www.czasopisma.pan.pl PAN POLSKA AKADEMIA NAUK www.journals.pan.pl

Polish Journal of Veterinary Sciences Vol. 22, No. 3 (2019), 531–540

DOI 10.24425/pjvs.2019.129961

Original article

Development of a recombinant NP protein based indirect ELISA for the detection of antibodies against Newcastle disease virus and differentiation of infected or recombinant vaccine immunized chickens

K. Domańska-Blicharz[1], J. Tyborowska[2], J. Sajewicz-Krukowska[1], M. Olszewska-Tomczyk[1], Ł. Rąbalski[2], K. Kucharczyk[3], B. Szewczyk[2], K. Śmietanka[1]

[1] Department of Poultry Diseases, National Veterinary Research Institute, Al. Partyzantów 57, 24-100 Puławy, Poland
[2] Department of Recombinant Vaccines, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Abrahama 58, 80-309 Gdańsk, Poland
[3] BioVectis Ltd., Pawińskiego 5A, 02-106, Warsaw, Poland

Abstract

Newcastle disease (ND) is a highly contagious and economically important disease in the poultry industry caused by avian avulavirus-1, historically known as Newcastle disease virus (NDV). Control of ND primarily relies on prophylactic vaccination of flocks, and many vaccines are available on the market, both conventional and more recently introduced new generation recombinant types. To assess the protection level achieved by vaccination ELISA tests are typically used, they also are to track an infection with field strains in non-vaccinated flocks. Special modifications of ELISA can be used as a screening tool to detect infection in flocks vaccinated with new generation vaccines. In this study, we have developed an ELISA test for the detection of antibodies against the nucleoprotein (NP) of NDV and for differentiation of chickens vaccinated with commercial and prototype in-house recombinant vector vaccines from those infected with field NDV strains. The NP gene of LaSota NDV strain expressed in a baculovirus vector was used as a coating antigen in the ELISA. The developed test was optimized, validated and compared to other serological tests. The sensitivity, specificity and accuracy of recombinant NP protein-based ELISA were respectively 96.1%, 96.3%, and 96.2%. Inter-rater (kappa) agreement between the NP-ELISA and the gold standard HI test was calculated to be 0.995. In our comparisons, commercially available ELISA tests revealed different specificities ranging from 95.5–100% and sensitivities at variance, ranging from 90.1 to 99.0%. A high level of maternally derived antibodies was measured in the serum of 1-day-old broilers in the NP-ELISA assay. These antibodies had disappeared and were undetected at 3, 5 and 6 weeks post-vaccination but birds became positive again at 2 weeks after control infection with a velogenic NDV strain. In SPF chickens, antibodies against NP protein were detected only after a challenge. The recombinant NP protein-based ELISA test is sensitive, specific and accurate when compared to the gold standard HI test and commercially available kits. Moreover, the method could be also used for the differentiation between vaccinated and infected birds.

Key words: Newcastle disease virus, serology, Enzyme-Linked Immunosorbent assay, nucleoprotein, differentiation

Correspondence to: K. Domańska-Blicharz, e-mail: domanska@piwet.pulawy.pl

Introduction

Avian avulavirus-1, historically known as Newcastle disease virus (NDV) is the causative factor for highly contagious and economically important Newcastle disease (ND) in poultry species. The virus, also abbreviated as AMPV-1 (avian paramyxovirus type 1) belongs to the *Orhoavulavirus* genus in the family *Paramyxoviridae*, order *Mononegavirales* (Amarasinghe et al. 2017, Rima et al. 2018). The NDV genome is a single-stranded negative-sense RNA of about 15.2 kb long. It encodes 6 essential proteins: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and large polymerase protein (L) (Krishnamurthy and Samal 1998, de Leeuw and Peeters 1999). The HN and F proteins are surface glycoproteins, mostly involved in virus virulence and tissue tropism while the NP protein is important viral structural protein and together with P, and L proteins form the polymerase complex protein. Recently, it has been shown that the polymerase complex also modulates the virulence and pathogenicity of NDV in chickens (Rout and Samal 2008). Depending on the severity of induced disease in chickens, NDV are divided into several pathotypes: asymptomatic, lentogenic (avirulent/mild respiratory symptoms), mesogenic (moderately virulent), and velogenic (highly virulent), the last being either viscero- or neurotropic.

Outbreaks of virulent NDV are reported every year in different parts of the world. In the EU countries, the last major epidemics occurred in the 1990s, with 239 outbreaks mainly in the Benelux countries and Germany, and in 2000 with 257 outbreaks in Italy (Alexander 2000, Capua et al. 2002). Of the recent outbreaks of NDV, a few cases were identified in Romania and Bulgaria in 2016 and 2017 and in Sweden in 2017 but due to swift action by the administrations of those countries, the virus did not spread to a larger geographical area (https://www.oie.int/wahis_2/public/wahid.php//Diseaseinformation). In Poland, ND appeared in 1942, causing an epidemic that lasted until 1974. Since 1975 Poland has been a country free from Newcastle disease (Marek 1972, Minta 1998). However, at the beginning of the 1990s NDV belonging to the lentogenic or mesogenic pathotypes were identified in the country (Minta 1998).

