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Research Article

Development of Inactivated Newcastle Disease Vaccine in Sudan

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ABSTRACT

Using 1-2 and La-Sota strains two inactivated Newcastle Disease (ND) vaccines were produced. The vaccine viruses were inactivated by treatment with 0.05% laboratory-grade formaldehyde, then each inactivated vaccine was preparedas water in oil (W/O) emulsion. For each emulsion, the aqueous phase ratio was (2.4:1) (allantoic fluid: tween 80) respectively. While the oil phase contains (9:1) (paraffin oil: Manidmonoleate (span 80)) as an oil emulsifier. The prepared vaccines were subjected to physical tests including stability, viscosity, and quality of emulsification completeness. The two vaccines were confirmed to be sterile, stable for 30 days at 37°C, and for 6 months at 4 co. the viscosity was 4 ml/8 second. Tests for safety, immunogenicity, and efficacy (challenge test) as well as cross-protection evidence for the two vaccines was performed in -one day- old broiler chicks. For phase I clinical trial both vaccines were found to be safe, immunogenic, and effective with 80% and 40% protection level for oil emulsion vaccines derived from ND I-2 and ND La-Sota strains respectively. Because of the relatively higher efficacy(80%) obtained from the I-2 strain in the phase I trial, this result validates further investigation for the I-2 strain in the phase II clinical trial. In phase II clinical trial, the protection reached 93% in a group vaccinated only with inactivated ND I-2 vaccine, compared with 100% protection against very virulent ND virus for the group vaccinated simultaneously with life and inactivated ND I-2 vaccines. Independent sample t.test was used to compare the GMT Abs titer for the post-vaccination sera with a statistically insignificant outcome (P>0.05). The results obtained in this study confirmed that the killed ND I-2 vaccine produced locally was safe and efficient, and could be used with high efficiency against the very virulent ND strains, and has the potential to replace the imported ND oil vaccines.

Keywords: Veterinary vaccinology; Poultry vaccines; Newcastle disease vaccines; Inactivated vaccine; I-2 thermostable strain; La-Sota strain; Health information;ELISA

INTRODUCTION

The presence of ND in Sudan was first reported in 1951 [1]. Since then ND has been periodically reported from all regions of Sudan, where heavy losses have been reported [2]. Diagnosis was based on clinical signs and post-mortem lesions as well as the general pictures of the disease. The first attempt to characterize the local isolates was done in 1979 and the isolates were found to be virulent [3].

Khalafalla et al. studied the pathogenic properties of ND virus isolates in Sudan and they found that all isolates were viscerotropic velogenic ND viruses [4]. In 2010 Wegdan et al. performed a phylogenetic analysis for the fusion protein gene of isolates obtained from outbreaks of ND in Sudan, and found that, all contemporary strains isolated between 2003 and 2006 were of genotype 5d [5].

In the commercial sector, losses due to virulent ND were 70%, 98% and 62% in chicks, growers and adults, respectively. In intensive poultry production, inactivated vaccines are usually applied after an initial priming vaccination with a live vaccine. To combat the disease a wide variety and types of vaccines have been developed including live lentogenic, live mesogenic and inactivated vaccines [6].

Despite the use of different vaccines including live and inactivated, (ND) is yet to be controlled in both village chickens and commercial flocks. However, given the upsurge in Newcastle Disease and the importance of inactivated vaccines, many poultry producers have been obliged for a number of years now to turn to the combined use of live and inactivated vaccines in young birds [7]. Inactivated vaccine is more capable of eliciting an immune response in the face of existing maternal immunity [8], and can be used in day-old chicks because the maternal antibodies do not affect the vaccine efficiency [9], as well as inactivated vaccines produce very high level of antibodies against ND virus, and provide good protection against the virulent viruses. Drawing on these facts we In the present study in order to potentiate the chickens immune responses against the Very Virulent (VV) NDV we utilized the thermostable seed virus of I-2 and La-Sota Strain to develop inactivated ND vaccines,(oil vaccines) and investigating these candidate vaccines in day-old

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broiler chicks with live I-2 ND vaccine via eye drop route or without live I-2 vaccine to obtain solid immunity until marketing of broilers at 6 to 7 weeks.

The I-2 virus was originally isolated in Australia with funding from the Australian Centre for International Agricultural Research (ACIAR) [10].

This strain was identified after testing of forty-five isolates of avirulent ND virus. It was chosen for its antigenicity and thermostability. The master seed stock of virus was derived from parent stock that had survived at 56°C for thirty minutes.

The master seed was then tested for safety and freedom from bacterial contamination [11]. The I-2 thermostable ND vaccine is similar to NDV4-HR but is free of commercial ownership, and the master seed virus is available to laboratories in developing countries wishing to produce the vaccine locally [12] for this study, the I-2 master seed virus was first supplied by the Department of Veterinary Pathology of the University of Queensland, Australia, which then handed over by the Department of Veterinary Virology of the Veterinary Research Institute, Sudan.

For our knowledge this is first time to produce an inactivated ND vaccine derived from a thermostable ND strain.

Inactivated vaccines are produced by growing ND virus in eggs, and then treating the infective allantoic fluid with an inactivating agent, such as formalin or betaproiolactone. An adjuvant, such as mineral oils, is usually added to make the inactivated virus more immunogenic.

Also in this study we end up with a simple and more robust vaccination programme which include killed vaccine and intraocular administration of live vaccine at day old, which proved to be more efficient and if it is followed by spray at the end of the second week of chicks, life it will be more immunogenic, and economically beneficiary to the broiler farmers than the conventional vaccination programme [7]. Therefore, it would be of good value if the local authority could recommend this vaccination programme to be launched in the commercial flocks from the very beginning as a hatchery vaccine, Such a synergistic hatchery vaccination program (live and inactivated) has been able to demonstrate its efficacy in building an efficient "wall of protection" in front of different wild pressure levels, from subclinical to clinical infections [7]. Then, the hatchery companies in Sudan would accept it as anew norms, and this will be helpful in our battle against the disease.

MATERIALS AND METHODS

Pre-clinical phase

Preparation of the I-2 Master Seed Lot (MSL): One out of two ampoules containing the master seeds virus was removed from -70°C storage and thawed at room temperature. Sound 10-day old-embryonated chicken eggs were used to prepare the Master Seed-Lot (MSL) from which the Working Seed Lot (WSL) was produced.

The embryonated chicken eggs were candled and cleaned with 70% alcohol. Holes were drilled on the eggs shell, and the allantoic cavities of 15 embryonated chicken eggs were aseptically inoculated with 0.2 ml of undiluted liquid allantoic fluid of the master seeds.

Five embryonated chicken eggs were inoculated with 0.2 ml of antibiotics solution and kept as a negative control group. The inoculation sites were then sealed with paraffin wax and the eggs incubated at 38°C for 120 hours. The eggs were candled daily any one with dead embryo was discarded. The infected allantoic fluid was harvested after the incubation period and chilled for 2 hours at 4°C, and then the allantoic fluid was collected, stored in 7 ml aliquots, and stored at -20°C.

Preparation of the I-2 Working Seed Lot (WSL): Using aseptic technique one aliquot of I-2 ND master seed bank prepared previously was thawed at room temperature. A 5 ml of the thawed I-2 master seed was diluted in 20 ml Normal Saline (NS) with antibiotic mixture. Seventy-10-day-old embryonated chicken eggs were inoculated with 0.2 ml of diluted I-2 master seed into the allantoic fluid using aseptic technique. A15 embryonated chicken eggs inoculated with 0.2 ml Normal Saline (NS) were kept as control group.

Inoculated eggs were incubated at 38°C for 120 hours, next day all eggs were candled for the evidence of nonspecific death. After 120 hours incubation, 400 ml of infected allantoic fluid was harvested, and immediately tested using the rapid haemagglutination test to determine the presence of the ND virus [13]. The harvested allantoic fluid centrifuged in a cold centrifuge at 10,000 RPM for 5 minutes, then the pooled allantoic fluid dispensed into aliquots of 1.5 ml under laminar flow system, the ampoules were lypholised, sealed and stored at

-20°C. The working seed bank was tested for bacterial, fungal, and mycoplasma contaminations [14].

Characteristics of the I-2 working seed virus: Identity test-(HI) test

Reference serum known to contain antibodies to ND virus was used to confirm the presence of Newcastle disease virus in three ampoules of the working seed virus.

Briefly a 25 µl of sterile Normal Saline (NS) was dispensed to all wells of a 96 microtiter plate, then 25 µl of the reconstituted lyophilized ND hyper immune serum were dispensed in to 1st column, and then two fold dilutions carried out across the plate. A 25 µl of the 4 HA unit (HAU) of I-2 virus suspension was added to each well of the 96 wells plate, then incubated at 37°C for 30 minutes, the test contains +ve control serum, then 25 µl of 1% chicken RBCs had been added and the plate incubated at room temperature (RT) for 30 minutes [14].

Assessment of I-2 virulence by Intracerebral Pathogenicity Index (ICPI)

Two ampoules of lyophilized I-2 working seed vaccine were reconstituted in 1 ml sterile NS with no antibiotics for each. Using aseptic technique the reconstituted vaccine was pooled and diluted 1/10.

A aseptically 50 µl of the diluted vaccine was injected intracerebrally into each of ten chicks of one- day old- chicks hatched from healthy flock. Further 5 one-day old- chicks were inoculated with 50 ul of the reconstituting diluent and observed as a control group. The birds were examined every 24 hours for 8 days, for each observation, the vaccine treated birds were scored 0 if they were normal, 1 if sick, and 2 if they died.

The final ICPI was calculated as mean score per bird per observation over the 8 days of observations.

Sterility testing

Tests for absence of bacteria, fungi, and mycoplasma, sterility tests have been done for the Master Seed Lot (MSL) and the Working

Seed Lot (WSL), as well as tests for safety [14].

The MSL and WSL used in this vaccine have been thoroughly investigated for its sterility, safety, and efficacy issues according to OIE terrestrial manual [14].

The vaccine batch

Quality of embryonated chicken eggs: This vaccine was produced using chicken embryonated eggs derived from a healthy flock and vaccinated against the major poultry disease [15].

Strains of vaccines production: For phase I clinical trial. The vaccines were prepared by using two strains of avirulent ND virus of I-2 strain (Australian strain), and La-Sota strain. The Intracerebral Pathogenicity Index (ICPI) of the I-2 strain is 0.125. Allantoic fluids of I-2 and La-Sota strains containing 109.1 EID50/ml and 108.5 EID50 /ml respectively were used as stock virus for the vaccine formulation.

