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Immunological study on production of an inactivated NDV vaccine by using Montanide™ISA71 and Chitocan Nanoparticle as adjuvants.

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ABSTRACT

Newcastle disease virus (NDV) as the causative agent of acute infectious disease that threatens the poultry flock worldwide. Vaccination could reduce the risk of an outbreak of ND virus. In this study chitosan nanoparticles were used as an antigen carrier for delivery into intranasal mucosa to enhance the immune response to the Newcastle disease (ND) vaccine in compare with Montanide oil 71 adjuvants. Results demonstrate that the success of the prepared vaccines to be satisfactory to all quality control parameters. Cell mediated immune response of vaccinated chicks showed high values from the 3rd day post vaccination (DPV) and persisted till 21st DPV for vaccine with chitosan nanoparticles and vaccine with Montanide oil 71 by lymphocyte blastogenesis and phagocytic assays. Serum antibody titer was increased from the 1st week post vaccination (WPV) and continued till 25th WPV against NDV for both vaccines by Hemagglutination inhibition (HI) test. Challenge test demonstrated that the vaccines protect vaccinated chicken in percentage of 90% and 95% in vaccine with chitosan and vaccine with Montanide oil 71, respectively. In conclusion**,** the prepared ND vaccines stimulate early humoral and cellular immune responses and control virus shedding. NDV vaccine with chitosan nanoparticles could save cost and time for vaccine production.

1. INTRODUCTION

Newcastle disease (ND) is a highly contagious viral disease that affects a wide range of wild and domestic birds worldwide which frequently causes economic losses to the poultry industry (OIE, 2012). Viruses of genus Avian orthoavulavirus -1 (AOAV-1) frequently known as Avian paramyxoviruses -1 (APMV-1) or Newcastle Disease viruses (NDVs) and formerly designated as Avian avulavirus -1 (AAvV-1) (ICTV, 2019). The pathogenicity of ND virus through avian species is variable with high morbidity and mortalities in broiler and layer flocks (Wajid et al., 2016). The virus transmits horizontally through airborne droplets or exposure to virus contaminated materials or mechanical vectors and enters the host through the mucosal tissues of the respiratory tract (Alexander, 2012). Vaccination program using inactivated oil emulsion vaccines and live attenuated vaccines is the primary strategy to control the disease in countries in which virulent ND virus strains are endemic (Gallili and Ben, 1998). Mucosal immunization strategy could be effective method of vaccination in outbreaks in endemic areas and it should be suitable for mass application and effective after single application (De Wit et al., 2010) Chitosan is considered as a carrier for protein, whole particle and DNA-based vaccines (Zhao et al., 2014). The mucoadhesive biopolymer comprises repeating units of b-(1-4)-2-amino-2--deoxy-D-glucopy ranose is found to be biodegradable, biocompatible and non-toxic. Mucoprotein in mucus is positively charged, so chitosan and mucus are attracted to each other to prolong the in vivo retention and improve drug bioavailability (Ameer et al., 2020). Montanide oil 71 is a mineral oil-based adjuvant that has been used for manufacture of water-in-oil emulsion vaccine (El Naggar et al., 2017). This work aimed to prepare and evaluate the potency of an inactivated NDV vaccine using chitosan nanoparticles in compared to NDV vaccine with Montanide oil 71 adjuvants to save cost and time for vaccine production and enhance the immune responses against ND virus.

2. MATERIAL AND METHODS

The experiment was ethically approved under the following number BUFVTM02-07-22.

2.1. ND Virus strain:

Velogenic NDV (genotype VII) with a titer $10^{10}EID_{50}$ /ml and 9 Log_2 HAU /25 microliter) was used in the process of vaccines preparation and challenge after vaccination with titer$10^{6.5}EID_{50}$ /ml. Virus was provided by Central laboratory for evaluation of Veterinary Biologics (CLEVB).

2.2. Adjuvants:

2.2.1. Chitosan

Chitosan, as nanoparticle adjuvant, was proved to be non-toxic. As a polyatomic, biodegradable, and biocompatible polymer, chitosan has attracted significant attention and

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can encapsulate a range of bioactive agents including proteins and peptides, from (Primex® company).

2.2.2. Montanide Oil 71:

It is a mineral oil-based adjuvant that has been develop for production of water-in-oil (w/o) emulsion vaccine. It incorporates a specific enriched light mineral oil and an extremely purified emulsifier obtained from purified oleic acid of vegetable origin and mannitol. It was obtained from SEPPIC S.A, Paris La Defense. batch No. T21931.

