Development and field application of a competitive enzyme-linked immunosorbent assay for detection of Newcastle disease virus antibodies in chickens and ducks

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ABSTRACT A competitive enzyme-linked immunosorbent assay (C-ELISA) using a baculovirus-expressed recombinant nucleocapsid protein antigen (rNDV-N) and an rNDV-N-specific monoclonal antibody (5B3) was developed for the detection of Newcastle disease virus (NDV) antibodies, and its diagnostic performance was evaluated. The specificity and sensitivity of the C-ELISA was found to be 98.4 and 98.9%, respectively, for chickens, and 98.2 and 97.9% for ducks. However, the C-ELISA showed weak cross-reaction with hyperimmune antisera to some other avian paramyxovirus serotypes. In all experimentally vaccinated chickens, seroconversion rates at 7 d postinoculation were 100 and 40% when measured by C-ELISA and hemagglutination inhibition (HI), respectively. In field trials, the C-ELISA showed positive results in 98.9% of HI-positive sera and 40.8% of HI-negative sera from NDV-vaccinated chickens (n = 705). In domestic ducks (n = 158) from NDV-positive duck farms (n = 8), the positive rates according to C-ELISA were significantly higher than those according to the HI test. At the same time, 98.1% of ducks (n = 209) from NDV-negative duck farms (n = 11) were also negative by C-ELISA. Our results indicate that C-ELISA could be a useful alternative to HI testing for detecting NDV antibodies in different avian species such as chickens and ducks.

Key words: Newcastle disease virus, competitive enzyme-linked immunosorbent assay, recombinant N protein, antibody detection

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INTRODUCTION

Newcastle disease (**ND**) virus (**NDV**) is an avian paramyxovirus (**APMV**) belonging to the genus *Avulavirus* of the family *Paramyxoviridae* (Mayo, 2002). The APMV are classified into 10 distinct APMV serotypes (APMV-1–10; Miller et al., 2010). Newcastle disease virus is the sole member of APMV-1. The virus possesses a negative-sense, single-stranded RNA genome, which encodes at least 6 main proteins: nucleocapsid protein (**N**), phosphoprotein (**P**), matrix protein (**M**), fusion protein (**F**), hemagglutinin-neuraminidase protein (**HN**), and the large (**L**) polymerase protein (Krishnamurthy and Samal, 1998; de Leeuw and Peeters, 1999; Lamb and Kolakofsky, 2001). The N protein, a major component of the herringbone-like nucleocapsid core (Yusoff and Tan, 2001), is the most abundant protein. As the fundamental structural protein of the viral particles, it is responsible for induction of a strong immune response in the host (Makkay et al., 1999; Choi et al., 2003b; Matsubara et al., 2012).

Newcastle disease virus has a wide host range, with 27 of the 50 orders of birds reported to be capable of infection by NDV (Kaleta et al., 1979). Chickens are highly susceptible to NDV, and the virus is so virulent in this host that NDV causes ND, which results in high mortality in unprotected chickens. The disease still remains endemic in poultry in many regions of Asia and Africa, despite extensive vaccination efforts for prevention of the disease (Alexander and Gough, 2003). By contrast, in general, duck species may show subclinical signs and act as carriers, even when exposed to highly virulent NDV strains (Alexander and Gough, 2003). Most of the NDV isolates from ducks are low-virulence viruses (Stanislawek et al., 2002; Zanetti et al., 2005; Kim et al., 2007; Jindal et al., 2009; Lee et al., 2009). However, one NDV variant that was highly pathogenic to fowls and waterfowls, known as goose paramyxovirus (**GPMV**), emerged in 1997 in China and has caused frequent outbreaks in geese and ducks (Liu et al., 2000; Zou et al., 2002; Zou and Gong, 2003; Jinding et al., 2005).

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The early detection of NDV infection and the assessment of the immune status induced due to vaccination are important for effective control measures. The hemagglutination inhibition (**HI**) test is the serological standard method and is still the most widely used method for NDV antibody detection in multiple bird species in many laboratories. Nevertheless, the HI test has limitations in terms of its low sensitivity and a high incidence of false-positive results (Czifra et al., 1996; Williams et al., 1997; Xu et al., 1997). Several ELISA have been developed as superior alternatives to the HI test due to their capacity to test a large number of samples at one time, and their rapid turnaround of results (Jestin et al., 1989; Brown et al., 1990; Cvelic-Cabrilo et al., 1992; Errington et al., 1995; Czifra et al., 1996; Williams et al., 1997; Xu et al., 1997; Cardoso et al., 1999; Sousa et al., 1999; Hauslaigner et al., 2009).

In the present study, we sought to develop a competitive ELISA (**C-ELISA**) capable of detecting NDV antibodies sensitively in multiple bird species. To that end, the N protein of NDV was expressed by a recombinant baculovirus in insect cells as the coating antigen and NP-specific monoclonal antibody was used as a competition antibody in the C-ELISA. The C-ELISA developed in this study was evaluated using experimental and field sera from domestic chickens and ducks and its results were compared with those of the HI test.

