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Original Article

Comparative Study on the Efficacy of MF 59, ISA70 VG, and Nano-Aluminum Hydroxide Adjuvants, Alone and with Nano-Selenium on Humoral Immunity Induced by a Bivalent Newcastle+Avian Influenza Vaccine in Chickens

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Abstract

Newcastle disease (ND) and Avian influenza (AI) are the major problems and the most economically important viral diseases in the poultry industry; therefore, vaccination against these diseases is considered one of the most effective ways of prevention. Extensive studies have been conducted to improve the performance of vaccines, and one of the major achievements of these studies is the preparation of adjuvants as stimulants of the immune system and one of the most important compounds in killed vaccines. An immunogenicity comparison of three adjuvants including, ISA70VG, Nano-Aluminum Hydroxide (Nano-Alum), and MF59 alone or with Nano-Selenium (Nano-Se), was performed using bivalent Newcastle plus Avian Influenza (ND+AI) killed vaccine. In this study, 105 specific-pathogen-free chicks (Ross-308) were divided into 7 treatments, including T1 (control group), T2 (ISA70VG), T3 (ISA70VG plus Nano-Se), T4 (Nano-Alum Hydroxide), T5 (Nano-Alum+Nano-Se), T6 (MF59), and T7 (MF59+Nano-Se). The vaccine was injected subcutaneously on day 21 in the back of the neck area. The blood samples were taken on days 14, 21, 28, 35, 42, and 49 post-vaccination. Serums of the samples were titrated by the haemagglutination inhibition (HI) test against Newcastle and Avian influenza. Based on the results, the highest HI test titers were observed for the T2 and T3 treatments, while the T6 and T7 treatments had the lowest titers. Moreover, regardless of the type of the adjuvants, adding Nano-Se increased the antibody titer in the vaccinated groups. In conclusion, a combination of the ISA70VG adjuvant and Nano-Se induced excellent antibody titers using bivalent ND+AI killed vaccine.

Keywords: Newcastle disease, Avian influenza, Adjuvant, Nano-Selenium, MF59, ISA 70 VG, Nano-Aluminum Hydroxide

1. Introduction

Newcastle disease (ND) and Avian influenza (AI), due to economic (i.e., mortality and decreased growth rate) and ecological importance, are the major problems of viral diseases of poultry worldwide. Vaccination against these diseases is the most important method of effective control in the poultry industry (1). The clear understanding of immune system development with including adjuvants has contributed to the efficiency of inactivated vaccines. Adjuvants are chemical or

biological compounds that are effective in various ways in stimulating a nonspecific immune system with antigens used as vaccines. The reduction in the side effects of the vaccine and stimulation of different types of immune systems lead to the development of different types of adjuvants (2). Mineral oils are one of the most commonly used adjuvants in veterinary vaccines, especially in poultry vaccines (2). The main important features of the adjuvants that should be considered are safety and effective efficacy in poultry and humans with the ability to induce protective immune responses. According to the recommendation of a minimum 6-week interval from the injection time of killed vaccines to slaughter time of broiler chickens (1), adjuvants' residues in meat may have side effects for humans in case of consumption (3). For this reason, more effective adjuvants with a powerful stimulation ability, rapid absorption time with fewer residuals in meat, and completely safe for poultry and humans must be replaced by existing adjuvants used in vaccines of the poultry industry. Moreover, the dosage of the adjuvants can be reduced by using the combination of adjuvants capable of inducing a desirable protective immunity. Therefore, in this study, the immunogenicity of two mostly used adjuvants in human vaccines, including Nano-Aluminum (Nano-Alum) Hydroxide and MF59,andone routinely used adjuvant (ISA 70 VG) in poultry vaccines alone or with Nano-Sein chickens infected with Newcastle virus strain V4 and the H9N2 strain of influenza virus were compared.

