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Single Step Detergent Assisted Extraction and Solubilization of the Recombinant Matrix Protein of Newcastle Disease Virus (NDV) and Development of ELISA for Detection of Anti-NDV Antibodies in Chickens

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Matrix protein, Newcastle disease virus, ELISA, Chicken, LDAO

ABSTRACT

The matrix (M) protein is the most abundant structural protein in Newcastle disease virus (NDV), the causative agent of Newcastle disease (ND) in chickens. Owing to its highly conserved nature among NDV strains and also due to its pivotal role in the viral life cycle, the M protein can be employed as a promising diagnostic antigen for reliable detection of NDV infection in chickens. In the present study, we have devised a strategy for extraction and solubilization of the NDV M protein from *Escherichia coli* in a single step using a non-ionic detergent, lauryl-dimethylamine oxide (LDAO), enabling the purification of the detergent-solubilized M protein in a soluble form through affinity chromatography without compromising the structural integrity of the protein. Using the purified M protein as a diagnostic antigen, an indirect enzyme-linked immunosorbent assay (ELISA) has been developed for the detection of anti-NDV antibodies in multiple serum samples collected from different poultry farms of district Faisalabad, Pakistan. The data presented here reveal that the recombinant detergent-solubilized M protein is an active, promising diagnostic antigen and can be exploited in an indirect ELISA for rapid and reliable detection of anti-NDV antibodies in chickens. Owing to the conserved nature of the M protein and the ease of extraction and solubilization in a single step using LDAO, the developed ELISA can be up-scaled for rapid and reliable detection of anti-NDV antibodies and/or NDV infection in a large number of serum samples collected from chickens originating from different sources (poultry farms).

INTRODUCTION

Newcastle disease (ND), also known as Ranikhet disease, is a highly contagious and deadly viral disease of chickens which poses a constant threat to the poultry industry worldwide (Suarez *et al*., 2019). ND is caused by Newcastle disease virus (NDV) (Suarez *et al*., 2019), a member of the Paramyxoviridae family (Fernandes *et al*., 2014). Like other members of the Paramyxoviridae family, NDV harbors a lipid bilayer envelope which is derived from the membrane of the host cell that the virus infects to replicate (Lamb and Parks, 2007). NDV displays polymorphisms in morphology and the viral particle size (Suarez *et al*., 2019). NDV exists either in the form of a sphere, with a diameter ranging from 100 nm to 500 nm, or in the form of a filament which varies in length but typically has a width of 100 nm (Suarez *et al*., 2019). The virus is extremely stable over a wide range of pH and at sub-optimal temperatures but is less stable at elevated temperatures (above 56 °C) (Ganar *et al*., 2014).

The genome of NDV consists of a negative-sense, non-segmented and single-stranded RNA molecule of approximately 15.2 kb that encodes several non-structural and six structural proteins (Ganar *et al*., 2014). The structural proteins include matrix (M), hemagglutinin-neuraminidase (HN), nucleoprotein (NP), fusion (F), phosphoprotein (P), and large polymerase (L) proteins (Gaikwad *et al*., 2019) (Fig. 1). Among the viral proteins,

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the M protein is a highly conserved structural protein encoded by different NDV strains (Shtykova *et al*., 2019), and is composed of 364 amino acids with a molecular weight of 40 kDa (Lamb and Parks, 2007). The dimeric M protein forms a grid-like array on the inner surface of the NDV membrane and interacts with the cytoplasmic tails of HN and F proteins in host cells (Russell and Almeida, 1984). In the virion, the M protein is associated with the lipid membrane, surface glycoproteins and nucleocapsid proteins (Panshin *et al*., 1997). The M protein is implicated in viral assembly and budding from the host cell surface as well as in the maintenance of the spherical structure of virions (Ganar *et al*., 2014; Shtykova *et al*., 2019). However, it has been found that the M protein does not play a significant role in enhancing the virulence of NDV (Iram *et al*., 2014).

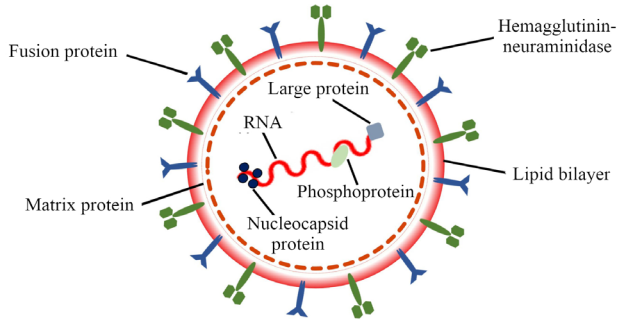

Fig. 1. Schematic representation of Newcastle disease virus (NDV) structure.

A rapid and reliable diagnosis of NDV is highly important to prevent sudden ND outbreaks and the spread of the disease. Furthermore, it is also important to distinguish NDV infections caused by velogenic (highly virulent) strains from vaccination or lentogenic (less virulent) strains (Khan *et al*., 2010). Multiple conventional methodologies such as pathogenicity testing, serological tests, polymerase chain reaction (PCR)-based tests, and the HN assay can be used to diagnose ND (Getabalew *et al*., 2019; Khan *et al*., 2010). A diagnostic strategy that involves observing clinical signs and subsequently confirming NDV infection by conventional methods is not feasible for the characterization of ND in a large number of field samples, as conventional diagnostic methods take 4 to 7 days to confirm and validate an NDV outbreak (Bello *et al*., 2018). So, there is an urgent need to develop rapid and reliable laboratory-based diagnostic tests for the detection of NDV infection in a large number of field samples.

