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THE EFFICACY AND POTENCY OF HEAT RESISTANT STRAIN OF NEWCASTLE DISEASE VIRUS IN PREPARATION OF AN INACTIVATED VACCINE

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SUMMARY

Inactivated oil emulsion vaccine of Newcastle disease virus (NDV) was prepared using heat resistant (HR) strain of NDV. Formalin and binary ethyleneimine (BEI) were used as inactivator with different concentration to determine the best inactivator and best concentration from each one. Experimental batches of inactivated HR strain of NDV suspension were prepared by using BEI and formalin as inactivator of concentration of 0.001% and 0.03%, respectively. Paraffin oil adjuvant was used in the prepared oil emulsion vaccine composed of different antigen concentration from HR strain. The efficacy and potency of inactivated HR strain of NDV were evaluated by haemagglutination test (HA), cell mediated immune response, macrophage activity, haemagglutination inhibition test (HI) and challenge test in all chicken groups. The results revealed that BEI was the best inactivator which did not affect the activity of HA titres, in addition the economic use of HR strain of NDV in oil emulsion inactivated vaccine either inactivated with BEI or formalin gave good immune response and 100% protection either in low or high antigen concentration due to its high EID_{50} over 10^{20}/ml.

INTRODUCTION

Newcastle disease virus (NDV) is a highly contagious septicaemic, fatal and destructive disease which attacks chiefly chickens and turkeys usually in an acute form, but sometimes in subacute or even chronic form. Newcastle disease (ND) remains a hazard to the poultry industry as it causes great losses in many countries where poultry are reared. The economic losses are mainly due to high mortality rate especially in subacute and

acute forms of the disease. Economic losses are encountered, also in losses of weights in broiler and in severe drop in eggs production, quantity and quality (Larenz and Newion, 1944; Khox, 1950 and Biswal and Narril, 1954). Newcastle disease virus is in genus paramyxovirus of the family paramyxoviridae. There are nine sero-groups of avian paramyxovirus designated PMV-1 to PMV-9 and NDV is belonging to PMV-1 (Alexander, 1986). NDV is grouped into three pathotypes based upon the type and severity of disease produced in chickens and the mean death time of inoculated chicken embryos. The most virulent viruses are called velogenic and cause an acute disease with high mortality.

Visccrotropic Velogenic Newcastle Disease (VVND) virus produces haemorrhagic lesions of the internal organs, particularly the digestive tract. Neurotropic Velogenic Newcastle Disease (NVND) virus produces a sever disease characterized by neurological signs and often respiratory signs. Mesogenic Newcastle disease viruses cause a disease similar to NVND in clinical appearance but are generally less lethal. Lentogenic viruses cause inapparent infection or mild respiratory signs (Beard and Hanson, 1984).

Vaccination has an important role in the prevention of ND. However, an ideal vaccination program against this disease can not be easily achieved as it would depend on level of challenge in the field, disease control polices in the country, type of birds (broiler or layers), vaccine strain, availability of vaccine, use of other vaccines, the vaccination equipment, route of administration, workmanship of applicant, climatic conditions and past performance of vaccination programs (Alexander and Jones, 2001 & 2003; Al-Garib et al., 2003, and Butcher et al., 2004).

Fifty years or more have passed since vaccine was first used to protect village poultry against Newcastle disease (ND) (Palcidi and Santucci, 1952). During this time, a wide variety of types of vaccine have been developed.

Inactivated oil emulsion vaccines are not as adversely affected by maternal immunity as live vaccines (Box et al., 1976) because the oil adjuvant acts as stimulus of defense mechanism and disperse antigen slowly (Bennejean et al., 1978). Inactivated vaccine produces very high levels of antibodies against ND virus, and provides a good level of protection against the virulent virus (Alexander and Jones, 2001 and 2003).

Some asymptomatic enteric viruses have been noted for their greater heat resistant than more conventional lentogenic viruses. This property has been enhanced by selection and cloning in the laboratory to produce heat tolerant vaccine. These have the distinct advantage in the village situation that it is possible to transport the vaccine without

necessarily having refrigerators along the way. The most extensively used has been the NDV4-HR vaccine (Ibrahim et al., 1992). More recently, a similar vaccine to NDV4-HR, called 1-2, has been made available for local production in developing countries which adds the significant advantage of low cost (Tu et al., 1998).

Therefore, this study was planned to fulfill the following:

1- Preparation of oil inactivated NDV vaccine using heat resistant strain of NDV.

2- Using two different inactivator (formalin and Binary ethyleneimine BEI) in different concentration and determination of the inactivated curve.

