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THE USE OF BINARY ETHYLENIMINE AND FORMALIN AS INACTIVANTS FOR PRODUCTION OF INACTIVATED NEWCASTLE DISEASE VACCINE

(With 3 Tabls and One Figure)

By

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استخدام مادة البيزى ايثلين أمين والفورمالين كمثبطات لأنتاج لقاح النيوكاسل الميت

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تم إستخدام البيزى ايثيلين أمين كمثبط للفيروس المسبب لمرض النيوكاسل وقد أعطى نتائج مرضيه على أساس أنه لايوجد له تأثير على وبائيه الفيروس عند استخدامه بتركيز ٠٫٠١، ٠٫٠٣ وزن جزيئ، وفى نفس الوقت قد قضى كلية على ضراوة الفيروس خلال ٦-٨ ساعات وذلك تبعا للتركيز. وتعد هذه النتائج أعلى كفاءة من النتائج التى حصل عليها عند إستخدام محلول الفورمالين كمثبط فى تركيز ١٫٠، ٢٫٠٪ والتى أنقصت بوضوح قوه التلازن الدموى من ١٠٢٤ إلى ٣٢ (HA titer). وقد أثبتت الدفعات التجريبيه المحضرة من اللقاح الميت المثبط بكل من البيزى والفورمالين مع استخدام (زيت- جيل) كمحسنات (منشطات مناعيه) أن البيزى ايثيلين أمين ٠٫٠١ وزن جزيئ مع الزيت أفضل على اساس انهم اعطوا اعلى معدل للاجسام المناعية المثبطه للتلازن (٣٫٥) $\log_2$ H1 titer كما انهم أعطوا أعلى نسبه حمايه وهى ٩٤٪.

SUMMARY

The use of Binary Ethylenimine as inactivant for Newcastle virus give a promising result as it has no effect on the antiginecity of the virus when used in a concentration of 0.01M or 0,03 M at the same time it had completely reduced the virulence of the virus within 6 to 8 hours according to the concentation. This results were superior than the results obtained when using

formaldehyde solution as inactivant in concentration of 0.1% or 0.2% which significantly reduced antigencity from 1024 to 32 HA titer. The experimental prepared batches of Binary in- activated vaccine as compared with formalin inactivated prepared vaccine by different adjuvents (oil-gel) proved that the BEI 0.01M with oil as adjuvent was the best as it gives the highest $10g_2$ HI titer (5.3) as well as the highest protection percentage 94%.

Key words: Newcastl disease- vaccine- Inactivation

INTRODUCTION

Newcastle disease viruses cause high economic losses in poultry industry, as the most important property of its ability to produce disease and death of young and old birds, specially velogenic strains *(HANSON et al., 1973).* Eradication was attempted in few areas, but most countries relied an intensified vaccination program *(DEXTER 1973).*

All NDV stains are capable of provoking an antibody response in chicken, the antigens that induce the neutralizing and haemagglutinating antibodies are associated with the envelope of the virus *(ROTT 1964).* Inactivated NDV vaccines are prepared by growing suitable antigenic strain of the virus (usually lentogenic strain) in embryonating chicken egg, the harvest was inactivates by formaldehyde *(ALLAN et al., 1978 and NADIA et al., 1992)* or by beta-propriolacton *(KEEL & WADE 1965).*

These chemicals mainly affecting the envelope of ND *(KING 1991),* Azirdin derivatives specially bromoethylamine hydropromid that is relatively harmless and transformed at pH above 8 was widely used as inactivant for many vaccines as it is not affected by the proteins found in the virus suspension and its action is directly to the viral nucleic acid *(BAHNEMANN 1975 & Part et al !985).*

Specific immunity against NDV develops within a week after vaccination and reachs to maximum by third week that can be measured by HI assay *(FAO 1978).* In this study a trial for preparation of ND inactivated vaccines using different inactivants with different concentration was caried out to choose the best one give the highest immunogenic vaccine.

MATERIAL and METHODS

1- Virus strains

a- Lentogenic seed strain of HB1 was originally supplied by the poultry vaccine research laboratory at Weybridge to Newcastle disease department. SVRI abbasia and used as seed virus.

b- Virulent veloginic viscerotropic NDV (VVNDV) strain was locally isolated and identified by *SHEBLE and REDA (1967).*

2- Emyreonated chichen eggs and chickens

a- Commercial 10 day-old ECE were obtained from a commercial source.

b- One day old chicks were supplied from the same source of the eggs and held in isolation to be used as susceptible chickens.

