ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 3 (4): 279-283, 2004

An Aluminium Hydroxide Gel Adsorbed Inactivated Egg Drop Syndrome Vaccine

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Abstract: An inactivated vaccine was prepared from local isolates of egg drop syndrome (EDS) virus using aluminum hydroxide as an adjuvant. A total of 30 commercial layers at the age of 16 weeks were procured and divided in to two groups, A and B having 20 and 10 birds, respectively. The birds of group A were vaccinated with 0.5ml aluminium hydroxide gel adsorbed EDS vaccine. The birds of group B were kept as unvaccinated control. The serum samples were collected at a 14-day interval till 6 weeks post vaccination. At 22 weeks of age group A was again divided into two groups A_1 and A_2. Birds of group A_2 and group B were challenged with a virulent EDS virus. The results revealed that birds of group A had HI titer ranging from 1:128 to 1:512 at different times of sample collection with a geometric mean titer 116. Highest antibody response appeared on 4th week post vaccination. Results also revealed that the vaccination protected the birds against challenge with the virulent EDS virus. Where there was a significant drop in egg production in unvaccinated challenged birds. The egg quality was also deteriorated as miss-shapened, soft-shelled and shell-less eggs were laid in EDS unvaccinated layers.

Key words: Egg drop syndrome, aluminium hydroxide, hemagglutination inhibition, vaccine, adjuvant

Introduction

Egg drop syndrome causes decreased egg production (Van Eck *et al*., 1976). It is caused by a duck adenovirus belonging to the genus Atadenovirus. The EDS virus is nonenveloped, hemagglutinating, DNA virus, 74-80 nm in diameter, which replicates in the nucleus of the host cells (Jordan, 1990).

The infected birds lay soft-shelled or shell-less, discolored and miss-shapened eggs. EDS virus may cause 40-50% decrease in egg production. If the disease is due to reactivation of latent virus, the fall usually occurs when production is between 50% and peak level. In acute cases there may be mild depression, however general appearance, feed and water intake of the affected birds remain normal (Yamaguchi *et al*., 1980). EDS virus spreads both vertically through the embryonated eggs (Adair *et al*., 1979) and horizontally. The horizontal transmission of the disease in a flock is usually faster. The virus can survive in the litter of an infected poultry house for many weeks. Both domestic and wild ducks may act as a carrier and play a vital role in the transmission of disease (McFerran, 1999).

The aluminium hydroxide gel has been used by Hassan *et al*. (1992), who prepared New castle Disease vaccine and compared it with other oil based vaccines experimentally. Blackall *et al*. (1992) evaluated the efficacy of aluminium hydroxide gel adsorbed Infectious coryza vaccine. Trampel *et al*. (1997) evaluated aluminium hydroxide gel adsorbed *Eschrichia coli* (*E. coli)* bacterins in turkeys against homologous challenge. The aluminium hydroxide gel adjuvanted EDS-76 vaccine had advantages over the oil adjuvant vaccine that it is easy to inject, induces lesser inflammatory response at the site of injection and is economical to produce vaccine commercially (Garg and Garg, 1994). So this study was carried out to see the effect of aluminium hydroxide as adjuvant on the efficacy of egg drop syndrome virus under experimental condition.

Materials and Methods

Virus Preparation: A local isolate of egg drop syndrome virus was inoculated in 11-day-old duck embryos through allantoic cavity route (Senne, 1989). After 6 days of incubation, the allanto-amniotic fluid (AAF) was harvested and pooled together. The hemagglutination activity of the virus suspension, i.e., AF was determined (Allan *et al*., 1978).

Washing of Chicken Erythrocytes: The chicken blood containing anticoagulant was centrifuged at 400 xg for 1 minute. The supernatant was discarded and sedimented RBCs were resuspended in 10 volumes of normal saline (pH 7.2). The suspension was centrifuged again at 400 xg for 1 minute. The supernatant was discarded while the sediment was resuspended in the saline. This process was repeated three times until supernatant was clear. Finally, RBCs were resuspended in phosphate buffered saline (PBS).

