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Development and Validation of an Indirect Whole-Virus ELISA Using a Predominant Genotype VI Velogenic NewCastle Disease Virus Isolated from Lebanese Poultry

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Abstract

Commercial ELISA kits are commonly used to assess the levels of chicken antibodies against NewCastle Disease Virus (NDV) and trace a field strain infection. Nevertheless, the specificity of these kits vis-à-vis endemic strains in Lebanon remains in question. This study developed an in-house indirect ELISA system to evaluate the level of chicken antibodies against a predominant velogenic NDV strain belonging to Genotype VI. A checkerboard analysis comprised a five-factorial multivariate experiment to optimize the protocol: coating antigen concentration, blocking buffer utilization, serum and conjugate dilution levels, and OD reading wavelength. The developed test was optimized and then validated through parallel testing of the sera of 20 broilers and 5 layers using standard serological assays. There was a strong correlation between the developed ELISA results and those obtained with the Hemagglutination Inhibition test ($P < 0.01$), and a commercial NDV ELISA kit ($P < 0.05$). The specificity, sensitivity, and reproducibility of the developed ELISA suggest that it can be used as the test of choice for the assessment of chicken antibody titers against locally circulating velogenic NDV strains, specifically those belonging to Genotype VI. It also offers better help in the serological detection of birds' exposure to the said strains.

Keywords

ELISA Development, Genotype VI, NewCastle Disease, Poultry, Lebanon

1. Introduction

Vaccination against NewCastle disease virus is a common practice since the

1950s in poultry, primarily aiming at protecting the birds against infection with circulating mesogenic and velogenic strains [1]. In developing countries, live lentogenic vaccines are widely used, and their ability to induce specific immune responses against velogenic strains is questionable. The same applies to other heterologous types of vaccines that are commercially available. These vaccines are made to achieve substantial immunity levels in flocks in order to prevent high mortality and morbidity resulting from serious NDV infections [1] [2].

Serological assays have been always on the frontline of evaluating protective immunity against NDV following a specific vaccination strategy that should be tailored for each poultry farm. These tests include, among others, Hemagglutination Inhibition (HI), Complement Fixation Test, Gel Immunodiffusion, Viral Neutralization Test, Enzyme-Linked Immunosorbent Assay (ELISA), Immunofluorescence Test, and Western Immunoblotting [3]. Because these serological tests are used as a first line of testing, they should be, therefore, highly sensitive and specific. HI and ELISA are the most frequently used tests for evaluating poultry's immunological response to NDV [4] [5]. Both are simple tests that do not require highly skilled personnel or an advanced and costly setting. Although HI is still considered the standard laboratory test for NewCastle disease, the lack of specificity of the HI test in discriminating antibodies against various strains makes ELISA the favored serological test in evaluating the efficacy of commercial vaccines against this disease. Moreover, ELISA is superior to HI due to its rapid results turnaround and its capacity to test a larger number of samples at a time [4] [5] [6].

Various imported ELISA kits are commercially available to assess the antibody titer to NDV in Lebanese poultry as a routine process to evaluate the efficacy of the utilized NDV vaccines. Most of the microtiter plates used in these kits are pre-coated with specific antigens such as the Nucleoprotein (NP) which is a structural viral protein with a highly conserved N-terminal region. However, the main proteins that are involved in viral pathogenicity are the Fusion (F) and Haemagglutinin (HN) which contain hypervariable domains, enabling the virus to escape the host immune response [7] [8] [9]. That is, knowing that DIVA vaccines are not very commonly used in Lebanese farms, little attention is given to whether the cut points set in commercial ELISA kits reflect true protective immunoglobulin levels against the local NDV strains circulating among poultry farms. Therefore, this study was undertaken to develop an accurate and efficient in-house indirect ELISA to assess chicken sera antibody levels against a predominant velogenic NDV strain of Genotype VI that was isolated from Lebanese Poultry. The performance of this indirect ELISA, including specificity, sensitivity, and reproducibility, was also evaluated. Sera collected from various vaccinated and non-vaccinated chickens were used to validate the developed ELISA by comparing the generated results with those obtained by the HI test and a commercial NDV ELISA kit.