Control of ND primarily relies on prophylactic vaccination of flocks and administrative measures (culling and establishment of protection and surveillance zones). In the EU, the available vaccines include live vaccines based on lentogenic NDV strains, inactivated (killed) vaccines used for booster immunization of laying birds and recombinant vector vaccines, the last having been used more often recently (Miller and Koch 2013). Serological tests are broadly used to assess the protection level achieved by vaccination and to track the infection with field strains in non-vaccinated flocks. Among serological tests used in the case of ND, virus neutralisation and hemagglutination inhibition (HI) assays are considered the gold standard. However, they are time-consuming and labor intensive (Czifra et al. 1996, Williams et al. 1997, Xu et al. 1997). On the other hand, the enzyme linked immunosorbent assay (ELISA) is fast and easy tool for the detection and quantitative measurement of anti-NDV antibodies and is used in routine diagnosis in many commercial services (Miller and Koch 2013). Additionally, special modifications of ELISA can be used as a screening tool to detect infection in flocks vaccinated with new generation vaccines (Errington et al. 1995).

In this study, we have developed an ELISA test for the detection of antibodies against NP protein of NDV and differentiation of chickens vaccinated with recombinant vector vaccines from those infected with field NDV strains. The recombinant vaccines used in our investigations included both a commercial vaccine [turkey herpesvirus (HVT) with a cloned F gene of NDV, Vectormune ND, Ceva, Hungary, abbreviated to HVT-F here] and our prototype in-house vaccine (HVT with cloned F and HN genes of NDV, abbreviated as HVT-F-HN). The NP gene of LaSota NDV strain expressed in a baculovirus vector was used as a coating antigen in the ELISA. A panel of chicken sera previously determined as negative or positive by HI test as well as sera from chickens vaccinated with recombinant HVT-F and HVT-F-HN and subsequently infected with pathogenic NDV were analysed using the ELISA test developed in this study.

Materials and Methods

Virus and its NP gene preparation

NDV LaSota strain was inoculated in embryonated SPF chicken eggs by the allantoic route. The allantoic fluids were then collected from dead and surviving eggs and checked for the presence of NDV by hemagglutination assay (HA) using 1% chicken red blood cells (OIE 2017). Viral RNA was extracted from pooled allantoic fluid using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's recommended procedure. The cDNA was prepared from the extracted RNA using the Thermoscript RT kit (Invitrogen, USA). Further, amplification of the NP gene with incorporated *SalI* and *HindIII* restriction sites in NP gene-specific forward and reverse primers (N-for: 5'-AAAAGTCGACATGTCTTCCGTATTTGATGA-3',

N-rev: 5’-AAAAAAGCTTTCAATACCCCCAGTCGT-3’) was carried out. PCR amplification conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 10 min.

Cloning, expression and purification of recombinant NP protein

The PCR product obtained as described above was cloned into pJET plasmid (ThermoFisher Scientific, USA) using CloneJet PCR cloning kit (ThermoFisher Scientific, USA) resulting in pJET-NP-NDV. Its sequence was confirmed by sequencing and used for obtaining recombinant baculovirus. The pFastBac1 shuttle vector (ThermoFisher Scientific, USA) and NP gene contained in the pJET-NP-NDV plasmid were digested simultaneously with *SalI* and *HindIII* and connected with T4 DNA ligase under the promoter of the polyhedrin gene. The resulting pFastBac1-NP-NDV plasmid was transformed into *Escherichia coli* DH10Bac competent cells for transposition into a bacmid. The appropriate bacterial colonies grown on LB agar with 100 µg/ml kanamycin, 7 µg/ml gentamycin, and 150 µg/ml X-gal (Carl Roth, Germany) were selected and obtained bacmid was purified with the PureLink HiPure Plasmid Midiprep kit (ThermoFisher Scientific, USA). Purified recombinant bacmid was used for *Spodoptera frugiperda* Sf9 (ATCC) insect cell transfection using Lipofectin (ThermoFisher Scientific, USA). The Bac-NDV-NP recombinant baculovirus was propagated in monolayers of insect cells grown at 27°C to get a higher amount of virus and concomitant abundantly expressed NP protein used subsequent for the design of the NP-based ELISA test. The identity, quantity and purity of the recombinant NP protein were analyzed using SDS-PAGE electrophoresis and Western blot. The resulting NP protein was mostly produced in the membranes of insect cells and was insoluble, and the approach of its preparation for the newly developed ELISA test is described below.

Chicken serum samples

A total of 396 chicken serum samples were collected from flocks from different regions of Poland. They originated not only from broiler chickens but also from commercial layers and broiler–breeder flocks sampled at different ages. Some of these chickens were immunized against ND. Blood samples were centrifuged at 1200 x g for 5 min for separation of the sera which were then stored below −20°C until use. All of them were first checked in an HI test and then in the recombinant NP protein based ELISA. As some of them were in small volumes, 147 sera were used to compare the results obtained in other commercial tests.

Vaccination experiment

One-day-old Specific Pathogen Free (SPF) White Leghorn (VALO BioMedia, Germany) and commercial Ross 308 chickens were separated into three groups of ten birds each. One group of each chicken type was vaccinated on the first day of life with an in-house recombinant turkey herpesvirus expressing the F and HN proteins of NDV (HVT-F-HN) (manuscript in preparation). The second group of chickens was immunized with the commercial Vectormune ND (HVT-F) vaccine with the dose recommended by the manufacturer. The remaining group was PBS-inoculated. At 6 weeks of age, all birds were infected via intraocular and intranasal routes with $10^6 EID_{50}$ of APMV-1/chicken/Poland/Radom/70 (velogenic NDV strain, class II, genotype IV). Blood samples were collected at day 1 (before vaccination) and at 3, 4, 6 and 8 weeks post vaccination (wpv). Serum samples were tested using HI, three commercially available ELISA kits and the in-house NP-ELISA test.