The detection of NDV infection by measuring the titer of anti-NDV antibodies in serum samples of unimmunized chickens using rapid and sensitive diagnostic assays is an attractive approach. Based on the level of the antibody titer, this approach also allows for estimating the time of infection as well as differentiation of birds exposed to even a mild NDV attack at any stage of their life from vaccinated chickens (Marquardt *et al*., 1985). Various types of serological tests have been reported which use NDV or its parts as diagnostic antigen(s) to detect anti-NDV antibodies in serum samples from infected birds (Berinstein *et al*., 2005; Choi *et al*., 2013; Koch *et al*., 1998; Makkay *et al*., 1999). Among these, two highly preferred tests for analyzing antibody titers are: the hemagglutination inhibition (HI) assay and the enzyme-linked immunosorbent assay (ELISA) (Yune and Abdela, 2017). In general, HI assays are not feasible for serodiagnosis of field samples consisting of different serotypes co-circulating in the field (Bello *et al*., 2018; Long *et al*., 2004). On the other hand, most of the reported ELISA tests for NDV have been developed using either the whole NDV or less conserved proteins (such as F and HN) of NDV as diagnostic antigens (Bello *et al*., 2018). Importantly, indirect ELISA tests which are based on the whole NDV or less conserved NDV proteins are not suitable for analyzing the titer of anti-NDV antibodies in serum samples of birds collected from different sources, most likely due to variations in NDV strains. To address these shortcomings, the conserved M protein of NDV can be exploited as a reliable diagnostic antigen to detect anti-NDV antibodies in serum samples of poultry birds infected with different NDV strains, since the M protein plays an important role in virus assembly and the amino acid sequence of the M protein remains conserved among different strains of NDV (Ganar *et al*., 2014; Seal *et al*., 2000; Shtykova *et al*., 2019). In the present study, we have evaluated the performance of the detergent-solubilized M protein of NDV as a diagnostic antigen in an indirect ELISA for rapid and reliable detection of anti-NDV antibodies in serum samples of chickens collected from different poultry farms. We previously reported a multi-step method for obtaining the NDV M protein expressed in *Escherichia coli* that consisted of refolding the M protein from inclusion bodies using urea, solubilization of the M protein using a non-ionic detergent (lauryl-dimethylamine oxide (LDAO)), and affinity purification using elution buffer that contained Tween-20 instead of LDAO (Iram *et al*., 2014). In the present study, we demonstrate that extraction and solubilization of the NDV M protein from *E. coli* using LDAO in a single step not only enhances the solubility and the yield of the recombinant M protein of NDV but also renders the detergent-solubilized M protein suitable for use in an indirect ELISA as a reliable diagnostic antigen for the detection of anti-NDV antibodies in multiple serum samples of chickens. Due to the ease of

extraction and solubilization of the M protein in a single step using LDAO, the ELISA described here can be up-scaled, allowing rapid and reliable detection of NDV infection and/ or anti-NDV antibodies in a large number of serum samples of chickens collected from the field.

MATERIALS AND METHODS

Ethical approval

This work has received approval from the ethics committee of National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan, and the work was performed following the guidelines of the institutional ethics committee.

Expression of the M protein

A widely used strategy to enhance the expression of recombinant proteins in *E. coli* is the use of engineered bacterial strains (Jana and Deb, 2005; Sørensen and Mortensen, 2005). To get the maximum yield of the M protein, therefore, we expressed the pHis$_8$-MBP-Matrix plasmid, available in Structural Biology Lab (NIBGE) (Iram *et al*., 2014), in different strains of *E. coli* in order to overcome the codon usage bias for expression of the viral protein in the prokaryotic host (Mädje *et al*., 2012; Rosano and Ceccarelli, 2009). For this, pHis$_8$-MBP (the vector-only control) and the recombinant plasmid construct (pHis8-MBP-Matrix) were transformed into Rosetta 2(DE3) and BL21 (DE3) strains of *E. coli*. Transformed cells were selected on LB agar media (2% agar, 1% tryptone, 0.5% yeast extract, 1% NaCl and 1% glucose) supplemented with antibiotics (25 µg/mL chloramphenicol and 100 µg/mL ampicillin for the Rosetta 2(DE3) strain, and 100 µg/mL ampicillin for the BL21 (DE3) strain). A bunch of identical colonies containing the vector-only control and the recombinant plasmid construct were inoculated in 10 mL LB broth supplemented with appropriate antibiotics, in separate tubes, and were incubated overnight in an orbital shaker at 37 °C and 225 rpm. Then, 500 mL of expression media (LB broth supplemented with 0.2% glucose and relevant antibiotics) were inoculated with 2% of the overnight culture and incubated at 37 °C and 225 rpm until the OD_{600} reached 0.6. The culture was then placed on ice for 20 minutes. Expression of the recombinant M (His$_8$-MBP-Matrix) protein was induced by addition of 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After induction for 8 h at 22°C and 225 rpm, cells were harvested by centrifugation at 4000× *g* at 4°C for 20 minutes. Expression of the recombinant M protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Extraction and solubilization of the recombinant M protein

