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DEVELOPMENT OF STANDARD PROTOCOLS FOR PREPARATION AND EVALUATION OF LIVER HOMOGENATE VACCINES AGAINST HYDROPERICARDIUM SYNDROME VIRUS IN POULTRY

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ABSTRACT

Twelve different vaccines were prepared from hydropericardium syndrome (HPS) infected liver homogenate by using two different virus concentrations ($1x10^2$ and $1x10^3$ ALD_{50}) and two virus inactivants (0.1% formalin and 0.01% binary ethyleneimine) with and with out different adjuvants. These vaccines were evaluated in 13 groups of broilers (8 in each group) for their comparative immunogenicity and protection. At day 14 of age, chicks of groups A1, B1, C1 and D1 were vaccinated with 4 oil based vaccines (OB-HPSV) with two different virus concentrations ($1x10^2$ & $1x10^3$ ALD_{50}) and two different inactivants (0.1% formalin and 0.01% binary ethyleneimine), respectively. Chicks of groups A2, B2, C2 and D2 were vaccinated with 4 aluminized vaccines (AH-HPSV) using two virus concentrations and two inactivants. Similarly, chicks of groups A3, B3, C3 and D3 were vaccinated with non adjuvanted vaccines (NA-HPSV) with two virus concentrations and two inactivants. Group E was kept as unvaccinated control. All the vaccinated birds were found sero-positive 7 days post vaccination (PV). IHA GMT results indicated no difference for different virus concentrations and different virus inactivants with same adjuvant ($P>0.05$). The IHA GMT recorded weekly during 0-28 days PV was the highest and more consistent (52-181) for OB-HPSV, followed by AH-HPSV (52-147) and NA-HPSV (73.3-104). The protection recorded for OB-HPSV vaccine was 100%, for AH-HPSV vaccine it 87.5% and with NA-HPSV vaccine it was 62.5% when challenged at 21 days post vaccination (PV). It was concluded that $1x10^2$ ALD_{50} oil based vaccines inactivated with either formalin or binary ethyleneimine can be recommended for commercial use.

Key words: Hydropericardium syndrome, ALD_{50}, oil based vaccine, alum based vaccine, protection.

INTRODUCTION

Hydropericardium syndrome, (HPS), also known as Angara disease, is a common viral disease of growing broilers (3-5 weeks of age). This disease is caused by avian adenovirus serotype-4 (Rabbani and Naeem, 1996) and characterized by high morbidity and mortality (up to 80%), with hydropericardium, necrosed and enlarged pale liver, enteritis, reactive spleen, congestion of lungs, haemorrhages on heart and kidneys as major post mortem findings (Anonymous, 1988; Chishti *et al*., 1989; Rabbani *et al.*,1998). In Pakistan, first out break of HPS occurred at Angara Goth, near Karachi in August, 1987. During 1989, more than 25% of broiler farms were closed due to this disease and losses due to HPS were estimated as more than Rs. 80 billions/annum (Khalid, 2003). HPS was first characterized as a hydropericardium-pulmonary edema with hepato-nephritis (Anonymous, 1988). Later Rabbani (1997) suggested the etiopathological name of the disease as infectious hydro-pericarditis-hepatitis syndrome (IHH).

Diagnosis of the disease before the appearance of symptoms is difficult since the birds do not show specific clinical signs. Sudden mortality at the third week of age and necropsy findings such as hydropericardium and demonstration of basophilic intranuclear inclusion bodies in hepatocytes are considered as pathognomonic. Indirect haemagglutination assay (IHA), enzyme linked immunosorbant assay (ELISA), agar gel precipitation test (AGPT), virus neutralization test (VN), dot immunobinding assay (DIA) and immuno-electrophoresis using infected liver homogenate filtrate as crude antigen, with specific antiserum have also been used in HPS diagnosis (Rabbani, 1997).

Standard vaccines are available against most of the poultry diseases, but the literature regarding the standard protocols for development and evaluation of avian HPS virus vaccines have been scanty. Therefore, the present project was designed to produce HPS virus vaccines from infected liver homogenate using two virus inactivants, with and with out different adjuvants. These vaccines were evaluated in experimental broilers for their comparative immunogenicity and protection.