MATERIALS AND METHODS

Generation of Recombinant Baculovirus

The NDV La Sota strain was used for cloning of the entire N gene of NDV. The oligodeoxynucleotide primer set was designed to amplify the open reading frame (**ORF**) of the N protein gene of the La Sota strain (GenBank accession no. AF077761). The sequences and locations of the primers were as follows: forward primer NDV-Nf: 5′-TAAGGCCTCTGTCGACTCCTCCGTATTTGATG-3′ (nt 122–140) and reverse primer NDV-Nr: 5′-CAGAATTCGCAAGCTTATACCCCCAGTCGGTG-3′ (nt 1588–1573). Sixteen nucleotides of flanking sequence (underlined) were added to the 5′ ends of the forward and reverse primers to facilitate cloning. The N gene fragment, 1,496 bp in size, was amplified by reverse-transcription (**RT**) PCR and then cloned into the In-Fusion Ready BacPAK Vector (Clontech Laboratories Inc., Mountain View, CA) according to the manufacturer's instructions. The constructed plasmid, pBacPAK/NDV-N, was cotransfected with BacPAK6 viral DNA (Clontech) to generate recombinant baculovirus in *Spodoptera frugiperda* 9 (Sf9) cells (Invitrogen, San Diego, CA). Recombinant baculovirus expressing N protein (designated as rBAC/NDV-N) was purified using a plaque assay. Expression of the recombinant virus in infected insect cells was confirmed by Western blot analysis using hyperimmune NDV chicken antiserum.

Preparation of Recombinant N Protein

The recombinant N (**rNDV-N**) antigen was prepared from lysates of infected cells in the presence of proteinase inhibitors, as reported previously (Choi et al., 2003a). In brief, the Sf9 cells were infected with the recombinant baculovirus expressing N protein at a multiplicity of infection of 5. The infected Sf9 cells were harvested after 4 d postinoculation (**dpi**). The cell pellets were collected by centrifugation at 500 × *g* for 20 min at 4°C, resuspended in 1/20 volume of a lysis buffer (0.01 *M* PBS containing 1% NP-40 and 0.05% Tween 20) containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany), and sonicated briefly. The lysate was clarified by centrifugation at 500 × *g* for 20 min at 4°C and then the supernatant was used as an ELISA antigen. The rNDV-N antigen preparation in 50% (vol/vol) glycerol was aliquoted and stored at −20°C before use.

Protein Separation and Western Blot Analysis

Proteins extracted from infected Sf9 cells were fractionated by 10% SDS-PAGE under reducing conditions and visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA). For the Western blot, the separated proteins were blotted onto an Immun-Blot polyvinylidene fluoride membrane (Bio-Rad) using a wet transfer system (Bio-Rad). The membrane was blocked with 5% skim milk in PBS containing 0.1% Tween 20 (**PBST**) at room temperature for 1 h. After washing 3 times with PBST, the blocked membrane was subsequently incubated with NDV chicken antiserum (diluted 1:100) for 1 h, rinsed in PBST, and incubated with horseradish peroxidase-conjugated goat anti-chicken immunoglobulin (Pierce, Rockford, IL) for 1 h. Protein bands were visualized by enhanced chemiluminescence, as previously described (Penna and Cahalan, 2007).

Sera and Monoclonal Antibodies

The APMV-1, −2, −4, −6, −8, and −9 antigens and reference antisera to each of the APMV serotypes were purchased from National Veterinary Service Laboratories (Ames, IA). Reference chicken antisera to infectious bronchitis virus, infectious bursal disease virus, avian influenza virus, chicken anemia virus, and avian reovirus were purchased from Charles River SPAFAS Laboratories (North Franklin, CT).

Hyperimmune NDV chicken antiserum was produced in 4-wk-old specific-pathogen-free (**SPF**) chickens experimentally vaccinated with a commercial oil-emulsion NDV (La Sota strain) killed vaccine (CAVAC, Daejeon, Korea). The chickens were bled 3 wk after vaccination. The antiserum was further diluted in negative control serum (SPF chicken serum) to prepare strong positive

(1:3 dilution) and weak positive (1:25 dilution) control sera for the ELISA.

A set of known-positive and known-negative sera, kept at our laboratory, was used to determine cut-off values and relative sensitivity and specificity of the C-ELISA in the study. The known-positive sera (n = 320) consist of (i) sera from ND-vaccinated chickens (n = 272), and (ii) sera from domestic ducks (n = 48) that were confirmed as NDV antibody positive by HI test. Known-negative sera (n = 253) originated from (i) sera from SPF chickens (n = 262), and (ii) sera from domestic ducks (n = 113) that were confirmed as NDV antibody negative by HI test. A set of sera from experimentally vaccinated chickens (n = 135), kept at our laboratory, was also used. These sera were taken at 0, 7, and 14 d postvaccination (**dpv**) from 45 chickens of 3 groups (15 birds per group), each of which received a different commercial inactivated ND vaccine. In addition, a set of previously obtained field serum samples (n = 1,072) from chickens (n = 705) and ducks (n = 367) were used. Chicken sera were collected at slaughterhouses from broilers of 55 flocks that had been vaccinated 2 times with live NDV vaccine. Duck sera (n = 367) were collected from randomly selected NDV-unvaccinated domestic ducks from 19 duck farms in Korea, including NDV-positive farms (n = 8) and APMV (including NDV)-negative farms (n = 11). The NDV isolates from the 8 positive farms were previously published by Lee et al. (2009).

