein (3 µL) was dotted onto nitrocellulose membrane and allowed to bind for an hour at 37°C. The membrane was blocked in 5% skimmed milk for 30 min. The membrane was immersed in 1:400 diluted primary chicken antibodies (pooled serum from rVP2 vaccinated birds, pooled serum from birds vaccinated with the commercial vaccine and IDVet anti-rVP2 IgY from ELISA kit) for 30 min. Anti-chicken horse reddish peroxidase conjugate (Sigma, USA) was allowed to react for half an hour. The membrane was subjected for spot development in 3'3' Diaminobenzidine (0.3%, Sigma, USA) and hydrogen peroxide (0.6%) for 10 min.

Immunogenicity of rVP2

Purified rVP2 protein (50 µg/dose) was blended with adjuvant grade montanide oil (1:2, Sigma, USA), and montanide oil with AgNP (20 mg/kg body wt., particle size 50 to 80 nm, zeta potential –21.03 + 2.46 mV, Sigma, USA). The blends showing no growth on bacteriological and mycological media were used for further study. The ethical approval was obtained from Institutional Animal Ethical Committee vide letter No. NVC/IAEC/3769/2018 dated 25/01/2018.

One day-old chicken birds were randomly divided into seven groups consisting of 20 birds per group. Sera were randomly collected from 12 birds/group before vaccination. The birds were vaccinated (subcutaneous route) with the above blends keeping placebo as negative control and commercial vaccines (IBD Ventri and Ventri plus) as a positive control. Each preparation was evaluated with and without a booster dose. Post-vaccination sera were collected on days 7, 14, 21 and 28 (Fig. 1). The sera were

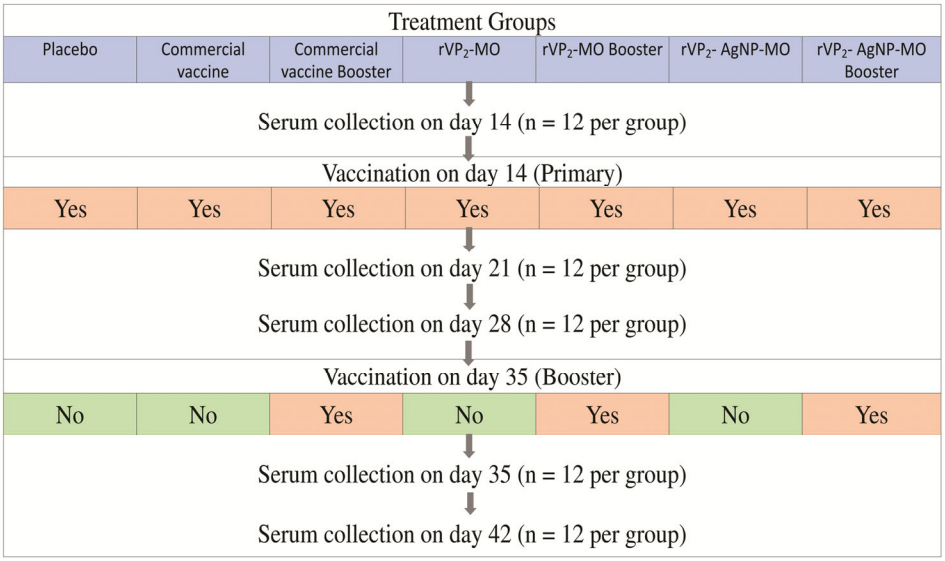

Fig. 1 — Experimental design for testing rVP_2-MO and rVP_2-AgNP-MO immunogen preparations [CV: Commercial vaccine; CV-B: Commercial vaccine booster; rVP_2-MO: truncated rVP_2 protein blended with montanide oil; rVP_2-MO-B: truncated rVP_2 protein mixed with montanide oil booster; rVP_2- AgNP-MO: truncated rVP_2 protein mixed with AgNP and montanide oil; and rVP_2- AgNP-MO-B: truncated rVP_2 protein blended with AgNP and montanide oil booster]

subjected to IBDV rVP2 ELISA to detect IgY (IDVet, France) following the manufacturer's instructions. The OD was taken at 450 nm in an ELISA reader (BioTech, India). The results were interpreted by calculating the S/P ratio and antibody titer. The ELISA antibody titer greater than 1324 was taken as positive as indicated by the manufacturer.

