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PREPARATION AND EVALUATION OF NEWCASTLE DISEASE VIRUS INACTIVATED VACCINES ADJUVANATED WITH CRUDE OR FRACTIONIZED NIGELLA SATIVA OIL

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SUMMARY

Different types of inactivated oil emulsion Newcastle disease vaccines were prepared using different extractions of the Nigella Sativa oil. The physical properties of emulsions were carried out and included emulsion type, emulsion stability and emulsion viscosity. The vaccinated chicks were bled at one-week intervals post-vaccination over six weeks and the collected sera were tested by the HI test. After that, they were challenged 21-days and 42-days post-vaccination by the intramascular inoculation with VVNDV. From this study we can conclude that the non-specific immunostimulant effect of Nigella Sativa oil is acquired when it is used as a crude oil and this improved its ability as a good adjuvant for viral vaccines.

INTRODUCTION

Newcastle disease (ND) is a highly contagious and fatal destructive disease which attacks chiefly chickens and turkeys usually in acute form, but sometimes in subacute or chronic form. It occurs worldwide and has a considerable economic impact on the world poultry industry, ranging from losses due to disease and the expensive of vaccination to the significant cost of diagnostic laboratory investigation (Sousa et al., 2000). The disease is caused by avian paramyxovirus type I (PMV-1) serotype and for many years Newcastle disease virus (NDV) strains were considered to form a serologically homogenous group and this has been the basis of vaccination procedures employed prophylactically in most countries (El-Kady & Madbouly 1997). However, some studies based on the use of monoclonal antibodies have shown that considerable antigenic variation exists

between different strains of NDV (Russell & Alexander, 1983; Russell, 1988 and Alexander, 1991). Other study depend upon the pathogenicity of NDV beside cross challenge test was done (El-Kady & Madbouly 1997). In Egypt, epidemics of ND among vaccinated flocks has been reported, and a virulent strains of NDV has been isolated showing viscerotropic characteristics (Reda, 1982; Khafagy, 1983; Amal Eid, 1988; Bekhit and Abd-El-Hamid,1990 and Manal Afifi et al., 1997).

The strategy of prevention of ND in chickens depends mainly on vaccination, by using either live attenuated or inactivated NDV vaccines, which usually protects the chickens from the more serious consequences of the disease, but the most used one of them is the live attenuated vaccine which has adverse post vaccinal reactions such as mild respiratory disease or transient decrease in egg production and these can be minimized or avoided by using inactivated vaccines. Besides, inactivated vaccines countered the problem of egg-transmitted pathogens and eliminate the unnecessary hidden costs often associated with the widely used live vaccines. Furthermore, and for minimizing and eliminating the undesired losses offered by using live attenuated vaccines, comprehensive approaches for using inactivated vaccines that considered safe and more economic especially when vegetable plant extract has the ability to induce cellular immune response nonspecifically were done using crude Nigella Sativa oil as adjuvant (Madbouly et al., 20000; Madbouly & Tamam, 2000; Madbouly et al., 2001 and Madbouly et al.,2002). This study aimed to compare crude and different fractions of Nigella Sativa oil by chemical compounds for preparing inactivated NDV vaccines.

MATERIAL AND METHODS

Chicks : Commercial broilers from vaccinated flocks of local breeds from Takamoly Poultry Project at El-Fayoum Governorate . These chicks were housed under strict hygienic measures and used for vaccination trials using the prepared vaccines.

Embryonated chicken eggs (ECE): Fertile specific pathogen free (SPF) embryonated chicken eggs were purchased from SPF-farm, Kom Oshim, El-Fayoum Governorate incubated and used at 9-10 days old for propagation & titration of the velogenic viscerotropic Newcastle disease virus (VVNDV).

Newcastle disease virus (NDV):A field strain was isolated from a sever outbreak of ND. The strain was identified and characterized as VVNDV. The strain was used for vaccine preparation and for challenge experiments (El-Kady and Madbouly, 1997).

Live Newcastle disease virus vaccine :The lentogenic living Hitchner B1 strain was prepared lo-

cally in Vet. Serum and Vaccine Res. Inst., Abbassia, Cairo.

Specific anti-NDV hyperimmune serum :It was locally prepared in the department of Newcastle disease, Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo. It was used as positive control in the applied serological techniques.

Buffers :-physiological buffer saline : was prepared as 0.85% NaCl according to Hudson & Hay, (1980) and used in haemagglutination inhibition test.

Phosphate buffer saline : was prepared according to Hudson & Hay (1980), sterilized by autoclaving and used diluent when ECE were used for virus titration.

Nigella Sativa oil (whole crude oil) : was prepared from the Nigella Sativa seeds (commercial source) by pressing the seeds.

