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EVALUATION OF THE EFFICACY OF DIFFERENT FORMULAE OF CONCENTRATED INACTIVATED RABIES VACCINE

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A B S T R A C T

There is increased cases of rabies worldwide and be afraid in everywhere. Such vaccine aimed to induce high level of more prolonged immunity in a safe manner. So the present work evaluate all prepared vaccine formulae which from different adjuvant and different doses and also by concentrated virus by PEG6000, were found free from foreign contaminants and safe induce no abnormal general or local clinical sings .The induced rabies antibodies were followed up in the sera of each puppies up to one year using serum neutralization test (SNT), indirect enzyme linked immune sorbent assay (ELISA). Both of SNT and ELISA indicated that the highest antibody level was induced by the vaccine adjuvant montanide ISA206 and inactivated by beta propiolactone and concentrated by poly ethylene glycol 6000.

Key Words: Montanide ISA206, PEG, SNT.

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1. INTRODUCTION

Rabies is an acute highly fatal infectious disease affecting all worm blooded animals and man. It is usually transmitted through biting of rabid animals to healthy one or man, where the causative virus is often present in the saliva of the victim [3]**.** Rabies is caused by a prototype of the genus *Lyssavirus* in the family *Rhabdoviridae*. All warm-blooded animals are vulnerable to infection with rabies virus, but mammals are the only known vectors and reservoirs in nature. Factors such as the viral variant, the quantity of virus inoculated and the bite site affects the host susceptibility. In addition, the degree of species susceptibility varies considerably [12]**.** Regarding Egypt, rabies is enzootic in jackals and common in dogs as reported by [11, 25] who concluded that dogs and wolves are the primary vector animals for transmission of rabies to cattle in the Middle East.

Vaccination is a powerful tool in combating the disease since rabies known as an incurable disease. For control of rabies in dog populations, vaccination of a minimum 50-70% of dogs is theoretically necessary [12]. Eliminating wildlife populations is nearly impossible and is expensive. Therefore the control measures spotted on vaccination of dogs and cats are most appealing and effective. Many types of vaccines were produced since [20] who carried out the first trial of antibodies immune prophylaxis.

WHO prevented the usage of all live rabies vaccines to be administrated for vaccination either in human beings or in veterinary use to avoid the possible reverse to the virulence of the vaccine strain and to avoid the incidence of a focal infection in clean area [28]. In order that elucidated the production of a safe inactivated vaccine for

immunization of pet and farm animals has been recommended.

In Egypt, inactivated vaccines were prepared from mice brain using Beta probiolactone (BPL) and binary ethyleneimine (BEI) [10, 19] succeeded in production of inactivated rabies tissue culture vaccine using binary ethtyneimine and adjuvanted by aluminum hydroxide gel. The later vaccine was found to be safe and potent for pets and farm animals [15]**.** OIE prefer rabies vaccines inactivated by ßPL.

An adjuvant or immunopotentiator should stimulate high antibody titers, but in the process it should have low toxicity and not induce harmful side effects after injection into either animals or human beings. The main function of an adjuvant is to stimulate antibody production against a range of antigens, even with small quantities of poorly antigenic substances, preferably in a small number of injections or administrations. These objectives would seem to be easy to achieve, but after much research the perfect adjuvant is still elusive to vaccinologists. More than 100 adjuvants have been described [10], but many of these would not be routinely included in vaccines because of a variety of reasons, e.g., cost and the complex preparation of the injection mixture, and many are too reactive in toxicology tests.

So, the present study aimed to evaluate different formulae of rabies inactivated vaccine including un-concentrated and concentrated virus antigen, gel and oil adjuvant forms.

2. MATERIALS AND METHODS

2.1. Animals

2.1.1. *Mice*

Albino Swiss mice (n=300), 3- 4 weeks old, were supplied by the Department of Pet Animal vaccine Research, Veterinary Serum and Vaccine Research Institute, Abassia, Cairo. These mice were used in the quality control tests of prepared rabies vaccine formulae.

