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# Immunogenicity and Efficacy Study on Newcastle Disease Vaccine Using Many Adjuvants and Chitosan Nanoparticles

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**Abstract:** To potentiate the immune responses of the Newcastle Disease vaccine, many adjuvants such as, Saponin, Paraffin oil, sunflower oil, Nigella Sativa oil, and Chitosan Nanoparticles “CHNPs”, were prepared with live and inactivated I-2 vaccines. The formulated vaccines were tested for their biological and physical characteristics, including sterility, safety, Immunogenicity protective efficacy, protein estimation for CHNPs, and the completion of the emulsification. The protein concentrations of pre and post encapsulation for CHNPs were 0.23g/100 ml and 0.065g/100 ml respectively. The encapsulation efficiency was 71.7%. To test the safety, immunogenicity and efficacy, a 180 white leghorn day-old-chicks divided randomly in to 9 groups of A, B, C, D, E, F, G, H, and I, a 20 chicks for each. Group A (Saponin + Inactivated I-2), group B (Paraffin oil + Inactivated I-2), group C (CHNPs+ Inactivated I-2), group D (Saponin + Live + Inactivated), group E (Nigella Sativa oil+Live I-2), group F (Sunflower oil + Live I-2), group G (Saponin+Live I-2) each groups from A to G simultaneously and inrtaocularly (I/O) inoculated with  $10^7$  EID<sub>50</sub> I-2 live vaccine. Group H (control group I) received only (Saponin+Inactivated), group I (control group II) received the placebo (Normal Saline) control group, in this group only 5 chicks out of 20 inoculated I/O with  $10^6$  EID<sub>50</sub> of live I-2, and the remaining chicks were left unvaccinated. There was no evidence for bacterial growth for the tested and control groups, nor post vaccination reactions neither ND clinical signs observed (except for Nigella Sativa oil). Immunogenicity was estimated using ELISA test. The highest Abs mean titer was for group E While the lowest Abs mean titer was for group C. Mean values were analyzed using one way analysis of variance test (ANOVA). Mean Differences were considered to be statistically significant at  $P < .05$ . The nine comparisons were associated with statistically insignificant effect ( $P > .05$ ). Groups A, B, C, D, and H challenged via mixing with clinically ill chickens for 14 days. The highest protection level was 90% for group D, while the lowest protection 50% was for group B (Paraffin oil+ Inactivated).

**Keywords:** Adjuvant, Saponin, Mineral Oils, Vegetable Oils, Chitosan Nanoparticles, Newcastle Disease Vaccine, Immune Response, ELISA Test

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## 1. Introduction

Newcastle disease (ND) is a contagious disease infecting manly avian species. The most important form of this disease is caused by virulent strains belong to the genus Avulavirus and serotype paramyxovirus-1. the virus genome is single stranded negative sense RNA virus [1] The disease is economically important and pose major threat to the poultry industry worldwide [2].

The general approaches to control the Newcastle disease are hygienic and vaccination approaches [3]. There are many types of vaccines used for combating the disease, live lentogenic, live mesogenic and inactivated vaccines as well as recombinant technology vaccines. The live lentogenic vaccines are usually derived from field viruses that have been shown to have low pathogenicity for poultry but produce an adequate immune response. Many adjuvants and immunopotentiators have been introduced to improve the immunogenicity of these

vaccines, for example water-in-mineral oil (W/O) emulsion adjuvants have been extensively used for inactivated ND vaccines formulation. These adjuvants are indeed have strong evidences that induce long-term efficacy in poultry vaccines [4]. However, there are some concerns. More adjuvants that also increase the cellular immune response and extend the ND vaccines, cross protection against different strains or serotypes have been successfully attempted. For example scientists have tested the extremely refined emulsifier obtained from Manitol and purified oleic acid of vegetable origin to enhance immune response against ND. This adjuvant i.e. Manitol was specifically formulated to stimulate cell-mediated immunity, and has been shown previously to induce strong humoral and cellular immune response in subunit vaccines as well.[5-7]. Chitosan Nanoparticles (CHNPs) also have been used effectively to enhance long and potent immune response against the deadly vv NDV. For instance after three vaccinations with inactivated NDV in combination with CHNPs an increase in antibody titers in blood and mucosal samples has been observed in chickens when compared with the administration of NDV antigen only [8]. Moreover, chitosan microsphere have also been tested and the result showed that NDV chitosan microsphere vaccine was safe, and could induce humoral and cell-mediated immune response and mucosal immunity strongly [9]. Despite the use of these different vaccines approaches, the disease (ND) is yet to be controlled. However, the use of oil adjuvants which encourages lasting Haemagglutination-inhibition (HI) antibody titer has been reported in different parts of the world [10].