2. Materials and Methods

2.1. Preparation of Inactivated Newcastle Disease and Avian Influenza Viruses

Newcastle virus of V4 strain and the H9N2 strain of influenza virus were used in this study, and inactivated vaccines of ND and AI were prepared according to the procedures suggestedby the World Organization for Animal Health (4). Inactivated QV4 strain (Asymptomatic, ICPI: 0.0) of ND virus and inactivated H9N2 strain of AI virus {A/chicken/IRAN/101/1998(H9N2)}2. were obtained from the Razi Vaccine and Serum Research Institute (RVSRI), Karaj, Iran and used according to the procedures recommended by the OIE (5). Briefly, under sterile conditions, 103-104 EID_{50}/0.1 mlof Newcastle virus of V4 strain and the H9N2 strain of influenza viruswere inoculated separatelyintothe allantoic cavity ofspecific-pathogen-free (SPF) 9-11-day-oldembryonated eggs (Ross-308) andincubatedat 37°C for72 h. Subsequently, infected eggs were chilled at 4°C for 24 h, and an allantoic fluid containing the virus was harvested,tested for bacterial contamination, and deactivated by formaldehyde (a typical final concentration is 1/1000). The employed Newcastle Antigens had a titer of HA 9 with an EID_{50} of 10.1, while the influenza antigen had a titer of HA 10 with an EID_{50} of 10.24.

2.2.Vaccine Development

2.2.1. Preparation of ND+AI Bivalent Vaccine with the Adjuvant ISA 70 VG

The Montanide ISA 70 VG (Montanide™, Paris, France; listed on Annex 2 of the European Council regulation 2377/90/ EC) was added to inactivated ND and AI virusesafter filtering, and then, sterilized. To prepare the vaccine with this adjuvant, 210 ml of ISA70 VG adjuvant and 90 ml of antigen (ratio of 70 to 30 adjuvants to antigen) were used. Given that the HA titerswere obtainedat 600 and 297 respectivelyin Newcastle and influenza virus in this study, the amount of inactivated Newcastle and Avian influenza were usedat 65.6 ml and 24.4 ml in vaccines, respectively. The dose for injection was 0.2 ml. Adjuvant ISA70 VG was placed inside a sterile Bécher under a rotary homogenizer underneath a laminar hood and placed on a container of dry ice. The purpose of the ice was to prevent the temperature of the vaccine from increasing to 35°C during the build-up. Afterward,the homogenizer started at 12,000 rpm and the antigen was added slowly to the adjuvant drop by drop. The adjuvant and antigen solution were then homogenized for 10 min with a circumference of 22,400 rpm and a

temperature variation of 30-35°C. After this step, the prepared vaccine was filled in a sterile vial and transferred to the refrigerator at 5°C.

2.2.2. Preparation of ND+AI Bivalent Vaccine with the adjuvant ISA 70 VG+Nano-Se

To prepare a combined adjuvant,15 ml of the Nano-Selenium (Nano-Se) solution (Iranian Nanomaterials Pioneers Company, Mashhad, Iran) after filtration by 450nm sterile filter wasadded slowly as well as dropwise (at 12,000 rpm) into 285 ml ISA 70 VG prepared according to section 2.2.1. Subsequently, the vaccine solutionwas homogenized at 22,400 rpm for 10 min. The solution temperature (not higher than 35°C) of the vaccine was also controlled. Finally, it was filled in sterile vials and stored at 5°C until being used.

2.2.3. Preparation of ND+AI Bivalent Vaccine with Nano-Alum

According to the guidelines of the World Health Organization (WHO), 0.85-1.25 mg/doseof Alum adjuvants was used in vaccines (6-8). To obtain the Nano-gel, the aluminum hydroxide with no allergic reaction was used at 10% (9-11). For this purpose, 30 gof aluminum hydroxide nanoparticles (American Elements 10884 Weyburn Ave, Los Angeles, CA 90024) was dissolved in 300 ml of PBS at 56°C (12, 13). At the next stage, the solution was mixed for 30 min and transferred to a 121°C autoclave at a specified pressure for sterilization (14). The pH of the solution was 7.4, which washighlysuitable for viral activity when combined with the desired adjuvant (15).

Due to the ratio of 10 to 90 of the amount of nano-aluminum hydroxide adjuvant in the vaccine formulation, 270 ml of antigens and 30 ml of prepared adjuvant were required to make 300 ml of the final volume. According to the hemagglutination unit antigens obtained in this study, for 270 ml of aqueous phase antigens, 58.6, 21.8,and 189.7 ml Newcastle antigen, Avian influenza antigen,phosphate-buffered saline (PBS) were used. Newcastle and influenza antigens and PBS were combined, and then, the Nano-Alum Hydroxide gel was added and mixed with 8,000 rpm speed of butterfly mixer for 30 min. Afterward, in a sterile vial, the vaccine was filled and placed in a refrigerator at 5°C. The vaccine needed tobe shaken and become completely homogeneous due to the precipitating properties.