The heat-inactivated sera were subjected to a virus neutralization test (VNT) as per OIE guidelines. In brief, the serum (in triplicate) was twofold diluted with 100 TCID50 CEF adapted IBDV in 96 well tissue culture plates in 50 µL volume and incubated at 37°C for 30 min. The CEF cells were adjusted to 10^6 cells/mL and added to the wells (200 µL/well). The plates were sealed and incubated at 37°C in a CO_2 incubator. The cytopathic effects were recorded after 72 h. The reciprocals of the highest dilution of serum showing no cytopathic effects were taken as the virus-neutralizing titers.

Statistical analysis

The VNT titers were converted to log2 values. Mean ELISA/VNT titers and SE of each group were calculated. The one-way analysis of variance analyzed antibody titers between groups. The titers at different time intervals in each group were analyzed by repetitive measures analysis of variance using IBM SPSS-20 software.

Results

Amplification VP2 gene and construction of recombinant plasmid

The RT-PCR amplified 664 bp nucleotide sequence specific to the VP2 gene hypervariable region from position 548 to 1248 on segment A of the IBDV genome. The homology of deduced amino acid sequence with other IBDV sequences is shown in Fig. 2.

The VP2 gene segment (aa 178-385) was ligated into *EcoR*I restriction sites of the pGEM-T Easy vector. The recombinant plasmid was transformed