As detergents such as LDAO can enhance the solubility and prevent aggregation of the M protein (Arachea *et al*., 2012; Columbus *et al*., 2006; Seddon *et al*., 2004), either urea or LDAO was used in the lysis buffer in the present study to refold and obtain the recombinant M protein in a soluble form. For this, pelleted cells from a 20 mL culture of the transformed *E. coli* Rosetta 2 (DE3) cells, containing the over-expressed recombinant M protein, were resuspended in pre-chilled lysis buffers (1 g wet cell biomass/7 mL buffer) either containing urea (20 mM Tris HCl, pH 7.9, 300 mM NaCl, 2 mM β-mercaptoethanol (β-ME), 8 M urea) or LDAO (20 mM Tris-HCl, pH 7.9, 300 mM NaCl and 2 mM β-ME supplemented with 1%, 5% or 10% LDAO (v/v)). The cell suspension was homogenized at high pressure, 20 kpsi, using a cell disruptor. The cell lysate was centrifuged at 12,000× *g*, 4 °C for 20 min to separate soluble and insoluble (inclusion bodies) fractions, and analyzed by SDS-PAGE. The urea-solubilized fraction was directly used for nickel-affinity purification under denaturing conditions. The concentration of LDAO in the LDAO-solubilized fraction was decreased from 5% (v/v) to 1% (v/v) using dilution buffer (20 mM Tris-HCl, pH 7.9, 300 mM NaCl, and 2 mM β-ME) before purification of the recombinant M protein through affinity chromatography.

Purification of the recombinant M protein

The LDAO-solubilized fraction was passed three times through the nickel-nitrilotriacetate (Ni-NTA) agarose affinity resin (QIAGEN), pre-equilibrated with buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole, 0.04% LDAO (v/v), and 2 mM β-ME). The resin was washed with 30x column volumes (CV) of wash buffer (20 mM Tris-HCl, pH 7.9, 150 mM NaCl, 25 mM imidazole, 0.04% LDAO, and 2 mM β-ME). The recombinant M protein was eluted with 5× CV of elution buffer (20 mM Tris-HCl, pH 7.9, 150 mM NaCl, 250 mM imidazole, 0.04% LDAO, and 2 mM β-ME). The purity of the protein was qualitatively analyzed through SDS-PAGE. Elution fractions containing the purified recombinant M protein were pooled and dialyzed against the dialysis buffer (20 mM Tris- HCl, pH 7.9, 150 mM NaCl, and 0.03% LDAO) in order to reduce the concentration of imidazole. The dialyzed protein was concentrated using a concentrating unit (Amicon Centrifugal Filter Unit, 30 kDa molecular weight cutoff), and glycerol (50% final concentration) was added to the concentrated protein. The protein was stored at -20 °C until further use.

Development of a recombinant M protein-based ELISA

An indirect ELISA was developed using the recombinant M protein as an antigen. The antigen (200

µL; 4 µg/well) was used to coat the wells of a micro-titer plate, and the plate was incubated overnight at 4°C. Wells were then washed three times with phosphate buffer saline-Tween 20 (PBS-T) supplemented with 0.04% LDAO (200 µL/well). Non-specific binding to the surface can be minimized by saturating these unoccupied binding sites with a blocking reagent in order to prevent non-specific binding, 200 µL of the blocking buffer (5% BSA in PBS) was added to each well, and the plate was incubated at room temperature for 60 min. Wells were then washed as above and dried. Serum samples collected from naturally infected birds, showing clinical signs and symptoms of ND, from various poultry farms (namely, Rana Amjad Ali poultry farm, AAA poultry farm, NM poultry farm, SF poultry farm, NIAB), in district Faisalabad, Pakistan, were diluted (i.e., 1:50, 1:100, 1:200, 1:400) in PBS-T, and applied to the wells (100 µL/well). After incubation at room temperature for 2 h, wells were washed three times with PBS-T containing 0.04% LDAO (200 µL/well) and dried. The anti-chicken IgY (IgG) (whole molecule), alkaline phosphatase antibody produced in rabbits (A9171 Sigma) was diluted (1:25000) in PBS-T and added to each well (200 µL/well). After incubation at room temperature for 2 h, wells were washed three times with PBS-T supplemented with 0.04% LDAO (200 µL/well) and dried. To develop the signal, 100 µL of the alkaline phosphatase substrate (p-nitrophenyl phosphate disodium salt hexahydrate), freshly prepared in 0.1 M glycine buffer (pH 10.4) (0.1 M glycine, 1 mM $MgCl_2$ and 1 mM $ZnCl_2$), was added to each well. The plate was incubated at room temperature for 30 minutes, and observed for the development of the yellow colored product (para-nitrophenylphosphate (pNPP)). The reaction was stopped by addition of 3 M NaOH (25 µL/well). To analyze the antibody titer, the absorbance was measured at 405 nm using a microplate reader (Dia 710-Microplate Reader).

RESULTS

Expression analysis of the recombinant M Protein in different E. coli *strains*

To obtain the M protein in sufficient quantities, the protein was expressed in fusion with a C-terminal His_8-MBP tag in Rosetta 2(DE3) and BL21 (DE3) strains of *E. coli*. In both the cases, the analysis of the protein expression by SDS-PAGE revealed the appearance of a dense band at ~83 kDa. However, the expression level of the recombinant M protein was higher in *E. coli* Rosetta 2(DE3) cells than in *E. coli* BL21 (DE3) cells. Furthermore, the expression of the recombinant M protein (His_8-MBP-Matrix) in a soluble form increased by lowering the post-induction temperature of the culture to 22 °C, as analyzed through SDS-PAGE (Fig. 2).