Inoculation of vaccine strains: A three vials of each lyophilized I-2 and LaSota strains were obtained from their Working Seed Lots (WSL) and diluted in sterile normal saline i.e. 0.2 ml containing 103 EID50 was inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs, and then incubated at 37°C for 120 hours. Dead embryos were discarded within 24 hours.

Harvesting: At the end of the incubation period the infected eggs were chilled at 4°C over night before being harvested. The Allantoic Amniotic Fluid (AAF) was aspirated using 10 ml pipette, then the harvested AAF was centrifuged using cold centrifuge at 1000 RPM for 7 minutes, pooled and stored at 4°C.

Test of the live virus content:

The HA test: This test was conducted according to OIE terrestrial manual [14].

Virus titration: The virus content of the WSL had been defined by making ten-fold dilution and inoculated in to 10-day-old embryonated chicken eggs. Titer estimated as embryo infectious dose of fifty (EID50).

Inactivation of the viruses: The two vaccines strains of I-2 and la-Sota were inactivated by treatment with 0.05% laboratory grade formaldehyde; this was according to Wisanu et al. and OIE Manual [16]. A 75 µl and 50 µl of absolute laboratory grade formaldehyde were added to 150 ml, and 100 ml AAF of I-2 and La-Sota strains respectively, and then the two bottles were shaken well, and incubated at 37°C for 16 hours. After incubation the bottles were stored at 4°C. Later on for the product escalation, a larger amount of 600 ml AAF of I-2 strain has been inactivated by adding 300 ul of the concentrated formaldehyde using the same procedure.

Control tests on raw vaccines:

Test for complete inactivation: The test has been performed on the formaldehyde treated allantoic fluid, immediately after the incubation for inactivation has been completed, The allantoic fluids of the two strains of I-2 and La-Sota were inoculated into 10-day old- embryonated chickens eggs and incubated at 37°C for 120 h [15].

Sterility test for raw and final product

Sterility test for raw and final product: After verifying completion of the inactivation, the raw allantoic fluids and final products were subjected to a simple bacteriological sterility test. 10 vials of thioglycolate broth media were each inoculated with 0.2 ml inactivated allantoic fluid and oily vaccines respectively and incubated aerobically at 37°C and at room temperature for 7 days.

Formulation of the water in oil (W/O) emulsion vaccines:

Phase I: The two formulations of the water in oil (W/O) emulsion vaccines were prepared according to Wisanu et al. [16]. The aqueous phase of the two emulsions consisted of the allantoic fluids according to the type of strain. For each formulation the aqueous phase composes of 9.6 ml allantoic fluid, and 0.4ml tween 80 mixed in sterile plastic containers and stirred with magnetic stirrer adjusted at low speed pace for 2 minutes.

The oil phases were purchased ready as incomplete Freund's adjuvant (sterile, oil-arlacel mixture) containing 9 ml of paraffin oil plus 1ml manidmonoleate (span 80) as an oil phase emulsifier.

For each preparation the aqueous phase was added drop wise to the oil phase while the oil phase was constantly stirred at low speed in sterile container.

After the addition was completed, the mixture was emulsified by mixing with disposable 10 ml syringe with 0.5 mm needle gauge. This process was repeated until all the aqueous phase has been incorporated into the oil phase.

On standing for a few moments no accumulation of the water phase has been seen at the bottom of the bottle.

Phase II: For phase II clinical trial, about 600 ml of W/O emulsion was prepared using a pharmaceutical grade, white mineral oil (paraffin oil) as follow: the aqueous phase was made up by adding 12 ml of tween 80 to the 288 ml allantoic fluid 1:24 respectively, then gently stirred for 30 minutes for proper mixing.

Equal volume of the oil phase was prepared by adding 30 ml manidmonoleate (span 80) to the 270 ml of the purified paraffin oil 1:9 ratio respectively, then gently mixed for 45 minutes using magnetic stirrer. Using the same procedure 300 ml aqueous phase was added drop wise to the 300 ml oil phase i.e. 1:1 ratio and thoroughly mixed by stirring for 20 minutes, then, using the rotary machine method, the product emulsified by using the machine Silverson emulsifier with head suitable for larger volumes, this process last for 20 minutes then a very homogenous product had been obtained [15].

The physical characteristics of the emulsions:

Testing the water in oil (W/O) emulsion: To determine the vaccine integrity, the finished W/O emulsion was tested by allowing a few drops to fall on the surface of tap water in a Petri dish.

Stability test: The stability of this oily preparation was tested by incubating and observing the vaccines at 4°C for 6 months in tightly screwed tubes.

Viscosity test: Relative viscosity was determined as a flow time at 24°C by discharging of 0.4 ml of the emulsified vaccine from a vertically mounted 1ml pipette, emulsion was drawn up to 1 ml mark. The time for discharging 0.4 ml was measured in seconds.

Clinical phase

Randomized-controlled trials for the two formulated inactivated Newcastle disease vaccines of I-2 and LaSota lentogenic strains in day-old-broiler chicks:

Study design: This phase I, and phase II double blinded randomized controlled trials were conducted in 80 and 120 healthy day-old

Commercial broiler chicks respectively at the Central Veterinary Research Laboratory, Department of Viral Vaccine Production.

Experimental design: This study was parallel randomized controlled design.

Sample size: The sample size of this study was determined according to the method of manufacture, and the requirement for authorization stated by OIE terrestrial manual 2012. To prove that the ND formulated inactivated vaccines were safe and immunogenic, an overall sample size of 80, 120 chicks was generally considered appropriate for safety, and efficacy evaluation in phase I and phase II clinical trials respectively.

Chick's inclusion criteria: Healthy day-old chicks were included regardless of their maternal antibodies level. Chicks were excluded if they were layer breed or more than 48 hours age or derived from flocks that have had any clinical signs attributed to ND or ND like diseases.

The vaccines: The formulated ND inactivated vaccines prepared in this study were from ND I-2 and La-Sota lentogenic strains, and according to the manual of production of ND vaccine [15].

Intervention: Tests for safety, immunogenicity, and efficacy were performed in host animal and had been done for the two vaccine interventions used in this study. Each dose for immunogenicity and safety tests was 0.2 ml or 0.4 ml respectively.

Safety groups: Twenty and thirty Chicks in phase I and phase II trials respectively received the double recommended dose of 0.4ml using subcutaneous route (s/c).

Efficacy groups

Phase I: The chicks in group I, and group II, designated the efficacy groups, received inactivated ND vaccines derived from I-2, and La-Sota lentogenic strains. Chicks in group I receive 0.2 ml by s/c route at the nap of the chick's neck. Chicks in group II received also 0.2 ml via the same route of administration. Control group of 10 chicks designed group V received no treatment, and remain as non-inoculated control.

Phase II: One hundred and twenty-one day- old- chicks obtained from the same source of the phase I, were randomly assigned to four groups as follow, 30 chicks as a safety group, 40 chicks as efficacy group, 20 chicks as enhanced efficacy group, and 30 chicks as non-inoculated control group, named group S, E1, E2, and C respectively.

Using the one ml Syringe group E1 received 0.2 ml of the candidate vaccine which derived from 1-2 strain subcutaneously and in the nap of chick's neck (field dose), while, chicks in group E2 vaccinated simultaneously at day old with live 1-2 vaccine via intraocular route and the candidate 1-2 inactivated ND vaccine via the subcutaneous route, chicks in group C kept as a non-inoculated control group.

Outcomes

Safety: Occurrence of local or systematic adverse events or tissue reaction was observed for 21 days post inoculation. Chicks which inoculated with double recommended dose observed for any local or general adverse events, the test was done according to the OIE terrestrial manual [14].

Immunogenicity: Chicks were bled 3 times, first at day-old- to evaluate the maternal antibodies level, and after 21, and 45 days post vaccination in phase I trial and at 18, 30 days post vaccination for phase II trial to evaluate the vaccines derived antibodies. Accordingly the antibodies titers were measured on day 1, 21, and 45 post vaccinations for phase I, and on day 1, 18 and day 30 for phase II clinical trial.

Chicks were inoculated with dose of 0.2 ml s/c, and then the seroconversion levels were evaluated using HI and ELISA tests [14].

Cross protection evidence: Virus antigens prepared from live La-Sota, and 1-2 strains were used to test the Abs derived from 1-2, and La-Sota inactivated vaccines respectively in both immunogenicity and safety groups using HI test.

Efficacy test (Challenge test)

Phase I: Three weeks post vaccination (21 days old) 5 chicks from group I and II (efficacy group), and 4 chicks from unvaccinated control group were selected randomly and challenged intraocularly with 106 EID50 of vv strain of (Shdi /12) according to the OIE terrestrial manual [14].

Morbidity and mortality rates were estimated after 10 days post challenge according to Wisana et al. [16].

Phase II: 30 days post vaccination, 15 chicks from group E1, and 8 chicks from group E2, and 12 chicks from group C were randomly selected and challenged intraocularly with 107 ELD50 of vv (Shdi /12) strain [14].

Randomisation

Sequence generation:

Phase I: 80 one-day-old chicks were randomly allocated to 5 groups.

Phase II: 120 one day old chicks were randomly assigned to 4 groups.

Phase I immunogenicity group:

Group I inoculated with 0.2 ml of I-2 inactivated vaccine (n=20)

Group II, inoculated with 0.2 ml La-Sota inactivated vaccine (n=20)

Safety group

Group (III) 0.4 ml I-2 inactivated vaccine (n=15) was used

Group (IV) 0.4 ml La-Sota inactivated vaccine (n=15) was used

Control group

Group (V) unvaccinated chicks served as control (n=10)

The sequence generation for the randomization was performed manually.

Phase II Immunogenicity group

Group EI: received 0.2 ml of I-2 inactivated vaccine (n=40)

Group EII: received 0.2 ml of I-2 inactivated vaccine plus 40 ul of live I-2 vaccine (n=20)

Group S: received 0.4 ml of I-2 inactivated vaccine (n=30)

Group C: neither vaccinated nor inoculated (n=30)

Allocation concealment and implementation

The intervention and control groups were assigned by research assistant who completely oblivious about the previous sequence generation to avoid the selection bias.

