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Cloning and Expression of the Nucleoprotein Gene (NP) of Newcastle Disease Virus (NDV) in *Escherichia coli* for Immunodiagnosis Application

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Abstract: The nucleocapsid protein (NP) of newcastle disease virus (NDV) is an important antigen to develop a serologic assay on account of its highly conserved sequences and high immunogenicity. This study aimed to express the gene of the NP of NDV in a heterologous system (*Escherichia coli*), using the appropriate vector. The NDV-NP protein was expressed as a fusion recombinant protein containing SUMO peptide and poly-histidine tags. This recombinant nucleocapsid protein (rNP) was expressed in a soluble form which was easily purified and showed the ability to react with chicken anti-NDV polyclonal antibodies. An indirect ELISA method based on the adsorption of an antigen composed by NP (rNP-NDV-ELISA) was developed. By comparing this rNP-NDV-ELISA with haemagglutination-inhibition test (HI) high and significant correlation with the HI (r = 0.83) was found. In addition, high sensitivity (88.9%), specificity (95.5%), accuracy (90.4%) and agreement (0.85) were obtained. In conclusion the results indicated that the cloning and expression procedures used in this study provided a rNP that shared the major epitopes with the homologous viral protein and has the potential to be applied in ELISA for the immunodiagnosis of the Newcastle Disease.

Key words: ELISA, expression, newcastle disease virus, recombinant nucleocapsid protein, SUMO, Cloning of NDV-NP gene, Immunodiagnosis Application

INTRODUCTION

Newcastle disease (ND) is an important infectious disease affecting poultry, since it can cause severe lesions and high mortality when non-vaccinated birds are infected by strains of high virulence (OIE Terrestrial Manual, 2012; Yusoff and Tan, 2001). This disease is worldwide distributed and besides the huge economic impacts on poultry production, it imposes to the countries which are suffering a ND outbreak, restrictions for the international trade of poultry products (OIE Terrestrial Manual, 2012).

The ND is caused by Newcastle disease virus (NDV) that is an Avian Paramyxovirus type 1(APMV-1). This virus is classified as belonging to the Mononegavirales order, Paramyxoviridae family, Paramyxovirinae subfamily and Avulavirus genus (Alexander, 2009). NDV particles are enveloped, pleomorphic, often spherical and has a diameter between 100 and 500 nanometers. The NDV genome is composed by a single-stranded non-segmented RNA of negative polarity and containing about 15.2 kilobases (Alexander, 2003.) Six major structural proteins are encoded by this genome; the nucleocapsid protein (NP), the phosphoprotein (P) and the large protein (L) which are associated with the nucleocapsid, the fusion glycoprotein (F), which constitutes the smallest projections in the surface of the virus particles, the glycoprotein hemagglutinin-neuraminidase (HN) and the matrix protein (M) (Mast and Demeestere, 2009; Yusoff and Tan, 2001).

Early diagnosis is critical point to achieve a more effective control of ND. Despite the importance and efficiency of direct diagnosis techniques, the immunodiagnostic methods are still important and useful to handle a large number of samples generating results in a short time. Immunodiagnostic techniques such as haemagglutination-inhibition test (HI test) and enzyme linked immunosorbent assay (ELISA) are widely used for the detection of anti-NDV antibodies (OIE Terrestrial Manual, 2012). Nowadays, HI test is the gold standard technique recommended by OIE Terrestrial Manual for ND diagnosis, (OIE Terrestrial Manual, 2012) while ELISA is more appropriate to analyze a high number of chicken serum samples providing a high sensitivity for detection of anti-NDV antibodies (de Oliveira *et al*., 2013; Singh *et al*., 2012; Yusoff and Tan, 2001).

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Currently, whole purified virus particles are used as the coating antigens in commercially available ELISA kits for detect anti-NDV antibodies. The purification of these viral particles requires the propagation of large quantities of virus in specific pathogen free (SPF) embryonated chicken eggs and depends on difficult and expensive ultra-centrifugation procedures (Miers *et al*., 1994; Rivetz *et al*., 1985) One alternative to this is the use of recombinant NDV proteins, such as NP or HN proteins, in an ELISA assay (OIE Terrestrial Manual, 2012; Yusoff and Tan, 2001). In fact, these NDV proteins were successfully expressed after cloning in baculovirus or *E. coli* system (Makkay *et al*., 1998; Errington *et al*., 1995) and used in indirect ELISA for measuring anti-NDV antibodies (Kim *et al*., 2009). However, no correlation was determined between those ELISA using recombinant NDV proteins and HI test in the studies performed so far. In addition, recombinant NP (rNP) has some positive attributes for application such as high amino acid sequence conservation and immunogenicity (Ahmad-Raus *et al*., 2009; Makkay *et al*., 1998; Kho *et al*., 2001; Errington *et al*., 1995).