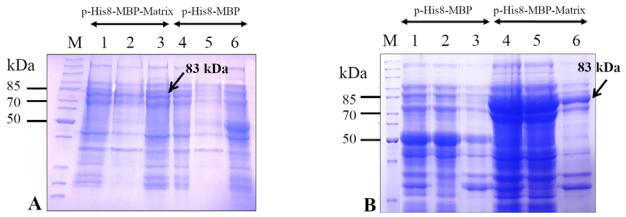

Fig. 2. Expression analysis of the recombinant M (His_8-MBP-Matrix) protein in *Escherichia coli* strains (BL21 (DE3) and Rosetta 2(DE3)) at 22 °C using 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a comparison of coomassie brilliant blue stained gels containing separated protein expressed in *E. coli* BL21 (DE3) (panel A) and *E. coli* Rosetta 2(DE3) (panel B) indicates that the target protein (His_8-MBP-Matrix) is better expressed in *E. coli* Rosetta 2(DE3) cells (panel B) than in *E. coli* BL21 (DE3) cells (panel A) using the expression plasmid, $pHis_8$-MBP-Matrix. The $pHis_8$-MBP plasmid was used as the vector-only control. Lanes 1 and 4 depict the whole cell lysate, lanes 2 and 5 represent soluble fractions, and lane 3 and 6 show insoluble fractions. Arrows indicate the band corresponding to the target (His_8-MBP-Matrix) protein (~ 83 kDa). Lane M depicts the mobility of a protein molecular weight marker for estimating the size of separated proteins.

Extraction and solubilization of the recombinant M protein

In our previous studies, the purification yield of the refolded M protein was too low (Iram *et al*., 2014). Therefore, *E. coli* Rosetta 2(DE3) cells containing the recombinant M protein were first lysed in the presence of 8 M urea (see Materials and Methods) in an attempt to improve the purification yield of the M protein. The soluble cellular content (supernatant) was applied to a Ni-NTA column, and the bound protein was eluted using elution buffer containing 250 mM imidazole and 8 M urea. However, the SDS-PAGE analysis revealed that the recombinant M protein could not be purified in the presence of 8 M urea (data not shown). To overcome the issue and obtain the protein in a soluble form, cell lysis and protein purification steps were performed in the presence of a non-ionic detergent, LDAO. For this purpose, different concentrations (1%, 5% and 10%) of LDAO were used in the cell lysate to enable the extraction and solubilization of the target protein. To this end, *E. coli* Rosetta 2(DE3) cells containing the overexpressed M protein were lysed in the presence of 1%, 5% and 10% LDAO in dilution buffer (20 mM Tris-HCl, pH 7.9, 300 mM NaCl, and 2 mM β-ME). The SDS-PAGE analysis revealed that the recombinant M protein was equally distributed in soluble and insoluble fractions obtained from cells lysed in the presence of 1% LDAO. On the other hand, the protein was predominantly

extracted in the soluble form from the cell lysate treated with 5% LDAO (Fig. 3A, B). The SDS-PAGE analysis of samples from cell lysis with 10% LDAO showed diffused bands, forming streaks on the gel which may be attributed to a high concentration of LDAO (Fig. 3C).

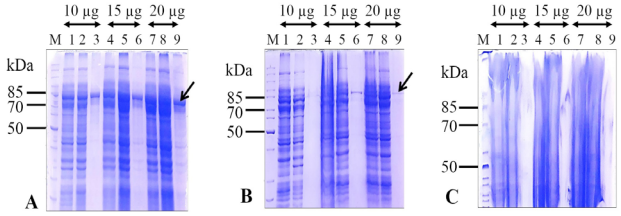

Fig. 3. SDS-PAGE analysis of the recombinant M protein solubilized using various concentrations of lauryl-dimethylamine oxide (LDAO). The protein was solubilized using 1% (panel A), 5% (panel B), and 10% (panel C) LDAO. For SDS-PAGE analysis, samples containing different quantities (10 µg, 15 µg, and 20 µg) of the total protein were loaded. It is evident that the target protein is best solubilized using 5% LDAO. Lanes 1, 4, and 7 represent the whole cell lysate, lanes 2, 5, and 8 depict soluble fractions, and lanes 3, 6, and 9 show insoluble fractions of lysed *E. coli* Rosetta 2(DE3) cells containing the expressed recombinant M protein. Arrows indicate the band corresponding to the target (His_8-MBP-Matrix) protein (~ 83 kDa). Lane M depicts the mobility of a protein molecular weight marker for estimating the size of separated proteins.

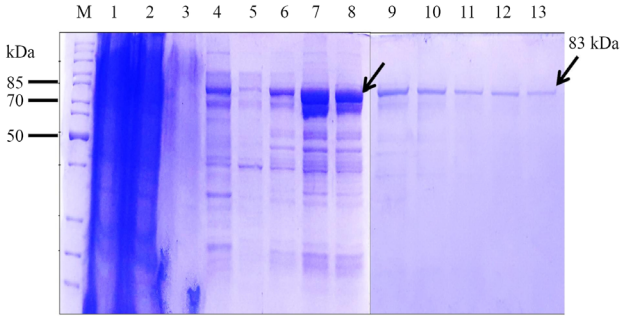

Fig. 4. Analysis of the recombinant M protein purified by affinity chromatography using nickel-nitrilotriacetate (Ni-NTA) columns. Samples collected at different stages of affinity chromatography were analyzed through SDS-PAGE. Lane 1 represents the whole cell lysate, lane 2 depicts the soluble fraction, lane 3 denotes the insoluble fraction, lane 4 represents the flow-through from the column, lane 5 shows the fraction collected during stringent washing of the column, lanes 6 to 13 represent elution fractions. Arrows indicate the band corresponding to the target (His_8-MBP-Matrix) protein (~ 83 kDa). Lane M depicts the mobility of a protein molecular weight marker for estimating the size of separated proteins.

