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IMMUNIZATION POTENCY AND PROTECTIVE EFFICACY OF A BIVALENT INACTIVATED FOWL CHOLERA AND AVIAN INFLUENZA VACCINE IN POULTRY

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Abstract

Introduction: Vaccines are very Important to prevent disease outbreaks. Vaccines have special importance in the prevention of diseases such as Avian influenza (AI) and Fowl cholera (FC) which are economically significant afflicting poultry in Pakistan as well as around the world. The immunogenicity of an experimental bivalent inactivated Avian Influenza H5 and Fowl Cholera *(P. multocida)*(PM) vaccine is tested in the field in this study.
Methodology: A total of 100-day-old broiler chicks were selected. The chicks were divided into four groups of 25 birds each. Group 1 injected with monovalent H5, Group 2 with PM. Group 3 with Bivalent H5 and *P. multocida* vaccine in equal dose of both vaccines at the rate of 1:1. Group 4 was kept as control. Group 1, 2 and 3 received 1st Vaccine shot at 7th day of age and 2nd dose at day 14th day of age.
Results: The immunization potency by ELISA and protective efficacy by challenge study of a bivalent inactivated fowl cholera and AI vaccine is determined through experimental application on broiler flocks in this study. ELISA test was used to determine the peak of the humeral immune response against PM at the 1st, 3rd, 4th, and 6th weeks after vaccination, same testing for Influenza H5 titer was done by using HI after vaccination. ELISA titer of Fowl cholera in monovalent vaccine group at 6th week was 2900 while in bivalent vaccine group was 4056. Similarly, HI test of H5 titer was 7log in bivalent while in monovalent was 5.3 log in 6th week.
Conclusions: It was observed that bivalent vaccine has relatively higher titer (protection) against H5 and PM than monovalent, with same dose (0.5ml dose/ pullet).

Keywords: Vaccine, Monovalent, Bivalent, *P. multocida*, Avian influenza, HI, ELISA.

Introduction:

Vaccination is a critical approach that aids in maintaining poultry flock's health by empowering the birds to fight off infections and providing effective preventive and control measures. A disease epidemic can seriously jeopardize the welfare of the flock, its ability to produce, and the security of its finished products. Understanding the principles of vaccination, comprehending its benefits, implementing vaccination program, utilizing improvements in vaccination procedures are always aiming in improving disease prevention, boost flock health, and ensure sustainable output. Through concerted efforts and study, the power of vaccination may be leveraged to protect chicken populations and the future of the poultry industry [1].

Vaccine-based immunization in poultry lowers death and morbidity rates. Birds' immune systems are more equipped to fight off diseases after vaccination. As a result, disease severity is frequently diminished, lowering mortality rates and improving flock health [2].By halting illness development and spread, vaccination support the well-being and longevity of poultry. In addition to improve the health of poultry birds, vaccination also has positive economic effects. The administration of vaccination to a flock is a useful and economical disease management strategy. Typically, the costs associated with administering vaccines are much lower than the potential losses resulting from disease outbreaks [3].

By preventing illnesses, farmers can avoid decreased production, slower development, higher mortality, and a demand for expensive treatments. Consequently, vaccinations support sustaining optimal output and ensures steady income for chicken breeders [1].

Bacteria and influenza virus complications are a common cause of high morbidity as well as mortality, hence specific preventative measures have to be developed. An outbreak was reported in 6000 Broiler respiratory disease occur at age of 4th week after eight-day bird was recovered with 50% morbidity [4]. In another study from broilers a total of 123 isolates of Fowl cholera (FC) were isolated [5]. FC prevalence percentage was recorded which was 4.25 % in Gazipur in Bangladesh [6].

In another experiment it was observed that bivalent vaccine of Influenza and bacterial streptococci vaccination improved mice's protection against influenza and associated bacterial complications [7]. At the global, national, and local levels, several tactics can be used to prevent and control the introduction and spread of poultry diseases [8]. The high prevalence of poultry illness places significant restrictions on the growth of the poultry industry, necessitating immunization of birds prior to disease introduction [9]. The first chicken vaccine was developed towards the end of 1800 for fowl cholera and today, most significant contagious poultry illnesses are prevented and controlled by the application of poultry vaccinations [10].