2.3. Vaccine formulation:

2.3.1. Preparation of NDV vaccine with Motanide oil 7l:

Egg adapted vaccinal strain of ND virus (genotype VII) was titrated on specific pathogen free-embryonated chicken eggs (SPF-ECEs) (Reed and Muench, 1938). The seed virus had titers of $10^{9.5}EID_{50}$ /ml and 10 Log_2 HAU /25 microliter using HA test. ND viruses were inactivated using formalin in a final concentration 0.1% according to (OIE manual, 2004***)***. Sample from the inactivated virus before addition of motanide oil adjuvant was tested by two passages in 10-day old SPF-ECEs (0.1 ml /egg) via the allantoic cavity (Garcia et al., 1998). All the embryos alive up to 6 days should be examined by the rapid Hemagglutination (HA) on the allantoic fluid for the presence of virus. If no mortality or HA activities were observed the vaccine was considered safe. NDV vaccine adjuvanted with Montanide ISA 71 were prepared as water in oil (W/O) emulsion at a ratio of (30 / 70) aqueous /oil ratio according to the standard protocol of the manufacturer.

2.3.2. Preparation of NDV vaccine with chitosan nanoparticles:

Chitosan nanoparticles were prepared by ionic cross-linking method, cross linking of chitosan solution with Tripolyphosphate (TPP) (Liang et al., 2017). The Newcastle disease vaccine with chitosan nanoparticles were prepared by ionic cross-linking method (Zhao et al.,2012), as followed: 2.5 ml of NDV solution were added drop by drop under magnetic stirring to 5ml of chitosan solution, and 2.5ml of TPP solution was added to the above solution at room temperature. The chitosan-NDV nanoparticles (NDV-CS-NPs) were separated by centrifugation for 30 min at 10,000g/min at 4°C and the supernatant was discarded.

2.4. Quality Control of the prepared vaccines:

2.4.1. Sterility test:

Experimental samples from the prepared vaccines were confirmed to be free from any contaminants according to the Federal Regulation USA, by the inoculation of thioglycolate broth and nutrient agar with the vaccine and incubation at 37 ºC for 72 hours. Also, inoculation was made on sabaroud maltose agar plates and incubated at 25ºC for 14 days.

2.4.2. Safety test:

Two groups (each has 10 chicks of 2 weeks old) 1st group inoculated with two field doses (1ml) of inactivated NDV vaccine with Montanide oil 71 subcutaneously at the nap of the neck and 2nd group inoculated with two field doses (0.2 ml) of NDV vaccine with chitosan by intranasal route in addition to a control negative group, chicks were observed for two weeks for appearance of any clinical signs or any signs of local reaction. Some birds were subjected to postmortem examinations after five days of inoculation to detect any pathological lesions.

2.4.3. Characterization of (NDV- CS-NP):

NDV-CS-NPs morphological characteristics were examined using high resolution transmission electron microscopy (TEM) (Jeol, JEM 1010, Tokyo, Japan). Dynamic light scattering technique was used to determine polydispersity index, the average hydrodynamic diameter of the particles in the freshly prepared dispersions using a Zetasizer® Nano-ZS (Malvern instruments, UK). This was performed using a scattering angle of 173° at 25 °C (Zhao et al., 2012).

2.4.4. Physical tests for NDV vaccine with Montanide oil 71:

2.4.4.1. Drop test to identify the type of emulsion (Khalil, 2015).

2.4.4.2. Viscosity tests.

1. Relative viscosity test was done according to (Cessi and Nardelli, 1973).

2. Syringeability test that gives an idea of the dynamic viscosity through A needle 21 G (Seppic, 2012).

2.4.4.3. Stability test: Centrifugation test was done according to Stone, (1991).

2.5. Experimental design:

One hundred, 21-day old SPF chicks were housed in brooder units within isolation facilities, then it divided into 3 groups (30 chicks/each group). Group (1) chicks vaccinated with NDV vaccine with Montanide oil 71. Group (2) chicks vaccinated with NDV vaccine with chitosan nanoparticlies. Group (3) non-vaccinated chicks kept as negative control.

2.6. Evaluation of cellular immune response:

2.6.1. Lymphocyte proliferation test:

Separation of lymphocytes, calculation of viable cells number, and adjustment of lymphocytes was performed depending on the information of cell proliferation kit (XTT). The test was done according to Scudiero et al., (1988).