Extractions and fractions of the Nigella sativa oil:

1-Un-saponifiable fraction (mostly steroids).
2-Hexan-extract of Nigella Sativa seeds. (A&B)
3-Fatty acids of Nigella Sativa oil.
4-Methanol-extract of Nigella Sativa seeds.

The oil, extractions and fractions were used as adjuvant in locally prepared inactivated NDV vaccines.

Emulsifiers :

- Sorbitan mono-oleate (SPAN 80) : it is oil-soluble surfactant and was kindly obtained from Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo,
- Polyoxy Ethylene Sorbitan (Tween 80) : non-ionic detergent was used as emulsifying agent to decrease the viscosity of the prepared vaccines.

Beta propiolacton :It was kindly obtained from Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, in a liquid form (2ml/vial), stored at -20°C and used as viral inactivator in a final concentration of 0.2%.

Infectivity titration in embryonated chicken eggs : Estimation of 50% end point was carried out by the method of Reed & Muench, (1938) .

Haemagglutination test (HA): was done according to the slandered procedure given by Anon, (1971)

Inactivation of virus suspension with beta-propiolactone :It was carried out according to Schamidt & Lennette, (1967).

Safety and potency of beta-propiolactone inactivated NDV : This was accomplished by inoculation of undiluted beta-propiolactone-inactivated suspension into 20, nine-day-old SPF-ECE (0.2ml/egg) via the allantoic sac. 6 days post inoculation eggs were individually tested for haem-

agglutination activity by the haemagglutination test. Fluids harvested from dead and survivor embryos were pooled and passaged blindly for 2 successive further passages before the batch of vaccine prepared was considered safe.

Also, ten 4-week-old birds were inoculated I/M with the inactivated suspension, another ten birds were left as control. The birds were observed for two weeks for any clinical signs.

Preparation of the extracts:

Seed powder of Nigella Sativa (250g) was refluxed with n-hexane (60-80°C) of three hours, the solvent was then distilled off to leave a yellowish brown oil (53.2g, 21.2%), EL-Sayed et, al., (1998).

Preparation of the fatty acids and the sterol fractions:

The fatty acids and the sterol fractions were prepared as previously reported (EL-Sayed et, al., 1998), n-hexane extract (20g) of the Nigella sativa was partitioned between 90% methanol and n-hexane to give 100ml of 90% MeOH extract, and 100ml of n-hexane extract.

Vaccine preparation:Water-in-oil-emulsion vaccines were prepared with both the oil-soluble surfactant (SPAN 80) that added to the oil phase and aqueous-soluble surfactant (Tween 80) that added to the aqueous phase then both phases were emulsified manually. Seven types of inactivated oil-emulsion NDV vaccines were prepared using the oil of Nigella Sativa, different extractions and fractions of the Nigella sativa oils as follows:

Vaccine 1 consisted of inactivated NDV(aqueous phase) + fatty acids of N.sativa (oil phase).

Vaccine 2 consisted of inactivated NDV(aqueous phase) + un-saponifiable fraction (oil phase)

Vaccine 3 consisted of inactivated NDV(aqueous phase) + hexane extract of N.sativa (oil phase) [A].

Vaccine 4 consisted of inactivated NDV(aqueous phase) + hexane extract of N.sativa (oil phase) [B(

Vaccine 5 consisted of inactivated NDV(aqueous phase) + methanol extract of N.sativa (oil phase)

Vaccine 6 consisted of inactivated NDV(aqueous phase) + crud oil of N.sativa (oil phase).

The prepared vaccines were examined for the absence of aerobic bacteria, anaerobic bacteria, fungal and mycoplasma contaminants for being sterile.

Physical properties of the prepared vaccines:

Observation of the physical properties of the prepared vaccines were carried out according to Stone et al., (1983). Observation included emulsion type, emulsion stability and emulsion viscosity.

Experimental design for studying the Efficacy and potency of the prepared vaccines: One hundred and sixty, one-day-old chicks were used

in this experiment. They reared under good hygienic measures till 21 days as they became fully susceptible to NDV after 20 random blood samples examination using HI test. The birds were divided into eight equal groups each of 20 chicks and were treated as follow:

Groups 1,2,3,4,5,6 & 7 were vaccinated with vaccines 1,2,3,4,5,6 & 7 respectively while group 8 was left unvaccinated as control. Each chicks was inoculated S/C in the mid-dorsal neck area with a 0.3ml vaccine dose.

Serology : All chicks were bled at one week intervals post vaccination over six weeks for individual HI test which was carried out according to the procedures adopted by Beard and Wilkes, 1973).

Challenge test :Ten birds from each group were subjected to challenge test 21 days post vaccination by I/M inoculation of 2X105 ELD50 of VVNDV The birds were observed for 15 days post inoculation and deaths within this period were subjected to post mortem examination. The remaining birds in each group were challenged 42 days post vaccination.