In current study bivalent inactivated vaccine of FC (Fowl Cholera) and H9 was evaluated in field trial. Serum was tested to evaluate humeral response against vaccine by ELISA for FC and HI test for AI. ELISA and HI was perform after 5th week of primary and 3rd week of booster dose.

Methodology:

Identification of Influenza Virus H5

For the molecular identification of suspected influenza virus infection, RNA extraction was performed by using RNA extraction Kit produced by VIVANTIS (catalog no. GF-TRD-100). Thermofisher's cDNA synthesis Kit(Catalog no. K1021) was used to convert the extracted RNA into complementary DNA (cDNA) and stored at -20°C to ensure its stability. Polymerase Chain Reaction (PCR) assays was performed to check for the presence of H5 avian influenza virus. Reaction mixture was prepared as follows.

Table 1: Reaction Mixture for H5 PCR

Reaction Mixture Recipe	Volume
Master Mix (Bases, dNTPs and $MgCl_2$)	12.5 µl
Forward Primer	2 ul (20pm / µl)
Reverse Primer	2 ul (20pm/ µl)
RNAse/DNAse free Water	6.5 µl
Sample Cdna	2 µl

Three reactions were run as Positive control, Negative control and non-template control respectively, in 0.2 ml PCR tube in thermocycler (ABI). For PCR amplification following Primer set used: Forward primer 5'ACT ATG AAG AAT TGA AAC ACC T3'Reverse primer 5' GCA ATG AAA TTT CCA TTA CTC TC3' [11]. Positive control indicated an amplicon length of 456bp. Trachea and tracheal swabs were found Positive for H5 [12].

Amplification Protocol:

Initial Denaturation	Cycle	Denaturation	Annealing	Extension	Final Extension
96ºC/ 5:00 min.	40	95ºC/1:00 min.	53ºC/5:00 min.	70ºC/1:00min	72ºC/10:00 min.

Propagation of Influenza H5 Virus:
First step of this research was how the influenza H5 virus propagate. At first step H5 Influenza virus was propagated in embryonated eggs. As the host for viral replication, selection of embryonated chicken eggs that were 9 to 11 days old. The influenza H5 virus can develop and reproduce in these eggs' favorable environment.

Positive Sample Collection:
Positive sample fluid containing the H5 influenza virus was meticulously collected to start the propagation process. To protect the workers and avoid any unintentional contamination, this sample was handled inside a Biosafety Level 2 (BSL-2) Cabinet.

Centrifugation for Purification:
The positive sample fluid was centrifuged at 14,000 relative centrifugal force (RCF) for thirty minutes to eliminate any pollutants and impurities. All bacteria, including mycoplasma, were successfully pelleted by this procedure, enabling us to deal with a clean sample.

Syringe Filtration:
A 0.2-micron syringe filter was used to further purify the supernatant fluid after it had been cleaned of all bacterial contamination. The eradication of any left-over bacteria or fungi throughout this filtration process was essential for maintaining the virus suspension's purity.

Gentamycin Injection:
Gentamycin was injected into the prepared sample fluid at a 1:10 ratio as an additional precaution to protect the sterility of the sample and prevent bacterial or fungal growth during the propagation procedure. This measure was performed to protect the viral culture's integrity [13].

Inoculation of Embryonated Eggs:
0.1 ml of the positive sample fluid, which was now supplemented with gentamycin, was then cautiously injected via the allantoic route into 9-day-old embryonated chicken eggs.

Incubation Period:
For two days, the contaminated eggs were then kept in an incubator maintained at a temperature of 37°C. The virus might spread inside the eggs because of the regulated environment [14].

Sterility of collected fluid:
10 ml from harvested fluid was centrifuge at 14000 RCF and pellet was used for sterility test Total Plate Count (TPC), Coliform, *Salmonella* and Fungal Growth according to FAO manual [14]. Harvested fluid was found negative for all tested bacteria. After conformation that harvested allantoic fluid is free of any other pathogen, EID_{50} was calculated.

HA Test:
Rapid slide HA test was perform with 10% washed chicken red blood cells [15].