The mAb 5B3 (IgG1, kappa), specific to the N protein of NDV (La Sota strain), was produced in-house at our laboratory according to a protocol previously described (Choi et al., 2003a). The hybridoma cell line secreting 5B3 antibody was generated from BALB/C mice immunized with recombinant NDV-N protein. The 5B3 antibody showed broad reactivity to the NDV isolates but not to any other APMV serotypes, according to indirect ELISA (data not shown).

C-ELISA

For C-ELISA, a checkerboard titration was performed to predetermine the optimal working dilutions for the coating antigen, mAb 5B3, serum, and horseradish peroxidase-labeled goat anti-mouse immunoglobulins. Maxisorp ELISA plates (Nalge Nunc International, Roskilde, Denmark) were coated with 100 μL of the rNDV-N antigen at predetermined optimal concentrations in carbonate-bicarbonate buffer (pH 9.6) for 1 h at 37°C with constant shaking. Plates were washed 3 times with PBST (0.002 *M* PBS containing 0.05% Tween 20) and then incubated for 1 h at 37°C with 100 μL of a mixture of equal volumes of the mAb 5B3 and test serum in blocking buffer (0.01 *M* PBS containing 0.05% Tween-20 and 6% skim milk). Strong positive, weak positive, and negative control sera were included. In each run, all sera, including the serum controls, were tested in duplicate. Following a washing step, the plates were incubated at 37°C for 1 h with 100 μL of horseradish peroxidase-labeled goat anti-mouse immunoglobulins (Kirkegaard-Perry Laboratories Inc., Gaithersburg, MD) prepared in blocking buffer for 1 h at 37°C. Following another washing step, the plates were incubated for 10 min with the substrate *o*-phenylenediamine (Sigma, St. Louis, MO) in 0.05 *M* citrate phosphate buffer (pH 5.0) containing 0.015% hydrogen peroxidase. The colorimetric reaction was stopped by adding 100 μL of 1.25 *M* sulfuric acid to all wells. Optical density (**OD**) readings were measured at 492 nm wavelength. The OD value was converted to the percent inhibition (**PI**) induced by the competition between the mAb and serum antibodies by using the following formula: [1 − (OD of serum-mAb mixture/OD of mAb alone)] × 100. The cut-off value for the assay was calculated as 3 SD above the mean PI value of known-negative serum samples.

HI Test

The HI test was performed in V-bottom microtiter plates. Briefly, a 25-μL volume of 2-fold dilutions of test sera in 0.01 *M* PBS, pH 7.4, was mixed with the same volume containing 4 HA units of NDV La Sota antigen at room temperature for 30 min. An equal volume of 0.5% chicken red blood cell solution was added and incubated at room temperature for 40 min. The HI titer of test serum samples was determined as the reciprocal of the highest dilution that showed complete inhibition of HA activity. All tests were repeated twice. Serum samples with HI titers of 4 or greater were considered positive.

Statistical Analysis

Specificity and sensitivity were calculated by a method described previously (Altman and Bland, 1994). Agreement between the C-ELISA and HI test was calculated using the kappa quotient (Martin et al., 1988). Multiple comparisons between groups were done using one-way ANOVA and the Tukey's honestly significant difference test (Tukey, 1949). A P-value of < 0.05 was considered statistically significant.

RESULTS

Expression of Recombinant N Protein in Insect Cells

Recombinant baculovirus (rBAC/NDV-N) expressing the entire N protein of NDV was generated by recombination between pBacPAK/NDV-N and BacPAK6 viral DNA in Sf9 cells. The rBAC/NDV-N-infected Sf9 cells were harvested 4 dpi and lysed to release the rNDV-N protein from the cells. The rNDV-N protein expressed by the rBAC/NDV-N had a molecular mass of approximately 53 kDa and was specifically recognized by the hyperimmune NDV chicken antiserum on Western blot analysis after SDS-PAGE (Figure 1A). No proteins from the uninfected Sf9 cells reacted with