2.6.2. Evaluation of phagocytic activity by using Candida Albicans:

Separation by ficol hypaque and cultivation of mononuclear cells were performed according to Antley and Hazen, (1988). Phagocytic % was done according to by the method of Harmon and Glisson, (1989), method was improved by El-Enbawy, (1990) and Phagocytic index was performed according to Richardson and Smith, (1981).

2.7. Evaluation of the humoral immune response in serum:

Blood samples were collected weekly from each vaccinated group till 25 weeks post-vaccination and centrifuged for 10 min to collect serum for detection of antibodies using hemagglutination inhibition test according to the recommendation of OIE-Manual, (2004). Positive and negative control antisera and antigens should be run with test.

2.8. Evaluation of the potency of prepared vaccines post challenge with VNDV:

Vaccinated chicks were challenged at 28 days after vaccination, each bird received a dose of (0.1) ml of a virulent NDV strain (I/M) ($10^{6.5}$ ELD_{50}/ml). Challenged birds were observed daily for 2 weeks post challenge and the mortality rates was recorded for each group to measure the protection %.

2.9. Detection of shedding NDV after challenge:

Tracheal swabs from chicks were collected at 2-, 4- and 6-days post challenge from each group to determine the shedding of virus using Real Time-PCR (RT-PCR). ND virus RNA extraction was carried out using Qiamp Viral RNA Mini kit (QIAGEN) catalogue No.52904.Primer and probes were designed as follow:

(M+4100 AGTGATGTGCTCGGACCTTC -3') (M-4220 CCTGAGGAGAGGCATTTGCTA-3') (M+4169 (FAM) TTCTCTAGCAGTGGGACAGCCTGC (TAMRA)-3'). Preparation of PCR Master Mix according to Quanti-Tect probe (RT-PCR) kit handbook while thermal cycling

condition for gene-specific Probe and Primer sets was carried out according to Wise et al. (2004).

3. RESULTS

3.1. Sterility and safety of the prepared vaccines:

Prepared vaccines were confirmed to be sterile and free from any bacteria and fungi, also vaccines were found to be safe in immunized birds, where they didn't induce any abnormal clinical signs and no local reaction.

3.2. Characterization and optimization of (NDV-CS-NPs):

Chitosan nanoparticles containing an inactivated NDV (NDV-CS-NPs) showed a spherical and polydisperse nature with good morphology using the TEM. The measurement of particles size showed a fairly even distribution with a zeta potential of 2.7 mV and a main diameter of 389.1 nm and an encapsulation efficiency rate of 78%.

3.3. Evaluation of the physical characters of NDV vaccine with Montanide oil 71:

3.2.1. Drop test:

It was performed immediately after manufacturing. It was found that, prepared vaccine was water-in-oil (W/O) emulsion type.

3.2.2. Rheology test

1- Relative viscosity:

The stable vaccines showed acceptable flow time indicating suitable injectability for inactivated vaccines adjuvanted with montanide™ oil ISA 71 as shown in table (1).

2- Dynamic viscosity by Syringability test:

The stable vaccine showed acceptable syringability time indicating suitable injectability for Montanide™ ISA 71 as shown in table (1).

Table 1 Evaluation of relative and dynamic viscosity of the prepared inactivated vaccine.

Prepared vaccine	Viscosity test	Flow time per second
NDVvaccine with Montanide ISA 71	Relative	9.28
	Dynamic by syringability	34.6

3.2.3. Centrifugation test.

It was noticed that the vaccine adjuvanted with Montanide™ ISA 71 were stable. Unstable vaccines showed water release and separation of oil phase upon centrifugation.

3.4. Cell mediated immune response:

Lymphocyte blastogenesis test showed that cell proliferation started to increase (0.459, 0.486) from the 3rd day post vaccination (DPV) for NDV vaccine with Montanide oil 71 and NDV vaccine with chitosan, respectively. It reached maximum value (0.814, 0.743) at the 10th DPV for both vaccines, respectively and it persisted in high values (0.474, 0.425) till 21st DPV for NDV vaccine with montanide oil 71 and NDV vaccine with chitosan vaccines, respectively (table 2).

Table 2 Lymphocyte blastogenesis using XTT reagent for chicks vaccinated with inactivated NDV vaccines.