Calculation of EID50:
One EID_{50} is dilution of virus at which 50% of inoculated embryonated eggs get infected. H5 virus was diluted in serial dilution with dilution ratio of 10^{-2} 10^{-3} $10^{-4}10^{-5}10^{-6}$ 10^{-7} 10^{-8} 10^{-9} and 10^{-10} in a sterile manner. Ten eggs from each dilution was used for inoculation with a volume of 0.1ml dilution each. Then eggs were incubated at 37ºC for 2 days. Allantoic fluid was harvested from each egg and spot HA was performed with 10% chicken washed RBCs. HA test was positive in 9 eggs out of 10 at 10^{-3} dilution while 1 egg out of 10 was HA test positive in 10^{-4} dilution. Accordingly, EID_{50} calculated according to the formula for proportionate distance (PD) given below;

$$EID_{50} = \frac{\text{Percentage infected at dilution immediately above 50\%}}{\text{Infected at dilution above 50\% - Infected at dilution below 50\%}}$$

EID_{50} = 90-50/90-10 = 0.5. So, the HA test was 90% positive at 10^{-3} dilution, EID_{50} will be $10^{3.5}$ /0.1ml accordingly, one ml it will be $10^{4.5}$/1ml [16].

Virus inactivation and Vaccine preparation:
In the procedure, a 0.4% formalin solution was used to inactivate the H5 virus, which was then incubated at 4°C for the overnight duration. A 9-day-old embryonated egg was then injected with 0.1 ml of the inactivated virus to ensure full inactivation. After that, mixture of the inactivated H5 virus and Montanoide oil ISA-71 was mixed at ratio of a 1:1, and the resulting mixture under-went 5 minutes of emulsification in a mixer running at 3000 RPM. Gentamicin was incorporated into the emulsified solution at a dosage of 20 mg per 10 ml. In order to prepare it for use later on, the finished emulsion was then kept at 4°C [17].

Identification of *Pasteurella multocida* (PM):
PCR was performed for identification of PM from tracheal tissues/tracheal swabs and liver samples. DNA extracted by using DNA extraction Kit (Thermo Fisher Scientific). DNA stored at -20ºC. Reaction mixture was prepared as follows.

Table 2: Reaction mixture for *Pasteurella multocida* PCR

Reaction Mixture Recipe	**Volume**
Master Mix (Bases, dNTPs and $MgCl_2$)	12.5 µl
Forward Primer	2 µl (20pm / µl)
Reverse Primer	2 µl (20pm/ µl)
RNA/DNA free Water	6.5 µl
Sample DNA	2 µl

Three reaction mixtures run as Positive control, Negative control and non-template control in 0.2 ml PCR tube in Thermocycler (ABI). A virulent local strain of *P. multocida* identified with PCR by using following primer sequence (Townsend et al. 2001) amplifying target fragment of 460bp. F primer: 5'-GCT GTA AAC GAA CTC GCC AC-3' R Primer: 5'-ATC CGC TAT TTA CCC AGT GG-3' [18]

Propagation of *Pasteurella multodica:*
Using a strict and regulated process, *Pasteurella multodica* (PM) was successfully propagated for this research. A number of critical procedures were taken during the propagation procedure in order to guarantee the culture's viability and purity.

Initial Growth and Inoculation:
The propagation process started when PM was incubated in the Brain Heart Infusion (BHI) broth at 37C^{o} for 24 hours [19].

Subculture on BHI Agar:
After a 24-hour incubation period in BHI broth, a subculture was carried out onto BHI agar plates. To isolate specific PM colonies, this transfer was necessary to guarantee a pure culture for further analyses, and isolate single colonies [19].

Secondary Subculture:
From the BHI agar plates, a single isolated colony was carefully chosen and sub-cultured onto a different set of BHI agar plates. It was a preventative action to better purify the culture at secondary sub-culture phase.

Microbiological Tests:
Microbiological tests were carried out to confirm the features of the culture after obtaining the secondary subculture on BHI agar. These assays comprised the determination of cell wall properties using Gram's staining, the measurement of metabolic activities using Oxidase and Catalase tests, and the visualization of PM morphology using Giemsa staining.

Molecular Confirmation:
Polymerase Chain Reaction (PCR) was used to positively identify the isolated culture as *Pasteurella multodica.* This molecular method includes amplifying particular DNA sequences that are specific to PM. For confirmation of the culture, identifying the species and ensuring the integrity of the culture was done through PCR [18].