2. Materials and Methods

2.1. Preparation of the Inactivated Velogenic NDV (v-NDV) Stock

The prevalent v-NDV strain (Genotype VI-GenBank accession number KC425723) has been previously isolated from broiler outbreaks in the Bekaa region which is an endemic area located at 34.5440˚N, 36.0798˚E [10]. The original stock suspension was re-inoculated in 100 µl volumes in 9 days old chicken embryonated eggs through the allantoic route. Eggs were incubated at 99.5˚F for three days, and their allantoic fluid was harvested and tested for Hemagglutination (HA) activity against 1% of chicken RBC suspension. Positive HA fluids were pooled and the confirmation of the v-NDV presence in the tested fluids was done by PCR, using forward and reverse primer sequences targeting the viral fusion gene [11]. The viral stock was centrifuged at 5000 ×g for 15 min to remove cellular debris. The viruses in the supernatant fluid were further purified via filtration through a 0.22 µm pore size filter. Viruses in the filtrate were inactivated using 0.3% formaldehyde with continuous mixing for 16 hours. The viability of the viruses was checked by inoculating 9 days old embryonated eggs with 100 µL of the presumably inactivated suspension whereby the absence of Hemagglutination activity in the harvested allantoic fluids indicated viral inactivation. Inactivated viruses were centrifuged at 30,000 rpm for 3 h using an ultracentrifuge (Sorvall Discovery 100 SE, Hitachi). Viral pellets were collected and resuspended in 20 mL Phosphate-Buffered Saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 1L distilled water). Protein quantification of the inactivated viral suspension was performed using Bio-Rad protein assay reagents (1000 Alfred Nobel Drive, Hercules, California 94547, USA).

2.2. Development and Optimization of ELISA

Inactivated velogenic NDV suspension was prepared into two working dilutions, namely 20 and 40 µg/100µL, in carbonate buffer that was used as a coating buffer (8.4 g $NaHCO_3$, 3.56 g Na_2CO_3, 1L distilled water; pH = 9.6). Working dilutions were added to microtiter plates in volumes of 100 µl/well and incubated at 4˚C for 20 h. The plates were washed four times with PBS containing 0.05% Tween-20 (PBST) and then incubated with or without a blocking solution of 5% bovine serum albumin (BSA) in PBS for 2 h at 37˚C. After three washes with PBST, NDV negative or positive antisera (KPL, Gaithersburg, MD) were diluted 1:50, 1:100, 1:200, and 1:500 in PBS and added in volumes of 100 µL/well. The plates were incubated at 37˚C for 30 min, and the well contents were then aspirated before washing the plate four times with PBST. The rabbit anti-chicken IgG horseradish peroxidase-conjugated antibodies (KPL, Gaithersburg, MD) were prepared in three working dilutions of 1:2500, 1:5000, and 1:10,000 in PBS. Each dilution was added in volumes of 100 µL/well and incubated at 37˚C for 30 min. The well contents were aspirated, and the wells were washed four times with PBST. After washing, 100 µl of Tetramethylbenzidine (TMB) substrate (Amresco, Solon, USA) were added to each well, and the plates were incubated

in the darkness for 15 min at room temperature. A volume of 100 µL of stop solution (0.15% sodium dodecyl sulfate) was added in each well to stop the enzymatic reaction, and the Optical Density (OD) was determined at 580, 600, or 630 nm using Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific™). Samples of each treatment were run in six replicated wells. The optimal conditions were determined by assessing the OD Positive/Negative (P/N) ratio of the samples.