Days post vaccination	Cell proliferation expressed by optical density: NDV vaccine with montanide oil 71	NDV vaccine with chitosan	Control
3rd	0.459	0.486	0.191
5th	0.751	0.647	0.179
7th	0.780	0.630	0.165
10th	0.814	0.743	0.174
14th	0.764	0.646	0.123
21th	0.474	0.425	0.142

The phagocytic % started to increase (56.25%, 43.75%) from the 3rd DPV for NDV vaccine with Montanide oil 71 and NDV vaccine with chitosan, respectively. It reached maximum value (83.33%, 80.00%) at the 7th DPV for NDV vaccine with montanide oil 71 and NDV vaccine with chitosan, respectively. It persisted in high values (56.25%, 53.33%) till 21st DPV for NDV vaccine with montanide oil 71 and NDV vaccine with chitosan, respectively (table 3).

Table 3 Evaluation of Phagocytic percent for chicks vaccinated with inactivated NDV vaccines.

Days post vaccination	Phagocytic percent: NDV vaccine with montanide oil 71	NDVvaccine with chitosan	Control
3rd	56.25%	43.75%	5.2 %
5th	66.66%	54.50%	5.4%
7th	83.33%	73.90%	7.6%
10th	80.00%	80.00%	7.6%
14th	76.47%	75.00%	5.4%
21th	56.25%	53.33%	5.4%

The phagocytic index started to increase (0.50, 0.44) from the 3rd DPV for NDV vaccine with montanide oil 71 and NDV vaccine with chitosan, respectively. It reached maximum value (0.73, 0.75) at the 10th DPV for NDV vaccine with montanide oil 71 and NDV vaccine with chitosan, respectively. It persisted in high values (0.44, 0.50) till 21st DPV for vaccine with montanide oil 71 and vaccine with chitosan vaccines, respectively (table 3).

3.5. Humoral immune response:

Serum antibody titer against NDV using HI test was increased ($4.3\log_2$ and $4.0\log_2$) from the 1st week post vaccination (WPV) for NDV vaccine with montanide oil 71 and NDV vaccine with chitosan and persisted in high values ($6.0\ \log_2$ and $3.6\log_2$) till 25th WPV for vaccine with Montanide oil 71 and NDV vaccine with chitosan, respectively compared with control one as shown in table (4). It reached maximum value ($9.3\ \log_2$ and $7.3\ \log_2$) at the 6th WPV and 5th WPV for vaccine with montanide oil 71 and NDV vaccine with chitosan, respectively.

Table 4 Evaluation of Phagocytic index for chicks vaccinated with Inactivated NDV vaccines.

Days post vaccination	Phagocytic index: NDV vaccine with monanide oil 71	NDV vaccine with chitosan	Control
3rd	0.50	0.44	0.04
5th	0.56	0.45	0.04
7th	0.67	0.61	0.10
10th	0.73	0.75	0.06
14th	0.59	0.65	0.06
21th	0.44	0.50	0.10

3.6. Protective efficacy of the prepared vaccines upon challenge:

Vaccinated chicks were challenged with the corresponding virulent ND virus showed that vaccine with montanide oil 71 and NDV vaccine with chitosan nanoparticles gave 95% and 90% protection percent, respectively (table 5).

Detection of NDV RNA using real time RT-PCR in tracheal swabs of vaccinated chicks after their challenge demonstrated that only one bird showed shedding of NDV from chicks vaccinated with NDV vaccine with Montanide oil 71 while vaccinated chicks with NDV vaccine with chitosan nanoparticles demonstrated that two bird showed shedding of NDV, only one at 2nd day post challenge (DPC) and only one bird at 4th DPC (table 6).

Table 5 Mean log2 serum antibody titers against NDV in vaccinated chicks with inactivated NDVvaccines using HI test.

Weeks post vaccination	Mean log_2 HI serum antibody titer for NDV /ml		
	NDV vaccine with montanide oil 71	NDV vaccine with chitosan	Control
1	4.3	4.0	0
2	6.3	4.3	0
3	7.0	6.3	0
4	7.6	6.7	0
5	8.7	7.3	0
6	9.3	7.0	0
7	9.0	7.0	0
8	8.7	6.7	0
9	8.3	6.3	0
10	8.0	5.7	0
12	8.7	5.3	0
14	7.3	5.0	0
17	7.7	5.3	0
19	6.7	4.7	0
21	7.3	4.3	0
25	6.0	3.6	0

Table 6 Protection% in chicks vaccinated with inactivated NDV vaccines after challenge with virulent NDV.