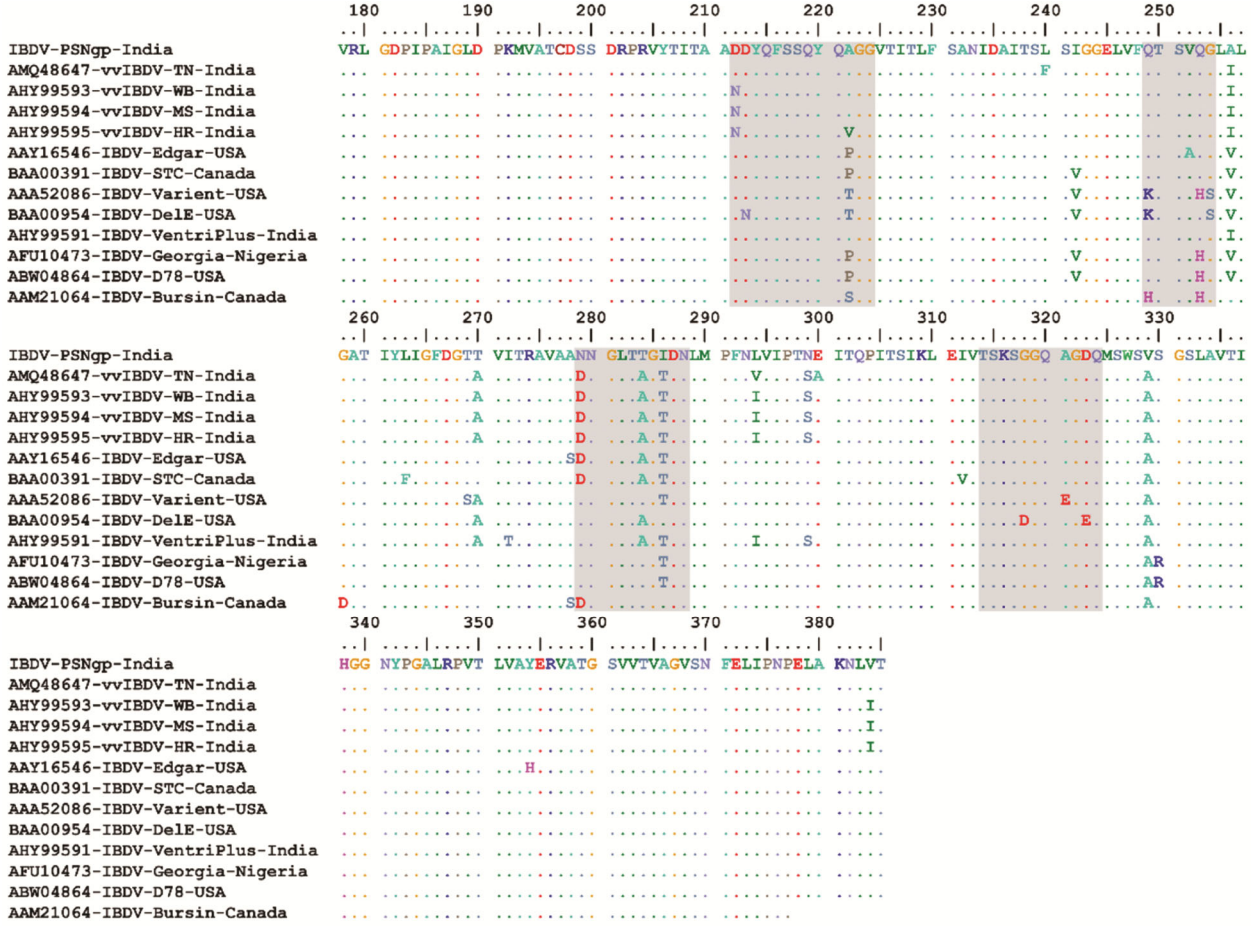

Fig. 2 — Alignment of deduced amino acid sequences for the hypervariable region of VP2 gene of very velogenic, classical, variant, and vaccine strains of IBDVs. [Dots and differences by single letter show identity. The major and minor hydrophilic peaks are highlighted, Virulent: TN, WB, MS, HR; Classical: Edgar, STC, Georgia, D78; Variant: Variant, DelE, Vaccine: VentriPlus, Bursin]

into JM109 *E. coli* cells. The plasmid DNA was extracted and digested with *Eco*RI. The excised VP2 gene insert was subcloned into *Eco*RI restriction sites of dephosphorylated pET32a expression vector. BL21DE3 *E. coli* cells were transfected with this recombinant pET32a plasmid. The recombinant pET32a plasmid yielded two fragments of 6336 bp and 64 bp on endonuclease digestion using *BamH*I. The digestion patterns showed correct insert orientation in the expression vector (Fig. 3).

Expression, purification, and characterization truncated rVP2

The truncated rVP2 protein of IBDV was expressed into a prokaryotic expression system under the control of the T7 promoter. The induced clones were lysed, and rVP2 was purified from cell-free supernatant using His tag purification. The Ni-NTA purification yielded rVP2 protein with a concentration of 1600 µg/mL. The induction and purification of rVP2 protein were monitored by SDS-PAGE (Fig. 4). The recombinant clones expressing a specific 19 kDa protein were selected for further evaluation. The immunoblot identified the expressed protein as IBDV rVP2 with no specific reaction in negative controls (Fig. 5), indicating that the protein is expressed correctly and has a reaction with chicken anti-IBDV antibodies.

Humoral response rVP2 protein blends

The mean serum antibody titers are shown in Table 1. The results showed that the commercial vaccines and rVP2 protein blend preparations induced significant (P <0.01) titers after the 14th day of vaccination compared to the titers on the 0 and 7th days. The titers of birds from the placebo group were non-significant throughout the experiment. The birds vaccinated with the commercial vaccine showed a significant decrease in the serum IgY titers on the 7th day post-vaccination. The titers on the 7th day post-vaccination were low in all the treatment groups. However, from the 14th day onwards, an exponential and significant increase (P <0.01) in serum IgY level was recorded till the 28th day post-vaccination. Similar trends were observed for all the treatment groups. The birds from groups vaccinated with rVP2-montanide oil showed