Growth for High Density:
After the isolated strain's identity was established, it was sub-cultured in BHI broth and given another day at 37°C to continue growing. The goal was to create a dense culture in a 50ml growth culture that contained roughly $2x10^{8}$ Colony Forming Units per millilitre (CFU/ml). A dilution approach was used to calculate the CFU/ml count in order to accurately measure the bacterial population [20].

Bacterial inactivation and vaccine preparation:
To get the bacterial strain ready for more testing and use, a special process was applied. This strain preparation's process summed up as follows:

Bacterial Inactivation:
The first action included rendering the bacteria by adding a 0.4% formalin solution to the bacterial culture. To ensure the deactivation of the strain while maintaining its structural integrity, formalin was added in a regulated manner [21].

Evaluation of Purity, Safety, and Sterility:
After the inactivation procedure, a series of tests were carried out to check the established bacterial culture's purity, safety, and sterility. These tests were essential to make sure that the culture was devoid of pollutants, did not pose any threats to safety, and did not include any live bacteria [22].

Emulsification using ISA-71 Montanide Oil:
The formalized bacterial culture was then precisely blended 1:1 ratio with Montanide oil ISA-71. In order to improve the stability and efficiency of the finished product, this stage attempted to form an emulsion in which the bacterial components were evenly dispersed inside the Montanide oil [23].

Emulsification Procedure:
The emulsification procedure was completed in a high-speed mixer that ran for 5 minutes at a speed of 3000 revolutions per minute (rpm). This meticulous mixing process made sure that the bacterial culture was well incorporated into the Montanide oil, creating a uniform emulsion [23].

Preservation at 4°C:
The emulsified culture was then cautiously preserved at 4°C. The stability and viability of the emulsion had to be maintained at this low temperature until it was prepared for use in later tests or applications [24].

Preparation of Bivalent Vaccine:
For the preparation of bivalent vaccine, 25ml of the bacteria culture has been inactivated and 25ml of H5 inactivated Avain influnza H5 virus culture mixed with 50 ml of Montanide oil 71 as adjuvant.

Combine adjuvant and component:
50ml of Montanide oil 71 added to the combined bacterial and H5 mixture. The mixture agitated at a speed of 3000 rpm for five more minutes and store at 4ºC.

Sterility test:
An experimental batch of the prepared vaccine tested for sterility and freedom from any fungal or bacterial contaminants by culturing on specific media like MacConkey agar, Standard Method Agar, Baird Parker Agar and Sabouraud Dextrose Agar (SDA) [14].

Experimental design
A total of 100-day-old broiler chicks were selected. The chicks were divided into four groups with 25 birds in each group: Group 1 injected with monovalent H5, Group 2 with *P. multocida*, Group 3 with Bivalent H5 and *P. multocida* vaccine. Group 4 for control. Group 1, 2 and 3, received 1st Vaccine shot at 7th day of age and 2nd dose at day 14th day of age.

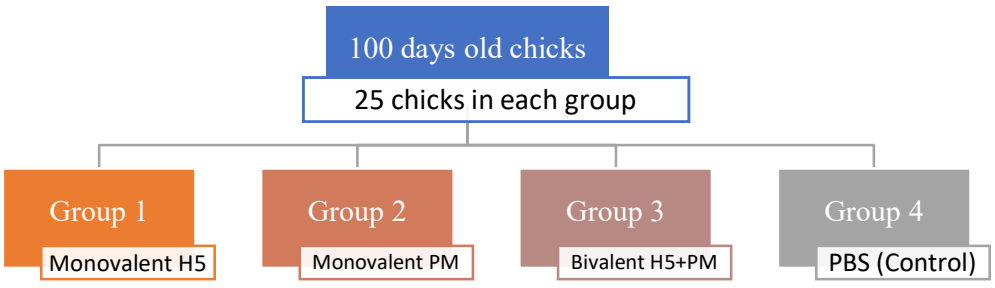

Figure 1: Experimental design for current study

Dose rate was 0.5 ml s/c. Chickens were housed in good facilities till they became 42 days of age with enough supply of water and feed. Blood samples were collected from each group at Day 1st, 7th

21th, 28th and 40th days post-vaccination. HI was used for detection of antibodies titer against H5, it was carried out using 4 HA Units of homologous antigen AIV, H5 strain, to estimate antibody titers in sera of vaccinated and unvaccinated chickens. ELISA (IDEXX) was used to detect titer against PM.

