RESEARCH ARTICLE AGROBIOLOGICAL RECORDS
ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
Open Access Journal

PREPARATION AND EVALUATION OF AVIAN INFLUENZA (H9) AND NEWCASTLE DISEASE (THERMOSTABLE I-2 STRAIN) BIVALENT VACCINE FOR COMMERCIAL POULTRY

M. Shahid Mahmood* and Rabia Sabir

Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan

*Corresponding author: shahiduaf@gmail.com

ABSTRACT

The goal of current research was production of bivalent adjuvant based inactivated I-2 strain of Newcastle Disease (ND) and Avian Influenza (AI) vaccine and to compare efficacy with commercially available bivalent inactivated vaccine. Experiment was carried out on one-day-old broiler chicks by dividing them into 3 groups A, B & C. Vaccine was injected on 7, 14, 21 and 28 day. Blood samples were collected on 7, 14, 21, 28 and 35 day. Results of Hemagglutination Inhibition (HI) test presented the geometric mean titers of group A and B were <1:4, 1:18.8, 1:115.2, 1:358.4, 1:128 & <1:4, 1:8.4, 1:36.8, 1:56, 1:22.4 respectively for AI. Similarly the geometric mean titers of group A and B were <1:4, 1:19.6, 1:89.6, 1:320, 1:115.2 & <1:4, 1:9.2, 1:25.6, 1:36.8, 1:22.4, respectively for ND. However, control group showed <1:4 geometric mean titer. The challenge was given on 28th day and birds were monitored for next 7 days for clinical signs. The chicks of group A showed no clinical signs of diseases (AI, ND). However, in group B 60% morbidity and 90% mortality was recorded on 4th and 6th day of post challenge, respectively. While 100% mortality was recorded in control group.

***Keywords*:** Avian influenza, Bivalent vaccine, Broilers, HI test, Newcastle Disease.

Article History (2020-0725) || Received: 05 Jul 2020 || Revised: 26 Aug 2020 || Accepted: 15 Sep 2020 || Published Online: 07 Oct 2020

1. INTRODUCTION

Avian Influenza virus (AIV) and Newcastle Disease virus (NDV) are responsible for causing Avian Influenza (AI) and Newcastle Disease (ND), respectively in poultry, both of which adversely affect the poultry industry in terms of massive economic losses (Hurtado et al. 2014). The genome of NDV and AIV are single stranded, negative sense RNA. AIV primarily infects the respiratory tract while NDV may infect the respiratory tract, visceral organs and nervous system of birds. Therefore, birds infected with AIV or NDV show decreased production of meat and eggs even if they survive (Iqbal et al. 2013; Richard et al. 2014).

Avian Influenza belongs to Orthomyxoviridae family, consists of genera that are influenza type A, B and C. Avian infections are mostly instigated by influenza type A (Cheema et al. 2011). Genome of AIV consists of enveloped, negative sense, single stranded, eight segments of RNA encoding eleven types of viral proteins (Saleem et al. 2011). Surface of enveloped AIV is covered with two types of glycoproteins spikes or projections that are Hemagglutinins (HA) and Neuraminidase (NA) (which is compulsory for infection). There are eighteen and eleven antigenically distinct HA and NA, respectively which leads to 198 different combinations (serotypes) of AIV so genetic drift and shift is common in AIV (Iqbal et al. 2013). AI is caused by different serotypes e.g. H3N2, H5N1, H7N3 and H9N2.

Newcastle Disease (ND) is another main infection of poultry due to the massive economic losses related to the pathogenic form of NDV (on both domestic and commercial poultry). Causative agent of ND is Newcastle Disease Virus (NDV) also known as Avian Paramyxovirus serotype-I (APMV-I) which belongs to family Paramyxoviridae and genus Avulavirus. It is an enveloped, single stranded, negative sense and non-segmented RNA virus (Chukwudi et al. 2011). Surface of enveloped NDV is covered with different types of antigenic structures but most important are Fusion Protein (F Protein), Hemagglutinins (HA) and Neuraminidase (NA) which is glycoprotein in nature. NDV is antigenically stable so genetic re-assortment of NDV not takes place (Feizi et al. 2011).