Blinding

This study is double blinded Randomized Control Trial (RCT), the participant were unaware about either they received the product or the placebo because of their animal nature.

Outcome evaluators and follow-up were masked about the group's allocation and their interventions.

Objectives

Phase I: This study designed to demonstrate the levels of safety and immunogenicity of the two prepared inactivated ND vaccines administered S/C to 1-day-old commercial broiler chicks at the nap of the neck as well as the survival rate after the challenge with vv ND strain.

Phase II: This trial conducted to confirm the results obtained from phase I clinical trial about safety and efficacy issues. Moreover, in this study only I-2 inactivated vaccine which needed to be further investigated was produced in a larger scale for safety and efficacy concerns.

Statistical analysis

The data collected from various groups were compared by independent sample t. test at 5% probability level.

RESULTS

Pre-clinical testing

Characteristics of the 1-2 working seed virus: Identity test (HI) test: The presence of Newcastle disease virus has been confirmed in a reconstituted sample of the Working Seed Lot (Figure 1).

Safety and sterility testing: The Master Seed Lot (MSL) and the Working Seed Lot (WSL) demonstrated freedom from microbial contamination including bacterial, fungal, and mycoplasma contaminations.

The 1-2 strain also proved to be safe, and effective ICPI=0.125 (Table 1).

The vaccine batch

Test of live virus content:

Virus titration and HA test: The virus content of I-2 and La-Sota strains was more than 1024 HAU/25ul for I-2 and La-Sota strains respectively, while virus titer was 9.1 EID50 (Figure 2 and Table 1).

Control tests on raw vaccines

Test for complete inactivation: The Rapid HA test revealed complete inactivation for the formaldehyde treated ND virus (Figures 3 and 4).

Sterility test for raw and final product: After 7 day's observation there was no bacterial growth observed in any of the thyoglycolate broth media inoculated with the inactivated allantoic fluid or the oil vaccines.

The physical characteristics of the emulsions

Testing the water in oil (w/o) emulsion: The vaccine's drops remained discrete on the surface of tape water without any dispersion (Figures 5 and 6).

Stability and viscosity tests: The vaccine preparations were confirmed to be stable for 30 days at 37°C and for 6 months at 4co. The viscosity was 4 ml/8 seconds.

Clinical phase

Outcomes:

Safety test: The two preparations of the I-2 and La-Sota inactivated vaccines were safe with no adverse reactions in 1-day-old broiler chicks when administered by S/C route.

Immunogenicity test:

Phase I: The mean Haemagglutination Inhibition (HI) Abs titers for group I after 21 and 45 days post vaccination were 128 HIU, and 256 HIU respectively, also the mean HI Abs titers for group II after 21 and 45 days were 128 and 256 HIU respectively. The mean HI Abs titers for the group V-control group - after 21 and 45days were 16, and 0 HIU respectively (Table 2 and Figure 8).

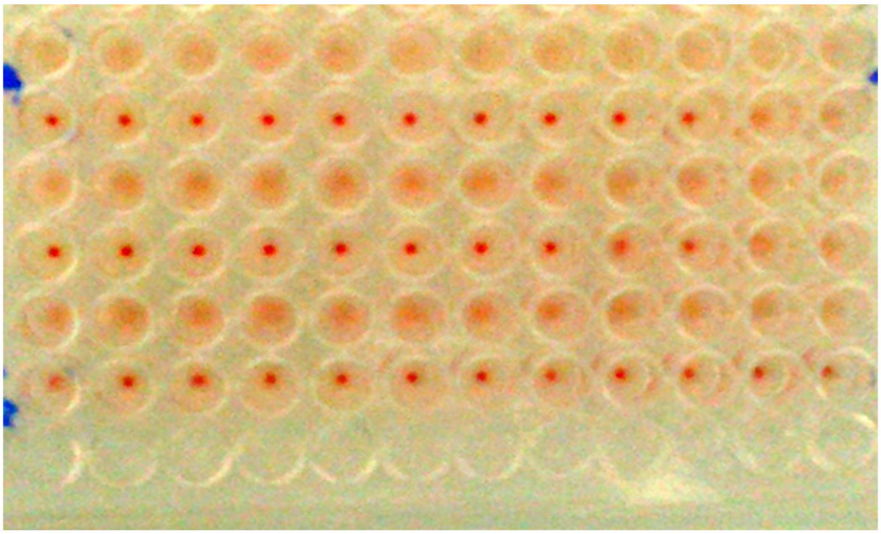

Figure 1: (Identity test) The Haemagglutination Inhibition test (HI) carried out for the Working Seed Lots (WSL) using reference hyperimmune serum (diluted more than 1024 times) and 8 Haemagglutination Unit (HAU) to test the identity of both I-2 and La-Sota strains. the top row shows the result of negative control serum while the bottom row shows the result of positive control serum, the rows in the middle show the result of the tested WSL of I-2 and La-sota strains respectively.

Table 1: This table compare the results of *in vitro* potency of I-2 working seed bank, and vvSHDI strain using the (HA) test and the 50% chicken's embryo infectivity titer.

ND virus strain	ICPI	Titer in EID50/ELD50	HA titer	Virulence
I-2	0.125	9.1 EID50	>512HAU/25 ul	Avirulent
SHDI	1.9	7.9 ELD50	>512HAU/25 ul	Very virulent

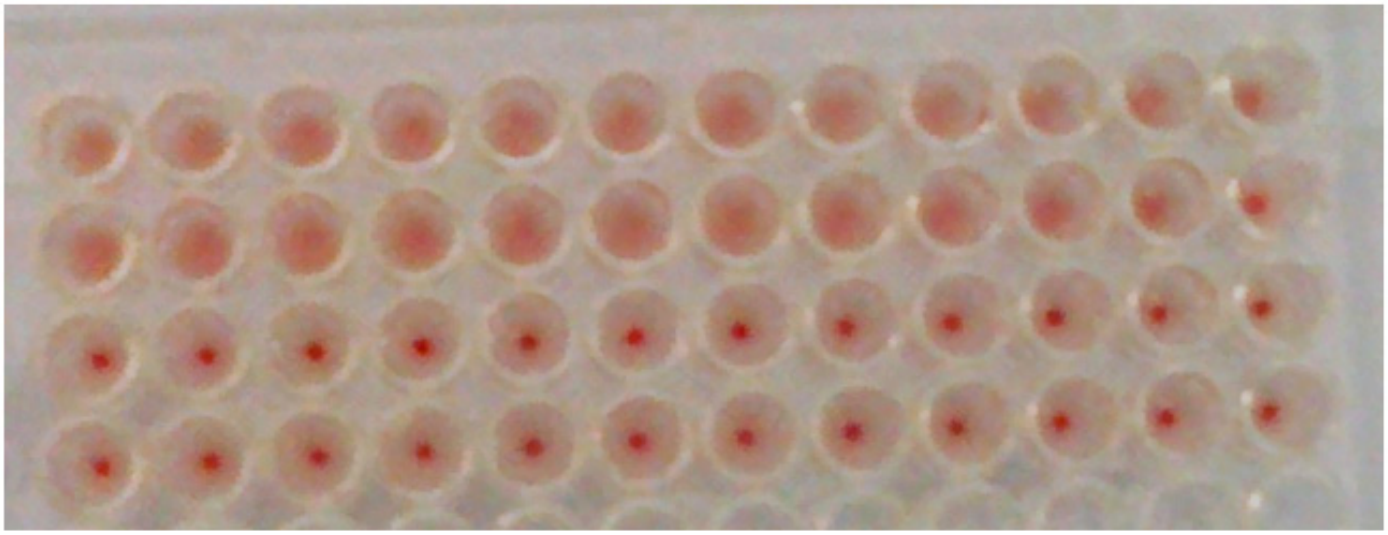

Figure 2: The first and secound rows show the virus content of I-2 and La-Sota strains using plate Haemagglutination HA test. HA titers>1024 HAU/25 ul while the negative control (the two bottom rows) show the button like precipitation of RBCs.

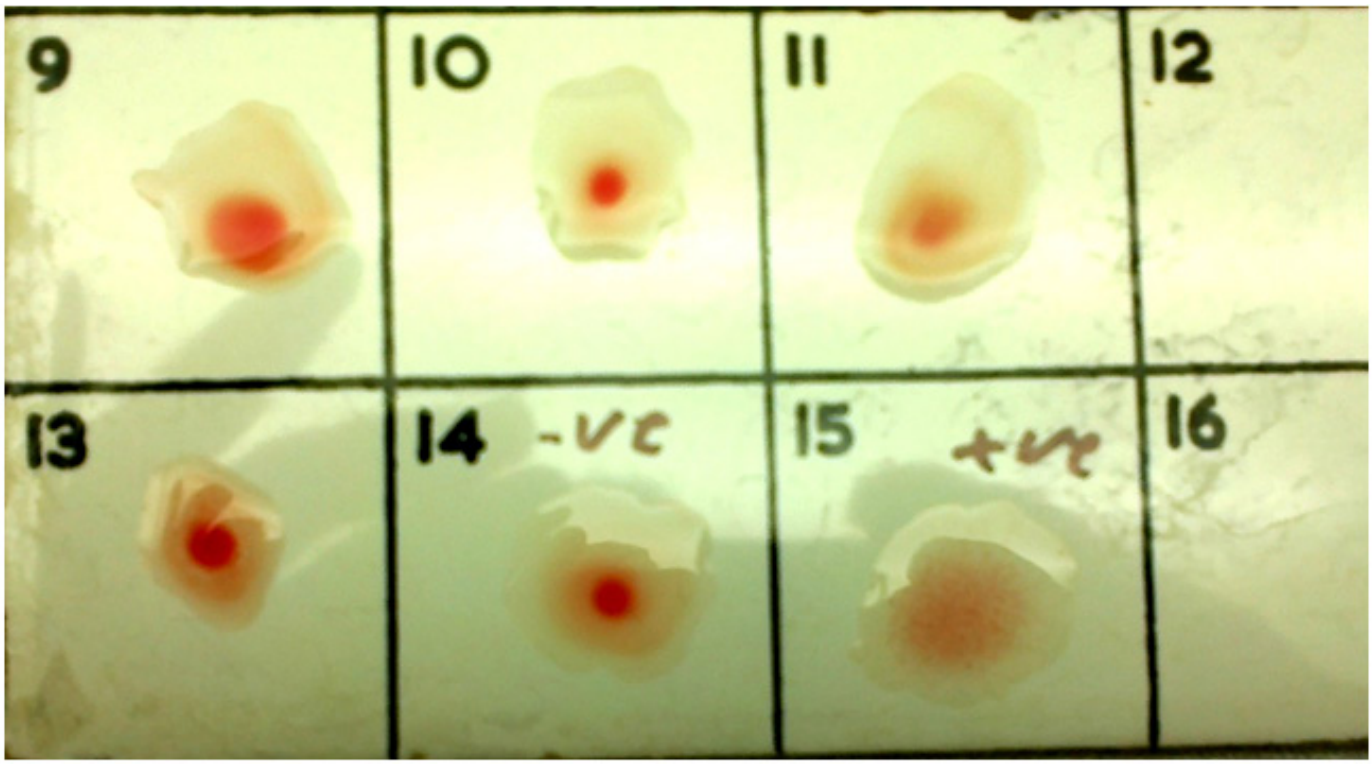

Figure 3: The Rapid HA test revealed complete inactivation for the formaldehyde treated ND virus, the positive and negative allantoic fluids were also respectively tested for the presence or absence of RBCs and the HA evidences.

