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PREPARATION AND EVALUATION OF AN INACTIVATED INFECTIOUS BURSAL DISEASE (IBD) VIRUS VACCINES FROM RECENT EGYPTIAN VIRUS ISOLATE ADJUVATED WITH *NIGELLA SATIVA* OIL.

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SUMMARY

Inactivated IBD virus vaccines were prepared from a recent Egyptian isolate and adjuvated with Nigella sativa oil. The first passages of propagated viruses in SPF- embryonated chicken eggs (ECE), Vero and chicken embryo fibroblast (CEF) cell cultures were inactivated with binary ethylenemine (BEI), and supplemented with *Negilla sativa* oil as adjuvant

The prepared vaccines proved to be highly immunogenic and elicited high titers of neutralizing antibodies (17-20 $\log_2$) at weekly interval till 7 months post-vaccination (PV) and high values of lymphocyte blastogenesis (0.598 versus control 0.06). Besides they were able to protect vaccinated chickens (100 % protection) when challenged 21 days PV. The superior potential effect of these vaccines, when compared with imported one, may be due to the use of recent local IBDV isolate and *Nigella sativa* oil for its non specific immune stimulation effect. In addition, the keeping quality of prepared vaccines proved to be sterile, safe, stable and potent when preserved at 4°C for 6 months (the end of the experiment) as they produced 100 % and 80 % protection after 3 and 6 months of preservation respectively. Each chick received 0.3 ml as one dose subcutaneously. From these studies we conclude that the use of *Nigella sativa* oil as adjuvant is considered a new trend in preparing inactivated vaccines

INTRODUCTION

In spite of using different types of vaccines in Egypt, serious outbreakes of IBD were observed since 1982 (El-Batrawi, 1990, Mousa and Saif El-deen 1990, Modbouly, et al 1992, Aly, et al 1996, Bekhit 1996 and Saif, et al 1996). The economic importance of IBD is manifested in two

ways. The first is due to the clinical disease in 10 -20 % and can reach to 100 % of the affected flock and cumulative mortality was 10 - 20 % (Cosgrov, 1962, Hitchner, 1970 and Fragher, et. al. 1974). The second and most important manifestion is a severe prolonged immunosuppression of chicken infected at an early age (Allan, et al . 1972). The appearance of antigenic variants of IBDV in USA (Saif, 1984) and very virulent strains in Europe and other countries (Brown, et. al. 1994) ensures that the economic importance of IBDV will continue to be a very complex problem because of the recent field isolates of IBDV have been found to be antignically different from previously isolated vaccinal strains of serotype I with 30 - 70 % relatedness which provide an explanation for failure of maternal immunity and vaccination programmes against IBDV using conventional vaccines (Jackwood and Saif, 1987) .

Allover the world, several studies were done to minimize the risk of this virus by using different types of vaccines and programmes of vaccination but the destructive and immunosuppressive effects of the virus are still inprogresse.

Therefore the aim of this study lies in developing inactivated vaccines from recent local isolate adjuvated with Nigella sativa oil for its non specific immunostimulation effect (Madbouly and Tamam 2000 and Madbouly, et al 2000)

MATERIAL AND METHODS

Virus isolation, propagation and inactivation :-

Bursae of naturally infected broiler flocks located at El-Fayoum governorate during 1997 were collected and crushed to from 10 % bursal homogenate. The bursal homogenate were subjected for three cycles of freezing and thawing then centrifugated for 15m at 3000 rpm. The supernatant fluid of this homogenate was used for virus isolation according to Allan et al (1973). The virus was propagated in SPF-ECE for 3 passages followed by 3 passages in young susceptible chicks then 3 passage in SPF-ECE followed by 12 passages in SPF-ECE. The first passage of last propagation was subjected for propagation on CEF and Vero cell cultures (60 passage for each). The isolated virus designated Fc-97 as the capital litter " F " denote to the locality at which the broiler flocks were reared, the small litter " c " denote to the host from which the virus was isolated (chicken), and the number " 97 " denote to the year in which the outbreaks were occurred. The first passages of last propagation either on SPF-ECE and passages 1, 20 , 40 , and 60 on either CEF or Vero cell cultures were inactivated with BEI according to Girand et al (1977). The inactivated Fc-97 IBDV propagated viruses were treated with sodium thiosulphate to neutralize the effect of BEI.