3-Using three different concentration of HR NDV strain in preparation of the oil emulsion vaccine.

4- Evaluation the prepared vaccines by injection in chickens and determination of the immune response of chickens by measuring the cell mediated immune response, macrophage activity, haemagglutination inhibition test (HI) as well as by challenge test.

MATERIAL AND METHODS

1- Virus:

Heat resistant strain of Newcastle disease virus (HR) was kindly supplied by Dr. Nadia Hassan from Manissa institute Azmir Turkey.

2- Embryonated chicken eggs (ECE):

9-11 day old embryonated chicken eggs. The eggs was purchased from Arab Republic of Egypt, Ministry of Agriculture, Specific Pathogen Free Egg Production Farm, Nile SPF eggs, Koum Oshiem, Fayoum, Egypt. These eggs were used for propagation, preparation, titration and testing of complete inactivation of the prepared batch of heat resistant strain of Newcastle disease virus (NDV).

3- Chickens:

One hundred and forty, one-day-old chicks were used. They were obtained from commercial poultry farm and reared under strict hygienic measures till four weeks old.

4- Chemicals:

a- Binary ethyleneimine (BEI):

It was prepared according to Hans (1990).

b- Sodium thiosulphate:

It was prepared as 20% solution in sterile distilled water. The solution was sterilized by autoclaving and stored at 4°C until used to stop the action of BEI.

c- Formalin:

It was secured as 40% formaldehyde solution from BDH limited (Poole, England). It has been mainly used in final concentration of 0.1%, 0.07%, 0.05% and 0.03%.

d- Paraffin oil:

Paraffin oil (white oil) MICBIL, Alexandria Whiterex 309, May 400.

e- Emulsifiers:

- Sorbitan mono-oleate (span 80) supplied by Ubichem Ltd.
- Polyxyethene sorbitan (tween 80) supplied by Sigma Company.

5- Virus propagation:

Virus was propagated in 9-11 day old SPF eggs according to Allan et al., (1973).

6- Virus titration:

It was carried out according to FAO Publication (1978). The EID_{50} was calculated according to Reed and Muench (1938).

7- Virus inactivation:

a- Inactivation by BEI:

The harvested infected amnioallantoic fluid was inactivated with BEI at a final concentrations of 0.001, 0.002 and 0.003 M, which were obtained from 0.1M BEI. The virus-BEI mixture for each concentration was incubated separately with continuous stirring at 37°C for 20 hours. From the inactivated virus suspension 2ml were collected in sterile tubes containing 0.4ml of 20% sodium thiosulphate solution to stop the action of BEI at different intervals.

All samples were assayed for virus infectivity by titration in ECE to determine the rate of virus inactivation and the best concentration of BEI, which inactivate the virus completely.

b- Inactivation by formalin:

The inactivation process was carried out according to Rozhdestvenskii (1984). The harvested infected amnio-allantoic fluid was treated with formalin at a final concentration 0.1%, 0.07%, 0.05% and 0.03% and put on a magnetic stirrer for continuous stirring during inactivation process at 37°C for 30 hours. Samples from each the virus formalin mixture were collected every 2 hours in a screw capped tube, for virus titration and HA activity to determine the rate of virus inactivation and the best concentration of formalin which inactivate the virus completely.

8- Vaccine preparation:

An experimental batch of inactivated heat resistant strain of NDV suspension by BEI at 0.001M for 16 hours at 37°C (the chosen concentration) and by formalin at 0.03% for 10 hours at 37°C (the chosen concentration).

The vaccine was prepared by mixing one part of aqueous phase and 2 parts of oil phase according to Stone et al., (1983). Two batches of inactivated oil emulsion NDV were prepared using the heat resistant strain (HR), the first was prepared after 0.001% BEI inactivation while the second was used after 0.03% formalin inactivation. 150ml of each batch were prepared composed of different antigen concentration. Composition of each emulsion was summarized as shown in table (1).

Table (1): Composition of 6 different prepared oil emulsion HR strain of NDV vaccines

Emulsion No.	Antigen concentration	Type of inactivator	Normal saline	Oil	Aqueous/oil ratio	Emulsion type
1	10 ml	BEI	40ml	100ml	1:2	W.O
2	15 ml	BEI	35ml	100ml	1:2	W.O
3	20 ml	BEI	30ml	100ml	1:2	W.O
4	10 ml	Formalin	40ml	100ml	1:2	W.O
5	15 ml	Formalin	35ml	100ml	1:2	W.O
6	20 ml	Formalin	30ml	100ml	1:2	W.O

W.O: Water in oil

9- Evaluation of the prepared vaccine (OIE, 2000):

a- Residual infective virus activity:

Undiluted inactivated virus was inoculated into the amnioallantoic cavities of live ECE 9-11 day old, haemagglutination activity should not be detected in these eggs after 5 days incubation. This test was repeated for two blind passages.

b- Purity test:

Samples from the prepared vaccine were cultured on different media to insure that the vaccine free from bacterial or fungal contaminant (Code of American Federal Regulation, 1985).