Hemagglutination inhibition and commercial ELISA tests

An HI test was carried out according to the procedure in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE 2017). The HA titer of the NDV LaSota antigen was adjusted by dilution to contain four units of HA activity. The collected serum samples were also screened with the commercially available ELISA tests: Newcastle Disease Virus Antibody Tests (IDEXX Laboratories, USA), ProFlock NDV Antibody Tests (Synbiotics, USA) and ID Screen ND Competition (IDvet, France) according to the manufacturer’s procedures.

NP-ELISA procedure

The resulting NP protein was insoluble, so whole Bac-NDV-NP infected insect Sf9 cells were washed with PBS and cell pellets were frozen at −80°C. Before being coated onto the plate, cell pellets were resuspended in PBS and NP antigen was released into a more homogenous form by triple ultrasonification at 8 microns of amplitude for 45 s (MSE, Poland). Flat-bottom polystyrene microtiter ELISA plates (Nalge Nunc International, Denmark) were coated with different amounts of cell lysates containing recombinant NP antigen using carbonate bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. The plates were washed three times with phosphate buffer saline (PBS) containing Tween

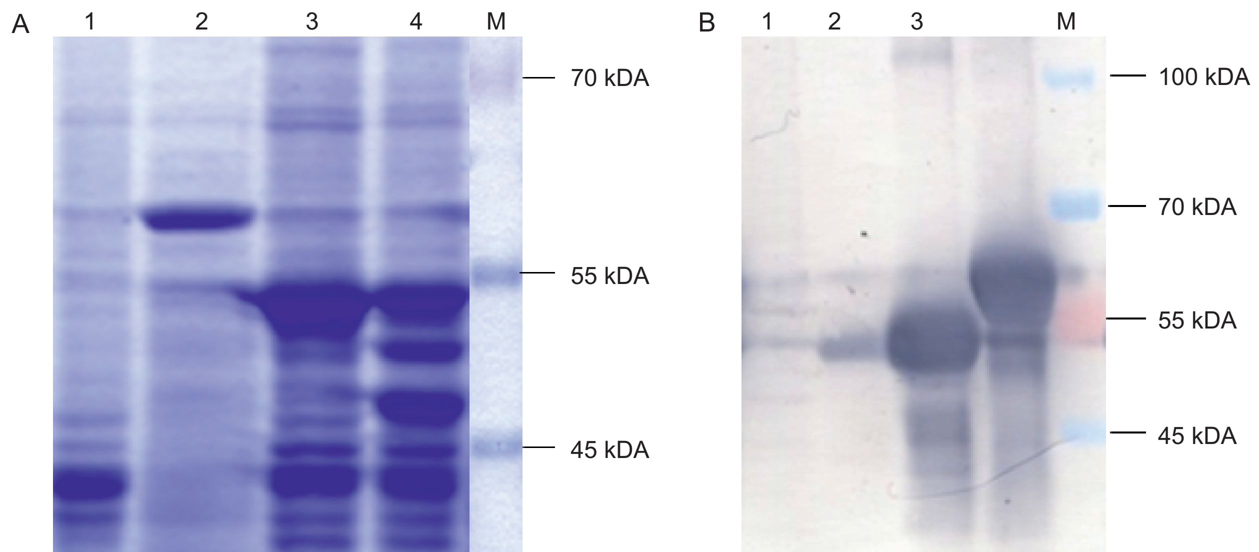

Fig. 1. Expression analysis of recombinant nucleocapsid protein (NP) of Newcastle disease virus (NDV).
A. The SDS-PAGE (stained with Coomassie Brilliant Blue dye) analysis of NP protein of NDV expressed in Sf9 insect cell line. Lane 1: membranes of uninfected insect cells; Lane 2: cytosol harvested from culture of insect cells; Line 3: membranes of insect cells harvested 2 days post infection (dpi); Lane 4: membranes of insect cells harvested 4 dpi; Lane M: protein molecular marker.
B. Western blot analysis of NP protein of NDV LaSota strain expressed in insect cells using chicken anti-NDV polyclonal serum. Lane 1: membranes of uninfected insect cells; Lane 2: membranes of insect cells harvested 2 days post infection (1:100 dilution); Lane 3: membranes of insect cells harvested 2 days post infection (undiluted); Lane M: protein molecular marker.

20 (PBST, 0.05%) and then blocked with 10% fetal calf serum (ThermoFisher Scientific, USA) for another 1 h at 37°C and again washed three times with PBST. Chicken sera samples were diluted serially and incubated for 1 h at 37°C. After subsequent washing with PBST in three iterations, the plates were filled with 100 µl of rabbit anti-chicken IgG horseradish peroxidase conjugate (Sigma, USA) diluted 1:5000 and stored for 1 h. This concentration of anti-chicken antibodies was selected after prior checkerboard titration. Tetramethylbenzidine substrate for horseradish peroxides was applied to each well of the plate in 100 µl volume and the color reaction was stopped after 30 min of incubation by adding 100 µl of 0.5M H_2SO_4. The plates were then read at 405 nm in an ELISA Sunrise microplate reader (Tecan, Switzerland). The optimal coating antigen concentration, serum and IgG horseradish peroxidase conjugate were determined by serial dilutions.

Determination of ELISA antibody titers

ELISA antibody titers determination was based on the method described by Snyder et al. (Snyder et al. 1983). Briefly, negative in HI test serum samples were tested in a few serial dilutions and obtained OD values used for the construction of a positive-negative threshold (PNT) baseline (the mean and 3 standard deviation). Obtained PNT baseline was then used for calculation of the observed antibody titers. The absorbance of tested serum was corrected by subtracting the average absorbance of internal negative control serum dilutions from the same trial. The positive sera samples were titrated serially and similarly corrected. The presence or absence of specific antibodies in tested serum was determined using the formula:

$$SP\,ratio = (OD_{of\,sample} - OD_{of\,negative}) / (OD_{of\,positive} - OD_{of\,negative})$$

The coefficient of correlation (r) between log 10 end titre and S/P ratio (at serum dilutions of 1:100 – 1:16,000) indicating maximum value of the regression equation [y=a+bx, where y is the log 10 (S/P ratio) of the tested serum at selected dilution, a is the intercept of regression quotation, and b is the slope of line] for prediction log 10 end titre by single dilution ELISA. Predicted titre was determined from standard curve by solving the regression line equation (Snyder et al. 1983).