2.1.2. *Dogs***:**

Native breed puppies (n=14) of 3 months age were used in the safety and seroconversion tests. These puppies were found to be free from rabies antibodies as screened by serum neutralization test

2.2. *Rabies virus strains:*

2.2.1. *ERA strain:*

ERA strain of rabies virus was kindly supplied by Prof. P. Sureau, the director of WHO Collaborating Centers for References and Researches in Rabies Institute Pasteur, Paris, France. It was supplied in a lyophilized form with a titer of $10^{3.5}$ $TCID_{50}$ / ml. This virus was used for vaccine preparations, serum neutralization test.

2.2.2. *Challenge virus strain (CVS):* it is a fixed virus strain derived from the original Pasteur strain. It was propagated and fixed in mice brain. The virus strain was also obtained from Pasteur Institute, Paris, in a lyophilized form. CVS had a titer of 10^5MILD50/ml and used in the test of the National Institute of Health (NIH test) during evaluation of the prepared vaccine formulae.

2.2.3. *Reference Rabies Vaccine*

Delcavac Rabies vaccine was obtained from Mycofarm UK Limited, Science Park, Milton Road, Cambridge CB44FP. It was used as reference vaccine in application of NIH test. The vaccine is containing cloned rabies virus strain RIV/PTA/78/BHK clone 8 Batch 75894 A.

2.3. *Vaccine adjuvant* (*Alumenium hydroxide gel):*

Aluminum hydroxide gel 2% was supplied by Superfos Biosector a/s. Frydenlands Denmark. The aluminum hydroxide gel was sterilized by autoclaving and kept at room temperature till used. It was used as adjuvant to an inactivated rabies vaccine form

2.4. *Preparation of rabies virus suspension***:** In order to prepare the virus suspension, ERA virus strain was replicated at MOI rate of 2:1 of virus/BHK21 cells. Titration of the obtained virus was performed using the micro-titer technique and the virus titer was calculated according to [21].

2.5. *Virus inactivation:*

2.5.1. *Virus inactivation by Binary ethylenamine (BEI):*

It was added at 37˚C to the viral suspension with a final concentration of 0.01M.The mixture was stirred continuously at 37˚C for 3.5 hours according to [27**]** and [10]. Inactivation process was stopped by addition of cold sodium thiosulphate with a final concentration of 2%.

2.5.2. *Inactivation by Beta propiolactone (ßPL):*

The clarified harvested virus fluid was treated with ßPL as 1:5000, on a magnetic stirrer for continuous stirring at 37ºc according to [1] for 4 hours.

2.6. *Virus concentration by polyethylene glycol 6000***:**

The inactivated virus was cooled to 4oC, then polyethylene glycol 6000 (PEG – 6000) was added (100 gm/1 liter virus suspension). The mixture was placed on a stirrer (for complete mixing and solubilization) at 4oC for 2 hrs and kept at 4oC overnight. The mixture was centrifuged at 6000 rpm for 20 minutes and the supernatant was discarded. This procedure was repeated using 8% PEG-6000 for further concentration of the viral antigen. The precipitate was re-suspended in 0.6 Mol. Potassium phosphate buffer (8µl KPB/liter of virus suspension), and mixed well by vortexing and the volume was brought to 100 ml using the elution buffer (tris-KCI). Then centrifugation at 1000 rpm for 15 minutes and the supernatant was collected in clean sterile tubes (concentrated purified rabies virus antigen). Addition of N Z amine A S 1/1000 to the final volume of virus suspension.

2.7. *Estimation of total protein content in the prepared antigen***:** the test was carried out as described by Bradford (1976).

2.8. *Addition of aluminum hydroxide gel as adjuvant to the prepared vaccine formulae***:** this adjuvant was added as 20% to the prepared inactivated rabies virus suspensions according to [10].

2.9. *Quality control tests of the prepared rabies vaccine formulae*:

Each of the prepared vaccine formulae was subjected to the following tests:

2.9.1. *Sterility test***:**

According to [23], samples of the vaccine batches were inoculated in thiogluconate broth, nutrient agar and then incubated at 37°C for 7 days. Also the test included inoculation of Sabouraud's agar, yeast and mould broth which were preserved at room temperature for 15 days. The results of sterility tests must be negative for any bacterial, fungal or yeast growth before allowing the vaccines to next steps.

