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The effect of *Spirulina algae* on the immune response of SPF chickens to commercial inactivated Newcastle vaccine in poultry

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The objective of this study was to investigate the effects of *Spirulina platensis* (SP) powder supplementation on immune response in SPF chickens. For this purpose, 120 SPF chicks were randomly clustered into six groups consisting of 20 birds each which assigned to five groups vaccinated by commercial inactivated Newcastle disease (ND) vaccine at 21 days of age. The four groups were supplemented with 0.5, 1, 1.5 and 2 g of SP per kg of ration at 7 day of age and other group as control treatment group. Control unvaccinated group still without any treatment. Individual blood samples were collected weekly from all groups, and NDV-HI antibodies were measured using Hemagglutination inhibition (HI) test. After 28 days post-vaccination, ten birds from all groups were challenged intramuscularly at a dose 0.5 mL/bird containing 10^6 EID_{50} of local NDV genotype VII. Challenge virus shedding was detected using real time qrt-PCR of oropharyngeal swabs that were collected from all challenged chicken groups of at 3, 5, 7 and 10 days post challenge. Obtained results showed that vaccinated groups of SPF-chickens either supplied with Spirulina or control treatment group induced positive serological response as NDV-HI antibody were measured in sera of immunized chicks (7.6, 8, 8.3, 8.9 and 7.4 log_2, respectively) at 4 weeks post vaccination (WPV). Significant differences were observed at 2 WPV in the vaccinated SPF chickens consumed 1, 1.5 and 2 g of SP/kg of ration, compared to untreated vaccinated group ($p<0.05$). Immunized SPF chickens supplied with different SP concentration confer satisfactory protection against heterologous challenge virus (90%, 100%, 100% and 100% respectively), in contrast to untreated vaccinated chickens. Different percentages of reduction of viral shedding (55%, 65%, 76% and 87%) of treated vaccinated chickens with different concentration of SP were detected, despite untreated group were reduced 46% from total viral shedding. These findings suggest that dietary Spirulina has immune-stimulatory effects on the immune system of SPF chickens. One gram from SP per kg of ration was minimum recommended concentration that able to exhibit optimum immune response, increase protection against heterologous strains and able to reduce viral shedding.

Keywords: Spirulina; Newcastle disease virus; humoral immune response; real-time PCR; vaccination; serological tests.

Introduction

Spirulina platensis (SP) is a blue-green alga having diverse biological activity. Early, due to high content of highly valuable proteins, indispensable amino acids, vitamins, beta-carotene and other pigments, mineral substances, indispensable fatty acids and polysaccharides, SP has been found suitable for use as bioactive additive.[1] Recent attention has been given to immune-stimulant role of SP.[2]

In 1994, the first report for immune-modulatory effect of this alga on mice through enhanced IL-1 antibody production.[3] Chicken diets were contained less than 1% Spirulina lead to significantly enhance the defense systems for increased microbial killing, antigen processing and greater T-cell activity. Subsequently, more researches were investigated the role of Spirulina to enhance immune system in different animal models as dogs and cats as well as human.[4] Newcastle disease virus (NDV) stands behind most of mortalities and morbidities in poultry.[5] The infection control depends mainly on vaccination of chickens with the commercial inactivated NDV vaccine. Many of these vaccine batches are submitted annually to the Central Laboratory for

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Evaluation of Veterinary Biologics (CLEVB), Cairo, Egypt for reviewing its quality. Potency of this type of vaccine is evaluated routinely by vaccination-challenge test (efficacy) in susceptible SPF chickens following the protocols.[6]

In this study, protective efficacy of an inactivated Newcastle vaccine (Ulster 2 strain) is determined in SPF chickens that were supplied with different concentrations of SP (0.5, 1, 1.5 and 2 g/kg ration) in comparison with SPF chickens fed with the same ration without any supplement against heterologous circulating NDV genotype VII.

Materials and Methods

Preparation of Spirulina extract: A crude extract was prepared from SP using a patent-pending procedure. Raw material was extracted two times with 50% ethanol at 70°C, 45 min each time. Supernatants from both extractions were combined following centrifugation for 5 min at 1500 g.

The ethanol concentration of the extract was adjusted to 75% by addition of 1 volume of cold ethanol. Following incubation for several hours at -20°C, perceptible material was collected by centrifugation at 1500 g and subsequently washed with cold ethanol. The final extract material was dried and represented a 15% yield of raw material dry weight.