3- Mice

Four weeks old healthy swessalbino mice were used.

4- Chickens RBCs

5- ND antisera: an international ND- reference antisera is available from WHO/FAO *ALLAN et al (1978).*

6- Preparation of virus suspension.

ND virus was inoculated by allantoic route to 10 day- old ECE,allantoic fluid were harvested 4 days later and assayed for sterilty, homogencity (by using specific immune sera) and infectivity according to the methods for examination of poultry biologies (1965).

7- Inactivants and inactivation:-

a- Tow- bromoethylamine hydrobromide (BEA) F.W.204.9, supplied by Aldrich chemical copman, Milwaukee, Wis., USA was used for preparation of BEI (Binary ethylinamine). The cyclization of bromoethylamin is a monomlecular first order reaction requiring OH ions and the reaction rate is temperature dependent *(BAHNEMANN 1975).* The formation of BHI apart from the virus suspension was affected by preparation of 0.1M BHE in 0.2N NaOH. The cyclization was allowed to proceed in a water bath 37°C for two hours then add the virus suspension at a concentration of 0.01, 0.03 and 0.05% samples collected at different intervals. (1,2,3,4,5,6 and hours) virus inactivation in these samples was stopped by addition of cold sodium thiosulphat to a final concentration of 2%. Inactivation rates were determined from invectivity titers.

b- Formaldehyde solution 37-41% was obtained from BDH limited pool England. Formalin used as 0.1 and 0.2% final concentration for virus inactivation (at 37°C for 24 hours).

8- Vaccines

Inactivated NDV suspension either by BHI at 0.01M for 6 hours at 37°C or formalin at concentration of 0.2% for 18 hours at 37°C were used for vaccine preparation.

Two types of adjuvants were used:

a- Aluminum hydroxide gel.

Sod- hydroxide and aluminum potassium sulphat were supplied by EL-NASAR pharmaceuticals prepared as ALOH3 gel of 2% strength which had 1.3% ALO3. The vaccine was prepared by the addition of equal amount of Al(OH)3 and inactivated virus suspension then adjust the pH to 7.8.

b- Oil adjuvant.

Formed from Sapn 80, Twin 80 (supplied by Niederlassung deer deutsche ICI GmbH. Goldsch midtstrasse. 100D.4300 Essen1 W. Germany) and Marcol 52 (Esso) mineral oil (The nationalformally USA 14th edition 1975). The vaccine was prepared by mixing one part of aqueous phase (inactivated virus suspension + 1% tween 80) and 3 parts of oil phase (Marcol 52+ 10% spain 80).

9- Toxicity, safety and potency tests.

a- Toxicity of oil adjuvants was tested individually or mixing. A group of 5 healthy mice weight 17-22 gm was inoculated with 0.5ml s/c of tested preparation. Animals were kept under observation for 7 days for any inflammatory reaction, erthyma or any thickening of skin at the site of inoculation.

b- Safety test was done by inoculation of one week old chicks S/C and intramuscular I/M routes by the prepared vaccines. The chicks were observed for at least 2 weeks for systemic or local reaction at the site of inoculation, 5 weeks later post mortum examination were done on each birds *(NADIA et al., 1972).*

c- Potency and endurance of immunity. Susceptible groups of 3 weeks old chicks (50 each) were inoculated I/M with 0.5 ml of the prepared vaccines in addiation of control non vaccinated group. Twenty one days post vaccination, blood samples were collected from all groups and subjected to Hl test. Challenging was done with 0.5ml ($10^{5.5}$ EID) of virulent NDV strain. All groups were observed for 2 weeks, specific ND morbidity and mortality were recorded.

10- Infectivty assay.

Samples from the initial ND non treated virus suspension were assayed for virus infectivity to determine the titer of the initial pool, also samples from

undiluted ND virus suspension treated with BHI or formalin were assayed for residual virus infectivity *(KING 1991)*.

11- Haemagglutination and haemagglutination inhibition tests.

Samples were collected before and after inactivation at different times for detecting HA virus titer as recorded in table (1), also blood samples were collected from vaccinated groups 3 weeks post vaccination for measuring of HI antibody titers. These methods were carried out according to the standard methods described in the *FAO publication (1978)*.

12- PH tests.

Chemically treated samples were tested to detect any PH changes attributed to the treatment.