2.3. Evaluation of the Specificity, Sensitivity, and Reproducibility of the Developed ELISA

Different positive antisera were used to evaluate the specificity of the optimized ELISA namely those against *Mycoplasma gallisepticum*, Infectious Bronchitis (IB) Arkansas strain, IB Massachusetts strain, Infectious Bursal Disease, and H9 and H5 avian influenza subtypes (KPL, Gaithersburg, MD). The sensitivity of the developed ELISA was evaluated by using various dilutions of the positive anti-NDV serum in PBS namely: 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12,800, 1: 25,600, 1:51,200 and 1:102,400. The specificity and sensitivity of the assay were determined via assessing the OD S/P ratio of each dilution in six replications.

In addition, twenty serum samples collected from three broiler farms (Table 1) were used to determine the reproducibility of the developed ELISA based on three separate runs that were carried out at the Animal Research and Diagnostic Lab-FAFS/AUB. Samples were run in triplicates, once every two weeks, based on the optimized ELISA protocol and the S/P values recorded for each farm were compared among various runs.

2.4. Screening of Sera Collected from NDV-Vaccinated and Non-Vaccinated Poultry by Various Serological Methods to Validate the In-House ELISA Results

In total, 25 chicken serum samples were examined by the developed ELISA for the evaluation of antibody titers against the local Genotype VI velogenic NDV and for subsequent comparison with the results obtained using the HI test and a commercial NDV ELISA kit. The samples were collected from four broiler farms in North Lebanon adopting classical NDV vaccination and from non-vaccinated backyard layer chickens, as indicated in Table 1. Healthy birds that had similar live body weights were randomly selected in each farm for blood withdrawal. Around two mL of blood were collected from the bird's wing vein in non-heparinized tubes and handed to the research team. Blood was then transferred to the lab, and centrifuged at 2000 rpm for 10 min. NDV antibody levels in each of the collected sera were evaluated using the developed and commercial ELISA kits, run in six replicated wells, and HI test run in triplicates.

2.5. Statistical Analysis

The OD Positive/Negative (P/N) ratios obtained in the optimization part were

Table 1. Farms and birds involved in the validation and reproducibility assessment of the developed ELISA.

Farm/Market	Location	Birds' Age (days)	Number of Serum Samples Collected	Vaccination with NDV	Rearing System
Farm 1	North Lebanon	28	10	Clone-d1 ND/Flu (oil)-d1 Lasota-d14	Closed
Farm 2	North Lebanon	22	5	Modified Live Vaccine-d1 LaSota-d12	Semi-open
Farm 3	North Lebanon	30	5	Modified Live Vaccine-d1 LaSota-d12	Semi-open
Backyard Layers	Beirut Suburbs	65	6	None	Extensive

evaluated using multivariate analysis (factorial) with five treatments: 1) antigen level in coating buffer: 20 or 40 µg/100µL; 2) blocking buffer usage: none or 5% BSA in PBS; 3) serum dilution: 1:50, 1:100, 1:200, or 1:500; 4) conjugate dilution: 1:2500, 1:5000, or 1:10,000; wavelength used: 580, 600 or 630 nm. One-way ANOVA was used to compare the Sample/Positive (S/P) ratios obtained in the specificity, sensitivity, and reproducibility assays at a confidence level of 95%. Tukey's test was used in Post-Hoc analysis for mean separation whenever a $P < 0.05$ was detected. Pearson correlation was used to associate antibody titers as revealed by HI, commercial, and developed ELISA kits computed at a 95% confidence level (IBM SPSS Statistics for Windows, Version 25.0, Armonk, NY: IBM Corp.).

3. Results

3.1. ELISA Optimization Checkerboard

The optimal working ranges for sera and conjugate dilution, coating antigen level, usage of a blocking buffer, and reading wavelength were determined by a checkerboard evaluation and decided by the greatest P/N values obtained using NDV positive and negative sera. All the studied factors and their interactions had a significant impact on the P/N OD readings at $P < 0.05\%$. The mean separation results for the optimization of the ELISA system targeting the highest P/N ratio are presented in Table 2. Based on these results, the final optimized ELISA protocol was decided as follows:

Formalin-inactivated Genotype VI v-NDV particles were added into microtiter plates in volumes of 100 µl/well containing 20 µg inactivated viruses, and incubated at 4˚C for 20 h. The plates were washed four times with PBS containing 0.05% Tween-20 (PBST) and then incubated with 5% BSA in PBS as a blocking solution at 37˚C for 2 h. After three washes with PBST, chicken sera to be tested

were diluted 1:50 in PBS and added in volumes of 100 µL/well. The plates were incubated at 37˚C for 30 min and the well contents were then aspirated before washing the plate four times with PBST. The rabbit anti-chicken IgG Horseradish peroxidase-conjugated antibodies diluted 1:10,000 in PBS were added in volumes of 100 µl/well and incubated at 37˚C for 30 min. Well contents were aspirated and the wells were washed four times with PBST. After washing, 100 µl Tetramethylbenzidine (TMB) substrate (Amresco, Solon, USA) were added to each well and the plates were incubated in the darkness for 15 min at room temperature. A volume of 100 µL of stop solution was added in each well to stop the enzymatic reaction, and the Optical Density (OD) was determined at 580 nm. These optimized parameters were applied for further evaluation and validation of the in-house ELISA.

Table 2. Checkerboard results for optimization of the in-house ELISA system.

Coating Antigen Concentration (/100 µL/well)	Wave-length (nm)	Conjugated Antibody Dilution	P/N of the Optical Density (OD) Reading at Different Levels of the Positive Serum Dilution								SEM
			Blocked Wells				Non-blocked Wells				
			1:50	1:100	1:200	1:500	1:50	1:100	1:200	1:500	
20 µg	580	1:2500	2.41^{b1}	2.43^{a1}	2.24^{b2}	$2.32^{b1,2}$	0.52^{4}	0.58^{4}	0.56^{c4}	1.38^{c3}	0.036
		1:5000	2.55^{b1}	2.45^{a2}	2.45^{a2}	2.34^{b3}	$0.55^{5,6}$	0.57^{5}	0.51^{c6}	1.49^{a4}	0.017
		1:10,000	**2.75^{a1}**	2.53^{a2}	2.49^{a2}	2.47^{a2}	0.62^{5}	0.60^{5}	1.65^{a3}	1.53^{a4}	0.020
	600	1:2500	1.85^{d1}	$1.79^{c1,2}$	1.67^{e2}	1.81^{e1}	0.58^{5}	0.58^{5}	0.76^{b4}	1.40^{bc3}	0.039
		1:5000	1.99^{d1}	$1.92^{bc1,2}$	$1.89^{d1,2}$	1.87^{cde2}	0.55^{5}	0.56^{5}	0.76^{b4}	1.48^{ab3}	0.039
		1:10,000	2.22^{c1}	2.02^{b2}	$2.13^{c1,2}$	1.97^{c2}	0.61^{4}	0.59^{4}	1.68^{a3}	1.52^{a3}	0.050
	630	1:2500	1.83^{d1}	1.80^{c1}	1.58^{e2}	1.79^{e1}	0.55^{5}	0.58^{5}	0.72^{b4}	1.39^{c3}	0.034
		1:5000	1.95^{d1}	1.88^{bc1}	1.83^{d1}	1.85^{de1}	0.52^{4}	0.56^{4}	0.74^{b3}	1.45^{abc2}	0.038
		1:10,000	2.17^{c1}	$2.01^{b2,3}$	$2.14^{c1,2}$	1.96^{cd3}	0.60^{6}	0.59^{6}	1.68^{a4}	1.51^{a5}	0.047
40 µg	580	1:2500	2.00^{d1}	2.00^{b1}	1.82^{d2}	1.90^{cde2}	0.60^{3}	0.58^{3}	0.59^{3}	0.70^{4}	0.020
		1:5000	2.10^{cd1}	2.11^{b1}	$2.02^{c1,2}$	1.95^{cd2}	0.58^{3}	0.60^{3}	0.55^{3}	0.73^{4}	0.033
		1:10,000	2.34^{bc1}	2.12^{b2}	2.10^{c2}	2.02^{cd2}	0.65^{3}	0.63^{3}	0.60^{3}	0.60^{3}	0.034
	600	1:2500	1.42^{f1}	$1.37^{f1,2}$	1.29^{g2}	1.42^{g1}	0.59^{3}	0.62^{3}	0.69^{3}	0.63^{3}	0.042
		1:5000	1.58^{e1}	$1.50^{de1,2}$	1.42^{f2}	1.45^{fg2}	0.58^{3}	0.61^{3}	0.73^{4}	0.72^{4}	0.039
		1:10,000	1.83^{d1}	1.65^{d2}	$1.75^{d1,2}$	1.53^{f3}	0.63^{4}	0.59^{4}	0.63^{4}	0.75^{5}	0.041
	630	1:2500	1.42^{f1}	1.40^{ef1}	1.23^{g2}	1.41^{g1}	0.57^{3}	0.58^{3}	0.64^{3}	0.65^{3}	0.037
		1:5000	1.56^{e1}	$1.47^{e1,2}$	1.42^{f2}	$1.46^{fg1,2}$	0.53^{3}	0.57^{3}	0.64^{3}	0.75^{4}	0.035
		1:10,000	1.79^{d1}	$1.63^{d2,3}$	$1.72^{de1,2}$	1.59^{f3}	0.64^{4}	0.64^{4}	0.71^{4}	0.72^{4}	0.039
SEM			0.053	0.052	0.028	0.036	0.037	0.023	0.032	0.025	

