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Research Article

Preparation and evaluation of oil-adjuvanted inactivated bivalent vaccine against Avian Influenza Virus (AIV) and Newcastle disease virus (NDV)

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ABSTRACT

The poultry sector is economically significant but faces many challenges. Avian Influenza Virus (AIV) and Newcastle Disease Virus (NDV) pose a great threat to susceptible birds. Vaccination strategies are useful in controlling infections caused by AIV and NDV. The goal of the current study was the production of a bivalent adjuvant-based inactivated vaccine against AIV and NDV and to compare the efficacy of an experimentally prepared vaccine with commercially available inactivated bivalent vaccine. Experimental trials were carried out on 14-day-old broiler chickens. Chickens were divided into three groups I, II, and III, with each group containing 20 birds. The first dose of vaccine was injected at 0 days (14 days of age) and the second dose was injected on the 7th day post-vaccination (21 days of age). Blood samples were collected from each group on the 7th and 14th day post-immunization. Results of the Haemagglutination Inhibition (HI) test at 7th and 14th day post-vaccination presented the geometric mean titers of group I and II were 42.2, 84.4 & 97.0, 168.9 respectively for AIV. Similarly, the geometric mean titers of groups I and II were 48.5, 97.0, and 84.4, and 168.8 respectively for NDV. However, the control group showed a geometric mean titer of <4 against AIV and NDV. The challenge was given on the 14th day post-vaccination and birds were monitored for the next 10 days for clinical signs. The chicks of group I and II showed no clinical signs of AI. However, in group I, 10% morbidity and 10% mortality were recorded for ND whereas, group II showed no clinical signs for ND. 100% mortality was recorded in control group.

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Introduction

Birds are being bred and raised rapidly at a commercial level to meet the nutritional needs of society worldwide. Chickens are prioritized because they are the chief source of good protein content. But microbes or parasites causing illnesses in the poultry sector are major challenges faced by domesticated birds. Avian Influenza virus (AIV) and Newcastle Disease virus (NDV) is causing Avian Influenza (AI) and Newcastle Disease (ND) in

poultry birds and are responsible for huge economic losses (Augustine and Shukla, 2015). AIV and NDV are enveloped viruses that fall under Class V according to the Baltimore classification because they contain a single-stranded, negative-sense RNA genome (Spackman 2020). AI is a respiratory illness, whereas; infection in visceral organs, and respiratory and nervous systems characterize ND. Infected birds show decreased oogenesis and meat production even if they survive (Alders et al. 2018).

AIV belongs to *Orthomyxoviridae* family, consists of 3 genera (A, B and C) out of which type A only affects avian species. Its hereditary material is composed of a single minus strand of RNA which is divided into eight parts. Its nucleocapsid is surrounded by lipid bilayer membrane, coated with glycoproteins including haemagglutinin and neuraminidase which are major virulent factors, responsible for developing disease (Spackman, 2020). Out of 16 H subtypes and 9 N subtypes, only H5, H7 and H9 subtypes are commonly seen to be primarily responsible for infecting avian species. About 144 different combinations of H and N are formed because each virus bears one H antigen and one N antigen, so genetic drift and shift is common in AIV. Depending upon H-N combinations there are two major subclasses of AIV; highly pathogenic (HPAIV) which causes systemic infections and low pathogenic (LPAIV) which is responsible for causing infection in respiratory tract (Suarez and Schultz-Cherry 2000). LPAI infections may be non-identifiable, but it may manifest symptoms which include lethargy, loss of appetite, reduced ova production, infections in mucosal associated tissues and organs. HPAI symptoms are short lived but severe, because it has 100% lethality rate and cause rapid death of the targeted bird (Blagodatski et al. 2021).

NDV first appeared in 1926 and was recognized in 1927. It comes under the family *Paramyxoviridae* and is of serotype- 1. It consists of single stranded, negative sense, non-segmented RNA genome (Jatav and Verma 2021). It is surrounded by lipid bilayer envelope on which particular surface antigens are attached among which most important are Fusion protein (F protein), Haemagglutinin (H) and Neuraminidase (N). NDV is antigenically stable so genetic re-assortment is not observed in case of NDV (Ganar et al. 2014). On the basis of its pathogenicity and lethality, there are three pathological subclasses of NDV including lentogenic which is asymptomatic and causes respiratory and enteric infections, mesogenic which causes respiratory infections with moderate mortality (<10%) and velogenic which is a highly virulent strain of NDV and causes systemic infections with high mortality (100%). Velogenic strains are further sub classified as viscerotropic (infection in major organs) and neurotropic (infection in the nervous system) depending upon the site of infection (Dortmans et al. 2011). ND infection is characterized by greenish diarrhea, enteric abrasions, immobility, anorexia, and hemorrhages in the proventriculus (Ashraf et al. 2016).

