

## Vaccination of Chicks with Experimental Newcastle Disease and Avian Influenza Oil-emulsion Vaccines by *In Ovo* Inoculation

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Received 17 May 2000; accepted 16 Jul 2000

### *Summary*

The immunologic efficacy of inactivated oil-emulsion vaccines against Newcastle disease (ND) and avian influenza (AI) by use of embryo vaccination technology was evaluated. The vaccine antigens were prepared from LaSota strain of ND and H9N2 strain of AI viruses. These antigens were emulsified with an oil adjuvant. 18-day-old embryonated eggs from Aryan broiler breeder were divided into three groups and were delivered inactivated oil-ND and -AI. Group 3 was considered as unvaccinated control. After incubation period, no significant difference ( $P < 0.05$ ) was observed in hatchability between two treatment and control groups. At intervals of experimental procedure up to 6 weeks the haemagglutination-inhibition antibody titer of groups 1 and 2 were higher than control group. The results of our study indicate that vaccination of chicks against ND and AI vaccines by *in ovo* technique, were successful under laboratory conditions.

**Key words:** embryo vaccination, haemagglutination-inhibition, Newcastle disease, avian influenza

### *Introduction*

The commercial poultry industry continues to grow due to increasing in meat and egg consumption. In Iran chicken meat consumption increased from 5.4 kg in 1985 to 9.6 kg in 1996. Egg consumption showed a similar ingestion pattern, with 4.5 kg in 1985

to 6.4 kg in 1996 (Asian Crisis Hits Consumption, 1999). Due to increase risks in order to extend poultry productions, many outbreaks of major diseases like ND and AI have been reported. Recently an AI virus strain (A/chicken/Iran/259/1998/H9N2) has been found in commercial poultry industries (Pourbakhsh *et al* 1999) and caused up to \$11 million cost (Shariatmadari 2000).

Since chicks are susceptible to ND in any age groups, the prevention will only focus on vaccination as the most important component of flock health program. To protection of hatch chicks, live ND vaccines are administered through spray, eye-drop, drinking water. However, to induce a uniform prolong protection an inactivated oil-emulsion (OE) vaccine is used subcutaneous and intramuscularly injections (Kouwenhoven 1993). In the area which disease is highly spread, the adjuvanted vaccine can be used. The adjuvant added to the antigens might stimulate the immune responses either by increase the immune response or by reducing the quality of antigen, and resulted in prolonging immunization (Payla 1991).

As well as, an inactivated OE vaccine is used against AI infection (Brug *et al* 1979). The disease is produced by an antigenically diverse group of type A influenza viruses, and protection induced by influenza vaccine is primarily dependent on the antigenic type of the viral haemagglutinin. Because only one subtype of AI virus isolated from chickens in Iran, AI vaccine, which made by the local strain can be expected to immunize chickens against influenza.

There is no doubt successful poultry production depends on significant prevention of the most important poultry diseases, particularly ND and AI. Vaccines are powerful tools in the diseases control (Alexander 1998, Swayne 1998). Regarding the economic importance of ND and AI, the development of vaccine-induced immunity depends on early production of antibody can result in decrease of poultry losses. Advancing in vaccine technology can be done either by improving the efficacy of available vaccines or developing new range of vaccines. New vaccination techniques should improve the immune response, qualitatively and quantitatively (OIE 1996). Studies on embryo vaccination (EV) in the last few years have indicated that this technology is the most efficient means for early immunization and induce a wide

spectrum of protection against these respiratory diseases (Stone *et al* 1997, Stone 1988, Stone *et al* 1980, Stone *et al* 1978). The advantages of this technique should produce early immunization, easier delivery and lower cost of administration. In this study the possibility of chick immunization against ND and AI, by use of EV technique in laboratory conditions was examined.

