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Effect of *In Ovo* Injection of VG/GA Vaccine, an Apathogenic Enteric Strain of Newcastle Disease Vaccine and Aluminum Hydroxide as an Adjuvant on Hatchability and Immune Response of Commercial Pullets

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Abstract
Current vaccination strategies for commercial poultry using live attenuated and inactivated Newcastle disease (ND) vaccines have some limitation and difficulties, and new vaccines with distinct features are needed. Recently, *in ovo* vaccination technology is concerned as a safe, efficacious, and convenient method. Common ND vaccines used in chickens cannot be employed *in ovo* due to embryo toxicity and high early mortality. One of the agents that may lead to attenuate ND virus (NDV) strains is aluminum hydroxide (AH) as an adjuvant. The objective of this study was to evaluate AH ability to attenuate NDV for *in ovo* administration of commercial pullets. Three hundred sixty fertile eggs of a Bovans strain as a factorial arrangement of six doses of the ND vaccine (50% egg infectious (EID50) of 0, 10^2, 10^3, 10^4, 10^5, and 10^6) with or without AH were ordered into 12 groups. At 18 d of incubation 0.1 mL of the inoculums was injected into the amniotic fluid of eggs. On the farm, each treatment group was further subdivided into two groups and one of these groups received ND-B1 vaccine on day seven post-hatch. Lowest hatchability was recorded in groups vaccinated with doses of 10^5 and 10^6 EID50. On day 21, the highest hemagglutination inhibition (HI) was detected for group vaccinated with dose 10^2 EID50. Furthermore, hatchability and ND-HI titer were found to be up for pullets received AH *in ovo* on day 42 posthatch. The results of this study indicated that aluminum hydroxide as an adjuvant could significantly improve hatchability and immune efficacy of pullets when used *in ovo*. Further, lentogenic VG/GA strain-Avinew will have the potential for application as *in ovo* vaccine against Newcastle disease, if the vaccine is prepared with sufficient dose.

Introduction

Newcastle disease (ND) is a highly contagious, fatal infection of economic importance in poultry production because of its potential to cause devastating losses in the poultry industry (Kapczynski *et al*., 2012). Its causative agent is avian paramyxovirus type 1, also designated as Newcastle disease virus (NDV), with a non-segmented, negative-sense RNA genome (Mayo, 2002). The NDV isolates can be classified by pathogenicity as highly virulent (velogenic), moderately virulent (mesogenic), low virulent

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(lentogenic) and apathogenic on the basis of the clinical signs observed in infected chickens (Ramp *et al*., 2012). Although ND vaccination is heavily practiced for control and management of velogenic ND outbreaks in developing and under-developed countries, these vaccines have a number of drawbacks including subclinical mild infection or acute respiratory side effects caused by live vaccines (Senne *et al*., 2003), effort required to handle each chick and ensure that it receives the optimal vaccine dose (Kapczynski *et al*., 2012) and faulty vaccination schedule (Manna *et al*., 2007). Accordingly, *in ovo* vaccination has been proposed to minimize these problems.

In ovo vaccination is being used for hatchery administrating of Marek's disease, and infectious bursal disease vaccines worldwide (Ricks *et al*., 1999). There are machines available and capable of injecting up to 70,000 eggs per hour. This technology entails precise, uniform and fast delivery, needle sanitation, stimulation of earlier immunity and reduction of chick stress and labor costs (Okwor *et al*., 2014). It is rapidly expanding globally with the investigation of *in ovo* vaccines for fowl pox and ND. Conventional live ND vaccines of low virulence such as LaSota and Hitchner B1 are highly lethal for chicken embryos, and thus in their current form are not acceptable for *in ovo* application (Kapczynski *et al*., 2012).

In order to reduce the lethality of NDV strains to the embryo, classical vaccine strains were either modified by an alkylating agent (Ahmad and Sharma, 1992) or injected *in ovo* as inactivated oil emulsion preparations (Stone *et al*., 1997). Furthermore aluminum hydroxide (Ohta *et al*., 2009) and chicken interferon I or II (Rautenschlein *et al*., 1999) were examined for this purpose. Adjuvants are added to vaccines to enhance the immunogenicity of antigens. They have potential benefits like inducing higher antibody production, increasing the duration of antibody response, reducing the number of immunizations and sparing the dose (use less antigen, increase global vaccine supply) (Reed *et al*., 2013).