Microhemagglutination: With the help of titer-teck, 50µl of the PBS was added to each of the 1 to 12 wells of all

the rows of the microtiteration plate. The AAF was added to first well of all the rows of the two plates. With the help of titer-teck, 2 fold dilutions of fluid were made from well 1 to 12, so as to achieve dilutions such as 1:2, 1:4, 1:8 to 1:4096 in A and B rows and diluted further back from 12th to 3rd well of row C and D, while leaving 1st and 2nd well of C and D rows as RBCs control. 50µl of the washed RBCs was added to each well containing PBS or PBS diluted virus. The plates were incubated at 37°C for 25 minutes and results were recorded (Allan *et al.*, 1978).

Hemagglutination Test with RBCs of Different Species: 2.0ml of blood each of hen, sparrow, parrot, pigeon, duck, horse, rabbit, buffalo and sheep was obtained in sterile 3ml disposable plastic syringes after the addition of EDTA. The RBCs of all the species were washed. The RBCs (1.0% suspension) of all the species were used to determine the hemagglutination activity.

Hyper immune Serum against Egg Drop Syndrome (EDS) Virus: Out of five rabbits, the imported oil-based EDS vaccine was inoculated in four rabbits with increasing dosage on alternate days (0.1-1ml intraperitoneally) while fifth rabbit was kept as control. After 14 days of last injection, blood was collected aseptically and serum was separated. Serum was heat inactivated in water-bath at 56°C for 30 minutes. Finally the serum was stored at –20°C for further use.

Hemagglutination Inhibition (HI) Test: The Hyper immune serum was titerated in 96 wells round bottom microtiteration plate using the four hemagglutination unit (4 HAU) of positive hemagglutinating AAF (Buxton and Fraser, 1977). With micro dispenser, 50µl of PBS was added to all the 8 rows of 96 wells of microtiteration plate. The 50µl of Hyper immune serum was added to the first well of row A, C, E and G. The serum was diluted as 2 fold from first well of row A, C, E and G to the second last well of row B, D, F and H leaving their last well as RBCs control. An equal quantity (50µl) of 4 HAU of AAF was added to each dilution of serum leaving the second last well of row B, D, F and H as serum control. The contents of the wells were mixed thoroughly by tapping and the microtiteration plate was incubated at 37°C for 30 minutes. The 50µl of 0.8% washed chicken RBCs suspension was added to each well of microtiteration plate. After gentle mixing, the plate was again incubated for 30 minutes at 37 °C and results were recorded.

Agar Gel Precipitation Test (AGPT): Positive samples were further confirmed through AGPT (Sulochana and Lalithakunjamma, 1991). Noble agar (0.7%) was prepared in physiological saline and boiled until completely dissolved. The solution was poured in Petri dish at 40-45°C to give a layer of 3-5 mm thickness and allowed to set. On solidification, circular wells of 4-mm diameter (one central and eight peripheral) were cut at equal distance. The bottom of each well was sealed with a drop or two of molted noble agar. The central well was loaded with EDS antiserum and peripheral wells with hemagglutinating AAF samples along with known positive and negative controls. The plates were incubated at 37°C in humidified chamber for 48-72 hours.

Calculation of Egg Infective Dose$_{50}$: Tryptose broth was added aseptically in eleven glass test tubes. One ml of the AAF was added in the first tube, mixed well and serially diluted up to the tube number 10. The tube number 11 was kept as negative control. 0.1ml suspension from each dilution of AAF 10^{-5} to 10^{-10} and negative control was inoculated in 35 embryonating eggs. The inoculated eggs were reincubated at 37°C for 120 hours and then chilled at 4°C. The allantoic fluid of each egg was subjected to HA test.

Inactivation of the Virus: The AAF was transferred to a 500 ml sterilized glass bottle. Formalin at the rate of 0.12% was added to it. The contents were properly mixed and incubated at 37°C for 48 hours. The fluid was transferred to refrigerator (4°C) and processed for safety and sterility tests.

Sterility Test: 10ml of the AAF was centrifuged at 600 xg for 15 minutes. The sediment was streaked on the MacConkey's agar and Nutrient agar. While a loopful of this material was transferred to the each of the set of 5 tubes containing mycoplasma broth. The agar plates were incubated at 37°C for 48 hours while the mycoplasma broth was incubated at 37°C for 10 days but it was examined daily for microbial growth (Takai *et al.*, 1984)

Safety Test: 0.2 ml of the AAF was inoculated into 10 fertile duck eggs of 10-day-old via allanto-amniotic route. The eggs were incubated for 96 hours and then the eggs were transferred to refrigerator. After overnight storage at 4°C the allantoic fluid was harvested. The HA activity was determined (Solyom *et al.*, 1982).