It is very important to protect birds from life-threatening agents like NDV and AIV to reduce economic loss. The main step to overcome the risk of infection is to decrease the susceptibility of birds to viruses. Biosecurity and vaccination are one of the cost-effective and safest approaches that can be opted to minimize the prevalence of AI and ND in the poultry industry (Brown and Bevins 2017). Inactivated bivalent water in oil vaccines are proving best in terms of efficiency and inducing immune response. They are inexpensive, safer to use, highly effective, not labor intensive and confer long-established defense against infectious agents. Birds experience less pain during the administration of one shot of bivalent vaccine as compared to two shots of monovalent vaccines against both agents (Mahmood and Sabir 2021).

Materials and Methods

Sample collection and processing

Infected organs were sampled from dead and infected birds from Poultry Diagnostic Laboratory, University of Agriculture Faisalabad. Proventriculus, intestines and gizzard were collected for NDV isolation whereas; lungs, liver and spleen were collected for the isolation of AIV and were further processed in Cell Culture Laboratory (CCL), Institute of Microbiology, University of Agriculture Faisalabad. Organ samples for AIV and NDV were chopped and weighed separately. Chopped organs were mechanically ground using sterile sand in mortar and pestle. 20% (w/v) tissue suspension was prepared in PBS for each virus separately. Homogenized tissue suspensions were centrifuged at 12000 rpm for 12 minutes at 4°C. Supernatant was collected and filtered using syringe filter having pore size of 0.2μm. 200μg/ml ampicillin was added in tissue suspension to avoid bacterial contamination. Temperature was maintained up to 4°C using crushed ice throughout the process. Tissue suspensions of both viruses were then stored at -20°C accordingly (Hammad *et al.*, 2022).

Cultivation of AIV and NDV

For virus inoculation, 9-10 days old chicken embryonated eggs were obtained from Poultry Research center, University of Agriculture Faisalabad. Eggs were candled for checking viability of the embryo. Dead and non-embryonated chicken eggs were removed from the batch. Air space and embryo head of the remaining eggs were labeled. Total 120, 9-10 days old chicken embryonated eggs were sterilized with 70% alcohol and divided in to 3 groups (I, II and III) with each group containing 40 eggs. Holes were

created in the allantoic cavity using an egg puncture. Eggs in group I and II were inoculated with 200µl suspension of AI and ND virus, respectively and eggs in group III served as negative controls and were inoculated with 200µl Phosphate Buffer Saline (PBS) through allantoic route of inoculation. The holes were sealed using paraffin wax. Inoculated eggs were incubated in an egg incubator at 37°C with up to 70% humidity and aeration for 48-72 hours. Eggs were candled daily for 3 days to check viability of embryo. Embryos that died after 48-72 hours of inoculation were kept overnight at 4°C in the refrigerator. Allantoic fluid was harvested and checked for haemagglutination activity (Mahmood and Sabir 2021).

HA test, HI test, AGPT, ICPI, sterility test and virus titration

Hemagglutination (HA) test was performed and hemagglutination activity of AIV and NDV was checked as described by Spackman (2008). The end point titer and 4HAU were calculated for both viruses separately. Both viruses were re-confirmed by performing Hemagglutination Inhibition (HI) test as described by Spackman (2008). The Agar Gel Precipitation Test (AGPT) was performed as described by Cheema et al. (2011) for virus confirmation. ICPI test was performed as described in OIE (2013) for assessing the pathological subtype of NDV. Sterility test of harvested allantoic fluid was performed as described in OIE (2013) for checking the presence of any bacterial or fungal contamination. Virus titration (EID_{50}) was calculated by the method as described by Sauerbrei et al. (2006).