10- Characterization of the vaccines:

For evaluation of the emusification process of vaccines; drop test, emulsion viscosity and emulsion stability were done according to Geneidy et al., (1971); Becher (1965) and Gessi and Nardelli (1973).

11- Virological and serological examination:

a- Rapid slide haemagglutination test:

It was carried out according to Anon (1971) for quick detection of haemagglutination in the amnioallantoic fluid of virus-inoculated eggs.

b- Quantitative haemagglutination test:

This test was done to determine the haemagglutination titre (HA) of samples, which collected before and after inactivation at different time.

c- Haemagglutination inhibition (HI) test:

It was done using beta procedure (constant virus plus different diluted serum) as described by Anon (1971). This test was used for measuring the antibody response of vaccinated chickens.

12- Evaluation of cell mediated immune response:

a- Estimation of lymphocyte blastogensis:

This test was carried out by tetrazolium calorimetric assay according to Mosmann (1983).

b-Macrophage activity test:

Phagocytic percentage:

It was performed by the method of Barry et al., (1988) which was modified by El-Enbawy (1990).

Phagocytic percentage =

$$\frac{\text{No. of phagocytes which ingest Candida X 100}}{\text{Total No. of phagocytes}}$$

Phagocytic index:

It was done according to Richardson and Smith (1981).

Phagocytic index =

$$\frac{\text{Total No. of phagocytes which ingest more than two Candid}}{\text{Total No. of phagocytes which ingest Candida}}$$

13- Challenge test:

Ten chickens from each group were challenged after 4 weeks post vaccination using 0.5 ml of velogenic viscerotropic Newcastle disease virus (VVNDV) containing 10^6 EID_{50}. The chickens were observed for 10 days post challenge. Dead chickens and those showing symptoms through the period of observation were kept for post mortem examination.

14- Experimental design:

One hundred and forty chickens were divided into 7 groups, 20 chickens for each as shown in table (2).

Table (2): Experimental Design

Groups	No. of Chicken	Inactivation	% Conc. of the virus in vaccine	Route	Dose	Age	Blood collection
Group (1)	20 for each	BEI	10 %	I/M injection	0.5ml	4 weeks	From the first week post vaccination till 12 weeks from the first vaccination
Group (2)		BEI	15 %				
Group (3)		BEI	20 %				
Group (4)		Formalin	10 %				
Group (5)		formalin	15 %				
Group (6)		Formalin	20 %				
Group (7)		Control non vaccinated group					

Table (3): Inactivation of HR strain of NDV using different concentration of BEI at different times

Time of sampling in hours	Concentration of BEI								
	0.001M			0.002M			0.003M		
	No. of inoculated eggs	No. of HA positive eggs	Log_2 HA	No. of inoculated eggs	No. of HA positive eggs	Log_2 HA	No. of inoculated eggs	No. of HA positive eggs	Log_2 HA
0	5 eggs for every hour	5/5	13	5 eggs for every hour	5/5	13	5 eggs for every hour	5/5	13
1		5/5	13		5/5	13		5/5	13
2		5/5	13		5/5	13		5/5	13
3		5/5	13		5/5	13		5/5	13
4		5/5	13		5/5	13		5/5	13
5		5/5	13		5/5	13		5/5	13
6		5/5	13		5/5	13		5/5	13
7		5/5	13		5/5	13		5/5	13
8		5/5	13		5/5	13		5/5	13
9		5/5	13		5/5	13		0/5	13
10		5/5	13		0/5	13		0/5	13
11		5/5	13		0/5	13		0/5	13
12		5/5	13		0/5	13		0/5	13
13		5/5	13		0/5	13		0/5	13
14		5/5	13		0/5	13		0/5	13
15		0/5	13		0/5	13		0/5	13
16		0/5	13		0/5	13		0/5	13

The times of complete inactivation of HR strain of NDV were at 5, 10 and 9 hours at concentrations of 0.001, 0.002 and 0.003, respectively without any effect on the activity of HA titres

Table (4): Results of inactivation of HR strain of NDV using different concentration of formalin at different times