Quality control of the prepared inactivated virus:

sterility test:

The locally prepared vaccine batches were examined for the absence of aerobic bacteria, anaerobic bacteria, fungal and mycoplasma contaminants using nutrient agar, thioglycollate broth . Sabouraud glucose agar and Frey's media for testing the sterility of the vaccines

Safety test on ECE : (complete inactivation)

Samples of the inactivated viruses were examined for the presence of active virus particles by 3 passages on the chorioallantoic membrane of ECE (11 - 13 days old). The inoculated eggs were incubated at 37°C and 80 % humidity for 7 days. Dead embryos within the first 24 hours were discarded. After 7 days all living and dead embryos were examined for the presence of specific IBDV lesions

Safety test on susceptible chicks :

After preparation of the inactivated vaccines 95 chicks of 21 day old were inoculated with 0.5 ml/ chick via S/C route (10 chicks for each inactivated passage and 5 as control non inoculated). These chicks were observed for 3 weeks for any signs of local reactions as abscess formation, irritation or systemic reaction. After five days of inoculation, 2 chicks from each group were subjected to postmortem examination to detect any pathological lesions especially in the bursa of Fabricius gland .

Potency test :

Ninety five - 3 weeks old chicks were inoculated with a full vaccinal dose 0.5 ml S/C (10 chicks for each inactivated vaccine passage and 5 chicks were kept as non vaccinated control). Three weeks post vaccination, blood samples were collected from the wing vein and all birds were challenged through conjunctival instillation of 10^2 TCID50 of local virulent IBDV. The challenged chicks were observed for 21 days. Every week 5 chicks were scarified and examined for specific IBDV lesions

Virus concentration was done according to killington, et al.,(1996)

The seeded IBD virus was concentrated to 10 % by ultra centrifugation at 26000 rpm for 2.5 hours.

Preparation of Nigella adjuvant :

Nigella sativa oil was used as adjuvant for preparation of inactivated IBDV vaccine according to Madbouly et al (2000). Nigella sativa oil was mixed with span in a ratio of nine parts oil to one part span (by weight or volume) with thoroughly mixing before sterilization by passing them through a Seitz filter. The oil span mixture was stored at room temperature and used within few weeks of preparation.

Preparation of inactivated IBDV vaccines adjuvated with Nigella sativa oil :

The inactivated first passages of last propagation

on SPF-ECE, CEF and Vero cell - cultures were used as aqueous phase for preparing the vaccine. Stable emulsion was prepared according to Madbouly et al (2000) by thoroughly mixing of aqueous and oil phase in ratio of 1 : 4, where one part of aqueous phase (containing 96 % inactivated IBDV suspension mixed with 4 % tween 80) was mixed with three part of prepared Nigella sativa adjuvant with continuous mixing. The mixtures were gently dispersed in a tube in a homogenizer till preparing a stable oil emulsion vaccine with low viscosity of 1 : 4 .

Evaluation of the prepared vaccines:

The physical properties of emulsions were investigated by the following :

1. Emulsion type : was applied according to Becher (1957).

This test was determined by the drop test in which 2 drops of emulsion were placed separately on a clean glass microscopic slide and each drop was mixed with either one drop of oil or one drop of water. A water in oil emulsion blend readily with oil but not with water

2. Relative viscosity : was applied according to Cessi and Nardelli. (1973).

It was determined as the flow time at 24°C for discharge of 0.4 ml of emulsion from vertical 1.0 ml serological pipette filled to the 0 mark

3. Emulsion stability :

It was expressed as weeks of storage time during which the oil and aqueous phases did not separate .

Keeping quality of inactivated locally prepared IBDV vaccines:

Locally prepared inactivated (SPF.ECE, Vero and CEF propagated) Fc-97 IBDV vaccines were kept at 4°C then tested at monthly interval for about 6 months for its protective effect in susceptible chicks. Five young chicks were used monthly. Each chick received 0.3 ml S/C. Vaccinated chicks were challenged 21 days PV. with hot IBDV .