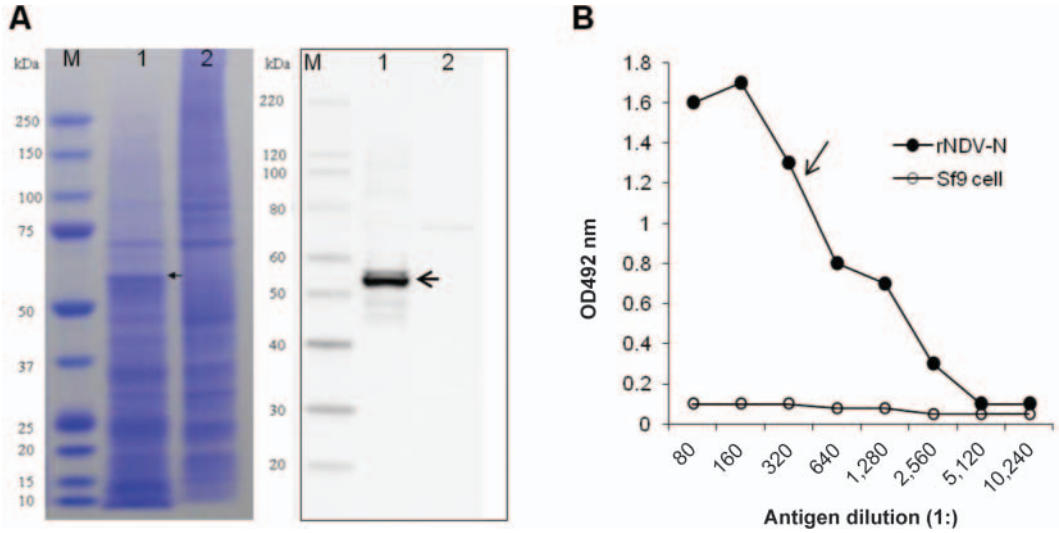

Figure 1. The SDS-PAGE/Western blot analysis (A) and ELISA titration (B) of the recombinant Newcastle disease virus (NDV) nucleocapsid (rNDV-N) preparation. Western blot analysis and ELISA titration were performed using hyperimmune NDV antiserum (diluted 1:100) and 5B3 monoclonal antibody (diluted 1:10,000), respectively. M, molecular marker. Lane 1, rNDV-N; lane 2, Sf9 cell lysate. Arrow in panel A indicates the N protein expressed by recombinant baculovirus. Arrow in panel B represents the optimal concentration of coating antigen for competitive ELISA. OD492 nm = optical density at 492 nm. Color version available in the online PDF.

the NDV antiserum. The molecular mass of the protein was in agreement with the predicted molecular mass for the N protein of NDV (Errington et al., 1995). For use as the antigen in the C-ELISA, a 20-mL volume of the rNDV-N protein in 50% (vol/vol) glycerol was prepared from 200 mL of Sf9 cells (2×10^6 cells/mL) that had been infected with the rBAC/NDV-N in a spinner apparatus. The N protein (r-NDV-N) preparation showed reactivity to mAb 5B3 up to a dilution of 1:2,560 when titrated by indirect ELISA (Figure 1B).

Cut-Off Value, Sensitivity, and Specificity of C-ELISA

Optimal conditions for C-ELISA were determined through checkerboard ELISA titration. As a result, rNDV-N antigen diluted 1:500 (approximately 2.5 μg/100 μL of total protein), 5B3 mAb diluted 1:10,000, test serum diluted 1:5, and peroxidase-labeled conjugate solution diluted 1:1,500 were determined to be optimal for C-ELISA.

The cut-off value, sensitivity, and specificity of the assay were determined using known-positive and known-negative serum samples. In chickens, the mean and SD of PI values from 192 known-negative chicken sera were 12.7 and 12.2, respectively. Thus, the cut-off value (mean PI + 3 SD) of the C-ELISA for chicken sera was set at 50 (Figure 2A). In ducks, the mean and SD of PI values from 113 known-negative duck sera were 27.6 and 11.1, respectively. Thus, the cut-off value of the C-ELISA for duck sera was set at 60 (Figure 2B). Based on the cut-off values, specificity of the C-ELISA was estimated to be 98.4% (189/192) for chickens and 98.2% (111/113) for ducks. The sensitivity of the C-ELISA was assessed using known-positive chicken sera and known-positive duck sera. In chickens, 98.9% (259/262) of HI-positive sera were also positive by C-ELISA (Figure 2A), whereas 97.9% (47/48) of HI-positive sera were positive by C-ELISA (Figure 2B).

For the assessment of analytical specificity, reference antisera to other APMV serotypes and other viral pathogens were evaluated by C-ELISA. As expected, antiserum to NDV was strongly positive (PI value of 99) in the C-ELISA. Reference antisera to other APMV serotypes such as APMV-2, −4, −6, −8, and −9 were also positive in C-ELISA, although P values of sera to other APMV were relatively low (PI values of 69 to 81) compared with NDV antiserum, indicating the presence of cross-reactive antibodies (Figure 3). However, antisera to infectious bronchitis virus, infectious bursal disease virus, avian influenza virus, chicken anemia virus, and avian reovirus were all negative in the C-ELISA.

Early Detection of NDV Antibodies in Experimentally Vaccinated Chickens

We next compared the dynamics of the humoral immune responses to NDV obtained with the competitive c-ELISA and the reference HI test (Figure 4). We tested a total of 135 sera that were collected from experimental chickens (n = 45) at 0, 7, and 14 dpv with 3 different commercial NDV vaccines (groups 1 to 3, 15 birds per group). The antibody dynamics in each group showed similar patterns to each other when measured by C-ELISA and HI tests, as shown in Figure 4. All of the chicken sera (n = 45) at 0 dpv tested negative by both HI test and C-ELISA. Seven days after vaccination, the C-ELISA detected seroconversion in all birds. However, the HI test failed to detect seroconversion in 40% (18/45) of these birds on the same day (Figure 4).

