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Original Research Article

Enhancing the Immune Response of Broilers Using Newcastle Disease Vaccine-Chitosan Nanoparticles

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Abstract: This study included one hundred and fifty one day old chicks divided into five groups each group contain 30 chicks G1 control negative , G2 control positive (deionized water), G3 Intranasal vaccine with CS-NPs, G4 Intranasal vaccine with NDV (La Sota), G5 Intranasal vaccine with NDV-CS-NPs(EID_{50}:$10^{7.5}$), each groups were vaccinated at 10 and 21 days old, blood samples were collected at 16 , 26 and 30 days old and examined by ELISA, the highest IgA level were in G5 (21.87 pg/ml) at 30 day old chicks. The infectivity of virulent ND virus were used to measure the titration of 70 embryonated eggs at ten-days old embryonated chicken eggs, the lethal dose (LD_{50}) of local ND virus and the result was $10^{7.34}$/1ml. G5 chickens vaccinated with the NDV-CS-NPs vaccine were completely protected against the ND virus challenge, while the chicks in G1 were dead following the virulent NDV challenge and displayed clinical signs such as conjunctivitis, sudden death, and ruffled feathers, as well as postmortem lesions such as hemorrhage the many positions of the trachea, caecal toncil, and on the tips of glands in proventiculus. These findings suggest that, in comparison to regular and nano-chitosan, the use of Newcastle disease vaccine-chitosan nanoparticles in experiment two (in group 5) produced a stronger immune response and better protection.

Keywords: Chitosan Nanoparticles, Newcastle Disease, IgA, Chickens.

INTRODUCTION

Newcastle disease (ND) is a highly contagious and disastrous disease of poultry and wild birds that causes significant economic losses (Alexander *et al*., 2012). It is caused by Newcastle disease virus (NDV) which is classified into 9 serotypes designated avian paramyxovirus (APMV)-1 to APMV-9 (Amarasinghe *et al.*, 2017). Newcastle disease virus NDV have ability to infected a wide variety of avian species and the pathogenicity of NDV through species is variable, poultry are most susceptible to NDV, with high morbidity and mortalities were notes in broiler and layer flocks (Wajid *et al*., 2016).

Because of their small size, nanoparticles—solid colloid particles that range in size from 1 to 100 nm—have greater mobility than micron-grade particles and can readily enter cells to gather at the site of a lesion. When polyanion, such as tripolyphosphate, is combined with chitosan solution while being continuously stirred, chitosan nanoparticles are created spontaneously (Singh *et al*., 2017). The primary technique for creating nanochitosan is ionic gelation, which involves the interaction of oppositely charged macromolecules to create chitosan nanoparticles. Tripoliphosphate (TPP) is frequently used to create chitosan nanoparticles due to its nontoxicity, multivalent nature, and ability to form gels through ionic interactions. The charge density of TPP and chitosan can regulate this interaction, which is reliant on the pH of the solution. (Zhao *et al*., 2017).

By improving antigen distribution, the use of nanochitosan in vaccination formulations can lower the number of booster doses needed to elicit a suitable immune response (Mohammadi *et al*., 2017).

METHODS

Preparation of chitosan nanoparticles and mixing these particles with Newcastle disease vaccine to formation Newcastle vaccine-chitosan nanoparticles that use in this study and measuring the best level that used as vaccine to give high immune response is based on study of Ameer *et al.*, 2020.

After being cleaned with 70% alcohol, the egg shell was left to dry and was numbered. Each dilution uses 60 eggs, five of which are from 10–1 to 1–10, five of which are inoculated with 0.2 ml of PBS (pH 7–7.2) as a control positive group, and five of which are left uninoculated as a negative control group (Wegdan *et al*., 2015).

The injection was carried out using a pinhole formed at the broad end of the eggs when they were completely sanitized, and as soon as it was finished, the inoculation site was sealed with sterile paraffin wax.

After the implanted eggs were incubated for 24 hours at 37°C and 70% relative humidity, the dead embryos were disposed of. The remaining embryos were re-incubated for seven days, while the weak and dead embryos were separated and stored in the refrigerator.

Experimental Design

A total of 150 broiler chicks subjected in the five groups as the following:

- Group 1 (control negative): Unvaccinated group.
- Group 2 (control positive): Vaccinated with 0.2ml deionized water at 10days old chicks and repeated at 21 days old chicks via intranasal route.
- Group 3: Vaccinated with 0.2ml chitosan nanoparticles (CS-NPs) at 10 days old chicks and repeated at 21days old chicks via intranasal route.
- Group 4: Vaccinated with 0.2ml ND La Sota (Volvac) (EID50: 109.5) (Ordinary Vaccine) at 10days old chicks and repeated at 21 days old chicks via intranasal route.
- Group 5: Vaccinated with 0.2ml NDV-CS-NPs (EID50:107.5) (Experimental Vaccine) at 10days old chicks and repeated at 21 days old chicks via intranasal route.