RESULTS

(1)Composition and physical properties of the prepared vaccines: All prepared vaccines were emulsified in a ratio of four oil-phase and one aqueous-phase. The virus titer in aqueous-phase was $10^{9.75}\log_{10}EID_{50}$/ml. The oil phase contains the fractionized Nigella Sativa oil.

Table (1): physical properties of the prepared vaccines

Emulsion status	Type of vaccine					
	1	2	3	4	5	6
Stability days*	<1	1	7	6	13	30
Viscosity second**	40	36.8	25.8	25.5	19.4	15

37°C during the oil and aqueous phases did not separate.
**Relative viscosity is given as flow time in seconds at 25C for discharge of 0.4ml from a vertical 1 0ml serological pipette filled to the 0 mark.

(2)Efficacy of the prepared vaccines in vaccinated chickens: Sera of vaccinated chickens with the prepared vaccines were collected weekly for 6 weeks post vaccination and tested by HI test. The mean log2 HI antibody titers were depicted in table (2).

Table (2): Mean log2 HI antibody titers weekly post-vaccination:

Emulsion status	Type of vaccine					
	1	2	3	4	5	6
1	0.7	2.5	4.8	5.6	4.3	3.5
2	0.65	2.6	5	5.3	4.4	4.1
3	0.95	3.2	5.5	5.5	4.9	4.3
4	0.85	3.4	5.7	5.2	5	4.7
5	1.05	3.6	6.2	6.3	5	5
6	2.15	4.12	7.07	7.45	6.95	6.5
7*	2.1	3.2	5.4	4.4	4.1	3.8
Control non-vaccinated	0	0	0	0	0	0

* Inactivated NDV withoyt adjuvant

(3)The quality control of the prepared vaccines: The prepared vaccines are sterile (not contained any bacterial, fungal, and/or mycoplasmal contaminants when cultured on the corresponding media used for their cultivation). They also are safe when inoculated in either SPF-ECE or in susceptible chicks.

(4)Potency (protection level) of the prepared vaccines: Vaccinated chicks were challenged with NDV by injecting2X105 ELD50 of VVNDV log10 EID50/ml and the challenged chicks were observed for 14 days post-challenge. The protection levels of the prepared vaccines were presented in table (3).

Table (3): Protection percentages of the prepared vaccines in chicks challenged at given days post-vaccination:

Type of vaccine used	21-days post-vaccination			42-days post-vaccination		
	No.of birds	No.of survived	Protection percentage	No.of birds	No.of survived	Protection percentage
1	10	6	60%	9	3	33.3%
2	9	5	55.5%	10	4	40%
3	10	6	60%	7	3	42.85%
4	10	7	70%	10	5	50%
5	10	8	80%	10	6	60%
6	10	10	100%	10	9	90%
7*	10	6	60%	10	4	40%
Control non-vaccinated	10	0	0	10	0	0

DISCUSSION

Oil adjuvants are readily adapted to many poultry disease antigens and are widely used in water-in-oil (W/O) emulsion vaccines through the poultry industry. Field and laboratory evidence suggests that inactivated ND vaccine with mineral oil emulsion-adjuvant can induce high levels of protection against clinical velogenic viscerotropic Newcastle disease (Stone, 1987).

Many different types of compounds are known to stimulate vaccine efficacy but most of the commercially available products are still supplemented with classical adjuvants including mineral oil emulsions (Hilgers et al., 1998). Tissue reactions in chickens injected with mineral oil emulsion vaccine remain a source of poultry condemnations and are of financial concern to the poultry industry. The mineral oil phase alone, which persists for months, may cause undesirable tissue reactions and is considered to have carcinogenic potential for consumers (Yamamaka etal., 1993). Another concern is that accidental injection of operators with mineral oil emulsion is a potential source of liability claims for personal injury (Stone, 1997). On the other hand, the main problem of inactivated vaccines adjuvated with mineral oils is the inability of them to activate cellular immunity like that induced by live attenuated vaccines.

For the above-cited reasons, the development of suitable replacements for the mineral oil portion of the vaccines is desirable. The replacement must have high potency, low viscosity, long shelf life, and minimal tissue reactivity. Also, they must be compatible with mass production techniques, homogenous in appearance, cost effective and has the ability to activate cellular immunity to compensate the effect induced by live attenuated vaccines. Therefore, the aim of this study is the production of inactivated NDV vaccine supplemented with Nigella Sativa oil or its fractions as adjuvant which has the ability to activate macrophages and lymphocytes non-specifically (Basil & Erwa, 1993 and Haq et al., 1995).