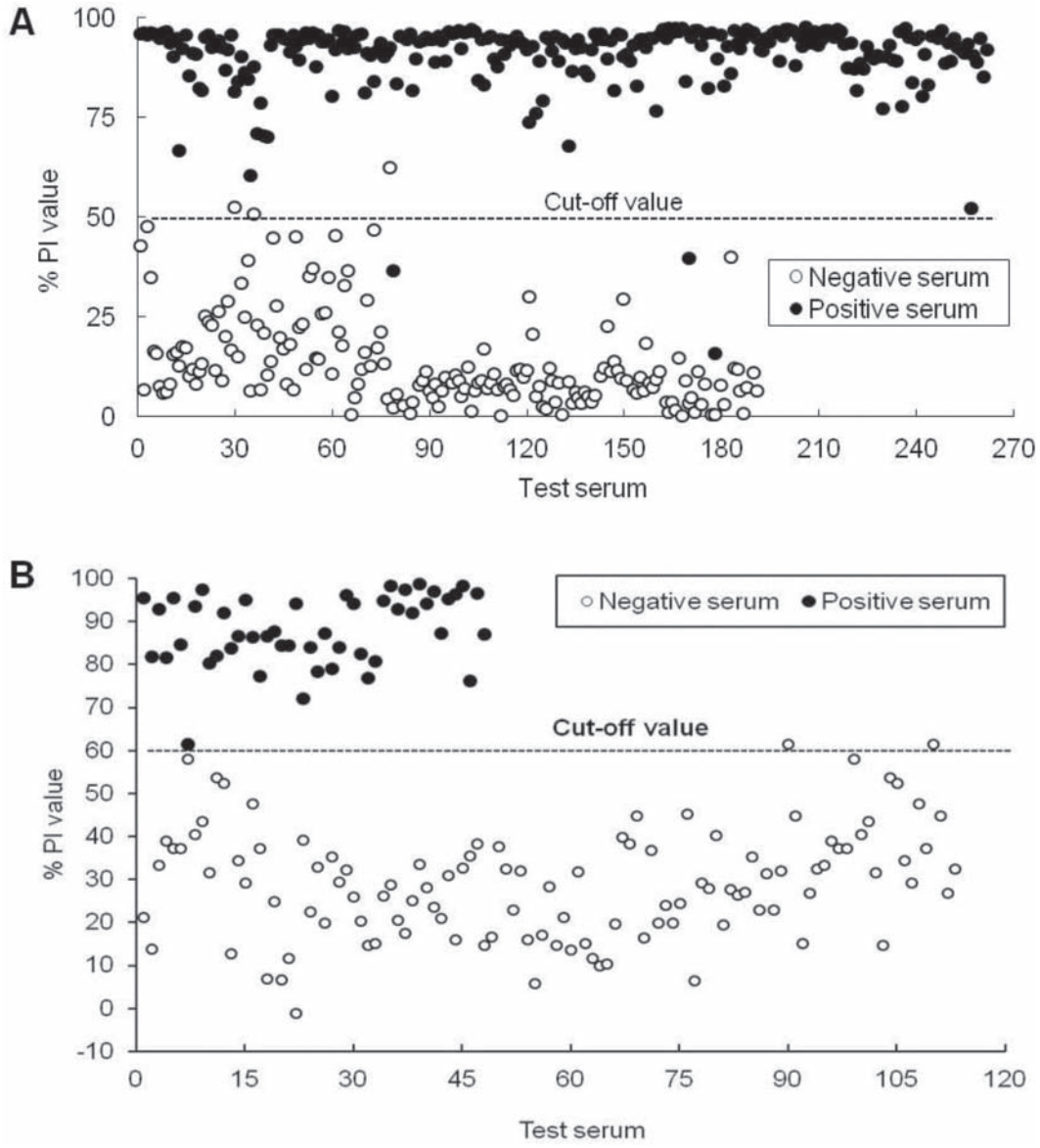

Figure 2. Competitive-ELISA (C-ELISA) results of known-positive (●) and known-negative (○) sera from chickens (A) and ducks (B). Cut-off percent inhibition (PI) values (mean PI + 3 SD) determined using known-negative sera were 50 and 60 for chickens and ducks, respectively.

The NDV antibodies were detected 14 dpv in all of the vaccinated birds by both C-ELISA and HI test. This indicates that in vaccinated chickens, C-ELISA can detect seroconversion to NDV earlier than the HI test.

Application of C-ELISA in Slaughtered Broiler Chickens

Chicken field serum samples (n = 705) collected from slaughtered broilers from 55 flocks, which had been vaccinated 2 times with live NDV vaccine before being slaughtered, were tested by C-ELISA, and the results were compared with those from the HI test (Table 1). By C-ELISA, 98.9% (564/570) of the HI-positive sera (HI titers of $\geq$4) gave positive results, whereas 40.8% (55/135) of HI-negative sera (HI titers of <4) also tested positive for NDV by C-ELISA. The HI-positive and ELISA-negative sera (n = 6) had low HI titers of 16 or less. The kappa value between the 2 tests was 0.68 when calculated according to the method described by Martin et al. (1988).