Preparation of aluminium hydroxide vaccine: A vaccine was prepared from inactivated AAF using aluminium hydroxide (Hassan *et al.*, 1992) as adjuvant. First of all the aluminium hydroxide gel was prepared by dissolving 1000ml of 10% potassium aluminium sulphate solution in a flask containing 1000ml of 1N solution of sodium hydroxide. After thorough mixing a white gelatinous precipitate of aluminium hydroxide was formed, which was allowed to settle down at 4°C. The supernatant was discarded and the precipitate was reconstituted with distilled water after every 24 hours for 3-4 times. For adsorption of sterilized virus on the gel three aliquots (A, B, C) were made. 0.5, 1.0 and 2.0ml of virus was added

Table 1: Antigenic response in layer to egg drop syndrome virus vaccine

Source of vaccine	Time post vaccination (Weeks)	Distribution of birds on the basis of EDS antibodies (HI)* titers									GMT
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	
(A) vaccinated with	2	1	2	2	2	3	6	4	0	0	30
Experimental vaccine	4	0	2	0	0	2	2	6	4	4	116
(20 birds)	6	0	0	1	2	4	5	5	2	1	70
(B) Un-vaccinated (10 birds)	2	0	0	0	0	0	0	0	0	0	0.00
	4	0	0	0	0	0	0	0	0	0	0.00
	6	0	0	0	0	0	0	0	0	0	0.00

* means Hemagglutination Inhibition

into 12.5ml of aluminium hydroxide gel. The adsorption of virus was checked after 48 hours. After every one week the pH of the vaccine was checked for any change. Viscosity and stability of vaccine was also observed by its storage at 4°C for 15 days.

Immunogenicity of the vaccines: Efficacy of the aluminium hydroxide gel adsorbed EDS vaccine was evaluated in 16 weeks old layers by hemagglutination inhibition test. Each of 20 layer were injected with 0.5 ml of the vaccine intramuscularly in pectoral muscle, ten birds were kept unvaccinated control. The antigenicity was evaluated in serum over a period of 6 weeks fortnightly. The HI titers of the samples were determined and geometric mean titer (GMT) was calculated. All the birds in control group and half of the vaccinated birds were challenged with 0.2ml of live EDS virus at the age of 22 week (Holmes *et al.*, 1989).

Results and Discussion

The isolated virus grew well on 11-day-old embryonated duck eggs. The infected duck embryos were found live 144 hours post inoculation. The HA titer of AAF harvested from the embryos ranged between "1:128 to 1:512". A compact button formation in hyper immune serum was considered as HI positive and the highest titer was 1:512. A clear precipitation line in AGPT at the junction of homologous antigen and antibodies was considered as positive. The study revealed that EDS virus cannot replicate in chicken embryos. It may be due to the fact that the cells in developing embryos are devoid of receptors for adsorption with ligand of the virus, because adsorption is the primary stage of EDS virus replication (Zsak *et al.*, 1982).

The EDS virus agglutinated avian erythrocytes but not the mammalian erythrocytes, due to the presence of hemagglutinin molecule on its surface (Baxendale *et al.*, 1980; Jordan, 1990). It was also observed that EDS virus mediated HA did not show elution on 24 hours post incubation. This property might be due to the lack of neuraminidase molecules on the surface of EDS virus (Spalatin *et al.,* 1970).

The selective growth in duck embryos, hemagglutination potential to only avian erythrocytes, HI and AGPT confirmed that the test virus was EDS virus.

