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Production of Recombinant N Protein of Infectious Bronchitis Virus Using the Baculovirus Expression System and Its Assessment as a Diagnostic Antigen

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Abstract The avian coronavirus-infectious bronchitis virus (AvCoV-IBV) is recognized as an important avian pathogen, and new viral variants are a continuous threat to the poultry industry worldwide. Sensitive diagnostics and efficacious vaccines are necessary to combat IBV infections in chickens. The aim of this study was to produce recombinant N protein of IBV in the baculovirus system to use in ELISA diagnostic tests in order to enable the assessment of

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the sero-prevalence and risk of IBV infections in chickens in Turkey. For this, the gene encoding the N protein of the Beaudette strain of IBV was expressed using a recombinant baculovirus expression system. The recombinant N protein was purified using Ni-NTA affinity chromatography. An estimated 50-kDa recombinant protein corresponding to the expected molecular weight of IBV N including the 6xHis tag was detected using an anti-His monoclonal antibody. Specific immunoreactivity of the recombinant protein was confirmed by Western blot using antiserum obtained from vaccinated and naturally infected chicken from Turkey as well as using a monoclonal antibody raised against the N protein of the IBV Massachusetts strain. The results obtained with the *in-house* ELISA had high agreement with a commercial ELISA. Immunoreactivity analysis using antisera in Western blotting and the *in-house* ELISA suggests that the recombinant IBV N protein could be broadly cross-reactive with antisera produced against different IBV strains. We conclude that the recombinant baculovirus expressed IBV N protein could serve as a useful diagnostic antigen for detection of IBV infections in chickens by ELISA.

Keywords Infectious bronchitis virus · Baculovirus · Expression · Recombinant · N protein

Abbreviations

AvCoV	Avian coronavirus
IB	Infectious bronchitis
IBV	Infectious bronchitis virus
IBV-N	Infectious bronchitis virus N
PCR	Polymerase chain reaction
WB	Western blot
ELISA	Enzyme-linked immunosorbent assay
SFM	Serum-free medium

Introduction

Infectious bronchitis virus (IBV) is the causal agent of infectious bronchitis (IB), an acute and highly contagious disease, threatening the poultry industry worldwide [5, 11, 13, 17, 19]. Avian coronavirus–infectious bronchitis virus (AvCoV-IBV) is an enveloped, positive

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single-stranded RNA virus, and belongs to the genus Gamma-coronavirus within the family of *Coronaviridae* in the order *Nidovirales* [2]. The virus is spread mainly by aerosol, consumption of contaminated feed and water, and contact with infected feces or equipment. IB is characterized by various clinical signs in broilers and layer hens: coughing, sneezing, and decreased weight gain [4, 13]. Specifically, in layers, egg production can drop up to 70% with eggs that have shells that are wrinkled, thin, and soft. In young chicks, IBV infection can lead to oviduct cysts and reduced laying potential [2, 4, 9].

The 27 to 28 kb genome of IBV encodes nine genes, which includes spike (S), membrane (M), envelope (E), and nucleocapsid (N) [2]. The spike protein, a viral surface glycoprotein, was shown to induce neutralizing antibody response, and the nucleocpasid protein was shown to elicit strong antibody responses [2, 5]. Despite the presence and application of IBV vaccines in poultry, there is a high rate of emergence of antigenic variants and recombinant strains, and the lack of cross-protection between different viral genotypes, making disease control difficult and vaccine development rather challenging [15, 16]. Therefore, genetic characterization of circulating strains of IBV, appropriate vaccination programs, and application of sensitive diagnostic tests to detect and assess disease risk are important regional, national, and international strategies to control IBV infections [2, 8, 10, 21, 23]. Several ELISAs have been developed for detection of antibodies to IBV in chickens. Recombinant antigens used in these ELISAs were either based on the S1 protein [20], which is highly variable, or the N protein and often produced in an *E. coli* expression system [15]. *E. coli* is a common flora or pathogen in chickens, thus creating the possibility of serological cross-reactivity and detection of false positives in these diagnostic assays. Thus, it is necessary to assess the suitability of other expression platforms for production of diagnostic antigens for use in IBV serology. The objectives of this study were to clone and express IBV N protein using the recombinant baculovirus expression system and assess its use as diagnostic antigen for serological diagnosis of IBV infection in chickens in Turkey.