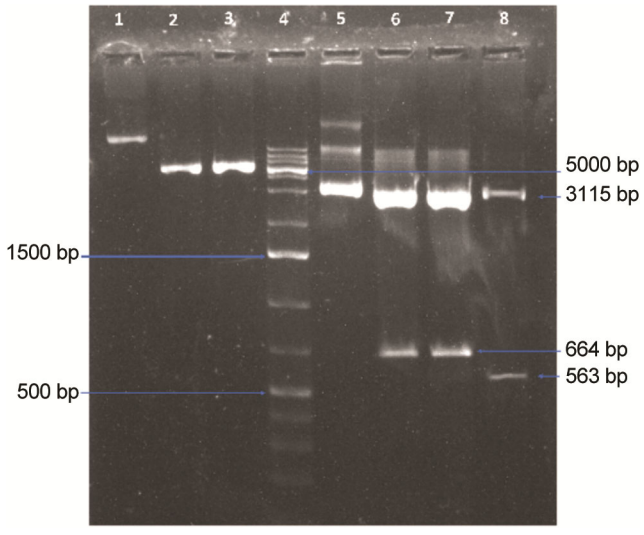

Fig. 3 — Restriction digestion of pET32a prokaryotic expression vector and pGEM-T Easy-VP2 recombinant cloning vector [Lane 1: Undigested pET32a; Lane 2 & 3: *Eco*RI digested pET32a; Lane 4: DNA Marker (1 kbp plus); Lane 5: Undigested pGEM-T Easy VP2; Lane 6 & 7: *Eco*RI digested pGEM-T Easy-VP2; and Lane 8: *Pst*I digested pGEM-T Easy-VP2]

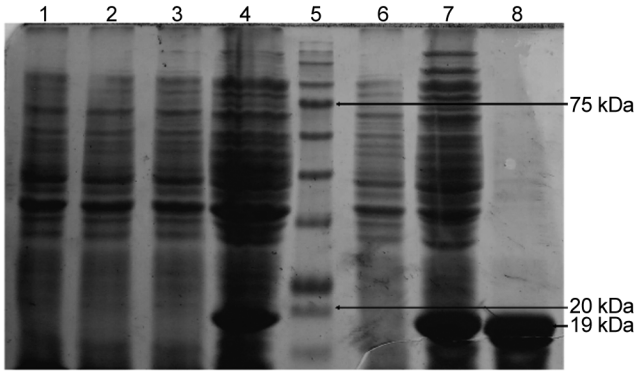

Fig. 4 — Expression and purification of rVP2 [Lane 1: BL21DE3 cell lysate; Lane 2: pET32a transformed BL21DE3 cell lysate induced with 0. 50 mM IPTG; Lane 3: pET32a-VP2 transformed BL21DE3 uninduced; Lane 4: pET32a-VP2 transformed BL21DE3 induced with 0. 50 mM IPTG; Lane 5: Protein Marker; Lane 6: Pellet after sonication; Lane 7: Supernatant after sonication; and Lane 8: Ni-NTA purified rVP2]

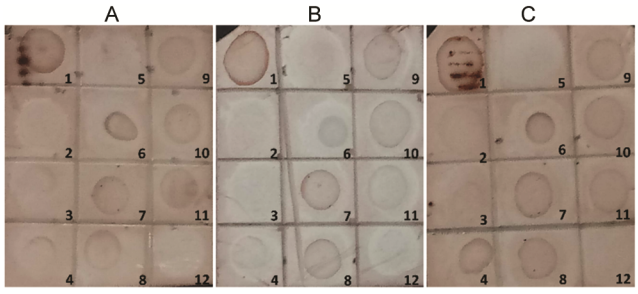

Fig. 5 — Dot blot showing reactivity of rVP2 with polyclonal and anti rVP2 antibodies (A) Pooled serum from rVP2 vaccinated birds; (B) Pooled serum from birds vaccinated with the commercial vaccine; and (C) Anti-rVP2 antibodies (IDVet), 1: Purified rVP2, 2: Uninduced BL21DE3 cell lysate, 3: IBDV infected cell culture supernatant, 4: Commercial IBD vaccine, 5: pET32a transformed BL21DE3 cell lysate induced with IPTG, 6: Conjugate at a working concentration from the kit as a positive control, 7: rVP2 + 7 M Urea (4:1), 8: rVP2 + 7 M Urea (3:2), 9: rVP2 + 7 M Urea (2.5:2.5), 10: rVP2 + 7 M Urea (2:3), 11: rVP2 + 7 M Urea (1:4), and 12: negative control]