It is been obvious that the development of safe, novel adjuvants is crucial to maximize the efficacy and the immunresponse induced by ND vaccines, Chitosan is one of them, it is a non-toxic, biocompatible, biodegradable and natural polysaccharide derived from the exoskeleton of crustaceans and insects. It has been demonstrated to be an effective absorption enhancer to improve delivery of peptide and protein drugs in human and mice in addition to vaccines [11]. In this study we utilized the very same material and many other locally available adjuvants to develop a good and potent immune response against ND virus.

To potentiate the immune response of the commercially available ND vaccines, there are many attempts that have been made by different scientists. They tested different adjuvants in different parts of the world.

Ezeifeke et al [10] investigated the adjuvant effects of many plant oils like Groundnut oil, Palm oli, Olive oil, and Soya bean oils on the immune response of (ND) vaccine. Parimal et al [12] also incorporated liquid paraffin and groundnut oil in to the live ND mesogenic vaccine of RDVK another attempt to promote the immune response of ND lentogenic vaccines was made by the Samina et al [13] who incorporated live and killed lentogenic strain of VH in to mineral oil as well as waheed et al [14]did who successfully prepared an oil emulsified vaccine from apathogenic strain of VG/GA. Another vaccine delivery system based on chitosan nanoparticles has been used by Zaho et al [15], who

encapsulated ND LaSota starin in to chitosan nanoparticles using an ionic cross-linking method. We in this study utilized the same technique to deliver inactivated I-2 thermostable ND vaccine in to Chitosan nanoparticles (CHNPS). To our best of knowledge this is a first attempt to investigate the immunogenicity and efficacy of lentogenic thermostable strain of ND with different adjuvant systems including different plant oils, mineral oil, CHNPS, as well as Saponin.

## 2. Material and Methods

### 2.1. Preparation of ND Vaccine

Five ampules of the freeze dried I-2 vaccine containing at least 4 log of HI tighter and more than  $10^9$  EID<sub>50</sub> infectivity titer per ampule were reconstituted using 1 ml NS, then pooled and mixed thoroughly, then added to sterile 200ml of cold deionized distilled water (DDW) mixed well, and kept cool at 4°C. The diluted vaccine was inactivated by 1% formaldehyde, and then incubated at 37°C for 20 hours and stored at 4°C [16].

### 2.2. Confirmation of Virus Inactivation

A 0.2ml of formaldehyde inactivated virus was inoculated into the allantoic fluid of 10 day-old-embryonated chicken eggs, and incubated at 37°C for 120 hours.

More embryonated chicken eggs were used to inoculate 0.2ml of  $10^3$  EID<sub>50</sub> of I-2 live vaccine and kept as positive control group. A 0.2ml of DDW was inoculated into 10-day-old embryonated chicken eggs, and designated as negative control group.

Both positive, and negative control groups were incubated at 37°C for 120°C and were tested after the incubation period for the HA activity [17].

### 2.3. Formulation of Live Adjuvanted (ND) Vaccines Using Sunflower and Neglia Sativa Oils as an Adjuvants

#### 2.3.1. Preparation of Oil Phases

A 0.5ml and 1ml of 1% tween 80 were added to 30 ml Neglia Sativa oil and 99 ml sunflower oil respectively, then these mixtures were mixed thoroughly using intermittent shot of ultrasonic pulses 50 watts/50 seconds for 3 minutes. Every care was kept not to heat or splash the mixture due to ultrasonic pulses [16].

#### 2.3.2. Emulsification of Oils Phases into the Aqueous Phases

A 30 ml of sunflower oil phase, and 30 ml of Neglia Sativa oil phase were respectively added to 30ml and 20ml (aqueous phases) of cold live I-2 vaccine dissolved in sterile distilled water. The adjuvant to vaccine ratio was 5:3 and 5:2 for the sunflower and Neglia Sativa oils respectively.

The emulsification was prepared by recycling the oils and aqueous phases mixture using disposable syringe.

#### 2.3.3. Emulsification Testing

The prepared emulsions of sunflower and Neglia Sativa oils were tested for well oil in water emulsification W/O, i.e.

0.5ml of the formulated vaccines were added drop wise to Petri dishes containing tap water.