Experimental Chicken: SPF chickens were obtained from Khom Oshem farm, El Fayoum as one day old. They were reared and housed in positive pressure stainless steel isolation cabinets with continuous light exposure.

Vaccine: The commercial inactivated NDV vaccine contained (Ulster2C strain) which was used for vaccination of SPF chickens with one field dose recommended for poultry that was administrated intramuscularly at a dose of 0.3mL/bird. The batch No. is (L469199) and it's expired by 23/11/2021.

Serum samples: Blood samples were collected from jugular vein of ten vaccinated SPF chickens from each group and sera were separated to conduct Hemagglutination inhibition test (HI test).

Virus: Local NDV genotype VII was obtained from Strain Bank of Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) which has (accession no. KM288609) to be used as challenge virus and as heterologous NDV antigen with a titer of 8 log 2 HA units/mL and used at a final concentration of 4 HA/mL in HI test for the tested serum samples.

Propagation and titration of NDV genotype VII: It was carried out according to the manual of the World Organization of Animal Health.[7]

Calculation of Egg infective dose/50 (EID_{50}) for local NDV genotype VII: It was done according to the manual of the World Organization of Animal Health.[7]

Serological tests: Hemagglutination (HA) and HI assays were performed using the standard microtiter plate method as recommended.[7] The HI tests was carried out with 4 HA units of NDV genotype VII per well.

Measurement of the protection efficacy %: By using challenge test through inoculation of 10^6 EID_{50}/SPF chickens of NDV genotype VII intramuscularly at a dose 0.5mL/bird.[7]

Measurement of viral shedding by RT-qPCR: Oropharyngeal swabs were taken from all groups of chickens at 3, 5, 7 and 10 days post challenge and prepared then kept at -80°C till use.[7]

Qrt-PCR: RNA was extracted from swabs using QIAamp Viral RNA Mini Kit that supplied from Qiagen, Valencia, CA, USA, Cat. No. 52906. Samples were amplified using Invitrogen superscript® III platinum® one- step Quantitative RT-PCR Cat. No 11732-088 to investigate the presence or absence of M gene of ND virus following the manufacture instructions using primers and probe and reaction condition.[8] The test was conducted in a CFX 96 touch TM Real time PCR (Table 1).

Experimental design: A total 120 SPF chickens at 7 days old of age were distributed randomly into three

Table 1. Oligonucleotide primers used in RT-PCR for detection of NDV M- protein gene.

Primer	Sequence (5′ - 3′)
Forward ND-M+4100	AGTGATGTGCTCGGACCTTC
Reverse ND-M-4220	CCTGAGGAGAGAGGCATTTGCTA
Probe	HEX-TTCTCTAGCAGTGGGACAGCCTGC-BHQ

groups. The first group (80 SPF chickens) was allotted into four replicates (20 SPF chickens/replicate) which were supplemented with different SP concentration (0.5, 1, 1.5, and 2 g/kg in ration), then vaccinated with NDV vaccine at 21 days old age.

The second group (20 SPF chickens) were fed without any supplement, then vaccinated with the same vaccine at the same age of the previous group despite, the third group control unvaccinated group (20 chickens) without any treatment.

Individual blood samples were collected from ten birds of each group weekly between the first and 4 weeks after inoculation and NDV-HI antibodies were measured in each collected serum sample by HI test. After 28 days post-vaccination, ten birds from all groups were challenged intramuscularly at a dose of 0.5 mL/bird by 10^6 EID_{50} of local NDV genotype VII. Oropharyngeal swabs were taken from all groups of chickens at 3, 5, 7 and 10 days post challenge.

Ethical approval: Institutional Animal Care and use committee at Central Laboratory for Evaluation of veterinary Biologics hereby acknowledge the research manuscript and it has been reviewed under our research authority and is deemed compliance to bioethical standards in good faith.

Statistical Analysis: Data generated from immune responses were subjected to one way analysis of variance (ANOVA). Variant means were separated post hoc using the least significant difference (LSD) method;[9] $p<0.05$ were accepted as significant.

Results

Hemagglutination inhibition test

The data revealed from Table 2, showed low mean HI antibody titers at one week post vaccination (WPV) for all vaccinated groups. Mean HI antibody titer increased gradually till reached its peak at 4th WPV to be 7.6, 8, 8.3, 8.9 and 7.4 log_2 in the sera of vaccinated SPF chickens supplemented with 0.5, 1, 1.5 and 2 g SP/kg ration and control treatment group, respectively.