Table 1 — Mean serum IgY titers induced by commercial vaccines and rVP_2-MO and rVP_2- AgNP -MO immunogen preparations

Treatments		Placebo	CV	CV-B	rVP_2-MO	rVP_2-MO-B	rVP2-MO-AgNP	rVP2-MO-AgNP-B	F	*P*
Age of the chickens	14th day	84.00 ± 32.37$^{NS/\overline{NS}}$	86.75 ± 28.86$^{d/\overline{NS}}$	85.42 ± 30.17$^{d/\overline{NS}}$	78.25 ± 28.12$^{c/\overline{NS}}$	84.00 ± 22.87$^{c/\overline{NS}}$	78.58 ± 21.63$^{d/\overline{NS}}$	87.58 ± 29.06$^{d/\overline{NS}}$	0.018	1.000
	21st day	116.50 ± 31.78$^{NS/\overline{NS}}$	74.83 ± 36.22$^{d/\overline{NS}}$	71.67 ± 39.81$^{d/\overline{NS}}$	126.83 ± 45.44$^{c/\overline{NS}}$	136.00 ± 49.35$^{c/\overline{NS}}$	25.17 ± 19.19$^{d/\overline{NS}}$	66.92 ± 29.89$^{d/\overline{NS}}$	1.141	0.347
	28th day	33.83 ± 17.92$^{NS/\overline{C}}$	2313.08 ± 152.77$^{c/\overline{B}}$	2315.42 ± 46.21$^{c/\overline{B}}$	2489.83 ± 79.17$^{b/\overline{AB}}$	2473.25 ± 83.53$^{b/\overline{AB}}$	2554.33 ± 97.25$^{c/\overline{AB}}$	2671.33 ± 108.91$^{c/\overline{A}}$	100.158	0.000
	35th day	84.17 ± 49.47$^{NS/\overline{E}}$	3493.00 ± 178.09$^{b/\overline{C}}$	5114.00 ± 48.61$^{b/\overline{A}}$	2920.25 ± 103.72$^{b/\overline{D}}$	2970.42 ± 302.24$^{b/\overline{D}}$	3942.92 ± 153.73$^{b/\overline{B}}$	4946.67 ± 70.86$^{b/\overline{A}}$	115.684	0.000
	42nd day	65.58 ± 31.34$^{NS/\overline{E}}$	12009.33 ± 375.67$^{a/\overline{D}}$	14557.17 ± 380.11$^{a/\overline{C}}$	11912.67 ± 415.89^{aD}	14071.92 ± 416.14$^{a/\overline{C}}$	16374.25 ± 347.89$^{a/\overline{B}}$	17845.83 ± 344.00$^{a/\overline{A}}$	274.818	0.000
	F	0.856	609.955	1549.363	663.683	635.43	1550.72	1900.854		
	P	0.498	0.000	0.000	0.000	0.000	0.000	0.000		

[Treatments found significant at P <0.01 (a-d: significance between days; A-E: significance between treatments; NS: non-significant. CV: Commercial vaccine; CV-B: Commercial vaccine booster; rVP_2-MO: truncated rVP_2 protein blended with montanide oil; rVP_2-MO-B: truncated rVP_2 protein mixed with montanide oil booster; rVP_2-MO- AgNP: truncated rVP_2 protein mixed with AgNP and montanide oil; rVP_2-MO- AgNP-B: truncated rVP_2 protein blended with AgNP and montanide oil booster]

Table 2 — Mean virus neutralizing titers (log2) induced by commercial vaccines and rVP_2-MO and rVP_2- AgNP -MO immunogen preparations