Biosecurity and vaccination are the most cost-effective measures for controlling ND and AI. In this regard, bivalent oil emulsion vaccines are considered most important due to their high efficacy and appropriate safety against infections. Single dose of bivalent formulations is of high impact and ensure long lasting immunity in poultry birds. Bivalent vaccines usage has relatively less labor cost and it also minimize the pain inflicted to the birds due to administration of monovalent vaccines for two diseases (Park et al. 2006).

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2. MATERIALS AND METHODS

2.1. Viruses, Adjuvant and Virus Cultivation

Well characterized Avian influenza virus serotype H9N2 was procured from Poultry Research Institute, Rawalpindi. While well characterized Newcastle disease virus was procured from Institute of Microbiology, University of Agriculture Faisalabad. Water-in-oil Montanite adjuvant ISA-206 was used for the preparation of research product. Total 180, 9-10 days old embryonated chicken eggs were obtained from Poultry Research center, University of Agriculture, Faisalabad. The eggs were first sterilized with 70% alcohol and divided into 3 groups. The groups A and B were inoculated with 0.2ml of AIV and NDV, respectively via chorio-allantoic route. While group C was kept as control. The eggs were then re-incubated at 37°C for 48-72 hours, refrigerated at -20°C for 4 hours, allantoic fluid was harvested and checked for hemagglutination activity.

2.2. Raising of Hyperimmune Sera

Twenty rabbits were raised in Animal House, Institute of Microbiology, University of Agriculture, Faisalabad and divided into 2 groups. One group received ND live virus vaccine and second group received inactivated AI (H9 serotype) vaccine. Both groups were vaccinated at the rate of 0.1, 0.3, 0.5, 0.7, 0.9 and 1.0 per rabbit at days 1, 3, 5, 7, 9 and 11, respectively. After 28 days of last inoculation, the rabbits were bled and 5ml of blood were collected from each rabbit in separate disposable sterile syringe and kept at slant position for an hour then refrigerated for 24 hours and serum were collected in sterile bottles and stored at -20°C until further use.

2.3. HA Test, HI Test, AGPT and Virus Titration

NDV and AIV were then checked for hemagglutination activity by performing Hemagglutination (HA) test as described by Sasaki et al. (2009). The end point titer and 4 HAU were calculated for both viruses (separately). Hemagglutination Inhibition (HI) test was performed as described by Shakal et al. (2013) for the re-confirmation of both viruses. Agar Gel Precipitation Test (AGPT) was also performed as described by Cheema et al. (2011), for virus confirmation. Virus Titration **(**EID_{50}) was calculated by the method as described by Kydyrbayev et al. (2010).

2.4. Virus Inactivation and Vaccine Preparation

Both antigens were separately inactivated with 0.12% formaldehyde for 48 hours at 37°C as described by Khalili et al. (2015) and then mixed for vaccine preparation. Vaccine was prepared by taking 3 parts (v/v) of antigen and 7 parts (v/v) of Montanide adjuvant ISA-206. The concentration of both antigen in aqueous phase was maintained at $10^{6.5}$ EID_{50}. First Montanide Adjuvant ISA-206 poured in the beaker and rotated at 20rpm then antigens were added slowly, and rotation was enhanced to 70rpm for 20 minutes and then homogenized at approximately 7000rpm for 5 minutes (Pour et al. 2006).

2.5. Vaccine Quality Control Tests

The Bivalent vaccine was subjected to quality control tests such as sterility and safety tests. In sterility testing the vaccine was inoculated on Nutrient agar, Sabouraud's agar, MacConkey agar and PPLO. Agar plates were incubated at 37°C and examined for any growth for 5 days. In safety testing the vaccine was inoculated into 9-10 days old embryonated chicken eggs and candled after 24 hours for 10 days, to check the livability of embryos and then allantoic fluid was harvested and subjected to HA test.

2.6. Experimental Design

A total of 150-day old broilers was reared in the Animal House, Institute of Microbiology, University of Agriculture, Faisalabad and divided into 3 groups. Group A and B were vaccinated at the rate of 0.3ml/bird via subcutaneous route, with research product and commercially available bivalent vaccine, respectively. While group C was kept as control. Vaccination was done at days 7, 14, 21 and 28. Blood samples were collected at day 7, 14, 21 and 28. Then birds were challenged with viruses (AIV and NDV) at day 28 and blood samples were collected at day 35. Antibody titers were then determined by performing HI test (Shakal et al. 2013).