Evaluation of test performance

Assessment against the gold standard (HI test) was made and a receiver-operating characteristic (ROC) curve was plotted to evaluate the diagnostic power of the newly developed ELISA test. The area under the curve (AUC), Youden's index cut-point value, sensitivity and specificity and test efficiency were measured using Epi Tools software available online (Diagnostic test evaluation and comparison, http://epitools.ausvet.com.au). The Epi Tools package was also used to compare the performance of the recombinant NP-protein ELISA and commercially available tests evaluated in the same population.

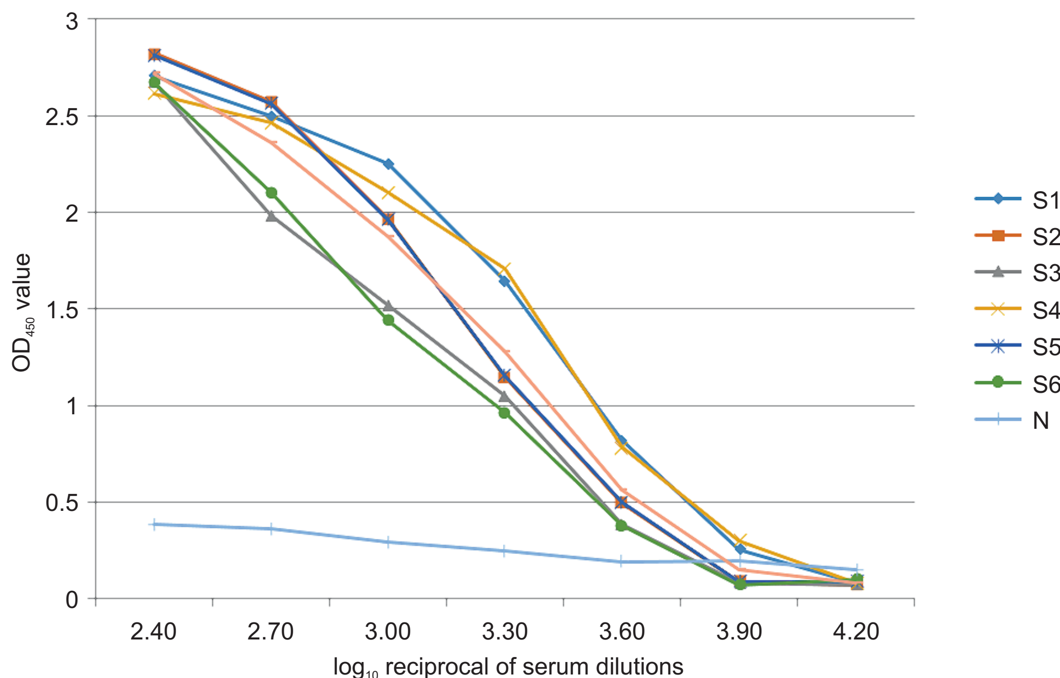

Fig. 2. Positive-negative threshold (PNT) baseline (N) with different field samples. S (1–6) represent different positive chicken samples at different dilutions.

Ethical statements

Serum sampling for ELISA optimization/validation was under the supervision of veterinarians, who took the samples used among different samples taken as part of their routine work (i.e. in screening flocks for evaluation of applied vaccine efficacy or presence of any infections) and did not require the approval of the Ethics Committee. All other experiments on chickens were approved by the Local Animal Ethics Committee in Lublin (permission numbers 51/2012 and 13/2015).

Results

Expression of the recombinant NP protein and its characterization

The gene amplified from NDV cDNA NP was successfully recloned from pJET plasmid to transfer into pFastBac1 shuttle vector. Subsequently, recombinant bacmids bearing the NDV gene under the baculovirus polyhedrin promoter were obtained by transposition of *E. coli* DH10 cells. The resulting baculovirus was used for transfection of Sf9 line insect cells. The recombinant NP protein was around 53-55 kDa in size and its highest amount was detected in membrane fractions of insect Sf9 cells on the 2nd day of infection and decreased in intensity over time. A much lower quantity of it was detected in the cytosol of Sf9 cells (Fig. 1A). Expression of NP NDV in insect cells was confirmed by Western blot (Fig. 1B).

ELISA optimization

The optimal concentration of coating antigen – recombinant NP protein was determined through checkerboard ELISA titration and the best was found to be diluted 1:200 (about 59 ng/well).

Serial dilutions of positive sera samples were tested and measured OD values were plotted on Y-axis against different sera dilutions given in logarithmic value in X-axis. The point where the sample line cuts the PNT line was taken as the titer of the sample (Fig. 2).