MATERIALS AND METHODS

Three weeks old broilers chicks (n = 21) were purchased from market and shifted to the experimental

shed of the Department of Microbiology, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. On day 26 of age, 15 birds were challenged with virulent hydropericardium syndrome (HPS) virus from frozen virus seed (obtained from University Diagnostic Laboratory, UVAS, Lahore) and remaining 6 birds were kept as non challenged control. Birds were observed for 5 days post challenge (PC) for any mortality and the results were recorded. The moribund livers of HPS infected dead birds were removed, placed in containers and stored at -20^0 C.

One gram of HPS virus infected liver was homogenized in an electric homogenizer with 9 ml of normal saline (pH 7.2) and centrifuged at 3000 rpm for 10 minutes. The clear supernatant was taken out and Gentamycin @ 200 ug/ml, Penicillin @ 1000 i.u./ml and Streptomycin @ 100 ug/ml were added to the supernatant fluid (Hussain *et al*., 1996). This 10% infected liver homogenate was processed for determination of animal lethal dose 50 (ALD_{50}), as described by Reed and Muench (1938).

Infected liver homogenate (10%) was diluted to achieve virus suspensions having $1x10^2$ and $1x10^3$ ALD_{50} HPS virus and inactivated by using formalin in the ratio of 1:1000 (Saif, 2003) and 0.001M binary ethyleneimine (BEI) in ratio of 1:10,000 (Anonymous, 1988). Each of the inactivated virus suspension was subjected to safety and sterility testing, as described by Khalid (2003).

Formalin inactivated virus suspensions ($1x10^2$ and $1x10^3$ ALD_{50}) were processed for vaccine preparation by admixing each virus suspension in ratio of 2:3 with oil base (span 80 = 7 parts, tween 80 = 3 parts and liquid paraffine = 90 parts), aluminium hydroxide gel (Malik, 2003) and no adjuvant and following 6 vaccines were prepared: (i) Formalized $1x10^2$ ALD_{50} oil base HPS vaccine (F-OB-HPSV-1), (ii) Formalized $1x10^2$ ALD_{50} aluminized HPS vaccine (F-AH-HPSV-1), (iii) Formalized $1x10^2$ ALD_{50} no adjuvant HPS vaccine (F-NA-HPSV-1), (iv) Formalized $1x10^3$ ALD_{50} oil base HPS vaccine (F-OB-HPSV-2), (v) Formalized $1x10^3$ ALD_{50} aluminized HPS vaccine (F-AH-HPSV-2) and (vi) Formalized $1x10^3$ ALD_{50} no adjuvant HPS vaccine (F-NA-HPSV-2).

Similarly, binary ethyleneimine (BEI) inactivated virus suspensions ($1x10^2$ and $1x10^3$ ALD_{50}) were admixed with oil base, aluminium hydroxide gel and no adjuvant and following 6 vaccines were again prepared; (i) BEI inactivated $1x10^2$ ALD_{50} oil base HPS vaccine (BEI-OB-HPSV-1), (ii) BEI inactivated $1x10^2$ ALD_{50} aluminized HPS vaccine (BEI-AH-HPSV-1), (iii) BEI inactivated $1x10^2$ ALD_{50} no adjuvant HPS vaccine (BEI-NA-HPSV-1), (iv) BEI inactivated $1x10^3$ ALD_{50} oil base HPS vaccine (BEI-OB-HPSV-2), (v) BEI inactivated $1x10^3$ ALD_{50} aluminized HPS vaccine (BEI -AH-HPSV-2 and (vi) BEI inactivated $1x10^3$ ALD_{50} no adjuvant HPS vaccine (BEI-NA-HPSV-2).