Figure 4: TThe prepared W/O emulsions of I-2 and La-Sota strains.

Figure 5: The I-2 candidate vaccine formulated as stable water -in oil-emulsion (W/O) using a pharmaceutical grade of light liquid paraffin oil.

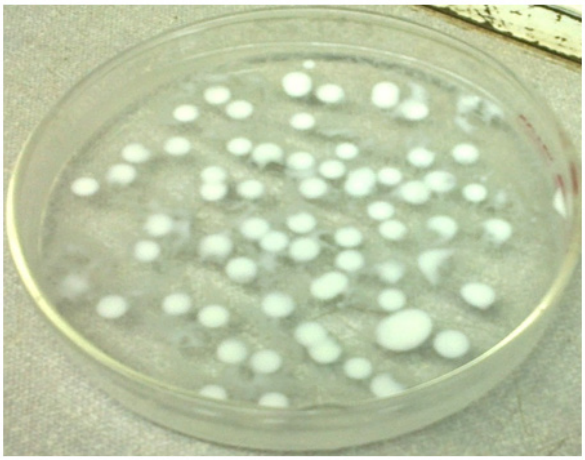

Figure 6: The candidate inactivated ND vaccine tested for complete water in oil emulsion, the vaccine drops remain discrete without any dispersion on the surface of the tap water.

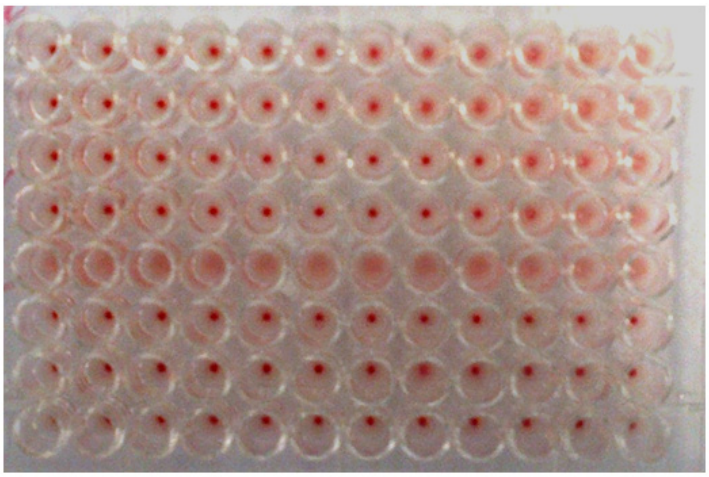

Figure 7: Shows the cross protection evidence between I-2 and La-Sota strains, the first two rows represent HI when La-sota strain used as antigen for ABS derived from I-2 vaccination, while the 3d and 4th rows correspond to I-2 strain used as antigen for Abs derived from La-Sota strain, the 5th row stand for +ve control, while the bottom three rows represent the -ve control.

Table 2: Comparison of Abs immunoresponses provided by vaccination of one day-old-broiler chicks vaccinated by inactivated Newcastle disease vaccines derived from I-2 and LaSota strains as measured by ELISA and HI tests.

			21 days post vaccination			45 days post vaccination		
				ELISA			ELISA	
Group No	ND strain	Group name	HI Titer	G.M.T.	C.V	HI log_2	G.M.T.	C.V.
I	I-2	Immunogenicity	128	1380	75	256	2565	53.13
II	La-Sota	Immunogenicity	128	1415	82	256	2929	58.56
III	I-2	Safety	-	-	-	256	-	-
IV	LaSota	Safety	-	-	-	128	-	-
V	N.A	Control 21days	16	59	156	0	0	173.2
VI	N.A	Maternal immunity	4096	6469	43		1214	
VII	Shdi/12	Challenge		-	-	2084		29.25

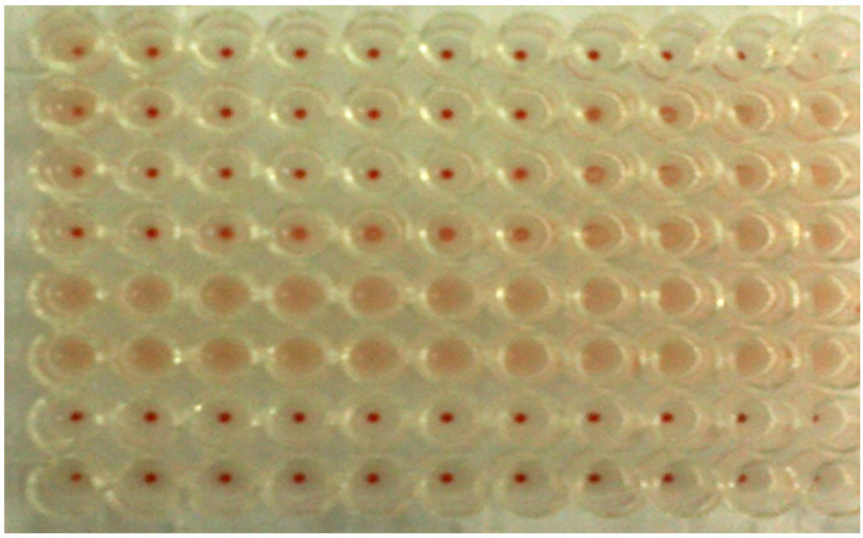

Figure 8: Shoes HI test of the pooled serum samples derived of I-2 strain (2d row) and La-Sota strain(3d row) the 4th row show the unvaccinated control, all were in day old broiler chicks and estimated 21days post vaccination. The 1st row shows the day old maternal immunity while the 5th and 6th rows show the negative control serum and the 7th and 8th show the +ve control serum.

Elisa mean Abs titers after 21 days post vaccination were 1380, 1415, and 59 for group I, II, and C respectively, while the maternal immunity level was 6469 (Table 2 and Figures 9 and 10).

The mean Abs levels for ELISA after 45 days were 2565, 2929, and 0 for group I, II, and C respectively (Table 2 and Figures 11-12).

Phase II: The mean (HI) Abs titers for group EI after 18 and 30 days post vaccination were 35.11 HIU, and 28.3HIU respectively, also the mean HI Abs titers for group EII after 18 and 30 days were 28.6 and 12.6 HIU respectively. The mean HI Abs titers for the group C- control group - after 18 and 30 days were 29.1, and 6.2 HIU respectively (Table 5 and Figures 15-19)

Elisa mean Abs titers after 18 days post vaccination were 5688, 4683, and 6360 for group EI, EII, and C respectively, while the maternal immunity level was 20003 (Table 5 and Figure 20), while the mean Abs levels for ELISA after 30 days were 270, 52, and 73 for group EI, EII, and C respectively (Table 5 and Figures 21 and 22).

Cross protection evidence:

High level of cross protection was observed. Abs derived from chicks immunized with I-2 inactivated vaccine neutralized La-Sota virus, and vice versa (Figure 7).

Efficacy (challenge test):

Phase I: After 4 days post challenge 1 out of 5 chicks in group I (1-2 inactivated vaccine), and 2 chicks out of 5 in group II (La-Sota inactivated vaccine) developed ND clinical signs of depression, ruffled feather, prostration and sleepiness, but no chicks died. In the control group 1 out of the 4 chicks was found dead.

On the seventh day post challenge, two out of the remaining 3 chicks of the control group were found dead with excessive salivation. After the first week of infection group I and group II of 1-2, and La-Sota chicks demonstrated 80% protection for each group with 1 chick out of 5 died from each group, later on the 10th day post challenge all control group of unvaccinated chicks died. At the end of observation period 1-2 inactivated vaccine of group I demonstrated 80% protection. The group of inactivated La-Sota vaccine demonstrated only 40% protection after 10 days post challenge (Table 3 and Figure 13).

Table 3: Efficacy tests.