Challenge Study:
For challenge study, 5 chicks from the vaccinated and non-vaccinated groups were challenged with H5 and PM antigen at 42-day post vaccination in chicken isolater, 100 µl/chick of allantoic fluid containing 100 EID 50 of the virus and PM Culture antigen $2x10^2$ CFU/ml of 0.1ml intramuscular were injected . Tracheal swabs were collected at 2 to 3 days post challenge to determine the virus shedding. Protection percentage and mortality were observed [25].

Hemagglutination Inhibition Test:
The V-bottom microwell plate was used for micro hemagglutination test. This approach used 1 percent red blood cell solution and benefits from the faster cell settling in V-bottom plates, which improves the discrimination between positive and negative results in comparison to U-bottom plates [26].
For HA test, took the microtiter V bottom shape plate, added 50 µL of PBS (Phosphate Buffered Saline) from well 1 to 12th. Then, added 50ul of H5 antigen virus in first well and perform two-fold serial dilution up to 11th well in first row, in the next step, 50ul of 1% washed chicken RBCs were added from well 1st to 12th then incubated the Plate for 15 min. at 37ºC , read the plate and determined the 4HA unit well concentration, well no. 8th (1:256) was found to be 4HA units. Accordingly, prepared the 4HA unit concentration of virus for HI test with ratio of 1:256 with normal saline. For HI test, added 50ul PBS solution from well 1st to 12th in whole plate. Then added 50ul serum in first wells column wise [27].
Performed two-fold serial dilution from well 1st to 11th.. Added 50ul 4HA unit antigen of H5 in each well from well 1st to 11th. Incubated the plate for 30 min. at 25ºC. Added 50 ul of 1% washed chicken RBCs in each well from 1st to 12th, and last well used as RBCs control. Again Incubated for 30 min. at 25ºC. Read the Plate, considered the last tearing in well as HI titer.

ELISA Test Protocol:
ELISA is a widely-used immunoassay technique employed to quantify the levels of specific antibodies produced in response to vaccination. This protocol outlines the steps required to determine the humoral immune response against the target pathogens after vaccination.

Procedure:
Preparation of Microplate:
Labelled the microplate accordingly, indicating the sample identification.

Sample Preparation:
Serum samples were collected from vaccinated birds in blocking buffer at an appropriate dilution, typically 1:500.

Incubation with Serum Samples:
Added 100 µL of each diluted serum sample to the respective antigen-coated wells. Included the blank well with blocking buffer. Incubated the plate for 30min at room temperature. Washed the wells three times with PBS-T with 350ul volume.

Secondary Antibody Incubation:
Added 100 µL of the appropriate secondary antibody conjugated to HRP to each well. Incubated the plate for 1 hour at room temperature. Washed the wells three times with PBS-T with 350ul volume.

TMB Substrate Reaction:
Added 100 µL of TMB substrate solution to each well, including the blank well. Incubated the plate in the dark for 15-30 minutes or until color developed. The reaction was stopped by adding 100 µL of stop solution to each well.

Measurement:
Measured the absorbance of each well at 450 nm using an ELISA plate reader. Recorded the absorbance values (OD) for data analysis.

Data Analysis:
Calculated the antibody titer for each serum sample using appropriate statistical methods, such as a standard curve generated from known positive and negative control samples.

Interpretation:
Higher absorbance values indicate a stronger humoral immune response against the target pathogens [28].

$$\text{Titer calculation(S/P)} = \frac{\text{Sample OD} - \text{Negative control OD}}{\text{Positive control OD} - \text{Negative control OD}}$$

$$Log\ titer = 3.36\ x\ (log\ of\ S/p) + 1.09$$

S/P is Sample to Positive ratio

Results:
Sterility and safety of the prepared vaccines:
Prepared vaccine was checked for the presences of any contaminant (bacteria and fungus), both bivalent and monovalent vaccine were found free from any contaminants by culture technique. 0.1 ml formalized H5 antigen was injected into 9-day old embryonic egg and incubate at 37^0C for up to 6 days for presence of any mortality and lesions. No mortality was observed and antigen was found inactivated.