Aluminum-containing adjuvants (alum) are widely used in commercial vaccines due to their good safety, low cost and compatibility with a variety of antigens (Kool *et al*., 2012). They promote a Th2 response and induce high titers of serum antibodies (Hogenesch, 2013). Despite its historicaluse, its mechanism of immunopotentiation still remains unclear (Maughan *et al*., 2014). Adsorption of antigens to aluminum adjuvants enhances the immune response by facilitating phagocytosis and causes the initial assumption for the formation of a depot of antigen, whereby it provides prolonged exposure of the antigen to the immune system and results in a higher antibody titer than antigen alone. However, some evidence indicates that depot effect and antigen adsorption are not necessary for immunopotentiation of aluminum adjuvants (De Gregorio *et al*., 2013).

The objective of this study was to examine the effect of AH as an aluminum-containing adjuvant through its ability to attenuate NDV. In addition, the hatchability and antibody responses of the pullets were measured.

Materials and Methods

This experiment was conducted at the Ferdowsi University of Mashhad, Iran, and was approved by the Ferdowsi University animal research committee.

Preparation of inoculum

Aluminum hydroxide was obtained from Merck (Darmstadt, Germany). The Avinew (VG/GA strain, enteric a pathogenic virus) was used as Newcastle vaccine. Aluminum hydroxide at the dosage of 2 mg/mL was prepared and sterilized by autoclaving at 121°C for 15 min. Ten mL of the solution of AH was mixed with every 10 mL of a 0, 10^2, 10^3, 10^4, 10^5, and 10^6 (EID50) to prepare NDV VG/GA strain solutions. The prepared solution was further mixed on a stirrer at 4°C overnight.

In ovo injection and experimental design

A total of 400fertilised commercial eggs of a Bovans strain from 40-week-old parents were incubated at 37.8°C with RH of 60%. All eggs were candled. Unfertilized eggs were discarded at day 18 of embryonic incubation. Afterwards, 360 eggs were randomly allocated to 12 hatching trays with three replicates of 10 eggs each. The experiment had a factorial design consisting of different doses of the ND vaccine (50% egg infectious (EID50) of 0, 10^2, 10^3, 10^4, 10^5, 10^6) with or without AH. The eggs were disinfected with 70% ethanol before injection and then eggshells were punched and 0.1 mL of the prepared inoculum was delivered into amniotic fluid using a 25-gauge needle. The eggs were injected

in a room with a controlled temperature and relative humidity. Upon completion of all injections, eggs were returned to the incubator for hatching. Hatchability was calculated by dividing the number of chicks hatched by the number of fertile eggs set at 22 d of incubation.

After hatching, chicks were transported to Ferdowsi university poultry research farm (Ferdowsi University, Mashhad, Iran), with free access to feed and water, and raised to 42 d of age. On the farm, each group of chicks was further subdivided into two subgroups, so that one of these groups received B1 Newcastle vaccine via an eye drop on day seven whereas the other subgroup did not. All the hatched chicks received vaccination for NDV on day 14 and 21 (B1 strain) by eye drop route.

Subsequently, blood samples were collected from the chicks on day three by cardiac puncture (four birds/group) and on days 21 and 42post-hatch through the wing vein (three birds/group) for HI assay. Hatchability and antibody titer data were statistically analyzed using the Generalised Linear Model (GLM) procedure from SAS 9.1 and the means were compared by Duncan test allowing all pairwise comparisons at $P < 0.05$.

Results

The effect of *in ovo* injection with different levels of ND vaccine and AH on hatchability of fertile eggs is shown in Table 1. The hatchability changed ($P < 0.05$) between groups that were inoculated with different doses of Avinew vaccine, so lowest hatchability was observed in eggs vaccinated with 10^5 and 10^6 EID50 doses (91.67% and 90%, respectively). Thus, *in ovo* administration of Avinew vaccine in correct doses did not compromise hatchability. There was a significant difference in hatchability of eggs received AH as compared with those which did not receive AH ($P < 0.05$). Hatchability was 96.67% for eggs received *in ovo* AH administration. There was not a significant interaction between different doses of ND vaccine and AH administration.