A single serum dilution (1:500) using 6 randomly selected samples and observed titers determined by serial dilution was checked in the recombinant – NP-based ELISA (Fig. 3). Regression analysis gave a regression line with the slope and intercept of 0.5402 and 3.3882 respectively. The correlation coefficient was calculated to be 0.962. The predicted titers were determined using the equation

$$\log10(S/P) = 0.5402x\log10titre+3.3882.$$

Diagnostic sensitivity, specificity and accuracy of the recombinant – N-protein-based ELISA

Comparisons of the results from 396 serum samples obtained from the newly developed recombinant NP-ELISA and the HI test as the gold standard are given in Table 1. The sensitivity, specificity and accuracy of recombinant – NP-protein-based ELISA were calculated as 96.1, 96.3% and 96.2% respectively. Examina-

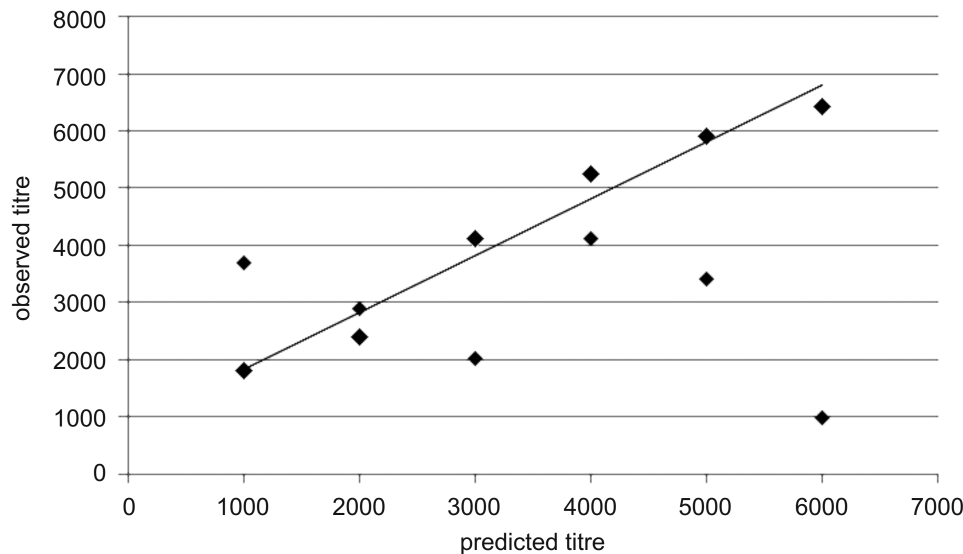

Fig. 3. Relationship between the observed antibody titers of the serum samples obtained from serial dilution ELISA and their corresponding predicted antibody titers obtained from single dilution ELISA at a 1:500 dilution.

Table 1. Results obtained with recombinant nucleocapsid protein (NP) - based ELISA assay developed in this study and hemagglutination inhibition (HI) test of field serum samples from chicken flocks in Poland.

Recombinant NP-ELISA	HI		
	Positive	Negative	Total
Positive	276	4	280
Negative	11	105	116
Total	287	109	396

Sensitivity = 276/287 x 100 = 96.1%
Specificity = 105/109 x 100 = 96.3%
Accuracy = 381/396 x 100 = 96.2%

tion of serum samples collected from different vaccinated and unvaccinated field flocks showed an agreement of 99.5% with the recombinant – NP-ELISA and HI assays (kappa value = 0.995).

ROC analysis for recombinant – NP-based ELISA calculated an AUC value of 0.983. The 95% confidence interval of the AUC ranged from 96.4 to 100%. The assay was validated further using the commercial ELISA kits previously referenced. Table 2 presents parameters evaluated in ROC analysis of three selected commercial ELISA kits. All assays were tested on the same pool of 147 serum samples (102 positive and 45 negative in the HI test).

Detection of NP antibody level following NDV infection and vaccination

To assess the recombinant – NP-protein-based ELISA and its possibility to discriminate between birds vaccinated with recombinant vaccines expressing the F or F-HN proteins and birds infected with NDV, we followed the humoral immune response in SPF and broiler chickens immunized with the recombinant commercial and in-house HVT-NDV vaccines on the first day of life and then infected with a Polish velogenic strain of NDV at 6 weeks of age. The results obtained were compared with those of the HI test (Table 3). The antibodies against NP protein were not detected in sera of immunized SPF chickens but they appeared after 2 weeks post challenge and the mean titer was 1305 ± 635 and 4531 ± 1093 (mean ± SD) in birds vaccinated with the Vectormune and in-house vaccines, respectively. HI antibodies were present in SPF chickens vaccinated with recombinant HVT-F-HN vaccine with the geometric mean log2 titer between 3.9 and 5.5 at 3–6 weeks after vaccination and they increased to geometric mean log2 titer of 5.9 after 2 weeks post infection with velogenic field NDV strain. The mean geometric level of HI antibodies in sera of Vectromune immunized SPF chickens was 5.1 at 6 weeks after vaccination and similarly it increased to 6.3 after 2 weeks post challenge. A slightly different model of antibody dynamics was observed in broilers. In serum of 1 day-old chickens a high level of maternally derived antibodies were observed using both HI and NP-ELISA tests. In subsequent terms, titers of NP antibodies de-

Table 2. Receiver-operating characteristic (ROC) analysis of the commercial and recombinant nucleocapsid protein (NP) - based ELISA tests.

Parameter	Recombinant NP-ELISA	Commercial ELISA 1	Commercial ELISA 2	Commercial ELISA 3
AUC[a]	0.983	0.983	0.989	0.990
AUC at 95% confidence interval	0.964–1.0	0.969–0.998	0.976–1.0	0.978–1.0
Sensitivity (%)	94.2	90.2	98.0	95.1
Specificity (%)	97.8	97.8	95.6	100
Youden's Index cut-point	0.23	0.183	1.977	0.532

[a] AUC: area under concentration-time curve

Table 3. Detection of antibodies to hemagglutinin-neuraminidase (HN) and nucleocapsid protein (NP) in sera specific pathogen free (SPF) and broiler chickens vaccinated on the 1st day of life with commercial (Vectormune ND®, Ceva) or in-house recombinant turkey herpesvirus with a cloned F and HN genes of Newcastle disease virus (HVT-F-HN) vaccines and challenged 6 weeks after vaccination.