Purification of the recombinant M protein

Based on the protein solubilization results, 5% LDAO was selected as the optimal concentration for solubilization of the recombinant M protein from lysed cells. The diluted supernatant (containing 1% LDAO, final concentration) was allowed to pass through the Ni-NTA resin and the bound recombinant protein was eluted with elution buffer containing 250 mM imidazole. Elution fractions were analyzed through SDS-PAGE, and the results showed that the recombinant M protein was purified in a stable form, with reasonable purity (Fig. 4), and in quantities sufficient for conducting serological studies.

Development of the M protein-based ELISA

An indirect ELISA was developed using the purified M protein as the coating antigen. Antisera samples collected from NDV-infected birds were used in 1:50 dilution for measuring the antibody titer against NDV (whole virus). Our results confirmed the presence of anti-NDV antibodies in all of the collected samples (Fig. 5), suggesting that the developed ELISA can reliably detect anti-NDV antibodies in serum samples of NDV-infected birds from different sources (poultry farms). Notably, the antibody titer in the serum samples from the Rana Amjad (layer) farm (serum samples 1 and 2) showed that a significant amount of the anti-NDV antibodies was produced in the chickens. Moreover, anti-NDV antibodies were confirmed in the serum samples of the chickens obtained from AAA farms (serum samples 3, 4) and NIAB (serum sample 7) but the antibody titer was less than that of the Rana Amjad (layer) farm. Antiserum samples from NM (serum sample 5) and SF farms (serum sample 6) were also confirmed for the presence of NDV-specific antibodies. None of the ELISA controls gave any significant signal at 405 nm. Antibody titers along with their standard deviations for the tested samples are shown in Figure 5. Based on these results, it can be concluded that the conserved M protein, which is detergent (LDAO)-solubilized, can be used as a reliable diagnostic antigen for the detection of anti-NDV antibodies in serum samples of chickens collected from different poultry farms.

DISCUSSION

Every year, infectious diseases of chickens adversely affect the growth of the poultry industry across the globe. ND, caused by NDV, is a highly devastating disease confronting the poultry industry (Suarez *et al*., 2019). One of the approaches for mitigating the economic losses due to ND is the timely diagnosis of the disease in chickens so that preventive measures to control the spread of the disease to uninfected birds can be undertaken timely. A

highly preferred technique for the detection of NDV or anti-NDV antibodies in antisera of infected birds is ELISA due to its reliability, high reproducibility, sensitivity and specificity (Makkay *et al*., 1999). To date, ELISAs based on various proteins of NDV have been developed for the detection of anti-NDV antibodies and/or NDV in infected birds. These ELISAs are either based on the structural proteins of NDV or the whole NDV (Gaikwad *et al*., 2019; Zhao *et al*., 2018).

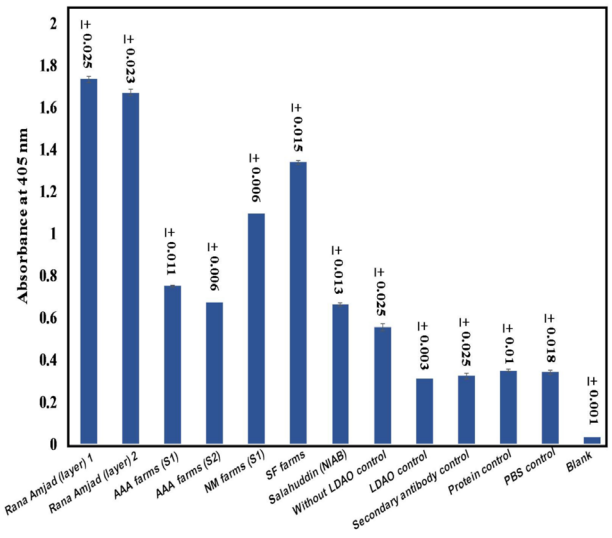

Fig. 5. Analysis of the NDV-specific antibody titer in chicken antisera through ELISA. The anti-NDV antibody titer and standard deviations (SD) among serum samples collected from chickens of different poultry farms are shown along with controls. Serum samples from NDV-infected chickens displayed a significanlty higher anti-NDV antibody titer than negative controls, confirming that the recombinant M protein can be used for detection of anti-NDV antibodies and/or NDV infection in chickens.