Microbiological Analysis:
Microbiological analysis was performed to confirm the sterility of the vaccine. This analysis sought to locate and get rid of any possible fungal, aerobic, or anaerobic bacterial contamination in the prepared individual vaccine or its bivalent combination. To avoid external contaminants, representative samples from each vaccination batch were aseptically collected in a controlled setting. The collected samples were subjected to microbiological culture in BSC-2. Sabouraud agar was used for the evaluation of fungi, Tryptic Soy Agar for the detection of aerobic bacteria, and MacConkey agar for the detection of coliform bacteria. Following incubation, the plate of agar colonies was closely inspected under a microscope to ascertain their features. To precisely distinguish between microorganisms, morphological characteristics and staining methods were used. To confirm the sterility of the culturing procedure, a control group of samples was kept alongside sterile growth medium.

Assessment of Pathological Lesions:
The embryos were carefully examined for any indications of clinical lesions to confirm presence or absence of any symptoms of viral replication or injury from the immunization. The absence of lesions offered compelling proof that vaccines were safe.

Hemagglutinin (HA) Activity:
To confirm that the virus in the vaccination was completely inactivated, HA activity for the H5 strain was evaluated. Successful inactivation was demonstrated by decreased HA activity by one log [26].

Mortality Assessment:
In 9 days old chicken embryos, the mortality rates were noted. The lack of fatalities provided more evidence of the vaccination's safety.

Comparative analysis of H5 titer:
Selected day-old chicks which were H5 free maternally derived Antibodies MDAs, therefore it was noticed that MDAs were zero at day one. First dose of vaccine was at 7th day and second was at 14th days. Dose rate was 0.5ml/ birds s/c. At 3rd week, after two weeks of first dose and one week of second dose, average titer of 15 samples in monovalent group (G-1) was 3.2 log, while in bivalent group (G-3) titer was 4.3 log. At 4th week after 3 weeks of first dose and 2 weeks of second dose the average titer of 15 samples in monovalent group (G-1) was 4.7 log while in bivalent group (G-3) is was 6.3, which mean within one week more than 50% titer increased. At 6th week, average titer of 15 samples in monovalent group was 5.3 log while in bivalent group was 7.1. In this study it was observed that H5 titer of bivalent vaccine have higher titers than monovalent at 6th week [29].

Monovalent (*Pasteurella multocida*):
Selected day-old chicks were *Pasteurella multocida* free MDAs, therefore it was notice that MDAs were below cut-off value of 396 titers in IDEXX ELISA kits. First dose of vaccine was applied on 7th day and second on 14th day. Dose rate was 0.5ml/ birds s/c. After two weeks of first dose and one week of second dose (21st day), average titer of 15 samples was 1000. After 3 weeks of first dose and 2 weeks of second dose (28th day) the average titer of 15 samples was 1600 which means within one week, the titer increased by ½ from 21st day to 28th day. Average titer of 15 samples was 2900 at 6th week of age.

Bivalent (*Pasteurella multocida* and H5):
First dose of vaccine was at 7th day and second was at 14th days, dose rate was 0.5ml/ birds (sub-cut). After two weeks of first dose and one week of second dose (21th days), Average titer of 15 samples was 1642. After 3 weeks of first dose and 2 weeks of second dose (viz. 28th day), the average titer of 15 samples was 2730, which mean within one week almost half of the titer was increased. Average titer of 15 samples was 4056 at 6th week of age as shown in graph and tables.

Comparative Trend analysis of Titers (Monovalent vs Bivalent)

Table 3: Comparative Analysis of Monovalent and Bivalent Vaccine Titer

Age	Group	ELISA- PM		HI H5
		Average Titer	CV	Average log 2 titer
6 weeks	G-3 (Bivalent)	4056	11	7.1
4 weeks		2730	12	6.3
3 weeks		1642	21	4.3
1 week		0	0	0
6 weeks	G-2 (Monovalent) *P. multocida*	2909	11	--
4 weeks		1700	30	--
3 weeks		1015	31	--
1 week		0	0	--
6 weeks	G-1 (Monovalent) H5	--	--	5.33
4 weeks		--	--	4.7
3 weeks		--	--	3.2
1 week		--	--	0

