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Comparative Immunological Response of Commercial Oil Based and Liposomal Vaccines of Avian Influenza H7

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Abstract: Avian Influenza (AI) has been recognized as a highly contagious and lethal generalized viral disease of birds. In this study, immune response of layers to the commercial oil based and liposomal vaccines of avian influenza H7 was evaluated. Thirty commercial layers were divided into three groups, T1, T2 and T3 with 10 birds in each group. Group T1 served as control, Group T2 was immunized with conventional AI oil-based vaccines, 0.5 mL/bird and Group T3 was immunized with AI Liposomal vaccines 0.5 mL/bird through sub/cut injection. Blood samples were taken and sera were separated at day 0, 7, 14, 21, 28 and day 35. Each time at least 6 samples were taken for antibody titration through AGPT. The geometric mean titre (GMT) of birds in T1, T2 and T3 was 4 ±1.02 at day 0. No significant difference was observed in the titres at day 0 in all the groups. The GMT (Geometric Mean Titer) of control group was 4 ±1.02 at day 7, 6.79 ±1.02 at day 14 and 8 ±1.02 from day 21 to 35. The antibody titre increased slowly from 32 on day 7 to 630.3 on day 35 in birds vaccinated with oil based vaccine, whereas a somewhat quick increase in immune response from 64 on day 7 to 891.4 on day 35 was observed in birds vaccinated with liposomal vaccine. The results showed that immune response of layers in term of GMT was well established with AI liposomal vaccine as compared to that of oil based vaccine. The present study will be helpful in preventing the commercial losses of the farmers and preventing the flock mortality due to the high efficacy of liposomal vaccine against avian influenza.

Key words: Avian influenza, vaccines, adjuvant, oil base, liposomes, immunity

INTRODUCTION

Avian Influenza (AI) is a serious, highly contagious and lethal generalized viral disease of poultry since 1901(Anonymous, 2004), resulting severe mortality in chickens and major disruption to production and trade (FAO, 2004). Low pathogenic AI viruses cause respiratory and gastrointestinal infections without infecting the meat. By contrast, HPAI viruses produce infection of respiratory and gastrointestinal tracts and virus is present in the meat and internal contents of eggs during the acute stages of the infection (Swayne, 2004). In highly pathogenic AI, the disease appears suddenly in a flock and many birds die either without premonitory signs or with minimal signs of depression, inappetence, ruffled feathers and fever (Anonymous, 2004; Alexander, 2000). There is extensive subcutaneous oedema, particularly around the head and hocks. The carcass may be dehydrated. Yellow or grey necrotic foci may be present in the spleen, liver kidneys and lungs. After infection poultry, excreted virus from both the respiratory and the digestive tracts, resulting in rapid spread through a population of susceptible host. Bird to bird transmission is very efficient via aerosol, contaminated feces, or various fomites and the virus can cause a wide range of disease symptoms (Alexander, 2000; Bankowski, 1981; OIE, 1996; Easterday, 1997; Swayne and Suarez, 2000).

In 1955, a specific type (A) of influenza virus was identified as the causal agent of fowl plague (Anonymous, 2004). All Avian Influenza (AI) viruses belong to the *Influenza virus A* genus of the *Orthomyxoviridae* family and are single stranded, negative polarity, segmented RNA viruses. *Influenza A* viruses can be divided into subtypes on the basis of the possession of one of 15 antigenically distinct haemagglutinin (HA) antigens (H1 to H15) and one of nine neuraminidase (NA) antigens (N1 to N9) (WHO, 1980; Swayne, 2004; Anonymous, 2004). HPAI viruses are not normally present in wild bird populations and only arise as a result of mutation after H5 or H7 LPAI viruses have been introduced to poultry from wild birds (Garcia *et al*., 1996; Perdue *et al*., 1998).