Two structural proteins (NP and P) of NDV have been extensively exploited for ELISA development (Zhao *et al*., 2018). The NP protein is the most abundant viral protein expressed in infected cells, whereas the P protein is associated with the nucleocapsid in the virion (Ahmad-Raus *et al*., 2009; Errington and Emmerson, 1997; Kho *et al*., 2004; Panshin *et al*., 2000; Zhao *et al*., 2018). However, the variability and serotype specificity of both the proteins limit the use of the N or P-based ELISAs for the detection of different NDV serotypes (Zhao *et al*., 2018). Recently, structural proteins other than NP and P (such as HN and F) of NDV have also been used for the development of ELISAs. An NDV genotype 2.VII-specific concanavalin A (conA)-ELISA has been developed by targeting the HN protein (Moharam *et al*., 2021). For this purpose, the HN protein obtained through biophysical enrichment from purified NDV was used as an antigen (Moharam *et al*., 2021). However, the genotype specificity of the HN protein limits the use of the developed ELISA for the detection of NDV in samples from different sources. Based on the purified F protein as an antigen, an indirect ELISA has been reported for the detection of NDV in poultry (Astuti *et al*., 2020). Serum samples were collected from birds vaccinated with either the purified recombinant F protein or a commercially available vaccine (Astuti *et al*., 2020). Results of the study confirmed that the recombinant F protein can be used as an antigen for the detection of a specific immune response against the F gene/protein-based NDV vaccines (Astuti *et al*., 2020). However, it remains to be seen if the developed F protein-based ELISA can be used for the detection of NDV infection in unimmunized birds.

Indirect ELISAs which are based on the whole NDV have also been reported for the detection of anti-NDV antibodies (Häuslaigner *et al*., 2009; Oliveira *et al*., 2013). For instance, an indirect ELISA which is based on the LaSota strain of NDV has been used to detect antibodies against NDV in the antisera collected from immunized domestic geese and ducks (Häuslaigner *et al*., 2009). However, a major factor which limits the widespread use of indirect ELISAs is that these tests are not suitable for analyzing multiple antisera of birds which originate from different sources such as antisera collected from birds of different poultry farms, most likely due to variations in NDV strains.

In the present study, we have targeted the M protein of NDV for ELISA development due to a high degree of conservation of the M protein among different NDV strains. For this purpose, the recombinant M protein was extracted and solubilized in a single step using a non-ionic detergent, LDAO, and purified in large amounts in a soluble form using affinity chromatography. The purified M protein was used as a coating antigen to develop indirect ELISA as a rapid diagnostic test for NDV. Our results show that the anti-NDV antibody titers in the serum samples of infected birds collected from different poultry farms are significantly higher than that of uninfected (control) birds (Fig. 5), confirming that the detergent-solubilized M protein is a promising diagnostic antigen for the detection of anti-NDV antibodies and/or NDV in infected chickens from different sources. It is worth-noting that we previously documented a multi-step procedure for obtaining the NDV M protein from *E. coli* that involved refolding the M protein from inclusion bodies using urea, solubilization of the M protein using LDAO, and affinity purification using an elution buffer that contained Tween-20 instead of LDAO (Iram *et al*., 2014). In the current study, we have

devised a strategy that allows extraction and solubilization of the recombinant M protein in a single step using LDAO, enabling the affinity-purified M protein to be used as a diagnostic antigen in an indirect ELISA for reliable detection of anti-NDV antibodies in serum samples of birds collected from different poultry farms. The single-step extraction and solubilization of the recombinant M protein using LDAO reduces the cost and time needed for obtaining the same amount of the protein through the multi-step procedure, thus opening up the possibility for up-scaling the developed ELISA presented in the current study for analyzing a large number of serum samples of NDV-infected birds from different poultry farms.

CONCLUSIONS

In summary, the recombinant M protein of NDV was successfully extracted and solubilized using LDAO in a single step and purified in large amounts in a soluble form through affinity chromatography. Considering the conserved nature of the M protein and an increased yield of the protein obtained without compromising its structural integrity, an indirect ELISA was developed using the LDAO-solubilized M protein as a diagnostic antigen. We demonstrate that the purified M protein can be used for the development of a cost-effective, rapid, and reliable ELISA for the detection of anti-NDV antibodies and/or NDV in serum samples of poultry birds collected from different sources.

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Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Ahmad-Raus, R., Ali, A.M., Tan, W.S., Salleh, H.M., Eshaghi, M., and Yusoff, K., 2009. Localization of the antigenic sites of Newcastle disease virus nucleocapsid using a panel of monoclonal antibodies. *Res. Vet. Sci.*, **86**: 174–182. https://doi.org/10.1016/j.rvsc.2008.05.013

Arachea, B.T., Sun, Z., Potente, N., Malik, R., Isailovic, D., and Viola, R.E., 2012. Detergent selection for enhanced extraction of membrane proteins. *Protein Expr. Purif.*, **86**: 12–20. https://doi.org/10.1016/j.pep.2012.08.016

Astuti, R.W., Wijayanti, N., and Haryanto, A., 2020. Expression of recombinant fusion protein from local isolate of Newcastle disease virus and antibody response to recombinant fusion protein in broiler chickens post-vaccination. *J. Indones. Trop. Anim. Agric.*, **45**: 78–90. https://doi.org/10.14710/jitaa.45.2.78-90

Bello, M.B., Yusoff, K., Ideris, A., Hair-Bejo, M., Peeters, B.P.H., and Omar, A.R., 2018. Diagnostic and vaccination approaches for Newcastle disease virus in poultry: The current and emerging perspectives. *Biomed. Res. Int.*, **2018**: 7278459. https://doi.org/10.1155/2018/7278459

Berinstein, A., Vazquez-Rovere, C., Asurmendi, S., Gómez, E., Zanetti, F., Zabal, O., Tozzini, A., Grand, D.C., Taboga, O., Calamante, G., Barrios, H., Hopp, E., and Carrillo, E., 2005. Mucosal and systemic immunization elicited by Newcastle disease virus (NDV) transgenic plants as antigens. *Vaccine*, **23**: 5583–5589. https://doi.org/10.1016/j.vaccine.2005.06.033