There are a number of approaches toward the expression of heterologous viral recombinant proteins, such as mammalian and insect cells, yeast and bacteria and this latter has shown the ability to combine several advantages over the other procedures. However, bacterial expression systems present some drawbacks, specially the tendency to express most of the recombinant proteins in insoluble form and to provide fewer amounts of soluble recombinant proteins. Thus, several vectors harboring different gene-fusion systems were developed and used for the expression of heterologous recombinant proteins in bacterial systems, although the results have not been significantly better. Small ubiquitin like modifier (SUMO) tag has proved to be an efficient alternative to increase the expression of viral recombinant proteins, such as nucleocapsid, 3CL protease and membrane proteins from severe acute respiratory syndrome coronavirus (SARS-CoV) (Zuo *et al*., 2005).

Therefore, the present study aimed to express the NP of NDV strain LaSota in a soluble form to be later used as a target antigen in an indirect ELISA for the immunodiagnosis of NDV infection in chickens. Furthermore, we also compared the performance of this new ELISA with the gold standard HI test.

MATERIALS AND METHODS

Viral propagation: The NDV strain LaSota was inoculated into the chorioallantoic cavity of SPF embryonated chicken eggs, as recommended (Moro *et al*., 2000; OIE Terrestrial Manual, 2012). After 40 h of inoculation, the eggs were cooled at 4°C for at least 4 h and the allantoic fluid was harvested and immediately centrifuged at 5000 x g for 10 min, then stored at -70°C until use.

RNA extraction and NDV-NP gene amplification: Total RNA was extracted from 500 µL of the NDV infected allantoic fluid using the TRIzol method[a], according to the manufacturer's instructions. The reverse transcription reaction to obtain cDNA from the extracted viral genomic RNA was performed with a suitable kit[b] according to the manufacturer's instructions. Using a primer design software[c], specific primers [5- ATG TCT TCC GTA TTT GAT GAG-3 (forward) and 5- TCA ATA CCC CCA GTC GGT-3 (reverse)] were designed from the nucleotide sequence of the NP gene from LaSota strain (GeneBank: AY845400) to amplify the entire open reading frame of this gene. The PCR consisted of denaturation (94°C/1 min), annealing (57.5°C/1.5 min) and extension (72°C/2.5 min), for 35 cycles, followed by a final extension step of 72°C/10 min. The PCR products were analyzed by electrophoresis in a 2% agarose gel with ethidium bromide followed by imaging.

Cloning of NDV-NP gene and expression of recombinant NP (rNP): The purified PCR product was inserted in the pET SUMO vector[d] following the manufacturer's indications and transformed into TOP10F' *E. coli* competent cells[e]. Transformant cells were grown in Luria-Bertani (LB) medium containing 50 µg/mL of kanamycin[f] and the constructed plasmids were analyzed after purification by sequencing[g] in order to confirm the presence of NP gene of NDV strain LaSota. To express the rNP, a single colony of the *E. coli* ER 2566 containing the plasmid with the NP gene inserted was inoculated into 10 mL of 2 x TY media added to kanamycin[f] (50 µg/mL). Cells were grown at 37°C for 16 h under shaking (150 rpm). Two milliliters from this culture was inoculated into 100 mL of sterile 2 x TY media in order to reach the exponential growth (DO_{600} between 0.6-0.8). Protein expression was induced by the addition of 0.4 mM of Isopropyl-β-D-thiogalactopyranoside (IPTG)[h], followed by prolonged growth at 37°C for 16 h. For rNP purification, cultures were scaled from 0.5 to 1.0 L in 2 x TY media, purified in nickel-agarose resin[i] and characterized by SDS-PAGE and Western-blot (Laemmli, 1970). Purified rNP concentration was determined through the method described by Bradford[j]. (Bradford, 1976).

Positive and negative anti-NDV chicken sera: The NDV negative control serum was obtained from a pool of 20 serum samples collected from 21-days-old White Leghorn specific pathogen free (SPF) chicks. The NDV-positive control serum were obtained from a pool of 20 serum samples collected from SPF chickens hyperimmunized with NDV strain LaSota, according to the method previously described (Adair *et al*., 1989.)