Table 1. The effect of *in ovo* injection of Newcastle disease vaccine and aluminum hydroxide (AH) on hatchability

Treatment			
In ovo vaccine (EID50)	No. of eggs		Hatchability (%)
0	60		98.33[a]
10^2	60		95.00[abc]
10^3	60		98.33[a]
10^4	60		96.67[ab]
10^5	60		91.67[bc]
10^6	60		90.00[c]
P-value			0.02
SEM			1.92
AH			
+	180		96.67[a]
-	180		93.33[b]
P-value			0.04
SEM			1.11
Interaction effect		*P*-value	*SEM*
In ovo vaccine × AH		0.50	2.72

[a-c] Means within columns followed by different superscript within each factor are different ($P < 0.05$).

Inoculation of eggs with different doses of Avinew vaccine did not significantly change the HI antibody titers to ND on days three and 42 of posthatch (Table 2). With regards to the fact that antibodies in blood usually appear within 6-10 days and peak 21–28 days after inoculation of live virus vaccine, the *in ovo* vaccine was not effective on days three and along with subsequent booster of B1 vaccine did not induce significant HI titer on day 42. On day 21, the lowest ND-HI titer (3.17 $\log_2$) was recorded in chicks which hatched from eggs inoculated with 10^6 EID50 and the highest HI (4.42 $\log_2$) was detected in chicks hatched from eggs vaccinated with 10^2 EID50. The HI titer was significantly increased when pullets hatched from eggs received *in ovo* AH (4.97 *vs*. 4.00 $\log_2$) in day 42 of post-hatch. Farm vaccination with B1-ND on day seven did not cause significant differences of HI between groups.

Table 2. Effects of *in ovo* injection of Newcastle disease vaccine and aluminum hydroxide (AH) on antibody titer against Newcastle disease in commercial pullets

Treatment			Antibody (HI) titer (log_2)		
In ovo vaccine (EID50)	No. of eggs	No. of birds	Day 3	Day 21	Day 42
0	60		6.00	3.58^{abc}	4.08
10^2	60		5.00	4.42^{a}	4.25
10^3	60		5.75	3.75^{abc}	5.25
10^4	60		5.00	4.08^{ab}	4.25
10^5	60		5.75	3.33^{bc}	4.58
10^6	60		6.12	3.17^{c}	4.50
P-value			0.26	0.03	0.39
SEM			0.42	0.28	0.40
AH					
+	180		5.83	3.72	4.97^{a}
-	180		5.38	3.72	4.00^{b}
P-value			0.19	1.00	0.01
SEM			0.24	0.16	0.23
Farm vaccine (on day seven)					
+		180	-	3.58	4.47
-		180	-	3.86	4.50
P-value			-	0.23	0.93
SEM			-	0.16	0.23
Interaction effect				*P*-value (*SEM*)[1]	
In ovo vaccine × AH			0.21 (0.59)	0.92 (0.39)	0.43 (0.57)
In ovo vaccine × Farm vaccine			-	0.62 (0.39)	0.63 (0.57)
AH × Farm vaccine			-	0.09 (0.23)	0.11 (0.33)
In ovo vaccine × AH × Farm vaccine			-	0.01 (0.56)	0.09 (0.80)

^{a,b,c} Means within columns followed by different superscript within each factor are different ($P< 0.05$).

[1]Numbers in parentheses are related to *SEM*.

Discussion

Newcastle disease caused by velogenic strains always leads to poor productivity and economic losses in poultry industry all over the world. Vaccination is common practice in many countries to control the ND. Despite the advantage of ND vaccines in control of the outbreaks and enhancing the production, they have unwanted side effects such as post administration reaction after the use of attenuated live vaccines reducing the production (Lowenthal *et al*., 2000; Lowenthal *et al*., 2005) and inadequate immune response with inactivated vaccines (Gupta *et al*., 2014).