Parameters

- Collecting blood sample in 5 day old to measure the maternal immunity.
- Collected blood samples in (16, 26 and 30 days).
- ELIZA for IgA.
- Clinical signs, morbidity and mortality.
- Challenge with ND virus at 30 day old.
- Pathological study: gross lesion examination.

Titration of Virulent Viruses on Chicken Embryonated Eggs (Embryo Lethal Dose 50)(ELD_{50})

The virus was kindly provided by Lectu. Mohammed Abdulkadhim Hussein / Karbala Province. The approach outlined by Ramakrishnan (2016) was used to determine the embryo lethal dose 50 (ELD50) of NDv. 70 embryonated eggs (Breed: Rose 308, Origin: Belgium) were obtained from AL-Anwar Hatchery-Baghdad and inoculated with the virus. The eggs were separated into seven groups, each of which had ten eggs. The inoculum dilution that resulted in 50% of the embryonated eggs dying was calculated. The following calculations were made using the Muench formula and the Ramakrishnan (2016) technique, which is dependent on reed:

$$Index\ \% = \frac{\%\ Lethal\ at\ dilution\ immediately\ above\ 50\%-50\%}{\%Lethal\ at\ dilution\ immediately - \%Lethal\ at\ dilution\ immediately\ above\ 50\%-50\%\ above\ 50\%-50\%}$$

This formula was used to determine the index for the dilution that resulted in a deadly concentration of greater than 50%.

Challenge Test

At day 30, ten birds from each group—including the control group—were isolated and challenged with one milliliter of the virulent ND virus, which had an ELD50 of 107.34 per bird. Blood was drawn, clinical symptoms, morbidity and mortality rates, and gross lesions were documented, and all birds were monitored for seven days after the challenge.

RESULTS

Evaluation of Maternal Immunity against ND:

The result of maternal immunity of experiment two for 10 serum samples that selected from 150 chicks (before division in to groups) at 5 day old chicks, recorded the Mean ± SE was (3737.90±384.13) data obtained in the present study concerning high antibody titers against ND.

Results of ELISA Test of IgA of Different Titers for NDV-CS-NPs Vaccine

The results of current study showed a significant statistically difference ($P \leq 0.01$) among vaccinated groups and control group in IgA concentration explained in different periods (16 , 26 and 30) days old, at 16 days old chicks the highest mean titer in G5 was (12.64 ± 0.62) followed by G4 , G3 and G2 (control positive) which were (10.58 ± 0.28 , 8.38 ± 0.53 and 7.91 ± 0.41) respectively , while the lowest IgA concentration was G1 (control negative) was (7.58 ± 0.45).

At 26 days old chicks there was significant statistically difference ($P \leq 0.01$) the highest mean titer in G5 was (14.91 ± 0.98) afterwards G4, G3 and G2 (control positive) which were (11.91 ± 0.39, 7.56 ± 0.51 and 3.89 ± 0.31) respectively, whereas, the lowest IgA concentration was in G1 (control negative) was (3,76 ± 0.19).

At 30 days old chicks there was significant statistically difference ($P \leq 0.01$) the highest IgA concentration was in G5 (21.87 ± 0.38) subsequently G4, G3 and G2 (control positive) which were (16.17 ± 1.47, 10.31 ± 0.61 and 0.80 ± 0.45) respectively, whereas, the lowest IgA concentration was G1 (control negative) was (0.428 ± 0.13) (table 1).

Table 1: The means value of antibody titer (IgA) after vaccinations (Mean ± SE) at 16, 26 and 30 day old of chicks

Groups Age(day)	G1	G2	G3	G4	G5
16 day	7.58 c ± 0.45	7.91 c ± 0.41	8.38 c ± 0.53	10.58 b ± 0.28	12.64 a ± 0.62
26 day	3.76 d ± 0.19	3.89 d ± 0.31	7.56 c ± 0.51	11.91 b ± 0.39	14.91 a ± 0.98
30 day	0.428 d ± 0.13	0.80 d ± 0.45	10.31 c ± 0.61	16.17 b ± 1.47	21.87 a ± 0.38

* Means having with the different letters in same column differed significantly ($P \leq 0.01$).
* G1: Control group (negative).
* G2: Control group (positive) (intranasal vaccine with deionized water).
* G3: Intranasal vaccine with chitosan nanoparticles (CS-NPs).
* G4: Intranasal vaccine with ND vaccine (La Sota).
* G5: Intranasal vaccine with NDV-CS-NPs.