Virus inactivation and vaccine preparation

Both antigens (AIV and NDV) were inactivated using 0.2% formalin at 37°C for 16-48 hours and inactivation of both antigens was confirmed by the method described by Pawar et al. (2015). Both inactivated antigens were mixed in an equal ratio of 1:1 for vaccine preparation. Vaccine was prepared by mixing 3 parts (v/v) of antigens and 7 parts (v/v) of Montanide adjuvant ISA-206 VG. Firstly, 14ml of Montanide ISA 206 VG was placed in a sterile beaker and rotated at 20 rpm then 6ml of aqueous solution of inactivated antigens was added dropwise and mixed properly. The prepared vaccine was homogenized at 7000 rpm for 5 minutes. After homogenization, the vaccine was transferred to sterile glass vial which was sealed afterwards under sterilized conditions.

Vaccine quality control tests

The prepared bivalent vaccine underwent rigorous quality control assessments, including sterility and safety evaluations. For sterility testing, the vaccine was inoculated on Nutrient agar, Sabouraud agar and MacConkey agar plates. These plates were then incubated at 37°C and closely observed for any signs of microbial growth over a 5-day period. In safety testing, the vaccine was inoculated in 9-10 days old chicken embryonated eggs. These eggs were examined under a candling process after 24 hours and observed for the subsequent 10 days to assess the viability of the developing embryos. Furthermore, the allantoic fluid obtained from these eggs was collected and subjected to a haemagglutination (HA) test to verify its safety and efficacy.

Experimental design

A total of 14-day old broilers were reared in the Animal House, Institute of Microbiology, University of Agriculture Faisalabad. All experimental birds were divided into three groups (I, II, III) with each group having 10 birds. Group I and II were vaccinated at the rate of 0.5ml per bird via subcutaneous route with commercially available bivalent vaccine and experimental vaccine, respectively. While birds in group III served as positive controls and were injected subcutaneously at the rate of 0.5ml per bird with a mixture of PBS and oil adjuvant to check if there is any side effect of oil adjuvant used. 1st dose of vaccine was injected at 0th day (14 days of age) and 2nd dose was injected on 7th day post-vaccination (21 days of age). Blood samples were collected from each group at 7th and 14th day post-immunization. Serum was separated from collected blood and was subjected to titration against AIV and NDV. HI assay was performed for assessing antibody titers and with the help of which geometric mean titer (GMT) was also calculated (Spackman, 2008). On the 14th day post immunization, 50% birds of each group (5 birds per group) were injected separately with 0.1ml AIV and remaining 50% with 0.1ml velogenic NDV intramuscularly. Birds were monitored for up to 10 days for morbidity and mortality. Clinical observations were recorded and protection rate (%) was determined.

Results

In the current study, the Haemagglutination (HA) titers for AIV and NDV were determined to be 1:128 and 1:512, respectively. To confirm the presence of these viruses, a (HI) test was conducted. The HI test results demonstrated that the antiserum specific to AIV bound exclusively to AIV, while the antiserum specific to NDV bound exclusively to NDV. This specificity indicated that the antibodies were accurately targeted. Additionally, when a 1% suspension of chicken red blood cells was added, it resulted in "button formations," which is considered a positive outcome in the context of the HI test. This positive outcome served as confirmation that both AIV and NDV were present in the samples.

To further confirm the presence of AIV and NDV, an Agar Gel Precipitation Test (AGPT) was conducted. The results revealed that the interaction between the antigen and antibody was highly specific, leading to the formation of a distinct white precipitation line between the test wells. This line became visible when exposed to

ultraviolet (UV) light. Sterility tests conducted on the vaccine confirmed the absence of contamination.

Table 1: HI antibody titers of Avian Influenza vaccine and Newcastle Disease vaccine in Broilers