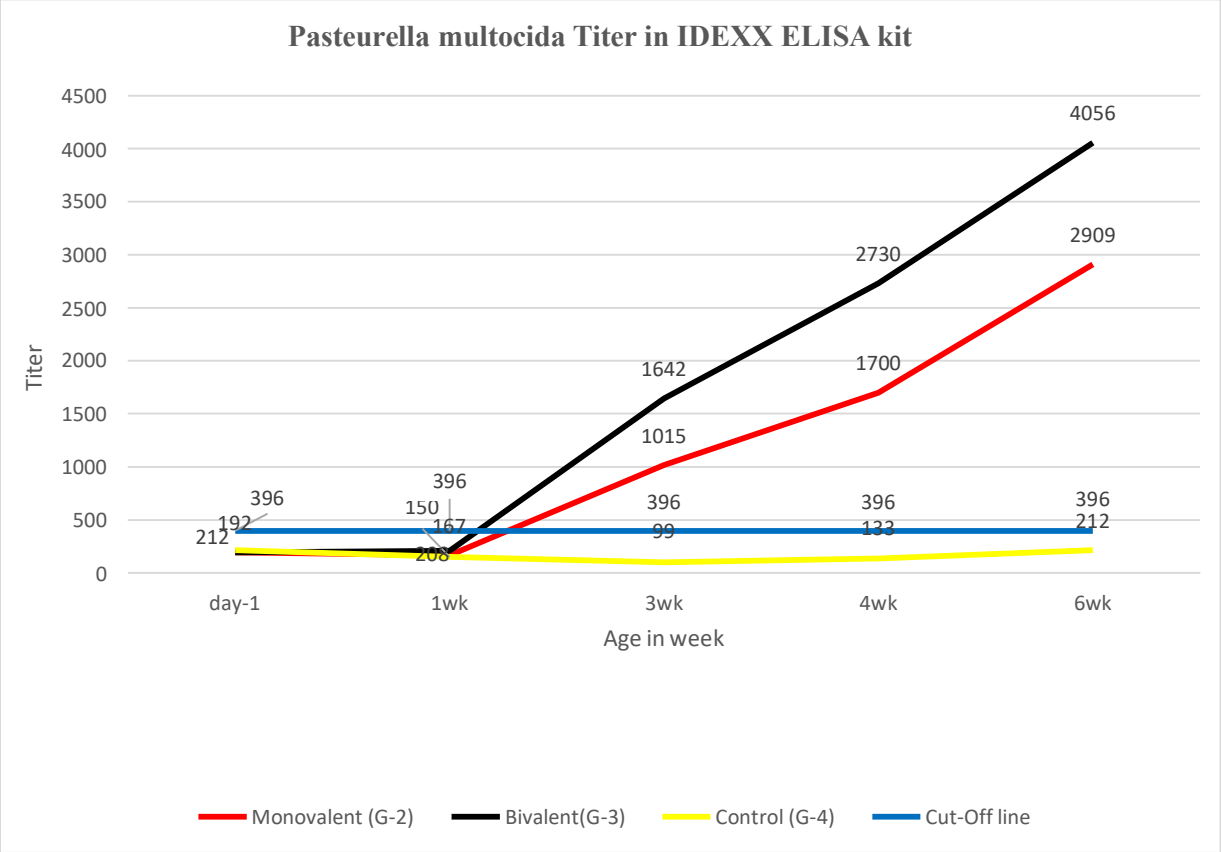

Figure 2: Comparative Analysis of Monovalent and Bivalent Vaccine Titer

Statistical analysis:

Table 4: Statistical analysis values of ELISA and HI test

Age	ELISA Titer (Arithmetic Mean)		HI Titer (Arithmetic Mean)	
	Monovalent	Bivalent	Monovalent	Bivalent
1wk	167	208	0	0
3wk	1015	1642	3.2	4.3
4wk	1700	2730	4.7	6.3
6wk	2909	4056	5.3	7.1
Chi-square value	1.5*		0.46	

*Marginally significant
Chi-square value >1 marginally significant, Chi-square value <1 significant

Discussion and Conclusion:

This study encompasses the humoral immune responses elicited in vaccinated chicks, the level of protection against AIV H5 and FC, and the potential role of bacterial proteins as adjuvants in the bivalent vaccine.