Titration of Virulent Viruses on Chicken Embryonated Eggs (Embryo Lethal Dose 50) (ELD_{50}) (for Challenge)

The lethal dose of virus to (50%) of embryonated eggs was calculated according to Reed and Muench (1938). The ELD_{50} of ND virus was $10^{7.34}$ ELD_{50} / 0.1ml. As shown in the figure (1).

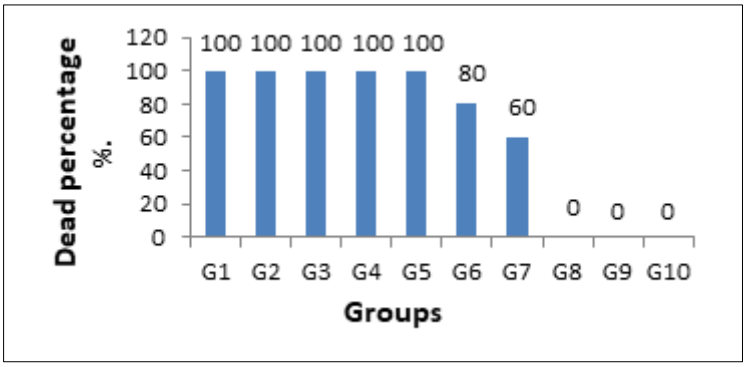

Figure 1: Show the comparison between difference groups in result of ELD_{50} test

Examination of Morbidity and Mortality Rate after Challenge

The development of the clinical signs and mortality were monitored during 7 days post challenge with Newcastle virus. The morbidity and mortality were recorded every day after challenge, the results confirmed that the group vaccinated with NDV-CS-NPs (G5) gave no morbidity percentage compared to other groups, respectively (figure 2).

On the other hand, the mortality percentage was 0 % of NDV-CS-NPs group G5 in comparison with other groups G4 and G3 which recorded medium percentage of mortality 30 - 50%, while, the control groups G2 and G1 registered the highest percentage with morbidity 80% and mortality 100% (figure 3).

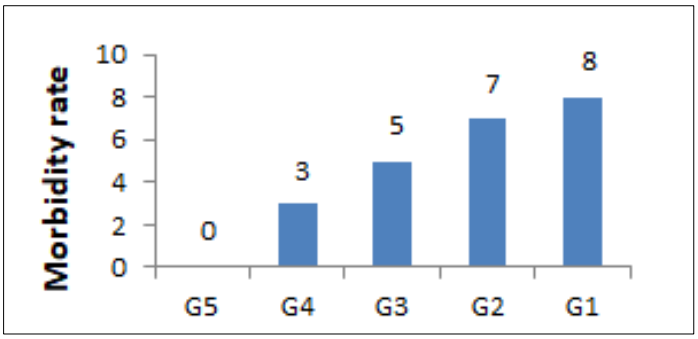

Figure 2: Morbidity rate induced after challenge with Newcastle virus (ELD_{50} $10^{7.34}$) at 30 days of age

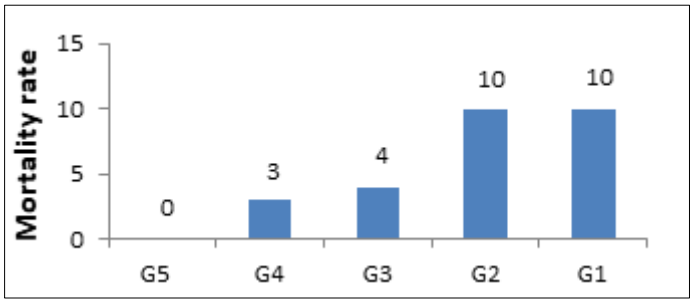

Figure 3: Mortality rate induced after challenge with Newcastle virus (ELD_{50} $10^{7.34}$) at 30 days of age

Macroscopic Examination

A total of 50 of the 150 chicks from the second experiment of this investigation were monitored daily for ND diagnosis based on case history, clinical symptom, and post-mortum (PM) lesion following ND virus inoculation from natural orifices (nose, mouth, and eyes).