Groups	Chicks challenged with virulent NDV		
	Challenged	Dead	Protection%
NDV vaccine montanide oil	20	1	95%
NDV vaccine with chitosan	20	2	90%
Control	20	20	0%

Table 7 Detection of shed of NDV using real time RT-PCR from vaccinated chicks after challenge with vNDV.

Days after challenge	Detection of NDV using real time RT-PCR		
	NDVvaccine montanide oil	NDVvaccine withchitosan	Control
2nd	1/5	1/5	5/5
4th	0/5	1/5	5/5
6th	0/5	0/5	5/5

4. DISCUSSION

The successful control of infectious viral diseases depends on a well-designed vaccination program using high potent and safe vaccines. This study demonstrates the efficacy of chitosan nanoparticles and montanide oil ISA 71 on immune response of vaccinated SPF chicks. The chitosan nanoparticles and montanide oil 71 adjuvants can enhance the efficacy of vaccination against infectious viral diseases (Corbanie et al., 2006). Adjuvants are an important role to enhance efficacy of vaccines and should be safe, not be immunogenic themselves, biodegradable and ease of manufacture (Pulendran et al., 2006).Chitosan consider a suitable polymer to be used as a delivery vehicle for vaccines production due to its biocompatibility, biodegradability, non-toxic nature and easily modified into desired sizes and shapes (Van der Lubben et al. 2001). Factors affecting NDV-CS-NP preparation are chitosan concentration, NDV/CS ratio and TPP concentration. The optimal combination for NDV-CS-NPs was a NDV/CS ratio of 1:2 that came in agreement with (Zhao et al., 2012). Montanide ISA 71 adjuvant was used to develop and manufacture of inactivated oil emulsion vaccines against NDV (Khalil, 2015**).** The prepared vaccines are completely sterile when tested on specific bacteriologic and fungal media. In addition, there is no mortalities were recorded in inoculated chicks and no local or systemic reaction, these indicate the safety of the prepared vaccines. These are coming parallel to the recommendation of (OIE, 2004).

Cellular immune response of vaccinated chicks was evaluated using the lymphocyte proliferation test, which showed that the cell proliferation started to increase from the 3rd day post vaccination DPV and persisted in high values till 21th DPV for NDV vaccine with montinoide oil 71 and NDV vaccine with chitosan nanoparticles. These results indicated positive effect of montanide ISA oils on cellular immune response as recorded by (Habjanec et al., 2008).and also positive effect of chitosan on immune response as recorded by (Zhao et al., 2012).

The phagocytic activity started to increase from the 3rd DPV and persisted in high values till 21th DPV for ND vaccine with Montanide oil 71 and NDV vaccine with chitosan. These results came in agreement with that of Madkour, (1992) who explained that chicken vaccinated with inactivated oil emulsion vaccine greatly stimulated the cellular immune response. The results also showed that values of cellular immune response at later stages came in agreement with Timms and Bracemell, (1983) who clarified that once the humoral immune response becomes established there is similar decrease in the cellular immune response.

Evaluation of humoral immune response induced in vaccinated chicks was carried out using heamagglutination inhibition (HI) test as assessed by Adrianus and Richard, (2009).

Serum antibody titer against ND was increased from the 1st WPV and persisted in high values till 25th WPV for NDV vaccine with Montanide oil 71 and ND vaccine with chitosan. These results were like that of Pour et al., (2006)**.**

Challenge of vaccinated chicks with the corresponding virulent NDV showed that the NDV vaccine with Montanide oil 71 and NDV vaccine with chitosan vaccine gave 95% and 90% protection percent, respectively. Detection of NDV using real time RT-PCR in tracheal swabs of vaccinated chicks after their challenge showed that only one bird showed shedding of NDV at 2nd day post challenge from chicks vaccinated with NDV vaccine with Montanide oil 71 while chicks vaccinated with NDV vaccine with chitosan showed that only one bird showed shedding of NDV at 2nd day post challenge and only one bird at 4nd day post challenge, compared to the control group showing 100% positive shedding for RNA copies of NDV. This result agrees with Khalil, (2015) and fulfill with OIE; (2012)**.**

5. CONCLUSION

In conclusion, the results of the present study demonstrate the immunoenhancing effects of chitosan nanoparticles and Montanide oil 71 on immune response of vaccinated birds and document the potentiality of chitosan nanoparticles as safe and nontoxic adjuvant when used via intranasal route. Application of such vaccine will improve the vaccination strategies against ND virus which save cost and time for vaccine production.

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