Materials and Methods

Cloning and Construction of Recombinant Bacmid

The complete coding sequence (1230 bp) of the IBV N gene of Beaudette strain (GenBank Accession No. M28565.1) was initially amplified by PCR using primers, JAR170F: 5′-CAC CAT GGC TTC CGG TAA GGC TG-3′ and JAR171R: 5′-CAG CTC GTT CTC ACC CAG AGC AGC-3′. The PCR product was cloned into pFastBac vector (Life Technologies), to create a recombinant donor plasmid, pFastBac-N, which was transformed into One Shot Mach1 T1 Chemically Competent *E. coli* (Life Technologies). The donor plasmid was double digested with restriction enzymes *BamHI* and *PstI* to determine the presence of the correct insert in the right orientation. The accuracy of the sequences was confirmed by DNA sequencing. The donor plasmid was transformed into MAX Efficiency DH10Bac Competent *E. coli* to construct a recombinant bacmid via site-specific transpositioning.

Expression and Purification of Recombinant IBV-N Protein

To rescue recombinant baculoviruses encoding the IBV N gene, recombinant bacmids were purified using HighPure MiniPrep Kit (Life Technologies) and used to transfect *Spodoptera*

frugiperda (Sf9) cells grown in Sf-900 II Serum-free Medium (SFM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin as manufacturer's instruction (Life Technologies). Transfection was carried out using Cellfectin II reagent as previously described [8] and according to the manufacturer's instructions (Invitrogen-Life Technologies). Recombinant IBV N protein was expressed using passage 2 or higher passage recombinant baculovirus stocks ($> 10^7$ pfu/ml). The protein was expressed with a carboxy-terminal 6xHis tag, and purification using Ni-NTA Superflow resin (QIAGEN Inc., Valencia, CA) was performed as described previously [7]. Concentration of the purified protein was measured by the method of bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL) at an absorbance of 562 nm, using bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as the protein standard. Aliquots of the protein were stored at − 80 C until used.

Western Blot Analysis

Western blot analysis was performed to verify specific protein expression. For this, approximately 5 μg of purified recombinant IBV N protein was resolved in 12% Bis-Tris polyacrylamide gel (Life Technologies) as described previously [7]. Following electroblotting of the proteins onto PVDF membrane, the blots were probed with mouse anti-His (C-Terminal)-HRP monoclonal antibodies (1.2 mg/ml) (Life Technologies) diluted 1:5000 to determine expression of the correct molecular size protein. Further confirmation of expression was performed using a mouse anti-IBV N (Massachusetts strain) monoclonal antibody (6 mg/ml) diluted 1: 2000 (Cat No. MBS312776; MyBioSource, San Diego, CA) and antiserum (at 1:50 dilution) collected from chickens naturally infected with local Turkish wild-type IBV strains. The membrane was then incubated with anti-species IgG-HRP secondary antibody conjugate (Santa Cruz Biotechnology, Dallas, TX) at 1: 5000 dilution, and specific signals were detected using enhanced chemiluminescent (ECL) detection system. To assess the induction of specific IBV N-specific antibodies in the immunized chickens (described below), the antisera were tested in immunoblot assays. Approximately, 100 μl of sample containing approximately 5 μg of recombinant IBV N protein was diluted in 50-μl Laemmli sample dilution buffer (Santa Cruz Biotechnology, Dallas, TX) and resolved in 12% Bis-Tris polyacrylamide gel as described above. Following transfer, the blot was probed with IBV N antisera obtained from the chicks immunized with the recombinant IBV N protein at a dilution of 1:50 and with antisera from wild-type IBV-infected chicken at 1:50 dilution [19]. IBV negative chicken sera, at a dilution of 1:50, and mouse anti-IBV N monoclonal antibody (2 mg/ml) (Thermo Fisher, Cat. No. 7500891) at a dilution of 1:500 were used as negative and positive controls, respectively. Thereafter, the blots were probed using goat anti-chicken HRP-conjugated secondary antibody (200 μg/0.5 ml) (Cat. No. sc2428; Santa Cruz Biotechnology, Dallas, TX) at a dilution 1:5000, and goat anti-mouse HRP conjugated secondary antibody (200 μg/0.5 ml) diluted 1:5000 (Cat. No. sc2005; Santa Cruz Biotechnology, Dallas, TX). Reactivity was detected using 3′-3′-Diamino benzidine peroxidase substrate system (Sigma Cat no: D4418-5) as recommended by the manufacturer.