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Various vaccine have been used for immunization against avian influenza, including conventional inactivated oil-adjuvanted whole AI virus (Halvorson, 1995; Pomeroy, 1995; Naeem, 1998; Garcia and Alvarez, 1999; Swayne and Suarez, 2000), Inactivated whole Avian Influenza (AI) virus, baculovirus-derived AI haemagglutinin, vectored virus, subunit protein and DNA vaccines (Swayne, 2003). AI vaccines can prevent clinical signs, deaths and reduce respiratory and intestinal replication of a challenge virus, increase resistance of birds to infection and decrease the amount of virus shed in the environment (Swayne, 2004). Adjuvanted vaccines elicit higher immune response, higher rates of sero-conversion and sero-protection compared to non-Adjuvanted vaccines (Podda, 2001). Historically, inactivated whole viruses using various adjuvant systems have been used (Swayne, 2004). IL-6, IL-2 Suplemented, negatively and positively charged, Polymerized and non polymerized and pure Liposomes (Lachman *et al*., 1995; Yehuda *et al*., 2003; Alonso-Romanowski *et al*., 2003; Verma *et al*., 2004; Huckriede *et al*., 2003; Voinea and Simionescu, 2002; Baldo *et al*., 2001; Budai and Szogyi, 2001; Sandhu *et al*., 1998; Fatunmbi *et al*., 1992; Kraaijeveld *et al*., 1984). Strictly stabilized Liposomes (SSL-IL-2) (Kedar *et al*., 1994), MF59 (Stephenson *et al*., 2002; Nicholson *et al*., 2001; Gasparini *et al*., 2001; Podda, 2001; Baldo *et al*., 2001; Minutello *et al*., 1999), Immunostimulating complex (Iscom) (Deliyannis *et al*., 1998; Coulter *et al*., 1998), Oil in water containing squalene (Podda, 2001; Coulter *et al*., 1998), Oil emulsion (Stone, 1987, 1993), Mineral oil emulsion (Stone, 1993), Alum (Gluck, 1999), Aluminum (Hehme *et al*., 2004) may be used to boost vaccines potency. Immunopotentiating, reconstituted influenza virosomes (IRIV) (Gluck, 1999), co-polymer adjuvant (CRL1005) (Katz *et al*., 2000) and Freunds complete adjuvant (Hjorth *et al*., 1997) have been used experimentally.

The present study was designed to evaluate the comparative immunological response of commercial oil based and liposomal vaccines of Avian Influenza. Commercial oil based and Liposomal AI vaccine (MediExcel Pharmaceuticals, Islamabad, Pakistan) were evaluated in commercial layers. The present study will be helpful in restoring the commercial losses of the farmers and preventing the flock mortality due to the high efficacy of liposomal vaccine against avian influenza.

MATERIALS AND METHODS

Antigen source: Commercial oil based (Rue Merial, France) and Liposomal AI vaccine (Medi Excel Pharmaceuticals, Islamabad, Pakistan) strain H_7 were evaluated in commercial layers.

Immunogen-containing liposomes was prepared by mixing, 20 mg of sphingomyelin with 8 mg of cholesterol and dissolved in 3 mL of chloroform and 3 mL of ether was added. The mixture was bathed and sonicated to get uniform water in oil emulsion with 10 mg of immunogen already dissolved in a balance salt solution (BSS) in 5.5 mM glucose, 0.4 mM KH_2PO_4, 1.2 mM $Na_2HPO_4.7HPO_4.7H_2O$, 1.3 mM $CaCl_2.2H_2O$, 5.4 mM KCl, 136 mM NaCl, 1 mM MgCl2. $6H_2O$, 0.8 mM $MgSO_4.7H_2O$.

- Liposomes were made up to 2 mL with BSS and treated with 0.1% osmium tetrodxide (final concentration) in saline.
- The suspension was held for 30 min at room temperature and then dialyzed it extensively against a continuous flow of deionized, double distilled autoclaved water.
- Liposomes were separated from free immunogen by centrifugation at 1000 g.
- Liposome suspension was made up in BSS to appropriate incorporated immounogen concentration for injection.