#### **2.4. Formulation of Live and Inactivated Vaccine with Saponin Adjuvant Preparation and Vaccine Formulation**

A 50ml inactivated ND virus prepared previously was incorporated into 50 ml of saponin adjuvant. The saponin adjuvant was added intermittently in 10 ml aliquot, and mixed thoroughly up to the last part of the saponin, and then the whole mixture was well mixed to produce a very homogenous solution of virus and Saponin adjuvant. The formulated vaccine was dispensed into 20 ml vials, and then stored cold at 4°C. To formulate a combination of live, and inactivated saponin adjuvanted vaccines a 30 ml of cold live I-2 vaccine contains at least  $10^{7.4}$  EID<sub>50</sub> was added in 5 ml each time to other 30ml of cold inactivated saponin adjuvanted ND vaccine.

##### **2.4.1. Incorporation of Paraffin Oil in to Inactivated Vaccine**

Small volume of inactivated ND oil emulsion had been prepared. A 30 ml of previously prepared inactivated ND virus dissolved in physiological saline, and tested in chicken embryonated eggs for its infectivity activity was used as antigen source.

One part of the inactivated virus was homogenized with two parts of ready prepared oil-Aralcel mixture of incomplete Freund's adjuvant [18].

The inactivated virus (water phase) was 30 ml added to 60 ml of the incomplete Freund's adjuvant (oil phase), the emulsion was formulated by vigorously injecting the antigen solution into the oil phase by small volume each time. Between additions the aqueous phase and the progressive oil emulsion were vigorously recycled several times using disposable syringe in order to increase the emulsion viscosity, and the dispersion of the antigen phase.

Each ml of used incomplete Freund's adjuvant contains 0.85ml paraffin oil, and 0.15 ml mannide monooleate.

Each dose of this vaccine formulation consists of 0.2ml oil emulsion, which contains the equivalent of  $10^8$  EID<sub>50</sub> antigens.

##### **2.4.2. Testing the Water in Oil Emulsion**

In order to confirm that the water and oil phases had completely emulsified, the prepared oil emulsion was tested by dropping 0.25ml of the emulsion into tap water in a Petri dish.

##### **2.4.3. Sterility Test**

The sterility test of the formulated emulsions accomplished using the thioglycolate medium broth. Ten vials were inoculated with 0.2ml vaccine preparation each. Four non- inoculated vials were kept as a negative control. Both tested and control groups were incubated at 37°C and room temperature for 7 days respectively.

#### **2.5. Preparation of Inactivate (ND) Vaccine Encapsulated in Chitosan Nanoparticles**

Chitosan (with a molecular weight of 71.3 KDa and

deacetylation degree of 80%), and Sodium tripoly phosphates (TPP) were provided by the State key laboratory of veterinary biotechnology, Harbin Veterinary Research Institute, China.

##### **2.5.1. Preparation of Chitosan and (TPP) Solution**

According to the principle of ionic cross linking, nonparticles can be formed by intra and inter molecular cross linking between positively charged chitosan and negatively charged TPP [15].

In this study, we used ionic cross linking method to develop a delivery system based on chitosan nanoparticles. A 0.25% W/V chitosan solution was prepared by dissolving 0.25 g in 100ml aqueous solution of 2% (V/V) acetic acid then mixed thoroughly via sonicator at 50 watt for 10 seconds until complete dissolving to produce chitosan solution of 2.5mg/ml.

A 15% W/V TPP solution was prepared by dissolving 15g of TPP in 100 ml DW to produce solution of 150 mg/ml. this solution was then autoclaved at 121°C for 15 minutes then cooled down to Room temperature (R. T.) and mixed thoroughly until the solution become transparent.

Every 5ml of the chitosan solution added to the TPP solution mixed via sonification at 50 watt for 10 seconds.

The formed Chitosan-Nonparticles (CHNPs) were allowed to stand in this solution for 30 minutes then after incubation the solution was centrifuged at 3000 rpm for 10 minutes.

After discarding the supernatant, the CHNPs were reconstituted in 100ml deionized distilled water for washing, then once again centrifuged at 3000rpm for 10 minutes. The remaining chitosan pellet was kept wet under the supernatant, and stored at 4°C.

The chitosan-NDV nonparticles were prepared using an ionic cross linking method. After discarding the supernatant off, a 10 ml of cold inactivated ND virus solution was added to the whole pellet of the CH-NPs. The protein concentration of inactivated vaccine was 2.2 mg/ml, the encapsulation capacity expected to be 4.4 mg to 5mg protein /ml.

The mixture of CHNPs and the inactivated NDV was then incubated at 26°C for 24 hours under gentle stirring to immobilize the NDV by adsorption, then after incubation the chitosan nonparticles containing NDV were centrifuged at 3000 rpm for 10 minutes, and then the supernatant containing the free NDV was removed. The remaining pellet was named NDV-CHNPs.