Evaluating the humeral and cell mediated immune responses

Four groups (50 chicks per each) were vaccinated with the three locally prepared vaccines beside the imported one and fifth group was left as control unvaccinated. Serum samples were collected at weekly intervals PV till 7 months (the end of the experiment). These sera were tested by virus neutralization test. Peripheral blood samples on anticoagulant were collected for lymphocyte blastog -enesis assay.

Virus neutralization test was applied according to Rossiter, et. al (1985) for determining the elevated amount of antibodies using constant virus variable serum mixture on CEF cell cultures.

Lymphocyte blastogenesis assay was applied according to Garn et al (1994).

RESULTS AND DISCUSSION

The preparation of inactivated IBD virus vaccines from recently isolated Egyptian isolate are considered a goal for preventing escape of IBDV variants and consequently an important step for controlling of IBD. Initially the first passages of IBDV isolate propagated on SPF-ECE, Vero and CEF- cell cultures have taken 44 hours for complete inactivation by BEI (Table 1) .Furthermore the subsequent passages either on CEF or Vero cell cultures (P_{20} , $_{40}$ and $_{60}$) showed decrease in time needed for their inactivation than the first passages. The hours needed for inactivation decreased from 44 as in first passage to 28 hours as in passage 60. This decrease in time for inactivating Fc-97 IBDV isolate after long passages either on CEF or Vero cells can be explained by reduction of virus virulence by passages, deletion of some genomic sequences and mutagenesis of the propagated virus . The quality controls of the prepared inactivated vaccines were assessed. The prepared vaccines are completely sterile and haven't any bacterial fungal, and or mycoplasma contaminants (Table 2). No residual live virus particles were detected either in inoculated ECE, cell cultures or injected young susceptible chicks (Table 1). There is no any pathological lesions on the inoculated embryos and no CPE on the inoculated cell cultures. Besides, there is no clinical symptoms, bursal lesions or deaths were recorded in injected chicks and these results denote to the safety of the prepared vaccines. Thereafter the inactivated vaccines were supplemented with Nigealla sativa oil as adjuvant. The advantages of Nigella sativa oil for its non-specific immunostimulating, effect (El-kadi et al .. 1990, Basil and Erwa 1993, Haq et al 1995, and Madbouly et al 1999 a and b); anti-inflammatory and antioxidant activity (Elliot et al 1989, and Houghton et al 1995,); growth promoting effect (Abdel -Aziz , et al 1995, Khodary et al 1996, and Madbouly et al 1999); anti-microbial effect (Topozada et al 1965, Agarwal, et al 1979, Namba, et al 1985, Akgul 1989, and Hanafy and Hatem 1991) and using it as an adjuvant in preparing infectious laryngotracheitis inactivated virus vaccine (Madbouly, et al 2000, and Madbouly & Tamam 2000) beside its safe natural vegetable oil, triggered us to use this oil as adjuvant for preparing the IBDV vaccines. On comparing the humeral and cell- mediated immune responses offered by the locally prepared inactivated IBDV vaccines with each others and with the imported one, the prepared vaccines proved to be highly immunogenic and still elicited high titers of neutralizing antibodies (17 - 20 log2) at weekly interval till 7 months post-vaccination (the end of the experiment) and high values of lymphocyte blastogenesis (0.598 versus control 0.06) as in table (4) besides their ability to produce 100 % protection when vaccinated chicks were challenged 21 days post-vaccination (table 5). The superior potential effect of these vaccines when compared with the imported one may be due to the use of a recent local IBDV isolate that circulates among the reared flocks and the use of Ni-

Table (1): Hours for inactivation and safety of propagated Fc-97 IBDV isolate on SPF-ECE,CEF, Vero cell cultures and chickens.