Immunization of hens: Thirty commercial layers were partitioned in isolated bio-secure room and were divided randomly and were given the same keeping and feeding facilities. Layers were divided into three groups, T1, T2 and T3 with 10 birds in each group. Each group was marked with a specific color, as each group had to receive a different antigen preparation. Group T1 served as control, Group T2 was immunized with conventional AI oil-based vaccines 0.5 mL/bird and Group T3 was immunized with AI Liposomal vaccines 0.5 mL/bird through sub/cut injection . Blood samples were taken and serum was separated at day 0, 7, 14, 21, 28 and day 35. Each time at least 6 samples were taken for antibody titration through AGPT test.

Serological quantification

Preparation of agar gel plate: Noble Agar gel was prepared by adding 0.9% of Noble agar, 8% of sodium chloride, 0.01% of Sodium azide in distilled water. All ingredients were mixed in distilled water and then heated to boil until a uniform suspension was obtained. Then uniform suspension of the agar gel was cooled to 45°C and poured into Petri-plate. The plate after pouring was kept at room temperature until gel was solidified. The solidified gel was transferred to refrigerator until use.

Punching of wells: Well guiding plate was used for punching wells in the agar gel plate. The diameters of wells were 5.3 mm and distances between wells were

2.4 mm. The lid of plate was removed and template was placed on the agar gel plate. Care was taken that the template should not touch the surface of agar gel. A gel borer was used to punch wells agar gel. The punched gel in the wells was removed with great care with the help of suction pump using a sterilized micropipette tip. Bottom of all wells was sealed with 20 μL of melted agar. This was done to minimize the leakage of antigen and antiserum between gel and glass plate.

Charging of wells: For qualitative assay the five wells were punched in a circle around a center well. Unknown samples of antigens were dispensed in the peripherals wells while known sample of antibodies was dispensed in the center well. The plate was incubated for 96 h in humid chamber. Between wells of specific antigen and antibodies a precipitation line was developed. The dilutions of these samples can be used laterally in experiment.

For quantitative assay wells were punched in row and column fashion. Numbers of wells were equal in all rows and columns. Two fold dilutions of antibodies were made in micro-titer plate. The diluted antibodies were transferred into the wells of first and third row and undiluted antigen was dispensed into the second row. The charged agar plate was transferred to a humid chamber at room temperature and kept there for 96 h. Appearance of precipitation line was recorded.

The Geometric Mean Titre (GMT) of each group was calculated by the formula:

$$GMT = \sqrt[n]{X_1, X_2, X_3 ..... X_n}$$

Where:
X = Value of the observation
n = No. of observations

Statistical analysis: All the data for the experiment was analyzed by using the one way analysis of variance (ANOVA). Significant differences among different treatments were analyzed with Least Significant Difference (LSD).

RESULTS

Titres of serum samples at 0 day: GMT of birds in T1, T2 and T3 were 4±1.02 at day 0. The GMT of the entire groups were low and were considered as the background reading. No significant difference was observed in the titres at day 0 in all the groups (Table 1 and Fig. 1).

Titres of serum samples at 7 day: GMT titre were low in group T1 (4 ±1.021), whereas it was high in T2 (32 ±0.722). The effect of Avian Influenza (AI) oil based vaccine in T2 was significantly higher in comparison with control group (T1). However Avian Influenza (AI) Liposomal vaccinated group (T3) was highly significant (64 ±0.722) as compared with T1 (4±1.021) and T2 (32±0.722). The data comparatively indicated that immune response in term of GMT was well established with Avian Influenza (AI) Liposomal vaccine as compared to Avian Influenza (AI) Oil based vaccine (Table 2 and Fig. 1).

Table 1: AGPT titres of Avian Influenza antibodies in birds, with different regimens of immunization at day 0 post treatment (n = 6)

Treatments	AGPT titres (log_2)		AGPT titres	
T1 (Control)	1:8	1:8	3	3
	1:4	1:4	2	2
	1:2	1:2	1	1
GMT±SDM	EM 2.00±0.8944		GMT 4±1.021[A]	
T2 (AI oil based)	1:8	1:8	3	3
	1:4	1:4	2	2
	1:2	1:2	1	1
GMT±SDM	EM 2.00±0.8944		GMT 4±1.021[A]	
T3 (AI liposomal)	1:8	1:8	3	3
	1:4	1:4	2	2
	1:2	1:2	1	1
GMT±SDM	EM 2.00±0.8944		GMT 4±1.021[A]	