### **Materials and Methods**

**Eggs.** 300 fertile eggs were obtained from an Aryan broiler breeder type flock. Ten of those were selected randomly. The routine bacteriological examinations for pathogenic germs, *Salmonella*, *Staphylococcus aureus*, pathogenic *Escherichia coli* and *Mycoplasma* spp. were carried out according to Appendix 2 of the "Merck brochure for microbiological analysis of food", 1992. Under sterile conditions the egg was broken into a Petri dish, and the yolk was separated from albumin then they were collected separately in centrifuge tubes and tested. 9ml distilled water was added to 1ml yolk, and a homogenous solution was prepared using an ultra-turrax. The dilution series of that were cultured on Plate count agar (30°C, 72h), Brilliant-green phenol-red lactose saccharose agar [BPLS agar] (37°C, 24h), and *Mycoplasma* isolation (PPLO) agar. The latter was obtained from Difco and the others were obtained from Merck. The same procedure was carried out for bacteriological examination of albumin.

**Egg-yolk serological test.** 30 fertile eggs were selected randomly. The entire yolk of each egg was separated from albumin and was placed in a polypropylene centrifuge tube. An equal volume of PBS (v/v) was added to the yolk, and a homogenous solution was made using a vortex shaker. One ml of the 1:2 (v/v) diluted egg-yolk suspension mixed with 2ml of reagent-grade chloroform. After 30-60min incubation at room temperature, tubes were centrifuged at 3000 rpm for 20min. The upper clear layer was used for serologic testing (Piela *et al* 1984). The chloroform extracted was examined for ND and AI antibodies using haemagglutination inhibition (HI) test.

The HI test for ND and AI, performed by microtiter method using eight and four haemagglutination units of antigens respectively. Titers are expressed as the

reciprocal of the highest yolk dilution showing 100% inhibition of haemagglutination.

**Incubation of eggs.** The eggs were placed on setter machine after shell quality control and were disinfected by formalin plus potassium permanganate, stored up to 10 days at 37.5°C and 65% relative humidity. Afterward, candled for viability and dead or non-fertile embryos were removed, then the embryonated eggs were incubated up to 18 days.

**Antigen preparation.** The LaSota strain of ND virus was injected into the allantoic cavity of 10-day-old specific-pathogen-free (SPF) chick embryos (Lohmann Co., Cuxhaven, Germany). The infective amnio-allantoic fluid (AAF) was harvested, pooled, titered (HA=1:512, EID<sub>50</sub> =10<sup>10.50</sup>/ml) and inactivated with 0.1% formalin (Bahnmann 1997).

Inactivation of ND virus was confirmed by inoculating 0.1ml of inactivated AAF into five 10-day-old SPF chick embryos. AAFs were harvested and injected into an additional five eggs. The procedure was repeated for three times. Finally, the harvested fluids were tested for inactivity of the virus.

Strain A/chicken/Iran/259/1998(H9N2) of AI virus which confirmed by central veterinary laboratory, Weybridge, Surrey, UK, was injected in SPF chick embryos and infective AAF was harvested, pooled, titered (HA=1:256, EID<sub>50</sub>=10<sup>9.83</sup>) and inactivated with 0.1% formalin. Inactivation of AI virus was confirmed by the same procedure, which was used for ND virus. Clarification of these antigens was done by centrifugation at 5000rpm for 30min at 4°C. Then were frozen (-20°C) until used.

**Vaccine preparation.** The ND and AI antigens were emulsified in ratio of 3:7 with ISA-70 oil adjuvant (SEPPIC, Cosmetics/Pharmacy Division, Paris, France) and then homogenized in which 0.5ml of the vaccines were contained 10<sup>8.5</sup> EID<sub>50</sub> viruses (before inactivation).

**Vaccination of embryos.** 18-day-old embryonated eggs were divided into three groups of seventy. Group 1 and 2 were given ND and AI vaccines respectively. Group 3 was considered as unvaccinated control chicks. Embryos in different vaccine treatment groups were delivered 0.1ml of vaccines by a 23-gauge needle at a 1.25-

inch depth and hatched at 37.8°C and 65% relative humidity. Hatched chicks were placed in three separate cages.

**Serology.** Ten chicks of three groups were bled five times at days 0, 10, 20, 30 and 40. All serum samples were tested individually for HI antibodies to AI and ND viruses by standard procedures in microtiter plates using four HA unites for AIV and eight for NDV. The antigens, which prepared by Razi Ins., Iran, were used in the assays. A geometric mean titer (GMT) was determined for the sera within each group (Brugh 1978).