Treatments	Age (days) of chickens					P value
	14	21	28	35	42	
Placebo	6.3219±0.13$^{NS/A}$	4.9175±0.12$^{NS/B}$	0.1174±0.09$^{d/C}$	0.0000$^{d/D}$	0.0000$^{e/D}$	0.0000
CV	6.5025±0.14$^{NS/D}$	4.9386±0.08$^{NS/E}$	9.9798±0.02$^{c/C}$	11.1876±0.09$^{b/B}$	12.5305 ±0.13$^{cd/A}$	0.0000
CV-B	6.4150±0.14$^{NS/D}$	4.9175±0.08$^{NS/E}$	10.1520±0.06$^{bc/C}$	11.7000±0.14$^{a/B}$	13.0000 ±0.11$^{bc/A}$	0.0000
rVP_2-MO	6.2563±0.11$^{NS/C}$	4.8524±0.11$^{NS/D}$	10.4150±0.08$^{ab/B}$	10.7370±0.11$^{c/B}$	12.0000 ±0.14$^{d/A}$	0.0000
rVP_2-MO -B	6.3219±0.12$^{NS/D}$	4.9594±0.03$^{NS/E}$	10.3847±0.09$^{ab/C}$	11.0000±0.12$^{c/B}$	12.7370 ±0.14$^{bc/A}$	0.0000
rVP2-MOAgNP	6.2563±0.11$^{NS/D}$	4.9386±0.05$^{NS/E}$	10.5305±0.13$^{a/C}$	11.3219±0.12$^{b/B}$	13.0000 ±0.08$^{ab/A}$	0.0000
rVP2-MOAgNP-B	6.4448±0.14$^{NS/D}$	4.8524±0.11$^{NS/E}$	10.6374±0.13$^{a/C}$	12.0000±0.14$^{a/B}$	14.0000 ±0.03$^{a/A}$	0.0000
P value	0.7978	0.8113	0.0000	0.0000	0.0000	

[Treatments found significant at P <0.01 (A-E: significance between days; a-e: significance between treatments; NS: non-significant. CV: Commercial vaccine; CV-B: Commercial vaccine booster; rVP_2-MO: truncated rVP_2 protein blended with montanide oil; rVP_2-MO-B: truncated rVP_2 protein mixed with montanide oil booster; rVP_2-MO- AgNP: truncated rVP_2 protein mixed with AgNP and montanide oil; rVP_2-MO- AgNP-B: truncated rVP_2 protein blended with AgNP and montanide oil booster]

significantly increased but low titers on the 7th day post-vaccination. On the 14th day, a significant increase in the serum antibody titers was recorded in the group vaccinated with rVP2- montanide oil, commercial vaccine, and rVP2-montanide oil-AgNP compared with the placebo group. On the 21st day, serum antibody titers were more significant in the group vaccinated with commercial vaccine booster followed by rVP2-montanide oil-AgNP booster, rVP2-montanide oil-AgNP, commercial vaccine, rVP2-montanide oil booster, and rVP2-montanide oil. The non-significant increase was recorded in serum IgY titers in groups vaccinated with rVP2-montanide oil (both primary and booster) on the 21st day compared with the respective titers on the 14th day. On the 28th day, a significant increase in the serum antibody titers was recorded in the group vaccinated with rVP2-montanide oil-AgNP booster followed by rVP2-montanide oil-AgNP, commercial vaccine booster, rVP2-montanide oil booster, commercial vaccine, and rVP2-montanide oil.

The virus neutralizing titers are shown in Table 2. Non-significant titers were recorded on the 1st and 7th days post-vaccination. Significantly high titers (P <0.01) were recorded in all the treatment groups compared to placebo on and after the 14th day post-vaccination. On the 28th-day post-vaccination, the virus-neutralizing titer in birds injected with rVP2-montanide oil-AgNP booster group was significantly higher. The birds received rVP2-montanide oil-AgNP, commercial vaccine booster, rVP2-montanide oil booster, commercial vaccine, and rVP2-montanide oil. The results indicated that rVP2 expressed in the prokaryotic expression system has immunogenic potential.