T1: Birds served as control, T2: Birds vaccinated with Avian Influenza oil based vaccine 0.5 mL subcutaneously, T3: Birds vaccinated with Avian Influenza liposomal vaccine 0.5 mL subcutaneously, A-C: Values with similar superscript do not differ statistically ($p<0.05$) While values with different superscript differ significantly

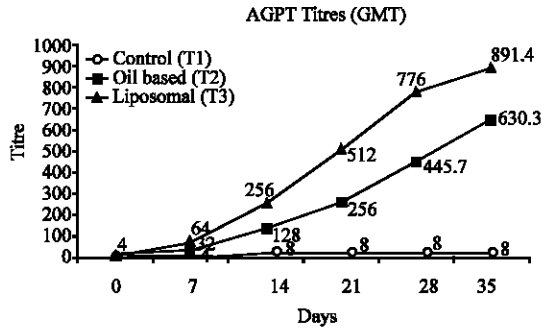

Fig. 1: Graph showing the over all AGPT titres of control, oil based and liposomal vaccinated groups from day 0 to 35

Titres of serum samples at 14 day: The GMT titres were marginal in group T1 (6.79±1.021) whereas, it was quite higher in T2 (128±0.722). The effect of Avian Influenza (AI) oil based vaccine in T2 was significantly high in comparison with control group (T1). However Avian Influenza (AI) liposomal vaccinated group (T3) was highly significant (256±0.722) as compared with T1 (6.79±1.021) and T2 (128 ±0.722). The data fairly indicated that immune response in term of GMT was well established with AI liposomal vaccine as compared to AI oil based vaccine (Table 3 and Fig. 1).

Titres of serum samples at 21 day: The GMT titres were marginal in group T1 (8±1.021) whereas, it was quite higher in T2 (256±0.722). The effect of Avian Influenza (AI) oil based vaccine in T2 was significantly high in comparison with control group (T1). However Avian Influenza (AI) Liposomal vaccinated group (T3) was highly significant (512±0.722) as compared to T1 (8±1.021) and T2 (256±0.722) (Table 4 and Fig. 1).

Titres of serum samples at 28 day: GMT titres were marginal in group T1 (8±1.022), whereas it was quite higher in T2 (445.7±0.46). The effect of AI oil based vaccine in T2 was significantly high in comparison with control group (T1). However Avian Influenza (AI) liposomal vaccinated group (T3) was highly significant (776±0.056) as compared with T1 (8±1.022) and T2 (445.7±0.46). The data fairly indicated that immune response in term of GMT was well established with Avian Influenza (AI) Liposomal vaccine as compared to Oil based vaccine (Table 5 and Fig. 1).

Table 2: AGPT titres of Avian Influenza antibodies in birds, with different regimens of immunization at day 7 post treatment (n = 6)

Treatments	AGPT titres (log_2)		AGPT titres	
T1 (Control)	1:4	1:2	2	1
	1:8	1:4	3	2
	1:2	1:8	1	3
GMT±SDM	EM 2.00±0.894		GMT 4±1.021^{A}	
T2 (AI oil based)	1:16	1:32	4	5
	1:32	1:32	5	5
	1:32	1:64	5	6
GMT±SDM	EM 5.00±0.6324		GMT32 ±0.722^{B}	
T3 (AI liposomal)	1:128	1:64	7	6
	1:32	1:64	5	6
	1:64	1:64	6	6
GMT±SDM	EM 6.00±0.6324		GMT 64±0.722^{C}	

T1: Birds served as control, T2: Birds vaccinated with Avian Influenza oil based vaccine 0.5 mL subcutaneously, T3: Birds vaccinated with Avian Influenza liposomal vaccine 0.5 mL subcutaneously, A-C: Values with similar superscript do not differ statistically ($p<0.05$) While values with different superscript differ significantly