Vaccinated SPF chickens were consumed SP algae in ration exhibited higher immune response than those induced by the other group. There were a significant difference at 2nd WPV in all groups consumed 1, 1.5 and 2 g of SP in ration to other vaccinated chickens fed ration without SP as shown in Table 3.

Protection efficacy

It was found that all vaccinated SPF chickens groups consumed SP in ration with different concentrations (0.5, 1, 1.5 and 2 g/kg ration) were protected the infection against local NDV genotype VII with a different percentage: 90, 100, 100 and 100%, respectively. The group fed without SP has less protection% reached 80% in contrast to the control group that showed 100% mortality (Table 4).

Viral shedding

Oropharyngeal swabs were collected from all groups after 3, 5, 7 and 10 days post challenge (DPC) examined by real time RT-PCR to calculate viral Shedding of

Table 2. Mean ND-HI antibody titer produced by sera of SPF chickens after 1, 2, 3 and 4 weeks post vaccination with one dose of commercial ND vaccine.

Group of chickens	Spirulina (g/kg) in ration	Mean HI antibody titer (log_2)			
		1st WPV	2nd WPV	3rd WPV	4th WPV
Vaccinated chickens were supplemented with different SP g/kg in ration	0.5 g/kg	1.2 ±1.09280	4.3 ±0.67495	6.4 ±0.69921	7.6 ±0.51640
	1 g/kg	1.4 ±0.96609	5.1 ±0.87560	7.3 ±0.67495	8 ±0.73786
	1.5 g/kg	1.6 ±0.84327	5.4 ±0.69921	7.4 ±0.51640	8.3 ±0.67495
	2 g/kg	2 ±0.94281	5.9 ±0.56765	7.8 ±0.63246	8.9 ±0.73786
Vaccinated chickens without SP supplement in ration		1.2 ±1.03280	4.1 ±0.87560	6 ±0.81650	7.4 ±0.69921
Control non-vaccinated chickens		0	0	0	0

Table 3. Significant values between vaccinated chickens without SP supplement in ration to Vaccinated chickens consumed different concentration SP g/kg of ration.

Group	Significant values				
	Vaccinated chickens supplied with different SP g/kg of ration				
	WPV	0.5 g/kg	1 g/kg	1.5 g/kg	2 g/kg
Vaccinated chickens without Spirulina supplement in ration	1 WPV	0.961	0.553	0.179	0.513
	2 WPV	0.649	0.005	0.000	0.000
	3 WPV	0.365	0.000	0.000	0.000
	4 WPV	0.074	0.000	0.000	0.000

Significance difference≤0.005.

Table 4. Protection % of SPF chickens after 4 weeks post vaccination with one dose of commercial ND vaccine following challenge by local NDV genotype VII.

Groups		Total mortality	Protection%
Spirulina g/kg in ration for vaccinated chickens	0.5 g/kg	1/10	90%
	1 g/kg	0/10	100%
	1.5 g/kg	0/10	100%
	2 g/kg	0/10	100%
Vaccinated chickens without Spirulina supplement		2/10	80%
Control non vaccinated		5/5	0%

Table 5. Reduction in viral shedding (log_{10}) from SPF chickens after 4 weeks post vaccination with one dose of commercial ND vaccine following challenge by local NDV genotype VII.

Group of chickens	Spirulina g/kg in ration	Titer of Shedding Virus titer (log_{10})					
		3rd DPC	5th DPC	7th DPC	10th DPC	Mean viral shedding	Reduction in viral shedding
Vaccinated chickens were supplemented with different SP g/kg in ration	0.05%	4	4.1	3	1.8	3.2	3.95 (55%)
	0.1%	3.3	3.1	2.3	1.4	2.5	4.65 (65%)
	0.15%	2.1	2.4	1.6	1	1.7	5.45 (76%)
	0.2%	1	1.3	0.9	0.4	0.9	6.25 (87%)
Vaccinated chickens without SP supplement in ration		4.8	4.6	4	2	3.85	3.3 (46%)
Control non-vaccinated chickens		7.1	7.2	-	-	7.15	0