For evaluation of these 12 vaccines, 104 day-old broiler chicks were purchased from market and divided into 13 groups (each having 8 chicks). Birds of each group were marked and vaccinated at 14^{th} day of age with 0.5 ml of vaccine subcutaneously as shown in Table 1. Birds of group-E were kept as non vaccinated control. Blood was collected randomly from 4 birds of each group weekly after vaccination (AV) up to 4 weeks, serum was separated and heated at 56^0C for 40 minutes to inactivate complement. Each serum sample was subjected to indirect haemagglutination assay (IHA) and geometric mean titer (GMT) for each group was calculated. On day 35 of age (21 days AV), all the birds were given challenge dose of virulent HPS virus (1 ml 40% w/v infected liver homogenate). Mortality and morbidity of all challenged birds were recorded up to 7 days post challenge (PC) and the relationship of IHA GMT with susceptibility was determined. Analysis of variance and DMR test were applied using SPSS software.

Table 1: Vaccination schedule for birds of different groups

Groups	No of chicks	Vaccine	Virus concentration (ALD_{50})
A1	8	F-OB-HPSV-1	1×10^2
A2	8	F-AH-HPSV-1	1×10^2
A3	8	F-NA-HPSV-1	1×10^2
B1	8	F-OB-HPSV-2	1×10^3
B2	8	F-AH-HPSV-2	1×10^3
B3	8	F-NA-HPSV-2	1×10^3
C1	8	BEI-OB-HPSV-1	1×10^2
C2	8	BEI-AH-HPSV-1	1×10^2
C3	8	BEI-NA-HPSV-1	1×10^2
D1	8	BEI-OB-HPSV-2	1×10^3
D2	8	BEI-AH-HPSV-2	1×10^3
D3	8	BEI-NA-HPSV-2	1×10^3
E*	8	Control	

E*= control non vaccinated group.

RESULTS AND DISCUSSION

HPS was reproduced in three weeks old broilers (not vaccinated against HPS) by injecting 40% w/v infected liver homogenate. After 42 hours, mortality started and reached to its peak 70-90 hours post challenge (PC). Dead birds showed characteristic hydropericardium with enlarged, pale, necrosed liver, congestion of lungs, enteritis and hemorrhages on heart and kidneys. These findings are congruent with those of Khwaja *et al*. (1988) and Anjum *et al*. (1989).

HPS infected liver homogenate (10%) was tested against known antiserum through AGPT for confirmation of HPS agent. After 24 hrs of incubation, a clear precipitation band was observed (Kumar *et al.*, 1997). ALD_{50} was calculated as $10^{-4.4}$/ml after this confirmation.

All birds vaccinated at day 14 of age were challenged with virulent HPS virus on day 35 of age and were observed for 7 days post challenge for mortality. Birds given oil based HPS vaccines (A1, B1, C1 and D1 groups) showed 0% mortality and 100% protection (Table 2). Birds vaccinated with aluminium hydroxide gel adjuvanted HPS vaccine (A2, B2, C2 and D2 groups) showed variable responses in terms of percent mortality and protection. The lowest mortality (0%) and highest protection percentage (100%) were recorded for group B2, C2 and D2, while in group A2 there was 12.5 per cent mortality Table 2). The protection percentage in vaccinated birds was found to be highest for oil based vaccine (100%), followed by aluminized vaccine (87.3-100 %) and non adjuvant vaccine (57.5%), when challenged with virulent virus inoculums at day 21 post-vaccination (PV). This may be due to higher antibody level in response to oil adjuvanted vaccine. These findings are in line with the observations of Hussain *et al*. (1996).

All vaccinated birds were found sero-positive 7 days AV when assayed by IHA. Hussain *et al*. (1996) and Noor-ul-Hassan *et al.* (1994) also found similar results, while Chishti *et al*. (1989) and Sarwar *et al*. (1995) reported seroconversion in 10-14 days after vaccination against HPS in broiler chickens. This difference in seroconversion rate is supposed to be due to composition of autogenous vaccines, breed of chickens, managemental condition, time of vaccination (bird immune status) or type of serological test.