In ovo vaccination technology has several advantages over the conventional methods including, uniform and fast delivery, neonatal resistance, reduction of chick stress and labor costs and also limited farmers involvement (Okwor *et al*., 2014). This method of vaccination is an attractive option for poultry producers. Nevertheless, studies have shown that most common post-hatch ND vaccines for chicks cannot be administered for *in ovo* vaccination in their current form due to their embryonic lethality (Ahmad and Sharma, 1992; Mebatsion *et al*., 2001; Saravanabava *et al*., 2005).

Okwor *et al*. (2014) divided embryonated eggs into five groups (A, B, C, D, and E). They vaccinated *in ovo* eighteen-day-old embryonated eggs into the chorioallantoic sac with avirulent I2 vaccine strain of ND (ND-I2) (group A) and ND-LaSota (group B). Groups C and D were vaccinated with ND-I2 and ND-LaSota in thirteen-day old, respectively. Group E served as unvaccinated control. The results of their study showed that hatchability between the vaccinated and control groups significantly differed and highest hatchability (64%) was recorded in group A when compared to other vaccinated groups. However, the hatchability in the control group was 83%. Romao *et al*. (2011) carried out a

factorial design to test the effects of four incubation time points (0, 5, 10, or 15) and three injection procedures (saline injection and Hitcher B1-ND plus saline or industrial diluent) on incubation of Japanese quail eggs. They indicated that the HB1 strain cannot be applied by *in ovo* route at any stage of incubation periods of quail eggs due to its high impact on hatchability and poor post-hatch antibody titers. Mebatsion *et al*. (2001) reported that hatchability was found to be about 93% and 23% for the eggs inoculating with NDV-P1 and either the parent rNDV or NDW (a lentogenic posthatching ND vaccine), respectively. Based on the results of these studies, it is critical to select a highly attenuated ND vaccine strain for the *in ovo* vaccination. Furthermore, determination of the inoculum with the least harmful effects on embryos or neonatal chicks is important.

Howeverunlike the studies mentioned above, Manna *et al*. (2007) injected F-strain virus in 2.84, 2.54, 2.24, 1.84, 2.24 Log_{10} EID50 per embryonated egg and described that *in ovo* administration of lentogenic F-strain of NDV did not hamper hatchability and successfully induced antibody response compared to the control group. Furthermore, in the present study we vaccinated Bovans eggs into amniotic fluid on day 18 and observed that using 10^2, 10^3, and 10^4 EID50 doses of Avinew ND, caused no significant change in the hatchability compared to the group injected without vaccine.

Different methods have been approached to reduce the virulence of NDV strains and make them more attenuated. For instance, Ahmad and Sharma (1992) mutated Hitchner Bl derived NDV strain by ethyl methane sulfonate. Moreover, Ramp *et al*. (2012) used 10^4 EID50 of recombinant viruses rNDV, rNDV49, and rNDVGu as well as vaccine strain NDV Clone 30 for *in ovo* vaccination into the allantoic cavity at 18 day of embryonic age. They indicated that NDV vaccine strains are pathogenic to chickens when inoculated *in ovo*.

Nowadays, the immune adjuvants have been defined as the agents employed with vaccines to make a robust immune response and long-lasting protection against some viral diseases like ND (Chen *et al*., 2010). Alum adjuvant especially aluminum hydroxide are the most common adjuvants for over 80 years (Kool *et al*., 2012). The mechanism by which these adjuvants selectively enhance the immune response is poorly understood (Maughan *et al*., 2014). Initially, it was thought that by the formation of an antigen depot, they illustrate their adjuvant effect. However, recently some evidence suggested that a depot effect is not so important for alum adjuvanticity and instead alum can activate the Nlrp3 inflammasome complex, which is required for the production of interleukin 1β (IL-1β)(De Gregorio *et al*., 2013).