Immunogenicity of Recombinant IBV-N Protein

Immunization of Chicks

To assess immunogenicity of recombinant baculovirus-expressed IBV N protein, embryonated SPF chicken eggs were obtained from the Bornova Veterinary Control Institute (Izmir,

Turkey). The eggs were incubated in an incubator for hatching. After hatching, 10-day-old IBV antibody negative chicks (determined by commercial ELISA-BioChek, IBV ELISA, Cat No: CK119IBV) were immunized with the recombinant IBV-N protein produced in this study. Four chicks were immunized intramuscularly with 15 μg each of the recombinant IBV-N protein mixed with ISA 71VG adjuvant (Seppic, France) at a ratio of 70:30 (adjuvant/IBV-N solution), and four chicks were given placebo as mock-immunized controls. On day 16, immunization was repeated using the same amount of adjuvant and antigen. Chicks were bled on day 0 (pre-immunization) and then on days 10, 16, and 23 to test for presence of antibodies to recombinant IBV-N protein.

Enzyme-Linked Immunosorbent Assay

Optimization of ELISA

An indirect ELISA protocol was first optimized by checkerboard titration following the methods as described previously [16, 18, 22]. For this, two-fold dilutions of IBV-N protein were made starting from 400 to 25 ng in a carbonate bi-carbonate coating buffer (Sigma, C-3041). The negative control serum was from the SPF chicks tested antibody negative by a commercial ELISA (BioChek). Positive control was from chickens either vaccinated or had field infection tested IBV-positive by PCR. The IBV positive and negative sera were also diluted two-fold (from 1:50 to 1:1600), and the conjugate (HRP-conjugated goat anti-chicken antibody, Santa Cruz, sc2428) was diluted 1:5000, 1:10,000, 1:20,000, and 1:40,000, in blocking solution. Per the results of the optimization, the optimal amount of the recombinant IBV protein for use in ELISA ranged from 50 to 100 ng, whereas the optimal dilution of IBV positive chicken serum ranged from 1:100 to 1:800, that of the conjugate at 1:5000 dilution.

Analysis of Test Sera

Following optimization of the ELISA protocol, 18 test sera were obtained from broiler chickens exposed to natural wild-type IBV infection, 18 test sera from broiler chickens vaccinated with a live-attenuated commercial IBV vaccine, and sera obtained at different time-points from chicks immunized with recombinant IBV N protein (described above) were analyzed to detect IBV N-specific antibodies. For this, a 96-well plate (Nunc MaxiSorb, Thermo Fisher Scientific) was coated overnight at 4 °C with 100 ng IBV-N protein/per well in 100-μl carbonate-bicarbonate coating buffer (Sigma, Cat. No. C-3041). The plate was then blocked for 1 h at room temperature with a blocking buffer (PBS containing 3% skimmed milk and 0.05% Tween 20). A volume of 100 μl of test sera, diluted 1:100 in blocking solution, was added and incubated for 1 h at 37 °C. Each serum sample was tested in duplicate, and each test plate included duplicate positive and negative control sera. The negative control serum was from the SPF chicks tested antibody negative by a commercial ELISA (BoChek). Positive control serum was from chickens either vaccinated or had field infection and tested IBV positive by PCR. Goat anti-chicken HRP-conjugated antibody (Santa Cruz Biotechnology, Cat. No. sc2428), diluted 1: 5000, was added to the wells and incubated at 37 °C for 1 h. Between each step, the plate was washed four times by using the wash buffer (PBS containing 0.05% Tween 20). Hundred microliters of TMB substrate solution was added to each well and

incubated at room temperature for 25 min. The reaction was stopped by adding 100 μl of 2 M H_2SO_4 and absorbance (optical density) was measured at 450 nm using a microplate reader (SLT-Spectra, SLT Lab instruments, Germany). The ELISA cut-off value was determined by addition of 3 standard deviations to measurements of the mean OD value of the negative control sera in each plate. OD values above cut-off value were taken as positive. Thus, a cut-off OD value of 0.2 was determined for the in-house indirect ELISA.