Test serum samples: A total of 265 serum samples were tested. A set of 100 serum samples was collected from NDV-multi-vaccinated layers of White Leghorn and

Lohmann Brown lineages, reared in commercial poultries. Another set of 69 serum samples was collected from a flock of broilers of the COBB lineage, vaccinated with 1 dose of NDV strain LaSota. Finally, another set of 96 chicken serum samples from commercial poultries were provided by the Brazilian Reference Laboratory LANAGRO (Campinas-SP). Serum samples were collected aseptically and were stored at -20°C until use.

Hemagglutination inhibition test: This test was performed as previously described, Allan and Gough (1974) using suspension of chicken red blood cells at 0.5% and four hemagglutinating (HA) units of the NDV strain LaSota.

Application of rNP in indirect ELISA (rNP-NDV-ELISA): The rNP-NDV-ELISA was performed according to the previously described methodology (Gibertoni, 2005). Initially, a checkerboard titration was tested in indirect ELISA in four different concentrations of rNP as coating antigen ranging from 2 to 16 µg/mL. Each rNP concentration reacted with six serial two-fold dilutions of both positive and negative control sera (1:100 to 1:3200) in order to determine the optimal concentration of antigen to be used and the chicken serum dilution. The microplates were coated with 50 µL of the rNP purified and diluted in carbonate/bicarbonate buffer (0.05 M, pH 9.6) to reach the defined concentrations and then incubated overnight at 4°C. The next day, microplates were washed four times with PBS added to 0.02% Tween 20 (PBST) and the non-specific binding sites were blocked with 10% skimmed milk powder in PBST (100 µL/well) followed by incubation for 45 min at 37°C. After a new washing cycle, the serum samples were diluted in blocking buffer, added to the microplates (50 µL/well) and incubated for 1 h at 37°C. Then microplates were washed again and treated (50 µL/well) with rabbit anti-chicken IgG horseradish peroxidase conjugate diluted 1:2000 in blocking buffer for 1 h at 37°C. The substrate-chromogen solution used consisted of 0.05 M citrate-phosphate buffer (pH 5.0), 0.04% *o*-phenylenediamine (wt/vol) and freshly added 0.006% H_2O_2. The colorimetric reaction was stopped by adding HCl 2 M after 15 min and the optical density (OD) was measured at 492 nm using an ELISA reader. For each test serum sample, the mean OD (OD_{MTS}) was expressed in relation to the positive reference serum mean OD (OD_{MPRS}) and the negative reference serum mean OD (OD_{MNRS}), according to the relation between OD_{MTS} and OD_{MPRS} (S/P), following the formula S/P = OD_{MTS}-OD_{MNRS}/OD_{MPRS}-OD_{MNRS}. An S/P mean value determined for the 20 NDV-negative serum samples coupled with the three standard deviations was defined as cutoff.

Statistical analysis: The levels of antibodies sample presenting positive values (S/P values) measured in the rNP-NDV-ELISA were analyzed, using a specific software, by linear regression with HI antibody titers, measured as Log_{10} HI titer in order to determine the correlation coefficient (Pearson's r) between these two serologic tests.

Sensitivity, specificity and accuracy values were determined in comparison to the HI test. The agreement was calculated by the kappa index. All these parameters were determined by the program of basic statistical calculations for diagnostic test Miers *et al*. (1994).

RESULTS

Construction and expression of the rNP in *E. coli*: The entire open-reading frame of the NP gene from the NDV strain LaSota was generated by RT-PCR, using N+ and N- primers and the PCR product was inserted into the pET SUMO vector. The correct insertion of the NP gene which was cloned in this vector was confirmed by sequencing and 100% of identity was observed between this sequence and that from NDV strain LaSota deposited in GenBank (AY845400). In addition, the cloned NP gene was in frame and downstream to SUMO-Poly-Hystidine tag sequence of this vector. The rNP of NDV was expressed, after IPTG-induction, in two forms of recombinant protein; full size (≅66kDa) and truncated forms (≅53 kDa) (Fig. 1). Both forms of rNP seem to encompass the whole NP peptide (≅66kDa) or a truncated NP peptide (≅53 kDa) and a poly-hystidine tag plus SUMO peptide (≅13 kDa), as shown by PAGE-SDS and Western blot analysis; in this latter probed with monoclonal anti-Hys antibody as well as with chicken anti-NDV polyclonal antibodies (Fig. 1). In addition, the analysis of protein yield showed that 5.7 mg (from insoluble fraction) and 5.4 mg of purified rNP (from soluble fraction) was retrieved after the purification through nickel-agarose resin from bacterial lysate.