**Statistical analysis.** Statistical analysis of the data was done using the Student *t*-test and variance analysis. Statements of statistical significance are based upon  $P < 0.05$ .

## Results

**Bacteriological examination.** None of pathogenic bacteria was isolated from the specimen cultures. Only *Micrococcus* spp. isolated from two yolk and albumin cultures that is not a pathogen.

**Comparison of egg-yolk and serum serology tests.** A marked rise in yolk antibody titers against ND (Table 1) and AI (Table 2) in comparison with 1-day-old chick serum titers was observed. There was a significant difference ( $P < 0.05$ ) between antibody levels of serum and chloroform-extracted yolk from individual chicks in the experimented groups.

Table 1. Comparison of egg-yolk and serum antibody titers against Newcastle disease

Yolk		Serum					
		G 1		G 2		G 3	
No.	HI	No.	HI	No.	HI	No.	HI
9	8.6	2	6	1	6	2	6
15	7.8	5	5	6	5	7	5
6	6.3	3	4	3	4	1	4
30	7.7	10	4.9	10	4.8	10	5.1

G 1: *In-ovo* vaccination against Newcastle disease

G 2: *In-ovo* vaccination against avian influenza

G 3: Unvaccinated group

Table 2. Comparison of egg-yolk and serum antibody titers against avian influenza

Yolk		Serum					
		G 1		G 2		G 3	
No.	HI	No.	HI	No.	HI	No.	HI
16	4.3	3	2	3	2	1	2
11	3.5	5	1	4	1	7	1
3	0	2	0	3	0	2	0
30	3.5	10	1.1	10	1	10	0.9

*Hatchability rates.* The result of hatchability was no statistically significant difference ( $P < 0.05$ ) in hatchability between unvaccinated group and treated ones (Figure 1).

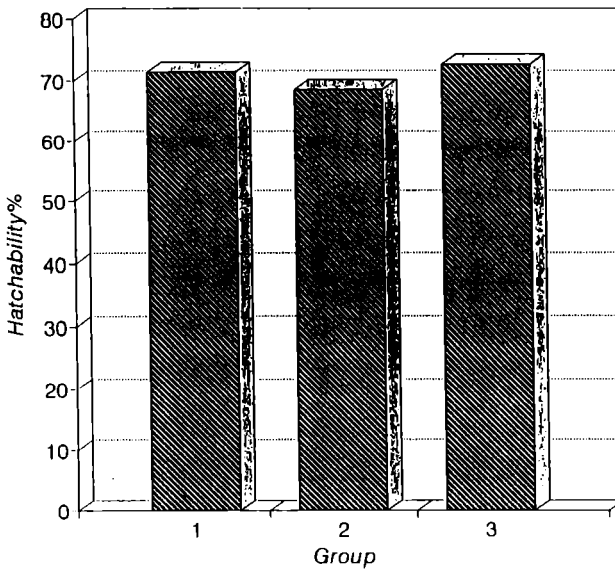


Figure 1. Comparison of hatchability rates in treatment groups

*Antibody titers.* An increase in HI titers was observed in groups 1 and 2, which were vaccinated against ND and AI, at intervals of experimental procedures (Table 3 and Figure 2).

Table 3. Geometric mean titers ( $\log_2$ ) for HI test on sera following an exposure to ND, AI and ND-AI vaccines

Groups	Days of trial															
	10				20				30				40			
	ND		AI		ND		AI		ND		AI		ND		AI	
	No.	HI	No.	HI	No.	HI	No.	HI	No.	HI	No.	HI	No.	HI	No.	HI
G 1	10	5.6	9	<1	10	6.3	10	<1	10	6.8	10	<1	9	7.2	10	<1
G 2	9	3.2	10	2.5	10	1.4	9	3.2	9	<1	10	4.1	10	<1	10	4.9
G 3	10	3.6	11	<1	9	1.8	11	<1	10	<1	10	<1	10	<1	10	<1