$^{a\text{-}g}$Means in a column with different alphabetical superscripts are significantly different ($P < 0.05$); $^{1\text{-}6}$Means in a row with different numerical superscripts are significantly different ($P < 0.05$). The highest P/N value, reflecting optimal conditions, is indicated in bold.

3.2. Specificity, Sensitivity and Reproducibility of the Developed ELISA

In the determination of the specificity of the developed ELISA, the positive ND reference serum showed a significantly higher S/P value of 1 in comparison to other reference sera, namely IB-Mas and IB-Ark, H9 AI, H5 AI, IBD and MG that recorded values less than 0.4 (Figure 1). Therefore, the S/P value of 0.4 was determined to be the cut-off value to depict positive vs negative sera samples. Regarding the sensitivity of the developed ELISA, The S/P and S/N ratios of the reference ND serum decreased significantly, following its serial dilution in PBS, and then stabilized at a dilution rate of 1:51,200 (Figure 2). Therefore, the limit of detection of the developed ELISA was equivalent to a 1:25,600 dilution of the positive serum used in this study.

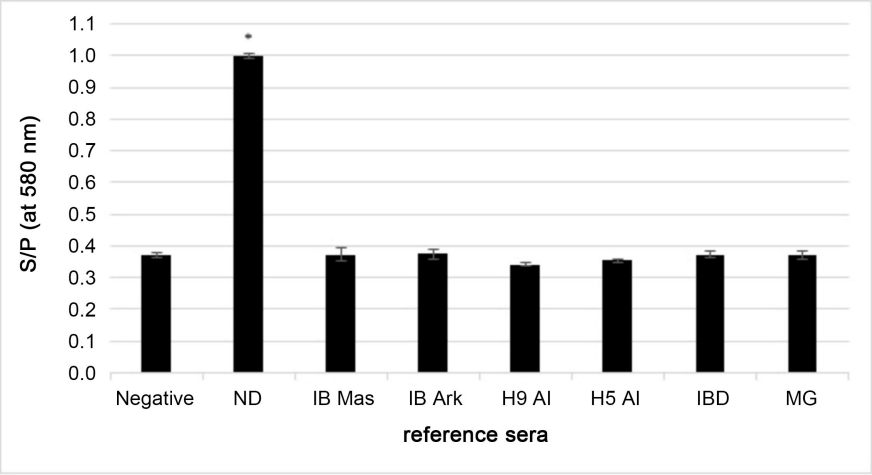

Figure 1. S/P values of various reference sera used in the specificity assay of the developed ELISA. The star indicates a significant difference at P-value of 0.05.