Time of sampling in hours	HA (log_2) of HR NDV for different concentration of formalin			
	0.03%	0.05%	0.07%	0.1%
0	13	13	13	13
2	13	13	13	13
4	13	13	13	12
6	13	13	13	12
8	13	12	12	11
10	13	12	12	11
12	ND *	ND	11	11
14	ND	ND	11	11
15	ND	ND	11	10
16	12	11	11	10
17	12	11	11	10
18	12	11	11	10
19	11	11	11	10
20	11	11	10	9
21	11	11	10	9
22	11	11	10	9
23	11	11	9	9
24	11	11	9	9
26	11	11	9	9
28	11	11	9	8
30	11	11	9	8

* ND: Not Done

HA activity of HR slightly decreased when treated with different concentrations of formalin at different times

Table (5): Evaluation of cell mediated immune response of vaccinated groups by macrophage activity using candida albicans expressed by phagocytic percentage.

Groups of chickens	Phagocytic % / Days post vaccination		
	5	12	19
1	92.5	87.2	75
2	90.6	90.9	75
3	90.92	89.5	73.3
4	90.6	90	88.2
5	92	76.6	77.7
6	91.6	70.6	69.9
7	13.9	14.1	13.1

Phagocytic percentage = No. of phagocytes which ingest candida X 100

Total No. of phagocytes

Table (6): Evaluation of cell mediated immune response of vaccinated groups by macrophage activity using candida albicans expressed by phagocytic index

Groups of chickens	Days post vaccination		
	5	12	19
1	0.92	0.85	0.66
2	0.86	0.73	0.66
3	0.9	0.88	0.55
4	0.93	0.77	0.53
5	0.86	0.78	0.5
6	0.85	0.75	0.49
7	0.09	0.06	0.04

Phagocytic index = Total No. of phagocytes which ingest more than two candida

Total No. of phagocytes which ingest candida

Table (7): Evaluation of cell mediated immune response of vaccinated groups by lymphocyte transformation expressed by optical density

Groups of chickens	Days post vaccination			
	5th	12	19	26
1	0.0337	0.0452	0.159	0.231
2	0.093	0.155	0.186	0.194
3	0.149	0.221	0.156	0.226
4	0.107	0.145	0.120	0.339
5	0.109	0.193	0.213	0.435
6	0.095	0.218	0.209	0.276
7	0.014	0.012	0.011	0.011

Table (8): The mean $\log_2$ HI titer to HR strain in vaccinated groups

Groups of chickens	Weeks post vaccination								
	1	2	3	4	5	7	8	9	12
1	6.33	7	10.25	10.33	10.5	10.5	9.5	8	8
2	5.33	7.33	10.25	10	10	10	9.25	9	8.25
3	7.33	7.66	7.75	10.33	10.2	10.33	9.5	8.7	8
4	4.66	7.66	8.25	10	10	9.33	9	8	5
5	5	6.33	7.8	9.33	9.4	10	10.2	7	6
6	9	9.5	10	11	11	10.5	10.25	8	7
7	0	0	0	0	0	0	0	0	0

Table (9): Protection and efficiency of vaccinated and non-vaccinated chickens with virulent NDV 4 weeks post vaccination.

Groups	No. of chickens	No. of survival	No. of dead	Protection %
1	10	10	0	100
2	10	10	0	100
3	10	10	0	100
4	10	10	0	100
5	10	10	0	100
6	10	10	0	100
7	10	0	10	0

DISCUSSION

Newcastle disease (ND) is a highly contagious poultry disease which varies widely in the type and severity of symptoms. Vaccination has an important role in the control of ND.

All of the usual commercial NDV vaccine will protect village chickens against NDV, if the vaccine reached the chickens in a potent form. There are special problems; many of commercial vaccines are thermolabile and sometimes extremely thermolabile. Cold chains are impossibly expensive to develop and maintain. Village flocks are small, scattered and multi-aged. Thermostable vaccine seems to be a partial solve the problem of NDV in village chickens.

This study briefly reviews the advantages of the heat resistant strain of NDV in preparation of inactivated oil emulsion vaccine.

The titration of propagated HR strain in embryonated egg showed high titre which recorded more than (1020/ml EID50), and 213 by microtitre haemagglutination technique. The results agree with Naglaa (2004).

Three concentration of BEI were used (0.001, 0.002 and 0.003) in the inactivation of HR. The results shown in table (3) revealed that there were differences in time of inactivation of HR between the three concentration of BEI, which reduced the infectivity titres to zero. The times of complete inactivation were at 15, 10 and 9 hours for concentration of 0.001, 0.002 and 0.003, respectively. The activity of HA titres is not affect by different concentrations and the time of inactivation. BEI (0.001) concentration was chosen as the lowest dilution which completely inactivated the virus in suitable time. The results agree with Soliman et al. (1998), who reported that there was no effect on the antigenicity of the virus when used BEI used at a concentration of 0.01 or 0.03 M.