2.9.2. *pH test***:** In this test, the aluminum hydroxide gel pH was adjusted at 8-9 and thoroughly mixed with the inactivated virus.

2.9.3. *Safety test:*

*2.9.3.1. In mice***:**

According to [16], the vaccine batches were I/P inoculated into 20 weaned mice. These animals were observed for any signs of rabies for 21 days. The vaccine was approved as safe if all inoculated mice remained alive.

*2.9.3.2. In dogs***:**

Tow puppies were used to test the safety of each vaccine formula. Each animal was inoculated with 10 ml of the tested vaccine via the S/C route. The animals were held under clinical observation for 15 days,

meanwhile the body temperatures were polled daily for each animal. The vaccine batch was considered to be safe when the animals remained healthy, without any rise of body temperatures.

2.9.4. *Potency test*:
Each vaccine formula was subjected to the following potency tests.
2.9.4.1. *National Institute of Health (NIH) test*:
According to [22] NIH test was carried out using the volumetric method. The survived mice were recorded for 14 days followed by application of the following special equation to estimate the antigenic value (AV) of the vaccine which must not be less than 0.3.

$$AV = \frac{\text{ED50 of reference vaccine}}{\text{ED50 of test vaccine}}$$

$$RP = \frac{\text{Reciprocal of ED50 TV}}{\text{Reciprocal of ED50 RV}} \times \frac{SDTV}{SDRV}$$

AV = Antigenic Value, RP = Relative Potency

TV= Tested vaccine, ED= Effective Dose. SD= Single Dose, RV= Reference Vaccine and AV= Antigenic Value.

2.9.4.2. *Potency tests in puppies*: The puppies were divided into 5 groups where each of the first 3 groups includes 3 puppies while group 4 included 2 puppies while the 5th group included 6 puppies as follow:
Group-1: injected with 2ml of vaccine inactivated by binary ethylenamine and adjuvanted with aluminum hydroxide gel (gel vaccine).
Group-2: injected with 0.5ml concentrated rabies vaccine by polyethylene glycol 6000, inactivated by binary ethylenamine and adjuvanted with aluminum hydroxide gel.
Group-3: injected 1ml concentrated rabies vaccine, inactivated by binary ethylene-amine and adjuvanted by aluminum hydroxide gel.
Group-4: two puppies were kept without vaccination as control
Group-4: included 6 puppies used for safety test for each vaccine.

Table (1): Illustration of puppy's groups

Group number	No. of dogs	Dose	Inactivator	Adjuvant	PEG 6000
1	3	2ml	↑ Binary ↓	Gel.	Non
2	3	0.5ml		AL (Hco_3) gel	Conc.
3	3	1ml		AL (Hco_3) gel	
4	2	Kept without vaccination as control			
5	6	Used for safety tests of the prepared vaccine formulae (2 puppies for each vaccine formula)			

2.9.5. *Seroconversion*:
Blood samples were aseptically obtained from vaccinated puppies through vein puncture using disposable sterile syringes and replaced into sterile screw capped vials according to [18]. These samples were allowed to clot at room temperature then kept at 4°C overnight. The formed serum was separated aseptically and centrifuged at 2000 rpm for 15 minutes. The obtained clear serum samples were kept at –20°C till subjected to serological studies. Serum samples were obtained before and weekly after vaccination for 4 weeks then monthly up to 6 months post vaccination. These samples were subjected to serological tests to estimate the induced antibodies.

2.9.6. *Serum Neutralization test (SNT):*
It was carried out using the micro titer technique according to [2]. The antibody titer was expressed as the reciprocal of the final serum dilution which neutralized and

inhibited completely the CPE of 100 $TCID_{50}$ of the used virus according to [24].

2.9.7. *Keeping Quality:*
It was applied after [17], by storage of samples from each produced vaccine formula at different temperatures (4°C, Room temperature 22 - 25°C) and 37°C). Then the potency of each type of vaccine was monthly estimated using the NIH test.

3. RESULTS AND DISCUSSION

The present work is a trial to improve the produced inactivated rabies vaccine through use of concentrated virus.
The experimental results revealed that all prepared vaccine formulae were free from foreign contaminants (aerobic and anaerobic bacteria, fungi and mycoplasma) and safe in vaccinated animals where such animals remained healthy all over the experimental period without local reaction at the site of inoculation as shown in table (4).