Weeks post vacc.	HVT-F-HN vaccinated				Vectormune ND® vaccinated			
	SPF chickens		Broiler chickens		SPF chickens		Broiler chickens	
	HI test[a]	NP-ELISA[b]	HI test	NP-ELISA	HI test	NP-ELISA	HI test	NP-ELISA
0	n/a	n/a	6–8 20/20	3456 20/20	n/a	n/a	6–8 10/10	3456 10/10
3	2–6 18/20	- 0/20	2–4 4/20	- 0/20	- 0/10	- 0/10	3–4 7/10	777 5/10
5	3–7 19/20	- 0/20	2–3 0/20	- 0/20	2–4 1/10	- 0/10	3–5 9/10	- 0/10
6[c]	3–7 19/20	- 0/20	2–6 5/20	- 0/20	4–6 10/10	- 0/10	4–6 10/10	- 0/10
8	7–10 10/10	4531 10/10	7–9 10/10	1735 6/10	6–7 10/10	1305 10/10	n/a	1984 9/10

[a] Hemagglutination inhibition (HI) titers of 4 or greater were considered as positive, data shown: log2 range, pos/total
[b] recombinant ELISA titers calculated as described in the text, data shown: HI titer range, number positive/total
[c] challenge with a velogenic Polish field Newcastle disease virus (NDV) strain

creased to an undetectable level as measured in the NP-ELISA test in sera of broilers immunized with both recombinant HVT-NDV vaccines. In sera of broilers challenged with a velogenic Polish field NDV strain, antibodies against the NP protein increased in almost all animals (in the Vectormune-immunized group) or in single animals (vaccinated with the in-house recombinant HVT-F-HN). HI antibodies gradually decreased but from 5/6 week post immunization an increase in their titers was observed.

Discussion

Different agents are used for coating ELISA plates, the most broadly applied is the whole virus antigen but different recombinant viral proteins are also adopted (Jestin et al. 1989, Errington et al. 1995, Czifra et al. 1996, Makkay et al. 1999, Phan et al. 2013, Das and Kumar 2015). In general, the use of the whole virus as an antigen for the binding of anti-NDV antibodies gives good results but requires a large amount of live virus to be produced, inactivated and purified. The alternative is *in vitro* production of viral proteins which besides offering even better specificity and sensitivity could also grant the possibility to differentiate infected specimens from those immunized with recombinant vector NDV vaccines (Makkay et al. 1999). The NP, HN and P proteins of NDV were used as recombinant coating antigens (Errington et al. 1995, Makkay et al. 1999, Mohan et al. 2006, Phan et al. 2013, Das and Kumar 2015). The HN protein is highly conformational-dependent and thus problematic with proper expression in a prokaryotic system, which could influence ELISA sensitivity (Mohan et al. 2006). The P protein appears to be the most divergent protein among all the remaining six proteins present in the virus therefore the ELISA with it as coating antigen may have lower specificity (Das and Kumar 2015). Taking these disadvantages into account, the NP protein as the most abundant and conservative viral protein is probably the best choice for use as the ELISA antigen.

In the present study we constructed a recombinant baculovirus expressing NP gene. In this system, the NP protein was overproduced and it was used as a coating antigen in a diagnostic ELISA. The obtained protein

was of a size predicted by sequence analysis of the NP gene and also by Western blot analysis. The most abundant production of the recombinant NP protein was in membranes of Sf9 cells on day 2 of incubation and such results were consistent with Makkay et al. (1999) who reported the best yield of this protein between 36 and 48 hours post infection. Because recombinant NP protein is highly insoluble it was "released" from the membranes into suspension by ultrasonication.

The NP protein is the most abundant and also a highly immunogenic structural viral protein (Dortmans et al. 2011). It is involved in formation of a helical structure which includes the polymerase complex. The essential subunit of this structure is a single NP polypeptide of varying length depending on the strain, and the NP of LaSota strain is built of 489 amino acids resulting in molecular weight of 53.06 kDa. This protein is present in a large quantities in the virus, as it was suggested that about 2200–2600 NP subunits surround viral RNA and protect it against nuclease activity (Choppin 1992). The amino-terminus of NP protein interacts with the P protein forming a complex while the carboxy terminus plays an important role in a complex polymerization (Dortmans et al. 2010, Dortmans et al. 2011). It was shown that NP protein is also involved in virus virulence through its contribution to this complex although its role is much lesser known than those of the remaining two proteins (i.e. P and L proteins) in the virus replication machinery (Dortmans et al. 2010).

In recent years, different NDV proteins have been used as coating antigens in ELISA assays including NP, P and HN proteins (Errington et al. 1995, Mohan et al. 2006, Phan et al. 2013, Das and Kumar 2015). Among NP proteins used for ELISA assays were those originating from Ulster 2C, Hitcher B1 and LaSota NDV strains and all were expressed in baculovirus expression vectors (Errington et al. 1995, Makkay et al. 1999, Phan et al. 2013). They were then used in indirect (Ulster 2C, Hitcher B1) or competitive (LaSota) ELISA tests. Results obtained in both indirect ELISA tests had very good correlation with other assays. The Ulster 2C-based ELISA gave perfect agreement with HI results but some discrepancies in comparison with commercial ELISA tests were observed. The reason for some false-positive results in the commercial assay would be cross-reactivity of whole NDV antigen coated on commercial plates with antibodies against other paramyxoviruses such as PMV-2 or PMV-3 present in tested sera (Errington et al. 1995). The Hitchner B1-based ELISA was only compared with commercial ELISA and its specificity was 94.8% (Makkay et al. 1999).