Four concentration of formalin (0.03%, 0.05%, 0.07% and 0.1%) were used in inactivation of HR at different times.

The results shown in table (4) revealed that the haemagglutination activity of HR was slightly decreased when treated with (0.03% and 0.05% formalin) at 37°C during the different time of inactivation, but it was gradually decreased when treated with 0.07% and 0.1% formalin during the same different time of inactivation.

So, the best lowest concentration (0.03%) was used without any effect in HA titre. In addition, there was no any residual virus. The results agree with Park et al. (1985) when used 0.1% and 0.2% formalin at 37°C.

Since, the titration of HR strain of NDV vaccine revealed very high titre 1020, so three groups were prepared with different antigen concentration (10, 15 and 20) to achieve the lowest quantity of vaccine which give high immunity, therefore lowest cost will be obtained. The groups of chicken were injected with the prepared vaccine to evaluate its effect as shown in table (2).

The results shown in tables (5, 6) revealed that the phagocytic percentage and index gave high activity at 5th day and then decline through the other days. There was no significant difference between vaccinated groups but there was noticeable difference between vaccinated groups and control non-vaccinated group. The results agree with Ashraf et al. (2002).

On the other hand, the results of lymphocytes blastogenesis as presented in table (7) revealed gradual increase in response of T-cells expressed as stimulation indices (SI) for different groups till 26 days post vaccination, but there was slightly increasing in values in groups which vaccinated with HR treated with BEI. The results agree with Reynolds and Maraque (2000) who explained the role of cell-mediated immunity against Newcastle disease and reported that cell mediated immunity (CMI) is an important factor in the development of protection in chickens against ND. Also, it was reported that the first immunological response was detected as early as 2-3 days after ND vaccination and that T-lymphocytes are the principle cells involved in the cellular response and a part of well known local immunity which also comprises immunoglobulin A (IgA) and interferon.

Concerning the humoral immune response as shown in table (8), the peak of HI titres which were recorded varied from the 4th to 8th week post vaccination for all groups of chicken, then slightly decrease through other weeks of observation period for all 6 groups. Although slightly better HI titre was observed for group 6. The results agree with Vijayashree et al. (2000), who reported that the thermostable variants of thre lentogenic strain of NDV gave high HI antibod titre.

Regarding to challenge test against NDV post 4th week of vaccination (Table, 9) revealed that all vaccinated groups show 100% protection when challenged by VVNDV.

From the above results it could be concluded that the use of HR strain of NDV in oil emulsion inactivated vaccine either inactivated with BEI or formalin gave good immune response in low or high antigen concentration. So, for the all advantages of these strain HR of NDV, it is advised to be used in preparation of inactivated vaccine.

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كفاءة فاعلية العترة المقاومة للحرارة لفيروس مرض النيوكاسل فى تحضير لقاح مثبط

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تم إنتاج لقاح مثبط زيتى لفيروس مرض النيوكاسل بإستخدام عترة مقاومة للحرارة. إستخدام الفورمالين والبينرى إيثيلين أمين كمواد مثبطة للقاح مع إستخدام تركيزات مختلفة لإختبار أفضل مثبط وأفضل تركيز من كل واحد. تم تحضير اللقاح من العترة المقاومة للحرارة بتثبيطة بالفورمالين (٠,٠٣٪) وأخر بالبينرى (٠,٠٠١٪) وإستخدام زيت البرافين فى تخليط اللقاح الزيتى وإستخدام تركيزات مختلفة من الفيروس.

تم تقييم إستخدام اللقاحات المحضرة ومعايراتها بحفنها فى مجاميع من الدواجن ثم قياس الإستجابة المناعية لمدة ١٢ إسبوع وذلك بتجارب قياس المناعة الخلطية للمصل المتعادل وقياس المناعة الخلوية وتحور الخلايا الليمفاوية وقياس نشاط الخلايا البلعومية (macrophage) وإختبار التحدى . وقد أثبتت النتائج أن إستخدام البنرى أفضل من الفورمالين.

بالإضافة إلى أن إستخدام العترة المقاومة للحرارة لفيروس النيوكاسل فى تحضير اللقاح الزيتى المثبط أعطى مناعة عالية سواء بإستخدام الفورمالين أو البينرى كمثبط وأيضاً عند إستخدام الفيروس بالتركيزات المنخفضة والعالية وهذا له مدلول إقتصادى.