First passage of	Titers expressed in log_{10} ID_{50}	Hours for inactivation	Effect of inactivated virus on	
			SPF-ECE and cell culture	Chicks
Last propagation on SPF-ECE	8	P_1 44	Absence Of Pathological lesions, no death of embryos	Absences of : Clinical signs Bursal lesions Mortalities in injected chicks by any of the three inactivated virus
	ND	P_{20} ND		
	ND	P_{40} ND		
	ND	P_{60} ND		
CEF cell	8	P_1 44	Absences of cytopathic effect	
	8	P_{20} 36		
	7.8	P_{40} 35		
	7.2	P_{60} 28		
Vero cells	5	P_1 44	Absences of cytopathic effect	
	5	P_{20} 36		
	6	P_{40} 35		
	6	P_{60} 28		

The infectivity titers for first passage of first propagation on SPF-ECE was 6.3 log_{10} ID_{50}

ND = not done due to shorting in SPF-ECE supply.

Table (2): Sterility of the prepared inactivated passages of Fc-97 IBDV isolate

Media	SPF	Vero cells				CEF cells				
	P1	P1	P20	P40	P60	P1	P1	P20	P40	P60
Nutrient agar medium	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Thioglycollate broth	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Sabouraud glucose agar	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Gre'y media	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC

NC= No colonies appeared on the used medium
NT = No turbidity appeared in the used broth

Table (3): Log_2 mean neutralizing antibody titers in sera of vaccinated chicks with inactivated vaccines (locally prepared and imported)

Type of vaccine used in chicken	Weeks post vaccination																			
	1	2	3	4	5	6	7	8	9	10	11	12	14	16	18	20	22	23	24	28
Inactivated vero	6	8	19	18	18	20	19	19	20	20	19	19	20	20	20	19	20	19	20	19
Inactivated CEF	6	8	19	20	20	20	20	20	20	20	20	20	20	20	20	20	20	19	18	19
Inactivated SPF	6	8	19	20	20	20	17	17	20	20	20	20	20	20	20	20	20	19	20	19
Inactivated imported	8	8	8	8	7	7	7	6	6	6	5	5	4	4	4	3	3	2	2	2
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

From this table it is very clear that the locally prepared inactivated vaccines that either propagated in SPF-ECE or Vero and CEF cells produced higher antibody titers than those commercial inactivated ones.

Table (4): Lymphocyte blastogenesis of chicks vaccinated with inactivated vaccines (locally prepared and imported)

Chicken group	Type of vaccine used	Weeks post vaccination			
		1	2	3	4
1	Inactivated Vero	0.527	0.455	0.509	0.447
2	Inactivated CEF	0.513	0.432	0.493	0.230
3	Inactivated SPF-ECE	0.598	0.433	0.326	0.469
4	Inactivated imported	0.432	0.247	0.219	0.178
5	Control	0.04	0.02	0.03	0.06

Table (5): Rate of protection of locally prepared inactivated FC-97 IBDV vaccines after 6 months of preservation at 4°C.

Type of vaccine used in chicken	No. of chicks used per months	Months of preservation											
		1		2		3		4		5		6	
		Prot.	%	Prot.	%	Prot.	%	Prot.	%	Prot.	%	Prot.	%
Inactivated Vero	5	5	100	5	100	5	100	4	80	4	80	4	80
Inactivated CEF	5	5	100	5	100	5	100	4	80	4	80	4	80
Inactivated SPF-ECE	5	5	100	5	100	5	100	4	80	4	80	4	80
Control Unvaccinated	2	0	0	0	0	0	0	0	0	0	0	0	0

Prot = Protected % = percentage of protection

gella sativa oil as adjuvant for its previously mentioned advantages. In addition the keeping quality of prepared vaccines proved to be sterile, safe, stable, and potent when preserved at 4°C for 6 months (the end of the experiment) as they induced 100 % and 80 % protection after 3 and 6 months of preservation respectively. Each chick received 0.3 ml as one dose subcutaneously. Passages 20 , 40 and 60 either on CEF or Vero cell cultures were not used for vaccine preparation to avoid loss in antigenicity, immunogenicity and protectivity due to mutagenesis of the virus offered by long passages on cell cultures. Yamaguchi, et al (1996) showed antigenic diversity between the cell culture adapted highly virulent IBDV strains and classical strains by cross neutralization analysis. From these studies we conclude that the use of *Nigella sativa* oil as adjuvant is considered a new trend in preparing inactivated vaccines.

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