Application of C-ELISA in Domestic Ducks

Duck serum samples (n = 367) collected from domestic ducks from 19 farms, which had never been vaccinated against NDV before, were tested for NDV by the C-ELISA. The results were compared with those from the HI test (Table 2). Duck serum samples (n = 158) from 8 NDV-infected farms (A to H) were serologically tested for NDV using the 2 methods. Thirty-two ducks from these farms were positive in the HI test. Most (29/32) of these sera had low HI titers of <32. Three ducks from farms D (n = 1) and G (n = 2) had high HI titers of 64 or greater. All of HI positive serum samples (n = 32) were positive in the C-ELISA. By C-ELISA,

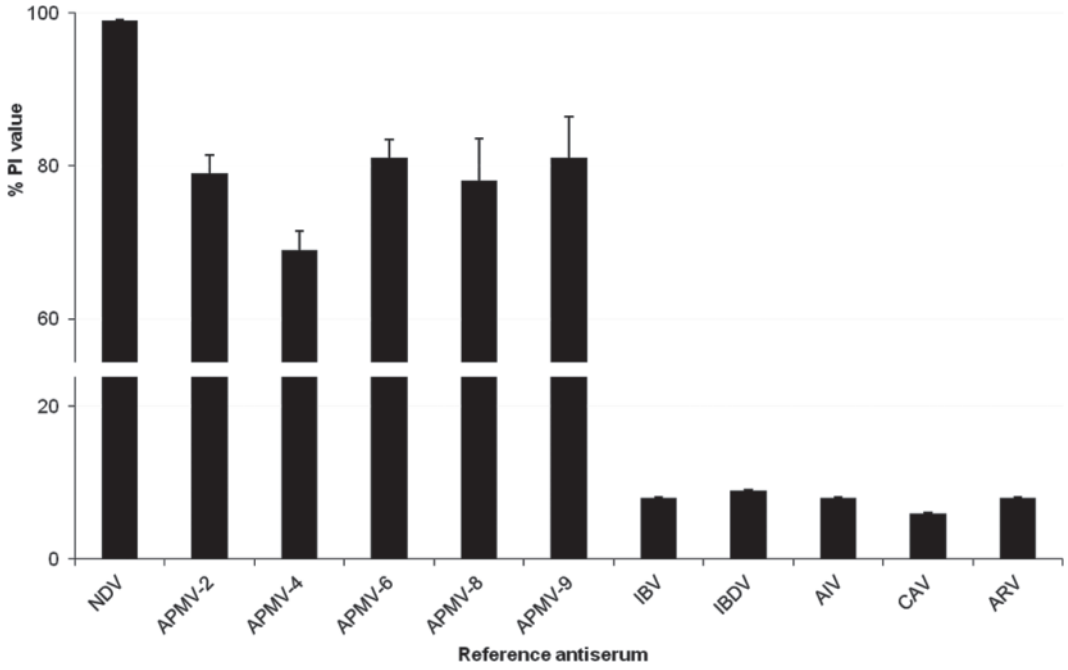

Figure 3. Competitive-ELISA (C-ELISA) results for reference chicken antisera to Newcastle disease virus (NDV) and other viral pathogens. The cut-off percent inhibition (PI) value was set at 50. NDV, Newcastle disease virus; APMV, avian paramyxovirus; IBV, infectious bronchitis virus; IBDV, infectious bursal disease virus; AIV, avian influenza virus; CAV, chicken anemia virus; ARV, avian reovirus.