Development of an Indirect ELISA with the rNP expressed in *E. coli*: The positive anti-NDV chicken serum strongly reacted with the rNP in the rNP-NDV-ELISA ($0.5 \leq ODs \geq 2.5$) and in a serum dilution-dependent manner, whereas the NDV-negative serum showed low reactivity in this test ($0.08 \leq ODs \geq 0.26$) (Fig. 2). The NDV-positive and negative chicken sera could be distinguished at coating concentrations as low as 2 µg/mL and at a serum dilution as high as 1:3200. Differentiation between NDV-positive and negative sera enhanced slightly as the coating-antigen concentration increased from 2 to 16 µg/mL. Thus, an antigen concentration of 4 µg/mL of rNP and a single serum dilution of 1:400 were determined for subsequent analysis of chicken test sera in rNP-NDV-ELISA (Fig. 2). Additionally, the analysis of 20 serum samples from NDV-negative poultry flock (unvaccinated and uninfected) showed a cutoff value of S/P = 0.112.

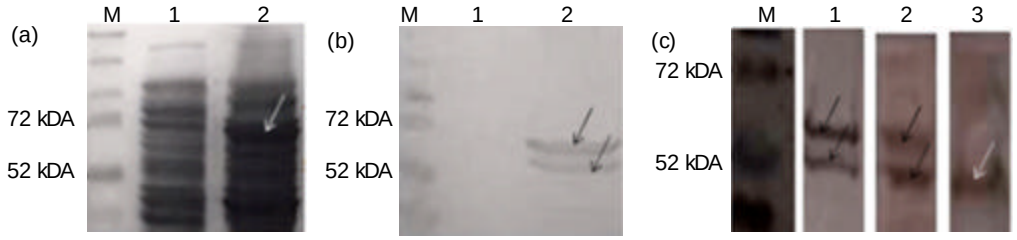

Fig. 1: SDS-PAGE and Western blot analysis of the rNP of NDV expressed in *E. coli*. (A) SDS-PAGE. Column M-molecular weight marker; ₀Column 1-Non-induced fraction of *E. coli* culture (negative control of the expression); Column 2-*E. coli* induced fraction contained the rNP expressed (white arrow). (B) Western blot of rNP expressed in *E. coli* probed with monoclonal antibodyₙ. Columns M, 1 and 2 received the same reagents as described for (A). Black arrows indicate rNP in a full size and in a truncated form; (C) Western blot of the NDV-NP; Column M-molecular weight marker; ₀Columns 1 and 2-purified fraction of rNP in the Nickel-Agarose resin, probed with monoclonal antibody and polyclonal chicken anti-NDV antibodies, respectively. Column 3-PEG concentrated suspension of NDV strain LaSota. Black arrows indicate rNP in a full size and in a truncated form. White arrow shows the native NDV-NP

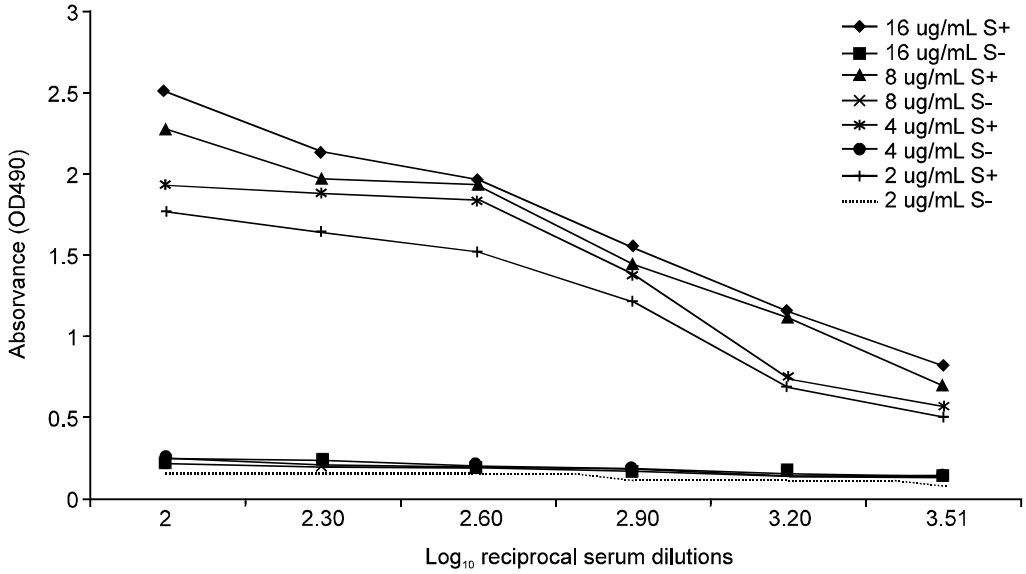

Fig. 2: Result of the checkerboard titration in Indirect ELISA of different concentrations of rNP antigen against different dilutions of NDV-positive and NDV-negative reference sera