Choi, K.-S., Kye, S.-J., Jeon, W.-J., Park, M.-J., Kim, S., Seul, H.-J., and Kwon, J.-H., 2013. Preparation and diagnostic utility of a hemagglutination inhibition test antigen derived from the baculovirus expressed hemagglutinin-neuraminidase protein gene of Newcastle disease virus. *J. Vet. Sci.*, **14**: 291–297. https://doi.org/10.4142/jvs.2013.14.3.291

Columbus, L., Lipfert, J., Klock, H., Millett, I., Doniach, S., and Lesley, S.A., 2006. Expression, purification, and characterization of *Thermotoga maritima* membrane proteins for structure determination. *Protein Sci.*, **15**: 961–975. https://doi.org/10.1110/ps.051874706

Errington, W., and Emmerson, P.T., 1997. Assembly of recombinant Newcastle disease virus nucleocapsid protein into nucleocapsid-like structures is inhibited by the phosphoprotein. *J. Gen. Virol.*, **78**: 2335–2339. https://doi.org/10.1099/0022-1317-78-9-2335

Fernandes, C.C., Varani, A.M., Lemos, E.G.M., de Miranda, V.F.O., Silva, K.R., Fernando, F.S., Montassier, M.F.S., and Montassier, H.J., 2014. Molecular and phylogenetic characterization based on the complete genome of a virulent pathotype of Newcastle disease virus isolated in the 1970s in Brazil. *Infect. Genet. Evol.*, **26**: 160–167. https://doi.org/10.1016/j.meegid.2014.05.014

Gaikwad, S.S., Lee, H.-J., Kim, J.-Y., and Choi, K.-S., 2019. Expression and serological application of recombinant epitope-repeat protein carrying an immunodominant epitope of Newcastle disease

virus nucleoprotein. *Clin. Exp. Vaccine Res.*, **8**: 27-34. https://doi.org/10.7774/cevr.2019.8.1.27

Ganar, K., Das, M., Sinha, S., and Kumar, S., 2014. Newcastle disease virus: Current status and our understanding. *Virus Res.*, **184**: 71–81. https://doi.org/10.1016/j.virusres.2014.02.016

Getabalew, M., Alemneh, T., Akeberegn, D., Getahun, D., and Zewdie, D., 2019. Epidemiology, diagnosis and prevention of Newcastle disease in poultry. *Am. J. biomed. Sci. Res.*, **16**: 50–59. https://doi.org/10.34297/AJBSR.2019.03.000632

Häuslaigner, R., Sonnenburg, J., Kothlow, S., Kaspers, B., Staubach, C., and Grund, C., 2009. Evaluation of an indirect enzyme-linked immunosorbent assay to study the specific humoral immune response of Muscovy ducks (*Cairina moschata*) and domestic geese (*Anser anser var. domestica*) after vaccination against Newcastle disease virus. *Avian Pathol.*, **38**: 89–95. https://doi.org/10.1080/03079450902737813

Iram, N., Shah, M.S., Ismat, F., Habib, M., Iqbal, M., Hasnain, S.S., and Rahman, M., 2014. Heterologous expression, characterization and evaluation of the matrix protein from Newcastle disease virus as a target for antiviral therapies. *Appl. Microbiol. Biotechnol.*, **98**: 1691–1701. https://doi.org/10.1007/s00253-013-5043-2

Jana, S., and Deb, J.K., 2005. Strategies for efficient production of heterologous proteins in *Escherichia coli*. *Appl. Microbiol. Biotechnol.*, **67**: 289–298. https://doi.org/10.1007/s00253-004-1814-0

Khan, T.A., Rue, C.A., Rehmani, S.F., Ahmed, A., Wasilenko, J.L., Miller, P.J., and Afonso, C.L., 2010. Phylogenetic and biological characterization of Newcastle disease virus isolates from Pakistan. *J. clin. Microbiol.*, **48**: 1892–1894. https://doi.org/10.1128/JCM.00148-10

Kho, C.L., Tan, W.S., Tey, B.T., and Yusoff, K., 2004. Regions on nucleocapsid protein of Newcastle disease virus that interact with its phosphoprotein. *Arch. Virol.*, **149**: 997–1005. https://doi.org/10.1007/s00705-003-0273-8

Koch, G., Czifra, G., and Engström, B.E., 1998. Detection of Newcastle disease virus-specific antibodies in ostrich sera by three serological methods. *Vet. Rec.*, **143**: 10–12. https://doi.org/10.1136/vr.143.1.10

Lamb, R.A., and Parks, G.D., 2007. *Paramyxoviridae*: *The viruses and their replication*. In: *Fields virology* (eds. B.N. Fields, D.N. Knipe and P.M. Howley). Lippincott, Williams, and Wilkins, pp. 1449–1496.