Groups	Broiler #	HA units/dose	HI antibody titers a week interval vaccination in broilers			
			AIV		NDV	
			7th Day	14th Day	7th Day	14th Day
I	1	**1:32** (for AIV) **1:16** (for NDV)	32	128	64	128
	2		64	128	32	64
	3		32	64	64	64
	4		32	128	64	128
	5		64	64	32	64
	6		64	64	32	128
	7		32	64	64	128
	8		32	128	64	64
	9		32	128	32	64
	10		64	128	64	64
GMT*			**42.2**	**97.0**	**48.5**	**84.4**
II	1	**1:128** (for AIV) **1:128** (for NDV)	128	128	64	128
	2		64	256	128	128
	3		64	256	128	256
	4		128	128	64	256
	5		64	128	128	128
	6		64	256	128	256
	7		128	256	128	128
	8		64	128	64	128
	9		64	128	64	256
	10		64	128	64	128
GMT			**84.4**	**168.9**	**97.0**	**168.8**
Control	1		2	2	2	2
	2		2	2	3	3
	3		3	2	2	2
	4		2	2	2	2
	5		2	2	3	2
	6		3	3	2	2
	7		2	3	2	3
	8		2	2	2	2
	9		2	2	3	2
	10		2	2	2	2
GMT			**<4**	**<4**	**<4**	**<4**

*Geometric Mean Titer

Geometric Mean Titer

ICPI of isolated strain of NDV was calculated as 0.7 which indicated the pathotype of harvested viral strain to be "lentogenic." The allantoic fluid having NDV and AIV was processed for the determination of EID_{50}. The EID_{50} of AIV and NDV was calculated as $10^{9.15}$/ml/bird and $10^{7.8}$/ml/bird, respectively. After virus titration, both viruses were subjected to inactivation with 0.2% formalin and inactivated adjuvant-based bivalent vaccine was prepared.

During safety testing, no embryo mortality was observed. Furthermore, the harvested allantoic fluid, when subjected to a HA test, exhibited no haemagglutination activity. This collectively indicated that the vaccine was both sterile and safe for use.

Then, efficacy of oil adjuvanted inactivated bivalent vaccine was evaluated in broilers through HI test. Post-vaccination HI antibody titers of experimental vaccine and commercially available bivalent vaccine are shown in Table 1. At 0th day (14 days of age) birds were injected with 1st dose of vaccine and at 7th day (21 days of age) 2nd dose of vaccine was injected, but before vaccination, birds were checked for AI and ND antibody titers through HI test and result show no antibody titer against them. Results of Haemagglutination Inhibition (HI) test at 7th and 14th day post-vaccination presented the geometric mean titers of group I and II were 42.2, 84.4 and 97.0, 168.9 respectively for AIV. Similarly, the geometric mean titers of group I and II were 48.5, 97.0 and 84.4, 168.8 respectively for NDV. However, control group showed geometric mean titer of <4 against AIV and NDV. The challenge was given on 14th day post-vaccination and birds were monitored for next 10 days for clinical signs. The chicks of group I and II showed no clinical signs of AI. However, in group I 10% morbidity and 10% mortality were recorded for ND whereas, group II showed no clinical signs for ND. 100% mortality was recorded in control group. In control group, no side effects were observed due to oil adjuvant used.

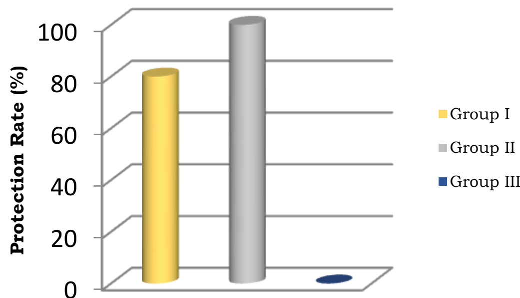

Fig. 1: Protection rate-based comparison of commercially available bivalent vaccine, experimental vaccine and PBS (control) among broilers of Group I, II and III.

Morbidity and mortality were observed for 10 days after challenging the broilers with HPAIV and velogenic NDV as shown in Table 2. No clinical signs were observed in group I up to 6 days with antibody titers ranges from 1:32 to 1:128 for both AI and ND. On 7th day post challenge, 20% broilers in group I showed clinical signs and symptoms out of which broiler # 8 died on 9th day post challenge and broiler # 6 died on 10th day post challenge. 80% broilers in group I were protected whereas, 20% morbidity and mortality were also recorded in group I. Absence of clinical signs and symptoms were observed in group II in which broilers were injected with experimental vaccine with antibody titers ranges from 1:64 to 1:256 for both AI and ND. 100% broilers in group II were protected and no morbidity and mortality were recorded at all. Broilers in group III (control) showed clinical signs and symptoms on 2nd day post challenge. 60% broilers in group III died on 3rd day post challenge whereas, remaining 40% died on 4th day post challenge. It was seen that the efficacy of the experimental vaccine was far better than commercially available bivalent vaccine.