Statistical analysis showed that antibody titers at day 7 PV in groups A3, B3 and C3 (F-NA-HPSV) were significantly higher (73.3) than for other groups (Table 3). At day 14 PV, there was no significant difference in IHA GMT values of groups A1, A2, B1, B2, C1, C2 and D1. The highest IHA GMT value (147) was recorded for these groups at day 14 PV. Similarly, the antibody titer was significantly higher for groups A1, B1, C1 and D1 than for other groups at day 21 PV. This highest antibody titer was maintained by groups A1, B1 and C1 for next 7 days (28 days PV). These IHA GMT values reveal the highest value (73.3) for non adjuvant vaccine during 1-2 weeks AV; it started to decrease in 3rd week and dropped to 4.6, at 4th week AV, probably due to immunity break (Afzal and Ahmad, 1990). This rapid increase and drop in GMT with non adjuvanted vaccine may be due to rapid absorption and rapid elimination of vaccinal agent. This is congruent with Hussain *et al*. (1999). The IHA GMT values at same time intervals for aluminized vaccine were 64, 128-147, 104-147 and 73-112. IHA GMT values were highest and more consistent for oil based vaccines inactivated either with formalin or BEI with peak GMT (181) in 3rd week AV (Hussain *et al*., 1999).

So in this study, two different virus concentrations ($1x10^2$ and $1x10^3$ ALD_{50} HPS antigen concentration) and two different virus inactivants (formalin and BEI) were used. It appears that neither virus concentrations nor inactivants make any difference in terms of protection percentage and IHA GMT values using same adjuvant. Ahmad *et al*. (1990) also recorded no difference in protection percentage and IHA GMT values of birds vaccinated with $5x10^1$ or $1x\ 10^{2.3}$ EID_{50} HPS virus containing vaccines.

From this study, it is concluded that oil based HPS vaccine containing $1x10^2$ ALD_{50} HPS virus, inactivated with formalin or BEI provided 100% protection in birds vaccinated at 14th day of age for at least 4-5 weeks AV. This vaccine can be recommended for commercial production.

Table 2: Mortality and protection in challenged vaccinated groups (challenged on day 21 post vaccination)

Days after virus challenge	Groups												
	A1	**A2**	**A3**	**B1**	**B2**	**B3**	**C1**	**C2**	**C3**	**D1**	**D2**	**D3**	**E**
1	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	1	2
4	-	-	1	-	-	-	-	-	1	-	-	-	-
6	-	-	-	-	-	-	-	-	1	-	-	2	2
7	-	-	1	-	--	2	-	-	-	-	-	-	-
8	-	1	-	-	-	-	-	-	-	-	-	-	3
9	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	0/8	1/8	2/8	0/8	0/8	2/8	0/8	0/8	2/8	0/8	0/8	3/8	7/8
Mortality (%)	0	12.5	25	0	0	25	0	0	25	0	0	37.5	87.5
Protection (%)	100	85.7	71.4	100	100	71.4	100	100	71.4	100	100	57	-

Table 3: IHA GMT values at 7, 14, 21 and 28 days post vaccination (DPV)

IHA GMT antibody titers at intervals	Formalized vaccine (F-HPSV)						BEI inactivated vaccine (BEI-HPSV)						
	Virus con.= $1x10^2$ ALD_{50}			Virus con.= $1x10^3$ ALD_{50}			Virus con.=$1x10^2$ ALD_{50}			Virus con.= $1x10^3$ ALD_{50}			
	A1	**A2**	**A3**	**B1**	**B2**	**B3**	**C1**	**C2**	**C3**	**D1**	**D2**	**D3**	**E****
7 days PV	52^a	52^a	73.3^c	52^a	64^b	73.3^c	52^a	64^b	64^b	64^b	52^a	73.3^c	0
14 days PV	147^e	147^e	84.4^b	147^e	147^e	73.3^a	147^e	147^e	104^c	147^e	128^d	128^d	0
21days PV	181^f	147^e	36^b	181^f	128^d	26^a	181^f	104^c	26^a	181^f	104^c	26^a	0
28 days PV	181^h	104^e	4.6^b	181^h	112^f	2.3^a	181^h	90.5^d	2.3^a	147^g	73.3^c	2.3^a	0

Figures with different superscripts across the row are significantly different from each other ($P<0.05$).
E** = Non vaccinated control group.
GMT* = Geometric mean IHA titre of 4/8 random samples.

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