Table 3: AGPT titres of Avian Influenza antibodies in birds, with different regimens of immunization at day 14 post treatment (n = 6)

Treatments	AGPT titres (log_2)		AGP titres	
T1 (Control)	1:4	1:16	2	4
	1:8	1:4	3	2
	1:16	1:8	4	3
GMT±SDM	EM 3.00±0.8944		GMT6.79±0.859^{A}	
T2 (AI oil based)	1:128	1:128	7	7
	1:64	1:128	6	7
	1:256	1:128	8	7
GMT±SDM	EM 7.00±0.6324		GMT 128±0.722^{B}	
T3 (AI liposomal)	1:512	1:256	9	8
	1:128	1:256	7	8
	1:256	1:256	8	8
GMT±SDM	EM 8.00±0.6324		GMT 256±0.722^{C}	

T1: Birds served as control, T2: Birds vaccinated with Avian Influenza oil based vaccine 0.5 mL subcutaneously, T3: Birds vaccinated with Avian Influenza liposomal vaccine 0.5 mL subcutaneously, A-C: Values with similar superscript do not differ statistically ($p<0.05$) while values with different superscript differ significantly

Titres of serum samples at 35 day: THE GMT titres in group T1 (8±1.022) were marginal, whereas it was quite higher in T2 (630.3±0.56). The effect of Avian Influenza (AI) oil based vaccine in T2 was significantly high in comparison with control group (T1). However Avian

Table 4: AGPT titres of Avian Influenza antibodies in birds, with different regimens of immunization at day 21 post treatment (n = 6)

Treatments	AGPT titres (log_2)		AGPT titres	
T1 (Control)	1:4	1:16	2	4
	1:8	1:4	3	2
	1:16	1:8	4	3
GMT±SDM	EM 3.00±0.894		GMT 8±1.021^{A}	
T2 (AI oil based)	1:512	1:256	9	8
	1:128	1: 256	7	8
	1:256	1:256	8	8
GMT±SDM	EM 8.00±0.632		GMT 256±0.722^{B}	
T3 (AI liposomal)	1:512	1:512	9	9
	1:256	1:512	8	9
	1:1024	1:512	10	9
GMT±SDM	EM 9.00±0.632		GMT 512±0.722^{C}	

T1: Birds served as control, T2: Birds vaccinated with Avian Influenza oil based vaccine 0.5 mL subcutaneously, T3: Birds vaccinated with Avian Influenza Liposomal Vaccine 0.5 mL subcutaneously, A-C: Values with similar superscript do not differ statistically ($p<0.05$) while values with different superscript differ significantly

Table 5: AGPT titres of Avian Influenza antibodies in birds, with different regimens of immunization at day 28 post treatment (n = 6)

Treatments	AGPT titres (log_2)		AGPT titres	
T1 (Control)	1:4	1:16	2	4
	1:8	1:4	3	2
	1:16	1:8	4	3
GMT±SDM	EM 3.00±0.894		GMT 8±1.022^{A}	
T2 (AI oil based)	1:512	1:512	9	9
	1:256	1:512	8	9
	1:512	1:512	9	9
GMT±SDM	EM 8.83±0.408		GMT 445.7±0.466^{B}	
T3 (AI liposomal)	1:1024	1:1024	10	10
	1:512	1:1024	9	10
	1:1024	1:512	10	9
GMT±SDM	EM 9.60±0.516		GMT 776±0.589^{C}	

T1: Birds served as control, T2: Birds vaccinated with Avian Influenza oil based vaccine 0.5 mL subcutaneously, T3: Birds vaccinated with Avian Influenza liposomal vaccine 0.5 mL subcutaneously, A-C: Values with similar superscript do not differ statistically ($p<0.05$) while values with different superscript differ significantly

Table 6: AGPT titres of Avian Influenza antibodies in birds, with different regimens of immunization at day 35 post treatment (n = 6)