Discussion

Poultry industry is providing us with a lot of animal protein. Over population leads to malnutrition, due to which the demand of poultry products has increased over time. Broilers constitute the major portion of world's poultry industry, as they are the cheap and easily available source of meat for people in all areas. Birds being bred under harsh environmental conditions face many challenges among which attack by infectious agents and spread of disease is the major factor which cause decline in the production rate of economically significant commercial birds (Sharma 1999). AIV and NDV had caused economic losses to poultry industry at a commercial scale. These diseases can be controlled by two main strategies, one is biosecurity, and the other is immunization. Use of vaccination aimed at reducing the risk of outbreak of clinical disease. Therefore, proper immunization programs should be carried out at a global, national, regional and farm level to control trans-boundary poultry diseases.

The goal of the current study was the production of bivalent adjuvant based inactivated vaccine (W/O) against AIV and NDV and to compare the efficacy of experimentally prepared vaccine with commercially available inactivated bivalent vaccine. Experimental trials were carried out on 14-day old broilers. Broilers were divided in to three groups I, II and III with each group containing 10 birds. First dose of vaccine was injected at 0th day (14 days of age) and second dose was injected on 7th day post-vaccination (21 days of age). Blood samples were collected from each group at 7th (21 days of age) and 14th day (28 days of age) post-immunization. HI titers and log_2 HI titers were measured using HI assay and geometric mean titers were calculated accordingly. The challenge was given on 14th day (28 days of age) post-immunization and birds were monitored for next 10 days for morbidity and mortality.

Zhao et al. (2017) prepared an inactivated bivalent vaccine against H9N2 AIV and NDV in China and evaluated its efficacy by performing experimental trials in chickens. Their findings suggested that inactivated bivalent vaccine against AIV and NDV triggered a strong humoral response in immunized chickens. The antibody titer increased at a greater level in a short period of time. Highest HI titer was observed at 4th week post-immunization with a

mean titer of 8.6 log_2 for NDV and 9.5 log_2 for H9N2. Thus, they concluded that inactivated NDV and H9N2 vaccine induced an immediate and strong antibody response in vaccinated chickens. Our study indicated somewhat better results. Experimentally prepared bivalent vaccine against AIV and NDV proved best in terms of generating immediate and strong humoral (antibody) response in vaccinated chickens. The antibody titers assessed after immunization by HI assay seemed to increase rapidly in a relatively short time duration. Our highest HI titer was observed at 2nd week post-immunization with a mean titer of 7.4 log_2 for both AIV and NDV. Such rapid and quick response could be due to unique formulation of prepared vaccine. In our experimentally prepared bivalent vaccine, AIV and NDV were inactivated using 0.2% formalin, which also possess an extraordinary ability to induce IgG1immune response. Moreover, Montanide ISA 206 VG oil adjuvant was used to preparing bivalent vaccine. Oil adjuvant increased the efficacy of the vaccine as it has the ability to retain itself and promote slow release of antigen thus, confer long-term and strong humoral immune response.

E1 Naggar et al. (2017) developed two newly formulated inactivated mucosal bivalent vaccines against NDV and H9N2 AIV, which differ in the type of oil adjuvant used. IMS1313 oil adjuvant was added in first vaccine and second vaccine contained GEL 01 oil adjuvant. Both vaccines were adminsitered intranasally and through spray route in respective groups of experimental SPF chickens. In addition to these two bivalent vaccines, Montanide ISA 71 based bivalent vaccine was also injected intramuscularly in a separate group containing SPF chickens and was also evaluated for its efficacy. Activity of T cells and antibody titer was observed after performing challenge trials. Results indicated that spray route of administration was a far better strategy as compared to intranasal administration of vaccine in inducing interferon-gamma and IL-6 response in vaccinated chickens. HI antibody titer of H9N2 in first 4 groups was as low as 5 log_2 as compared to the 5th group in which it exceeds 9 log_2 and retained for 15 weeks. Montanide ISA 71 immunized chickens revealed high HI antibody titer of 8 log_2 for NDV at 8th week which lasts till 15th week post-immunization. Our results fall close to the results obtained by the use of Montanide ISA 71 described in above study. In our study, Montanide ISA 206 VG oil adjuvant was used which generated mean HI titer of 7.4 log_2 for both AIV and NDV as compared to Montanide ISA 71 used in above mentioned study which produced HI titer of 8 log_2 for NDV. Findings in our research were far better than those obtained by the use of IMS1313 and GEL 01 oil adjuvants. Our vaccine was administered through subcut route in experimental chickens. Intranasal route didn't prove to be effective because there was no surety that the antigen reached its targeted area successfully. During research, our Montanide ISA 206 based bivalent vaccine was administered through subcut route which proved effective in terms of generating stronger immune response for longer period because nodule was formed subcutaneously due to the presence of oil adjuvant because of which antigen was released at a slow rate confering long-term immune response.