3. RESULTS

In present research, HA titer of AIV and NDV was 1:256 and 1:128, respectively. For the confirmation of viruses (AIV and NDV) HI test was performed and the result revealed that the antiserum of AIV and NDV bind with the AIV and NDV respectively as antigen and antibody was specific to each other so when 1% suspension of chicken erythrocytes was added then button formation was formed which confirm that both viruses were AIV and NDV.

AGPT was performed for the further confirmation of AIV and NDV. Results discovered that the antigen and antibody was specific to each other so white precipitation line was formed between the wells which were seen under UV light. The allantoic fluid having NDV and AIV was processed for determination of EID_{50}. The EID_{50} was

ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
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$10^{6.6}$/ml/bird and $10^{6.4}$/ml/bird for AIV and NDV, respectively. After virus titration, both viruses were subjected to inactivation with 0.12% formaldehyde and bivalent inactivated adjuvant-based vaccine was prepared.

Vaccine sterility tests revealed that there was no contamination. While in vaccine safety testing no embryo, mortality was recorded, and harvested allantoic fluid was subjected to HA test which show no hemagglutination activity, so vaccine was sterile and safe to use. Then bivalent inactivated vaccine efficacy was assessed in Broilers through HI test. HI antibody titers after vaccination with research product and commercially available bivalent vaccine are shown in Table 1 and 2. As day 7 was considered as zero day so before vaccination, birds were checked for AI and ND antibody titers through HI test and result show no antibody titer against them. A week interval geometric mean titers for AI was recorded as <1:4, 1:18.8, 1:115.2, 1:358.4 & 1:128 in A group, <1:4, 1:8.4, 1:36.8, 1:56& 1:22.4 in B group. Similarly the geometric mean titers for ND was recorded as <1:4, 1:19.6, 1:89.6, 1:320 &1:115.2 in A group, <1:4, 1:9.2, 1:25.6, 1:36.8 & 1:22.4 in B group. However, in control group all broilers show the <1:4 geometric mean titer. GMT based comparison among 2 vaccines are shown in Fig. 1.

Table 1: HI antibody titers of Avian Influenza vaccine in broilers

Groups	Broiler #	HA units/dose	HI antibody titers a week interval vaccination in broilers				
			1 (7th Day)	2 (14th Day)	3 (21st Day)	4 (28th Day)	5 (35th Day)
A	1	1:256	<4	8	64	256	128
	2		<4	32	128	512	128
	3		<4	8	64	128	64
	4		<4	16	256	512	256
	5		<4	4	128	512	128
	6		<4	32	128	256	64
	7		<4	8	64	128	64
	8		<4	32	128	512	128
	9		<4	16	64	256	64
	10		<4	32	128	512	256
		GMT*	**<4**	**18.8**	**115.2**	**358.4**	**128**
B	1	1.32	<4	4	16	64	32
	2		<4	16	64	32	16
	3		<4	4	16	64	16
	4		<4	4	16	16	16
	5		<4	8	64	32	32
	6		<4	16	32	64	32
	7		<4	4	16	64	16
	8		<4	8	64	32	16
	9		<4	4	16	128	16
	10		<4	16	64	64	32
		GMT	**<4**	**8.4**	**36.8**	**56**	**22.4**
Control	1		<4	<4	<4	<4	<4
	2		<4	<4	<4	<4	<4
	3		<4	<4	<4	<4	<4
	4		<4	<4	<4	<4	<4
	5		<4	<4	<4	<4	<4
	6		<4	<4	<4	<4	<4
	7		<4	<4	<4	<4	<4
	8		<4	<4	<4	<4	<4
	9		<4	<4	<4	<4	<4
	10		<4	<4	<4	<4	<4
		GMT	**<4**	**<4**	**<4**	**<4**	**<4**

*Geometric Mean Titer

Clinical signs were observed for 7 days after challenged the broilers with AIV (H9N2) and NDV as presented in Table 3. No clinical signs were observed in broilers of group A with antibody titers ranges from 1:128 to 1:512 for both AI and ND. However, broilers of group B show no clinical sins in first three days with antibody titers ranges from 1:16 to 1:128 for AI and 1:16 to 1:64 for ND. On 3rd day of post challenge (p.c) 60% of the broilers in group B exhibit typical clinical signs of AI and ND on 4th day of p.c and 90% broilers were dead on 6th day of p.c.