			12 days post challenge		
			Efficacy		
Group No.	ND strain	Group affiliation	Mortality rate	Survival rate	Efficacy %
I	I-2	Efficacy	1/5	4/5	80
II	La-Sota	Efficacy	3/5	2/5	40
V	N.A	Unvaccinated control	4/4	0/4	0

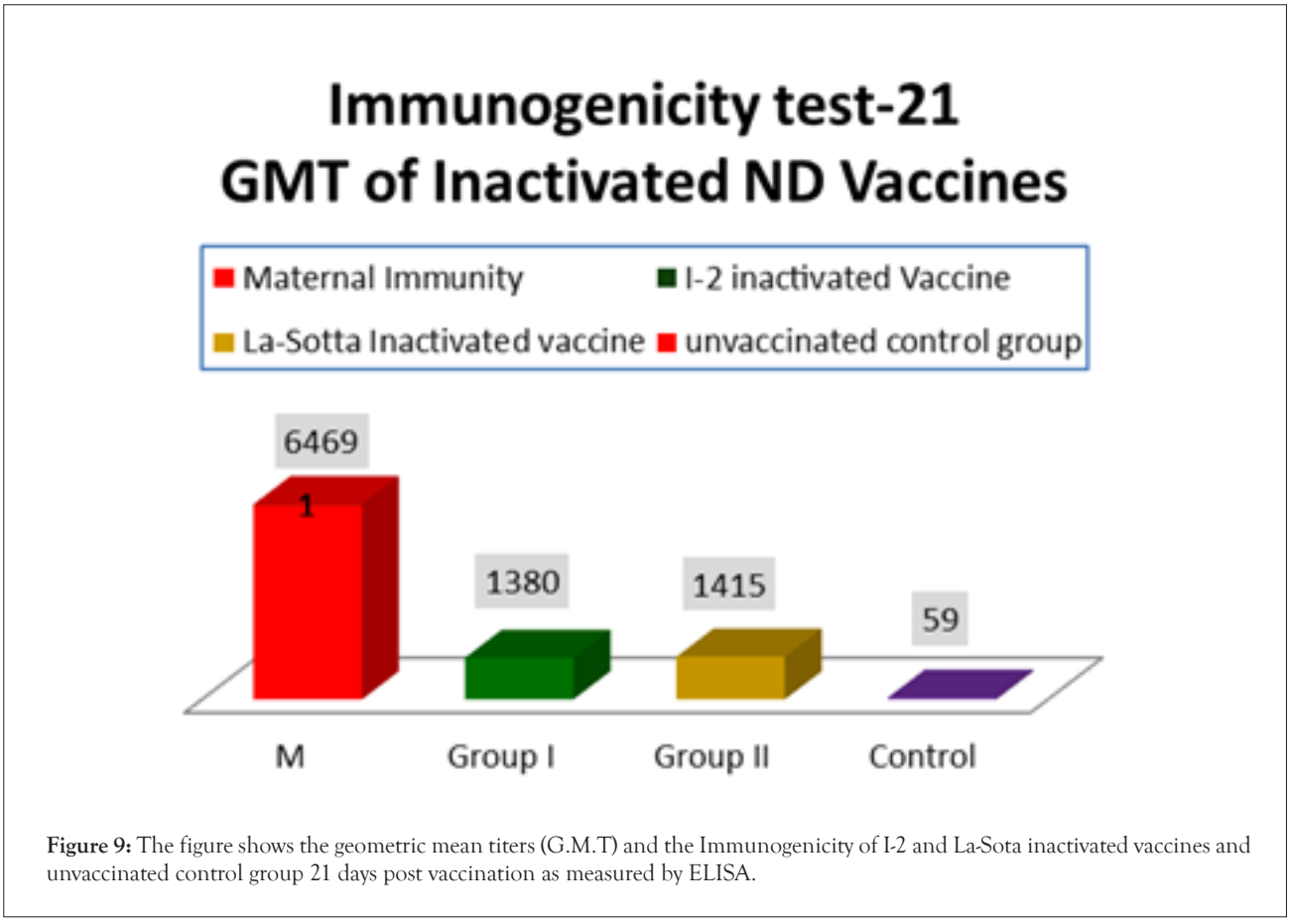

Figure 9: The figure shows the geometric mean titers (G.M.T) and the Immunogenicity of I-2 and La-Sota inactivated vaccines and unvaccinated control group 21 days post vaccination as measured by ELISA.

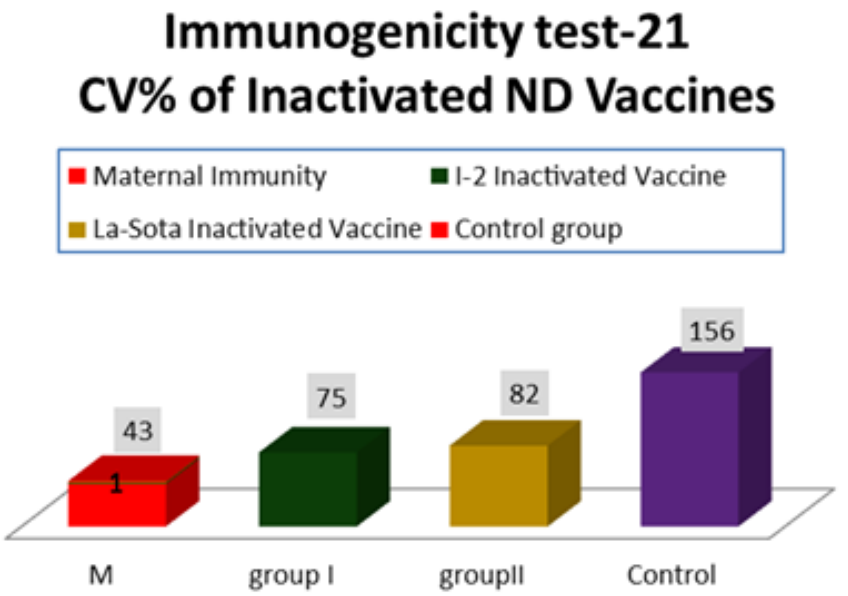

Figure 10: This figure shows the CO-efficient variation percentages (CVs %) of the immune responses developed by I-2 and La-Sota inactivated vaccines as well as the control group 21 days post vaccination as measured by ELISA.

Table 4: Descriptive statistics and result of independent sample t-test for I-2, La-Sota and control group (maternal immunity) 21 days post vaccination.

Vaccine	N	Mean	Comparison			P
I-2 inactivated vaccine	40	1380	I-2 inactivated vaccine	vs.	Maternal immunity (after 21 days)	0.075
La-Sota inactivated vaccine	40	1415	La-Sota inactivated vaccine	vs.	Maternal immunity (after 21 days)	0.077
Maternal immunity after 21 days (control group)	30	59	La-Sota inactivated vaccine	vs.	I-2 inactivated vaccine	0.848

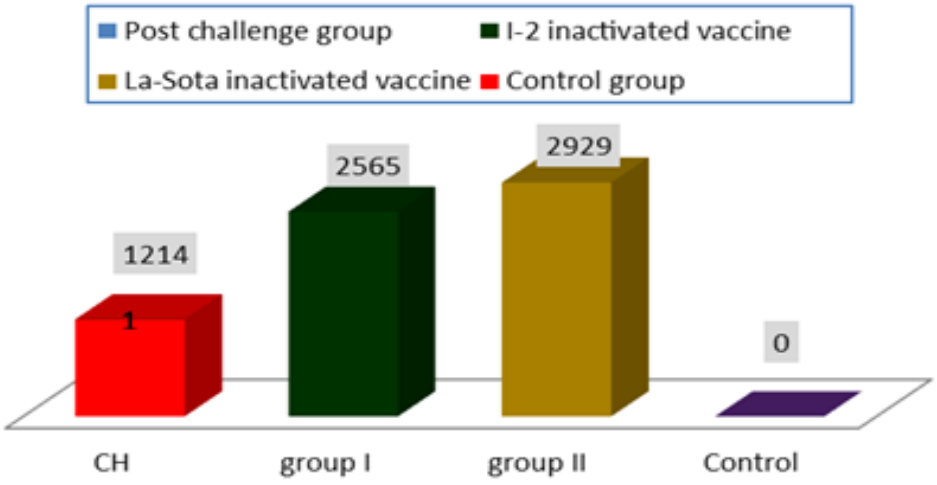

Figure 11: The Geometric Mean Titer (GMT) of the two efficacy group I-2 and La-Sota Inactivated and the control group 45 days post vaccination as measured by ELISA.

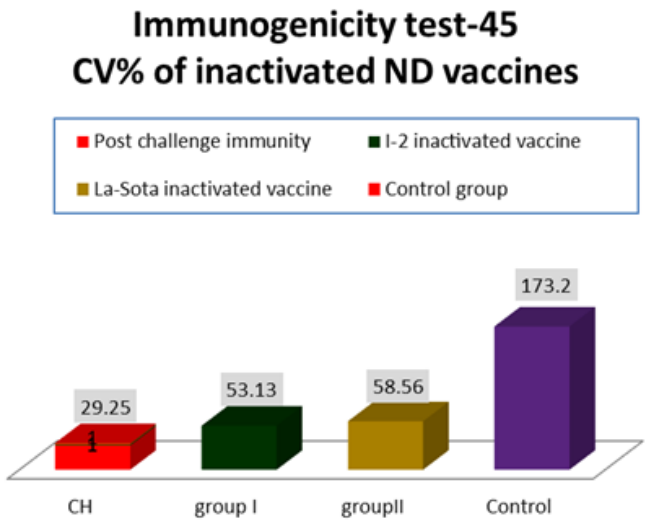

Figure 12: The CO-efficient variation percentages (CVs %) of the two efficacy group of I-2 and La-Sota Inactivated vaccines and the control group 45 days post vaccination as measured by (ELISA).

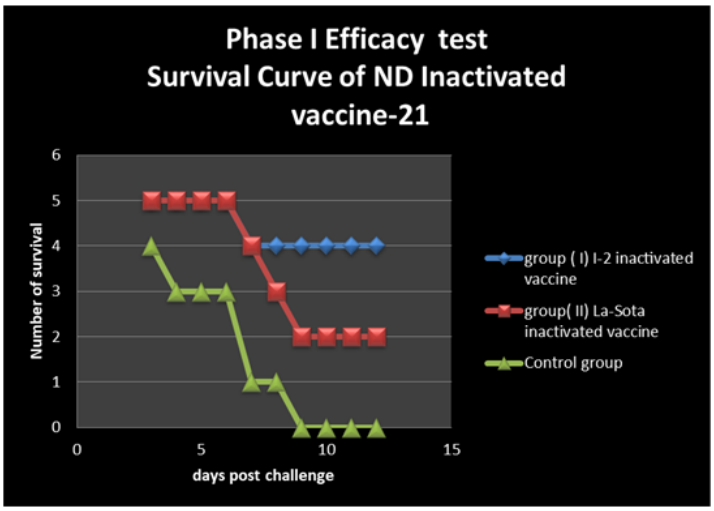

Figure 13: Survival curve of broiler chicken vaccinated at day old by I-2 and La-Sota inactivated vaccines and challenged at 21 days old by (vvNDV). Efficacy estimated 12 days post challenge.