Table 2 Virus protein in inactivated rabies virus

Item	Protein content (gm/dL)
Control MEM	1.8
Virus inactivated by BEI	0.7
Concentrated virus by PEG	1.2

The results from estimation viral protein found in the clear minimum essential media & comparing by inactivated virus by binary ethyleneimine & Beta propiolactone & concentrated inactivated virus as shown in table (5) it is clear that the concentrated virus by PEG (poly ethylene glycol 6000) is highly fourth time more than the inactivated virus, these results confirmed by [29] and [30].
The progress in vaccine production is directed towards the selection of proper adjuvant that can elaborate high and long last immunity, so adjuvant considered one of the important factors in vaccine formulation [8]. Both aluminum hydroxide gel vaccine and oil adjuvant vaccines have been used to control the disease [18].

Table 3 Potency of rabies vaccine formulae as determined by NIH test

Tested vaccine formula	Inactivator	Adjuvant	Polyethylene Glycol concentration	Relative Potency	Increase of relative potency
(1)	BEI	Al-hydra gel	PEG	2.6	2.2
(2)	BEI	Al-hydra gel	-	2.0	1.7
Reference (3)	BEI	Al-hydra gel	-	2.0	1.7

Table 4 Summarized results of sterility, safety and potency of the prepared inactivated rabies vaccine

Types of vaccines	Dose	Sterility			Safety / potency		
		Bacterial	Fungal	Mycoplasma	mice	dog	NI H
Bpl +oil	2ml	-ve	-ve	-ve	Safe	Safe	2.3
Bpl + gel	2ml	-ve	-ve	-ve	Safe	Safe	2.1
BEI + oil	2ml	-ve	-ve	-ve	Safe	Safe	2.2
BEI +gel	2ml	-ve	-ve	-ve	Safe	safe	2.0

Table 5 Mean rabies serum neutralizing antibody titers in vaccinated puppies

Group	Vaccine formulae	Dose	Mean rabies serum neutralizing antibody titer* on periods post vaccination													
			WPV**			MPV***										
			1	2	3	2	3	4	5	6	7	8	9	10	11	12
1	A	2ml	2	4	8	16	32	64	64	64	64	64	64	64	64	64
2	B	1/2ml	4	16	32	64	64	64	64	64	64	64	64	64	64	64
3	C	1ml	2	16	32	64	64	128	128	128	128	128	128	128	128	128
4	Control	2 ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*Antibody titer= the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100. $TCID_{50}$ of rabies virus **WPV= week post vaccination ***MPV= month post vaccination. A: Binary + Gel, B: Binary+ conc. Virus +gel, C. Binary +conc. Virus +gel.

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مجلة بنها للعلوم الطبية البيطرية
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تقييم كفاءة صور مختلفة من لقاح السعار المثبط

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الملخص العربى

حالات الإصابة بداء السعار فى زيادة فى كل العالم مما يسبب الرعب حيثما وجد,فقد تم اجراء هذا البحث لتحضير لقاح يعطى مناعة عالية ولمدة طويلةّ امن ,وهذا البحث تم تقييم و معايرة اللقاحات المختلفة المثبطة بمادتى البتا بروبيولاكتون والبينارى ايثيلينامين وباضافة محفزات مختلفة كزيت المونتوناید SA206 اوالامونيوم هيدروكسيد جيل والمركز بمادة البولى ايثيلين جليكول 6000 ,وعند تحصين مجموعات الجراء تبين ان هذه التركيبات المختلفة امنة لم تظهر اية اعراض عامة او مكان الحقن ,وكذلك اوضحت نتائج اختبارى المصل المتعادل والانزيم الممدص المرتبط المناعى ان كل من صور اللقاح المحضرة يعطى مناعة ذات مستويات جيدة الا ان اعلاها قدرا تم احداثه باللقاح المساعد بمادة زيت المونتناید ISA206 والمثبط بالبيتابروبيولاكتون والمركز بمادة البولى ايثيلين جليكول6000.

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