Broiler # 1, 4 and 8 showed typical signs of AI on 4th day of p.c and died on 6th day of p.c. Broiler # 2 & 3 showed typical signs of ND on 4th day of p.c. But broiler # 2 showed digestive signs of ND and died on 7th day of p.c. However, broiler # 3 showed nervous signs of ND and died on 5th day of p.c. Broiler # 5, 6 & 10 showed mixed type of signs for both diseases (ND and AI) and died on 7th, 5th and 6th day of p.c respectively. Broiler # 7 showed

ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
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no clinical signs and died on 5th day of p.c. Similarly, broiler # 9 died on 6th day of p.c without showing any clinical signs. Broilers of control group started exhibiting signs on 2nd day of p.c and all broilers were dead on 4th day of p.c.

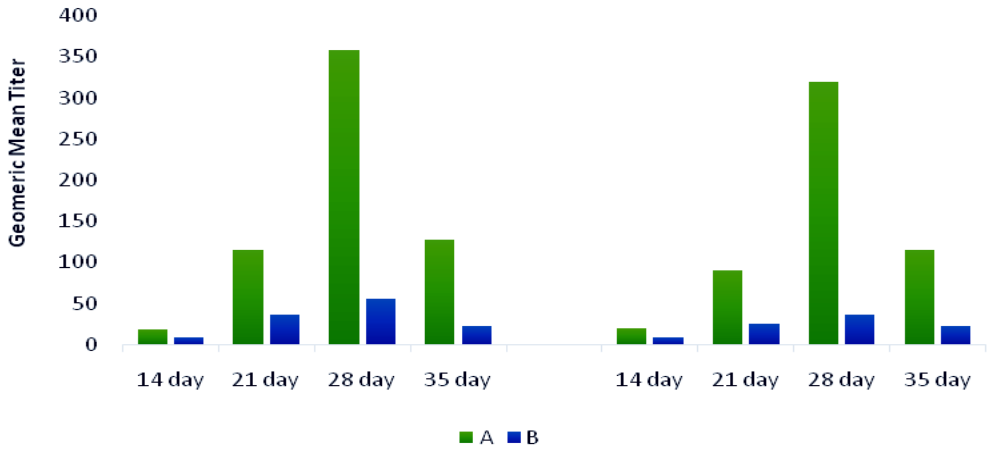

Fig. 1: GMT based comparison of research product and commercially available vaccine among broilers of Group A and B

Table 2: HI antibody titers of Newcastle Disease vaccine in broilers

Groups	Broiler #	HA units/dose	HI antibody titers a week interval vaccination in broilers				
			1 (7th Day)	2 (14th Day)	3 (21th Day)	4 (28th Day)	5 (35th Day)
A	1	1:128	<4	16	64	128	64
	2		<4	8	64	512	128
	3		<4	32	128	512	64
	4		<4	4	64	256	128
	5		<4	32	128	128	64
	6		<4	8	64	512	256
	7		<4	16	128	256	128
	8		<4	16	128	128	64
	9		<4	32	64	512	128
	10		<4	32	64	256	128
GMT*			**<4**	**19.6**	**89.6**	**320**	**115.2**
B	1	1:16	<4	8	16	16	16
	2		<4	4	32	16	32
	3		<4	16	32	32	16
	4		<4	8	16	64	32
	5		<4	16	16	32	16
	6		<4	4	16	16	16
	7		<4	8	64	32	32
	8		<4	4	16	64	32
	9		<4	16	32	64	16
	10		<4	8	16	32	16
GMT			**<4**	**9.2**	**25.6**	**36.8**	**22.4**
Control	1		<4	<4	<4	<4	<4
	2		<4	<4	<4	<4	<4
	3		<4	<4	<4	<4	<4
	4		<4	<4	<4	<4	<4
	5		<4	<4	<4	<4	<4
	6		<4	<4	<4	<4	<4
	7		<4	<4	<4	<4	<4
	8		<4	<4	<4	<4	<4
	9		<4	<4	<4	<4	<4
	10		<4	<4	<4	<4	<4
GMT			**<4**	**<4**	**<4**	**<4**	**<4**

* Geometric Mean Titer

ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
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4. DISCUSSION

Results of HI test presented that, there is correlation between antibody response of vaccinated broilers and the antigen level of vaccine. Veits et al. (2006), Hu et al. (2017) and Xu et al. (2019) reported the recombinant attenuated Newcastle disease virus expressing H9 subtype hemagglutinin protected chickens from challenge by genotype VII virulent Newcastle disease virus and H9N2 avian influenza virus. They have reported the promising protection against challenge. In case of research product vaccine, which was administered to broilers of group A, the minimum antibody titer which could protect the broilers from the AI and ND signs and death was deliberated as 1:32 (both). As the birds having antibody titers ranges from 1:4 to 1:16 died next day of p.c. However, birds having antibody titers 1:32 and above not show any clinical signs of AI and ND and remains protected from these diseases. Zhao et al., 2017 also reported the same findings for bivalent vaccine against ND and AIV.