Comparison with Commercial ELISA

To assess the reliability of the performance of our in-house indirect IBV N ELISA, a panel of sera was obtained from chickens naturally infected with local wild-type IBV strains, chickens vaccinated with live-attenuated commercial IBV vaccine, and chickens immunized with recombinant IBV N protein (expressed in a baculovirus expression system). The serum samples were tested simultaneously in the in-house indirect ELISA (using the procedure described above) and a commercial ELISA (Biocheck, San Francisco, CA) per manufacturer's instruction. The cut-off value for the commercial ELISA was defined according to the manufacturer and determined as samples with a sample to positive ratio (S/P ratio) of 0.2 or greater.

Results

PCR Amplification and Cloning

A 1203 bp fragment representing the expected molecular size of the IBV N gene was successfully amplified by high fidelity PCR (Fig. 1). The PCR amplicon was successfully cloned into pFastBac/CT plasmid resulting in the creation of a donor plasmid pFastBac-N (5985 bp), Restriction enzyme analysis of the recombinant donor plasmid confirmed the presence of the correct insert in the correct orientation by release of two restriction fragments of the expected size (5090 and 895 bp) (data not shown). The donor plasmid with the correct sequences was used to create recombinant bacmid for subsequent expression of the target protein.

Expression and Purification of Recombinant IBV-N Protein

In order to express the recombinant N protein of IBV, Sf9 cells were infected with a recombinant baculovirus encoding the N gene of IBV. At 72 h post-infection, cells were harvested and the recombinant protein was purified via affinity chromatography. An estimated 50 kDa recombinant protein was overexpressed, which corresponded to the expected molecular size of IBV N protein as determined by Coomassie staining (Fig. 2a) and Western blot analysis using anti-His (C-terminal)-HRP monoclonal antibody (Fig. 2b). To confirm specificity of the target protein, an immunoblot analysis was performed using monoclonal antibodies raised against the N protein of the Massachusetts IBV strain (Fig. 3a) and as well as antiserum obtained from local chickens naturally infected with Turkish wild-type IBV strain (Fig. 3b). In both cases, specific reactivity was confirmed by detection of a single band (50 kDa) of the expected molecular size.

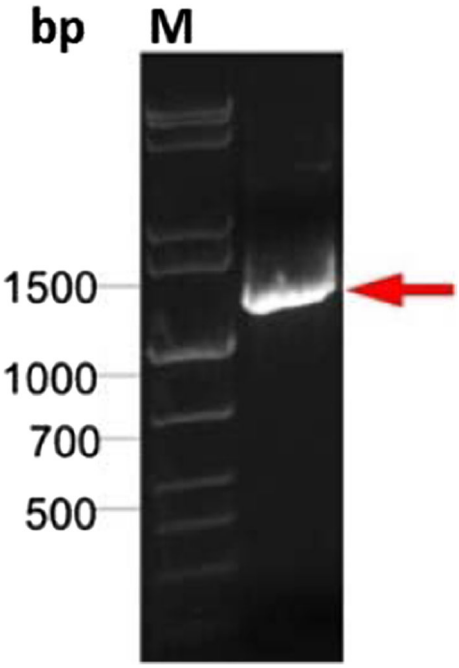

Fig. 1 PCR amplification of IBV N gene (1230 bp) indicated by an arrow. M = molecular base-pair marker

Detection of IBV N-Specific Antibody Responses in Immunized Chickens

To assess the ability of the recombinant IBV N protein to induce host specific antibody responses, four chicks (10-day old) were immunized with the recombinant protein and the sera examined for specific reactivity via immunoblot analysis. Specific reactivity was detected with serum samples obtained from 2 of the IBV N-immunized chickens at day 23 post-immunization (example see Fig. 4, lane 7). No reactivity was