Comparative analysis between rNP-NDV-ELISA and HI test: Antibody levels detected by rNP-NDV-ELISA in a collection of 265 sera from commercial poultry flocks that were multi-vaccinated against NDV were compared with those obtained in the HI test (Table 1). Sensitivity, specificity and accuracy values for the rNP-NDV-ELISA were 88.9, 95.9 and 92.8%, respectively. Additionally, the positive and negative predictive values were 94.5 and 91.5%, respectively and the agreement was 0.85 (kappa index). A total of 104 chicken serum samples were classified as positive for anti-NDV antibodies and 142 were classified as NDV-negative in both serological tests. Conversely, nineteen sera showed discordant results so that six serum samples were positive only by the rNP-NDV-ELISA, while thirteen serum samples were positive only by the HI test (Table 1).

A linear regression analysis showed a good correlation ($r = 0.83$) between the levels of antibodies detected by the rNP-NDV-ELISA as S/P values and those determined by the HI test as Log_{10} HI anti-NDV antibody titer ($p<0.0001$) (Fig. 3).

DISCUSSION

In poultry industry, a great number of serum samples must be analyzed by different serodiagnostic procedures in a simple and economical fashion. Thus, the availability of cheap, efficient and reproducible immune reagents is essential for the development of serological

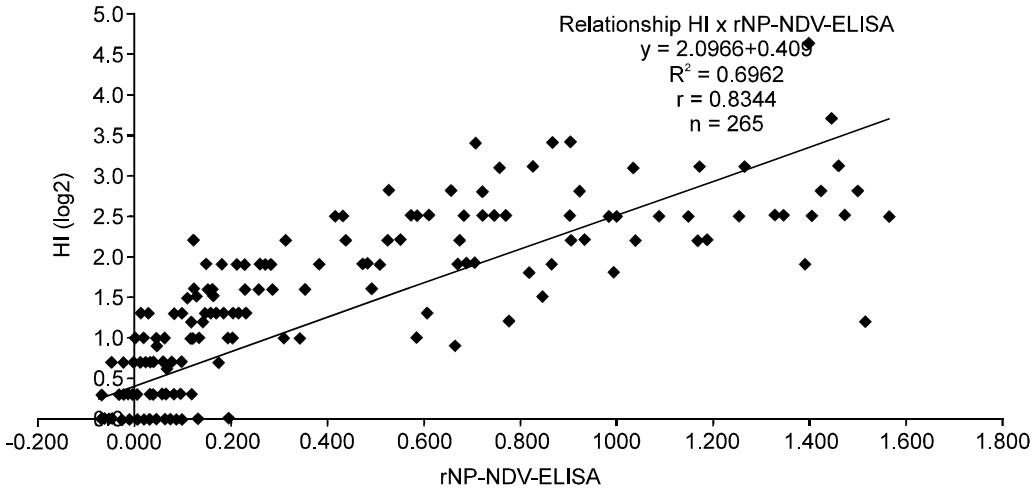

Fig. 3: Correlation between antibody levels detected by the HI test (Log_{10} Titre) and the rNP-NDV-ELISA (Value S/P)

Table 1: Comparison between the rNP-NDV-ELISA and HI test for the detection of anti-NDV antibodies in chicken sera

	HI		
rNP-NDV-ELISA*	Positive	Negative	Total
Positive	104	6	110
Negative	13	142	155
Total	117	148	265

*Relative sensitivity = 88.9%; relative specificity = 95.9%; relative accuracy = 92.8%; positive predictive value = 94.5%; Negative predictive value = 91.6%; kappa index = 0.85 and correlation coefficient (r) of rNP-NDV-ELISA using the HI as a reference = 83%

tests. Furthermore, the preparation of ideal serological reagents depends on readily availability of pure antigen suspensions. At the moment, most of the commercial kits for the detection and monitoring of chicken NDV antibodies are based on ELISAs that use purified whole viral particles as solid-phase attached antigens. The propagation of this virus in embryonated eggs and the purification of whole viral particles by ultra-centrifugation methods are very time-consuming and expensive. Therefore, molecular cloning and expression of recombinant NDV protein, as described here, provide a simple and efficient method for antigen preparation to be used in the serodiagnosis of ND.