In group B which was vaccinated with the commercially available vaccine, the minimum antibody titer was 1:64 for the protection of birds from infection. But this protection lasts for three days of p.c and then birds start showing signs of both diseases and eventually death occur on 6th day of p.c. Heba et al. (2017) studied the mucosal nanoparticles and polymer-based inactivated vaccine for Newcastle disease and H9N2 AI viruses.

Table 3: Clinical signs observed in Broilers after challenge with Avian Influenza (H9N2) virus and Newcastle Disease virus

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
A	1	1:256 (for AI)	256	128	-	-	-	-	-	-	-
	2		512	512	-	-	-	-	-	-	-
	3		128	512	-	-	-	-	-	-	-
	4	1:128 (for ND)	512	256	-	-	-	-	-	-	-
	5		512	128	-	-	-	-	-	-	-
	6		256	512	-	-	-	-	-	-	-
	7		128	256	-	-	-	-	-	-	-
	8		512	128	-	-	-	-	-	-	-
	9		256	512	-	-	-	-	-	-	-
	10		512	256	-	-	-	-	-	-	-
B	1	1:32 (for AI) 1:16 (for ND)	64	16	-	-	-	+	+	D	
	2		32	16	-	-	-	+	+	+	D
	3		64	32	-	-	-	+	D		
	4		16	64	-	-	-	+	+	D	
	5		32	32	-	-	-	-	-	+	D
	6		64	16	-	-	-	+	D		
	7		64	32	-	-	-	-	D		
	8		32	64	-	-	-	+	+	D	
	9		128	64	-	-	-	-	-	D	
	10		64	32	-	-	-	-	+	D	
Control	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 showed no abnormal signs, +) Birds showed typical signs of diseases (diarrhea, torticollis, anorexia, respiratory distress etc), D) Dead

Ali et al. (2017) reported the protective efficacy of combined trivalent inactivated ISA 71 oil adjuvant vaccine against avian influenza virus subtypes (H9N2 and H5N1) and Newcastle disease virus. Ali et al. (2016) evaluated the cross protection against avian influenza virus (AIV) and Newcastle disease virus (NDV) in broiler chickens after vaccination in Pakistan. Kai-Yue et al. (2020) reported the protection of chickens against hepatitis-hydropericardium syndrome and Newcastle disease with a recombinant Newcastle disease virus vaccine expressing the fowl adenovirus serotype 4 fiber-2 protein. As research product bivalent vaccine containing 1:256 HA unit/dose for AI and 1:128 unit/dose for ND, exhibit not only rapid and better immunological response but also provide long lasting and high level of immunity for several weeks after a booster shot of vaccine. However, the HA unit/dose for

ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
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commercially available bivalent vaccine was 1:32 and 1:16 for AI and ND respectively which could not provide rapid and long-lasting immunity to birds even after 2 booster doses. Birds sooner become ill and 60-70% mortality was recorded. Ahmed et al. (2019) studied the role herbal medicine additives as powerful agents to control and prevent Avian influenza virus in poultry. Herbal products may be the next target for the field treatment and control of Avian Influenza, Newcastle disease viruses and other viruses of veterinary and poultry importance.

Conclusion: In conclusion, the mechanism of immunization of broilers with bivalent vaccine was to aggravate the immune response deprived of causing infection itself against viral infections. Viral antigens should be neutralized by the antibodies, which were produced by vaccine. The vaccination of broilers was chiefly depending upon diverse factors such as quality and efficacy of immunization, birds' health status, levels of parental antibodies and methods of vaccination. So, the research product bivalent inactivated vaccine accomplished this basic mechanism but unfortunately the commercially available bivalent vaccine could not fulfil this basic mechanism of vaccination.

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