Humoral Immune Responses:

The primary objective of this study was to assess the humoral immune responses generated by bivalent AIV H5 and *P. multocida* in oil emulsion-based vaccine. The results demonstrated that both the monovalent and bivalent vaccines induced suitable humoral immune responses in the vaccinated chicks. However, there were differences in the magnitude and kinetics of the immune response between these two groups. In Group 3, which received the bivalent vaccine with Montanide ISA 71 adjuvant, a substantial increase in ELISA titers was observed within three weeks of vaccination. The ELISA titer of PM increased from 1302 to 3340 and H5 HI titer up to 5.8 at 6th week. Indicating a

robust and rapid antibody response to the antigens present in the vaccine. In Group 2, which received the monovalent vaccine, also exhibited an increase in ELISA titers but at a somewhat lower level, with titers rising from 1015 to 2909 within the same three-week period and H5 Hi titer at 6th week was 5.33. This suggests that the bivalent vaccine formulation with Montanide ISA 71 as well as bacterial proteins act as adjuvant in bivalent vaccine proving to be effective in stimulating an early and enhanced humoral immune response [30].

Protection against AIV H5 and FC:

An essential aspect of evaluating vaccine efficacy is the level of protection it provides against the target pathogen. In challenge study, we assessed the protective efficacy of the bivalent and monovalent vaccines against AIV H5 infection. The results demonstrated that Group 3, which received the bivalent vaccine with Montanide ISA 71 adjuvant, exhibited superior protection compared to the monovalent vaccine group and other control groups. A remarkable 88% protection rate was observed in Group 3 against H5 infection, indicating a high level of vaccine efficacy. In contrast, in monovalent vaccine group protection percentage was 75%. The control groups exhibited 100% mortality. These findings highlight the superiority of the bivalent vaccine in providing protection against AIV H5. This research suggests that bacterial proteins may act as potent adjuvants, augmenting the immune response triggered by the viral antigens in the vaccine. Future research could focus on elucidating the specific mechanisms through which these bacterial proteins enhance vaccine efficacy [31].

Implications and Future Directions:

Future research directions could include investigating the molecular mechanisms by which bacterial proteins enhance vaccine efficacy and exploring the safety and efficacy of this bivalent vaccine in different avian species. Additionally, further studies could assess the long-term durability of the immune response generated by this vaccine. The bivalent vaccine further demonstrated its effectiveness in fostering a strong humoral immune response by inducing superior antibody titers [31]. The utilization of locally isolated strains of *P. multocida* and H5 in the vaccine formulation is one of this study's remarkable features. This decision highlights the significance of creating regionally specialized vaccines that are adapted to the dominant pathogenic strains. Utilizing local isolates can boost the likelihood of a good response to the specific diseases prevalent in Pakistan's poultry populations. Significant ramifications for the poultry business and public health result from the successful development of a bivalent vaccination against Avian Influenza H5 and Fowl Cholera. In another study, bivalent vaccine against rabbit hemorrhagic disease virus and *Pasteurella multocida* was successfully developed and induced the good immune response [32].

In terms of the economy, the vaccine has the ability to lessen the losses brought on by disease epidemics, such as lower production and elevated mortality rates. A zoonotic threat, avian influenza must be controlled from a public health standpoint. The danger of transmission to humans can be decreased by preventing outbreaks in poultry. CpG motif DNA is prevalent in bacteria that act as superior adjuvant, it boosted the antigen specific antibody response combination with viral vaccine [33]. Fowl cholera is also a persistent threat to poultry health and may indirectly affect human health due to safety of food [34].

In conclusion, this study has shown how locally isolated strains can be used to successfully produce and test a bivalent vaccination against Avian Influenza H5 and Fowl Cholera. *Pasteurella multocida* proteins are effective and can act as adjuvant that trigger the immune response [35]. The vaccine has demonstrated significant immunogenicity, as demonstrated by strong humoral immune response, and it has proven to be very successful in defending broiler flocks against challenges with the relevant infections in experimental study. The results of this study hold enormous promise for the poultry business in Pakistan. It provided applied evidence to devise pathogen-specific strategy to minimize the negative effects of avian influenza and fowl cholera in poultry sector. Furthermore, studies are required to thoroughly evaluate the vaccine's performance in field situations to better asses the vaccine performance.

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