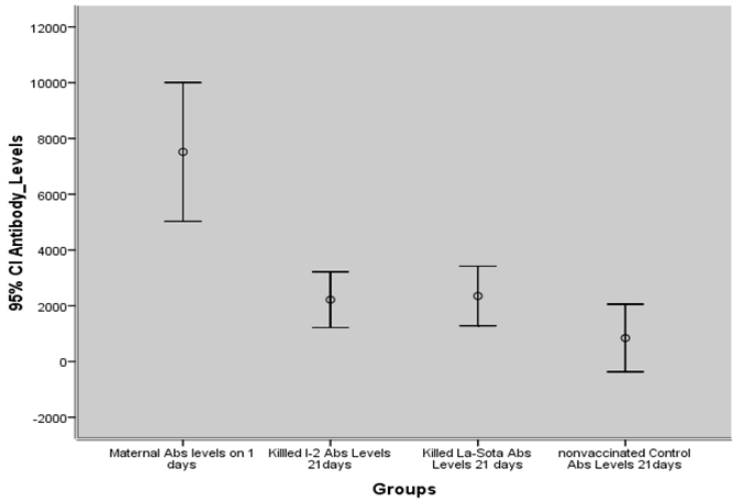

Figure 14: Graphical representation of the means and their coefficient variations of the I-2 and La-Sota vaccines preparations 21 days post vaccination displayed in bar charts diagram.

Table 5: The Abs levels of maternal immunity at day 1, 18, and, 30 days compared with the candidate vaccine Abs levels as measured by (ELISA) and (HI) tests at day 18 and 30 post vaccination.

	18 days post vaccination				30 days post vaccination			
	HI test		ELISA test		HI test		ELISA test	
Efficacy group	HIU	stDev	G.M.T	C.V%	HIU	stDev	G.M.T	C.V%
Inactivated vaccine (EI)	35.11	39.4	5688	20.94	28.3	14.5	270	46.95
Live+Inactivated vaccine (EII)	28.6	66	4683	29.56	12.6	4.3	52	63.44
Control group ©	29.1	32.2	6360	31.48	6.2	2	73	93.84
Maternal immunity					870.86	1381.58	20003	13.43

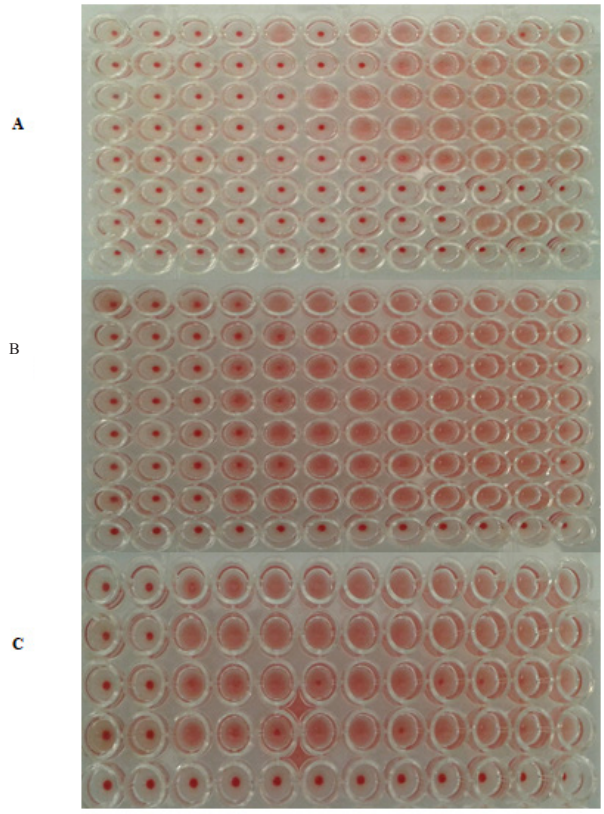

Figure 15: Shows the waning of maternal Abs over time in, day 1 , day 18 and day 30 A,B,C respectively, the mean Abs titers are 870.86, 29.1, and 6.2 HIU respectively.

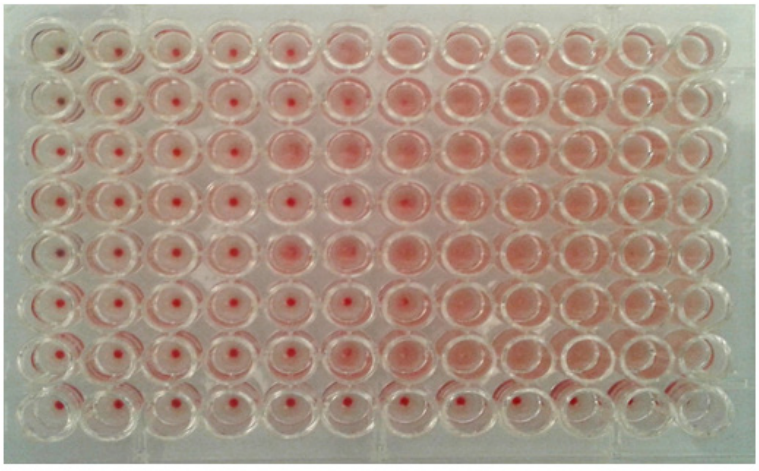

Figure 16: This figure shows the efficacy of the candidate vaccine (I-2) when day old broiler chicks vaccinated only by the recommended field dose (EI) via the S/C route, it demonstrates the Abs levels as measured by HI test 18 days post vaccination. The mean Abs level is 35.11 HIU.

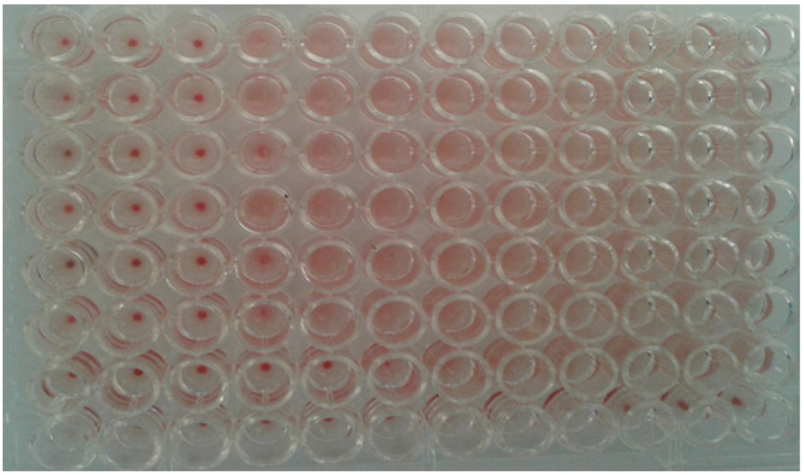

Figure 17: This figure displays the efficacy of both candidate vaccine and live I-2 vaccine when day old broiler chicks vaccinated simultaneously with the recommended field dose of live and candidate vaccines(EII) via eye drop and the S/C routes respectively, it demonstrates the Abs levels as measured by HI test 18 days post vaccination. The mean Abs level is 28.6 HIU.

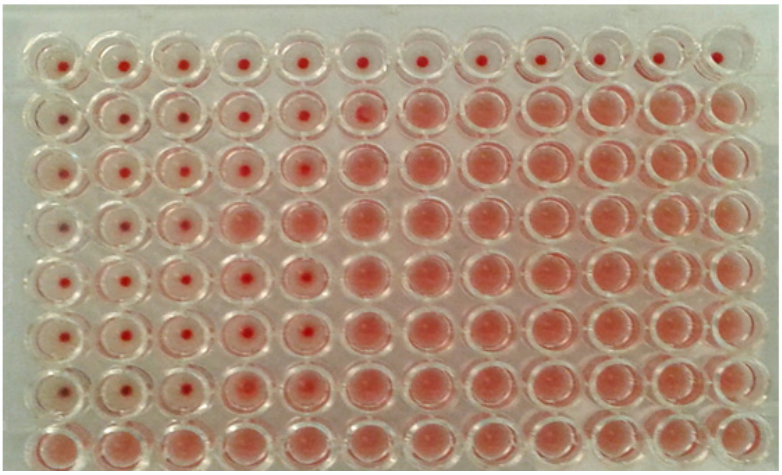

Figure 18: This figure shows the efficacy of the candidate vaccine when day old broiler chicks vaccinated only by (killed vaccine) using the recommended field dose (EI) via the S/C route, it demonstrates the Abs levels as measured by HI test 30 days post vaccination. The mean Abs level is 28.3 HIU.

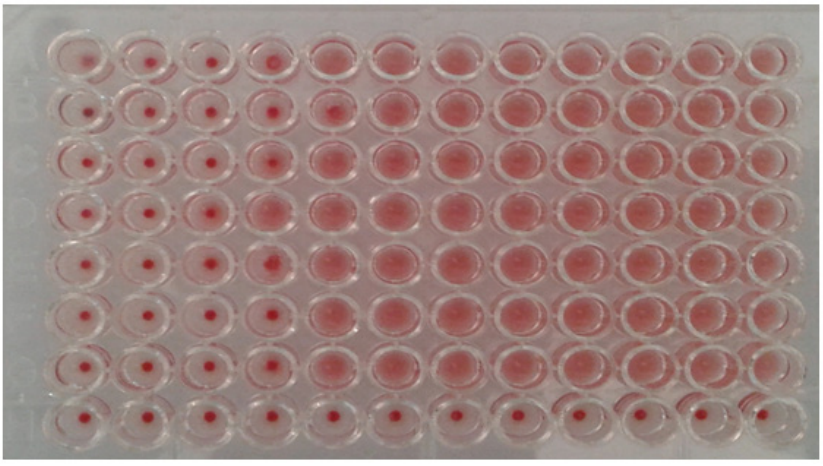

Figure 19: This figure presents the efficacy of both candidate vaccine and live I-2 vaccine when day old broiler chicks vaccinated simultaneously with the recommended field dose of live and inactivated ND vaccines (EI) via eye drop and the S/C route respectively, it demonstrates the Abs levels as measured by HI test 30 days post vaccination. The mean Abs level is 12.6 HIU.

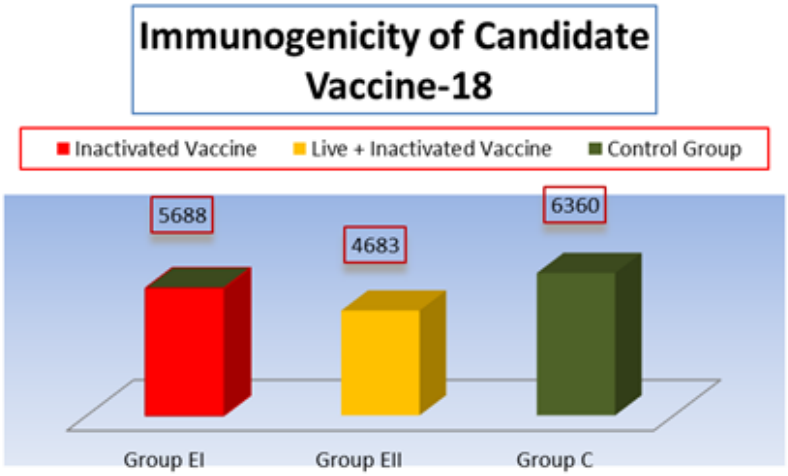

Figure 20: This figure shows the immune responses of the candidate vaccine in day old broiler chicks when administered alone group EI, or concurrently with live I-2 as in group EII and 18 days post vaccination as measured by ELISA.