2.2.4. Preparation of ND+AI Dual Vaccine with Nano-Alum+Nano-Se

All stages of manufacturing the vaccine were repeated exactly with the Nano-Aluminum Hydroxide adjuvant. After making the ND+AI dual vaccine with the Nano-Aluminum Hydroxide adjuvant to the final volume of the 285 ml vaccine, and considering the calculation of the required dose of Nano Selenium in section 2.2.1, 15 ml of Nano Selenium solution was added to the final volume of the vaccine drop bydrop and was mixed for 15 min with 8,000 rpm speed of a butterfly mixer after filling in a sterile vial in a refrigerator at 5°C.

2.2.5. Preparation of ND+AI Dual Vaccine with MF59 Adjuvant

The proofs of the safety of the ratio MF59 adjuvant and antigen in vaccines was reported to be50 to 50 (16-22). Therefore, according to the above information, for the manufacture of the vaccine, for thefinal volumes of 250 ml and 0.2 ml injection dose, 125 ml of MF59 and 125 ml of the antigens were required. Due to the formula ofcalculating the ratio of 125 ml of the aqueous phase, 48.8 ml Newcastle antigen, 18.2 ml influenza antigen, and 58 ml PBS wereneeded. After measuring 125 ml of MF59 oil in Becher and placing it under a rotary homogenizer and on dry ice to control the temperature, the desired antigens phase was slowly added at a rotational speed of 12,000 rpm. It was then homogenized at 22,400 rpm for 10 min. In none of these steps, the temperature exceeded 30°C. All of these steps were performed underneath the laminar hood. After construction, the vaccine was filled in a sterile vial and placed in a refrigerator at 5°C (9).

2.2.6. Preparation of ND+AI Bivalent Vaccine with MF59+Nano-Se

The entire process of making the ND+AI vaccine with MF59 was repeated exactly as the previous stage.

For this purpose, 12.5 ml Nano-Se was also slowly added to the final dose of 235 ml and re-homogenized with a rotating homogenizer for 10 min at a circumference of 22,400 rpm. After preparation, the vaccine was filled in a sterile vial and placed in a refrigerator at 5°C.

2.3. Animal Experiments and Laboratory Groups

A total of 105 unsexed one-day-old SPF chicks (Ross-308) were divided into 7 groups, including T1 (control group), T2 (ISA 70 VG), T3 (ISA70 VG+Nano-Se), T4 (Nano-Alum), T5 (Nano-Alum+Nano-Se), T6 (MF59), and T7 (MF59+Nano-Se). All the groups were reared at isolation units in the Marand Branch of RVSRI, Marand, East Azerbaijan Province, Iran, and all procedures involving chickens were carried out according to standard animal experimentation protocol of the Veterinary Ethics Committee of RVSRI.

2.4. Vaccination

Chicks of the groups T2 to T7 were vaccinated on 21-day-age, as shown in table 1. The vaccine was performed on the back of the neck with subcutaneous injection. The chickens were cultivated in terms of management and nutrition standards.

Table 1. Vaccination groups

Groups	Type of vaccines
T1	No vaccine (control group)
T2	ND + AI with ISA70VG
T3	ND + AI with ISA70VG plus Nano-Selenium
T4	ND + AI with Nano-Aluminum Hydroxide
T5	ND + AI with Nano-Aluminum Hydroxide plus Nano-Selenium
T6	ND + AI with MF59
T7	ND + AI with MF59 plus Nano-Selenium

2.5.Blood Sampling

Blood sampling was carried out 6 times on days 14, 21, 28, 35, 42, and 49 post-vaccination. The samples collected at each day were incubated at 37°C for 1 h; serum was used for other experiments after isolation.

2.6. Hemagglutination Inhibition Test

In the serum of numerous species, there are non-specific inhibitors, which may interfere with the hemagglutination inhibition (HI) test. Serum placeed in the bain Marie's bath before the test for 30 min at 56°C (23). Serial serum dilutions in PBS were subsequently mixed with equal volumes (25 µL) of the virus containing 4 hemagglutinating units (HAUs) for influenza and 8 HAUs for Newcastle antigen wereincubated 30 min at room temperature or 60 min at 4°C; afterward, 25 µL of washed chicken red blood cells was added. In this study, after that the HI titers were incubated for 30-45 min at room temperature, they were determined as reciprocals of the highest serum dilutions in which inhibition of hemagglutination was observed (23).

2.7. Statistical Methods

The data for all variables were analyzed in the SPSS software (Version 25; SPSS Inc., Chicago, USA) using one-way analysis of variance (ANOVA) as a completely randomized design. The means for treatments showing significant differences in the ANOVA were compared using post-hoc, Tukey, and paired-samples t-tests. Differences between the treatments with the p-values of < 0.05 were considered significant.