The NDV-NP gene has been cloned and expressed in baculovirus system and used as a coat antigen in different ELISA formats to detect anti-NDV antibodies (Errington *et al*., 1995; Kho *et al*., 2001). More recently, a rNP expressed in *E. coli* system was also used in indirect ELISA and compared with an in-house-ELISA performed with whole virus particles (Kim *et al*., 2009). However, parameters of sensitivity, specificity, accuracy and agreement were not determined in this prior study, including the comparison with HI test, which currently is the gold standard for the serology of anti-NDV antibodies (OIE Terrestrial Manual, 2012).

The cloning and expression of the NDV-NP in the SUMO system used in this study led to a significant increase in the amount of soluble rNP of NDV fused to the poly-Hys tag plus SUMO peptide. Indeed, this expression system has been successfully applied for the expression of soluble forms of envelope glycoprotein G of rabies virus Singh *et al.* (2012) and S, N and CL proteins of SARS coronavirus (Zuo *et al*., 2005).

The molecular mass from the two forms of rNP was determined by PAGE-SDS and Western blot analysis and was compatible with the molecular weight of the full size (66kDa) or the truncated (53kDa) forms of NP peptide fused with the poly-hystidine tag plus SUMO peptide (13kDa). The antigencity of rNP expressed by this system when characterized here by Western blot analysis, demonstrated similarity between these two forms of rNP and the natural viral NP, since the polyclonal antibodies generated by chickens immunized with NDV were cross-reactive with the recombinant *E. coli* expressed NP forms, as demonstrated in this study.

The results of rNP-NDV-ELISA generated through the analysis of 265 chicken sera showed good agreement index, high sensitivity, specificity and accuracy values in addition to a strong correlation with HI test. However, there were some discordant results between rNP-NDV-ELISA and HI test regarding to the identification of some NDV-positive or NDV-negative sera. This discordance between them could be explained by the differences in the viral antigenic target of each serological test, e.g., NP for the ELISA and HN for HI test used here. Thus, it could be supposed that there are two subsets of thirteen and six chicken serum samples, among the serum samples tested, that reacted preferentially with HN or NP and showed positive results only in HI test or in rNP-NDV-ELISA, respectively.

The rNP expressed in this study in a *E. coli* system showed similar performance with regard to antigencity and to the many other serological parameters as have been reported for other rNP preparations cloned and expressed in baculovirus (Makkay *et al*., 1998; Errington

et al., 1995) or other *Escherichia coli* systems (Kim *et al*., 2009) also used as solid-phase antigen in indirect ELISA methods. However, higher sensitivity was obtained in some of these previous studies, (Makkay *et al*., 1998; Errington *et al*., 1995) though a lower number of chicken serum samples were tested.

Conclusion: In conclusion the results indicated that the cloning and expression procedures used in this study provided a significant amount of a rNP protein of the NDV strain LaSota that shared the major epitopes with the homologous viral protein. In addition, the ELISA developed in this study using this rNP was able to efficiently detect and measure antibodies against NDV in sera of chickens. These data highlight the potential of this rNP-NDV-ELISA to be used in the serodiagnosis of NDV in poultry flocks, given its sensitivity, specificity and the ability to analyze more rapidly and practically a large number of serum samples.

Source and manufacturers:

a: Life Technologies, Grand Island, NY
b: Life Technologies, Foster City, CA
c: Gene Runner, version 3.05: http://www.generunner.com
d: Life Technologies, Carlsbad, CA
e: Life Technologies, Carlsbad, CA
f: Sigma-Aldrich, St. Louis, MO
g: ABI 3730 XL DNA Analyzer, Applied Biosystems, CA
h: Invitrogen, Carlsbad, CA
I: GE Healthcare, Buckinghamshire, United Kingdom
j: BioRad, Hercules, CA
k: Costar, Corning, NY
l: Sigma-Aldrich, St. Louis, MO
m: BioRad, Hercules, CA
n: Excel 2007, Microsoft Corp., Bellevue, WA
o: Sigma-Aldrich, St. Louis, MO
p: Thermo Scientific, Rockford, IL

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