Discussion

The VP2 protein is the major structural protein of IBDV and is routinely used in diagnosis and epidemiology[20]. It forms the outer surface of the viral capsid and possesses neutralizing epitope. The neutralizing antibodies and specific immune response

have been demonstrated against this protein[12,21]. The VP2 protein comprises the major conformational epitopes. These epitopes are responsible for the induction of virus-neutralizing antibodies. Therefore, the IBDV major capsid protein VP2 is utilized for developing diagnostics and novel subunit vaccines[12]. Recent studies used *Escherichia coli*[10,22], *Lactococcus lactis*[23], and plant[12] expression systems to express IBDV rVP2 based virus-like particles and had molecular mass of more than 40 kDa. This study elaborated the diagnostic and immunogenic potential of a truncated 19 kDa rVP2 protein.

These titers were comparable with the titers induced against commercial vaccines. In the placebo group, the purified rVP2 protein concentration of 50 µg/dose induced significant serum IgY titers. the titers remained low throughout the experiment. Recently, a 40 kDa IBDV rVP2 expressed in *Nicotiana benthamiana* demonstrated IBDV specific neutralizing antibody titers in chicken comparable to those induced by the commercial vaccine[12].

The significant (2.31 to 2.67 fold) increase in the serum IgY level was recorded on the 14th day post-vaccination in the birds vaccinated with rVP2 protein and commercial vaccines. On the 14th day, the titers against rVP2-Montanide oil (2.48 fold) were significantly higher than the titers produced by the commercial vaccine (2.31 fold). The birds vaccinated with rVP2-Montanide oil-AgNP showed equivalent but significantly higher serum IgY titers (2.55 to 2.67 fold) than the commercial vaccine.

On the 21st day, a significant rise in serum IgY level (4.95 to 5.11 fold) was recorded in birds vaccinated with booster dose compared to birds that did not receive booster dose (2.92 to 3.94 fold). Comparable but non-significant titers were observed in the birds with (2.97 fold) and without (2.92 fold) booster vaccination with rVP2-Montanide oil blends. However, the group receiving booster rVP2-Montanide oil-AgNP booster showed significantly higher titers (4.95 fold).

On the 28th day, titers were significantly higher in all the vaccinated groups except in placebo (0.06 fold) compared with the respective titers on the 21st day. All the groups receiving booster doses viz. commercial vaccine, rVP2-montanide oil, and rVP2-montanide oil-AgNP showed significantly higher levels of serum IgY titer (14.55, 14.07 and 17.84 fold, respectively) as compared to the corresponding groups which did not receive booster vaccination (12.00, 11.91 and 16.37 fold, respectively). The titers in the groups receiving rVP2-montanide oil-AgNP were significantly higher than those reported in the commercial vaccine booster group. At the same time, the titers in the group receiving rVP2-montanide oil booster were comparable with the titers recorded in the commercial booster vaccine group. The titers in groups not receiving booster doses of rVP2-montanide oil were comparable with the titers observed in the group receiving only a primary dose of commercial vaccine. Exceptionally, the titers in the group receiving only primary rVP2-montanide oil-AgNP were significantly higher throughout the experiment. Earlier reports documented significantly higher anti-IBDV antibody titer against rVP2 after two weeks of immunization[10,23]. Wang and co-workers[22] reported that the IBDV SH619-VLP vaccine induced comparatively lower antibody titers than the commercial vaccine. Our findings also demonstrated significantly high IgY titers after two weeks of vaccination. However, the titers produced against rVP2-Montanide oil were comparatively lower than those induced by commercial vaccines.

The results indicated that the immunogenic potential of rVP2 is comparable to commercial vaccines. Moreover, serum IgY response in birds receiving rVP2-montanide oil-AgNP was superior to commercial vaccines or rVP2-montanide oil without AgNP. Similarly, developing a recombinant DIVA vaccine could be possible as antibody response to VP3 structural protein will be absent in marker vaccine preparations. The commercial live vaccines could not differentiate the infected versus vaccinated birds due to the induction of similar immune responses. Recombinant VP2 vaccine may discriminate vaccinated versus naturally infected birds as naturally infected birds show antibodies to VP3 viral protein[24]. Earlier studies reported induction of specific immune response in chickens immunized with VP2 antigen[21]. The recombinant VP2 protein expressed in the prokaryotic expression system was utilized in diagnostic assays. Recombinant VP2 subunit vaccines have been experimentally demonstrated[25,26]. The available commercial vaccines are prepared from "Intermediate" and "intermediate plus" or "hot" strains, which lead to the bursal changes attributing the immunosuppression, which was being altered by the use of the recombinant VP2 protein-based vaccines. The benefits of using

recombinant VP2 immunogen are that it harbors most of the neutralizing epitopes with a crystalline structure and will not compromise sero-surveillance of IBD.