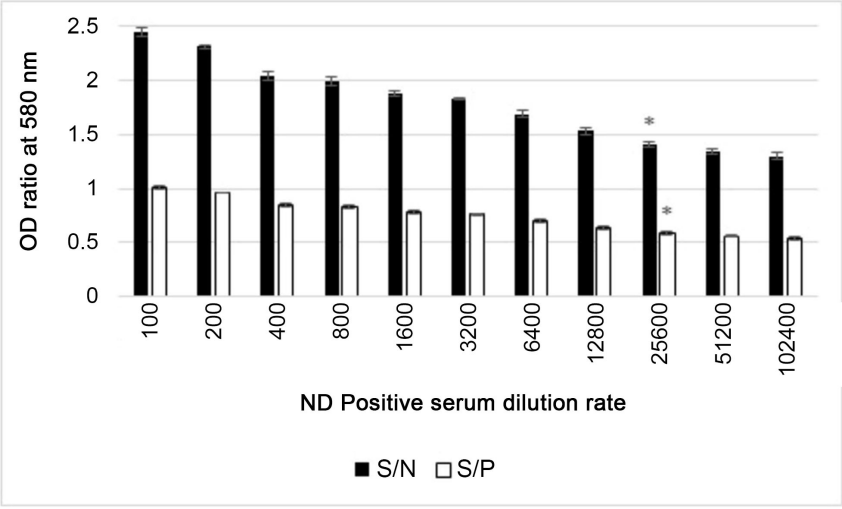

Figure 2. S/P and S/N values of the ND reference serum dilutions used in the sensitivity assay of the developed ELISA. The star indicates a significant difference at P-value of 0.05.

The 20 serum samples that were collected from broiler farms 1, 2, and 3 were used to determine the reproducibility of the developed ELISA based on three separate runs that were carried out within a time frame of eight weeks. For each farm, the average S/P values were not significantly different among the three runs as shown in Table 3 reflecting a consistency of results. Moreover, the S/P coefficients of variation (standard deviation*100/mean) of the inter-run reproducibility did not exceed 2.5%.

Table 3. S/P values of chicken sera recorded in three separate runs.

Farm	N	Mean S/P Value[1] Recorded with Different Runs			SEM*	CV** (%)
		1	2	3		
1	10	0.949	0.912	0.955	0.0126	2.48
2	5	0.963	0.961	0.970	0.0117	0.49
3	5	0.900	0.880	0.911	0.0056	1.75

*SEM = Standard Error of Mean; **CV = Coefficient of Variation; [1]Each sample was run in triplicate.

3.3. Validation of the In-House ELISA Results with Haemagglutination Inhibition and Commercial ELISA Tests

The S/P values of 25 serum samples collected from various chickens (Table 1), as revealed by the developed ELISA, were compared to those of the Haemagglutination inhibition test and the commercial ELISA kit using Pearson Correlation (SPSS). A significant correlation was observed between the S/P values of the developed ELISA and HI titers ($P < 0.01$). They were also significantly correlated, but to a lesser extent, with S/P values manifested by the commercial NDV ELISA kit ($P < 0.05$). Remarkably, no significant correlation was observed between the S/P values generated by the Commercial ELISA kit and HI titers of chicken sera collected in this study (Table 4).

Table 4. Correlation of chicken sera HI titers with S/P values of the developed ELISA system and the commercial ELISA kit.

	Coefficient of Correlation		
	Developed ELISA (S/P)	HI (titer)	Commercial ELISA (S/P)
Developed ELISA (S/P)	1.000	0.461**	0.330*
HI (titer)	0.461**	1.000	0.121
Commercial ELISA (S/P)	0.330*	0.121	1.000

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed).