Treatments	AGPT titres (log_2)		AGPT titres	
T1 (Control)	1:4	1:16	2	4
	1:8	1:4	3	2
	1:16	1:8	4	3
GMT±SDM	EM 3.00±0.894		GMT 8±1.021^{A}	
T2 (AI oil based)	1:512	1:512	9	9
	1:1024	1:512	10	9
	1:512	1:1024	9	10
GMT±SDM	EM 9.33±0.516		GMT 630.3±0.589^{B}	
T3 (AI liposomal)	1:1024	1:1024	10	10
	1:1024	1:1024	10	10
	1:1024	1:512	10	9
GMT±SDM	EM 9.83±0.408		GMT 891.4±0.466^{C}	

T1: Birds served as control, T2: Birds vaccinated with Avian Influenza oil based vaccine 0.5 mL subcutaneously, T3: Birds vaccinated with Avian Influenza liposomal vaccine 0.5 mL subcutaneously, A-C: Values with similar superscript do not differ statistically ($p<0.05$) while values with different superscript differ significantly

Influenza (AI) liposomal vaccinated group (T3) was highly significant (891.4±0.46) as compared with T1 (8±1.022) and T2 (630.3±0.56). The data fairly indicated that immune response in term of GMT was well established with AI liposomal vaccine as compared to Avian Influenza (AI) oil based vaccine (Table 6 and Fig. 1).

DISCUSSION

In view of the low immunogenicity and the relative inadequacy of the currently used commercial vaccines of avian influenza, especially in high risk groups (usually <50% efficiency), liposomes as a carrier/adjuvant system for novel influenza vaccine can be used as candidate vaccine. The use of liposomes as antigen/adjuvant carriers for vaccines has several distinct advantages. These include: (a) liposome bio-compatibility, biodegradability and low or lack of toxicity, (b) the effective targeting of encapsulated antigens to antigen-presenting cells (APC), (c) the slow release of the antigen(s), which may provide long-term protection using a single-dose vaccine, (d) the possibility of coentrapping several antigens or various adjuvants with antigen in the same vesicles, (e) induction of serum and secretory antibodies, as well as cellular responses and (f) the feasibility of large-scale production and the extended shelf life and excellent stability of freeze dried formulations. Indeed, liposome based influenza vaccines, of at least some strains, showed higher potency than nonliposomal vaccines in both rodents and humans (Conne *et al*., 1997; Powers *et al*., 1995).

For more than 30 years inactivated whole-virus avian influenza vaccines have been the only product available to control the spread of the disease from infected to susceptible birds (European Commission, 2000). Influenza virus vaccines formulated in either adjuvant are far superior to the non-adjuvanted aqueous vaccine in eliciting antibody and T-cell responses in mice (Deliyannis *et al*., 1998). Tissue reaction from injection of animal-oil and vegetable oil vaccines is less than that induced by mineral-oil vaccines (Stone, 1993). Traditionally, inactivated oil emulsion vaccines have been used worldwide to control Low Pathogenic Avian Influenza Virus (LPAI) infections in poultry and more recently, HPAI in Mexico and Pakistan (Swayne and Suarez, 2000; Garcia and Alvarez, 1999; Halvorson, 1995; Pomeroy, 1995; Naeem, 1998).

A single dose liposomal vaccine composed of the H3N2 proteins (derived from influenza A/Shangdong/9/93) and IL-2 or GM-CSF, used as adjuvants, elicits a rapid, strong and longlasting (one year) anti-viral humoral response (Babai *et al*., 1999). Influenza subunit vaccines formulated in liposomes have been tested on humans (Powers, 1997) and in most of these studies, the liposomal vaccines produced a greater humoral response than that evoked by the aqueous forms of the vaccine. In mice, the liposomal formulations effectively stimulate a wide spectrum of anti-viral Ig isotypes, including IgG1, IgG2a, IgG2b and IgG3, indicating the activation of both Th1 and Th2 cell lineages, whereas the nonliposomal vaccines almost exclusively trigger Th2 mediated responses (Ben *et al*., 1993, 1994). The combined liposomal influenza vaccines elicit high titers of serum IgG1, IgG2a, IgG3 and IgM antibodies and mucosal IgA, as well as DTH and cytotoxic responses, suggesting the activation of both the Th1 and Th2 pathways, are very effective following immunization by various routes (i.p., s.c., i.m., i.n.) and in myelosuppressed mice (Babai *et al*., 1999) and induce high titers of antibodies directed against neuraminidase (N2), which is less variable and less immunogenic than haemagglutinin, thereby according partial protection against various influenza A (N2) substrains (Babai *et al*., 1999).