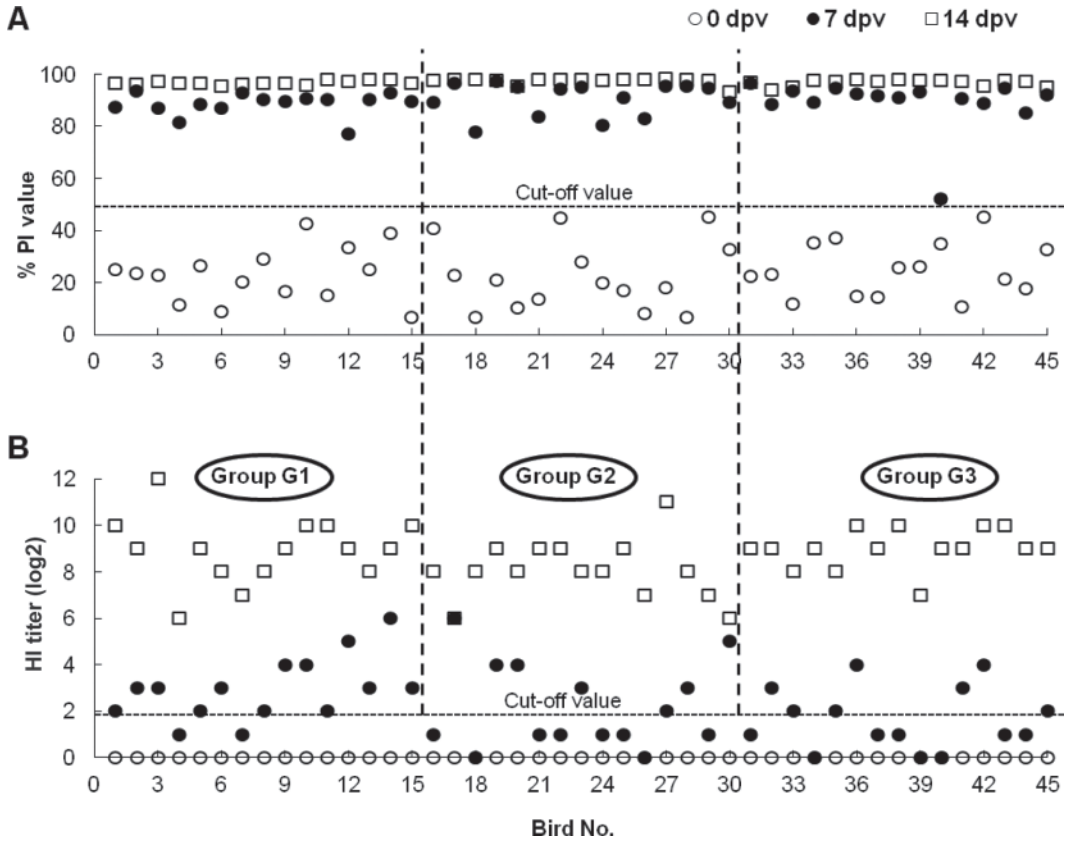

Figure 4. Competitive-ELISA (C-ELISA) results for sera collected 0 (○), 7 (●), and 14 (□) d postvaccination (dpv) from experimentally vaccinated chickens. Three different groups (groups 1 to 3, 15 birds per group) received vaccination. Each group was vaccinated with a different Newcastle disease virus (NDV) vaccine. PI = percent inhibition; HI = hemagglutination inhibition.

Table 1. Competitive-ELISA (C-ELISA) results of field serum samples obtained from Newcastle disease virus (NDV)-vaccinated chickens in Korea

	HI titer[2]						
ELISA[1]	≤2	4	8	16	32	≥64	Total
Negative	80	3	1	2	0	0	86
Positive	55	16	18	28	30	472	619
Total	135	19	19	30	30	472	705

[1]The cut-off value for C-ELISA was set at a percent inhibition of 50% for chickens. Relative sensitivity = 98.9% (564/570); relative specificity = 59.3% (80/135); kappa = 0.68.

[2]Hemagglutination inhibition (HI) titers of 4 or greater were considered positive.

38.9% (49/126) of HI-negative sera tested positive for NDV, so the C-ELISA (51.2% ± 29.7) showed significantly higher positive rates than the HI test (20.3% ± 16.0; $P < 0.05$). Duck serum samples (n = 209) from 11 NDV-uninfected farms (I to S) were also tested for NDV by C-ELISA and HI test. As a result, 98.9% of these sera were negative by C-ELISA. From these, 7 farms (J, M, N, P, Q, R, and S) gave negative results by both tests. In the case of farms I, K, and L, all except for one sample were also negative by both tests. A few positive samples (n = 4) were detected by C-ELISA but were all weakly positive (PI value of <90).

DISCUSSION

Currently available commercial ELISA kits are mostly of the indirect format, which is capable of quantifying NDV antibodies easily with a single dilution of test serum in chickens, especially vaccinated chickens. The application of indirect ELISA kits is limited when testing serum samples from multiple bird species, because of the use of anti-mouse conjugates in place of an anti-chicken conjugate (Cadman et al., 1997; Williams et al., 1997). Thus, in the present study, we instead used C-ELISA, which is designed to detect NDV antibodies, even in multiple bird species, through the use of competition between test serum antibodies and a murine monoclonal antibody specific for NDV. The N protein is a highly immunogenic structural protein (Makkay et al., 1999; Choi et al., 2003b; Matsubara et al., 2012), and this might improve sensitivity of the assay. Thus, the N protein expressed by the rBAC/NDV-N baculovirus was used as the target protein for C-ELISA in the study. The molecular mass of this rNDV-N protein was approximately 53 kDa, as detected by a single band of that size on SDS-PAGE. This band was strongly

Table 2. Competitive-ELISA (C-ELISA) results for field serum samples (n = 367) from Newcastle disease virus (NDV) unvaccinated domestic ducks in Korea

		No. positive[1] (%)		Virus isolation result[2]	
Item	No. of birds tested	HI test	C-ELISA	NDV isolated	Reference
Positive farm					
A	20	10 (2/20)	45 (9/20)	KR/duck/06/07	Lee et al., 2009
B	20	45 (9/20)	85 (17/20)	KR/duck/08/07	Lee et al., 2009
C	19	36.8 (7/19)	68.4 (13/19)	KR/duck/09/07	Lee et al., 2009
D	20	10 (2/10)	30 (6/20)	KR/duck/11/07	Lee et al., 2009
E	19	5.3 (1/19)	21.1 (4/19)	KR/duck/12/07	Lee et al., 2009
F	20	15 (3/20)	25 (5/20)	KR/duck/14/07	Lee et al., 2009
G	20	5 (1/20)	35 (7/20)	KR/duck/13/07	Lee et al., 2009
H	20	35 (7/20)	100 (20/20)	KR/duck/10/07	Lee et al., 2009
Mean ± SD[3] (%)		20.3 ± 16.0[a]	51.2 ± 29.7[b]		
Negative farm					
I	20	0 (0/20)	5 (1/20)	Negative	—
J	17	0 (0/17)	0 (0/17)	Negative	—
K	18	0 (0/18)	5.6 (1/18)	Negative	—
L	20	0 (0/20)	5 (1/20)	Negative	—
M	18	0 (0/18)	0 (0/18)	Negative	—
N	19	0 (0/19)	0 (0/19)	Negative	—
O	20	0 (0/20)	5 (1/20)	Negative	—
P	20	0 (0/20)	0 (0/20)	Negative	—
Q	19	0 (0/19)	0 (0/19)	Negative	—
R	18	0 (0/18)	0 (0/18)	Negative	—
S	20	0 (0/20)	0 (0/20)	Negative	—
Mean ± SD (%)		0[c]	1.9 ± 2.6[c]		

[a–c]Mean values within a column with different superscript letters are significantly different ($P < 0.05$).