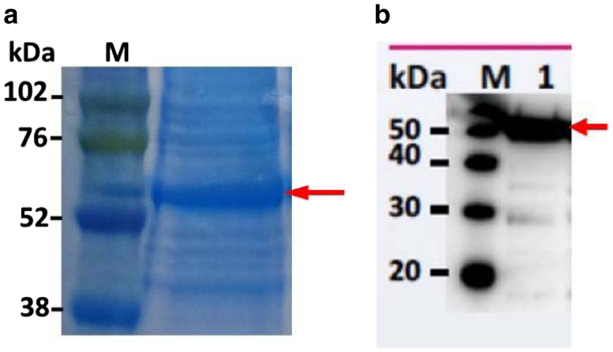

Fig. 2 Recombinant baculovirus expression of IBV-N protein. An estimated 52 kDa molecular weight band (including the histidine tag) was detected. **a** Coomassie blue staining of overexpressed and purified recombinant N protein (arrow). **b** Detection of recombinant IBV N protein using anti-His (C-Terminal)-HRP monoclonal antibody. Lane 1 shows specific reactivity of N protein. M = molecular weight marker; N = IBV N protein. Arrows show N protein or reactivity of N exhibiting the expected molecular size

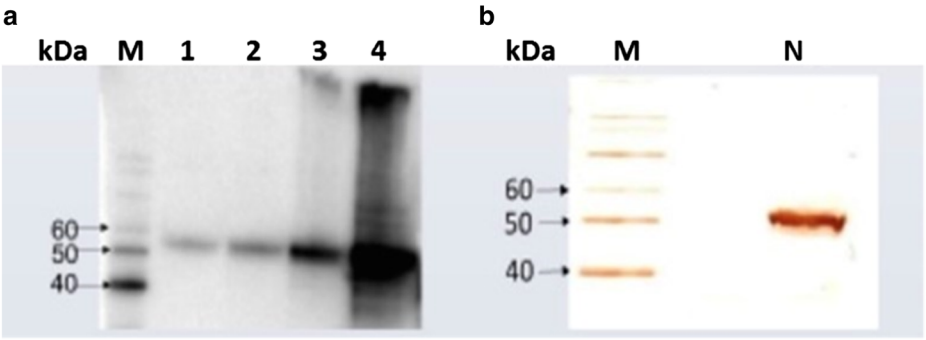

Fig. 3 Western blot confirmation of recombinant IBV N protein expression. An estimated 50 kDa molecular weight band was detected. **a** Image depicting two-fold dilution series (lane 1 = 0.625 μg, lane 2 = 1.25 μg, lane 3 = 2.5 μg, lane 4 = 5 μg) of recombinant N protein reactivity with monoclonal antibody, B819M, raised against the N protein of IBV Massachusetts strain. **b** Reactivity of recombinant IBV N protein with antiserum obtained from chicken naturally infected with Turkish wildtype strains of IBV; M = marker; N = nucleoprotein protein

seen with the other two chicken sera. This result was supported by detection of specific IBV N-reactivity with the IBV-specific monoclonal antibody (Fig. 4, lane 1) and with antisera from a chicken exposed to a natural field infection (Fig. 4, lane 3). No specific bands were detected in sera from mock-immunized chickens or sera taken from chicks before immunization (Fig. 4, lanes 2, 4, 5, 6, 8, 9, 10). Overall, sera from vaccinated and naturally infected (field-exposed) chickens that tested positive in the immunoblot assay were found to be correspondingly positive in the in-house indirect IBV N ELISA (data not shown).