RESULTS

Table No. 1 shows the effect of different concentration of formaline and BEI on the infectivity titer [inactivation rate], HA activity and pH.

The higher concentration of BEI [0.05m] inactive completely NDV with a titer of 2.5 in 4 hours with decrease in heamaggltination activity from 1024 to 512 with no change on pH, while the concentration of 0.03M inactivate the virus within 6 hours, with no change HA activity while pH increased from 8.5 to 9.4, conc of 0.01M cause complete inactivation after 8 h with no change on HA activity and the pH increased from 8.5 to 9.5.

Formaline at a conc. of 0.1% inactivate the virus titer within 24h with residual infectivity 0.9 and high significant decrease on the HA activity from 1024 to 128 with increase pH from 7.5 to 8.5 when rising a conc. of 0.2% formaline, the virus inactivated within 24h and high significant decrease in HA activity from 1024 to 32 and the pH decreased from 8.5 to 7.5.

Figure [1] shows the effect of BEI and formaline with different conc. on infectivity of NDV in a period ranging from 4hs to 7hs according to the concentration used. Formaline leave residual infectivity titer ranging from 0.7 to 0.9 during experimental period (24hs).

Table (2) presents the inactivation rates (decrease of viral infectivity per minute during all the inactivation period) of NDV when use a different concentration of BEI and formaline at 37%. It is clearly that the inactivation rate increased when high conc. of BEI used. In formaline a constant inactivation rate noticed inspite of the different conc used.

Table (3) shows the results of the vaccinated groups of chicks with vaccines prepared from chosen concentration of different inactivants (BEI 0.01M and formaline 0.2%) with either oil or AL (OH)3 gel adjuvants.

The oil adjuvants vaccine and BEI used as inactivants gave high log2 HI titer (5.3.) 3 weeks post vaccination with low morbidity and mortality with consequently high protection (94%).

On the other hand the oil adjuvant vaccine prepared by using formaline as inactivant gave lower log HI titer (1.52) than the previously mentioned vaccine 3 weeks post vaccination and high morbidity, low mortality and protection (82%).

Al (OH)3 gel adjuvant vaccine and BEI used as inactivant gave log2 HI titer (4.8) 3 weeks post vaccination with moderate morbidity, low mortality and protection (90%).

AL(OH)3 gel adjuvant and formline used as inactivant resulted in HI titer (3.2) 3 weeks post vaccination with high morbidity, moderate mortality and protection (80%).

Generally, vaccines prepared using BEI as inactivant gave the best results in immune response measuring by HI and challenge tests than the vaccines prepared using formaline but it is in permissible level.

DISCUSSION

Practice to avoid exposure of flocks to NDV and appropriate immunization programs are the most obvious and effective. There are three essential requirements for production of inactivated vaccines. First, the viral antigen must be available in high concentration, second, the virus preparation must treated in such way that no residual infectivity remain, third, adjuvants without being toxic to the immune response of the vaccinated chicks.

The chosen viral antigen of this study was obtained from inoculation of 10 day-old chicken embryos with lentogenic HB1 strain as it is the most suitable strain for preparation of ND inactivated vaccine *(GOUGH, et al., 1977).*

Until recently, most European producers used formaldehyde to inactivate viruses for preparation of different types of inactivated vaccines, but the safety of these vaccines was dubious specially for FMD *(BARTELING and VREESWIJK 1991).*

In this study two inactivants were chosen to inactivate ND virus suspension, formaldehyde and Binary ethylen- imine. Formaline used as inactivant for the NDV suspension in concentration of 1% and 0.2% decreased the infectivity titer with residual of log10 0.7 and 0.9 after 24 hours treatment (Table. 1), at the same time it caused decreasing of haemagglutinating activity (HA) specially for samples collected 12 to 24 hours from startment of inactivation. This may be related to the formaline

which was the only chemical inactivants that did not hydrolyze during treatment *(KING 1991)* as well as to the effect of formalin on the structure of viron envelop which contains 3,4 glycoproteins in two deferent kinds of spikes, the largest glycoprotein have both haemaglutinating and neuramindase activity, other spikes carry precursor glycoprotein *(MATSUMOTO 1982).* On the other hand, treatment with BEI as inactivant at various concentrations (o.o1M, 0.03M, 0.05M0 reduced infectivity titers to zero at 4,6 and 8 hours respectively without no apparent adverse effect on HA activity. This result is supported by *(KING 1991)* who found that the BEI did not affect the HA activity of NDV or avian influenza virus.