3. Results

The results of antibody titers against ND virus and AI are depicted in figure 1 and figure 2, respectively.

3.1. Humoral Immunity against ND

As shown in figure 1, HI antibody titers of group 1 (T1) were zero for the all sampling period. The comparison ofantibody titers of the vaccinated groups (T2-T7) showed that antibody titers of the groups increased gradually following vaccination and reached the highest level on day 28post-vaccination in theT2 and T3 groups andon day 21 post-vaccination in rest groups (T4-T7). Overall, as depictedin figure 1, antibody titers were higher inthe groups vaccinated with ISA 70 VG adjuvant (i.e., T1-T2) than in the groups vaccinated with Nano-Alum adjuvant (i.e., T4-T5), and the differences were significant ($P<0.05$). The results ofcomparisons between Nano-Alum and MF59 groups showed that the mean ND HI titers of Nano-Alum groups were higher than those of the MF59

groups, and the differences were significant on all days after vaccination (P<0.05). Based on figure 1, regardless of the type of used adjuvants, adding Nano-Se to them increased antibody titers of the groups; nevertheless, the elevated antibody titers of the T3 group treated with ISA 70 VG+Nano-Se vaccine was significantly (P<0.05) different from those of the group 2, which was treated with ISA 70 VG vaccine only on days 21 and 28 post-vaccination.

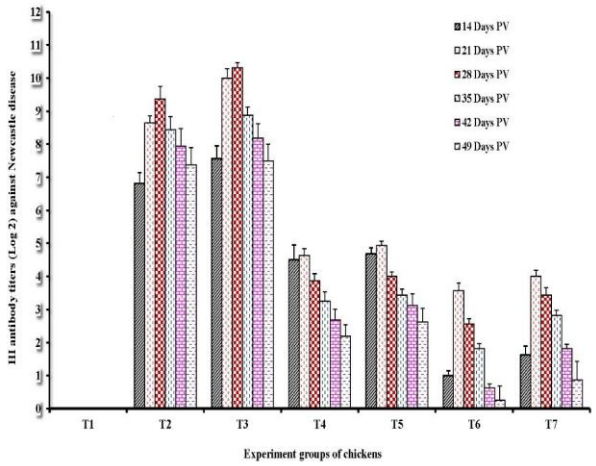

Figure 1. Average (Mean ±SEM) HI titers (sampling days) against Newcastle disease.
T1 (control), T2 (ISA70VG), T3 (ISA70VG+Nano-Se), T4 (Nano-Alum), T5 (Nano-Alum+ Nano-Se), T6 (MF59), T7 (MF59+Nano-Se).

3.2. Humoral Immunity against AI

As shown in figure 2, HI antibody titers of group 1 (T1) were zero for the all sampling period, indicating that there was not any environmental/cross contamination with AI viruses.

According to figure 2, antibody titers against AI of all groups increased gradually following vaccination and reach the highest level on day 28 of post-vaccination; however, it then gradually declined. The findings of comparing the groups indicated that the groups vaccinated with nano-aluminium hydroxied (T12-T3) had higher antibody titer than those (T4-T5) vaccinated with Nano-Alum adjuvant, and the latest had higher antibody titers than the groups vaccinated with MF59 adjuvant and the differences were significant (P<0.05). As shown in figure 2, regardless of the type of the employed adjuvants, adding Nano-Se to them increased antibody titers of the groups. Comparison AI HI antibody titers of T2 with T3, T4 with T5, and T6 with T7 indicated that Nano-Se increased the AI antibody titers on all days of post-vaccination; nevertheless, the elevated antibody titers of the groups were not significant (P>0.05) during the experimental period.

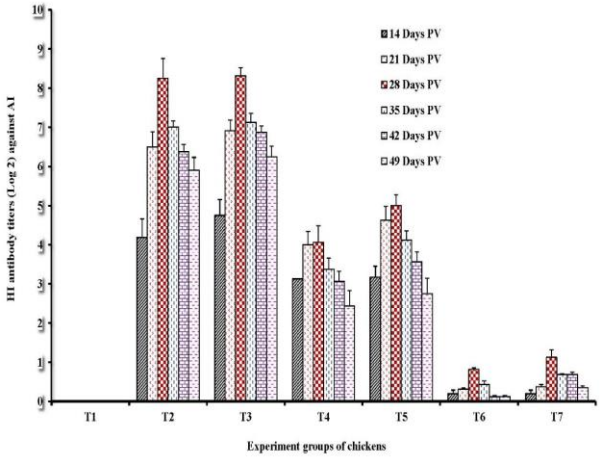

Figure 2. Average (Mean ±SEM) HI titers (Log 2) of the group against avian influenza.
T1 (control), T2 (ISA70VG), T3 (ISA70VG+Nano-Se), T4 (Nano-Alum), T5 (Nano-Alum+ Nano-Se), T6 (MF59), T7 (MF59+Nano-Se).