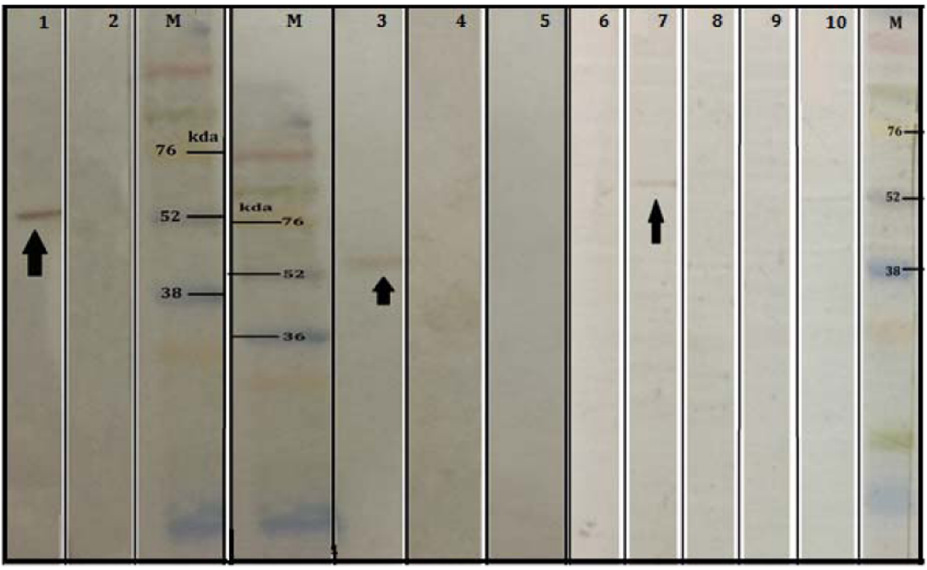

Fig. 4 Immunoblotting with IBV field infected chicken sera, anti-IBV-N protein monoclonal antibody, immunized chicken, and negative chicken sera. Arrows indicate positive reactions. Lane 1: monoclonal anti-IBV–N protein; lane 3: IBV field infected chicken serum; lane 7: immunized chicken serum; lanes 2, 4, 5, 6, 8, 9, 10: negative controls and negative chicken sera; M: rainbow marker

ELISA for Naturally Infected, Vaccinated, and IBV-N Protein Immunized Chicken Sera

To further assess the immunogenicity of the recombinant N protein and the reliability of our results, a panel of antisera from naturally infected (18 samples plus negative and positive controls), from chickens vaccinated with live-attenuated IBV (18 samples plus negative and positive controls) and from chickens immunized with recombinant IBV N protein (4 samples), were tested using both the *in-house* indirect ELISA and a commercial ELISA. With the *in-house* ELISA, cut-off value was set at OD of 0.2. There was relatively good agreement in performance between the two ELISAs in detecting IBV N-specific antibodies in the sera from naturally infected chickens (Fig. 5a) and live-attenuated vaccinated chickens (Fig. 5b). Of the 18 serum samples obtained from the naturally infected chickens, 72% (13/18) tested positive in the *in-house* indirect ELISA, whereas 83% (15/18) tested positive in the commercial ELISA detected (Fig. 5a). All 18 samples collected from vaccinated chickens tested positive in both the *in-house* and commercial indirect ELISAs (Fig. 5b). Of the 15 commercial ELISA-positive field serum samples, 13 tested positive in the *in-house* indirect ELISA, indicating a sensitivity of 87% (using the commercial ELISA as reference test). None of the sera obtained from preimmunized or naïve chicks tested positive in either ELISAs, indicating 100% specificity ($n = 4$). Of the four chicks immunized with the recombinant IBV N protein, two chicks seroconverted at day 16 after immunization. All four chicks seroconverted at 23 days post-immunization in both ELISA tests, indicating a 100% agreement between the two assays. The OD values of chicks immunized with recombinant IBV N protein were 0.3, 0.4, 0.41, and 0.44.

Discussion

Production of immunogenic recombinant proteins derived from pathogenic organisms represents a good strategy for identification of antigenic proteins that may serve as targets for sero-diagnostic and vaccine development [12, 14]. This represents the first study in Turkey that expressed recombinant IBV N protein in baculovirus and examined its reactivity against antisera obtained from Turkish chickens for potential use as antigen