In our study, using AH inoculation method *in ovo*, HI was significantly increased at d 42 post-hatch. However, the interaction was not significant between *in ovo* vaccination of different doses of the ND vaccine and AH during the period of experiment. According to our results, the role of antigen depot in alum's mode of action was not observed. Jafari *et al*. (2016) also vaccinated 1-week-old SPF chickens and determined that with the use of two concentrations of 10% and 20% adjuvant of alum in ND vaccines, they increased the immune response after 14 d of inoculation and remained steady until d 42. They assumed that the performance of vaccines containing 20% adjuvant of alum is better than the ones containing 10% of alum and this higher performance is related to a longer period of antigen depot. Nayan *et al*. (2015) compared the effectiveness of the NDV-Genotype VII along with three different adjuvants and LaSota vaccine. Their results suggested that with highest mean HI titer of log_2 6, the NDV-Genotype VII vaccine with AH gel and oil-emulsion complete freund's surpassed the LaSota. Mishra *et al*. (2014) investigated that alum-HBsAg formulation induced humoral immunity significantly because the IgG1 was increased in serum. In addition to aluminum adjuvants, potential of other adjuvants was considered. For instance, El Sabry *et al*. (2012) considered potential use of Interleukin2 (IL-2)-rich supernatant adjuvant in Fayoumi hens and demonstrated that ChIL-2-rich supernatant, when given together with NDV antigen, significantly enhanced humoral immune responses against NDV. In another study, Montanide TM ISA 71 VG as water-in-oil adjuvant was evaluated using ND vaccine model and it was found that this new adjuvant is safe and can improve vaccine efficacy (Arous *et al*., 2013). Wang *et al*. (2013) also observed that *Cordyceps militaris* polysaccharides could significantly improve the immune efficacy of ND vaccine.

The use of adjuvantin *in ovo* vaccination has

been proposed. Stone *et al*. (1997) in an experimental research vaccinated chicken embryos with ND and avian influenza oil-emulsion vaccines on day 18 of incubation by *in ovo* method. They demonstrated that if the vaccines are prepared with sufficient antigen and administered properly, acceptable hatchability, and protective immunity with *in ovo* inoculation of ND or avian influenza oil-emulsion vaccines can be attained. In research on *in ovo* delivered endosomal toll-like receptor-21 and -9 sense CpG DNA, Thapa *et al*. (2015) recorded hatchability rates of 86% following *in ovo* delivery of CpG DNA compared to 75% for the group that received PBS and determined that CpG DNA is safe in terms of hatchability of the incubated eggs. Rautenschlein *et al*. (1999) inoculated SPF turkey eggs on day 24 of incubation with recombinant fowl pox viruses (rFPV) vaccines (rFPV, rFPV-NDV, rFPVNDV-type II IFN, rFPV-NDV-type I IFN). These researchers observed no change in hatchability rate with rFPV vaccines in comparison to diluent inoculated embryos. Furthermore, they found that the rFPV-NDV-IFN-II induced the onset of NDV production in SPF birds at one weekpost hatch. Ohta *et al*. (2009) injected 18-day-old embryonated eggs with 10^4 and 10^2 EID50 of NDV D26 strain with or without AH. The hatchability of eggs inoculated with AH virus was improved compared with eggs inoculated with non-AH virus. Higher HI antibody responses were observed in administration group of the virus alone. Results of hatchability in our study also showed that eggs received AH as compared with the ones that did not received AH had higher hatchability.

Dilaveris *et al*. (2007) did not observe adjuvant effect of cytokines (IFN-γ, IL-4 and IL-18) when they were injected *in ovo* with NDW vaccine and were unable to find suitable doses for injection of NDW. Subsequently they stated that amniotic injection, in common with high doses of aerosol virus, may cause an adverse reaction or death due to the fact that ingested virus could deeply diffuse into the lung. However, it was verified that protection was highest (90%) when Marek's vaccine was applied in amniotic fluid compared to when it applied in allantoic fluid and air cell (Romao *et al*., 2011). In the present study we vaccinated Bovans eggs with different doses of the Avinew ND vaccine into amniotic fluid and did not observed death due to injection in the amniotic fluid.

Conclusion

In ovo administration of AH could significantly improve hatchability and immune response against ND. *In ovo* vaccination using 10^2 EID50 dose of enteric VG/GA strain-Avinew, it showed to be safe and effective in protecting Bovans pullets against ND. Newcastle disease vaccine delivery using *in ovo* technology needs to be further examined for other types and strains of ND vaccines with different adjuvants.

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