Some studies were planned to avoid the undesirable effect of the mineral oils by replace them with suitable animal, vegetable or synthetic oil as Stone., (1997) and Madbouly et al., (2000, 2001 & 2002). Nigella Sativa oil was one of these adjuvants that used as a replacement for the mineral oil due to its non-specific immunostimulant effect (Basil & Erwa, 1993 and Haq et al., 1995) besides other different desirable effects such as anti-microbial effect (Hanafy & Hatem, 1991) and anthelmintic effect (Akhtar & Riffat, 1991).

In this study, (6) types of inactivated oil emulsion Newcastle disease vaccines were prepared using different extractions of the Nigella Sativa oil. The quality control tests of these vaccines proved that they are sterile. Also, the prepared vaccines

are safe when inoculated in SPF-ECE for three passages after the virus had been inactivated with b-ptopiolactone or when injected in susceptible chicken.

Concerning the physical proties of the prepared vaccines, the data presented in table (1), showed that all of the prepared (6) vaccines were miscible in oil-emylsion stability for the two hexane extracts of the Nigella Sativa oil was nearly similar (6 & 7 days at 4∞C), while it was (<1, 1, 13 & 30) days for fatty acids, steroids, methane extract and crude- oil of the Nigella Sativa respectively. The viscosity of the prepared vaccines was relatively low for the vaccine that prepared from crude- oil of the Nigella Sativa (15 seconds at 25°C), moderate for methane extraction and both hexane extractions of Nigella Sativa oil (19.4, 25.5 & 25.8 respectively).

The haemagglutination inhibition (HI) test is still the most widely used conventional serological method for measuring anti-NDV antibody levels in poultry sera, and it is considered the standard laboratory test for this disease (Xu & Greiner 1997).

Concerning the efficacy of the prepared vaccines, data presented in table (2) revealed marked differences between the prepared vaccines. Generally, as shown in table (2) the HI-titers increase gradually from the second week post-vaccination and reached the peak at the fourth week post-vaccination then became to decrease gradually with low amounts from the fifth week post-vaccination. The obtained results that presented in table (2) revealed clearly that chicks vaccinated with Nigella Sativa oil adjuvant vaccine elicited higher antibody titer than other groups vaccinated with other types of the used vaccines. Methane extract vaccine elicited HI-titers near to that produced by the crude-oil vaccine while other vaccines like hexane extractions of Nigella Sativa oil, steroids of Nigella Sativa oil and fatty acids of Nigella Sativa oil produced low levels of HI-antibody titers. For example, the HI-antibody titers in sera of vaccinated chicks by fatty acids, steroids, both hexane extractions, methane extraction, crude-oil of Nigella Sativa and mineral oil were 2.5, 2.6, 3.2, 3.4, 3.6, & 4.12 respectively at the 2nd week post-vaccination and 5.6, 5.3, 5.5, 5.2, & 6.3 at the 4th week post-vaccination then these titers gradually decreased to reach 3.5, 4.1, 4.3, 4.7, 5, 6.5 & 3.8 at the 6th week post-vaccination. The sera of control non-vaccinated group were (0) while that of control group vaccinated with antigen only without adjuvant ranged from 3.2 at the 2nd week to 4.4 at the 4th week and 3.8 at the 6th week post-vaccination.

The protection percentage of the prepared vaccines was carried out by challenging all the vaccinated and control groups at 21 & 42 days post-vaccination and remained under observation for 14 days post-challenge for symptoms and mortalities. Protection was expected with the results of

HI-antibody titers induced in this study, results presented in table (3) support this finding. For example, the protection percentages 21 days post-vaccination was 60, 55.55, 60, 70, 80, 100 & 60% for chicks vaccinated with vaccine 1, 2, 3, 4, 5, 6 & 7 respectively while it decreased to 33.3, 40, 42.85, 50, 60, 90, & 40% when chicks challenged 42 days post-vaccination. The protection percentage for the control non-vaccinated group was 0%.

However, the relatively high HI-antibody titers induced by oily extract vaccine number 6 and number 5 may be related to the presence of all Nigella Sativa oil constituents which has synergistic action while the un-satisfactory level of protection against clinical disease seen in other oil-fractionized vaccines may be related to absence of one or more constituents from the oil phase of the adjuvant. This result comes in contact with that of Madbouly et al., (2000, 2001 & 2002) who used the whole Nigella Sativa oil as adjuvant for preparation of inactivated infectious laryngeotracheitits virus, infectious bursal disease virus and lumpy skin disease virus vaccines respectively and concluded that the Nigella Sativa oil is highly immunogenic in the choice of vegetable oil instead of mineral oil for preparation of inactivated avian and/or animal viral vaccines.

So, from this study we can conclude that the non-specific immunostimulant effect of Nigella Sativa oil is acquired when it is used as a crude oil and this improved its ability as a good adjuvant for viral vaccines.

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