Greenish-dark diarrhea, edema around the eyes and head, respiratory symptoms, drooping wings, torticollis, incoordination, and rapid death were among the clinical findings. Significant hemorrhagic ulcers in the intestinal wall and cecal tonsils, pinpoint hemorrhages at the tip of proventriculus glands, hemorrhagic lungs, tracheitis with congestion and hemorrhage corresponding to the ring of cartilage, and catarrhal exudates were among the post mortem findings that were thought to be diagnostic of ND., Figure (4) .

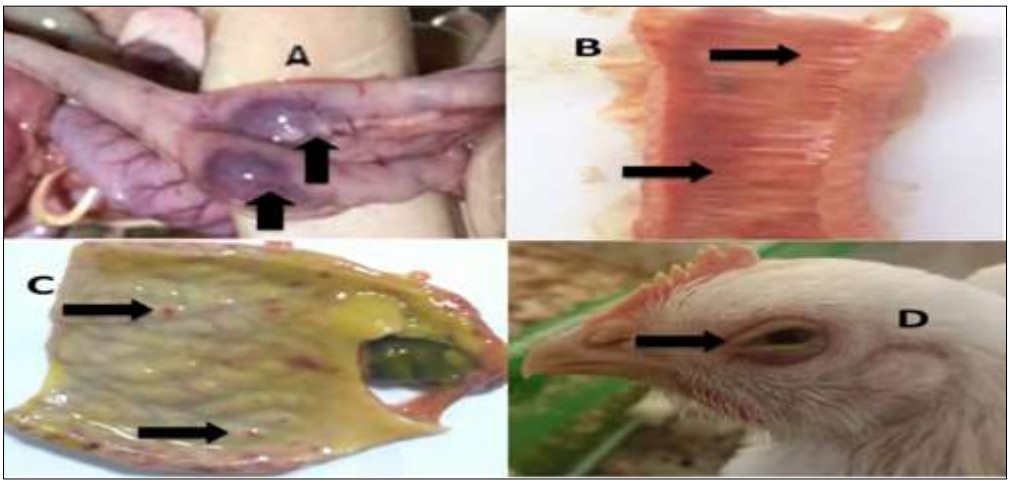

Figure 4: Clinical signs and gross lesions in chickens suspected to be naturally infected with NDV A) Hemorrhages on cecal tonsils, B) Congestion of trachea, C) Hemorrhages on proventriculus gland tips, D) Broiler chicken of 30 days infected with NDV showing conjunctivitis

DISCUSSION

Maternal Immunity against ND

Waheed *et al*., (2013) suggested that the serum sample results from the experiment before group division for evaluating maternal immunity to NDV findings are consistent with, who discovered that several ND vaccinations were

administered to breeders flocks via various routes during the rearing and production periods. Several ND vaccine strains, such as B1, La Sota, and oil immersion, were all used in vaccination prior to lying. Furthermore, it has been observed by Agrawal *et al*., (2016) that the cumulative effect of many vaccinations leads to greater titers, which means that the eggs would acquire a significant quantity of Ab from their own breeders, as evidenced by born chicks.

The respiratory and digestive systems are part of the avian mucosal immune system. While parenterally delivered vaccinations predominantly drive systemic responses, mucosal immunization produces a mucosal immune response with S-IgA antibody and systemic humoral and cellular immunological responses with IgG and IgM antibodies (Gupta *et al*., 2014). Mucosal administration has several benefits over conventional vaccination routes, such as a porous endothelium membrane, a wide epithelial surface with many microvilli, a highly vascularized mucosa that facilitates absorption, and easy accessibility (Turker *et al*., 2004).

The study based on nasal administration of ND vaccine coated with chitosan nanoparticles is in agreement with Arthanari *et al*., (2016), who found that the chitosan nanoparticles. The results of NDV-CS-NPs showed a significant ($p<0.01$) differences which was higher than ordinary vaccine (La Sota) in IgA concentration , in group (5 and 4) for IgA detections were (12.64 ± 0.62),(10.58 ± 0.28) respectively, as well as in G3 (chitosan nanoparticles vaccine) IgA level was (8.38 ± 0.53) at 16 days old chicks, while in 26 days old chicks the level of IgA in group 5 (NDV-CS-NPs vaccine) was high level (14.91 ± 0.98) from G4 (ordinary vaccine) and from G3 (chitosan nanoparticles vaccine) were (11.91 ± 0.39) (7.56 ± 0.51) respectively, in G5 the concentration of IgA was (21.87 ± 0.38) at 30 days old chicks and in group 4 and 3 were (16.17 ± 1.47) and (10.31 ± 0.61) respectively, in compared with control groups. This result is in agreement with Gavini *et al.*, (2006) and Khameneh *et al*., (2014) whom explained that the sIgA titers in mucosa extracts were determined with the nanoparticles was significantly higher than other groups, it shows the potential of chitosan nanoparticles for the induction of mucosal IgA against their coated antigen. This could be attributed to mucoadhesion potential of chitosan nanoparticles and their more prolonged presence in contact with mucosal surface, also Jankovic and Feng (2015) improved that the antigen uptake rate by the mucosa-associated lymphoid tissue, resulting in a good mucosal immune effect. Further, the enhanced proliferation of lymphocytes, the enhanced cellular response was also documented in the detection of the increased levels of IFN-c and IL-2, indicating a higher induction of Th-1 type responses.