The Geometric Mean Titre (GMT) of birds in T1, T2 and T3 was 4±1.02 at day 0. The GMT of the entire groups was low and was considered as the background reading. No significant difference was observed in the titres at day 0 in all the groups. Looking in to the data the GMT titres on day 7 were (4±1.021) in group T1 whereas, it was higher in T2 (32±0.722). The effect of Avian Influenza (AI) oil based vaccine in T2 was significantly higher in comparison with control group (T1). However Avian Influenza (AI) Liposomal vaccinated group (T3) was quite significant (64±0.722) as compared with T1 (4±1.021) and T2 (32±0.722). The data comparatively indicated that immune response in term of GMT was well established with Avian Influenza (AI) Liposomal vaccine as compared to Avian Influenza (AI) Oil based vaccine and is in agreement with (Joseph *et al*., 2002), stated that the HI titre as well as the seroconversion with liposomal ISS-ODN were significantly greater than those of mice vaccinated with antigen alone. The HI titres obtained with liposomal vaccine were 2-8 folds higher.

In the current study, the GMT titres were marginal in group T1 (6.79±1.021) on day 14 whereas, it was quite higher in T2 (128±0.722). The effect of Avian Influenza (AI) oil based vaccine in T2 was significantly high in comparison with control group (T1). However Avian Influenza (AI) Liposomal vaccinated group (T3) was highly significant (256±0.722) as compared with T1 (6.79±1.021) and T2 (128±0.722). The data fairly indicated that immune response in term of GMT was well

established with Avian Influenza (AI) liposomal vaccine as compared to Avian Influenza (AI) oil based vaccine.

In a clinical trial, liposomal influenza vaccine containing the viral hemagglutinin was found no more effective than the standard vaccine, as determined by serum HI titer (Powers, 1997). This outcome could result from the relatively high pre-vaccination antibody titers among the vaccines participating in that clinical study as the current experiment shows the high antibody titre of liposomal vaccinated birds.

The GMT titres on day 21 were marginal in group T1 (8±1.021) whereas, it was quite higher in T2 (256±0.722). The effect of Avian Influenza (AI) oil based vaccine in T2 was significantly high as compared to control group (T1). However Avian Influenza (AI) Liposomal vaccinated group (T3) was highly significant with GMT (512±0.722) as compared with T1 (8±1.021) and T2 (256±0.722).

The GMT of 8±1.021, 445.7±0.46 and 776±0.56 at day 28, were noted in T1, T2 and T3 respectively. GMT titres were marginal in group T1 (8±1.021) whereas, it was quite higher in T2 (445.7±0.46). The effect of Avian Influenza (AI) oil based vaccine in T2 was significantly high in comparison with control group (T1). However Avian Influenza (AI) Liposomal vaccinated group (T3) was highly significant (776±0.56) as compared with T1 (8±1.021) and T2 (445.7±0.46). The data fairly indicated that immune response in term of GMT was well established with Avian Influenza (AI) Liposomal vaccine as compared to Avian Influenza (AI) Oil based vaccine. Such like results are indicated by (Joseph *et al*., 2002), stated that at 3 weeks post vaccination, the seroconversion rate was 100% with liposomal vaccine. Liposome induced significant levels of antigen-specific IgG1, IgG2a and IgA in serum, lungs and nasal wash. The significant difference between Liposomal and conventional Oil based AI vaccine is in good agreement.