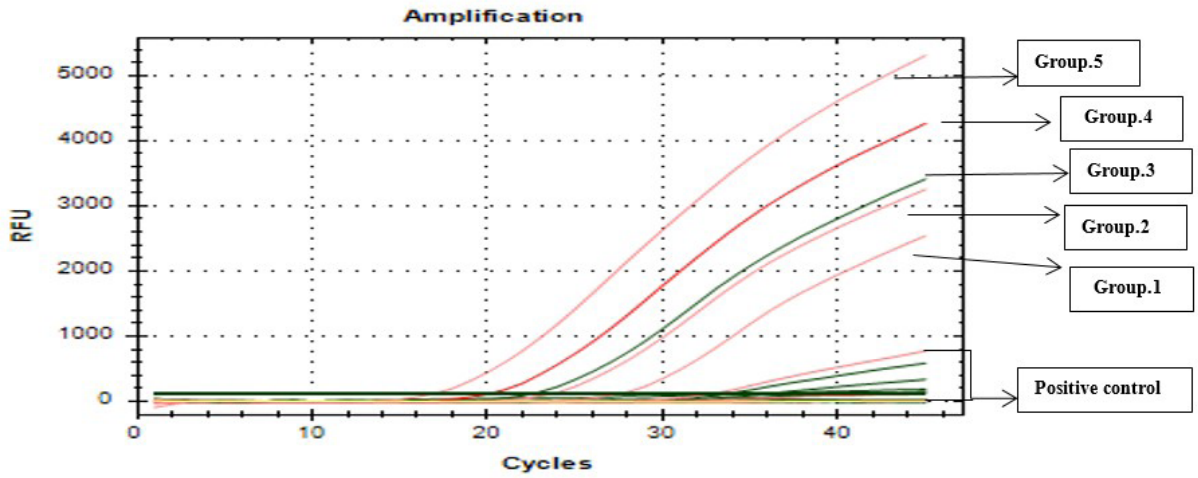

Fig. 1. Amplification curve obtained during RT-PCR Positive control (accession no. KM288609): (1) (challenged SPF chickens without treatment), (2), (3), (4) and (5) SPF chickens were treated with 0.5, 1, 1.5 and 2 g of SP/kg ration, and negative control (non-infected allantoic fluid).

Local NDV genotype VII. The exhibited data from Table 5 and Figure 1 showed the challenged vaccinated chickens released the virus with low titers in comparison to unvaccinated challenged chickens. The reduction of viral shedding reached 3.95, 4.65, 5.45, 6.25 and 3.3 $\log_{10}$ in challenged vaccinated chicken consumed Spirulina algae 0.5, 1, 1.5 and 2 g/kg of ration and the other challenged vaccinated not treated respectively.

Discussions

Spirulina is a cyanobacterium species.[10] In poultry, some recent studies have shown that feeding SP is responsible for improvement of immune functions, subsequently increased disease resistance, improved survival and growth rates.[3]

In this study, the immunostimulant effect of Spirulina was assessed through vaccination of SPF chickens with commercial inactivated NDV vaccine which clustered into five groups (20 SPF chickens/group). Five groups were supplied with different SP grams (0.5, 1, 1.5 and 2) per kg of ration respectively, in comparison with control treatment group which agreed with some researchers. [11] Blood samples were collected from vaccinated SPF chickens weekly till 4 weeks after inoculation, and ND-HI antibodies were measured in collected sera by HI test. The obtained results pointed that all vaccinated groups of SPF-chicks induced high seroconversion response when ND-HI antibodies were measured in sera of immunized chicks at 3 and 4 WPV.

An arithmetic mean of ≥6 log of HI antibodies in serum samples collected 3-4 weeks after vaccination is required for approval.[7] Despite, vaccinated SPF chickens were consumed SP in ration exhibited higher immune response than that induced by control treatment group which agreed with Egorova et al,[12] that proved the selenium enriched phycocyanin (Se-PC) from food microalgae Spirulina demonstrated significantly increased specific IgG response found the dietary SP increase IgG level in sera of vaccinated chickens.[13]

Significance between vaccinated SPF chickens groups consumed different SP grams to untreated group exhibited a significance difference ($p \leq 0.005$) at 2 WPV of all treated groups except SPF chickens consumed SP with a 0.5 g in kg of ration.

The protective efficacy of inactivated NDV vaccine containing (Ulster 2C strain) were measured through challenging all vaccinated chickens by heterologous local NDV genotype VII,(7) stating that an effective ND poultry vaccine should protect at least 90% of vaccinated chickens from death. Another interesting finding was all vaccinated SPF chickens consumed different SP grams confer satisfactory protection which ranged 90-100% against heterologous NDV genotype VII despite, 0.5 g from Spirulina did not revealed significance difference in HI titer to untreated group. These results matched with studies that reported that Spirulina is capable to enhancing non-specific immune responses as well as that the ingestion SP enhanced cell mediated immunity.[14,15,16] They revealed the SP produces an immunostimulating effect by enhancing the resistance of humans, mammals, chickens and fish to infections.