##### **2.5.2. Evaluation of Encapsulation Efficiency (EE %)**

The encapsulation efficiency of NDV-CHNPs was determined by measuring the amount of protein content in the supernatant pre and post encapsulation using Biuret method.

##### **2.5.3. Protein Estimation**

The protein content of inactivated ND vaccine stock solution, containing at least  $10^{7.4}$  EID<sub>50</sub> and supernatant free NDV named sample I, and sample II respectively were tested for protein content using Biuret method.

This method estimate protein content in gram/100ml i.e. 1ml

from sample I and sample II was added to 1ml Buriat main solution, then mixed well. The optical densities of the two samples were measured using spectrophotometer at 540 nm.

Utilizing the following formula, protein concentrations of the two samples were estimated against standard bovine serum albumin (B. S. A) solution.

$$\text{Protein /100 ml} = \text{test O. D} - \text{Blank O. D} \times 7.5$$

Standard O. D - Blank O. D

The encapsulation efficiency (EE) of the prepared nanoparticles was calculated according to Xuym *et al* [19] using the following formula

$$\text{EE\%} = \frac{\text{WO} - \text{WI}}{\text{WO}} \times 100\%$$

Where

WO: is the a total amount of the protein added

WI: is the amount of the protein in the supernatant after encapsulation.

#### 2.5.4. Preparation of NDV-CHNPs Inoculum

The prepared NDV-CHNPs was more viscous to be inoculated. Therefore, the solution was diluted in equal volume of DDW i.e. 10 ml of DDW was added to 10 ml NDV-CHNPs then mixed well, and dispensed in to 20 ml McCartney vial, and stored at 4°C. A 20 - day-old chicks were immunized subcutaneously with 200µl of the NDV-CHNPs chicks, and then observed for 21 days post vaccination.

### 2.6. Clinical Evaluation of ND Adjuvinated Vaccines Preparations Chickens

One hundred and eighty white leghorn day-old-chicks were obtained from commercial hatchery, then immediately after housing they were served with slightly sweetened water to decrease the transportation stress. On the next day they were divided randomly into 9 groups from A to I.

Groups A, B, C, D, E, F, G, H and I, 20 chicks each, all chicks were maintained separately with feed and water. Group I which receive the placebo was kept as a control group.

In group I only 5 chicks vaccinated I/O with 10<sup>6</sup> EID<sub>50</sub> ND vaccine, and the remaining chicks were left unvaccinated to prove that the I-2 can transmit horizontally.

#### 2.6.1. Samples Collection

A 24 samples of prevaccination sera were collected at random before grouping the chicks, and kept at -20°C, till tested.

#### 2.6.2. Immunization Procedure

All test groups from A to G were initially vaccinated intraocularly with 10<sup>7</sup> EID<sub>50</sub> of the I-2 vaccine before the commencement of vaccination, then just one hour later the chicks of all groups were simultaneously vaccinated accordingly into the back neck via the subcutaneous route. Chicks that belong to group A were vaccinated subcutaneously by 0.2 ml of the Saponin adjuvinated inactivated vaccine.

Chicks in group B received paraffin oil with inactivated vaccine while chicks in group C received chitosan NPS

loaded with inactivated ND vaccine. Group D Saponin with mixed live and inactivated vaccines, group E Neglia Sativa oil with live vaccine and group F sunflower with live vaccine also. Group G Saponin with live vaccine, group H (control group I) receive no intaocular live vaccine was only saponin plus inactivated vaccine and group I (control group II) live vaccine only.

#### 2.6.3. Protective Efficacy

After 21 days post vaccination 10 chicks were randomly selected from groups A, B, C, D and H (control group I) and challenged by mixing with chickens infected with very virulent NDV strain (SHD/12). The ND clinical signs and mortality rates among the challenged chicks were monitored on daily basis for 14 days.

Dead birds as well as birds showing either clinical signs or no clinical signs were recorded.

#### 2.6.4. Safety Test

Safety test was conducted according to OIE Manual [20], utilizing the potency test, all vaccinated groups were observed for 21 days post vaccination.

#### 2.6.5. Efficacy Test

After 21 days post vaccination, chicks from all 9 groups were bled, sera were collected, and stored at -20°C, and then ND antibody levels against every vaccine formulation were estimated using ELISA test.

### 2.7. Statistical Analysis

Mean values were analyzed using one way analysis of variance test (ANOVA). Mean Differences were considered to be statistically significant at P<.05.