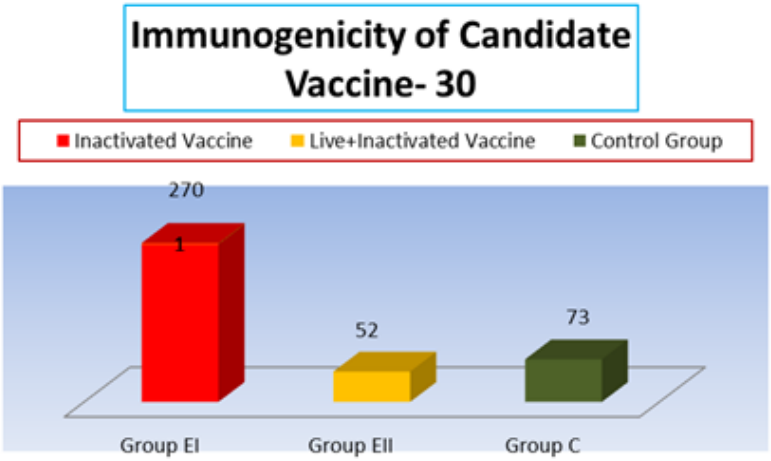

Figure 21: This figure shows the immune responses of the candidate vaccine in day old broiler chicks when administered alone group EI, or concurrently with live I-2 as in group EII in 30 days post vaccination as measured by ELISA.

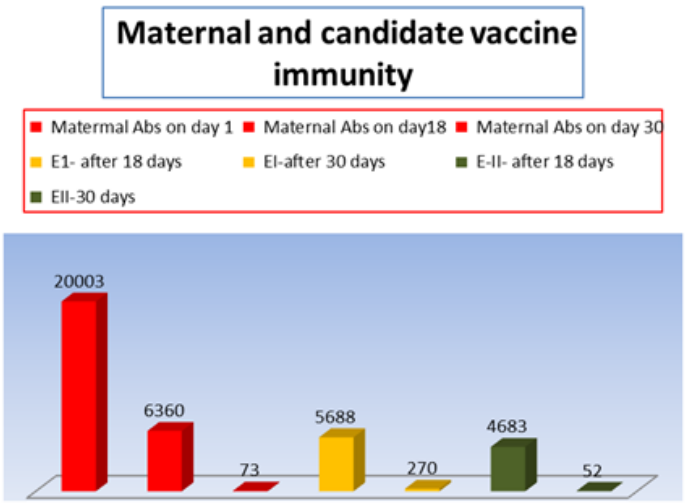

Figure 22: This figure compares the waning of maternal passive immunity over time against the active immunity of the candidate vaccine as measured by ELISA.

Phase II: Efficacy for group E1,E2, and C estimated after 21 post challenge, for group E1, within the first 3 days, 5 chicks out of 15 developed clinical signs, later on only one chicks died by the 7 th day post challenge while the remaining sick chicks recovered and withstand the test. For group E2 all chicks survived the challenge test and only 2 chicks showed mild clinical signs and then completely recovered. For group C- the control group- 9 out of 12 chicks died within 7 days post challenge. See (Table 6 and Figure 23).

Statistical analysis

Phase I:

Part I: After 21days post vaccination Chicks vaccinated with 0.2ml of the I-2 inactivated vaccine group I (N=40), associated with ELISA Abs levels M=1380. By Comparison, the non-vaccinated control, group C associated with numerically lower Abs levels, M=59.

Chicks vaccinated with inactivated La-Sota inactivated vaccine (N=40) associated with Abs levels M=1415. By Comparison, the same non vaccinated control group (N=30) group C was also associated with numerically lower Abs levels M=59 (Table 4 and Figure 9).

Part II: After 45 days post vaccination Chicks vaccinated with 0.2 ml of the I-2 inactivated vaccine, associated with ELISA Abs levels M=2565. By Comparison, the non-vaccinated control group C associated with numerically lower Abs levels, M=0 Chicks vaccinated with inactivated La-Sota inactivated vaccine associated with Abs levels M=2929 By Comparison, the same non-vaccinated control group C was also associated with numerically lower Abs levels M=0 (Table 2 and Figure 11).

To test the hypothesis that the non- vaccinated, and vaccinated groups were associated with statistically significantly different means Abs levels, an independent samples t- test was performed. Additionally, the assumption of homogeneity of variances was tested, and satisfied via levene's F-test.

In part I group I-2 against group CF (18)=0.466, P=0.075, for group La-Sota against group CF(20)=1.320, P=0.077

In part II group I-2 F (7)=3.337, P=0.072, for group La-Sota F (9)=13.781, P=.056

In part I group I-2 and group La-Sota when compared together F (26)=0.392, P=0.848 (Table 4).

In part II group I-2 and group La-Sota when compared together F (12)=1.738, P=0.533.

A Graphical representation of the means and their coefficient variation were displayed in bar charts diagram (Figure 14).

Phase II:

Part I: After 18 days post vaccination Chicks vaccinated with recommended dose of the candidate vaccine (N=40) group EI, associated with Abs levels M=5688 (SD=(1835.4), By Comparison, the non-vaccinated control group (N=30) group C was associated with numerically larger Abs levels, M=6360 (SD=3134).

Chicks vaccinated with live vaccine together with recommended dose of the candidate vaccine (N=20) group EII, associated with ELISA Abs levels M=4683 (SD=2057.9), By Comparison, the same non vaccinated control group (N=30) group C was also associated with numerically larger Abs levels, M=6360 (SD=3134) (Figure 20 and Table 5).

To test the hypothesis that the non-vaccinated, and vaccinated groups were associated with statistically significantly different means Abs levels, an independent samples t-test was performed. Additionally, the assumption of homogeneity of variances was tested, and satisfied via levene's F-test.

In part I group EI F (31)=3.95, P =0.056, for group E II F (28)=2.5, P=0.059

In part II group EI F (24)=.124, P=0.728, for group EII F (17)=4.9, P=0.041

In part I Group EI and EII when compared together F (31)=0.070, P=0.303 (Table 7).

Table 6: This table show the efficacy of the candidate vaccine compared with non-vaccinated control chicks 21 days post challenging with (vvNDV).

		21 days post challenge			
		Efficacy			
Efficacy groups	No of chicks	Morbidity rate	Morbidity rate	Morbidity rate	Efficacy%
EI	15	15-May	15-Jan	14/15	93.30%
EII	8	8-Feb	0/8	8-Aug	100%
C	12	12-Sep	12-Sep	12-Mar	33.30%

Table 7: Descriptive statistics and (ELISA) results of independent sample t-test for the candidate vaccine when administered by the recommended field dose or simultaneously with live I-2 vaccine 18 days post vaccination when compared against the nonvaccinated control group(maternal immunity).

Vaccine	N	M	Comparison			P
Candidate vaccine (EI)	40	5688	Maternal immunity after 18 days (control group)	Vs.	Candidate vaccine only	0.056
Candidate vaccine^{+} live I-2 (EII)	20	4683	Maternal immunity after 18 days (control group)	Vs.	Candidate vaccine^{+} live I-2	0.059
Maternal immunity (group C) after 18 days	30	6360	Candidate vaccine only	Vs.	Candidate vaccine^{+} live I-2	0.303

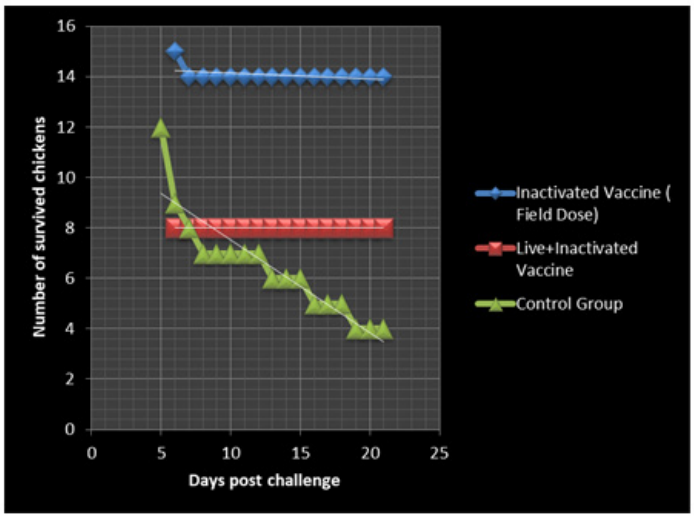

Figure 23: Survival curves of vaccinated Broiler chickens throughout out 21 days post challenge.

In part II group EI and E II F (21)=2.39, P=.137.

Part II: After 30 days post vaccination immune responses of group EI (40) was associated with Abs levels M=270, By Comparison, the non-vaccinated control (N=30) group C was associated with numerically smaller Abs level M=73 (SD=695.2)

After 30 days post vaccination Chicks vaccinated simultaneously with live vaccine along with recommended dose of the candidate vaccine (N=20) group EII, associated with Abs levels M=52.By Comparison, the same previous non vaccinated control group (N=30) group C was also associated with numerically larger Abs levels, M=73 (Figure 21 and Table 5).

To test the hypothesis that the non-vaccinated, and vaccinated groups were associated with statistically significantly different means Abs levels, an independent samples t-test was performed. Additionally, the assumption of homogeneity of variances was tested, and satisfied via levene's F-test.