The vaccinated SPF chickens still untreated couldn't induce the satisfactory protection level which agreed

with the same results obtained by Sedeik et al,[17] who said that the ND homologous vaccine containing the challenge virus was better for clinical protection than the heterologous vaccine in terms of mortality and body weight loss.

Detection of the oropharyngeal (tracheal) viral shedding post challenge in vaccinated and control groups revealed that, vaccinated SPF chickens groups consumed different SP concentration (0.5, 1, 1.5 and 2g/kg of ration) showed a decrease in the tracheal viral shedding than vaccinated untreated group with a percentage of 55%, 65%, 76%, 87% and 46% of the total collected samples, respectively, but cannot completely prevent viral shedding which agreed with Elshazly,[18] who tested the viral shedding in vaccinated chickens after challenge with a NDV genotype VII, and they showed that shedding was significantly reduced in vaccinated groups in comparison with the unvaccinated group but not completely prevented the ND outbreak.

These findings suggest that dietary Spirulina has immune-stimulatory effects on the immune system of SPF chickens. One gram from SP per kg of ration was minimum recommended concentration that able to exhibit optimum immune response, increase protection efficacy against heterologous strains and able to reduce viral shedding.

Conflict of interest

The authors whose names are listed immediately above certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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El efecto del alga Spirulina sobre la respuesta inmune de pollos SPF inducida por la vacuna inactivada comercial contra la enfermedad de Newcastle

Abstract

El objetivo de este estudio fue investigar los efectos de la suplementación con polvo de *Spirulina platensis* (SP) sobre la respuesta inmune en pollos SPF. Para este propósito se agruparon al azar 120 polluelos SPF en seis grupos de 20 aves cada uno, que se asignaron a cinco grupos vacunados con la vacuna comercial inactivada contra la enfermedad de Newcastle (ND) a los 21 días de edad. Cuatro grupos se suplementaron con 0,5; 1; 1,5 y 2 g de SP por kg de ración a los 7 días de edad, un grupo vacunado sin suplemento y un grupo sin ningún tratamiento. Semanalmente, se recogieron muestras de sangre individuales de todos los grupos y se midieron los anticuerpos hemaglutinantes contra el virus Newcastle (NDV-HI) mediante la prueba de inhibición de la hemaglutinación (HI). Veintiocho días después de la vacunación, fueron retadas diez aves de cada grupo por vía intramuscular a una dosis 10^6 EID_{50} del genotipo VII del NDV local en un volumen de 0,5 mL/ave. Se detectó la eliminación del virus mediante qrt-PCR en hisopos orofaríngeos que se recolectaron en todos los grupos a los 3, 5, 7 y 10 días después del reto. Los resultados obtenidos mostraron que los grupos vacunados de pollos y suplementados con Espirulina y el grupo de control vacunado, indujeron una respuesta serológica positiva cuando se determinaron los anticuerpos NDV-HI en los pollitos inmunizados (7,6; 8; 8,3; 8,9 y 7,4 log_2 respectivamente) a las 4 semanas después de la vacunación (SPV). Se observaron diferencias significativas a las 2 SPV en los pollos vacunados que consumieron 1, 1,5 y 2 g de SP/kg de ración, en comparación con el grupo vacunado no tratado ($p<0,05$). Los pollos inmunizados que recibieron diferentes concentraciones de SP mostraron una protección satisfactoria contra el desafío heterólogo viral (90%, 100%, 100% y 100% respectivamente), en contraste con los pollos vacunados no tratados. Se observaron diferentes porcentajes de reducción de la diseminación viral (55%, 65%, 76% y 87%) entre los pollos vacunados tratados con diferente concentración de SP. En el grupo no tratado se redujo al 46%. Estos hallazgos sugieren que la Espirulina en la dieta tiene efectos inmunoestimuladores sobre el sistema inmunitario de los pollos. Un gramo de SP por kg de ración fue la concentración mínima recomendada para una respuesta inmune óptima, y de esta forma aumentar la protección contra las cepas heterólogas y disminuir la diseminación viral.

Palabras clave: Spirulina; virus de la enfermedad de Newcastle; respuesta inmune humoral; PCR en tiempo real; vacunación; pruebas serológicas.

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