In our study the NP gene of LaSota strain was used for the construction of recombinant baculovirus and then used in the indirect ELISA assay. The high homology of 95.6–98.9% of the amino acid sequence between the NP proteins of LaSota, Ulster 2C and Hitchner B1 NDV strains suggests similar specificity of all ELISA assays developed with their recombinant forms obtained in a baculovirus expression system. The ELISA developed in this study had a sensitivity and specificity of respectively 94.2–96.1% and 96.3–97.8% relative to the HI results. These values indicate a very good correlation between the tested assays. The reason for the slight discrepancies between our ELISA and HI tests results may be due to the cross-reaction with antibodies against other paramyxoviruses, such as PMV-2 or PMV-3 present in tested sera. Such tests, which would confirm this suspicion, have not been carried out in the present study. On the other hand, commercially available ELISA tests revealed different specificities ranging from 95.5–100% and also various sensitivities from 90.1 to 99.0%. Such differences could result from the lower sensitivity of the HI test which did not detect antibodies against NDV detectable by ELISA tests. It could be related with the presence of a much higher titre of anti-NP than anti-HN antibodies in sera of NDV-infected birds (as the result of the high expression of NP protein during infection). Another reason may be that the cut-off values in these ELISA assays were set too low and samples readings very close to the cut-off sets were interpreted as false positives. Because the specificity and sensitivity of the newly developed ELISA test is within the values obtained by other such tests as well as the HI assay regarded as the gold standard, we believe it is a reliable test for detecting the presence of NDV-specific antibodies. Moreover, applying a developed regression equation and OD values from serum testing, a linear relationship was found between the predicted and observed antibody titers.

As conventional live and inactivated vaccines for ND control have some shortcomings, efforts have been made to develop new generation vaccines in which HVT acts as a vector expressing the F gene of NDV (Morgan et al. 1992, Esaki et al. 2013). Such vaccines are safe, can be administered even in the presence of maternally-derived antibodies and induce lifelong immunity. This type of vaccine is commercially available in several countries including Poland. Recently, our team has developed a prototype vaccine in which the two NDV genes F and HN are incorporated into the HVT genome. The serum samples from SPF and broiler chickens vaccinated with commercial and in-house recombinant HVT vaccines and challenged with velogenic NDV strain were examined using HI and NP-based ELISA tests. The birds exposed to the whole virus produce antibodies against all viral proteins,

including NP protein, and these could be detected by the developed ELISA which, in turn, will not identify any antibodies in sera of chickens immunized with recombinant vaccines (F and HN) and detectable by HI test. Similarly, antibodies derived from hens, induced by contact with the whole virus (or a conventional vaccine) should be detected by both tests during the first weeks of life of their progeny. In fact, a high level of maternally derived antibodies was observed in sera of 1-day-old broilers both in HI and NP-ELISA assays. These antibodies had disappeared and were undetected in sera of broilers in weeks 3, 5 and 6 post vaccination but the birds became positive again in week 2 after challenge as observed in NP-ELISA results. In contrast to the NP-ELISA results, the same sera were positive in the HI test. In turn, in sera of SPF chickens, antibodies against the NP protein of NDV were identified only after a challenge with a highly virulent NDV strain. The same sera were positive in the HI test starting from 3 weeks after vaccination with both vaccines. Makkay et al. (1999) also described the possibility of differentiating between vaccinated and infected birds. The authors used a recNP-ELISA test and did not identify antibodies in chickens vaccinated with recombinant fowlpox viruses expressing only the HN protein of NDV but detected high level of antibodies to NDV in infected chickens.

In conclusion, the recombinant NP protein-based ELISA test developed in this study was demonstrated to be sensitive, specific and accurate test for the assessment of ND serostatus. Moreover, the developed ELISA could be also utilized for differentiation between vaccinated with recombinant vaccines against NDV and NDV-infected birds.

Acknowledgements

This work was supported by a grant from the National Centre for Research and Development within the Applied Research Programme Grant No. PBS1/B8/2/2012. Publication costs were covered by the Polish Ministry of Science and Higher Education (Grant No. 05-1/ /KNOW2/2015 within the Scientific Consortium “Healthy Animal - Safe Food”). The funding guaranteed the purchase of reagents and equipment only.

The authors wish to thank Elżbieta Juszczuk and Łukasz Bocian for their excellent technical assistance.

References

Alexander DJ (**2000**) Newcastle disease and other avian paramyxoviruses. Rev Sci Tech OIE 19: 443-462.

Amarasinghe GK, Bao YM, Basler CF, Bavari S, Beer M, Bejerman N, Blasdell KR, Bochnowski A, Briese T, Bukreyev A, Calisher CH, Chandran K, Collins PL, Dietzgen RG, Dolnik O, Durrwald R, Dye JM, Easton AJ, Ebihara H, Fang Q, Formenty P, Fouchier RAM, Ghedin E, Harding RM, Hewson R, Higgins CM, Hong J, Horie M, James AP, Jiang DH, Kobinger GP, Kondo H, Kurath G, Lamb RA, Lee B, Leroy EM, Li M, Maisner A, Muhlberger E, Netesov SV, Nowotny N, Patterson JL, Payne SL, Paweska JT, Pearson MN, Randall RE, Revill PA, Rima BK, Rota P, Rubbenstroth D, Schwemmle M, Smither SJ, Song QS, Stone DM, Takada A, Terregino C, Tesh RB, Tomonaga K, Tordo N, Towner JS, Vasilakis N, Volchkov VE, Wahl-Jensen V, Walker PJ, Wang BB, Wang D, Wang F, Wang LF, Werren JH, Whitfield AE, Yan ZC, Ye GY, Kuhn JH (**2017**) Taxonomy of the order Mononegavirales: update 2017. Arch Virol 162: 2493-2504.