The protection and survival rates were also estimated.

## 3. Results

### 3.1. Confirmation of Virus Inactivation

The formalin treated virus was confirmed to be completely inactivated by absence of HA evidence in the tested allantoic fluid. The positive, and negative HA reactions for positive and negative control groups were recorded.

### 3.2. Testing of Water in Oil Emulsion

The drops of the prepared oil emulsions remained discrete without dispersion except for sunflower preparation.

### 3.3. Sterility Test

There was no evidence for bacterial growth in the inoculated thioglycolate medium for the tested, and the control groups of all ND vaccine preparations.

### 3.4. Protein Estimation

The protein concentrations of pre- and post- encapsulation were 0.23 g/100ml and .065g/100ml respectively. 023-.0065/.023x100%. The encapsulation efficiency was 71.7%.

### 3.5. Safety Test

After 21 days observation, there were no adverse reactions for chicks vaccinated with NDV-CHNPs, furthermore there were no nervous signs, clinical symptoms, and death observed.

There were no post vaccination reactions attributed to any vaccine formulations containing Saponin adjuvant.

For sunflower oil adjuvant, there were no post vaccination reactions. However, a severe reaction was observed in chicks vaccinated with Nigella Sativa oil after 12 hours post vaccination. Chicks developed a severe depression, and within 72 hours 17 out of 20 chicks died.

### 3.6. Analysis of Variance Test (ANOVA Test)

Descriptive statistics associated with antibody levels

across the nine groups reported in table 1. It can be seen that the chitosan nanoparticles loaded with inactivated ND virus was associated with numerically smallest Abs level (M=857.6) and Negllia Sativa oil with live ND vaccine associated with numerically highest Abs level (M=4381.7). in order to test the hypothesis that the nine adjuvant group means were equal, a series of single-step scheffe multiple comparisons were performed. Furthermore, the assumption of homogeneity of variances was tested and satisfied based on Levene, s *F* test  $F(8,107) = 1.93 P = .063$

As can be seen in table 1 the whole nine comparisons were associated with statistically insignificant effect ( $P > .05$ ) the independent between groups (ANOVA) yielded statistically insignificant result.

## 4. Efficacy Test

**Table 1.** Comparison of immuneresponses and protection in day old layer chicks vaccinated subcutaneously with (ND) vaccines incorporated in different adjuvants (Efficacy Experiment).

Adjuvanted (a) vaccines	14 days post challenge				Geometric mean titer 21 days post vaccination (ELISA)	
	Mortality rate %	No of survival /challenged	Protection	No of chicks	ELISA test	
					GMT*	%CV
A Saponin+inactivated	30%	7/10	70%	15	653	68.75
B Paraffin oil+inactivated	50%	5/10	50%	15	534*	72.22
C ChitosanNPS +inactivated	30%	7/10	70%	15	49	82.87
D Saponin+(inactivated+live)	10%	9/10	90%	15	281*	81.60
H (b) Control (saponin+inactivated	30%	7/10	70%	15	1347*	75.13
F Sunflower oil+(live)	-	-	-	15	1131	70.79
G Saponin+(live)	-	-	-	15	1354	43.62
E Nigella Sativa oil +(live)	-	-	-	15*	3767	41.95
I (c) Control group live only	-	-	-	8	1698*	36.56
J Maternal immunity	-	-	-	11	10929	29.26

Groups: A, B, C, D, and H were subjected to challenge test. Groups: F, G, E, and I were not subjected to challenge test. Group: J Maternal immunity Groups H(b) and I(c) controls groups.

GMT= Geometric Mean Titer

**Table 2.** Descriptive statistics of the antibody levels across the (ND) vaccine ajuvanted groups, and the results associated with Scheffe multiple-comparison test.

Adjuvant	N	GMT	Comparison	P value
Sap+(inact)	15	653	Sap+(inact) vs Para+(inact)	1.000
Para+(inact)	15	534*	Para+(inact) vs Chit+(inact)	.774
Chit+(inact)	15	49	Chit+(inact) vs Sap+(liv+ianct)	.959
Sap+(ianct+ liv)	15	281*	Sap+(liv+ianct) vs Cont I (Sap+inc)	.996
Cont I (Sap+inact)	15	1347*	Cont I (Sap+inc) vs Sun oil+ (live)	1.000
Sun oil+ (live)	15	1131	Sun oil+ (live) vs Sap+(live)	.996
Sap+(live)	15	1354	Sap+(live) vs Baraka+(live)	.886
Nigella Sativa +(live)	3	3767	Nigella Sativa+(live) vs Cont II (live)	.960
Cont II (live)	8	1698*	Cont II (live) vs Cont I (Sap+inc)	.994