Long, B.C., Goldberg, T.L., Swenson, S.L., Erickson, G., and Scherba, G., 2004. Adaptation and limitations of established hemagglutination inhibition assays for the detection of porcine anti-swine influenza virus H1N2 antibodies. *J. Vet. Diagn. Invest.*, **16**: 264–270. https://doi.org/10.1177/104063870401600402

Mädje, K., Schmölzer, K., Nidetzky, B., and Kratzer, R., 2012. Host cell and expression engineering for development of an *E. coli* ketoreductase catalyst: Enhancement of formate dehydrogenase activity for regeneration of NADH. *Microb. Cell Fact.*, **11**: 7. https://doi.org/10.1186/1475-2859-11-7

Makkay, A.M., Krell, P.J., and Nagy, É., 1999. Antibody detection-based differential ELISA for NDV-infected or vaccinated chickens versus NDV HN-subunit vaccinated chickens. *Vet. Microbiol.*, **66**: 209–222. https://doi.org/10.1016/S0378-1135(99)00016-4

Marquardt, W.W., Snyder, D.B., Savage, P.K., Kadavil, S.K., and Yancey, F.S., 1985. Antibody response to Newcastle disease virus given by two different routes as measured by ELISA and hemagglutination-inhibition test and associated tracheal immunity. *Avian Dis.*, **29**: 71–79. https://doi.org/10.2307/1590695

Moharam, I., Asala, O., Reiche, S., Hafez, H., Beer, M., Harder, T., and Grund, C., 2021. Monoclonal antibodies specific for the hemagglutinin-neuraminidase protein define neutralizing epitopes specific for Newcastle disease virus genotype 2. VII from Egypt. *Virol. J.*, **18**: 86. https://doi.org/10.1186/s12985-021-01540-0

Oliveira, E.S. de, Silva, K.R., Fernando, F.S., Gonçalves, M.C.M., Fernandes, C.C., Borzi, M.M., Santos, R.M. dos, Tamanini, M. de L.F., Montassier, M. de F. da S., and Montassier, H.J., 2013. A liquid-phase–blocking concanavalin A enzyme-linked immunosorbent assay for the detection of antibodies against Newcastle disease virus in serum of free-ranging pigeons. *J. Vet. Diagn. Invest.*, **25**: 720–726. https://doi.org/10.1177/1040638713503656

Panshin, A., Shihmanter, E., Weisman, Y., Örvell, C., and Lipkind, M., 1997. Antigenic epitope characterization of matrix protein of Newcastle disease virus using monoclonal antibody approach: Contrasting variability amongst NDV strains. *Comp. Immunol. Microbiol. Infect. Dis.*, **20**: 177–189. https://doi.org/10.1016/S0147-9571(96)00026-4

Panshin, A., Shihmanter, E., Weisman, Y., Örvell, C., Kydyrmanov, A., Asanov, N., Daulbaeva, K., Sayatov, M., and Lipkind, M., 2000. Antigenic characterization of the nucleocapsid protein

of Newcastle disease virus by means of a new panel of monoclonal antibodies. *Comp. Immunol. Microbiol. Infect. Dis.*, **23**: 209–220. https://doi.org/10.1016/S0147-9571(99)00077-6

Rosano, G.L., and Ceccarelli, E.A., 2009. Rare codon content affects the solubility of recombinant proteins in a codon bias-adjusted *Escherichia coli* strain. *Microb. Cell Factories*, **8**: 41. https://doi.org/10.1186/1475-2859-8-41

Russell, P.H., and Almeida, J.D., 1984. A regular subunit pattern seen on non-infectious Newcastle disease virus particles. *J. Gen. Virol.*, **65**: 1023–1031. https://doi.org/10.1099/0022-1317-65-6-1023

Seal, B.S., King, D.J., and Meinersmann, R.J., 2000. Molecular evolution of the Newcastle disease virus matrix protein gene and phylogenetic relationships among the Paramyxoviridae. *Virus Res.*, **66**: 1–11. https://doi.org/10.1016/S0168-1702(99)00119-7

Seddon, A.M., Curnow, P., and Booth, P.J., 2004. Membrane proteins, lipids and detergents: Not just a soap opera. *Biochim. biophys. Acta Biomembr.*, **1666**: 105–117. https://doi.org/10.1016/j.bbamem.2004.04.011

Shtykova, E.V., Petoukhov, M.V., Dadinova, L.A., Fedorova, N.V., Tashkin, V.Y., Timofeeva, T.A., Ksenofontov, A.L., Loshkarev, N.A., Baratova, L.A., Jeffries, C.M., Svergun, D.I., and Batishchev, O.V., 2019. Solution structure, self-assembly, and membrane interactions of the matrix protein from Newcastle disease virus at neutral and acidic pH. *J. Virol.*, **93**: e01450– e01468. https://doi.org/10.1128/JVI.01450-18

Sørensen, H.P., and Mortensen, K.K., 2005. Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *J. Biotechnol.*, **115**: 113–128. https://doi.org/10.1016/j.jbiotec.2004.08.004

Suarez, D.L., Miller, P.J., Koch, G., Mundt, E., and Rautenschlein, S., 2019. *Newcastle disease, other avian paramyxoviruses, and avian metapneumovirus infections*. In: *Diseases of poultry,* (eds. D.E. Swayne, M. Boulianne, C.M. Logue, L.R. McDougald, V. Nair, D.L. Suarez, S. de Wit, T. Grimes, D. Johnson, M. Kromm, T.Y. Prajitno, I. Rubinoff and G. Zavala). 14th edn. pp. 109–166. https://doi.org/10.1002/9781119371199.ch3

Yune, N., and Abdela, N., 2017. Update on epidemiology, diagnosis and control technique of Newcastle disease. *J. Vet. Sci. Tech.*, **8**: 429. https://doi.org/10.4172/2157-7579.1000429

Zhao, N., Grund, C., Beer, M., and Harder, T.C., 2018. Engineered recombinant protein products of the avian paramyxovirus type-1 nucleocapsid and phosphoprotein genes for serological diagnosis. *Virol. J.*, **15**: 8. https://doi.org/10.1186/s12985-018-0924-8