Capua I, Dalla Pozza M, Mutinelli F, Marangon S, Terregino C (**2002**) Newcastle disease outbreaks in Italy during 2000. Vet Rec 150: 565-568.

Choppin J (**1992**) Interactions between Peptides and Major Histocompatibility Complex-Molecules. Pathol Biol 40: 257-264.

Czifra G, Nilsson M, Alexander DJ, Manvell R, Kecskemeti S, Engstrom BE (**1996**) Detection of PMV-1 specific antibodies with a monoclonal antibody blocking enzyme-linked immunosorbent assay. Avian Pathol 25: 691-703.

Das M, Kumar S (**2015**) Recombinant phosphoprotein based single serum dilution ELISA for rapid serological detection of Newcastle disease virus. J Virol Methods 225: 64-69.

de Leeuw O, Peeters B (**1999**) Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae. J Gen Virol 80: 131-136.

Dortmans JCFM, Koch G, Rottier PJM, Peeters BPH (**2011**) Virulence of Newcastle disease virus: what is known so far? Vet Res 42.

Dortmans JCFM, Rottier PJM, Koch G, Peeters BPH (**2010**) The Viral Replication Complex Is Associated with the Virulence of Newcastle Disease Virus. J Virol 84: 10113-10120.

Errington W, Steward M, Emmerson P (**1995**) A Diagnostic Immunoassay for Newcastle-Disease Virus-Based on the Nucleocapsid Protein Expressed by a Recombinant Baculovirus. J Virol Methods 55: 357-365.

Esaki M, Godoy A, Rosenberger JK, Rosenberger SC, Gardin Y, Yasuda A, Dorsey KM (**2013**) Protection and Antibody Response Caused by Turkey Herpesvirus Vector Newcastle Disease Vaccine. Avian Dis 57: 750-755.

Jestin V, Cherbonnel M, Lhospitalier R, Bennejean G (**1989**) An Elisa Blocking Test Using a Peroxidase-Labeled Anti-Hn Monoclonal-Antibody for the Specific Titration of Antibodies to Avian Paramyxovirus Type-1 (Pmv1). Arch Virol 105: 199-208.

Krishnamurthy S, Samal SK (**1998**) Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. J Gen Virol 79: 2419-2424.

Makkay AM, Krell PJ, Nagy E (**1999**) Antibody detection-based differential ELISA for NDV-infected or vaccinated chickens versus NDV HN-subunit vaccinated chickens. Vet Microbiol 66: 209-222.

Marek K (**1972**) Newcastle disease. In: Marek K (ed.), Diseases of poultry, PWRiL, Warsaw, pp 83-89.

Miller PJ, Koch G (**2013**) Newcastle disease. In: Swayne DE, Glisson JR, L.R. M, Nolan LK, Suarez DL, Nair V (eds), Disease of poultry, 13th ed., John Wiley & Sons, Inc., Ames, Iowa, USA, pp 89-107.

Minta Z (**1998**) Newcastle disease - a disease constantly threatening poultry production. Polskie Drobiarstwo 3: 3-6.

Mohan CM, Dey S, Rai A, Kataria JM (**2006**) Recombinant haemagglutinin neuraminidase antigen-based single serum dilution ELISA for rapid serological profiling of Newcastle disease virus. J Virol Methods 138: 117-122.

Morgan RW, Gelb J, Schreurs CS, Lutticken D, Rosenberger JK, Sondermeijer PJA (**1992**) Protection of Chickens from Newcastle and Mareks Diseases with a Recombinant Herpesvirus of Turkeys Vaccine Expressing the Newcastle-Disease Virus Fusion Protein. Avian Dis 36: 858-870.

OIE (**2017**) Chapter 2.3.14. Newcastle Disease (infection with Newcastle disease virus). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals

Phan LV, Park MJ, Kye SJ, Kim JY, Lee HS, Choi KS (**2013**) Development and field application of a competitive enzyme-linked immunosorbent assay for detection of Newcastle disease virus antibodies in chickens and ducks. Poultry Sci 92: 2034-2043.

Rima B, Collins P, Easton A, Fouchier R, Kurath G, Lamb RA, Lee B, Maisner A, Rota P, Wang LF (**2018**) Problems of classification in the family Paramyxoviridae. Arch Virol 163: 1395-1404.

Rout SN, Samal SK (**2008**) The large polymerase protein is associated with the virulence of newcastle disease virus. J Virol 82: 7828-7836.

Snyder DB, Marquardt WW, Mallinson ET, Russek E (**1983**) Rapid serological profiling by enzyme-linked immunosorbent-assay .1. Measurement of antibody-activity titer against Newcastle-Disease Virus in a single serum dilution. Avian Dis 27: 161-170.

Williams R, Boshoff CH, Verwoerd D, Schoeman M, van Wyk A, Gerdes TH, Roos K (**1997**) Detection of antibodies to Newcastle disease virus in ostriches (Struthio camelus) by an indirect ELISA. Avian Dis 41: 864-869.

Xu H, Lohr J, Greiner M (**1997**) The selection of ELISA cut-off points for testing antibody to Newcastle disease by two-graph receiver operating characteristic (TG-ROC) analysis. J Immunol Methods 208: 61-64.