This may be due to that the BEI known to react primary with nucleic acid and its activity is not reduced by extraneous protein (protein found in virus suspension). This phenomena was described by *(PARK et al., 1985).*

The inactivation rates of NDV with BEI and formalin are given in table (2), the rates at 37°C for BEI are higher than formalin, at the same time the rates of inactivation by using BEI as inactivant depend upon its concentration (higher by about 2 times when conc. 0.05M was used and one time when used as 0.03M in relation to conc. of 0.01M).

On the other hand the inactivation rates are mainly constant by using formalin as inactivant inspit of different concentrations (0.1% & 0.2%).

The decreasing in activation rates seen in table (1) by using BEI as inactivant may be due to the increasing of the pH of the virus fluid and is not due to a reduced formation of BEI *(BAHNEMANN 1975).*

Most adjuvants incorporate two components, one is a substance designed to form a deposit protecting the antigen from rapid catabolism which means that much smaller doses of antigen can be used and that antibody responses are more persistent. The second component is the substance to stimulate the immune response nonspecifically *(HARLOW and LANE 1988).*

The most commonly used adjuvants which fulfill the obvious mentioned points are oil and aluminum hydroxide, so they were chosen for this study.

The prepared vaccines as shown in table (3) were inoculated into susceptible groups of 3 week-old chicks with 0.5M each, 3 weeks post vaccination the HI testes were done for each group, it is clear from the results shown in table (3) that the BEI oil vaccine give the highest titer followed by BEI gel then formalin oil and lastly formalin gel. We noticed that the inactivant plays a role in the immunogenicity of the prepared vaccine, this is may be due to destructive effect of the foramen on the haemagglutinin of the viral particle *(MOTSUMOTO 1982).* at the same time when these vaccinated groups challenged with ND velogenic strain I/M and the protection percent was calculated, it is clear that vaccine prepared from BEI

oil or gel gives higher protection than those using foramen as inactivant. These results agree with *(KING 1991).*

Finaly the use of oil adjuvant ND vaccine inactivated with BEI 0.01M is safe, and gave the highest antibody titer and therefore it is the best protection against virulant ND virus.

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Table # 1.

Effects of different concentrations from BEI, formalin on the residual infectivity and haemogglutination activity of NDV.

Type of inact.	Con.	Time	RInt	Inact. r.	HA	pH
BEI	0.05M	0	9.5		1024	8.5
		3	2.2	0.0405	512	8.5
		4	0	0.0122	512	8.7
	0.03M	0	9.5		1024	8.5
		3	3.3	0.0344	1024	8.8
		6	0	0.0183	1024	9.4
	0.01M	0	9.5		1024	8.5
		3	5.3	0.0233	1024	9.5
		6	1.2	0.0227	1024	9.5
		7	0.7	0.0833	1024	9.5
		8	0	0.0116	1024	9.5
Formaline	0.1%	0	9.5		1024	8.5
		12	1.8	0.0106	256	7.9
		18	0.9	0.0025	128	7.5
		24	0.9	0	128	7.5
	0.2%	0	9.5		1024	8.5
		12	1.8	0.0106	128	7.8
		18	0.7	0.0030	32	7.7
		24	0.7	0	32	7.5

inact. = *inactivator*
con. = *concentration*
RInt = *Residual infectivity titer*
HA = *Haemmogglutination titer*
pH = *after addition of Sodium Thiosulphate*
Inact. r. = *inactivation rate*

Table # 2.

Inactivation Rate of NDV virus by BEI, and Formalin at 37°C.

Inact	Preparation	Conc.	Inactivation Rate[1]
BEI	BEA in NaoH[3]	0.01M[2]	-0.0197 ± 0.0014
		0.03M	-0.0263 ± 0.0038
		0.05M	-0.0395 ± 0.0091
Formalin	Pure	0.1%	-0.0065 ± 0.0004
		0.2%	-0.0065 ± 0.0006

([1]) = Decrease of viral infectivity in Log_{10} per minute.
([2]) = Molarity of BEA.
([3]) = Preparation of 0.01M in 0.2N NaoH for 1 hour at 37°C and used as 0.01, 0.03and 0.05 Concentrations.
Conc = Concentration.
Inact = Inactivation

Table # 3.

Challenge results of vaccinated chicks with either locally prepared BEI 0.01M or Formalin 0.02% as oil emulsion and gel vaccines.