The AAF having EDS virus suspension gave high HA titer 1:8192 with the chicken, sparrow, pigeon and duck erythrocytes, while it gave no titer with buffalo, horse, rabbit and goat erythrocytes. This confirmed EDS virus was processed for preparation of aluminium hydroxide gel adjuvanted EDS vaccine. The EID_{50} of the virus was $10^{10.88}$/ml (Reed and Muench, 1938). The formaldehyde, when admixed in the EDS virus suspension at the rate of 0.12%, inactivated the virus with 48 hours incubation at 37°C. The lack of the HA activity of the fluid indicated complete inactivation of the virus in duck embryos showed that the formalin is effective virucidal. The higher concentration of the formalin presumably mitigates the antigenicity of the virus, while lower concentration may necessitate the prolonged incubation of the formalin-virus mixture.

There was no growth on Nutrient agar, Blood agar and MacConkey's agar after 24, 48 hours of incubation. PPLO broth also showed no growth after 10 days of incubation. These results showed that the virus suspension was completely sterilized. In 11-day-old fertile duck embryos, no growth of EDS virus in AAF showed the complete killing of virus by formalin. However, formalin is carcinogenic in nature if more than 12mg per dose is given (Solyom *et al.*, 1982).

In response to aluminium hydroxide gel adsorbed vaccine, which was given to birds of group A on 2nd week, maximum HI titer was 128 with GMT 30. On 4th week maximum HI titer was 512 with the GMT 116. Highest HI titer remained the same as it was on 4th week but the GMT was decreased up to 70. This GMT

Table 2: Challenge protection test in layer

Days post Challenge	Number of eggs produced by various groups at different days post challenge		
	Groups of Birds		
	(A1) Challenged (10 birds)	(A2) Non Challenged (10 birds)	(B) Non vaccinated but Challenged (10 birds)
1	8	8	4
2	8	8	3
3	8	8	4
4	8	8	3
5	9	8	4
6	8	8	5
7	8	8	3
8	8	8	3
9	7	8	3
10	8	9	4
11	8	8	3
12	8	8	3
13	6	8	3
14	7	8	3
15	8	8	3
16	8	8	3
17	7	8	3
18	8	9	4
19	6	9	3
20	8	8	3
21	8	8	3
22	8	9	3
23	8	8	3
24	8	8	2
25	7	8	3
26	7	8	3
27	8	8	1
28	8	8	3
29	8	8	3
30	6	8	2
% age	79%	86%	32%

trend we got in this study means that we can get maximum protective titer around 4th week after EDS vaccination. The unvaccinated birds of group B did not show any titer of antibodies on 2nd, 4th and 6th week.

All layers were challenged with local EDS virus intranasally with $10^{10.88}$ per ml at the age of 22 weeks. Chicken in all $Al(OH)_3$ groups were given challenge and egg production was decreased (Table 2). The group in which EDS vaccine was given, the challenge resulted in 6% decrease in egg production in face of challenge. The challenge was also given to unvaccinated control group. The production in that group remained 32% only. The challenge resulted in decreased egg production. The miss-shapened, soft-shelled and shell-less eggs were also seen in unvaccinated control group. Keto *et al.* (1994) used aluminium hydroxide gel and calcium phosphate gel as vaccine adjuvants for many years. They found a positive correlation between the hemolytic activity and adsorption capacity of aluminium hydroxide gel. Hassan *et al.* (1992) prepared inactivated aluminium hydroxide adjuvanted N.D. vaccine and oil emulsion N.D. vaccine. They compared these vaccines with commercial oil based vaccine. They found aluminium hydroxide gel adsorbed and oil adjuvanted vaccines safe and immunogenic. Hassan *et al.* (1992) reported that oil based vaccines are unsuitable for field use due to high viscosity of oils. They also reported that aluminium hydroxide adsorbed vaccine gave 93% protection for 6 months whereas oil emulsified vaccines gave only 80% protection. Usinger (1997) compared six adjuvant formulations for their ability to potentiate the primary and memory antibody response in mice to three

companion animal vaccines. The safety of aluminium hydroxide gel and the apparent absence of adverse reactions were proved, which made these vehicle/ adjuvant formulations worthy for additional study.

The results of the present experimental study revealed that aluminium hydroxide gel adsorbed EDS vaccine gave sufficient antibody titer, which protected the birds against clinical disease i.e., disease induced by challenge with a virulent EDS virus. The vaccine needs to be tested at commercial scale. A comparative study between aluminium hydroxide gel adsorbed vaccine and oil emulsified vaccine is also under way.

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