4. Discussion

Commercial NDV ELISA kits might not reflect true titer values against field strains that are predominant in a specific geographic zone. The main target of this

study was to tailor an indirect ELISA to quantitate the antibody titers of chicken sera against a local and major velogenic NDV strain belonging to Genotype VI that is endemic to Lebanon and many countries of the MENA region. The ELISA protocol optimization was significantly dependent on all the factors included in this study along with their interactions. These factors included the NDV coating antigens concentration, blocking the wells with bovine serum albumin, dilution rate of both the serum and the conjugated secondary antibodies, and the OD reading wavelength.

The use of whole ND viruses as coating antigens for the binding of corresponding antibodies gives generally adequate results but requires a considerable amount of live viruses to be propagated, purified, and inactivated [12]. The optimal coating antigen concentration for an ELISA assay could vary depending on the targeted specificity, sensitivity, and signal-to-noise ratio. In this study, the optimal viral concentration used for wells coating was 20 μg/100μL/well whereas a double antigen concentration resulted in mediocre outcomes. This could be due to the fact that higher concentrations of coating antigens open the door widely for false positive readings through non-specific interactions, particularly with multi-epitope antigens such as whole viruses, as is the case in our study [13]. When present in high concentrations, divergent NDV proteins, such as the P protein, might play a key role in lowering the specificity of the system through interaction with non-specific antibodies [14].

When optimizing solid phase ELISA, blocking the microtiter plate well-active sites prevents non-specific binding to reactants such as proteins and various immunoglobulins. Bovine Serum Albumin (BSA), at a concentration of 5% in PBS, was able to improve significantly the specificity of the developed ELISA. Non-blocked wells resulted in similar P/N OD readings for positive and negative anti-NDV sera, which means that the signal-to-noise values were significantly improved by the use of BSA as a blocking agent. BSA is one of the most used blocking agents in ELISA protocols, along with skimmed milk and whole serum in order to eliminate the residual binding capacity of the wells, stabilize the biomolecules bound to the well surface and reduce non-specific interactions [15] [16]. Nevertheless, the works of Xiao and Isaacs highlighted the fact that BSA preparations do not behave the same and that non-specific binding of proteins and antibodies to BSA might occur [15]. This might explain the fact that the NDV-negative serum used in this study presented a considerable S/P noise reading of around 0.4.

Animal sera are composed of an undefined mixture of macromolecules, including hormones, transport proteins, growth factors, lipids, minerals, and detoxifying factors. As a result, when using ELISA to quantitate antibodies against NDV or other poultry pathogens, undiluted serum samples may produce significant background noise and false-positive findings which could result in an erroneous diagnosis. Therefore, the dilution of chicken serum samples is an essential step when running ELISA assays. The optimal serum dilution of 1:50 documented in this study is in accordance with the findings of several researchers, whereby a dilution of 1:50 or 1:100 was found to be optimal for detecting IgG

antibodies against various pathogens in chicken serum samples using ELISA [17] [18] [19]. The said authors reported that using higher dilutions might result in reduced sensitivity, while lower dilutions entail higher background noise and lower specificity. This is probably why the highest serum dilutions of 1:200 and 1:500 used in this study resulted in poor outcomes. It is worth noting that most of the locally available commercial ELISA kits use a 1:200 - 1:500 dilution range which can be explained by the fact that these kits could reduce the background noise by using a single protein coating for the wells instead of whole viruses. In fact, various NDV proteins have been used as coating antigens in ELISA protocols such as the Nucleoprotein (NP), Phosphoprotein (P), and Haemagglutinin protein (HN) [12]. Again, the use of single proteins as coating antigens could compromise the specificity of the protocol in regard to endemic NDV escape mutants that might have undergone adaptive mutations in more than one protein.