[1]Hemagglutination inhibition (HI) titers ≥4 for HI test and percent inhibition values ≥60 for C-ELISA were considered positive.

[2]Virus isolation was conducted by egg inoculation from swabs collected from birds and the environment.

[3]Mean ± SD of % positive.

stained with hyperimmune NDV antiserum in Western blot analysis. This is consistent with results obtained from other recombinant N proteins of NDV as previously reported (Errington et al., 1995; Makkay et al., 1999). This indicates that the rNDV-N protein expressed by the BAC/NDV-N virus was prepared with little protein degradation during the protein extraction steps.

In the present study, the cut-off (PI value of 60) for duck sera was determined to be higher than that (PI value of 50) for chicken sera when C-ELISA was performed under the same conditions of antigen and antibody reagents. This suggests that duck serum samples might have nonspecific background and the cut-off value might be different depending on bird species. If so, use of single cut-off value in C-ELISA in multiple bird species is needed to improve availability of C-ELISA at the laboratory level. More effective blocking buffer, purified antigen, or pretreated sera would be helpful in the attempt to reduce nonspecific background and establish single cut-off value in multiple bird species. Competitive-ELISA has also a limitation of failing to quantify antibodies related with protective immune response especially in vaccinated birds. Nevertheless, C-ELISA showed an excellent specificity (98.4% for chickens and 98.2% for ducks) and sensitivity (98.9% for chickens and 97.9% for ducks) with known-negative sera and known-positive sera, respectively. Competitive-ELISA showed a tendency to detect seroconversion in experimentally vaccinated chickens earlier than the HI test (100% for 7 dpv sera for C-ELISA compared with 40% for 7 dpv sera for HI) as shown in Table 1. Similar results were demonstrated in slaughtered chickens that were vaccinated 2 times with live NDV vaccines on the farm. In vaccinated chickens, C-ELISA had positive results in 40.8% of HI-negative sera (HI titer of <4), whereas 1.1% of HI-positive sera were negative in C-ELISA. This indicates that C-ELISA, as used in this study, might be an appropriate screening test for surveillance of chickens and ducks, with advantages over the HI test. Perhaps this discrepancy between both tests might be associated with 2 factors: (i) different kinetics of anti-N and anti-HN antibody development produced due to NDV vaccination or infection (Matsubara et al., 2012) and (ii) differences in sensitivity between the ELISA and HI tests employed (Charan et al., 1981; Dumanova et al., 1983; Snyder et al., 1983; Folitse et al., 1998).

In the present study, the C-ELISA was also applied to 387 ducks, raised on 19 commercial farms, which had never been vaccinated against NDV. Eight farms (designated A to G), which had NDV isolated from ducks (Lee et al., 2009), were tested by both C-ELISA and HI. The C-ELISA showed significantly higher positive rates than the HI test. Meanwhile, in farms I through S, in which neither NDV nor HI antibodies had been detected, 98.1% (204/209) of the ducks tested were negative by C-ELISA, which is comparable with the level of specificity (98.2%) for ducks. Here, most (90%, 29/32) of the HI-positive sera had low titers of ≤ 32, suggesting that ducks with natural NDV infection might develop relatively weak antibody responses to the HN protein compared with infected chickens (Westbury, 1981). Thus, the C-ELISA might detect NDV antibodies in duck serum samples at levels that are undetectable by the HI test, as in chicken serum samples. If so, the C-ELISA would be a useful tool for early detection in ducks during disease monitoring.

In the present study, our C-ELISA was focused on chickens and ducks in NDV-positive farms only because other APMV have never been detected in domestic ducks in Korea. However, we should note that C-ELISA cross-reacts reference antisera to some of the APMV serotypes tested. The APMV might be present in numerous species of domestic and wild birds (Alexander and Gough, 2003). Waterfowl, in particular, such as ducks and geese, are considered to be natural reservoirs for NDV and other APMV, such as APMV-4, -6, -8, and -9 (Alexander and Gough, 2003). Thus, if other APMV besides NDV are present in the sera of birds tested, such sera can result in false-positive results in the C-ELISA due to the presence of cross-reactive antibodies. Thus, when C-ELISA is applied to chicken and duck farms in regions where APMV are present, the serological test results should be interpreted carefully together with virus isolation results in case of contamination of the duck farms with other APMV serotypes.

At present, genetically engineered vector or subunit vaccines without the N protein component of NDV have been developed for prevention of ND in poultry (Meulemans et al., 1988; Boursnell et al., 1990; Morrison et al., 1990; Nagy et al., 1991; Karaca et al., 1998; Perozo et al., 2008; Sun et al., 2008; Kumar et al., 2011). An indirect ELISA-based recombinant N protein was developed to differentiate between naturally NDV-infected animals and those vaccinated with the corresponding subunit vaccine (Makkay et al., 1999). If these vaccines become available in the field, our C-ELISA could have the potential to be applied as a serological tool for the differentiation of an infection from vaccination with vector or subunit vaccines.

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