Ismail et al. (2018) developed two different formulations of inactivated bivalent vaccine and worked on prime-boost vaccination strategy against NDV-genotype VIId and H5N1-HPAIV. Two different types of oil adjuvants were used for developing two different bivalent vaccines against NDV-genotype VIId and H5N1-HPAIV. Montanide-GelO_2 based bivalent vaccine was introduced in 10-days old experimental birds through oral route. It served as an initial dose followed by one or two booster doses of Montanide ISA 71 based vaccine. Prime-boost strategy proved best in terms of rising antibody titers in vaccinated chickens and rendered 50-60% protection against H5N1 and NDV challenge respectively. Montanide ISA 71 based vaccine protected 80% experimental chickens by up-regulating the levels of interferon-gamma and interleukin-6. 0% mortality in experimentally vaccinated chickens was observed due to prime-boost combination. This study highlighted the importance of vaccination strategies to maximize protection against AIV and NDV by inducing longer and stronger immune response. Similar results were obtained in our study in which initial dose of experimentally developed Montanide ISA 206 based inactivated bivalent vaccine against AIV and NDV was introduced in 14-days old chickens followed by a booster dose of the same vaccine at 7th day post-immunization. As a result, antibody titer in vaccinated chickens raised to a greater extent. Montanide ISA 206 based bivalent vaccine confered 100% protection in vaccinated chickens upon challenge against AIV and NDV.

Said et al. (2019) developed an inactivated recombinant H5-NDV vaccine in Egypt. Vaccine contained H5 gene segment of AIV integrated in baculovirus and expressed by insect cell and egg based LaSota strain of NDV. The efficacy of developed vaccine was evaluated by performing challenge studies in broiler chickens in which vaccinated and non-vaccinated groups were given single and dual antigen challenge with HPAI H5N1 and velogenic NDV antigens. Results indicated that recH5-NDV vaccine provided 90-100% protection to vaccinated chickens from single and dual challenges as compared to non-vaccinated group which showed 100% mortality rate upon a single challenge. Vaccinated groups showed highest haemagglutination Inhibition antibody

titer at 2nd week post-immunization with a mean titer of >6 log_2 against both H5N1 and NDV antigens.

Table 2: Clinical signs observed in Broilers after challenge with HPAIV and velogenic NDV.

Groups	Broiler #	HA units/dose	HI antibody titer at the time of challenge		Observed clinical signs after virus challenge									
			AI	**ND**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
I	1	**1:32** (for AI) **1:16** (for ND)	128	128	-	-	-	-	-	-	-	-	-	-
	2		128	64	-	-	-	-	-	-	-	-	-	-
	3		64	64	-	-	-	-	-	-	-	-	-	-
	4		128	128	-	-	-	-	-	-	-	-	-	-
	5		64	64	-	-	-	-	-	-	-	-	-	-
	6		64	128	-	-	-	-	-	-	+	+	+	D
	7		64	128	-	-	-	-	-	-	-	-	-	-
	8		128	64	-	-	-	-	-	-	+	+	D	
	9		128	64	-	-	-	-	-	-	-	-	-	-
	10		128	64	-	-	-	-	-	-	-	-	-	-
II	1	**1:128** (for AI) **1:128** (for ND)	128	128	-	-	-	-	-	-	-	-	-	-
	2		256	128	-	-	-	-	-	-	-	-	-	-
	3		256	256	-	-	-	-	-	-	-	-	-	-
	4		128	256	-	-	-	-	-	-	-	-	-	-
	5		128	128	-	-	-	-	-	-	-	-	-	-
	6		256	256	-	-	-	-	-	-	-	-	-	-
	7		256	128	-	-	-	-	-	-	-	-	-	-
	8		128	128	-	-	-	-	-	-	-	-	-	-
	9		128	256	-	-	-	-	-	-	-	-	-	-
	10		128	128	-	-	-	-	-	-	-	-	-	-
III	1		<4	<4	-	+	D							
	2		<4	<4	-	+	D							
	3		<4	<4	-	+	+	D						
	4		<4	<4	-	+	+	D						
	5		<4	<4	-	+	D							
	6		<4	<4	-	+	+	D						
	7		<4	<4	-	+	D							
	8		<4	<4	-	+	D							
	9		<4	<4	-	+	+	D						
	10		<4	<4	-	+	D							