Note: all groups were primed with live I-2 using the I/O route except control I (Sap+inact)

Sap+ (inact) = Saponin+ (inactivated ND vaccine)

Para+ (inact) = Parafin oil + (inactivated ND vaccine)

Chit+ (inact) = Chitosan- NPs+ (inactivated ND vaccine)

Sap + (liv+inact) = Saponin+ (live +inactivated ND vaccine)

Cont I (Sap+inact) = Control group I (Saponin+ (inactivated ND vaccine)

Sun oil+ (live) = sunflower oil + (live ND vaccine)

Sap+ (live) = Saponin + (live ND vaccine)

Nigella Sativa+ (live) = Nigella Sativa oil + (live ND vaccine)

Cont II (live) = Control group II (live ND vaccine only)

GMT= Geometric Mean Titer



Figure 1. Chitosan Nanoparticles preparation mixed with inactivated I-2 vaccine.



Figure 2. The three preparations of Saponin, Sunflower oil and Nigella Sative oil mixed with live I-2 vaccines.



Figure 3. The Nigella Sativa oil vaccine preparation showing a discrete drops on tap water.



Figure 4. Illustrates experimental infection by mixing clinically ill local breed with commercial chicks immunized by the adjuvinated ND vaccines preparation, some commercial chicks show typical clinical signs of resting head and neck on their back like the source of infection (local breed) while other withstand the challenge test.

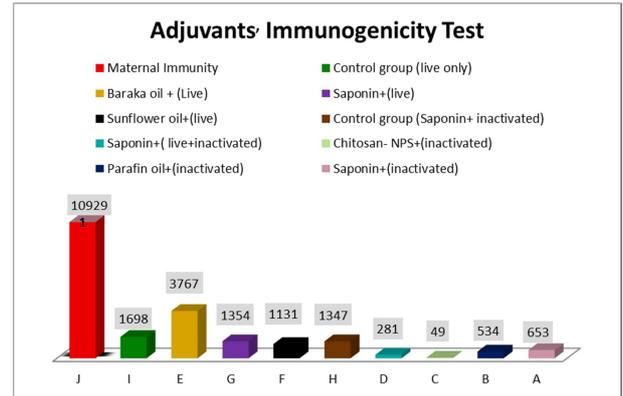


Figure 5. The geometric mean titers of the (ND) vaccine formulations 21 days post vaccination as measured by ELISA.

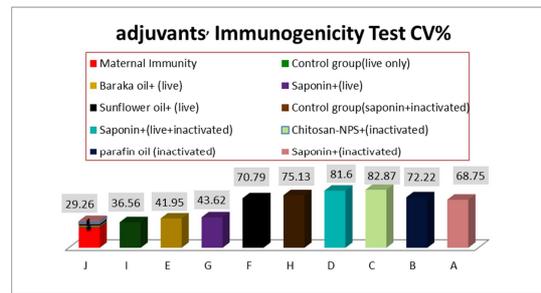


Figure 6. The CO-efficient variation percentage (CV %) of the vaccine formulations 21 days post vaccination as measured by ELISA.

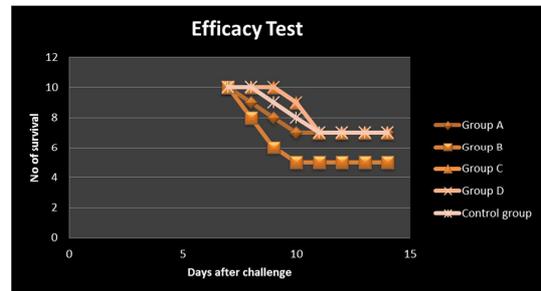


Figure 7. The survival analysis of layer chickens vaccinated at day old by live and inactivated ND vaccines incorporated in to many adjuvant systems.