In part I group EI F (31)=3.95, P =0.056, for group E II F (28)=2.5, P=1.25

In part II group EI F (24)=.124, P=0.728, for group EII F (17)=4.9, P=.041

In part I Group EI and EII when compared together F (31)=0 .070, P=0.794, in part II group EI and E II F (21) =2.39, P=0.137

In part I group E I, and group C the independent sample t-test was associated statistically with insignificant effect, t (18)=1.89 P=0.056, also in group EII, and C the independent sample t-test was associated statistically with insignificant effect (28)=1.969 P=0.059 (Tables 7 and 8)

In part II, group EI and group C the independent sample t-test was associated statistically with insignificant effect t (24)=1.135 P=0.268, also in group EII, and C the independent sample t-test was associated statistically with insignificant effect t(17)=1.175P=0.258 equal variance not assumed with groups. Data did not tabulated

For part I Group EI and E II were not associated statistically with significant effect after 18 days post vaccination t (30)=1.047, P=0.303, (Tables 7 and 8) for part II, 30 days post vaccination t(21)=2.297, P=0.032 associated with statistically significant effect. Data did not tabulated

The group vaccinated with the inactivated vaccine only, and those vaccinated concurrently with live, and inactivated vaccines were associated with statistically insignificantly Abs level than non-vaccinated control group. (Maternal immunity) except for group EI and EII in part II

A Graphical representation of the means and their coefficient variation were displayed in bar charts diagram (Figure 24).

Table 8: Descriptive statistics and (HI) results of independent sample t-test for the candidate vaccine when administered by the recommended field dose or simultaneously with live I-2 vaccine 18 days post vaccination when compared against the non-vaccinated control groups (maternal immunity).

Vaccine	N	M	SD	Comparison			P
Candidate vaccine only	40	35.11	39.4	Maternal immunity after 18 days (control)	*Vs.*	Candidate vaccine only	0.65
Candidate vaccine+live I-2	20	28.57	66.07	Maternal immunity after 18 days (control)	*Vs.*	candidate vaccine+live I-2	0.977
Maternal immunity	30	870.86	1381.6	candidate vaccine only	*Vs.*	Candidate vaccine+live I-2	0.73

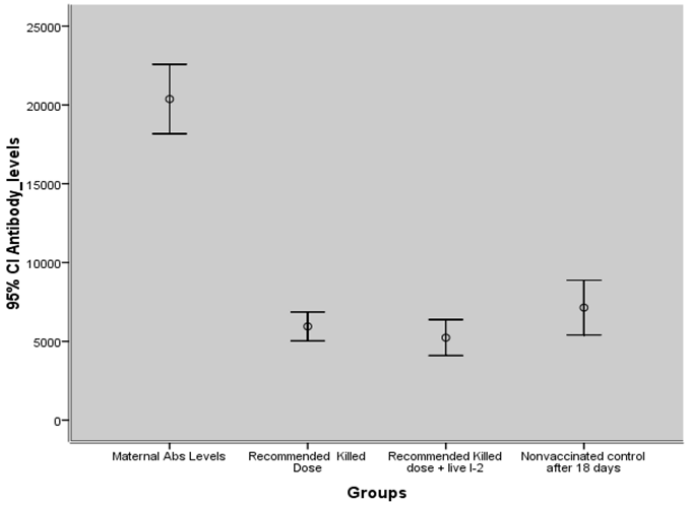

Figure 24: Graphical representation of the means and their coefficient variations of candidate vaccine 18 days post vaccination displayed in a bar charts diagram.

DISCUSSION

Newcastle Disease (ND) is most dangerous disease that continuously pose threat to chickens and poultry industry, and this because it is a highly contagious, and rapidly spreading disease among chickens and the other poultry species [17].

To combat this deadly chickens, disease an oil emulsion vaccines derived from I-2 thermostable strain and La-Sota strain have been tried in this study.

For preparing a plausible oil emulsions, firstly, the syringe technique had been utilized, and for better performance, stability and the duration of immunological activity of the prepared emulsions were greatly improved by breaking up the water phase into very small droplets i.e. less than 1μm in diameter. To avoid the unnecessary risks of using virulent ND strain these vaccines were prepared from lentogenic ND strains. The inactivated ND vaccine was developed according to the standard vaccine developmental procedure which mandates the vaccine clinical evaluation should undergo phases for safety and efficacy issues. In this study throughout phase I and phase II clinical trials the prepared vaccines were safe and immunogenic, the immunogenicity of candidate vaccines derived from La-Sota and I-2 strains was consistent when measured by HI, ELISA, and efficacy test, but 1-2 derived vaccine was more protective, and immunogenic which demonstrated by better protection and higher Abs levels respectively. While I-2 strain showed longer lasting immune response, the vaccine derived from La-sota gave slightly better homogenous immune response.

The results obtained from these trials confirm what the Italian and foreign workers have already found, i.e. that inactivated vaccine in oil emulsion evokes very high antibody levels and durable resistance to massive challenge [18-20].

High level of cross protection was observed between the two vaccine strains which indicated vaccination with either strain could be protective against the other. This finding alludes to the fact that ND vaccine derived from any strain can produce consistently protective immune response against any other ND strain.

In phase II clinical trials there was a dramatic decrease in the maternal Abs of the control groups in the first 18 days, but still higher than in its counterpart -the vaccinated group- within the same period of time. Over time more reduction of the Abs level was occurred in the unvaccinated control groups which might be due to the high metabolic rates of the broiler chicks, however, chicks still maintain protective levels, while active and rising immune response were noticed in the vaccinated groups according to the serological results obtained.

Although the GMTs derived from La-Sota vaccine was arithmetically slightly higher than that derived from the ND I-2 vaccine, the difference remains statistically insignificant $p>0.05$. Nevertheless, the 1-2 vaccine performed better in the challenge test 80% protection with higher survival rate,compared to 40% for La-Sota vaccine; this result might be due to higher virus yield of 1-2 strain than that of La-Sota strain and the better cellular immune response which might be developed in case of I-2 rather than of La-Sota strain. This result is consistent with Ezeifeka [21] who tested many adjuvant with ND vaccines with 100% protective HI titer among the 15 weeks of their study.

For all groups, the ELISA C.Vs were increased unproportionally against the level of immune response, with obvious decreasing among the vaccinated group which indicate a clear effect of the candidate vaccine upon producing more consolidated and homogeneous immune response. Generally the Abs driving immunity among vaccinated groups increased over time while the passive immunity of the non-vaccinated controls decreased over time.

After 45 days post vaccination, only the chicks which received the candidate vaccine remain with protective Abs level while the maternal Abs of the non-vaccinated group have already vanished. In phase II clinical trial only I-2 derived vaccine was further investigated.

The ELISA results were consistent with HI results; chicks vaccinated with candidate and live vaccine evoked Abs immune response lower than the chicks vaccinated only with the candidate vaccine, but achieved better protection than chicks vaccinated only with candidate vaccine. The decreases in the Abs levels might be due to the neutralizing effect of the live vaccine, but at the same time it might be provoked a better cellular and mucosal immunity than that of the candidate vaccine alone. Despite, there is a decrease in the Abs level for the combination of live and in activated vaccines,

the chicks perform better with higher weight gain and no deaths. This better protection might be due to the live vaccine effect which replicates quickly in the mucosal membrane of the conjunctiva (harderian gland) and nostrils and stimulate cellular and humoral immune responses.

The function of adjuvant was elaborated more by Al-Zubeedy [20] and Chedid et al. [22] and Chansiripornchai and Sasipreeyajan [23] who described the effects of the adjuvant is to stimulate the macrophages, which increases the antigen presentation capacity. In addition, another possible reason is that, unadjuvanted vaccines easy exposed to mop-up by neutralizing antibodies, whereas the oil adjuvant protect the unreleased antigens from the effect of antibodies [24].

Although the maternal Abs of the non-vaccinated control group keep vanishing over time, it remains higher than the group vaccinated with both live and inactivated vaccines at day 18 and day 30.However, only 33.3% of the chickens of the control group withstood the challenge, compared to 100% (no deaths) in chickens received live and inactivated combined vaccines. This finding is consistent with Folitse who explained the reasons for why vaccination of an inactivated NDV vaccine (s/c route) combined with live NDV vaccine (intranasal route) provided the higher HI antibody titer and this was because NDV from live vaccine can replicate rapidly on mucous membrane of ocular and nasal organs of chickens who had higher HI Abs titer than using only inactivated vaccine, this result Generally favoured the result of Wisanu et al. [16] who described the higher HI titer when combining live and inactivated ND vaccines, our finding in this study are partially goes with these findings regarding the better protection when combining live and killed vaccine and partially inconsistence regarding the higher HI titers, this might be due to the differences in chickens, age and species used, while they use laying chickens with more than 35 days old we only used -one- day old broiler chicks i.e. we had already included the effect of the maternal immunity while they did not.

When comparing a single live vaccine with combined live and inactivated vaccines, we found that chicks received a live and inactivated vaccine produced health information antibody titers higher than chicks received only a singly live vaccine. Also the considerable decrease in maternal immunity of control group might be due to rapid metabolic rate of the broiler chicks. While the increases in treatment group might be due to the active stimulation of the both arms of the immune response. On the other hand chicks received only inactivated vaccine stimulated higher Abs immune response compared to control group along the 30 days of the observations. This is due to the active effects of the candidate vaccine, and allude to fact that the candidate vaccine did not interfere with the passive immunity "working progressively while passive immunity waned" and stimulate active immunity to the levels that protect chickens from the challenge with vv NDV. This result is in favor of Al Zubeedy who considered the humoral immunity as a key component in the protection against ND [20], however, this study demonstrated further the goodness of the collaboration between humeral and cellular immunity when enhanced concurrently. These results is more consistent with Marangons and Busani who tested inactivated vaccine in the presence of passive immunity and found that, ND killed vaccines were more capable of eliciting an immune response in the face of existing antibody in spite of generally slower onset of immunity [8].

CONCLUSION

The non-antagonistic effect of inactivated vaccines over the maternal immunity was illustrated by the insignificant differences between the ELISA means titers between the active and passive immunity throughout 30 days.

This indicated that the passive immunity was always waned over time while the active immunity had increased. The fact that chicks with vaccination keeps developing protective immune response with increasing Abs levels despite presence of maternal Abs and along the broiler life span is now more than obvious ($P>0.05$). Moreover, the results of this study supported the concept that both humeral and cellular immunity are a key component in the protection against ND. Therefore, vaccination programs should always be directed toward eliciting and stimulating the both arms of the immune response in the birds' flocks.

From this study we also have strong evidence that, the immunogenicity of locally prepared inactivated vaccine is same either with the recommended or the double recommended dose, with similar level of humoral immune response and. thus, there is no need to over dose the chicks for better immune response. The candidate vaccine complied with the main quality control tests of sterility, viscosity, stability, safety and efficacy and has the potential to replace the imported inactivate ND vaccines.

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