(-) healthy birds showing no abnormal signs, (+) birds showing typical signs of disease (diarrhoea, anorexia, respiratory distress etc.), (D) dead birds

It was concluded that recH5-NDV successfully triggered strong humoral immune response in immunized chickens against single and dual challenge with HPAI H5N1 and vNDV. Analogous results were obtained in our study also in which we developed oil adjuvanted inactivated bivalent vaccine against AIV and NDV. Its efficacy rate was observed to be 100% as it confered 100% protection to immunized chickens upon challenge exposure to HPAI AIV and velogenic NDV (Fig.1) Highest Haemagglutination Inhibition (HI) titer in vaccinated group was observed at 2nd week post-vaccination with a mean titer of >7 $\log_2$ for both AIV and NDV. Such a minor difference in the HI titer of both vaccines could be due to the differences in their formulations. As we used 0.1% formalin for inactivation of AIV and NDV which perform dual functions, at first it inactivates viruses and secondly it can also induce IgG1 immune response in chickens. So, humoral antibody response was not only triggered by inactivated AIV and NDV but also by formalin to some extent. Moreover, oil adjuvant also increased the efficacy of AIV-NDV bivalent vaccine in a synergistic manner.

Cahyani et al. (2020) compared protection levels and efficacy of two different formulations of a bivalent vaccine against H9N2 strain of AIV and LaSota strain of NDV. Two oil adjuvanted inactivated bivalent vaccines were developed when inactivated LaSota strain of NDV and H9N2 strain of AIV were mixed and homogenized with Marcol white mineral oil (Vaccine A) and Montanide ISA 70 (Vaccine B) adjuvants. Both bivalent vaccines were tested for their safety by observing any physical and histological medical features at the site of injection. Efficacy of these two inactivated bivalent vaccines was evaluated in SPF chickens. Haemagglutination Inhibition titers and protection percentage was recorded accordingly. Highest mean HI antibody titer against ND antigen at 2nd week post-vaccination was found to be 4.5 $\log_2$ for Marcol white mineral oil based bivalent vaccine and 6.1 $\log_2$ for Montanide ISA 70 based bivalent vaccine whereas, highest mean HI antibody titer against AI antigen at 2nd week post-vaccination was found to be 5.6 $\log_2$ for Marcol white mineral oil based bivalent vaccine and 7.4 $\log_2$ for Montanide ISA 70 based bivalent vaccine. Single dose of both bivalent vaccines offered 100% protection in chickens that have been immunized and then exposed with virulent AIV and NDV antigens. Results declared that bivalent vaccine B induced stronger immune response in vaccinated chickens as compared to bivalent vaccine A. But, in the later stages of developing immune response no significant difference was observed between these two vaccines. In our study, we used Montanide ISA 206 VG oil adjuvant to prepare inactivated bivalent vaccine against AIV and NDV. Efficacy of prepared vaccine was evaluated in SPF chickens in terms of HI titers and protection percentage. Highest HI titer was observed at 2nd week post-immunization with a mean titer of 7.4 $\log_2$ for both AIV and NDV. Montanide ISA 206 based bivalent vaccine conferred 100% protection in vaccinated chickens upon challenge against AIV and NDV.

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Ethical statement
Experiments were conducted with the permission of the ethical committee.

Competing Interest:
The authors declare that they have no relevant financial or non-financial interests to disclose.

Consent for publication
All the Authors consented to publication of the paper.

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Author Contribution
TAF, MS and MM designed the study. AJ, MNA, and ZAB performed the research. MSM revised the manuscript.

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