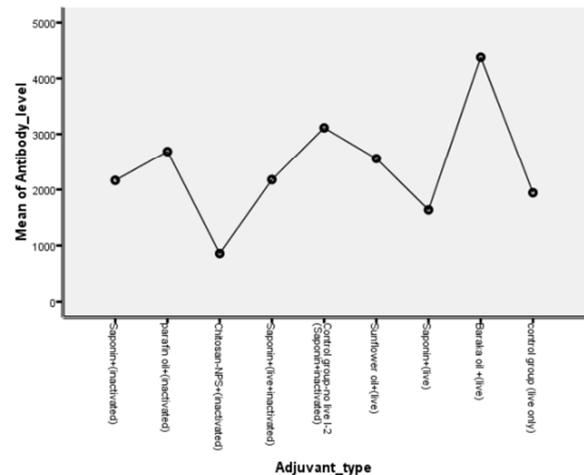
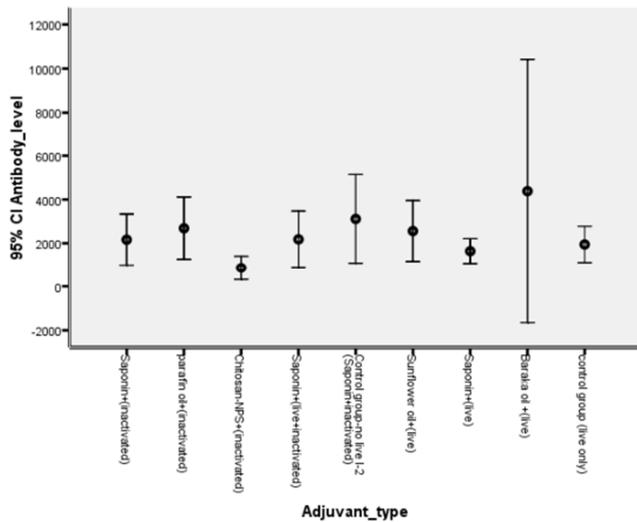


Figure 8. A visual depiction of the adjuvinated (ND) vaccines Abs mean titers in day old white leg horn chicks 21 days post-vaccination.



**Figure 9.** The 95% confidence interval of locally prepared (ND) vaccines formulation when vaccinated at day old and measured 21 days post vaccination.

## 5. Discussion

The I-2 is used usually as thermostable vaccine to combat the ND disease at different ages,

In this study we formulated many adjuvant systems, either mixed with live or inactivated I-2 vaccines and tested them in commercial layer flocks. These adjuvant preparations were used to stimulate better ND immune-responses; the results obtained confirmed that, all of the formulated vaccine preparations were safe and no post vaccination reactions were observed in all groups except in group E (Nigella Sativa oil) which showed excessive adverse reactions. The vaccinated birds were apparently healthy, no allergic reactions or clinical signs attributed to either ND virus or any other avian pathogens were seen, indicating that the locally formulated vaccines were safe and sterile. The integrity of the tested water in oil emulsion indicates that products have been successfully formulated.

The preparation NDV-CHNPs vaccine was safe at dose of 200 $\mu$ l, and when administered in day-old-chicks there were no clinical or nervous signs and the vaccinated chicks performed well during the observation period for up to 21 days post vaccination, A similar work done by Zhao et al [15], revealed that, the NDV-CHNPs adjuvanted vaccines have a high level of safety when 250  $\mu$ l of the formulated vaccine administered to 30 days old chickens, also no post vaccination reactions or ND clinical signs were recorded when live or killed ND I-2 vaccines with Saponin inoculated into one day old chicks. Also Rauw et al [11] showed that NDV-CHNPs had enhanced antigen-specific cell-mediated immune response in the spleen and it is a good enhancer for T. helper pathway of immunity. The Saponin adjuvanted vaccine tested in this study was also found safe with no reaction on day old chicks, and chicks remained healthy for up to 40 days post vaccination, chicks in all groups withstood the 10 times recommended field dose of the live I-2 vaccine.

The I-2 either alone or simultaneously with these adjuvants was also proof to be safe.

The Nigella Sativa oil vaccine formulation when dropped on the surface of tap water the vaccine remained discrete compared to sunflower vaccine preparation which partially dispersed. even though, they were treated similarly, and this might be either due to the higher viscosity of Nigella Sativa oil, or in sunflower oil case due to the formation of multi water in oil emulsion W/O rather than complete W/O or due to the missing span 80 (oil phase emulsifier).

In this study we noticed that plant oils are more difficult to be emulsified as W/O emulsions than the mineral oils. Nevertheless, the plant oil vaccine formulations prepared in this study were easier to be administered even via 1ml syringe, which indicated that the viscosity of these vaccines was reasonably good, although, it had not been tested objectively.

When Nigella Sativa oil was used as an adjuvant, excessive reactions were seen among the chicks, and 85% of the vaccinated chicks died. This might be due to the sever reactivity of the Nigella Sativa oil.

The remaining survived chicks developed a robust immune response against vvNDV. From the results obtained, Nigella Sativa oil was the most potent adjuvant with the highest Abs levels among all investigated adjuvants.