Type of Vacc.	Adj.	No. of Birds	Gm Log_2 HI 3 weeks post vaccination	Challenge Results		Protection %
				Morbidity	Mortality	
BEI 0.01M	Oil	50	5.3	2	1	94
BEI 0.01M	Gel	50	4.8	4	1	90
Formalin 0. 2%	Oil	50	3.78	7	2	82
Formalin 0. :2%	Gel	50	3.2	8	2	80
Non-vaccinated	-	50	0.3	2	48	0

Gm = Geometric mean Log_2 HI titer
Gel = Aluminum hydroxide gel.

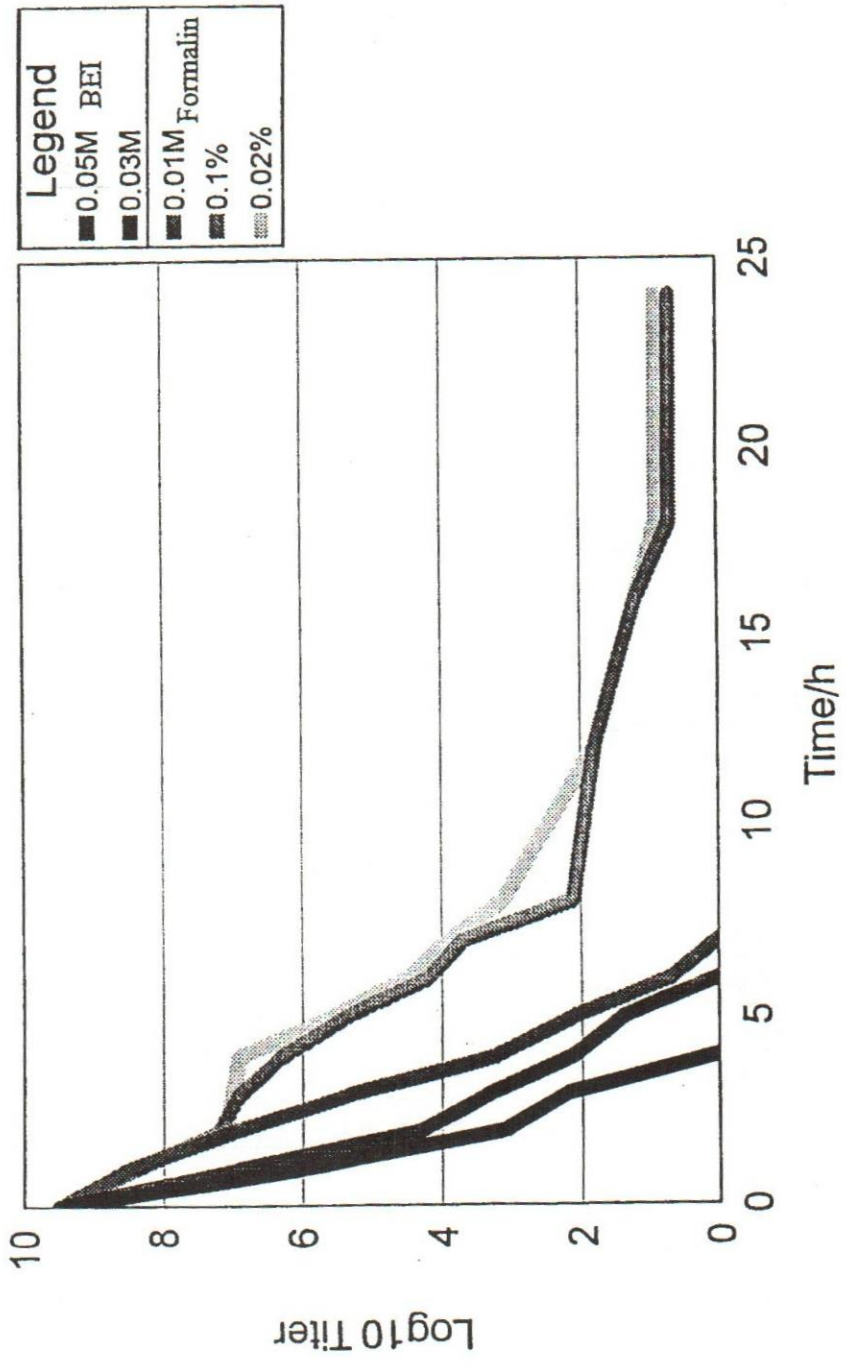

Fig. (1) Effect of BEI and Formalin with different concentration on the infectivity of NDV.