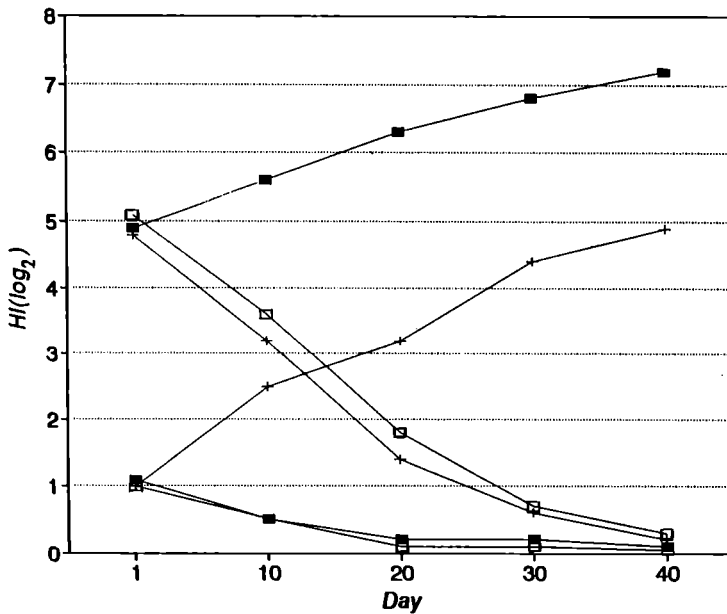


Figure 2. Comparison of antibody titers (HI test) in treatment groups. ■ group 1; + group 2; □ group 3. AI antibody titers designed by heavy and ND antibody titers by light lines.

### Discussion

The concept of embryo vaccination is based on an observation that chickens develop some immunologic maturity well before hatching (Jankovik *et al* 1975, Well & Reynoud 1987). Early protection from virulent Marek's disease virus, infectious

bursal disease virus, AI virus and ND virus has been achieved through EV in chickens and turkeys (Stone *et al* 1997, Sharma 1986, Sharma & Witter 1983). Similar observations have been reported after inoculation of birds against mentioned above viruses alone or in combination (Rautenschlen *et al* 2000, Ahmad & Sharma 1993, Sharma 1985).

In endemic areas, chickens must be protected against the diseases by vaccination. The protection of young chicks against ND and AI is an integral part of health management in most commercial poultry flock units. Thus the chickens raised for commercial consumption must be protected against viruses in the environment. EV provides more time for chicks to develop immune response before being placed in a contaminated environment.

Results on comparison study of egg-yolk and serum antibody titers against ND and AI, show that the transfer of passive antibodies into eggs is degraded in hatch period so that, their levels in chicks are lower than that in egg-yolk. The immobilization of immunoglobulins replication in yolk, and their short half-life results in titers reduction during incubation period. Often the absorption of yolk is not completed. It may be related to breeding and feeding of dam, conditions of incubation and quality of eggs (North 1990).

Results of our study obtained under laboratory conditions on the immunologic efficacy of EV against ND and AI, did indicate that inoculation of 18-day-old embryonated chick eggs with inactivated oil-ND and -AI vaccines did not affect hatchability of eggs. Our findings about hatchability did agree with those reported by Stone *et al*, 1997, who found adverse effect on hatchability when eggs were vaccinated with various vaccine volumes and needle gauges.

Vaccination of broiler breeder (Aryan breed) chickens as 18-days embryos with inactivated oil-ND and -AI vaccines, clearly resulted in initiation of immune response in hatched chicks. All chickens hatching from vaccine-injected eggs had HI antibodies at 6 weeks of age. Use of vaccines by *in ovo* vaccination procedure is likely to eliminate the stress from individual injection of newly hatched chicks and save the cost of vaccine administration. This study demonstrated that *in ovo*



vaccination may be a safe, because hatchability is not affected, and efficient way of vaccine delivery because of savings in time and labor associated with handling live birds. Also, *in ovo* vaccinated chicks develop early post hatch protective immunity.

The ultimate result will be obtained if we experience the success of lab procedure on the field condition.

### **Acknowledgment**

The authors would like to advance their appreciation to Mr.E.Sorour, S.R.Hosseini and P.Gitigard for their assistance.

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