4. Discussion

Vaccination against Avian pathogens, in particular viral infections, has an important role in the prevention and control of poultry diseases. In order to improve the immunogenicity of vaccines, different types of adjuvants have been evaluated in poultry as stimulants of the immune system (24). Since a period of 42 days is recommended between vaccinations days with inactivated vaccines and slaughter time (25), finding adjuvants with short absorbance, fewer residues in chicken meat, and good potency has been an interesting subject in vaccine production. The ideal adjuvant should maximize the immunogenicity of the vaccine without compromising tolerability, safety, or side effects (8). Therefore, in this study, the most commonly

used vaccine adjuvant in poultry (ISA70VG) was compared with the most common adjuvants of human vaccines (MF59, Alum) alone or with Nano-Se, and their advantages and disadvantages were evaluated.

In a study conducted by Liu, Liu (26), the evaluation of immunogenicity among mineral adjuvants, ISA70 VG, and ISA 206 VG showed that the adjuvant ISA 70 VG produced the highest antibody titers and immunization in the poultry influenza vaccine than ISA 206 VG, indicating the immunogenicity of this adjuvant, which was in line with the results of the present study (26) The MF59 adjuvant is among those adjuvants that have been approved and routinely used in human vaccines (23). Major immune effects of MF59 adjuvant increased and activated antigen presenting cell recruitment and promoted antigen uptake and migration of cells to lymph nodes (27).

The immunogenicity of oil-in-water adjuvants, like the MF59, is short, and the speed of release of antigen in these adjuvants is faster; according to the first blood sampling within 14 days of injection, the reason for the low titers of the T6 and T7 groups containing the MF59 adjuvant can be attributed to this issue (2). The immunogenicity of alum adjuvants is higher than oil-in-water ED01 and ED02 adjuvants (9), which, in the current study, the immunogenicity of Nano-Alum Hydroxide was found higher than that of MF59.Given that the MF59 was used for the first time in the Newcastle+Influenza vaccine in poultry, to the best of our knowledge, no studies have been conducted in poultry to compare their results with those of this study, which were the aspects of the novelty in this study.

According to the results, the addition of Nano-Se increased the titer of antibody against Newcastle and Avian influenza in all groups, indicating the effect of Nano-Se on boosting the titer of the antibody, which might be related to the effects of selenium compounds. The specific form of selenium nanoparticles is involved in the process of host immune responses, and given the routine use of selenium compounds in poultry nutrition, these results are not expected. The obtained data from the studies carried out by Gabalov, Rumina (28) suggested that Selenium Nanoparticles can be used as an adjuvant for immunization with cellular and extracellular *Escherichia coli* antigens (29). The effect of selenium on the activation of the poultry immune system has been demonstrated (30); furthermore, Baowei, Guoqing (31) showed that selenium was effective in stimulating the immune system in goose, which confirmed the results of the present study. Based on the results of other studies, Selenium immunogenicity can increase the protective rate and level of anti-HBs (32), Selenium supplementation increases the proliferation of lymphocytes in broiler chickens (29), and Selenium deficiency causes damage to cellular and humoral immunity (33); all of these findings were in agreement with those of the current study. The results of comparing these three adjuvants in this study showed that the combination of two ISA70VG and Nano-Se adjuvants produced the highest antibody titers and immunogenicity in the Newcastle+Avian influenza vaccine.

Authors' Contribution

Study concept and design: A. A. R.
Acquisition of data: M. R.
Analysis and interpretation of data: A. T.
Drafting of the manuscript: S. M. M.
Critical revision of the manuscript for important intellectual content: A. A. R.
Statistical analysis: M. J. G.
Administrative, technical, and material support: M. T.

Ethics

All experimental procedures were carried out according to the standard animal experimentation protocol of the Veterinary Ethics Committee of RVSRI.

Conflict of Interest

The authors declare that they have no conflict of interest.

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