The optimal dilution of the rabbit anti-chicken IgG secondary antibody conjugated to HRP recorded in this study (1:10,000) is supported by abundant literature documenting the use of a similar level of dilution in ELISA testing of NDV, infectious Bronchitis, avian influenza, and other diseases [20] [21] [22]. Higher dilutions of the conjugate enhance the specificity of the developed protocol by avoiding non-specific reactions with various components coating the wells also known as extraneous proteins [22]. Moreover, the fact that IgG whole molecules have a very broad specificity means that these immunoglobulins might cross-react, non-specifically, with other Ig classes and with IgG from other species. It is worth noting that the HRP enzymes conjugated to the secondary antibodies cleave the Tetramethylbenzidine used in this study as a substrate to produce a deep blue color during the enzymatic degradation of hydrogen peroxide. The sodium dodecyl sulfate used in this experiment preserved the blue color of the final product [23] which presented an optimal OD reading at 580 nm wavelength.

Optimizing the developed ELISA parameters resulted in a specific, sensitive, and reproducible diagnostic test for detecting NDV antibodies in chicken sera. The developed assay recorded negative results *i.e.* below the established S/P cut-off value of 0.4 for the ND negative serum and the heterologous antibodies, including IB Mas, IB Ark, H9 HI, H5 AI, IBD, and MG. Many researchers affirm the superiority of ELISA specificity in comparison to other serological methods used to diagnose animal diseases [24] [25] [26]. The numerous epitopes found on the whole velogenic NDV virus used in this study could be the reason behind the high specificity of the developed ELISA protocol. Interestingly, the sensitivity of the developed ELISA presented another confirmation of the reported S/P cut-off value and showcased a reliable tool for the detection of a wide range of anti-NDV antibody levels in chicken sera. Various researchers reported similar sensitivity levels corroborating a better efficiency in comparison to other serological methods including HI. Although the latter is commonly considered as a gold standard method, the HI titer can be influenced by the type of Red Blood Cells (RBCs), and the variable expression of neuraminic acid receptors on the

membranes of different RBCs, which may affect the binding affinity and consequently the sensitivity of this method [25]. That is why ELISA, in general, consistently detects significant levels of reactive antibodies against NDV in more birds than the HI does [26]. Nevertheless, after analyzing the chicken serum samples collected from various farms, the highest significant correlation was observed between the developed ELISA and HI results. Given that HI was regarded as a reference method [27], the correlation results emphasize the diagnostic validity of the developed ELISA. This validity was further manifested after the test was repeated three times within a period of 8 weeks, resulting in the obtention of very low CV values (less than 2.5%) for each farm. The correlation of NDV titers in chicken sera between the developed and the commercial ELISA was also significant but to a lesser extent. This reflects the potential variations that exist in the amino acid sequence of various proteins used in coating the commercial ELISA kit and those found on the Genotype VI velogenic field strain used in this study. It is well known that the Nucleoprotein (NP) or the Phosphoprotein (P) used to coat most of the indirect NDV ELISA plates has high variability between mesogenic and lentogenic strains used as live vaccines, and the velogenic strains circulating worldwide [28]. Therefore, not only does the incompatibility between field and vaccine strains explain the failure of vaccination protocols, but it also leads to an overestimation of protective NDV antibody titers revealed by commercial kits. The validity of the commercial NDV ELISA kit was further questioned when the obtained sera titers did not significantly correlate with those of a Genotype VI-based HI. The performance parameters of the developed ELISA kit suggest that it could be the test of choice for the assessment of antibody titers in chickens against predominant velogenic NDV strains in Lebanon and the region, namely those belonging to Genotype VI. It might also offer better help in the serological detection of birds' exposure to the said NDV strains. The developed ELISA protocol offers a simpler and more performing tool in comparison to HI that requires relatively high volumes of sera, and a more specific tool in comparison to the commercial ELISA kit used in this study. Nevertheless, the cross-reactivity with other NDV strains or potential interference from non-specific antibodies should be further studied. The inclusion of a controlled birds' challenge would have been an added value in providing a better insight into whether the antibody titers of vaccinated chickens, as revealed by the developed ELISA, are protective against infection with the local velogenic NDV strain.

5. Data Availability Statement

The datasets used and/or analyzed during this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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