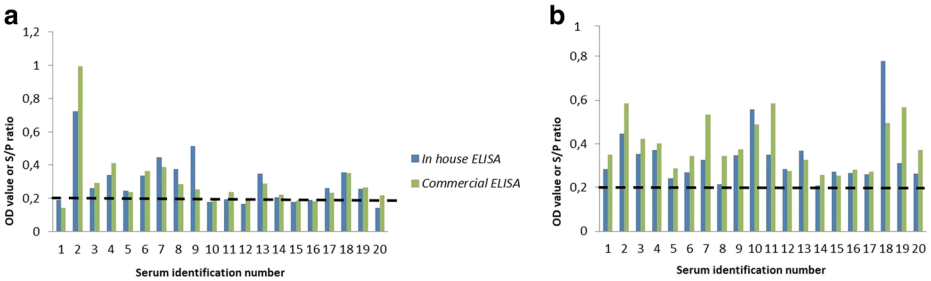

Fig. 5 Detection of IBV N specific antibodies in sera obtained from naturally infected chicken (**a**) and vaccinated chickens (**b**) using an *in-house* IBV-N ELISA and a commercial ELISA. The cut-off value for *in-house* indirect ELISA is set at OD 0.2, and for the commercial ELISA at S/P (sample to positive) ratio of 0.2

in serological investigation of IBV infection in domestic poultry. We have demonstrated that IBV N produced in a recombinant baculovirus expression system is immunogenic in local chickens and could detect IBV N-specific antibodies in sera obtained from chickens exposed to natural infection. The recombinant N protein was reactive in both immunoblot and indirect ELISA assays. The performance of the in-house indirect ELISA was comparable to the commercial ELISA tested in this study, indicating the potential suitability of recombinant baculovirus IBV N as a diagnostic antigen that could be used for serological risk assessment of IBV in domestic chickens in Turkey. Indeed, the N protein of IBV, in contrast to the S1 protein, has been associated with high stability and immunogenicity and has been used by others as a diagnostic antigen [1, 12]. The recombinant N protein used in the current study was based on nucleotide sequences of the Beaudette IBV strain. The reactivity of the recombinant IBV N protein with a monoclonal antibody (B819M) produced against the heterologous IBV Massachusetts strain, and with sera from local Turkish chicken indicate the conserved, cross-reacting nature of the N protein sequence and suggest potential broad cross-reactivity of the target protein among different IBV isolates and its suitability as a diagnostic antigen to detect IBV infection in local Turkish poultry. Other researchers have utilized recombinant IBV N expressed in various host systems in serological assays. For example, in a study performed by Pradhan and others [14], IBV-N protein was produced using a prokaryotic expression system. The protein was used for diagnostic purposes and similar to this study, compared the performance with a commercial ELISA kit (IDEXX). The authors reported a 96.8% sensitivity and 95.8% specificity for the *in-house* ELISA based on the recombinant IBV N antigen and they concluded that an indirect ELISA based on the recombinant antigen is suitable for use in development of serodiagnostic tools [14]. In another study, the IBV-N protein was produced in both *E. coli* and baculovirus expression systems [3], and the recombinant antigens were used in ELISAs to analyze chicken sera collected from farms [3]. Their data from screening of sera for the presence of IBV antibodies indicated that using the recombinant N protein as coating antigen could achieve equivalent performance to an ELISA kit based on extracts from infected material as coating antigen [3].

In a study performed by Ding and others [6] utilizing a multi-fragment antigen composed of S (spike), M (matrix), and N proteins produced in *E. coli* [7], a good reactivity with an anti-IBV chicken serum was demonstrated. An in-house ELISA using the multi-fragment protein exhibited a 95.4% concordance with a commercial ELISA [6]. In the present study, recombinant IBV-N protein was produced using a baculovirus expression system and utilized as a diagnostic antigen in an indirect ELISA. The chicken sera from farms and the sera from immunized chickens were reactive with the recombinant antigen with comparable performance to a commercial ELISA test kit.

Conclusion

This study describes the successful cloning and recombinant baculovirus expression of IBV nucleoprotein and its evaluation as a potential serodiagnostic antigen. The recombinant antigen was immunoreactive with hyperimmune sera from local Turkish chickens and the performance of an in-house indirect ELISA based on the antigen was comparable to a commercial ELISA, suggesting the potential utility for serological detection of IBV infections in chickens in

Turkey. Further studies to validate the assay using a larger sample size including comprehensive assessment of assay specificity will be performed.

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Compliance with Ethical Standards National and international ethical rules were followed during this study.

Conflict of Interests The authors declare that they have no conflicts of interest.

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