Nigella Sativa oil adjuvant might be safer at lower dose levels or concentrations. Therefore, further investigations for safety, immunogenicity, and efficacy are needed. The plants oils are cheap, and available locally at affordable prices. Consequently, utilization of this kind of oils as an alternative adjuvant will reduce the cost of the oil vaccines production. Moreover, the plant derived oils are safer with lower risks of carcinogenic hazard. thus, it could be a potential substitution for the carcinogenic and expensive mineral oils.

A reasonable levels of hummoral immune response were obtained when liquid paraffin oil used as an adjuvant with ND I-2 inactivated vaccine. This result is consistent with nature of the inactivated vaccines which produce better antibody derived immunity than cellular immunity [21].

The incorporation of Saponin with live and inactivated vaccine produced the highest level of protection 90% with relatively lower antibody levels (GMT), a similar result was obtained by Samina, et al [13].

Although the GMT of NDV-CHNPs (857.6) was the lowest one among the all formulations, the level of protection was relatively higher (70%) this might indicate that, a potent cellular or mucosal immunity had been taken place rather than humoral immunity. This prediction is in line with Volcova et al [22] who demonstrated changing in both humoral and cellular immunity after administering chitosan particles with inactivated ND vaccines. Also our findings is consistent with Yang et al [8] who clearly illustrated that CHNPs evoked better cellular immunity than Abs derived immunity when compared with commercial ND vaccines without chitosan nanoparticles. Zahi et al [9] successfully tested the ability of chitosan microsphere to produce both arms of immune response as well as mucosal immunity. The

ELISA result GMT (857.6) from group C (CHNPs + inactivated I-2), and GMT (2176.7) from group D (Saponin + live + inactivated), indicates that the protection against ND might correlate well with cellular immunity. Generally the Abs levels among all groups – table 2- associated with statistically insignificant effect ( $P>0.05$ ) while there are considerable differences in challenge test and protection, this finding allude to different cellular or mucosal immune responses among the different vaccine preparations. Moreover, the obtained Abs levels were generally low or equal to the non adjuvanted control (group I), this might be because of interference for humoral immunity which might be happened due to administration of live I-2 concurrently with adjuvanted vaccine preparations in to the same chick. Highest level of Abs observed in chicks received the vaccine with Saponin plus live and inactivated vaccine, while the lowest Abs level was detected in group vaccinated with the N.D.C.H.NPS. Moreover, the highest protection level (challenge test) of 90% and 70% had been obtained for Saponin and NDV-CHNPs respectively. The higher 95% confidence interval for group C figure 7 was because we adopt intention to treat analysis.

The levels of Abs in groups F, G, E which received sun flower oil, Saponin and Neglia Sativa oil plus live vaccines were relatively equal to those mixed with inactivated vaccines in groups A, B, C, this finding is inconsistent with Peleg et al [21] who described that the live-in-oil vaccine has both the effect of live and killed vaccines. thus, he -continue- some live viruses 'escape' the trapping of oil environment which infect various tissues, multiply and initiate immunoreponse, virus trapped in the oily environment killed and released slowly which provide booster effects and the duration of immunity is extended.

This inconsistency might be due to the interference between the live vaccine administered I/O simultaneously with vaccine preparations discussed above. Despite we believed in this fact our goal was to benefit from the very beginning -day old -from the mucousal and cellular immunity generated by the live vaccines.

In the line of this interpretation the early -day old- inoculation of live I-2 alongside with these vaccine preparations leads to lower Abs levels and inconsistency with Parimal et al [12] who suggested that the non-adjuvanted vaccine (like control group I) may fail to establish immunity in the presence of antibody because of the neutralisation caused by maternal Ab, but oil adjuvanted vaccines would induce higher Abs levels even in the presence of antibodies due to slow and persistent release of the virus from this oily environment.

On tap water, the drops of the prepared inactivated ND vaccine remained discrete without any dispersion; even when the vaccine had been stored at 4°C for more than 12 month. This indicated that, the water phase containing the antigen was entirely enclosed within the oil phase; and the type of emulsion meant has been achieved. Also, a good performance in the immune response and efficacy obtained indicate a good water in oil emulsification.

## 6. Conclusion

This study has explored the usefulness of some affordable and accessible adjuvants such as Saponin, sunflower oil and chitosan nanoparticles as a promising substitutions for the mineral oil and to potentiate the immune response against the vv ND strains. it also for the first time introducing the Nigella Sativa oil as a promising and potent adjuvant if it is safety and mechanisms of action are studied well in the future.

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