The GMT of 8±1.021, 630.3±0.56 and 891.4±0.46 were recorded in T1, T2 and T3 respectively at day 35. GMT titre was marginal in group T1 (8±1.021) whereas, it was quite higher in T2 (630.3±0.56). The effect of Avian Influenza (AI) oil based vaccine in T2 was significantly high in comparison with control group (T1). However Avian Influenza (AI) liposomal vaccinated group (T3) was highly significant (891.4±0.46) as compared with T1 (8±1.021) and T2 (630.3±0.56). The data fairly indicated that immune response in term of GMT was well established with AI Liposomal vaccine as compared to Avian Influenza (AI) Oil based vaccine and is in good agreement with (Yehuda *et al*., 2003), they vaccinated mice with liposomal antigen (HN) combined with liposomal IL-2 (INFLUSOME-VAC) attained higher HI antibody titers and seroconversion (percentage of mice that developed an HI titer = 40) than did mice injected with either the naked subunit vaccine. That experiment also shown that the vaccines containing IL-2 induced earlier seroconversion and that liposomal IL-2 is a more efficient adjuvant than free IL-2.

The clinical study of (Yehuda *et al*., 2003) demonstrates that an influenza vaccine composed of a subunit antigen preparation and IL-2 as an adjuvant, both contained in liposomes, induces an enhanced anti-hemagglutinin (HA) serological response in young adults, as compared with the response elicited by the current commercial vaccines and was found superior in all three parameters (i.e., GMT, seroconversion rate and seroprotection rate), as determined by the HI assay.

The combined liposomal vaccines are easy to prepare, stable, safe and far more potent than the oil based avian influenza vaccines, now in use. Liposomal HN induces an earlier, much stronger and longer-lasting response. Intranasal liposomal vaccine should be tested in poultry for its immune response and challenge protection (Babai *et al*., 1999).

Evaluation of liposomes as vehicle for oral vaccines and characterization, the stability of polymerized and non-polymerized liposomes was examined. It was suggested that polymerized and non-polymerized liposomes would serve effectively as an oral delivery vehicle (Alonso-Romanowski *et al*., 2003).

Liposomes are lipid vesicles. The external liposome membrane is composed of the same lipids as the cell membranes. This is very important, since the fact that the molecules used are not foreign to the host prevents the induction of immune rejection and the same liposome formulation can be used for repeated vaccinations. Iscom are solid particles generated by combining an antigen with a biocompatible detergent and the adjuvant Quil-A, thus giving rise to minute structures (35 nm,). These particles can only be used with antigens that can be mixed with lipids and with Quil-A (normally proteins) (FAO, 2002).

Liposome-vesicles have been introduced as drug delivery vehicles due to their structural flexibility in size, composition and bilayer fluidity as well as their ability to incorporate a large variety of both hydrophilic and hydrophobic compounds. With time the liposome formulations have been perfected so as to serve certain purposes and this lead to the design of intelligent liposomes, which can stand specifically induced modifications of the bilayers or can be surfaced with different ligands that guide them to the specific target sites (Voinea and Simionescu, 2002).

Budai and Szogyi, (2001) stated that, first the concept of the liposome as a microparticulate lipoidal vesicle separated from its aqueous environment by one or more lipid bilayers. Later, liposomes as drug carrier systems (Budai and Szogyi, 2001).

Liposomes offer an excellent opportunity to selective targeting of drugs which is expected to optimize the pharmacokinetical parameters, the pharmacological effect and to reduce the toxicity of the encapsulated drugs (Budai and Szogyi, 2001).

Several adjuvants were evaluated in order to enhance the immune response to influenza vaccines. Among these, oil in water adjuvant emulsion containing squalene, MF59, has been combined with subunit influenza antigens and tested in clinical trials in comparison with non-adjuvanted conventional vaccines. Immunogenicity analyses demonstrated a consistently higher immune response with statistically significant increases of postimmunisation geometric mean titres and of seroconversion and seroprotection rates compared to nosn-adjuvanted subunit and split influenza vaccines (Podda, 2001).

In conclusion, liposomal avian influenza vaccine has proved very effective in inducing strong immune response in commercial layers as compared with conventional oil based AI vaccine. Further, large studies are needed in order to test whether this advantage over the currently used commercial vaccine can translate in to better clinical outcomes, such as reduction in mortality and morbidity rates and to define optimal liposomal formulations and routes of administration.

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