This was in line with a study by Miller *et al*., (2013) that concentrated on allowing adequate time between vaccination and challenge in order to achieve adequate and consistent immunity and superior protection. The morbidity in the control unvaccinated group (G1 and G2) was 80% and 70% when the chickens were challenged with local isolated NDV at 30 days of age. This outcome supports the hypothesis of Ashraf and Shah (2014), who predicted that two to three days after infection, the percentage of morbidity in Newcastle disease would reach 100%. However, the morbidity rate rose to 50% in G3 recipients of chitosan nanoparticle vaccination. This is in line with Zhao *et al*., (2017), who found that chitosan nanoparticles activate cellular immunity and strengthen the immune response against viral infection.

The morbidity rate of 30% in G4 vaccinated with the standard Newcastle disease vaccine (La Sota) is consistent with the findings of Musa *et al*., (2010), who claimed that the commercial Newcastle disease vaccine was unable to prevent infection in chickens because of genotyping variations in the virus. The morbidity rate in G5 vaccinated with NDv-CS-NPs was 0%, which is consistent with Zhao *et al*., (2012), who explained that, as illustrated in figure (2), chickens immunized with the NDv-CS-NPs vaccine induced better immune responses than chickens immunized with the standard live attenuated NDV vaccine.

Group 5 had the lowest mortality rates among the groups, according to statistical analysis. The control unvaccinated groups G1 and G2 had high mortality rates of 50% to 60%, while both G3 and G4 had intermediate mortality rates of 30% to 40%, and G5 had no mortality (Panus *et al*., (2015) (Bu *et al*., (2019). Figure (3) illustrates the 60% protection rate in G3 that received the chitosan nanoparticle vaccination. This is consistent with Zhao *et al*., (2017), who noted that the chitosan nanoparticles boost the immune response against viral infection and enhance cellular immunity. Additionally, group four that received the regular vaccine (La sota) had a 70% protection rate. This is comparable to Ahmed (2018), who reported that even those who received the attenuated ND vaccine using heterologous (non-matching genotypes) live or inactivated ND vaccine had a high mortality rate against Newcastle disease. The failure to provide protection was caused by incorrect vaccination administration (Dortmans *et al*., 2011). However, as illustrated in figure (3), 100% of chicks survived in G5 following vaccination with the NDv-CS-NPs vaccine, which generated protective antibodies after vaccination. This result is comparable to that of Zhao *et al*., (2012), who reported that chicks injected with the NDv-CS-NPs vaccine exhibited greater immune responses than chickens immunized with the standard live attenuated NDV vaccine. Additionally, the data indicated that the NDv-CS-NPs vaccine, which was produced for this experiment, may have reduced viral shedding and protected the chickens during field outbreaks. According to research, the NDv-CS-NPs vaccination offers superior defense against the pathogenic NDV (Hu *et al*., 2009). The clinical indicators following the challenge were initially identified at two days in individuals who were either unvaccinated or had inadequate protection against virulent NDV. Susta *et al*., (2010) noted similar symptoms, which were caused by administering a high dosage of

viral titer via intraocular and intranasal routes (Sen *et al*., 2017). Following a challenge, the P.M. lesions developed in scarified chickens; similar findings were confirmed by Wakamatsu *et al*., (2006). As demonstrated by Bulbule *et al*., (2015), who noted that the current ND vaccinations do not provide protection against morbidity and mortality brought on by novel NDV variations, the results demonstrated that the standard vaccine was ineffective in preventing ND clinical symptoms and gross lesions. Additionally, Jeon *et al*., (2008) speculate that inadequate immunization may have led to the evolution of NDV variants in chicken.

CONCLUSION

These findings suggest that, in comparison to regular and nano-chitosan, the use of Newcastle disease vaccine-chitosan nanoparticles in experiment two (G 5) produced a stronger immune response and better protection.

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