The mineral oils are often used in a vaccine to form stable water in oil emulsions, ensuring depot formation and steady antigen release[9,27]. The montanide oil used in this study yielded stable emulsions after blending with rVP2 and rVP2-AgNP. It is a proven adjuvant and is safer than Freund's adjuvant, aluminum hydroxide, and aluminum phosphate and can be used in veterinary vaccines[28]. A recent report indicated that the chickens vaccinated with rVP2 neutralizing epitope antigen blended in oil emulsion adjuvant-induced more robust humoral immune response with no side effects[10,29]. This investigation reported the adjuvanticity of montanide oil in significant induction of anti VP2 antibodies when blended with rVP2.

In the present study, we reported enhanced adjuvanticity of montanide oil using AgNP in poultry vaccines. Availability of a novel adjuvant is the current need of veterinary vaccines with more safety, adjuvanticity, and targeted antigen delivery. Many adjuvants have been used in the development of veterinary vaccines. But issues like antigen-dependent adjuvanticity, their physicochemical properties, and toxicity limited their applications. Mineral oil like montanide oil and other derivatives are routinely used in vaccine formulations. However, nanoparticles showed more promising results in potentiating the immune response[19].

Moreover, AgNPs showed strong inhibitory effect (*in vitro*) against IBDV[30], and were experimentally used in vaccine preparations in laboratory animals like dogs, rabbits, and mice for viral antigens like Rabies[31]. There are proven reports of silver toxicity *in vitro* and *in vivo*; however, AgNP is considered non-toxic due to the negligible release of Ag ions from AgNP in water[32]. Asgary *et al*.[31] documented no toxicity of AgNP at the triple dose rate (60 mg/kg body wt.) in laboratory animals. The present study utilized the AgNP at the dose rate of 20 mg/kg body wt. in poultry, considered safe[31].

This study compares the adjuvanticity of montanide oil with and without AgNP and commercial live poultry vaccines. The results indicated a significant rise of serum IgY titers in birds who received rVP2 vaccine blended with montanide oil and AgNP compared with commercial vaccines and rVP2 vaccine mixed with montanide oil alone. The AgNP can be used in veterinary vaccine preparations for a more promising and long-lasting immune response with the additional advantage of less animal handling. Moreover, booster vaccination can be withdrawn because the primary vaccination could produce comparable IgY titers, as evident in the present investigation. The mechanism of AgNP in potentiating antigenicity is not well elucidated in the literature. However, the accumulation property of the AgNP in water is believed to be the primary mechanism involved in antigen trapping and its slow release. Other suggested mechanisms involve cytokine release, leukocyte recruitment, and upregulation of major histocompatibility complex (MHC II) expression of peripheral macrophages[33]. Xu *et al*.[18] reported follicular hyperplasia due to increased B cell number in rabbit spleen and increased humoral response after AgNP immunization.

Conclusion

The rVP2 protein has diagnostic potential as evident by dot blot assay and immunogenic potential as it induced serum IgY titers significantly. The silver nanopartices (AgNPs) had boosting effect on humoral response and produced IgY at par with the commercial vaccines.

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Ethics approval

The study protocol was approved by the Board of Studies, Maharashtra Animal and Fishery Sciences University, Nagpur (No. NVC/Micro/MAFSU-BOS/47/2017 dated 30/05/2017) and the Institutional Animal Ethical Committee, Nagpur Veterinary College, Nagpur (No. NVC/IAEC/3769/2018 dated 25/01/2018).

Conflict of Interest

Author declares no competing interests.

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